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JOURNAL OF OIL PALM RESEARCH

Vol. 33 (2) June 2021

C O N T E N T S

REVIEW ARTICLE

- 181** Oil Palm Economic Performance in Malaysia and R&D Progress in 2020
Ghulam Kadir Ahmad Parveez; Azmil Haizam Ahmad Tarmizi; Shamala Sundram; Soh Kheang Loh; Meilina Ong-Abdullah; Kosheela Devi Poo Palam; Kamalrudin Mohamed Salleh; Sheilyza Mohd Ishak and Zainab Idris

RESEARCH ARTICLES

- 215** Soil Fungal Composition and Diversity in Oil Palm Plantation at Sungai Asap, Sarawak, Malaysia
Sharron Wong; Farah Melissa Muharam; Osamah Rashed and Khairulmazmi Ahmad
- 227** A Proof-of-Concept Study: Determining the Geographical Origin of Crude Palm Oil with the Combined Use of GC-IMS Fingerprinting and Chemometrics
Goggin, K A; Brodrick, E; Wicaksono, A; Covington, J A; Davies, A N and Murphy, D J
- 235** Isolation and Characterisation of Gibberellin 2-oxidase 6 Gene from Oil Palm (*Elaeis guineensis* Jacq.)
Zatty Syamimi Adura, M S; Nurniwalis, A W and Zubaidah, R
- 248** Expression of Cadherin Gene in *Bacillus thuringiensis* Cry1A Toxin Resistant and Susceptible Strains of *Tirathaba mundella*
Calvin Tan Zhe Khai; Patricia King Jie Hung; Su Chong Ming; Ho Kok Lian and Lau Wei Hong
- 257** Soil Carbon Dioxide (CO₂) Efflux Rate and Oil Palm Yield from Different Peat Types in Sarawak, Malaysia
Hasimah Mos; Mohd Haniff Harun; Kho Lip Khoo; Nur Maisarah Jantan; Afifah Abdul Razak; Nur Amanina Shahabuddin; Hasnol Othman; Norman Kamarudin; Ghulam Kadir Ahmad Parveez; Ahmad Kushairi Din; Paramananthan Selliah; Ngab Dollah Salam and Yusri Yusup
- 267** The Dynamic of Carbon Dioxide (CO₂) Emission and Land Coverage on Intercropping System on Oil Palm Replanting Area
Sri Ambar Kusumawati; Sudirman Yahya; Hariyadi; Sri Mulatsih and Ida Nur Istina
- 278** Quantitative Approach for Irrigation Requirement of Oil Palm: Case Study in Chuping, Northern Malaysia
Mohd Shahkhirat Norizan; Aimrun Wayayok; Yahya Abd Karim; Ahmad Fikri Abdullah and Muhammad Razif Mahadi
- 289** Odour Exposure Level Measurement Surrounding Palm Oil Mill
Andrew Yap Kian Chung; Nastaein Qamaruz Zaman; Rohaya Mohamed Halim; Fatah Yah Abd Manaf and Nu'man Abdul Hadi
- 299** Investigation on the Effect of Monoglyceride Concentration on Palm Oil-based Alkyd Resin Preparation
Ridzuan Ramli; Huei Ruey Ong; Chi Shein Hong; Md. Maksudur Rahman Khan; Rosli Mohd Yunus; Rohaya Mohamed Halim; Astimar Abdul Aziz; Zawawi Ibrahim and Muhammad Remanul Islam
- 307** Xylan Recovery from Dilute Nitric Acid Pretreated Oil Palm Frond Bagasse Using Fractional Factorial Design
Nurul Aishah Mazlan; Kamaliah Abdul Samad; Hafizuddin Wan Yussof; Rozaimi Abu Samah and Jamaliah Md Jahim
- 320** Methanolic Extraction of Free Ferulic Acid from Oil Palm
Ng Mei Han
- 327** Growth Performance and Carcass Traits of Goats Fed with Oil Palm By-products Based Feed Pellet
Nur Atikah Ibrahim; Wan Nooraida Wan Mohamed; 'Abidah Md Noh and Mookiah Saminathan
- 335** Effect of Feeding Palm Oil Sludge as Partial Replacement of Maize in Growing-Finishing Pigs on the Growth Performance, Nutrient Digestibility and Blood Profiles
Temjemungsang; Samanta, A K; Ali, M A; Das, B K and Girin, G K
- 347** Phase Behaviour of High, Medium and Low Melting Fractions in Lauric Fat Blends Formulated Based on Melting Triacylglycerol Group
Bangun Nusantara; Yanty Noorzianna Manaf; Davy Van De Walle; Filip Van Bockstaele and Koen Dewettinck
- 360** Blended Palm Fractions as Confectionery Fats: A Preliminary Study
Norazura Aila Mohd Hassim; Sivaruby Kanagaratnam and Noor Lida Habi Mat Dian

SHORT COMMUNICATION

- 381** Potential of *Syncephalastrum racemosum* and *Rhizopus arrhizus* Isolated from Oil Palm Trunks Produce Chitosans That Inhibit the Growth of *Ganoderma boninense*
Fahrizal, M; Sarifuddin and Sabrina, T

Cover picture: Oil palm industry performance and development in 2020.

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OIL PALM ECONOMIC PERFORMANCE IN MALAYSIA AND R&D PROGRESS IN 2020

GHULAM KADIR AHMAD PARVEEZ*; AZMIL HAIZAM AHMAD TARMIZI*; SHAMALA SUNDARAM*; SOH KHEANG LOH*; MEILINA ONG-ABDULLAH*; KOSHEELA DEVI POO PALAM*; KAMALRUDIN MOHAMED SALLEH*; SHEILYZA MOHD ISHAK* and ZAINAB IDRIS*

ABSTRACT

The year 2020 faced unprecedented challenge for most of the global economic growth due to the outbreak of Coronavirus disease (COVID-19). Despite a downward trend performance of the Malaysian oil palm industry, particularly for the first half of 2020, the impact is less severe due to the encouraging palm oil export revenue through the National Economic Recovery Plan (PENJANA) which nurtures a notable increase in crude palm oil (CPO) price. In honouring the Malaysian pledge on forest conservation, land expansion for the oil palm cultivation remains stagnant over the years. The effort is now shifting towards enhancing the oil palm yield performance through new planting materials and good agricultural practices, coupled with systematic pest and disease management. Sustainability continues to be the key agenda of the oil palm industry, in moving forward to sustain the industry ecosystem. The industry is now open for innovative palm oil processes to comply with the dynamic and stringent food safety and quality standards and trade regulations. Owing to the distinction in food and feed applicability together with health prospects, translating the information into consumer-friendly language is becoming crucial for effective communication. Valorisation via the concept of 'waste to wealth' has compelled series of innovations in capitalising oil palm co-products for greener bioenergy and oleochemicals, and source of phytonutrients to generate higher earnings without having to heavily rely on palm oil trade as commodity. Mandatory enactment of the Malaysian Sustainable Palm Oil (MSPO) certification scheme has portrayed a success story of showing the utmost commitment towards sustainability. With persistent dedication, the oil palm industry is envisaged to be self-sustaining, amidst the never-ending challenges surrounding economy, well-being and environment.

Keywords: bioenergy, COVID-19, food safety and health, MSPO, oleochemicals, palm oil, sustainability, valorisation.

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INTRODUCTION

Palm oil sector is one of the crucial revenue earners for Malaysia's economy after the electrical and electronic, petroleum and chemicals industries (MATRADE, 2021). The challenges in confronting the COVID-19 pandemic did not jeopardise its ranking as the fourth world's largest producers of oils and fats in 2020 accounting at 8.1% of the

global total production (Oil World, 2020). Indeed, Malaysia is more prominent as the major exporter of palm oil, as reflected by 18.3% of the global oils and fats (17.37 million tonnes) while contributing to 34.3% of the total palm oil trade. The prevalence of COVID-19 pandemic, however, has shown less impact to most of the national oil palm industry performance indices particularly after the relaxation of movement restrictions, resumption of economic sectors in most countries and exemption of CPO export duty under the National Economic Recovery Plan or PENJANA. It is also worth to note that the aforesaid pandemic led to unexpectedly positive

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effects on the national oil palm industry evidenced tremendous increase of 29.2% from the previous year (RM2079.00 t⁻¹), and this further reflected by the improvement of total export revenue by 8.4%.

Over the past few years, palm oil research is geared towards the sustainable development goals across the oil palm supply chain. Despite all the efforts made, palm oil cultivation is recently reported to adversely impacting the environment associated to deforestation, biodiversity and carbon footprint (Meijaard *et al.*, 2020). Furthermore, the issues on nutrition and health relating to palm oil are imposed to suppress its applicability and potential use in food applications. Food safety concerns with regard to 3-monochloropropane-1,2-diol esters (3-MCPDE) and glycidyl esters (GE) have reached their pinnacles when the European Commission (EC) implemented the maximum limits of these contaminants in edible oils and fats on 1 January 2021 for 3-MCPDE (Commission Regulation (EU) 2020/1322, 2020) in addition to 19 March 2018 for GE (Commission Regulation (EU) 2018/290, 2018). Notwithstanding this, the world's reliance to palm oil is undeniable witnessing its significant global export in 2020 surpassing, 50% in comparison to other leading vegetable oils (Oil World, 2020). In order to remain sustainable and competitive globally, it is imperative to combat the aggravating environmental issues and move forward so that palm oil meets the quality and safety standards, hence gaining international recognition. Such effort can be seen from the commitment of the Malaysian Government to fully implement sustainable palm oil cultivation and processing practices across the whole value and supply chains through the Malaysian Sustainable Palm Oil (MSPO) certification scheme effectively from 1 January 2020. This initiative would enlighten the image of the national's palm oil in the arena of sustainability and to cater the demand of certified palm oil from the global markets. In order to safeguard the palm oil trade and authenticate the viability of palm oil industry, more proactive research and development (R&D) is needed to address outstanding issues particularly those requiring immediate action.

This article reviews the oil palm industry performance for the year 2020 as well as the key findings from some of the published scholars locally and internationally in the respective year. The discussion will emphasise on the current research advancement and innovation across the oil palm supply chain covering the upstream, midstream and downstream sectors so that the oil palm industry remains competitive and resilient. Finally, this review will highlight the strategies and directions to strive for the betterment of the oil palm industry performance in the coming years.

PERFORMANCE OF THE MALAYSIAN OIL PALM INDUSTRY

The year 2020 has been very tough as the COVID-19 pandemic disrupted the global political, social, economic and financial structures (Verma *et al.*, 2021). In the light of the Malaysian palm oil industry, the pandemic has led the industry to experience a slowdown in the production, export demand and prices particularly at the beginning of the year. Approaching second half of the year, many countries have re-opened their economic sectors through the relaxation of movement restrictions and the Malaysian government has exempted the export duty of crude palm oil (CPO), crude palm kernel oil (CPKO) and refined, bleached and deodourised (RBD) palm kernel oil (PKO) from 1 July 2020 until 31 December 2020 under the PENJANA. This positive environment has led to the recovery of export demand of palm oil and other oil palm products despite the tight supply of CPO. In the end, the palm oil stock level in the country has subsequently reduced, hence improving its price. Overall, the year 2020 had witnessed the declining of Malaysian oil palm planted area, CPO production as well as palm oil exports, imports and stocks decline but an unexpected improvement in the CPO price and export revenue of palm oil products in comparison to the previous year.

Planted Area

Oil palm planted area in 2020 had reached 5.87 million hectares, which denoted a marginal drop of 0.6% (5.90 million hectares) recorded in the previous year. This is mainly attributed by the reduction of the planted area in the Peninsular Malaysia by 1.1% or 13 280 ha (Table 1). The restriction of the oil palm expansion and the delay in the oil palm replanting are primarily associated with the imposition of movement control order (MCO) that contributes to the downturn in planted area. Sarawak remains the largest oil palm planted state owing 1.58 million hectares or 27.0% of the total Malaysian oil palm planted area, and subsequently Sabah and Pahang with 1.54 million hectares and 0.78 million hectares, respectively.

The oil palm matured area reported for 2020 is 5.23 million hectares which accounts for 89.2% of the total oil palm planted area (Table 2). Sarawak has a total of 1.43 million hectares of matured oil palm trees constituted by 90.3% of total oil palm planted area in the respective state. With regard to ownership, oil palm plantation consists of approximately 4.24 million hectares which accounts for 72.2% of the total planted area (Table 3). On the other hand, organised and independent smallholders only hold 11.5% and 16.3% of the total share of the oil palm planted area, respectively. In comparison from the previous year,

the total planted areas from both organised and independent smallholders have dropped for which the latter encountered 0.4% loss due to the shrinkage in the planted area.

Status of Mills and Plants

A total of 457 oil palm mills in Malaysia are in operation in 2020 with a total processing capacity of 116.81 million tonnes of fresh fruit bunches (FFB) per year of which 53.0% of the oil palm mills were located in the Peninsular Malaysia (58.16 million tonnes) (Table 4). The current year also witnessed a 4.40% depreciation in the milling capacity by 83.30% when compared to 87.16% in 2019 as a result of lower FFB production.

In the refining sector, a total of 50 palm oil refineries were in operation in 2020 with a total processing capacity of 25.35 million tonnes of CPO and CPKO. About 35 refineries are located in the Peninsular Malaysia with a total processing capacity

of 14.84 million tonnes. The refining capacity showed a reduction of 8.50% from 72.12% to 66.02% in comparison to the previous year due to lower CPO production but higher CPO exportation.

There are a total of 42 Malaysian palm kernel crushers in operation in 2020 with a total processing capacity of 7.17 million tonnes. About 61.90% or 26 palm kernel crushers are located in the Peninsular Malaysia with a total processing capacity of 4.40 million tonnes. The palm kernel crushing capacity declined by 1.50% from 65.47% in 2019 to 64.48% because of inferior palm kernel supply arising from lower FFB production.

In 2020, a total of 20 Malaysian oleochemical plants and 19 biodiesel plants are in operation with processing capacities at 2.63 million tonnes and 2.23 million tonnes, respectively. The oleochemical and biodiesel plants are mainly located in Selangor and Johor with eight and seven oleochemical plants respectively, and six biodiesel plants are located in Selangor and Johor, respectively.

TABLE 1. MALAYSIAN OIL PALM PLANTED AREA AS AT DECEMBER
(million hectares)

	Planted area		
	2020	2019	Difference (%)
Peninsular Malaysia	2.74	2.77	(1.1)
Sabah	1.54	1.54	(0.1)
Sarawak	1.58	1.59	(0.1)
Malaysia	5.87	5.90	(0.6)

Source: Department of Statistics Malaysia (2021).

TABLE 2. MALAYSIAN OIL PALM MATURED AREA AS AT DECEMBER
(million hectares)

	Matured area		
	2020	2019	Difference (%)
Peninsular Malaysia	2.46	2.44	0.5
Sabah	1.34	1.35	(0.7)
Sarawak	1.43	1.42	0.9
Malaysia	5.23	5.22	0.3

Source: MPOB (2021).

TABLE 3. DISTRIBUTION OF OIL PALM PLANTED AREA BY OWNERSHIP IN
MALAYSIA AS AT DECEMBER (million hectares)

Ownership	2020		2019	
	ha	%	ha	%
Plantation	4.24	72.2	4.23	71.7
Organised smallholders	0.67	11.5	0.68	11.6
Independent smallholders	0.96	16.3	0.99	16.7

Source: MPOB (2021).

TABLE 4. NUMBER OF OIL PALM MILLS, REFINERIES, PALM KERNEL CRUSHERS, OLEOCHEMICAL PLANTS AND BIODIESEL PLANTS AND THEIR CAPACITIES IN MALAYSIA IN 2020

Facility	No.	Processing capacity (million tonnes per year)
Oil palm mill		
Peninsular Malaysia	242	58.16
Sabah	132	34.65
Sarawak	83	24.01
Malaysia	457	116.81
Palm oil refinery		
Peninsular Malaysia	35	14.84
Sabah	9	7.24
Sarawak	6	3.28
Malaysia	50	25.35
Palm kernel crusher		
Peninsular Malaysia	26	4.40
Sabah	12	2.07
Sarawak	4	0.70
Malaysia	42	7.17
Oleochemical plant		
Selangor	8	0.80
Johor	7	0.68
Others	5	1.16
Malaysia	20	2.63
Biodiesel plant		
Johor	6	0.85
Selangor	6	0.67
Sabah	3	0.40
Others	4	0.32
Malaysia	19	2.23

Source: MPOB (2021).

CPO Production

Malaysian CPO production in the first quarter of 2020 was significantly lower by 22.1% when compared to the corresponding period of 2019. It is also reported that decreasing CPO production is contributed by lower FFB supply, and partly attributed by the dry season in early 2019, low fertiliser application during low CPO price in 2019 and flood in most areas in Malaysia due to northeast monsoon season (CPOPC, 2020; Leng, 2020). The highest CPO production was recorded in June 2020 (1.89 million tonnes) while the lowest production was observed in January (1.17 million tonnes). Overall, the CPO production in the current year portrayed a drop from 19.86 million tonnes in 2019 to 19.14 million tonnes where Sabah witnessed a notable reduction by 7.7% (Table 5).

The FFB yield for 2020 went down by 2.7% (16.73 t ha⁻¹) in comparison to 17.19 t ha⁻¹ in 2019 due to lower yield performance in the first quarter of 2020 (Table 6). Comparable to that of the CPO production, all regions experienced FFB yield decline, especially in Sabah and Sarawak, which recorded a notable decline of 4.6% and 3.7% to 16.84 t ha⁻¹ and 14.99 t ha⁻¹, respectively. Similarly, the oil extraction rate (OER) dropped by 1.4% from 20.21% (2019) to 19.92% in 2020 reflecting the inferior quality of FFB supplied to the palm oil mills (Table 7). On a monthly year-on-year comparison, OER performance for 2020 was found lower throughout the year in correspondence to the previous year; June 2020 recorded the lowest OER at 19.47% while the highest OER was achieved in October 2020 at the rate of 20.36%. Sarawak suffered severe OER reduction by 2.0% (19.62%) whereas OER loss in Sabah was published at 1.1% (20.74%).

TABLE 5. MALAYSIAN CRUDE PALM OIL (CPO) PRODUCTION (million tonnes)

	2020	2019	Difference	
			Volume	%
Peninsular Malaysia	10.44	10.58	(0.14)	(1.4)
Sabah	4.65	5.04	(0.39)	(7.7)
Sarawak	4.05	4.24	(0.18)	(4.3)
Malaysia	19.14	19.86	(0.72)	(3.6)

Source: MPOB (2021).

TABLE 6. MALAYSIAN FRESH FRUIT BUNCHES (FFB) PRODUCTIVITY (t ha⁻¹)

	2020	2019	Difference	
			Volume	%
Peninsular Malaysia	17.76	17.95	(0.19)	(1.1)
Sabah	16.84	17.66	(0.82)	(4.6)
Sarawak	14.99	15.56	(0.57)	(3.7)
Malaysia	16.73	17.19	(0.46)	(2.7)

Source: MPOB (2021).

TABLE 7. MALAYSIAN OIL EXTRACTION RATE (OER) (%)

	2020	2019	Difference	
			Volume	%
Peninsular Malaysia	19.68	19.93	(0.25)	(1.3)
Sabah	20.74	20.97	(0.23)	(1.1)
Sarawak	19.62	20.03	(0.41)	(2.0)
Malaysia	19.92	20.21	(0.29)	(1.4)

Source: MPOB (2021).

Exports

According to the Department of Statistics Malaysia, the total exports of oil palm products in 2020 stood at 26.59 million tonnes, down by 8.5% against 29.04 million tonnes reported in 2019 because of weakening in palm oil, palm-based oleochemicals and biodiesel exportation (*Table 8*). Exports of palm oil for 2020 was 16.22 million tonnes, which was reflected by a significant decrease of 7.0% from 17.43 million tonnes in 2019, primarily caused by lower CPO production. On the other hand, exports of PKO in 2020 had surged by 13.3% to 1.14 million tonnes from 1.01 million tonnes in 2019 due to the higher demand from the European Union (EU), China and USA. Meanwhile, exports of palm-based oleochemicals and biodiesel had also dropped substantially by 19.4% and 53.7% to 4.41 million tonnes and 0.33 million tonnes, respectively, due to lower demand from the EU and China.

In spite of low exports performance, the total export revenue for palm products had increased by

8.4% to RM73.25 billion from RM67.55 billion in 2019 due to higher export prices of palm oil and other oil palm products in 2020. Export revenue of palm oil and PKO surged by 16.7% and 25.6% to RM45.66 billion and RM4.15 billion in 2020, respectively. In the case of palm-based oleochemicals and biodiesel, the higher prices in 2020 eased the export revenue loss to 9.4% and 40.1%, respectively.

Imports

The importation of Malaysian oil palm products stood at 1.29 million tonnes in 2020, down by 1.7% from 1.31 million tonnes in 2019. Palm oil represents 73.6% of total Malaysian imports of oil palm products for 2020, followed by PKO (21.7%) and palm kernel (4.6%) (*Table 9*). Imports of palm oil declined by 3.1% to 0.95 million tonnes from 0.98 million tonnes in 2019 which was associated with lesser demand from domestic palm oil refining and processing sector by 13.9% to 17.51 million tonnes as against 20.34 million tonnes in 2019.

Closing Stocks

Palm oil closing stocks in December 2020 was lower by 37.0% to 1.27 million tonnes *vis-à-vis* 2.01 million tonnes recorded in December 2019, mainly due to lower palm oil production (Table 10). All the three regions in Malaysia experienced a substantial reduction in the palm oil stocks with major drop recorded in Sarawak and Sabah by 52.1% and 45.9% to 0.17 million tonnes and 0.35 million tonnes, respectively.

Price

All major oil palm products were highly traded in 2020 as opposed to 2019 (Table 11). Local CPO price peaked by 29.2% or RM606.50 t⁻¹ to RM2685.50 t⁻¹ when compared to RM2079.00 t⁻¹ in 2019. The highest traded CPO price for 2020 was reported in December amounting at RM3620.50 t⁻¹ while the lowest was recorded in May at RM2074.00 t⁻¹. The higher CPO price in 2020 was in line with the higher prices of other major vegetable oils in the global market due to the supply disruptions in key edible oils as well as lower palm oil supply, which in turn indicates lower palm oil stocks in 2020 (CPOPC, 2020).

Following the increase in CPO price, export prices of major processed palm oil products namely RBD palm oil, RBD palm olein and RBD palm stearin in 2020 had showed an increment by 28.6% (RM2887.00 t⁻¹), 27.2% (RM2844.00 t⁻¹) and 29.1% (RM2801.00 t⁻¹), respectively while the price of palm fatty acid distillate (PFAD) rose by 40.9% (RM2546.00 t⁻¹).

In the lauric-based market, the price of palm kernel increased by 26.2% (RM1532.00 t⁻¹) compared to RM1214.00 t⁻¹ in 2019 mainly due to the higher domestic CPKO price. Moreover, the CPKO price in 2020 improved by 23.6% (RM3247.00 t⁻¹) in comparison to RM2626.50 t⁻¹ as reported in the previous year. The higher CPKO price in 2020 was in agreement with higher PKO price in the global market by USD158.00 or 23.7% (USD826.00 t⁻¹) which coincided with higher price in coconut oil by USD276.00 or 37.4% (USD1014.00 t⁻¹).

Conforming to the substantial increase in CPO and palm kernel prices, FFB price at 1% OER in 2019 had increased by 34.7% to RM28.51 as opposed to RM21.17 in 2019. With reference to the national OER, FFB price in 2020 was equivalent to RM561.00 t⁻¹, which was higher by 32.9% when compared to RM422.00 t⁻¹ reported in 2019.

TABLE 8. MALAYSIAN EXPORTS OF PALM OIL AND OIL PALM PRODUCTS

	Volume (million tonnes)			Value (RM million)		
	2020	2019	Difference (%)	2020	2019	Difference (%)
Palm oil	16.22	17.43	(7.0)	45 656	39 128	16.7
Palm kernel oil	1.14	1.01	13.3	4 151	3 306	25.6
Palm-based oleochemicals	4.41	5.46	(19.4)	16 415	18 121	(9.4)
Biodiesel	0.33	0.72	(53.7)	1 194	1 994	(40.1)
Other palm-based products	1.96	1.84	6.8	4 542	3 951	15.0
Palm kernel cake	2.53	2.58	(2.1)	1 295	1 045	23.9
Total	26.59	29.04	(8.5)	73 253	67 546	8.4

Source: Department of Statistics Malaysia (2021).

TABLE 9. MALAYSIAN IMPORTS OF OIL PALM PRODUCTS (million tonnes)

	2020	2019	Difference	
			Volume	%
Palm oil	0.95	0.98	-0.03	(3.1)
Palm kernel oil	0.28	0.26	0.02	7.9
Palm kernel	0.06	0.07	-0.01	(18.0)
Total	1.29	1.31	-0.02	(1.7)

Source: MPOB (2021).

TABLE 10. MALAYSIAN PALM OIL CLOSING STOCKS AS AT DECEMBER (million tonnes)

	2020	2019	Difference	
			Volume	%
Peninsular Malaysia	0.74	1.00	(0.26)	(25.9)
Sabah	0.35	0.65	(0.30)	(45.9)
Sarawak	0.17	0.36	(0.19)	(52.1)
Malaysia	1.27	2.01	(0.74)	(37.0)

Source: MPOB (2021).

TABLE 11. MALAYSIAN PRICES OF OIL PALM PRODUCTS (RM t⁻¹)

	2020	2019	Difference	
			RM t ⁻¹	%
CPO	2 685.50	2 079.00	606.50	29.2
RBD palm oil	2 887.00	2 245.50	641.50	28.6
RBD palm olein	2 844.00	2 236.50	607.50	27.2
RBD palm stearin	2 801.00	2 169.00	632.00	29.1
PFAD	2 546.00	1 807.00	739.00	40.9
Palm kernel	1 532.00	1 214.00	318.00	26.2
CPKO	3 247.00	2 626.50	620.50	23.6
FFB at 1% OER	28.51	21.17	7.34	34.7

Note: CPO - crude palm oil; RBD - refined, bleached and deodourised; PFAD - palm fatty acid distillate; CPKO - crude palm kernel oil; FFB - fresh fruit bunches; OER - oil extraction rate.

Source: MPOB (2021).

R&D FOCUS AREAS IN 2020

Biotechnology and Green Approaches in Upstream

The demand of palm oil has been in continuous growing trend over the years. To meet this demand, large conversion of tropical peatlands in the last decade with more than 3.1 million hectares area have been cultivated with oil palm (Miettinen *et al.*, 2016). While tropical peatland in the South East Asia (SEA) cover more than half of the global peatlands area, it is Malaysia and Indonesia which occupy the largest areas (Page *et al.*, 2011). The significant peatland conversion has sparked an on-going debate internationally, predominantly due to the loss of biodiversity, ecosystem carbon emissions, reduced carbon sink source and loss of flood regulators (Kiew *et al.*, 2020; Lupascu *et al.*, 2020; Meijaard *et al.*, 2020). Most of the articles in 2020 narrated the negative impacts of tropical and peatland conversion for agricultural purposes. Several articles reported a significant loss in bacterial, macrofungi and spider community diversity (Kusai and Ayob, 2020; Potapov *et al.*, 2020; Shuhada *et al.*, 2020) while Cooper *et al.* (2020)

highlighted the importance to include the different stages of peatlands conversion in estimating global warming potential (GWP). The net impact of forest conversion to oil palm was found nearly doubling GWP over a period of 30 years.

Besides the general negative accounts of peatlands conversion by scientific findings, the role of media coverage in providing misleading claims on the peatlands sustainability; an article, *denialist narrative* as analysed by Liu *et al.* (2020) had heavily criticised the role of media in supporting unproven claims on peatland cultivation, influenced by political and socio-economic nuances respective to the country. On the contrary, a big data analytics study had found the same media specifically discusses the role of social media that drives negative public perception on palm oil through on-going online discussions and campaigns despite the continuous efforts by the oil palm industry to lift the negative perception (Teng *et al.*, 2020). Ultimately, we foresee the amalgamation of these reports with the conscious move by Malaysian government prohibiting future oil palm planting on peatlands in 2019, reflecting its commitment to establish oil palm as a sustainable crop. The oil palm industry strives

hard and continues to innovate and address the negative social impacts implied on palm oil trade with some of the perceptions illustrated in the 57 case studies reported by Ayompe *et al.* (2021).

The potential threat and impact of climate change on the environment and livelihood of people is acknowledged especially in the agricultural sector and the economy. A 30 years' time series data on oil palm production found a significant negative relationship between annual average temperatures and oil palm production (Sarkar *et al.*, 2020). A maximum of 41% of oil palm production decrease was recorded for a 4°C temperature rise and a similar impact was also recorded by the sea level rise. The authors suggest several mitigation strategies to methodically reduce the negative impacts but requires revamping the current water and agriculture policies while providing adequate trainings to planters with viable financial aid. The multibillion-dollar industry requires greater attentions to investigate impacts of palm oil production compared to alternatives for the trade-offs to be assessed at a global scale (Meijaard *et al.*, 2020).

Research in the past decade recognises the well-being of the below ground, soil as one of the most pertinent backbone of the agriculture sector. Looking back a few decades ago, the mainstream research in oil palm industry has always been predominantly focusing on the above ground which is the yield while unconsciously neglecting below ground. The scenario is further aggravated by activities such as agronomic practices, fertiliser and chemical applications, and land use changes that took place in agriculture which negatively impact the general soil health in the long run (Sundram *et al.*, 2019). With sustainable development sought after in each sector of the supply chain, research is emphasised more in sustainable approaches of fertiliser management in the field. This conventional assessment for nutrient deficiency requires *in situ* leaf sampling which then goes through laborious and lengthy protocols. This additional activity increases the production cost of the already overly dependent industry with foreign labour. Therefore, any reduction in labour and cost of production is highly welcomed by the industry. Yadegari *et al.* (2020) investigated the use of satellite imagery in determining nitrogen deficiency in field. The technique used vegetative indices (VI) via SPOT-7 satellite images and compared the predicted satellite data and actual leaf analysis with an accuracy of 77%. The approach allows appropriate dosage of nutrients to be applied to the palm, to avoid excessive application that will lead to environmental and ecological disruption. Whilst the application of agrochemicals is an integral component in the well-being of palm, it is still not recognised as a sustainable practice and affects the soil health in the long run. Leveraging on the

prospect of sustainable approaches, the mixing of 75% cow manure together with 25% nitrogen, phosphorus and potassium (NPK) compounds fertiliser was found to give the best vegetative growth among the five dosages examined on pre and main nursery seedlings (Adileksana *et al.*, 2020). These are some of the optimisations that can be incorporated into best management practices (BMP), which has the appropriate fertiliser application in achieving optimum production. Despite the fact that BMP in oil palm is well established, failing to adopt the practices will result in yield declines as seen in the two studies reported in Ghana and Cameroon (Kome *et al.*, 2020; Rhebergen *et al.*, 2020). The studies identified production issues such as labour constraints, and funding limitations apart from recognising characteristics such as variation in soil properties which contribute to compromised yield. Both studies identified the intrinsic details that led to the decline and recommended mitigation approaches in addressing the yield decline. Besides the need for the industry to establish sustainable farming, active agronomic research using advanced technological methods need to be performed, by focusing on yield improvements and incorporating green technologies for the sustainable well-being of the palm. One such research area which needs to be explored intensely in the oil palm cultivation is the identification of nutrient solubilising microbes (NSB) as a long-term programme, either to replace or to be used in combination with agrochemicals in the future.

The description of an undesirable presence of organisms in agriculture is commonly referred to as pest and diseases (P&D), particularly when grievous losses to agricultural sector is experienced, hence affecting the national economy. The oil palm industry is no exception in facing multifaceted challenges which include the severe yield decline incurred by the P&D infestation. In Malaysia for instance, P&D research is mainly driven by the significant economic losses suffered by the industry which then navigates investment to focus more on the biology, early detection and control of these P&D. Chemical application promises an almost immediate control of P&D outbreaks, but the approach is not sustainably commended due to its harmful effects on beneficial insects, microbial community and chemical residues. Therefore, green approaches using naturally occurring enemies are more preferred, which play a key role in integrated pest management (IPM). Natural predators such as *Sycanus dichotomus* STAL. and *Bacillus thuringiensis* are among the two naturally occurring enemies in the environment and can be formulated to control bagworm in oil palm fields. Ahmad *et al.* (2020b) found the adult stage of *S. dichotomus* as a potential predator of bagworm based on the voracity and predation rate. The study has completed its initial

laboratory stage and will be expanded to assess the dynamics of the control in the field.

Moving on to a more established biocontrol agent (BCA) of bagworm, the bacterium, *B. thuringiensis* (*Bt.*) performance as a BCA has been influenced by the fermentation protocols during its cultivation stage. Masri and Ariff (2020) reported that intermittent fed-batch cultivation recorded higher mortality rate at 80% after seven days of treatment. This is an interesting finding considering the fact that the method of *Bt.* cultivation influences its toxicity towards its target pest and establishes that the efficiency and performance of *Bt.* depend largely on the control and monitoring of every step of production and delivery of any *Bt.* are in fact crucial. A more innovative approach in pest control is explored by a group of researchers that investigated the transcriptome dataset from four different developmental stages of *Metisa plana* (Rahmat *et al.*, 2020). The study found seven regulatory genes in chitin biosynthesis; an important biosynthesis pathway across each development stage of the pest. RNAi-mediated pest management by targeting certain pathways to cease the chitin biosynthesis in the leaf defoliator is proposed. More than a decade ago, red palm weevil (RPW) emerged as a potential threat to the local coconut industry. Initial records only reported the infestation in 58 localities which then quickly spread to 858 localities in Terengganu within a short span of four years (DOA, 2011). The pest was then reported to be able to transit in oil palm with no damage or reports of outbreak. Whilst being mindful on the potential threat on oil palm, immediate surveys and research investigations were initiated. Based on the two studies reported in 2020, the pest preferred bait from pineapple tissue to oil palm or coconut tissues while the artificial infectivity of the pest in oil palm seedlings indicated compromised physiological readings (Haris-Hussain *et al.*, 2020; Harith Fadzilah *et al.*, 2020). More studies should be conducted to investigate and suggest measures to prevent, mitigate and to understand the potential threat imposed by the pest as part of the biosecurity plan of oil palm.

It is very interesting to note that the year 2020 had witnessed the overwhelming increase in studies conducted on one of the deadliest oil palm diseases in SEA countries namely basal stem rot (BSR) caused by a white rot fungi *Ganoderma boninense*. More than 60 articles covering various research areas of the pathogen were published and can be categorised as follows: (1) early detection methodologies; (2) chemical-based controls; (3) BCA articles; (4) plant-pathogen interactions at cellular and molecule; (5) genetic diversity of the pathogen; (6) economic impact of disease, and finally (7) soil characteristics on disease development. The oil palm industry is already witnessing the impact of the disease prevalence increase over the years with all evidence

leading to the significant reduction in productivity. Evidently, the highest number of publications recorded in 2020 was also coincidentally on the control and early detection of the disease. To begin with, Olaniyi and Szulczyk (2020) published an interesting forecast of the economic damage caused by BSR disease. The article discussed on the potential economic damage based on four infection rates and treatment costing models. Despite the fact that BSR is a slow-spreading disease, the model stimulation predicts significant economic losses by 2040. Nevertheless, the predictive models did not include the effect of prevention and surveillance as part of the disease mitigation measures which in turn reinforces the need to increase research activities in preventing and controlling the disease. Additionally, climate change was also noted to affect BSR incidences in oil palm cultivation by 2050 (Paterson, 2019).

Timely prevention with effective mitigation measures are important components of both integrated disease management (IDM) and integrated pest management (IPM) which also requires a reliable early detection of the P&D. Among the early detection tools, multispectral and hyperspectral remote sensing imaging have been explored quite extensively in the past decade with most reports reporting its efficiency on field palms (Izzuddin, *et al.*, 2020). A similar approach was then designed to detect early infection of the disease but at the seedlings stage via the spectral reflectance with an accuracy of 100% for disease classification (Noor Azmi *et al.*, 2020). This gave rise to the potential use of the multispectral technology to be utilised in quick screening of disease onset, especially in the selection of progeny tolerance, assessment of biological and chemical control agents with an added advantage of reduced experimental time. Nevertheless, this technique requires further exploration and validation before it can be recommended. Apart from the aerial detection tools, application of ground-based Light Detection and Ranging (LIDAR) also known as Terrestrial Laser Scanning (TLS) have been gaining popularity based on its high precision in detection and innovative plant phenotyping (Husin *et al.*, 2020a, 2020b, 2020c). The studies examined the use of the technology in the application, classification and characteristics of BSR disease in mature palms. The classification study for instance, gave a 100% accuracy in predicting actual disease severity using TLS. This is an emerging technology potential to be exploited further for disease detection, pest outbreak and other physiological assessments such as nutrient deficiency in palms. Aside from the contactless aerial and ground census tools, the use of deoxyribonucleic acid (DNA)-nanoparticle is another emerging technology with a huge potential for early detection devices (Rani *et al.*, 2020).

Ultimately, effective control measures are the backbone of any IDM programmes and can be categorised into three universal approaches: (1) biological, (2) chemical, and (3) cultural practices. In recent years, we have witnessed developments in greener technologies with most research attempting to control or prevent diseases in oil palm. It is an undeniable fact that greener technologies compliment Sustainable Development Goals (SDG) due to their added environmental benefits. However, the preliminary investigation of these technologies that focuses too much on the early stage, neglected the more crucial stage; delivery and persistence of the BCA in the environment for a continued control (O'Brien, 2017). Once again in 2020, we see a surplus of publications identifying potential microbes, their modes of actions and disease suppression either in the laboratory or in the nursery stages (Ahmad *et al.*, 2020a; Anggita *et al.*, 2020; Goh *et al.*, 2020a; Naeimi *et al.*, 2020; Parvin *et al.*, 2020; Pramudito *et al.*, 2020). There were no article focusing into the efficacy of an established product in the field. There has been an interesting development on the use of fungicide for BSR control through nanoformulation. A series of studies was conducted on encapsulated hexaconazole fungicide, using chitosan nanoparticles for their delivery as a sustainable alternative to the conventional fungicide application (Maluin *et al.*, 2020a; 2020b). The formulation emerges to be a successful application in controlling the BSR with 74.5% suppression compared to that of untreated seedlings.

Aside from addressing the potential control measures and early detection tools, deciphering the role of soil in predisposing the palms to BSR disease was also addressed. A study by Anothai and Chairin (2020) has put forward some interesting postulations on the role of soil physico-chemical properties; pH, fungal enzymes and plant defence enzymes in predisposing the palms to the disease. It was established that there is a correlation between soil organic matter (OM), organic carbon (OC) and pH influencing the fluctuation of enzymes in the soil. These enzymes include the cell wall degrading enzymes (CWDE) such as chitinase, β -1,3-glucanase, peroxidase (POX) and degradation enzymes (*e.g.* laccase, lignin peroxidase and manganese peroxidase) with the former enzymes dominant in the natural suppression of phytopathogens while the latter are influential in accelerating degradation. In a similar approach, the profiling of soil microbial community in two contrasting BSR disease affected areas were compared through a high-throughput sequencing (Goh *et al.*, 2020b). Interestingly, the soil community in the less affected BSR area had a higher abundance of rare metabolically diverse and versatile bacterial taxa as compared to the presence of predominantly disease-inducible bacterial taxa in the higher disease incidences area. The two studies identified the role

of physico-chemical properties such as pH and carbon content in driving the distinct differences in the soil microbial community. It is therefore recommended that more investigations should be conducted for the industry to move forward to a sustainable green management of the disease, with innovative technologies embedded along the way. Nevertheless, the increased number of publications in the varied research fields resonates with the industries' commitment to decipher, understand and formulate sustainable control for the disease in the future.

Mitigating the Changing Environment through Technology

The year 2020 started with some devastating news of fires raging the forests from Australia to West America, Amazon to Siberia (Cove, 2020; Pike, 2020) and reports of crop destruction by swarms of desert locust decimating crops in India and Pakistan (Agarwal and Jain, 2020; Khan, 2020). One common denominator that seems to link these incidences is likely, climate change. It is believed that these, once considered as natural disasters, but more so now as human-induced calamities (Union of Concerned Scientists, 2021), were triggered by general global warming. With rising temperatures, extreme weather has become a norm. We are now witnessing prolonged heat waves, cold spells as well as severe drought and flooding. Even the invasion by crop pests such as the desert locust was attributed to climate change (Khan, 2020).

Crop threatening extreme weathers have influenced some of the research direction undertaken in agriculture (Lesk *et al.*, 2016; Zandalinas *et al.*, 2020). Similarly, for the oil palm, there has been a concerted effort to address the impact of abiotic stresses on yields of this oil-bearing crop. To further dissect the biology involved in environment adaptation, accessibility to genome information is crucial. The publicly available oil palm reference genome (Singh *et al.*, 2013b) expedited the application of molecular breeding approaches for crop improvement, which has to date been further improved. The updated physical genome coverage of 79% from the earlier 43%, has successfully placed 1968 unassigned scaffolds onto the genome which extended the 16 pseudochromosomes thus, enhancing its overall quality (Ong *et al.*, 2020). Leveraging the available oil palm genome databases as well as next generation sequencing technologies, Zhou *et al.* (2020) and Wang *et al.* (2020) identified candidate genes, potential pathways as well as transcription factors likely to be involved in drought tolerance in the oil palm. Aside from drought, EgMYB transcription factors (Zhou *et al.*, 2020) were also found to be differentially expressed when exposed to other stresses such as cold and salinity.

Oil palm generally adapts well to high water table, but prolonged waterlogging has negative effect on its root system due to oxygen deprivation which could lead to lower yields. Nuanlaong *et al.* (2020) carried out differential gene expression studies of oil palm roots under waterlogging conditions using a less commonly used sequencing platform, the ion torrent as opposed to Illumina. This study established the Deli x Ghana variety to be tolerant to waterlogging with genes such as (Glutathione S-Transferase (*GST*), Serine/threonine-Protein Kinase (*SAPK10*) and NAC transcription factor 29 (*NAC29*) acting as potential markers.

Developing planting materials that are adaptive to the environment aligns well with the industry's aspirations. However, improved yields as well as quality of the palm oil produced remain the key criteria that directly tackles both consumer-driven sustainability and food security demands. That being said, a series of publications had addressed yield traits (Babu *et al.*, 2020; Teh *et al.*, 2020; Bhagya *et al.*, 2020) and oil quality based on fatty acids composition (Ting *et al.*, 2020a; Constant *et al.*, 2020) and successfully identified quantitative trait loci as well as candidate genes, using approaches such as Genome Wide Association Study (GWAS) and Genotyping-by-Sequencing (GBS). Herrero *et al.* (2020) explored the Single Primer Enrichment Technology (SPET) as an alternative to GBS and microarrays in constructing a high-density linkage map which apparently is a more cost-effective alternative.

As the oil palm industry continues to embrace technology in its research pursuit, genome editing has begun to gain traction due to its precision and less invasive nature supported by available whole genome information (Yarra *et al.*, 2020). This revolutionary technology also known as the CRISPR/Cas9 system is pioneered by Jennifer Doudna and Emmanuelle Charpentier, which won the 2020 Nobel Prize in Chemistry, touted as a game-changer for crop agriculture (Carpenter, 2019) and more so if coupled with speed breeding (Haroon *et al.*, 2019). Recently, with the introduction of prime editing, this has opened up more avenues for editing the plant genome (Lin *et al.*, 2020).

As the saying goes, 'Necessity is the Mother of Invention', and this holds true for Daza *et al.* (2020). Despite the fact that cultivating OxG interspecific hybrids requires assisted pollination due to its poor bunch formation, it is still a promising genotype to grow for its productivity, oil quality and partial resistance to some diseases. OxG hybrids tend to produce both types of fruits, with and without seeds. Hence, it was an attractive idea to stimulate the production of seedless or parthenocarpic fruits without assisted pollination, via the application of plant hormones. Daza *et al.* (2020) showed that with auxins, in particularly NAA and 2,4-D, it is

feasible to produce good bunch of parthenocarpic fruits without having to rely on pollination. With its striking characteristics and the need to diversify the current *guineensis*-based planting materials, more efforts are being channelled to further unravel the *oleifera* and its hybrids for future applications (Ting *et al.*, 2020b; Zulkifli *et al.*, 2020).

Research in tissue culture-derived oil palm is picking up pace with renewed confidence in utilising clonal oil palms as a choice for commercial planting (Nur Nadia *et al.*, 2020; Ang *et al.*, 2020). Through studies carried out by Aroonluk *et al.* (2020) and Sarpan *et al.* (2020), a better understanding of the mechanisms, both from the aspects of genetics and epigenetics could shed light on further improving the process. Nyouma *et al.* (2020) suggested that the genomic selection (GS) approach could be extended to improve selection of ortets prior to cloning as its predictive power is by far more accurate than phenotypic selection for most traits of interest.

Whilst it is argued that genomics, in particularly the Human Genome Project has yet to deliver any major clinical breakthroughs (November, 2018), and with agricultural genomics being well behind that of human genomics, it was envisaged that the latter would probably realise the benefits earlier, generally because it is easier and less controversial to put the technology into practice. One of the earliest demonstrations of the commercial use of genomics in oil palm is in addressing the non-*tenera* contamination in the industry (Ooi *et al.*, 2016). The discovery of *SHELL* gene (Singh *et al.*, 2013a) which subsequently led to the unearthing of more novel mutant alleles (Ooi *et al.*, 2016; Singh *et al.*, 2020) was the basis of the diagnostic assay. Despite the potential of the technology in improving overall yields, its broad adoption within the industry has been lukewarm. Although we are empowered to drive discovery as well as pursue new applications, we foresee the adoption of any future technologies requires a clear strategic advocacy plan without which the impact of the research will not make a difference to the industry (ASHG, 2020).

Sustainable Development for Smallholders

The implementation of Good Agriculture Practice (GAP) is considered as the baseline for the MSPO certification. Soil and water conservation (SWC) practices are of importance for the production of oil palm whilst preserving the quality of natural resources and environment protection. To date, there is no scientific study conducted to reflect the degree of awareness and adoption of SWC amongst independent smallholders (ISH). Realising this limitation, the study done by Johari *et al.* (2020) had revealed that adoption of SWC by the members of Saratok Sustainable Oil Palm Growers Cooperation by quantitative and qualitative analyses and through

in-situ assessment and face-to-face interviews were albeit high degree of awareness amongst ISH, and thus, recommends government assistance in promoting MSPO through the smallholders' cooperative.

Weed management at the oil palm plantation is also another aspect of GAP requirement. These unwanted plants intensely compete for nutrients, water, light and space with the oil palm. Ismail *et al.* (2020b) revealed that no oil palm seedlings are contaminated by *Bipolaris sorokiniana*. Other host plants such as strawberry, okra, sweet corn, chives, bananas, eggplants, chillies, sweet potatoes, spinach and Napier lawn exhibited a moderate *B. sorokiniana* manifestation between 10% and 20%. It was also suggested that an appropriate biological control agent is required to control *Eleusine indica* and *B. sorokiniana* at the oil palm plantation. According to Ibrahim *et al.* (2020a), a total of 3450.70 ha owned by the ISH is contaminated with BSR covering planting area in three states in Peninsula Malaysia namely Johor (1032.97 ha), Sabah (930.85 ha) and Perak (718.49 ha).

The requirement of optimum fertiliser application is crucial in capitalising the palm oil productivity. Research work by Tan *et al.* (2020) revealed that majority of ISH have a general understanding of good practice to fertilising oil palm. However, the rate of fertiliser applied by the ISH is relatively lower than the recommended dosage. Most of the ISH not only have inadequate information on oil palm nutrient deficiency but also failed to perform soil analysis prior to planting. There is a need to reinforce the extension service in providing awareness to the ISH on proper fertiliser application. More studies are needed to understand the impact of ISH decision on the application of fertiliser. A survey was done by Zaki *et al.* (2020a) which aimed to understand the behaviour of purchasing fertiliser amongst ISH in Sabah in term of price, distributor and fertiliser purchase intention. Almost two-third or 61.2% of ISH has applied fertiliser based on their own experience and expertise while the remaining ISH obtained technical advice from MPOB. It was also noted that about 40.0% of ISH have hired workers to fertilise their oil palm. Except for price, brand and dealer portrayed good relationship with fertiliser purchase. Factors such as types and dosage of fertiliser, number of harvesting rounds, weed control and farm management were all found to be affecting the oil palm yield.

In 2016, the Malaysian Government has allocated about RM37.60 million and RM1.03 billion through Oil Palm Seedling Assistance Scheme (SBABB) and Tenth Malaysian Plan, respectively, with the aim to facilitate ISH for replanting in order to improve productivity through proper adherence to GAP. Sheilyza and Abd Manaf (2020) correlated the influence of knowledge, attitude and skill on

GAP of the SBABB recipients towards the oil palm production, which clearly showed that most of the ISH aged between 48 and 59 years old have a moderate knowledge and skill operating at their farms. Majority of the respondents was reported to only harvest FFB between 10 and less than 20 t ha⁻¹ yr⁻¹, which are notably lower than the national average production (20 t ha⁻¹ yr⁻¹). Factors such as farm size, knowledge on farm management, farm operating skills, perception towards SBABB and perception towards MPOB as the implementer agency were positively correlated with the productivity. This observation is in agreement with the study done by Sheilyza *et al.* (2020) where another scheme known as the Oil Palm Replanting Scheme (TSSPK) and New Planting Scheme (TBSPK) were introduced to gauge its effectiveness towards oil palm replanting and hence improve the productivity and income of the ISH. The FFB harvested was found to be lower than 4.6 t ha⁻¹ yr⁻¹ for the first two years of replanting, but the ISH still enjoyed an uptrend price reflected by their income. The researchers also proposed that future schemes shall incorporate agricultural input distribution and clustering of the ISH into cooperatives for the betterment of input distribution and deliverability of extension services.

Clustering the ISH was found to be the best way to overcome issues on extension services. The Sustainable Oil Palm Growers Cooperative (KPSM) is responsible to boost the income of ISH whilst creating job opportunities in the Malaysian oil palm sectors. Based on the study by Zaki *et al.* (2020b), almost 70% of KPSM's Board Members possess experience in managing the business and had accomplished their cooperative-related training. Albeit most of them understood their positions in terms of communication with other Board Members, as well as internal control system policy and cooperative regulations, the researchers observed their lack of experience in financial literacy, and hence, suggested the Board Members to be equipped with appropriate knowledge to contribute new ideas in churning profit to the cooperative.

Biomass and Bioenergy Innovations

The research in palm oil midstream sector concentrates on utilisation and management of biomass in reducing environmental impacts. Two main by-products generated from the palm oil milling process are the solid oil palm biomass and liquid wastewater or palm oil mill effluent (POME). These abundantly generated lignocellulosic materials have been the main feedstock for the value-added conversion into bioenergy, biochemicals and bioproducts (Figure 1). Due to the inherited recalcitrance characteristics, pre-treatment is required before the biomass can be efficiently

exploited. Promising pre-treatment method of recent interest is supercritical carbon dioxide (SC-CO₂) has resulted in high diffusivity, which subsequently enhances the structural disruption, promotes sugar's solubilisation and eventually led to better enzymatic digestibility of the pre-treated biomass (Sohni *et al.*, 2020). Another method involving hydrothermal pre-treatment between 100°C and 200°C showed the ability to depolymerise oil palm biomass in producing lignin-based aromatic chemicals as precursors for protocatechuic acid (polyphenols antioxidant) synthesis (Ramachandran *et al.*, 2020). The pre-treated biomass was proven to be more effective. The hydroperoxide (H₂O₂)-assisted hydrothermal pre-treated empty fruit bunches (EFB) showed better degradation performance to enhance batch anaerobic digestion at mesophilic condition with 43% increase in methane yield and 30% lesser greenhouse gas (GHG) emissions when compared to the control (Lee *et al.*, 2020).

Furthermore, torrefaction (Sukiran *et al.*, 2020a) and microwave-assisted wet torrefaction pre-treatment (Sangjan *et al.*, 2020) of oil palm biomass demonstrate a promising route for improving energy content of solid biofuel and bio-oil yield via hydrothermal liquefaction, respectively. As moisture retention in the oil palm biomass influences the mass and energy yields of the torrefied final solid product, the ultimate recovered energy content could vary tremendously. This observation has been validated through a development of a least squares power regression

model based on quadratic equations (Sukiran *et al.*, 2020b) which showed strong correlation with the experimental values, and thus, can be practically applied to estimate the energy potential of torrefied products for possible fuel mix or displacement in biomass boilers. A kinetic modelling study of CO₂ gasification behaviour and reactivity profiles between 800°C and 900°C demonstrated an apparent activation energy of the torrefied oil palm biomass as affected by the physico-chemical compositions, in particular the inorganic mineral, carbon, moisture and volatile matter contents (Chew *et al.*, 2020).

Many bio-products have been innovated using pre-treated oil palm biomass. The cellulase-pre-treated EFB fibre is found as a good substrate for bioflocculant production using *Aspergillus niger* DWB (Mohd Luthfi *et al.*, 2020) via solid state fermentation. Previously, bioflocculant was produced from POME (Nurul-Adela *et al.*, 2016). A low oxalic acid (1% w/v) pre-treated oil palm trunk (OPT) biomass is favourable for succinic acid production albeit requiring longer reaction time. Oxalic acid exceeding 1% (w/v) seems to prohibit the fermentation of *A. succinogenes* 130Z, thus, should be avoided (Bukhari *et al.*, 2020). Indeed, palm oil has also been exploited as a renewable feedstock for bioprocessing. One example is production of bioplastic, *i.e.* medium chain length polyhydroxyalkanoates (PHA) which can further converted into biopolymers (Tanikkul *et al.*, 2020).

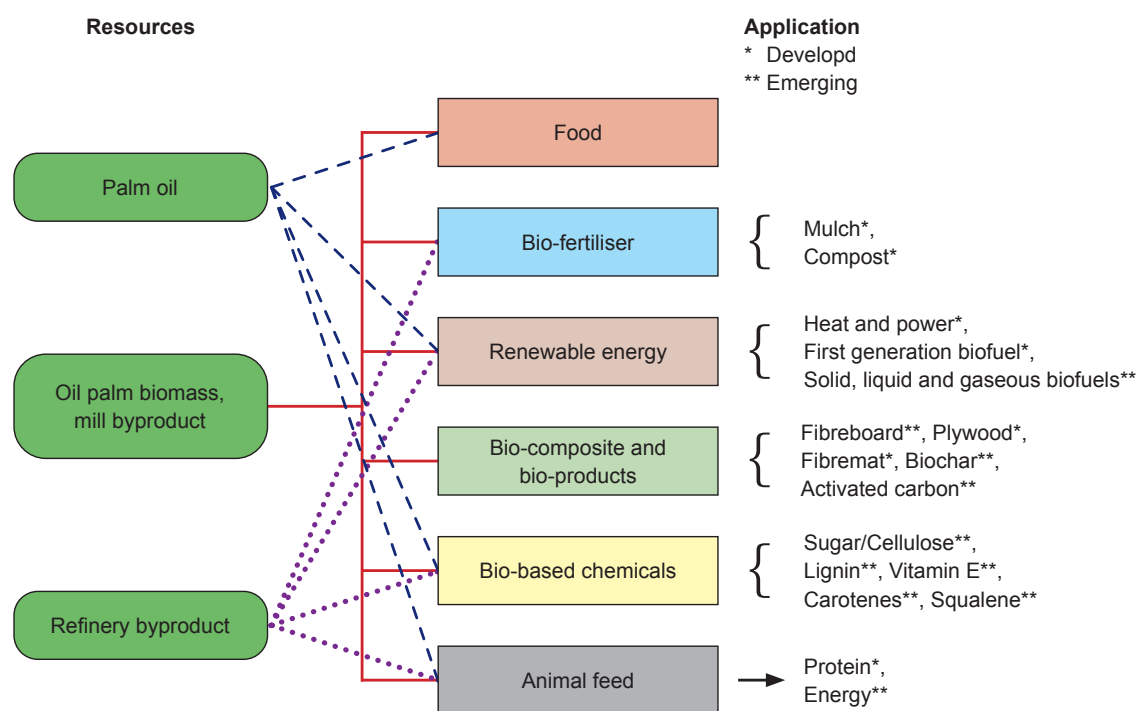


Figure 1. Resource optimisation and diversification strategy for food, feed, fuel chemicals and other bio-products.

Biochar research currently focuses on assessing carbon sequestration potential (CSP) as a soil amendment. The types of biomass feedstock and mode of production are important parameters in biomass-to-biochar valorisation. Table 12 shows the Brunauer-Emmett-Teller (BET) surface area and CSP of some biochars derived from oil palm biomass. Oil palm frond biochar with a BET surface area between 180 and 190 m² g⁻¹ has an average CSP of 0.27 t of CO₂ for every tonnage of biomass (Karananidi *et al.*, 2020). Palm kernel shell (PKS) biochar shows higher BET surface area and better CO₂ adsorption (Kong *et al.*, 2019; Dominguez *et al.*, 2020; Promraksa and Rakmak, 2020). Furthermore, the CSP of this biochar increases over surface properties. Higher temperature promotes volatilisation during biochar production leading to more porous materials and active sites that could improve the CO₂ sequester. However, further enhancement of this carbonaceous material such as chemical treatment is required for carbon sequestration. Pyrolysis using microwave under vacuum resulted in a highly porous PKS biochar with a very moist and suitable substrate that can promote mycelium colonisation for mushroom growth (Mahari *et al.*, 2020). Green carbon-based nanomaterials namely carbon quantum dots have been produced from EFB-derived activated carbon through hydrothermal carbonisation-activation processes. The materials were of good optical properties, water stability and solubility which offer versatile applications as bio-imaging and sensor materials (Mahat and Shamsudin, 2020).

Metal oxide impregnation of oil palm biomass-based magnetic biochar can be used as a heterogeneous catalyst to improve biodiesel production and eases catalyst separation (Quah *et al.*, 2020). In addition, a green tea-based catalyst called magnesium oxide (MgO)-clinoptilolite nanocomposite could transform used frying oil into biodiesel in compliance with international specifications with the advantages of relatively high

yield (approximately 9%) and high degree of catalyst recyclability (Basyouny *et al.*, 2020). In addition, the calcium oxide (CaO) catalyst derived from raw chicken eggshell is capable of reducing 95% of free fatty acids content in palm fatty acid distillate to be used as biodiesel feedstock (Idris *et al.*, 2020).

Cellulose nanofibre (CNF) extracted from oil palm biomass can be impregnated and sandwiched in the polyvinyl alcohol (PVA) film to produce PVA-CNF nanocomposite which encompasses of reinforced thermal stability, tensile strength and crystallinity (Okahisa *et al.*, 2020). The sodium hydroxide (NaOH)-treated oil palm fibres (OPF) have been added to cement paste containing 0.25% of superplasticiser to form less viscous cementitious matrix (Bonnet-Masimbert *et al.*, 2020). The reinforced cement is chemically and mechanically compatible, allowing cement hydration due to the enhanced fibre-and-cement interface properties. Nanosized lignin from oil palm biomass isolated through soda pulping process is a stable and non-toxic water-in-oil emulsifying agent (Seker *et al.*, 2020).

Of the many non-food applications, starch derived from the OPT has successfully been extracted and chemically modified to form a binder that reduces the usage of formaldehyde in wood composite production. However, the inclusion of small amount of conventional binder, *i.e.* urea-formaldehyde into the formulations is still required as it could further improve the moisture and fungal resistance of the products (Amini *et al.*, 2021). Moreover, OPT pre-treated with sodium chloride solution could potentially be used as fire-retardant particleboards with high thermal stability (Yusof *et al.*, 2020) when bonded with poly(vinyl) alcohol as well as additives such as citric acid and calcium carbonate. The OPT biocoal produced via pyrolysis emits nitrogen oxide (NO_x) and sulphur dioxide (SO₂) emission of 296 and 190 ppm, respectively, when co-combusted with sub-bituminous coal as a solid biofuel between 600°C to 900°C. The emission

TABLE 12. CARBON SEQUESTRATION POTENTIAL (CSP) OF OIL PALM BIOMASS-BASED BIOCHAR

Biomass source	Brunauer-Emmett-Teller (BET) surface area, m ² g ⁻¹	CSP, t CO ₂ t ⁻¹ biomass	Production mode	Reference
Oil palm fronds	180 - 190	0.27	Kon-Tiki flame curtain pyrolysis. 200°C-800°C, 100 min	Karananidi <i>et al.</i> (2020)
Palm kernel shell	389	0.46 mmol g ⁻¹ (CO ₂ adsorption)	Fixed bed pyrolysis at 500°C, 60 min	Promraksa and Rakmak (2020)
Palm kernel shell	135	0.398	Gasification at >700°C. Biochar was impregnated with NH ₄ NO ₃ and KH ₂ PO ₄ as a slow-release fertiliser	Dominguez <i>et al.</i> (2020)
Palm kernel shell	200 - 329	0.49 - 0.63	Multi-mode allothermal pyrolysis, 400°C-600°C, 30-90 min	Kong <i>et al.</i> (2019)

Note: NH₄NO₃ - ammonium nitrate; KH₂PO₄ - potassium dihydrogen phosphate.

of these gases is well below the legislative limits enforced by the Environmental Quality (Clean Air) Regulation 2014 (Nudri *et al.*, 2020).

One of the greatest challenges in POME treatment is the consistent performance of polishing plant in meeting the stringent biological oxygen demand (BOD) discharge standard set by the Department of Environment Malaysia (DOE). It depends largely on the hydraulic retention time (anaerobic stage), aeration system (aerobic stage) and availability of microbial cells in POME. Chin *et al.* (2020) concludes that the modified Stover-Kincannon model is most suitable to gauge the performance of anaerobic POME treatment through a covered lagoon with hydraulic mixing. The findings facilitate the selection of industrial-scale method and equipment sizing for wastewater treatment. The following are some of the adsorbents developed for POME treatment: (1) PKS activated carbon produced via a two-in-one carbonisation activation system and packed in a continuous system (Nahrul Hayawin *et al.*, 2020); and (2) steam-pyrolysed and pulverised oil palm frond biochar (Lawal *et al.*, 2020). The latter exhibits an increased new external surface area and less pore blockage, capable of adsorbing POME contaminants.

The capturing of biogas releasing from POME during anaerobic digestion should be pursued prior to discharging the treated final discharge into a watercourse (Loh *et al.*, 2017). As of December 2019, a cumulative of 125 biogas plants were installed corresponding to 28% of biogas implementation in the country (Loh *et al.*, 2020). The serial biogas capturing has shown the capability to mitigate an estimated GHG emission of about 5.52 million tonnes CO₂ eq. annually in comparison to without biogas capture (18 million tonnes CO₂ eq). In 2020, another five plants were established giving a total of 130 plants equipped with biogas capturing facilities across 457 palm oil mills in the country. Biogas has a huge untapped potential not only as a readily form of bioenergy but also could be upgraded into bio-compressed natural gas (bio-CNG). This approach could be made more economically viable if a biogas plant at the palm oil mill is integrated with a purifying plant to produce biomethane (>92% biomethane) or bio-CNG for industrial uses (Nasrin *et al.*, 2020). On that note, POME is proven as an effective solvent to pre-treat EFB fibre for fermentable sugars production (Tang *et al.*, 2020).

Environmental Sustainability of the Oil Palm Life Cycle

Findings from a life cycle impact assessment of refined palm oils with co-products allocated based on economic value (Yung *et al.*, 2020) call for the improvement on bleaching earth usage and transportation of CPO during refining and fractionation processes. Separately, in production of

palm oil, the main contributors to water footprint along the oil palm supply chain through the concept of cradle-to-gate are: (1) cultivation of oil palm FFB in plantations, (2) boiler and process water in mills, and (3) processing of palm kernel from kernel crushers. The water footprint assessment based on the water accounting and vulnerability evaluation (WAVE) method has been compared via mass, economic and energy allocation (Berger *et al.*, 2014; 2018). Mills with the highest water footprint impact are urged to avoid dilution after the screw press of CPO (Subramaniam *et al.*, 2020).

In evaluating the suitability of oil palm as bioenergy crop, Heidari *et al.* (2020) have looked into the hydrological impacts and water-biomass trade-offs associated with oil palm cultivation in Tabasco, Mexico using a calibrated Soil Water Assessment Tool (SWAT) model (Arnold *et al.*, 2012). The hydrologic-agronomic relation for palm biodiesel feedstock production has been modelled through different land use management scenarios. In a separate study, oil palm residues have been investigated via a weighted decision matrix to measure its bioenergy potential and technical feasibility by taking into account the sustainability criteria (Ordoñez-Frías *et al.*, 2020). Nevertheless, a more structured holistic research should be initiated through an integrated analysis of all the potential impacts to ecosystem and socioeconomic aspects in identifying the crop most suitably used for bioenergy production. In addition, an investigation on spatio-temporal analysis of oil palm biomass value chain or biomass-to-bioenergy in Indonesia has been conducted using an optimisation model called 'BeWhere Indonesia' which shows huge bioenergy potential for palm oil-based biorefinery configuration in two islands of Kalimantan and Sumatra until 2030 (Harahap *et al.*, 2020). The findings suggest that policy revision is required to attract investment in harnessing the untapped full potential of palm oil coupled with oil palm biomass. In particular, bioenergy exploiting multiple biomass feedstock shows great applicability at rural areas to overcome electricity shortage (Mahidin *et al.*, 2020).

In a detailed consequential and attributional life cycle assessment study, Schmidt and De Rosa (2020) show that certified palm oil through Roundtable on Sustainable Palm Oil (RSPO) scheme is more reputable with lesser GHG emissions than the non-certified. Certified palm oil features higher oil yield, lesser land use and peat cultivation, and higher biogas capturing rate at palm oil mill.

Challenges in Food Safety and Quality

Food safety and quality are often ascribed as the assurance of food produced for human consumption. The acceptance of food particularly for trading purposes much relies on the compliance

with the latest food safety legislations and quality standards. Pertinent issues related to 3-MCPDE and GE in processed edible oils continues to dwell amongst edible oil processors and food regulators for more than a decade as evidenced from an increase in research works related to toxicity impacts, mechanistic pathways, analytical methods and mitigation strategies (Gao *et al.*, 2020). The time has come for the EC to mandate the regulation of 3-MCPDE and GE thresholds in edible oils and fats with an effective date of 1 January 2021 (Commission Regulation (EU) 2020/1322, 2020). The imposition is certainly a challenge to the global palm oil producers, not only to comply with the EU importation prerequisite, but also countries that are adopting the similar requirement. As palm oil witnesses relatively higher 3-MCPDE and GE contents, remedial mitigation action is highly needed to ensure its acceptance globally.

There are many publications which detailed the mitigation strategies in minimising the formation of 3-MCPDE and GE in refined palm oil (Arris *et al.*, 2020). A recent study by Sim *et al.* (2020) demonstrated a remarkable reduction of more than 80% of 3-MCPDE and 65% of GE after optimising the refining conditions using micro- and macro-scale deodorisers with the capacity between 0.1 kg and 3.0 kg. It is worth noting that the majority of research works performed at laboratory scale often encounter obstacles when converting the optimum process conditions to a commercial plant setup.

On that note, Ramli *et al.* (2020a) have initiated series of commercial trials at the mill and refinery to attest the efficiency of washing CPO at the former premise and subsequently lowering the formation of 3-MCPDE and GE in processed palm oil at the latter premise. Washing 900 t of CPO lessened the total chloride content by 85% which yielded the final total chloride content of 0.9 ppm. Reduced chloride content in washed CPO further lowered 3-MCPDE content in the refined oil (1.4 ppm) when subjected to processing at a 500 t capacity physical refining plant. A reduction of 86% in GE content was prevailed when the refined oil underwent post-refining trials at lower deodourisation temperature in the absence of degumming stage and reduced dosage of acid activated clay.

Gao *et al.* (2020) highlighted the importance of integrating three mitigation approaches involving reactant control, inhibition of promoter, catalyst or intermediate, and minimising contamination occurrence during refining that should be undertaken to resolve the presence of 3-MCPDE and GE in both cooking oils and oil-based food products. Bognár *et al.* (2020) showed that different chlorinated salts influence the fate of 3-MCPDE in edible oil when subjected to frying. Frying of potato chips depicted by the increase in oil temperature and frying interval have significantly enhanced the

development of 3-MCPDE but displayed a declining trend in GE content over frying time (Wong *et al.*, 2020). The inclusion of external antioxidants such as butylated hydroxyanisole (BHA) and tocopherol in baked cakes have shown the ability to retard 3-MCPDE and GE formation while rosemary extract was demonstrated to be the most effective in minimising the presence of these compounds (Goh *et al.*, 2020).

The use of pesticides at the oil palm plantations could lead to the presence of pesticide residues in the FFB after harvesting. Cypermethrin, indaziflam, metsulphuron-methyl, glyphosate and glyphosate are amongst the 29 pesticides registered under Regulation 41 of Schedule Sixteenth of the Malaysian Department of Agriculture (DOA) that are allowed for use at oil palm plantations. Maznah *et al.* (2020) have established a method to detect the residue of metsulphuron-methyl, and observed that accumulation of metsulphuron-methyl gives a minimal impact to the soil, thanks to its rapid degradation with the half-life of up to eight days. The potential use of gas-chromatography mass-spectrometry (GC-MS) to detect the presence of trace cypermethrin has been established by Sulaiman *et al.* (2020) with satisfactory performance and acceptable recoveries and precisions. The application of indaziflam at the oil palm plantations can potentially lead to the migration of its residues through its root system and possibly accumulates in the raw agricultural commodities (RAC). This has led Halim *et al.* (2020) to develop a detection method of indaziflam residue and its three metabolites namely diaminotriazane, triazine indanone and carboxylic acid based on the application of a liquid-chromatography tandem mass-spectrometry (LC-MS/MS). The application of similar instrument was also beneficial to simultaneously detect glyphosate and gluphosinate residues including their metabolites (Shinde *et al.*, 2020).

Palm oil is often regarded as one of the ideal vegetable oils for culinary and frying sectors because of its nature to resist excessive heat under intensive operation. Recently, Hu *et al.* (2020) conducted a comparative intermittent frying trials using palm stearin and oil blend containing a mixture of high oleic sunflower oil and canola oil, and depicted that the combined level of primary and secondary oxidation in palm stearin was two folds lower than that of blended oil. According to Su *et al.* (2020), the oil types and frying cycles could also influence the fate of acrylamide in fried products. The consumption of naturally stable oil for frying is not only found to be cost-effective but also overcomes public concern towards oils produced from genetically modified crops (Qi *et al.*, 2020). Thermal resistance of red palm olein for different cooking techniques showed an apparent vitamin E and carotenes retention when

heating under conventional oven and frying conditions (Loganathan *et al.*, 2020a). A separate study by Loganathan *et al.* (2020b) demonstrated an excellent thermal stability of vitamin E and carotenes exceeding 87% in baked cupcakes made of red palm olein whilst preserving similar sensory characteristics as those containing conventionally refined palm olein.

Superiority of vegetable oils is somehow appreciable when they are properly handled during storage and food processing. An investigation on the shelf-life stability of red palm olein by Loganathan *et al.* (2020c) displayed minimal oil degradation associated to hydrolysis and oxidation while retaining vitamin E and carotenes contents even after four months of storage at relatively lower temperature in the absence of light and sealed bottles. A survey conducted at the selected fast-food restaurants in the East Coast of Peninsula Malaysia by Ismail *et al.* (2020c) monitored the quality of fresh, in-use and discarded oils for discrete frying of different food products. From all four fast-food brands evaluated, most of used frying oils collected from two premises had exceeded the threshold limit of total polar compounds (27%) while almost one-fifth of the used oils exhibited extremely high level of total polar compounds ranging from 35% and 48%. This experience clearly indicates that the insufficient oil management and handling could shorten frying oil usability.

Valorisation in Food and Feed Innovation

Recognition on the unique intrinsic characteristics and functionality of palm oil creates a vast opportunity to innovate myriad of food products. Valorisation of specialty fats derived from multiple fractionation processes could fetch premium price and marketability for niche food segments (Bruce, 2020). Characterisation of soft palm mid fraction (PMF) by Kanagaratnam *et al.* (2020) aims to distinguish it from soft palm stearin considering their overlapped iodine value and thus, fatty acids composition. Research efforts on oleogel development has been active in the recent years to enhance the thermal resistance, texture attributes, and structural binding capacity of lipids (Park and Maleky, 2020). Recent fundamental research work by Saw *et al.* (2020a) had found a marginal increase in olein yield of between 3% and 6% when different dosages of polyglycerol esters (PGE) of 0.1%-0.7% were added into palm oil before fractionation. This indicates the role of PGE to retard the growth of palm oil crystals following a possible mechanism as illustrated in Figure 2. In another study, Saw *et al.* (2020b) have screened several organogelators that can be potentially used to build oleogel network with palm superolein. Alteration of palm oil crystallisation can also be affected by the imposition

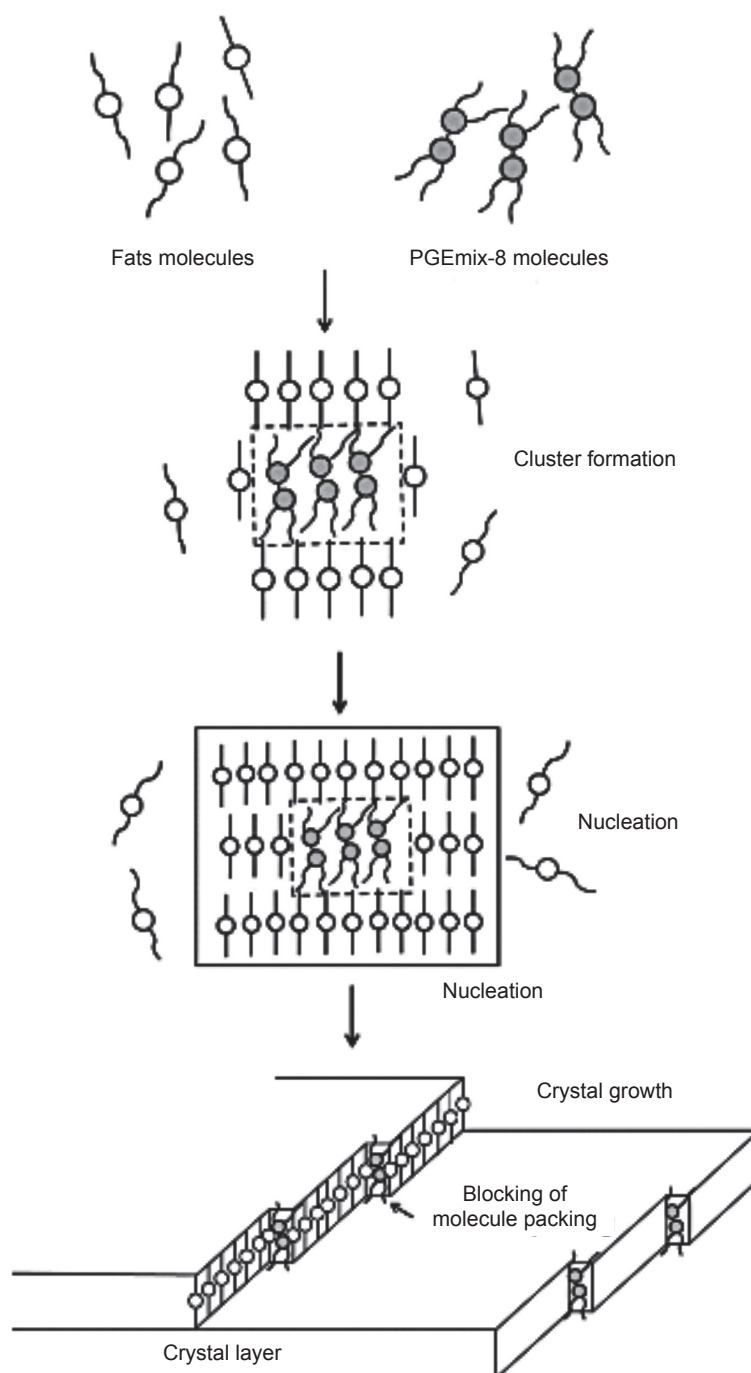
of shear and dispersion of particle phase (West and Rousseau, 2020).

The increase in demand for healthy food of meat-free products had motivated Chen *et al.* (2020) to revolutionise meat homologues from soya protein fortified with palm-fat. It was reported that a lower cholesterol content of 12.1 mg kg⁻¹ was observed in comparison to the conventional meat balls which ranged from 148.3 mg kg⁻¹ and 287.1 mg kg⁻¹, and furthermore, enhanced product texture and sensory characteristics. Similarly, Mohd Hassim *et al.* (2020) looked into the marketability of palm-based milk drink or lassi as an alternative to traditional milk-based fermented drink, and improved the product feasibility based on the quality characteristics and sensory acceptance. Exploration of the potential use of palm mid fraction and palm stearin blend in producing frozen Chinese pancake by Xie *et al.* (2020) yielded comparable texture but outperformed four bestselling lard-based pancakes, with respect to thermal stability and appearance. Notwithstanding that, palm oil which is the essence of food innovation, has somehow been bombarded by irresponsible allegations associated to health, deforestation and human rights, which in turn, advocates inversely to reduce reliance on palm oil (Parsons *et al.*, 2020; Ramli *et al.*, 2020b). In a study by Nicholson and Marangoni (2020), the ability of enzymatic glycerolysis for structuring liquid oils could offer alternative to saturated oils in formulating solid fat-based products. Of course, any attempts to construct liquid oil structure in favour to direct use of palm oil would incur unnecessary cost pertaining to additional processes related to enzymatic treatment. Likewise, substituting palm oil with high oleic oils would command higher premium attributed by the limited source and supply (Bruce, 2020).

Animal feed represents a significant portion of the total production cost in the livestock and aquaculture industry. The Malaysia's importation of raw ingredients for animal feed was reported to surpass 70% annually encompassing 2000 t of corn meal and 1000 t of soyabean meal (Nur Atiqah *et al.*, 2020). Utilisation of palm oil derivatives and oil palm by-products as shown in Figure 3 indicates an initiative to substitute some of the rations in feed formulations to ensure consistency in supply and cost-effective without the need to highly rely on imported ingredients. Exploration on the application of palm-based calcium soap in feed ration for broiler chickens portrayed similar growth performance and carcass characteristics with the diet without calcium soap (Villanueva-Lopez *et al.*, 2020). Comparable observation on growth performance and carcass traits was also witnessed by Nur Atiqah *et al.* (2020) when formulating goat feed pellets with oil palm by-products such as OPF, palm kernel cake (PKC), EFB and PFAD. Palomar *et al.*, 2020 performed

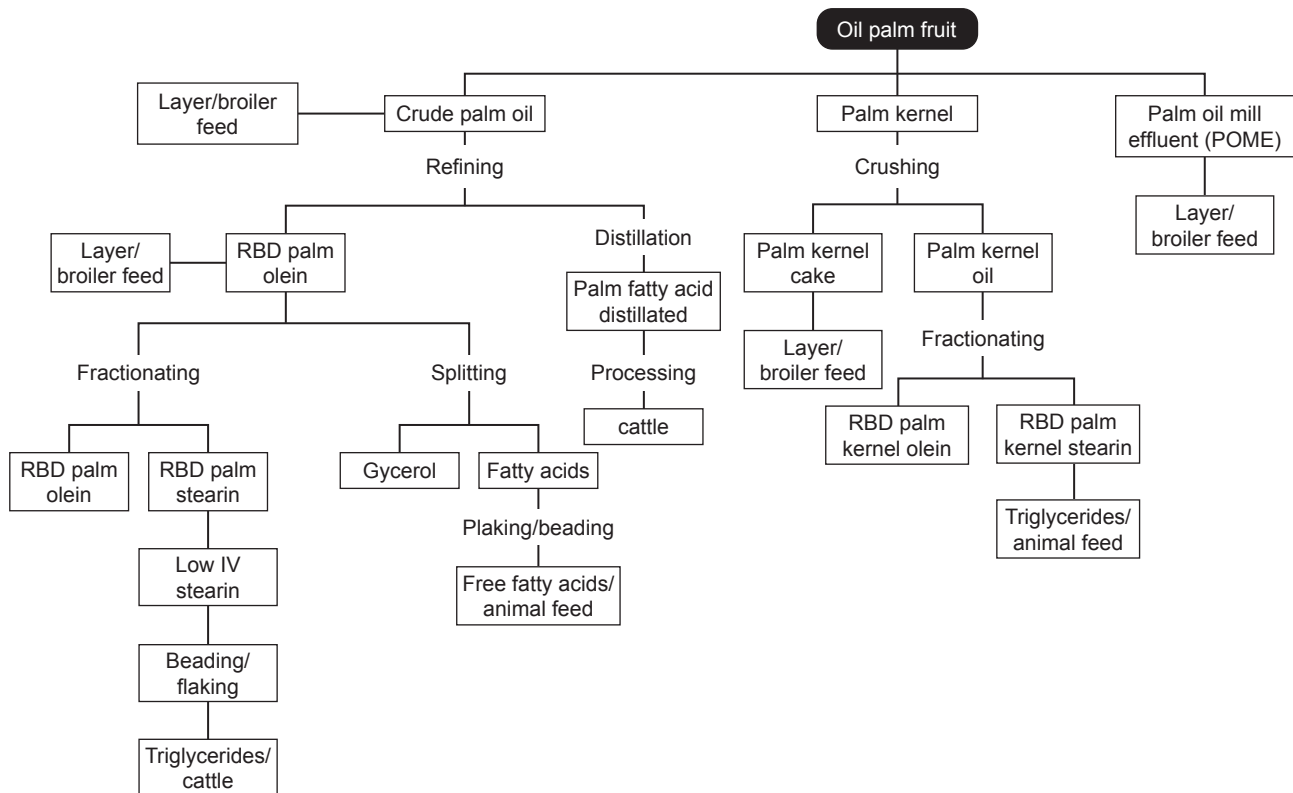
a comparative study on laying hens' dietary preferences toward feed diet formulations each with palm oil, PFAD and soyabean, and discovered that the hens have better preference towards diet containing palm oil and PFAD. Incorporation of encapsulated palm oil in the feed ingredient for piglets has been reported to enhance tract digestibility, lowers the incidence diarrhoea and increases high-density lipoprotein cholesterol (HDL-C) in the blood serum (Ren *et al.*, 2020). The prospect of CPO and its by-

products on the performance of laying hens and egg quality has been reviewed by Saminathan *et al.* (2020) and found that the inclusion of CPO in the feed ration stimulates the growth performance and metabolisable energy consumption. In the light of aquaculture segment, Wan Nooraida *et al.* (2020) appraised comparable sensory properties of tilapia (*Oreochromis niloticus*) fillets fed with diets containing emulsified PFAD and CPO, respectively, with those fed with commercial diet.



Source: Saw *et al.* (2020).

Figure 2. The proposed mechanism of interaction between polyglycerol esters (PGE) and oil molecules.



Note: RBD - refined, bleached and deodourised; IV - iodine value.

Source: Saminathan *et al.* (2020).

Figure 3. Potential use of palm oil and its co-products for poultry feed applications.

Nutrition and Health

The past two decades have marked a dramatic growth of research interest in the studies related to palm oil nutrition and health. A review on animal intervention studies by Syarifah-Noratiqah *et al.* (2020) deliberated on the role of palm oil to induce total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) profiles and further suggests the potential of palm oil as lipid-lowering agents. Another review with special reference to palm olein has reported a promising effect of blood lipid profiles in healthy adults and notably comparable with oils comprising of high monounsaturated fatty acids (Yilmaz and Ağagündüz, 2020). A research work on hypocholesterolaemic and anti-atherogenic effects of palm-based oils namely NoveLin I (oil with balanced ratio of saturated-, monounsaturated and polyunsaturated fatty acids) and NoveLin II (oil with moderate levels of monounsaturated fatty acids and lower in saturated- and poly-unsaturated fatty acids) in rabbit model by Che Idris *et al.* (2020) observed that both oils significantly lessen TC and

LDL-C when compared to rabbit fed with diet containing coconut oil.

Human intervention study on three confectionery products made of PMF, shea stearin (SS) and high oleic sunflower oil (HOSFO) showed that dietary fats rich in palmitic acid (PMF) and oleic acid (HOSFO) at sn-1,3 positions of triacylglycerol backbones exhibited similar postprandial lipid and glucose responses in comparison to SS with higher stearic acid (Voon *et al.*, 2020). It is a known fact that the intake of used oils through food poses the tendency of health incidences. Recently published findings revealed the effect of consuming overheated oils which lead to higher occurrence of non-alcoholic fatty liver disease based on the intervention studies using human hepatic carcinoma cell line (HepG2) (Chatuphonprasert *et al.*, 2020) and C57 mice (Li *et al.*, 2020). In a study comparing the effect of heated canola and palm olein on gut microflora in rats, Ruan *et al.* (2020) found that the latter oil significantly stimulates the proliferation of beneficial probiotics namely *Lactobacillus* and *Roseburia*.

Palm puree derived from oil palm mesocarp is abundant with phytonutrients such as vitamin E (in the form of tocopherols and tocotrienol homologues), carotenoids, phenolics, squalene, polyphenols and flavonoids, and thus, requires confirmative measure for human consumption. Based on this understanding, Zainal *et al.* (2020) performed a systematic acute and sub-chronic toxicity assessment and discovered that palm puree did not exhibit any signs of substantial toxicity or mortality. Driven by the growing evidence on the prospect of oil palm phenolic (OPP), many studies have been undertaken to gauge its bioactivity on health benefits. A review by Ibrahim *et al.* (2020b) explicated the current evidence of several mechanisms associated to cholesterol biosynthesis pathway, anti-inflammation and antioxidant capacity of OPP in providing cardioprotective effects to human. The OPP was also reported to improve bowel health in rats coincides with the increase in some essential bacteria that could aid the barrier turnover (Conlon *et al.*, 2020). A study by Leow *et al.* (2020) has successfully identified and characterised the reference genes from quantitative reverse transcription polymerase chain reaction (RT-qPCR) gene expression from Nile rats supplemented with OPP, which could further support future hepatic gene expression research particularly related to metabolic syndrome and type-2 diabetes melitus.

Palm oil is one of the natural sources of tocotrienols which generally accounts for at least two-third of the total vitamin E content. Tocotrienols are established as natural bioactive compounds with the ability to reduce cancer risk (Aggarwal *et al.*, 2019). The latest findings by Loganathan *et al.* (2020d) portrayed the functionality of palm tocotrienols to exert anti-proliferative and pro-apoptotic effects based on the manifestation of poly-(adenosine diphosphate)-ribose polymerase (PARP-1) cleavage and cyclooxygenase-2 (COX-2) inactivation of human breast cancer cells. A systematic review on the safety and neuroprotective efficacy of palm oil and tocotrienols-rich fractions (TRF) by Ismail *et al.* (2020a) inferred that the role of palm TRF or alpha-tocotrienol (α -T3) stimulates the cognitive performance of healthy animal and cognitive functions. A study by Fairus *et al.* (2020) discovered similarity in the overall antioxidant synergistic effect between TRF and alpha-tocopherol (α -T).

Oleochemical Innovations

Consumers' preferences for sustainable and bio-based products are the key factors fuelling the market with such products. Besides, favourable government policies regarding financial incentives and tax benefits for bio-based chemical producers are also positively impacting the industry. Bio-based products are exclusively or partly derived

from biological origin materials, and various methods are developed to detect bio-based content of these products. Bio-based content is defined as the weight ratio of the bio-based carbon to the total organic carbon in product. According to American Society for Testing and Materials (ASTM) D6866 Standard Test Method, the bio-based content should be calculated based on the percentage of modern carbon. The ASTM D6866 standard regulates three measuring methods using ^{14}C concentration such as a liquid scintillation counter (LSC), accelerator mass spectrometry (AMS) or isotope ratio mass spectrometry. Mohd Azmil *et al.* (2020) studied bio-based content of several vegetable oils along with polyols derived from the vegetable oils, mainly palm oil, using Sample Oxidiser and LSC. The optimised method was validated with respect to trueness (recovery and memory tests), linearity (quench curves) and precision. The recovery tests using ^{14}C standard verified the performance of sample oxidiser and LSC. All of the vegetable oils have 100% of ^{14}C , which signifies an entirely modern carbon (biomass) source. Polyols derived from castor oil, palm oil and fatty acid methyl esters (FAME) had lower bio-based content (71% to 96%) than their respective oils. This is due to the addition of reactants derived from the fossil carbon source during the ring-opening reaction of the epoxidised oils to obtain the polyols. Thus, the method based on LSC analysis of ^{14}C cocktails can be used as a tool to determine the bio-based content of palm-based polyols. It was proven to provide reliable results and is comparable to the more expensive AMS technique.

In another development, research on bio-based thermoplastic polyurethane (TPU) is continuously being carried out using dicarboxylic acids and diols from vegetable oils, fatty acids, FAME and PFAD produced via sustainable processes. Besides, the bio-content in TPU materials increases with incorporating bio-based polyols as an alternative to petrochemical polyols, which gives immense benefits. However, to find the suitability of bio-based polyester polyols for different applications, the effect of the structure-properties relationship between the diols and diacids to synthesise polyester polyols is essential (Zhou *et al.*, 2019). Tuan Noor Maznee *et al.* (2020) developed polyester polyols with 2000 g mol^{-1} molecular weight from 1,4-butanediol and linear dicarboxylic acid (DCA) having different carbon numbers (odd and even numbers). TPU prepared with DCA, which consists of even carbon numbers, possessed higher hardness, tensile strength, hysteresis, and tensile set than DCA with odd carbons due to stronger hydrogen bonds. The findings from this study were valid to select suitable polyester polyols in designing TPU for specific products.

Vegetable oil-based lubricants are environmental friendly and have been widely studied as an

alternative for mainstream petroleum-based lubricants. It is anticipated that the driving force of competitiveness among the commercially produced lubricants is the synthesis of biolubricant from cheaper feedstocks via a simple synthesis route. Herein, Hoong *et al.* (2020) successfully studied the synthesis of biodegradable lubricant using the following cheap feedstocks: oleic acid, acetic acid, and hydrogen peroxide via a simple one-pot process. The synthesised biolubricant, which consists of acetylated polyhydroxy estolide (AcPE) and polyhydroxy estolide (PE), was further modified for enhancing its functionality. The prepared AcPE and PE with ester and amide functionality, respectively, holds comparable physico-chemical properties to the commercial samples with respect to pour point, oxidation stability, viscosity index, and anti-wear. The outcomes of this study clearly showed that the inferior properties of plant oil-based lubricants such as poor oxidation stability and high pour point could be improved with chemically modified palm oil molecules. Besides their excellent lubricant properties as lubricant base oil for environmentally acceptable biolubricant, they are also inherently biodegradable.

In addition to the advancement in a new class of biolubricant, the development of useful tribological models to understand better the fundamentals of friction and wear, and fast characterisation of lubricants properties without overly relying on tribology test machines is necessary (Vakis *et al.*, 2018). Therefore, Chan *et al.* (2020) developed the first-ever friction-wear model for four-ball extreme pressure (EP) lubrication test (ASTM D2783) using 12 oil samples comprising of minerals, esters, and other formulated lubricants. It was rationalised using a single equation model based on non-equilibrium thermodynamics principles. The model estimated the EP performance of an unknown lubricant based on the model parameter, the dissipative coefficient that describes the proportionality between the friction and the wear phenomena based on the thermodynamic analysis. It is practical and easy to use since it involves only one parameter, the dissipative coefficient. In conclusion, this study provides useful tools and fundamentals for more advance studies related to the dissipative coefficient of various formulation or lubricant components to be investigated strenuously without over depending on the tribology test machine.

Utilisation of corrosion inhibitors is still one of the most practical techniques to combat corrosion. Moreover, replacement of toxic inorganic inhibitors with green, non-toxic and safe chemicals is always sought for. The effectiveness of corrosion inhibition largely depends on the presence of heteroatoms such as sulphur, nitrogen and oxygen and/or in the form of double or triple bonds between the aforesaid atoms (multiple bonds). Therefore,

Schiff-base with long-chain alkyl that can be produced from nitrogen-based compounds from derivatives of various vegetable oils, including palm oil, can be an interesting alternative. Imine functional group in Schiff-base molecules can act as active centres for adsorption on the metal surfaces. Noor Khairin *et al.* (2020), successfully synthesised a new Schiff-base compound known as N-cinnamalidene palmitohydridize (CHP) via one-pot reaction between palmityl hydrazide and trans-cinnamaldehyde. The inhibition efficiencies of CPH noticeably increased with increasing inhibitor concentration and temperature. Importantly, CPH acts as a suitable inhibitor on mild steel.

The technology to produce biosurfactants and bio-based surfactants using palm-based oleochemicals is continuously developed to meet the demanding requirements of major end-use industries. Sugar esters are classified as non-ionic bio-based surfactants with excellent emulsifying, conditioning, and stabilising properties, making it suitable for use in pharmaceutical, cosmetic, food and detergent industries. Typically, sugar esters are produced via chemical routes that are poorly sustainable, which requires metal, acid or base catalyst, high temperature, toxic organic solvents and reduced pressure conditions. In the present years, the application of enzymes has emerged as an interesting alternative for synthesis (Pyo *et al.*, 2019). The enzymatic synthesis route is desirable as it is green, safe, and environmental-friendly. Arniza *et al.* (2020) studied possible route to produce bio-based surfactants without using toxic and expensive solvents commonly employed for the derivatisation of sugars or pre-derivatisation of the substrate's molecules. The performance of commercial immobilised lipase from *Thermomyces lanuginosus* (Lipozyme® TL IM) as a catalyst was evaluated to produce palm-based sorbitol monoesters via esterification reaction in a non-toxic organic solvent. Seven types of sorbitol monoesters with acylation at C₁ and C₆ of sorbitol, were successfully synthesised from fatty acids found in palm and PKO. Approximately 76% of sorbitol conversion was achieved under optimal reaction conditions. The conversion was found higher for medium-chain fatty acids ($\leq C_{12}$) when compared to longer chain fatty acids. The lipase catalyst can be used for at least four batch reaction cycles before it undergoes inactivation. The synthesised sorbitol monoesters, the bio-based non-ionic surfactants, could be potentially used in foods, personal care products, and pharmaceuticals.

Biosurfactants are derived from living cells compared to chemically synthesised surfactants and possess similar surface-active properties allowing their applications as cleaning, emulsifying, foaming, and dispersing agents. Biosurfactants from various microbial species have been produced on a range

of carbon substrates. Most reported studies on biosurfactants are on glycolipids biosurfactants such as sophorolipid (SL) and rhamnolipids, but SL is the most studied biosurfactant. Abdul Rashid *et al.* (2020) utilised *Starmerlla bombicola* (*S. bombicola*) for the preparation of SL from palm oil. The SL component structure was elucidated using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) under positive and negative ion modes with 3-hydroxypicolinic acid (HPA) as the matrix. The use of HPA as the matrix for the structure elucidation of SL using MALDI-TOF-MS was never reported before. The 3-HPA has been established to be the superior matrix for the elucidation of SL structures using MALDI-TOF-MS. Furthermore, rapid analysis technique of 2 min run times identified the SL congeners produced, all of which contain 17-hydroxy *cis*-9-octadecenoic acid homologue. The determination of the distribution of SL congeners is essential as it regulates their self-assembly structures and, therefore, their application as glycolipid biosurfactants in environmental remediation, cosmetics, and personal care applications. Overall, favourable support from government regulatory bodies regarding the bio-based process and utilisation of bio-based raw materials act as a significant support system for the industry.

A study to investigate the potential of heterogeneous catalysts CaO, zinc oxide (ZnO) and MgO was conducted on transesterification reactions between methyl palmitate and triethanolamine to produce fatty esteramine an intermediate used in the production of esterquats (Haliza *et al.*, 2020). Among the metal oxides investigated, the CaO catalyst with low dosage (0.1%) showed the best catalytic activity towards the transesterification process as it gave the highest conversion (94.5%) of methyl palmitate and yielded fatty esteramine (46% diesteramine) compositions analogous to the conventional homogeneous catalyst within a relatively shorter reaction time. Therefore, the production of esterquats from esteramine becomes efficient and economically viable using the heterogeneous catalyst herein, CaO catalyst that can be recycled three times. Overall, the development of green process is positioned as an alternative to the conventional homogeneous approach for esterquats production.

Methyl ester sulphonates (MES) are known as an alternative green surfactant for the detergent market. The application of MES largely depends on the quality, which includes the composition, active content, purity and physical appearance. Growing emphasis are also given on eco-friendly manufacturing processes and raw material procurements. Moreover, the purification method of MES is of great interest to further expand the application of MES with high active content. Zulina *et al.* (2020) studied the factors influencing the

purification of MES derived from palm methyl ester with different chain lengths (C_{12} , C_{14} , C_{16} and C_{16-18}). Purification of MES powder was conducted via crystallisation process with ethanol as the solvent. Interestingly, the process successfully attained more than 97% active content and 96% crystallinity index without changing MES structure. The crystallised MES C_{16} and C_{16-18} attained excellent flow characteristics. Crystallisation also allows MES C_{12} and C_{14} conversion into solid powder, which was previously available, in the form of paste. The morphology, structural and its crystallinity analyses showed that the crystals MES had good solubility properties. The crystallised MES containing C_{12} , C_{14} , C_{16} and C_{16-18} with high active content have β -polymorphic form, triclinic lateral structure, which are the most stable arrangement. The brittleness of MES crystals increased from a β' to a β sub-cell. Crystal with high brittleness has the potential to ease the production of powder, which leads to a reduction in the cost of production and improves efficiency. Notably, the crystallisation of MES enable high active MES derivatives to be derived from palm methyl ester with different chain lengths.

CONCLUSION

The oil palm industry in 2020 was blessed with an upsurge of export revenue that counterbalance the depreciation of overall oil palm performance over the challenging landscape of COVID-19 pandemic. With the emphasis of achieving sustainable development goals, the latest research advancements are primarily driven towards oil palm cultivation, environmental management, food and health, renewable energy, green technologies and society. All of the misconceptions pertaining to palm oil have been taken positively for dynamic participation in research activities in addressing the international concerns. In fact, unwavering commitment by the Malaysian palm oil industry in combatting issues and critics related to sustainable development is reflected through various policies and regulations administered within the industry. The enhancement of oil palm productivity can be attained through precision agriculture for early pest and disease detection using non-invasive tools, aside from translating the knowledge of genomics research into cost-effective technology and application to end-users. In the perspective of the midstream sector, it is vital to improve milling efficiency via disruptive technology, process integration and digitalisation for higher extraction rate without compromising the safety and quality aspects of produced CPO. Moreover, the underutilised by-product streams at the palm oil mills can be potentially harnessed by ensuring the technologies and products attained are following

the environmental regulations. As the world's population continue to grow for the next decades, sustainable palm oil has its pivotal role to play to intensify the value-creation of the downstream sector for both food and non-food applications to cater the demand from global industries. Apart from nutrition and health, the palm oil industry has doubled their efforts to mitigate the concerns associated to food safety and quality via innovative technology and good manufacturing practices. On that note, it is now the time for the industry to gravitate towards strengthening the consolidation between upstream, midstream and downstream for the retainment of palm oil existing market, aside from capturing more trade opportunities at the international arena. Focus should be also directed in embracing the fourth industrial revolution in leveraging the oil palm productivity and blockchain technology to reflect the industry's commitment for sustainable and transparent supply chain.

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SOIL FUNGAL COMPOSITION AND DIVERSITY IN OIL PALM PLANTATION AT SUNGAI ASAP, SARAWAK, MALAYSIA

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 KHAIRULMAZMI AHMAD[‡]

ABSTRACT

A study was carried out to compare the soil fungal composition and diversity in oil palm plantation with the adjacent secondary forest at Sungai Asap, Sarawak, Malaysia using denaturing gradient gel electrophoresis (DGGE). Findings revealed that soil fungal composition in the oil palm plantation was significantly different compared with undisturbed secondary forest (SF1) and disturbed secondary forest (SF2) by forming three main clusters as shown in the principal component analysis (PCA). Furthermore, DGGE profile revealed that the banding pattern of oil palm planted area (OPPA) remained diverse throughout the assessment years. However, DGGE banding patterns of SF1 and SF2 showed similarities as noted in the dry season (June 2011) whereby both clusters were overlapped. The composition of soil fungal community in oil palm plantation was dominated by fungi from the phylum Ascomycota whereas secondary forests were dominated by fungi from Basidiomycota. Biodiversity indices showed that the soil fungal diversity in SF1 was higher than SF2 and OPPA. It was postulated that there was possible interactions between soil fungal communities with plantation activities thereby soil fungal communities were shifted in order to acclimatise with new environments in the plantation.

Keywords: Ascomycota, Basidiomycota, biodiversity, DGGE, soil fungi.

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INTRODUCTION

Oil palm (*Elaeis guineensis*) is cultivated commercially in Indonesia and Malaysia as a main source of cooking oil. Oil palm plantation is

a highly important industry because of significant contribution to the Malaysian economy. This sector occupies 5%-6% of Malaysia's gross domestic product (GDP) (Malaysian Palm Oil Council, 2015). Also, palm oil industry is very successful to eradicate poverty in Malaysia (Palm Oil World, 2011). Sarawak is one of the largest palm oil producers in Malaysia, and has registered a total of 1.44 million hectares of oil palm planted areas (MPOB, 2016). Due to the widespread of plantation, environmental issues such as deforestation and the loss of biodiversity have arisen (Fitzherbert *et al.*, 2008). Reduced biodiversity may have other negative impacts on ecosystem functions, especially on the soil microorganisms' which play vital roles in maintaining soil ecosystem.

Although, the oil palm industry is highly significant to the Malaysian economy but the establishment of vast monoculture system without proper legal control has created a number of

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environmental impacts. The two most serious are large-scale forest conversion and loss of critical habitat for endangered species. Other associated issues are soil erosion, air, soil and water pollutions as well as climate change. Furthermore, new plantations can also create social conflicts if the rights and livelihoods of local communities are ignored. By studying composition of soil microorganisms their diversity and their roles in ecological functions may help in improving quality of soil and ecosystem health. The outputs of the research findings can be used to develop specific programme and strategy in order to conserve and sustain our mother nature for the future generation.

Soil is one of the most diverse terrestrial habitats that support life forms from microorganisms to invertebrates, flora and fauna. Microorganisms are the largest and the most diverse biotic group in soil, with an estimated one billion microorganisms per one gram of soil (McCauley *et al.*, 2005). They are bacteria, fungi, actinomycetes, algae and protozoa. They mediate major processes occurring in the soil including decomposition activity, nutrient and energy cycling. Several difficulties involved in studying soil microbial community were reported. Besides, variation in environmental factors in each of the sampling sites may cause difficulties in comparing the results.

Meanwhile, advancement in research techniques especially in molecular biology has opened up a new dimension in studying environmental functions. Molecular approaches like polymerase chain reaction (PCR) techniques and the fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE) and amplified recombinant deoxyribonucleic acid (rDNA) restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP) are widely used to study the diverse community members in any given environment. These molecular methods do not require the extraction of individual (DNA) or to culture the organisms, yet can be targeted at a very broad or narrow range of organisms present in the community (Torsvik and Øvreås, 2002).

In soil ecosystems, fungi play fundamental roles during decomposition of plant residues which promotes nutrient cycling and stimulate plant growth. In addition, soil fungi along with other soil microbes are significant in conserving soil productivity and are sensitive to the small changes in soil environment. Hence, studying soil fungal community is a good way to determine soil health and quality. In other words, soil fungal communities have the potential role as a biological indicator for the soil quality and health. Thus, the aims of this study were to determine the short-term impacts on soil fungal composition and diversity in oil palm plantation using PCR-DGGE method.

MATERIALS AND METHODS

Soil Sampling

Soil samples were collected from the Malaysian Palm Oil Board (MPOB) Research Station at Sungai Asap, Sarawak, Malaysia. Samplings were carried out at a six-month interval (December 2010, June 2011 and December 2011). There were three sampling sites, the undisturbed secondary forest (SF1), disturbed secondary forest (SF2) and oil palm planted area (OPPA). The OPPA was 106.4 ha. SF1 and SF2 are two forests nearby, where SF1 was 6 km and SF2 was 9.7 km from OPPA. Size of the sampling sites for both SF1 and SF2 were approximately 30 ha. During the study time, the oil palms were three years old (December 2010), 3.5 years old (June 2011) and four years old (December 2011). OPPA was planted entirely with oil palms; ground vegetation was few as compared to SF1 and SF2. SF1 remained undisturbed after logging activities whereas SF2 was disturbed forest due to minimal human activities.

About 100 g soil samples were collected using soil auger at 10 GPS-tagged sampling points (P1 to P10) from OPPA, SF1 and SF2. Each sampling points was at least 100 m apart. Four replications were taken from each sampling point and pooled as a composite sample to reduce any bias. All samples were collected at 0 to 15 cm depth after the removal of top vegetation with trowel. Soil samples were kept in sterile falcon tubes (Corning Inc.). New trowel was used and sterilised with 70% ethanol after each sampling sites. Soil samples were stored at -20°C for further use. This was to ensure that there was no microbial growth which could affect the result of this study. Layout of the study sites and sampling points is illustrated in *Figure 1*.

Soil Chemical Properties Analysis

Soil samples were air dried and 10 sampling points (P1 to P10) of each of the sampling sites were pooled together as one sample. The soil samples were analysed by a local agro-chemistry laboratory, RH Biotech Sdn. Bhd. in Sibu, Sarawak, for basic soil chemical analysis. Soil pH, total nitrogen (TN), total phosphorus (TP), exchangeable potassium (exch. K), and cation exchange capacity (CEC) were determined. Analysis of variance (ANOVA) was performed for the selected soil chemical properties with SAS - Statistical Analysis System (ver. 9.0, SAS Institute, Inc., Cary, NC, USA). Differences between sampling time were analysed using Tukey test at $p \leq 0.05$.

Characterisation of Soil Fungi Using DGGE

Extraction of DNA from soil samples was carried out using the PowerSoil DNA Isolation Kit

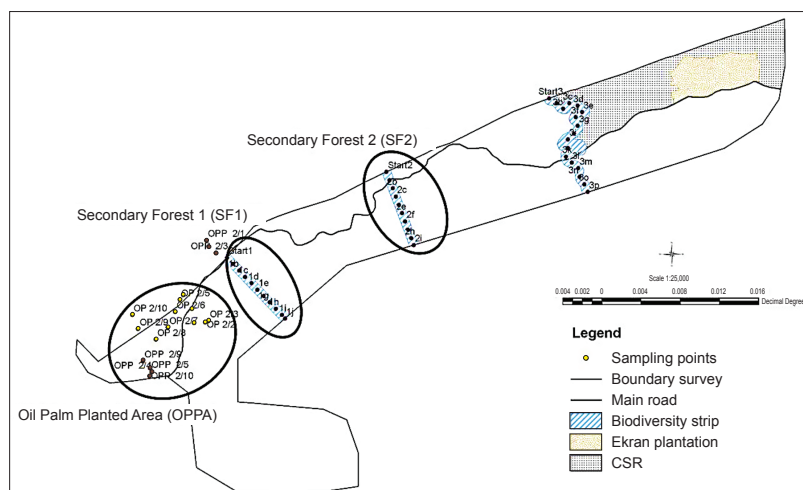


Figure 1. Sampling sites of oil palm planted area (OPPA), undisturbed secondary forest 1 (SF1) and secondary forest 2 (SF2) at Sungai Asap, Sarawak, Malaysia.

(MO BIO) with a modified protocol. The procedures of PCR and DGGE were according to Vainio and Hantula (2000) with some modifications.

PCR Amplification of 18S rDNA Gene

PCR amplification of fungal 18S rDNA gene was performed using the fungi specific primers, GC-Fung and NS1 (May *et al.*, 2001; White *et al.*, 1990) under PCR condition as follows, initial denaturation at 94°C for 130 s, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 130 s and final extension at 72°C for 7 min (Cornejo *et al.*, 2004). The sequences of the primers were as follows: primer NS1 (White *et al.*, 1990), 5'-GTA GTC ATA TGC TTG TCT C-3'; primer GC-Fung (May *et al.*, 2001), 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAT TCC CCG TTA CCC GTT G-3'. The 40-nucleotide GC clamp was added to the 5' end of the reverse primer, to stabilise the melting behaviour of the PCR product for DGGE. Each PCR was carried out in a final volume of 25 µl, which contained 1 µl of DNA template, 0.25 U Taq polymerase, 1 x Reaction Buffer (Invitrogen), 2 mM MgCl₂, 0.3 µM of each primers and 0.2 mM of dNTP. PCR products were analysed first by electrophoresis 1.0% agarose gel, PCR products loaded with EZ-Vision® (Amresco Inc., USA) and view under UV-illumination.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed in a D-Code system (Bio-Rad) with 16 cm x 16 cm x 1 mm thick of 7.5% polyacrylamide gels (acrylamide: bisacrylamide ratio 37.5:1) in 1x TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA; pH 8.0) and a 25%-40% gradient [100% corresponds to 7 M urea and 40% (v/v) deionised formamide]. Urea and formamide were used as the denaturing agents. Electrophoresis was

carried out for 16 hr at temperature of 60°C and 55 V (Aguilera *et al.*, 2006; Díez *et al.*, 2001). Marker was used as calibration among different lanes in each gel.

PCR of Excised Bands in TE Buffer

The targeted DNA was excised and eluted in TE buffer overnight. The entire eluted DNA was amplified with the same method as stated above, with primers without GC clamp. The PCR product was purified with GeneJET[®] (Fermentas). Later, the products were sent for DNA gene sequencing in commercial laboratory, First Base Laboratories Sdn. Bhd.

Analysis of DNA Sequences

Targeted gene sequence was edited and aligned using Bioedit software. After that the sequence was then compared with the gene sequences available in the NCBI database. List of blast results were compared to determine unique groups and common groups among assessment sites. Meanwhile, the number of phylum, class, order, family and genus were recorded as a way to monitor the composition of fungal community.

Analysis of DGGE Gel

Computer-assisted comparison of DGGE band profiles was analysed using Phoretix 1D gel analysis software (Total Lab Ltd., United Kingdom). Background noise was deducted by using the rolling ball algorithm with a radius of 50 pixels. First, automatic band detection was performed with a minimum slope of 75 to 100 and a noise reduction of 5, and peaks smaller than 2% of the maximum peak were eliminated. Bands positions were then compared with the DGGE marker to create a matrix containing the reference value (Rf) of each band.

The Rf of DGGE marker was set as 0.5 in every gel. The band matrix was then used to gain information for biodiversity indices and clustering (version 5; Primer-E Ltd, Plymouth, United Kingdom) to determine fungal communities in the OPPA and secondary forests, SF1 and SF2. Principal component analysis (PCA) was used to interpret each sampling because this analysis provides a way to reduce the complexity of our data to a lower dimensionality.

Biodiversity Indices

A diversity index is a measurement that shows how many different types (such as species) there are in the study area, and simultaneously takes into account how evenly the individuals are distributed among the community. The value of a diversity index increases as both the number of type increases and evenness increases. For a given number of types, the value of a diversity index is maximised when all types are equally abundant.

The Shannon diversity index, H' , a general diversity index, considers both species richness and evenness:

$$H' = - \sum p_i \ln p_i$$

where $p_i = n_i/N$, n is the total number of clones of the i^{th} , and N the total number of samples.

The Simpson's index of diversity was calculated as the following equation:

$$1-D = 1 - \{\sum n(n-1)/N(N-1)\}$$

where n equals to the total number of organisms of a particular species, N equals to the total number of organisms of all species. The value of D ranges between 0 and 1, thus 1 represents infinite diversity and 0, no diversity.

RESULTS AND DISCUSSION

Soil Texture and Chemical Properties Analysis

Both secondary forests of SF1 and SF2 were having the same soil texture, silty clay loam (SF1 = 32% clay, 48% silt and 20% sand; SF2 = 32% clay, 49% silt and 19% sand). Soil texture in OPPA was loam (OPPA = 15% clay, 36% silt and 49% sand). These were determined with Texture Auto Lookup program based on US Department of Agriculture classification schemes (Teh and Rashid, 2003).

The selected soil chemical properties including soil pH, organic carbon (C), TN, TP, CEC, for each sampling period are summarised in *Table 1*.

Results showed that the conversion from secondary forest (SF1 and SF2) to OPPA had caused modifications to the physico-chemical properties of soil. Findings revealed that OPPA soils were more alkaline and with reduced organic C content. Soil pH would directly affects other soil chemical properties and influencing composition and diversity of soil fungi. The requirements of oil palm nutrients were estimated at around 110-156 kg ha⁻¹ yr⁻¹ for nitrogen (N), 64-98 kg ha⁻¹ yr⁻¹ for phosphorus (P), 10-21 kg ha⁻¹ yr⁻¹ for magnesium (Mg), 365-705 kg ha⁻¹ yr⁻¹ for potassium (K), in order to obtain optimum growth. The concentration of selected nutrients (TP, TN) in OPPA showed slight increase over the years (*Table 1*). This findings suggested that agricultural practices such as balance fertilisation that have been adopted could improve soil nutrients for the examined areas. However, the selected nutrients in SF1 showed slight decrease over the years. This may be due to leaching or natural soil erosion in the forest. Similarly, SF2 showed fluctuation in the soil nutrients, suggesting that minimal disturbances can have impacts to the soil.

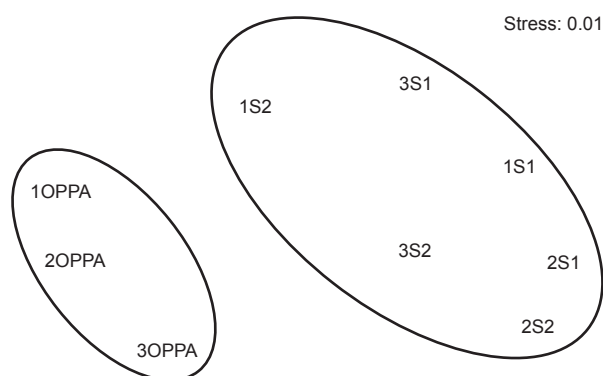
CEC is important as an indicator to soil fertility because it shows the ability of the soil to provide

TABLE 1. SELECTED SOIL PHYSICO-CHEMICAL PROPERTIES OF THE SAMPLING SITES AT SUNGAI ASAP, SARAWAK, MALAYSIA

Sample		pH (H ₂ O)	Organic carbon (%)	Total N (%)	Total P (mg kg ⁻¹)	CEC [cmol(+)/kg]
OPPA	Dec 2010	5.26±0.01c	0.67±0.02c	0.10±0.00b	150.60±3.93b	8.28±0.21b
	Jun 2011	5.03±0.02b	0.77±0.03b	0.11±0.00a	193.50±15.36a	8.34±0.25b
	Dec 2011	5.54±0.02a	0.84±0.02a	0.13±0.00a	232.33±25.18a	9.76±0.28a
SF1	Dec 2010	5.30±0.01b	1.63±0.03a	0.19±0.01a	294.21±13.59a	12.61±0.13b
	Jun 2011	5.30±0.01b	1.32±0.04b	0.18±0.00a	293.99±8.62a	13.11±0.38a
	Dec 2011	5.33±0.01a	1.10±0.03c	0.15±0.01b	283.95±8.38a	11.46±0.21c
SF2	Dec 2010	5.02±0.02b	0.84±0.01c	0.11±0.00c	150.56±8.49c	10.42±0.28c
	Jun 2011	4.89±0.00c	1.50±0.01a	0.19±1.01a	243.23±3.63b	12.78±0.45a
	Dec 2011	5.22±0.01a	1.06±0.04b	0.17±0.01b	320.32±31.34a	11.85±0.18b

Note: OPPA - oil palm planted area; SF1 - undisturbed secondary forest; SF2 - disturbed secondary forest; CEC - cation exchange capacity; mean ± standard of deviation, n - 4; mean with same letter within column and sampling sites were not significantly different by Tukey test ($p \leq 0.05$).

calcium (Ca), Mg and K to the plants. From the results, OPPA gave lower CEC compared to SF2, and SF2 had lower CEC than SF1. This indicated that soil in OPPA was less fertile compared to SF1 and SF2, and SF2 was less fertile than SF1. High pH (less acidic) gave higher negative charge to the soil and hence, higher CEC. The soil pH of OPPA, SF1 and SF2 soils were strongly acidic because they ranged from pH 4.89 to pH 5.33. However, discrepancies were observed when SF1 showed higher pH (less acidic) yet yielded lower CEC. The soil chemical properties result demonstrated that soil type of SF1 was silty clay loam and thus, gave higher CEC, which was more fertile than OP soil. The selected soil chemical properties were subjected to a cluster analysis according to Euclidean distance analysis and summarised in Multidimensional Scaling (MDS) (Figure 2). Soil chemical properties of OPPA over years were in tight cluster, whereas soil chemical properties of SF1 and SF2 were in another cluster. This showed that soil chemical properties between OPPA and SF1, SF2 were significantly different. This was probably due to the effects of agricultural practices carried out in the oil palm plantation such as liming, pest and disease control and fertilising.



Note: First number in the designation referring to sampling time (1 - December 2010; 2 - June 2011 and 3 - December 2011) and followed by sampling sites (SF1 - undisturbed secondary forest; SF2 - disturbed secondary forest and OPPA - oil palm planted area).

Figure 2. Multidimensional Scaling (MDS) analysis of soil chemical properties at OPPA, SF1 and SF2.

PCR Amplification of Partial 18S rDNA Gene

The 18S rDNA gene from all soil samples ($n=90$) were successfully amplified by primer pair NS1/GC-Fung (May *et al.*, 2001; White *et al.*, 1990) except one soil sample OP-3-P4. All the PCR products with the expected sizes of 370 bp were obtained. This indicated that no inhibition in the PCR products. No amplification in sample OP-3-P4 might be due to the sample was taken from

waterlogged area. Majority of soil fungi survive better in an aerobic conditions compared to anaerobic. This was explained by Ingham (1999) who stated that soil which is anaerobic due to waterlogged or compacted soils for significant periods, would lose their fungal component. Result of PCR products which successfully amplified for OPPA were shown in Figure 3.

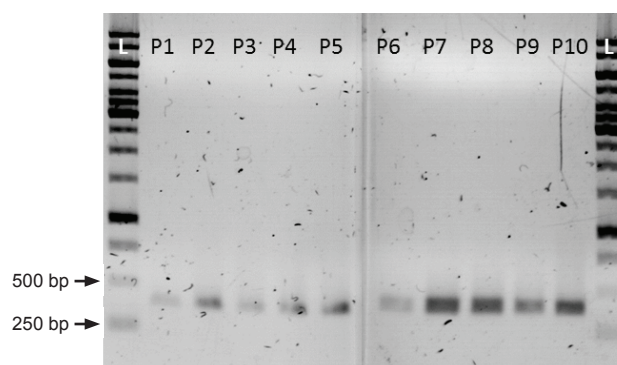


Figure 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) products obtained from oil palm planted area using primer NS1/GC-Fung. Molecular marker is GeneRuler 1 kb DNA ladder (Thermo Scientific, Lithuania).

DGGE Profiling of 18S rDNA Gene

The amplification of extracted soil DNA with fungal-specific primer pair, NS1/GC-fung (May *et al.*, 2001) yielded products of 370 bp to be further investigated in DGGE. DGGE successfully separated all the DNA of same length into different bands. Results from the DGGE gels showed mixtures of bands with different intensities. This DGGE profile, or known as fingerprinting profile, contained the whole fungal community amplified from soil total DNA. Each discrete band is referred as operational taxonomic units (OTU) or discrete fungal population. Band intensity gives an idea to the frequency or abundance of individual fungal biomass. The number of bands corresponds to the number of members in the soil fungal communities (Duarte *et al.*, 2012). Overall, high numbers of OTU were found in SF1. Whereas, lower OTU were recorded in both OP and SF2. Over hundreds of total number of bands were obtained in SF1 over the sampling time. Meanwhile, OPPA and SF2 recorded lesser total number of bands and most of the bands were separated at the middle part of the gels while fewer appeared at the top and bottom of the gels. Complex DGGE patterns were obtained, suggesting that complicated fungal communities existing in the soil.

Findings revealed that in agricultural land (OPPA), the fungal communities were much affected by their surroundings, including farm practices,

pesticides and fertilisers applied to the palms. These activities directly affect physical, chemical and biological factors which resulted in reducing soil productivity. In forest area (SF1), the growth of soil fungi was between evenness and richness and that may refer to the natural habitat, humidity, shrubby and shading condition (Jena *et al.*, 2015) and that matched with the finding revealed by OTU results in the current study. Whereby, OPPA in December 2010, there was less crop treatment being carried out while the oil palms were young and at new stage of growing. Bold dried land in OPPA was not a preferential habitat to the soil fungi. Therefore, soil fungal community was fewer.

Community Structure through Gene Sequence Analysis

A total of 175 selected bands were excised from DGGE profile for further analysis using gene sequencing. Tables 2 and 3 show taxonomic order and common soil fungal species found in the study sites. Ten species were commonly found in both SF1 and SF2. Three common species were found in all assessment sites and they were *A. polytricha*, *Clitocybula atroalba* and *S. cuboideum*. Based on taxonomic order, five fungal species were from the Phylum Ascomycota (*Aspergillus* sp., *A. ochraceus*, *S. cuboideum*, *Cochliobolus cynodontis*, *A. polytricha*) and the rest were from the Phylum Basidiomycota.

The common species shared between the secondary forests SF1 and SF2 were *Aspergillus* sp., *Antrodia xanthan*, *Cantharocybe gruberi*, *Cryptococcus podzolicus*, *H. cantharellus*, *Hygrocybe conica*, *Mycocalia*

denudata, *Ramaricium alboflavescens* and *Tricholoma subresplendens*. Results showed that shifting of land from forest to oil palm plantation reduced the abundance of *Aspergillus* sp., *A. ochraceus*, *Cantharocybe gruberi*, *C. podzolicus*, *H. cantharellus*, *H. conica*, *M. denudata*, *R. alboflavescens* and *T. subresplendens*. Whereas, *A. polytricha*, *C. atroalba*, *C. cynodontis* and *Rhizoctonia* sp. were found to be resistant to deforestation. Sequencing of the excised bands gave the picture of composition of fungal community. Fungi from the phylum Ascomycota, genus *Aspergillus* were found in both SF1 and SF2. These fungi are common associates of decaying plant materials (Cannon and Kirk, 2007).

Basidiomycota were found in both SF1 and SF2, they belong to the family of Fomitopsidaceae, Tricholomataceae, Tremellaceae, Hygrophoraceae, Nidulariaceae and Gomphaceae (corresponding to the closest match *A. xanthan*, *C. gruberi*, *C. podzolicus*, *H. cantharellus*, *H. conica*, *M. denudata*, *R. alboflavescens* and *T. subresplendens*). Among these families, Fomitopsidaceae are the parasitic fungi causing brown rot on trees (Bolhassan *et al.*, 2012). Hygrophoraceae are ectomycorrhizal or saprobic found in forests. Gomphaceae are widely spread in tropical climatic zones and formed ectomycorrhizal relationships with woody plants. Nidulariaceae are the saprobic on dead plant materials, especially on dead woods or grasses. Tremellaceae are often parasitic on other fungi. Tricholomataceae are ectomycorrhizal and occurs in broadleaved forests. All the ectomycorrhizals are dependent on the forest trees to survive, hence, they are absent from OPPA (Cannon and Kirk, 2007).

TABLE 2. TIME-COURSE NUMBER OF FUNGAL PHYLA, CLASS, ORDER, FAMILY AND GENERA FOR OIL PALM PLANTED AREA (OPPA) AND SECONDARY FOREST (SF1 and SF2) IN SOIL SAMPLED FROM SUNGAI ASAP, BELAGA, SARAWAK, MALAYSIA

Sites	Taxonomy	December 2010	June 2011	December 2011
OPPA	Phyla	2	2	2
SF1		2	2	2
SF2		2	2	2
OPPA	Class	6	5	5
SF1		2	5	5
SF2		2	4	6
OPPA	Order	7	7	9
SF1		4	8	9
SF2		4	9	11
OPPA	Family	9	11	11
SF1		9	15	14
SF2		5	16	14
OPPA	Genus	13	12	17
SF1		13	18	16
SF2		7	16	15

Note: SF1 - undisturbed secondary forest.
SF2 - disturbed secondary forest.

TABLE 3. COMMON SPECIES FOUND AMONG THE THREE STUDY SITES FROM DECEMBER 2010-2011

Closest match	OPPA			SF1			SF2		
	Dec-10	Jun-11	Dec-11	Dec-10	Jun-11	Dec-11	Dec-10	Jun-11	Dec-11
<i>Aspergillus</i> sp.						+			+
<i>Aspergillus ochraceus</i>					+	+		+	
<i>Auricularia polytricha</i>		+			+	+			
<i>Antrodia xantha</i>				+				+	
<i>Ascomycete</i> sp.			+				+		
<i>Cantharocybe gruberi</i>					+		+		
* <i>Clitocybula atroalba</i>		+		+		+		+	
<i>Cochliobolus cynodontis</i>		+				+			
<i>Cryptococcus podzolicus</i>					+	+			+
<i>Hygrocybe cantharellus</i>				+			+		
<i>Hygrocybe conica</i>					+			+	
<i>Mycocalia denudata</i>						+			+
<i>Ramaricium alboflavescens</i>					+		+	+	
<i>Rhizoctonia</i> sp.		+							+
<i>Scytalidium cuboideum</i>	+				+	+			
<i>Tricholoma subresplendens</i>				+				+	

Note: * Indicates species occurs at all study sites. + Indicates the presence of species at the study site.

OPPA - oil palm planted area.

SF1 - undisturbed secondary forest.

SF2 - disturbed secondary forest.

The composition of fungal orders increased over time as shown in the bar charts (Figure 4). SF1 and SF2 were having more diverse orders of fungi compared to OPPA. From the charts, it was obvious that fungi from the order Pleosporales were dominant in OPPA whereas the order Agaricales was dominant in SF1 and SF2. From the analysis of fungal orders, it was found that there were orders shared among OPPA, SF1 and SF2 and also there were orders uniquely exist at individual sites. Orders shared among OPPA, SF1 and SF2 were Agaricales, Polyporales, Pleosporales, Eurotiales and Cantharellales. They are terrestrial saprobes growing on fallen woods or grasses (Binder *et al.*, 2013; Castellano *et al.*, 2012).

Orders shared between OPPA and SF1 or SF2 are Auriculariales, Sordariales and Pezizales. These fungi are mostly saprobes on grasslands (Huhndorf *et al.*, 2004). Auriculariales are jelly fungi (Sotome *et al.*, 2014) whereas Pezizales are saprophytes and almost always occur on soil or dung (Harrington *et al.*, 1999; Moore-Landecker, 1972). Orders shared between SF1 and SF2 are Phallales and Tremellales. Phallales grow on leaf litters in damp woodland (Grgurinovic and Simpson, 2001). Tremellales are parasites of other fungi (Pippola and Kotiranta, 2008). These were the common fungi found among OPPA, SF1 and SF2.

Unique orders in OPPA were Xylariales, Ascomycete, Microascales, Microthyrales and Sebaciniales. Xylariales, Ascomycete, Microascales are saprobes found on terrestrial environments (De Beer *et al.*, 2013; Smith *et al.*, 2003). Sebaciniales are mycorrhizas (associated with plant roots for nutrition) (Weib *et al.*, 2011). Unique orders in

SF1 are Aphyllophorales, Coniochaetales and Botryosphaerales. Aphyllophorales are similar to Agaricales except they are not bearing gills (non-agarics) and they are root-rotting fungi in the soil (Rajchenberg, 1989). Botryosphaerales are sac fungi and usually are saprobes (Adesemoye *et al.*, 2014; Phillips *et al.*, 2013). Coniochaetales are saprotrophs, leaf and root endophytes, and plant pathogens.

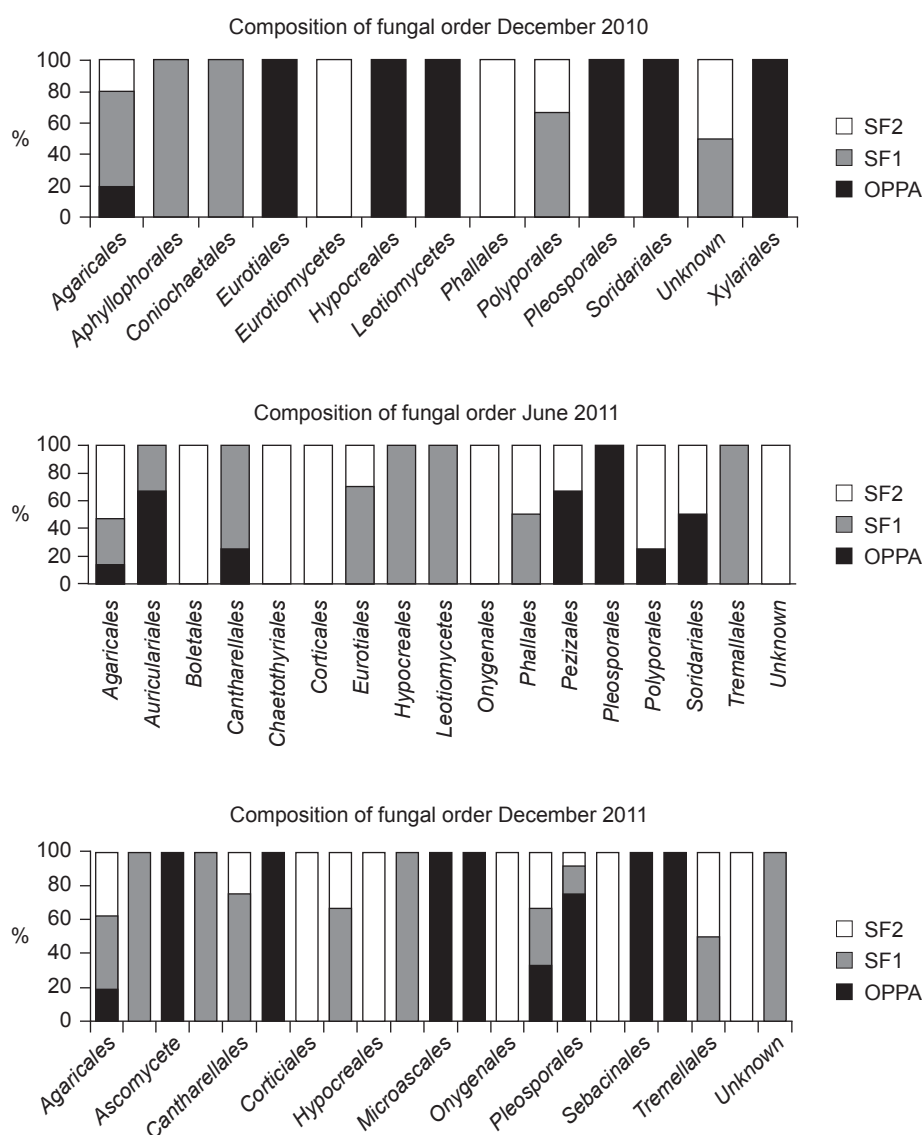
Unique orders in SF2 are Eurotiomycetes, Boletales, Corticiales, Onygenales, Russuales and Umbilicariales. Eurotiomycetes, Boletales, Corticiales are saprobes on wood and grasslands (Geiser *et al.*, 2006). Onygenales are able to digest keratin and may be found on animals' hooves in the forests (Johnston *et al.*, 2015). Russuales could be found on hardwoods. Umbilicariales are lichenized fungi (Admčik *et al.*, 2013; Miller *et al.*, 2006). From the results above, it was observed that saprobes were among the most common fungi that were present in OPPA and secondary forests. The importance of fungi as saprobes in ecosystem processes is undeniable.

Ascomycota are predominantly terrestrial, some live in fresh or marine waters. They are majority of saprophytes on decaying plant debris or parasites on plants, insects and animals (Moore-Landecker, 1972). Ascomycota are important as decomposers (saprophytes). Together with other fungi, they break down large molecules in the environment and contribute to the nutrients cycling in the ecosystems. Some Ascomycetes form symbiotic relationship with other plants for survival strategy. They associate with green algae to form lichens; they live at the plant roots as mycorrhiza; and in plants

to form endophytes. All these characteristics of Ascomycota make it a majority fungal composition in the oil palm planted areas.

Basically, Basidiomycetes follow the same roles as the other fungi; they are important decomposers, plant pathogens and symbionts with plants (mycorrhiza). Our results showed that Basidiomycota was the highest phylum in secondary forest. Our findings were in line with the reports of Moll *et al.* (2016), McGuire *et al.* (2015) and Dai *et al.* (2013). Basidiomycota are common wood decomposers or decomposers of leaf litters and animal dung. Some of them are mycorrhiza to the roots of forests trees (Moll *et al.*, 2016). Hence, this explained why Basidiomycota are dominating secondary forest.

Forests floor were covered with decaying leaves litters. A study by Vorířkova and Baldrian (2013) showed that towards the later stage of degradation processes, fungi from the phylum Basidiomycota particularly the cellulolytic fungi would replace Ascomycota. This observation also supports our results of high abundance of Basidiomycota in secondary forests compared to oil palm plantation area. In addition, Basidiomycota are slow growing fungi and are sensitive to soil disturbance (McGuire *et al.*, 2015). Therefore, Basidiomycetes are relatively low in oil palm plantation. Meanwhile, Ascomycota were recorded to be at higher abundance in stressful environments (Tripathi *et al.*, 2016). Thus, this report is in line with our results.



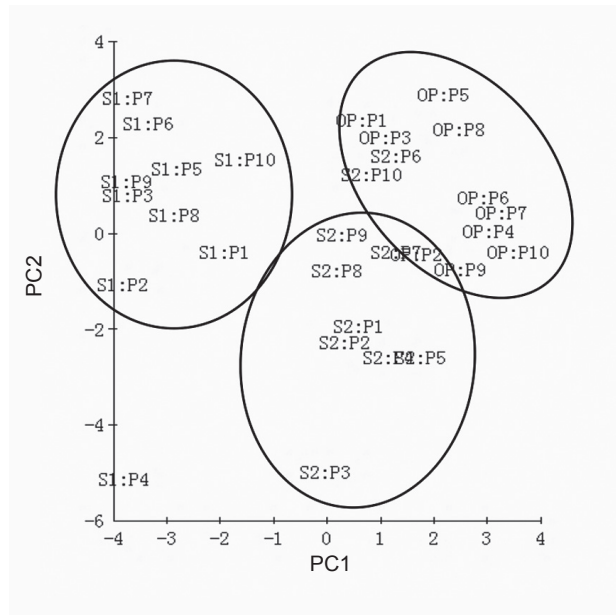
Note: OPPA - oil palm planted area.
SF1 - undisturbed secondary forest.
SF2 - disturbed secondary forest.

Figure 4. Comparison of fungal order composition as determined in December 2010-2011.

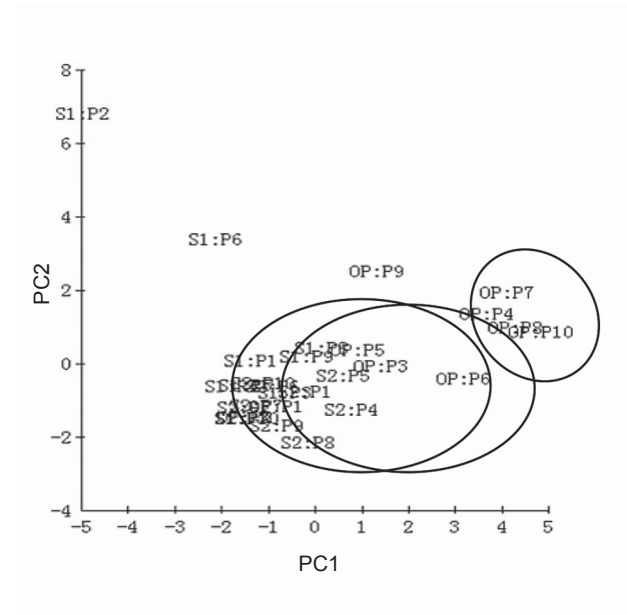
PCA of DGGE Profiles

The binary matrix of DGGE bands were subjected to PCA (Figure 5). PCA is good in interpreting complex DGGE data, giving a quick and simple view to study the distribution of biodiversity. Findings revealed that the secondary forests (SF1 and SF2) shared similar DGGE banding patterns, yielding similar distribution when the scattered plot was produced. OPPA obviously produced unique DGGE banding pattern, thus, separated themselves

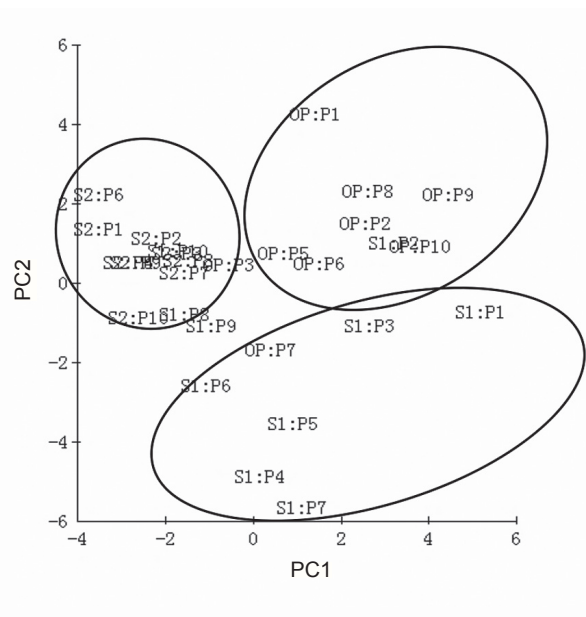
to form one unique cluster. Groupings of SF1 and SF2 were found to be overlapped in June 2011, as this period was dry season. In short, SF1 and SF2 shared about similar DGGE banding pattern whereas OPPA was significantly different and was not significantly affected by dry or rainy seasons. This result suggested that soil fungal communities in OPPA were heterogeneous compared to the fungal community in SF1 and SF2. This observation matched previous results, where OPPA and SF1, SF2 were distinctive in their dominant fungal phylum.



a) December 2010



b) June 2011



c) December 2011

Figure 5. Principle component analysis (PCA) of three assessment sites on the three sampling times from December 2010-2011.

TABLE 4. DIVERSITY INDEX ANALYSIS OF THE THREE STUDY SITES

Sites	Sampling time	Species richness	Shannon-Weaver index (H')	Simpson (1-D)
OPPA	Dec 2010	2.57±0.71 ^b	1.61±0.04 ^b	0.78±0.08 ^a
	June 2011	3.67±0.69 ^{ab}	2.17±0.32 ^{ab}	0.88±0.05 ^a
	Dec 2011	3.83±0.55 ^b	2.26±0.22 ^b	0.89±0.03 ^a
SF1	Dec 2010	3.95±0.92 ^a	2.27±0.39 ^a	0.89±0.05 ^a
	June 2011	4.33±1.40 ^a	2.36±0.51 ^a	0.89±0.06 ^a
	Dec 2011	5.45±1.02 ^a	2.75±0.28 ^a	0.93±0.02 ^a
SF2	Dec 2010	2.97±0.88 ^b	1.79±0.54 ^{ab}	0.804±0.13 ^a
	June 2011	2.98±0.83 ^b	1.82±0.47 ^b	0.82±0.09 ^a
	Dec 2011	4.12±0.81 ^b	2.34±0.31 ^{ab}	0.90±0.03 ^a

Note: Labels of the samples were as follows: OPPA - oil palm planted area, SF1 – undisturbed secondary forest 1 and SF2 – disturbed secondary forest 2. Sites with the different letter in a column in each sampling month were significantly different ($p < 0.05$) by Tukey's test.

Biodiversity of Soil Fungal Community

The total number of bands can be used for comparing fungal compositions (Duarte *et al.*, 2012). Biodiversity index could be calculated by taking one band as one species to estimate the species richness and diversity. Binary matrix generated using CLIQS 1D Pro (Total Lab Ltd., United Kingdom) was used in the calculation of species richness. Shannon-Weaver index (H') and Simpson (1- λ) were calculated by using software Primer-E (Plymouth). Table 4 shows the results of diversity index of the study sites. Species richness in OPPA, SF1 and SF2 at the beginning of the study were 2.57, 3.95 and 2.97, respectively. These indices increased to 3.83, 5.45 and 4.12 at the end of this study. In short, the species richness has increased significantly in every site from December 2010 to December 2011.

In Shannon-Weaver index, the increasing trend was observed in SF1 and SF2. OPPA recorded its Shannon-Weaver index as 1.6 (December 2010) to 2.26 (December 2011). However, there was no significant increment. SF1 and SF2 showed significant increment in Shannon-Weaver index. Shannon-Weaver index was higher in forest soils compared to OPPA. This was in line with a study by Grantina *et al.* (2011).

In Simpson index, there was no significant increment of soil fungal community in OPPA, SF2 and SF1. The diversity of soil fungal community showed increment over years in SF1 and SF2. SF1, SF2 and OPPA showed increment in species richness over the study. This showed that there was no significant disruption of composition of fungal community in SF1, SF2 and OPPA over the study period.

CONCLUSION

Apparently, our results showed that the composition and biodiversity of soil fungal communities in oil

palm plantation have shifted in order to acclimatise with stressful environments as compared to secondary forest. Biodiversity of soil fungal communities in oil palm plantation reportedly lower than secondary forests and this suggested that plantation activities can cause destruction on the ecological components in the assessment sites. Hence, sustainable agricultural practices should be adopted in oil palm plantation to ensure good soil health and quality in order to conserve healthy ecosystems.

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A PROOF-OF-CONCEPT STUDY: DETERMINING THE GEOGRAPHICAL ORIGIN OF CRUDE PALM OIL WITH THE COMBINED USE OF GC-IMS FINGERPRINTING AND CHEMOMETRICS

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ABSTRACT

Current administrative controls used to verify geographical provenance within palm oil supply chains require enhancement and strengthening by more robust analytical methods. In this study, the application of volatile organic compound fingerprinting, in combination with five different analytical classification models, has been used to verify the regional geographical provenance of crude palm oil (CPO) samples. For this purpose, 108 CPO samples were collected from two regions within Malaysia, namely Peninsular Malaysia (32) and Sabah (76). Samples were analysed by gas chromatography-ion mobility spectrometer (GC-IMS) and the five predictive models (Sparse Logistic Regression, Random Forests, Gaussian Processes, Support Vector Machines and Artificial Neural Networks) were built and applied. Models were validated using 10-fold cross-validation. The area under curve (AUC) measure was used as a summary indicator of the performance of each classifier. All models performed well (AUC 0.96) with the Sparse Logistic Regression model giving best performance (AUC = 0.98). This demonstrates that the verification of the geographical origin of CPO is feasible by volatile organic compound fingerprinting, using GC-IMS supported by chemometric analysis.

Keywords: chemometrics, fingerprinting, GC-IMS, geographical origin, palm oil, volatile organic compounds.

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INTRODUCTION

Palm oil obtained from the fruit of the oil palm (*Elaeis guineensis*), is the most consumed vegetable oil globally. In 2018, it was estimated that 68 million tonnes were produced globally (Statista, 2018). The oil palm plant originates from West Africa,

but now grows in wild, semi-wild and cultivated states right across the equatorial tropics, including Malaysia, Indonesia, Papua New Guinea, Western Africa and South and Central America (Corley and Tinker, 2008). Two different oils are extracted from palm fruits, namely crude palm oil (CPO) and crude palm kernel oil. CPO is the main oil of commercial interest, being semi-solid at room temperature and containing high proportions of both saturated and monounsaturated fatty acids. These properties make for a versatile oil that is predominantly used in foodstuffs as an ingredient in thousands of processed foods ranging from noodles to chocolate. Oil palm cultivation has grown rapidly in recent decades due to low production costs and high demands from the food industry, especially for CPO (Corley and Tinker, 2008; Paddison *et al.*, 2014).

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The initial rapid expansion of the oil palm industry in Malaysia occurred by conversion of land from other plantation crops, mainly rubber. However, after 2000, large tracts of primary and secondary rainforests, as well as peatlands, were also converted especially in Indonesia. This process often occurred in regions of high biodiversity and conservation value (Koh and Wilcove, 2008; Meijaard *et al.*, 2018). As a reaction to this process, the Roundtable on Sustainable Palm Oil (RSPO) was established in 2004 to improve sustainability and traceability of the industry. However, to a great extent, current traceability methods are largely based upon potentially fallible audit trails. Therefore, it is increasingly important that there are alternative methodologies that can be applied reliably within supply chains and which enable authentication of geographical provenance, to facilitate current traceability measures.

Chemically-based methods for the authentication of geographical provenance of vegetable oils has been well studied in the case of olive oil but less so for other oils (Janin *et al.*, 2014; Ou *et al.*, 2015; Portarena *et al.*, 2014). All vegetable oils are complex natural mixtures comprising of many components. Fatty acid composition is the most studied component for vegetable oil authentication (Janin *et al.*, 2014; Korifi *et al.*, 2011; Tres *et al.*, 2013). However, other important components can also be utilised for authentication including sterols, elemental isotope ratios, volatile organic compounds (VOC) and tocopherols. With the exception of isotope ratios, fingerprinting techniques are the most common approach for assessing such components, as they provide analytical information about a sample in a non-selective way (Ruiz-Samblás *et al.*, 2013).

However, fingerprinting usually generates issues of 'big data' analysis, which require the use of appropriate multivariate statistics to extract the most important information for characterising a particular sample (Cumeras *et al.*, 2015; Hauschild *et al.*, 2012; Szymanska *et al.*, 2014). Other studies have shown that VOC fingerprints can be useful for discerning vegetable oils by geographical origin because their quality and composition depend on several factors, including genetic variety, growing conditions, processing technologies and storage (Tres *et al.*, 2011). In the case of palm oils, the composition may be significantly affected by seasonal variation, fertilisation regime, oil processing techniques, *etc.*

In this work, gas chromatography-ion mobility spectrometer (GC-IMS) was used to generate VOC fingerprints. IMS was initially developed in the 1970s for detection of explosives and chemical warfare agents. It relies upon the separation of charged particles in an electric field, with separation depending upon mass, shape, size and

collisional cross-sectional area (Eiceman *et al.*, 2016) of each ion cluster. However, ion mobility spectrometers typically have low resolution due to overlapping signals resulting from ion-ion or ion-molecule reactions in the ionisation process (Garrido-Delgado *et al.*, 2011). In this work, we explored the use of an IMS. The method is therefore often coupled with other techniques for fast pre-separation, usually a standard GC column, as is the case in this study. GC-IMS is now increasingly applied in the environmental, biomedical and food and flavour industries due to its selectivity and sensitivity, time of analysis, small footprint, low cost and its ability for easy on-site implementation by relatively unskilled operatives, meaning it is potentially accessible to laboratories worldwide.

The present work is one of only a few studies that have sought to characterise CPO samples by geographical origin. Four previous studies have sought to do this on a continental scale (South-east Asia *vs.* South America *vs.* Africa) (Obisesan *et al.*, 2017; Pérez-Castaño *et al.*, 2015; Ruiz-Samblás *et al.*, 2013; Tres *et al.*, 2013) and one study was on a regional scale (central Malaysia *vs.* northern Malaysia *vs.* east coast Malaysia *vs.* southern Malaysia *vs.* east Malaysia) (Muhammad *et al.*, 2017). While, GC-IMS has previously been used to distinguish different olive oil samples by grade (Garrido-Delgado *et al.*, 2015; 2011), to our knowledge this study is the first time GC-IMS has been used for palm oil analysis. Here we describe the application of chemometrics to raw GC-IMS chromatograms to successfully establish models for the prediction of regional geographical provenance of CPO samples in Malaysia.

MATERIALS AND METHODS

Palm Oil Samples

A total of 108 palm oil samples were provided by the Wageningen University of Research (WUR), The Netherlands. These samples had been collected from various mills across Peninsular Malaysia and the state of Sabah in North Borneo. A total of 32 samples originated from Peninsular Malaysia whilst 76 originated from Sabah. Samples were stored at 4°C until analysis.

Sample Preparation

No pre-preparation or derivatisation of samples is required prior to GC-IMS analysis. CPO samples were melted at 45°C to enable aliquoting of 1 g to a 20 ml glass headspace vial and vials were secured with a magnetic screw cap, sealed with a PTFE/silicon septum. Samples were pre-conditioned at 60°C and 275 rpm for 15 min, via an integrated

sample introduction system (SIS) unit (CTC-PAL, CTC Analytics AG, Zwingen, Switzerland) to ensure equilibration between the sample and headspace. The 200 μ l of sample headspace was directly injected into the GC-IMS system via a 2.5 ml Hamilton syringe with a 51 mm needle.

GC-IMS Analysis

All CPO analyses were performed on a commercially available GC-IMS instrument (model, FlavourSpec[®]) from Gesellschaft für Analytische Sensorsysteme mbH (G.A.S., Dortmund, Germany). The headspace sample was injected via a heated splitless injector on to a low polarity GC column consisting of 9%-diphenyl – 95% dimethylpolysiloxane of 15 m length, an internal diameter of 0.53 mm and 1 m of film thickness (FS-SE-54-CB-1 of CS-Chromatographie Service GmbH, Düren, Germany) facilitated by Nitrogen (6.0) carrier gas. The analytes enter the ionisation region and undergo soft ionisation via a cascade reaction by a Tritium H³ radioactive ionisation source of 300 MBq.

Ion swarms are released into the drift region through a Bradbury Nielsen gate (grid pulse width

of 100 μ s and a sampling frequency of 150 kHz) when the electric field strength of the grid set of the shutter is weakened or eliminated. Ions travel towards the detector (Faraday plate) against an opposing drift gas (Nitrogen 6.0) and are separated based on mass, charge, size and cross-sectional collision surface area, due to the presence of an electric field. Subsequently, different ions reach the detector at different times, with each component having a specific IMS drift time.

IMS data were acquired in positive mode using Laboratory Analytical Viewer (LAV) software (v.2.0.0) from G.A.S (G.A.S, 2018). Each spectrum had an average of six scans, obtained using a repetition rate of 30 ms. Instrumental and experimental parameters for CPO analysis are displayed in Table 1. Working principles of the FlavourSpec[®] are displayed in Figure 1.

Data Analysis

GC-IMS spectral data was exported into comma separated values (CSV) format for data processing (typically 11 000 000 data points per file). The general workflow is summarised in Figure 2 and was developed in R (v 3.0.2). A number

TABLE 1. INSTRUMENTAL AND EXPERIMENTAL PARAMETERS FOR CRUDE PALM OIL (CPO) ANALYSIS

Parameter	Values and units
Sample Introduction System (SIS)	
Sampling type/volume	Headspace (200 μ l)
Agitation time	15 min
Incubation temperature	60°C
Syringe temperature	80°C
Column	
Injector temperature	80°C
Capillary column	SE-54 (low polar) ID 0.53 mm, 1 μ m
Column length	15 m
Column temperature	40°C
Gas chromatography (GC) run time	16 min
Carrier gas flow rate	T= 0-10 min: 2 ml min ⁻¹ to 50 ml min ⁻¹ T= 10-15 min: 50 ml min ⁻¹ to 150 ml min ⁻¹ T= 15-16 min: 150 ml min ⁻¹ (N ₂ 6.0)
Ion mobility spectrometer (IMS)	
Ionisation source	Tritium (30 MBq)
Voltage	Positive drift
Drift length	9.8 cm
Electric field strength	510 V cm ⁻¹
Drift gas flow rate	150 ml min ⁻¹
IMS temperature	45°C

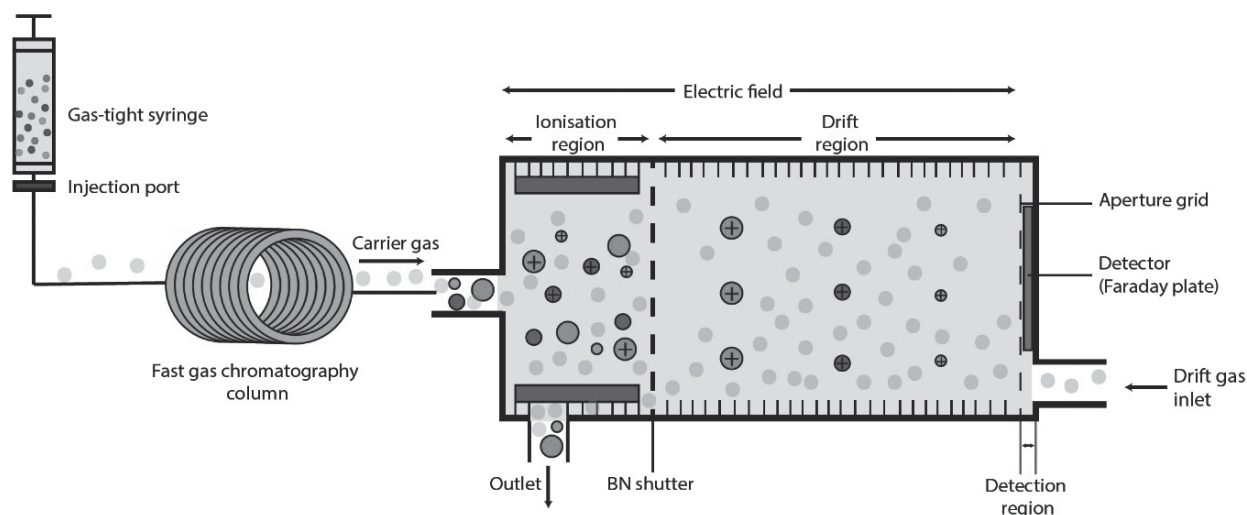


Figure 1. A diagrammatic overview of the working principles of gas chromatography-ion mobility spectrometer (GC-IMS).

of pre-processing steps were undertaken prior to chemometric analysis. The first step cropped an area of interest, reducing data points by a factor of 10. All data were aligned in the x-axis relative to the Reactant Ion Peak (RIP) position of the first file and thresholding to remove background was followed by x/y realignment, further reducing the number of data points to below 100 000. At this stage, a 10-fold cross validation technique was applied. In each fold, around 90% of the data was used as the training set. Within the training set, features were identified using a Wilcoxon rank-sum between the two groups (Sabah *vs.* Peninsular Malaysia). One hundred features (data points) with the lowest *p*-values were retained and used to construct the models. This model was then applied to the remaining test set and this was repeated until each sample has a prediction as a test sample (Martinez-vernon *et al.*, 2018). The five classification models used in this study are listed below:

- Sparse Logistic Regression;
- Random Forests;
- Gaussian Processes;
- Support Vector Machines; and
- Artificial Neural Networks.

RESULTS AND DISCUSSION

Examples of the GC-IMS spectra obtained from Sabah and Peninsular CPO samples are shown in Figure 3. GC-IMS analysis results in a three-dimensional topographic plot where the x-axis represents IMS drift time (ms), the y-axis represents GC retention time (s) and the z-axis represents peak height/intensity (V). Due to the three-dimensional nature of the data, each spectrum contains around 11 million data points making visual comparison

of different samples arduous and inefficient. Furthermore, distinguishing less intense but perhaps important signals is not possible as they may not be readily apparent above the background noise. This is why the application of chemometrics was required in order to process data automatically, to reduce dimensionality and size, and to build classification models for discerning CPO samples by geographical origin (Sabah *vs.* Peninsular Malaysia).

The five different classification models (Sparse Logistic Regression, Random Forests, Gaussian Processes, Support Vector Machines, Artificial Neural Networks) include both linear and non-linear methods. A single classification model was not selected for this study as the dataset was relatively small and until larger data sets can be tested, it is recommended that multiple classifier models should be used. In order to quantify the quality of classification results, several performance features were proposed as metrics. The estimation of such metrics is based upon the classifiers ability to distinguish classes correctly and to subsequently avoid classification failure (Martinez-vernon *et al.*, 2018). The different quality metrics used in this article for evaluating the classification results are shown below (Pérez-Castaño *et al.*, 2015):

- Area under curve (AUC): the area under the receiver operating characteristic (ROC) curve is a measure of the quality of classification models that can summarise the performance of a classifier into a single metric. Its value varies between 0 and 1, although values should generally be greater than 0.5.
- Sensitivity: also known as the true positive rate and measures the proportion of actual positives that are correctly identified as such. The range of values for this feature is 0 to 1.

- Specificity: also known as the true negative rate and measures the proportion of actual negatives that are correctly identified as such. The range of values for this feature is between 0 and 1.
- p -value: a measure to determine the significance of the results. p 0.05 typically indicates strong evidence against the null hypothesis meaning the result is significant.

All five models produced strong results for discerning Sabah and Peninsular Malaysia CPO samples (AUC 0.96) meaning they could correctly distinguish between samples at least 96% of the time. However, the Sparse Logistic Regression method performed best (AUC 0.98) (Table 2 and Figure 4). Since GC-IMS is a rapid, sensitive and selective, cost-effective and non-destructive technique, which can be readily implemented on-site, it could be proposed as an initial screening technique for the geographical origin of CPO, prior to the utilisation of more costly and time-consuming targeted techniques.

Whilst the aim of this study was to assess the use of GC-IMS as a fingerprinting approach for discerning samples by origin, the pipeline used in this study allowed for feature extraction to

identify significant data points involved in the classification. For example, several data points may have formed a single peak that was only present in one group of samples and not the other, therefore it might have been of interest to identify this peak using the NIST2014 database and IMS library. However, in this study, there was no correlation between specific features and individual spectral peaks. The features with the greatest variance were spread across the spectra and likely represented global changes in total profiles, making peak picking and subsequent compound identification difficult. Nevertheless, this study has shown GC-IMS combined with chemometrics to be a feasible fingerprinting approach for discerning between CPO samples from Sabah and Peninsular Malaysia. Further work should be conducted on a larger sample size to increase the likelihood of detection of a specific geographical marker using feature extraction, followed by compound identification using NIST2014 and IMS databases.

Sample and group size have been major limitations in all previously published studies in this area. Our study is only one of two which has successfully discerned CPO samples by region of

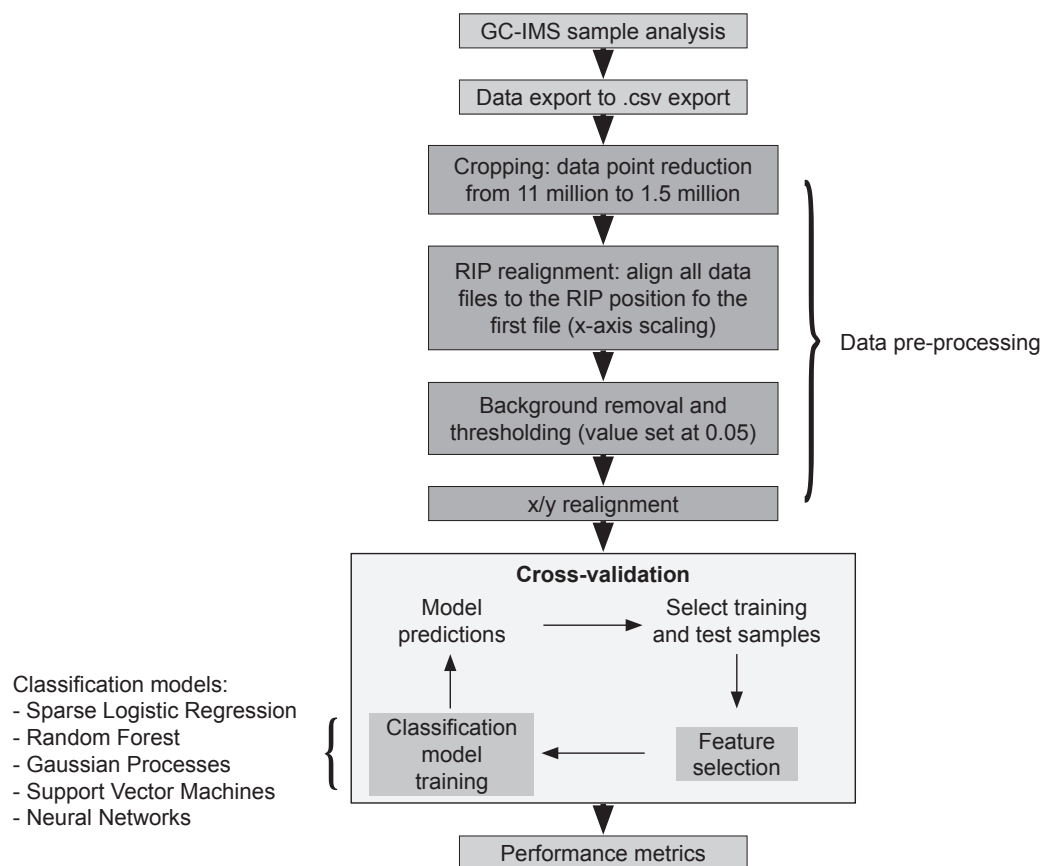


Figure 2. An overview of the general workflow used for classifying gas chromatography-ion mobility spectrometer (GC-IMS) data into Sabah or Peninsular Malaysia classes.

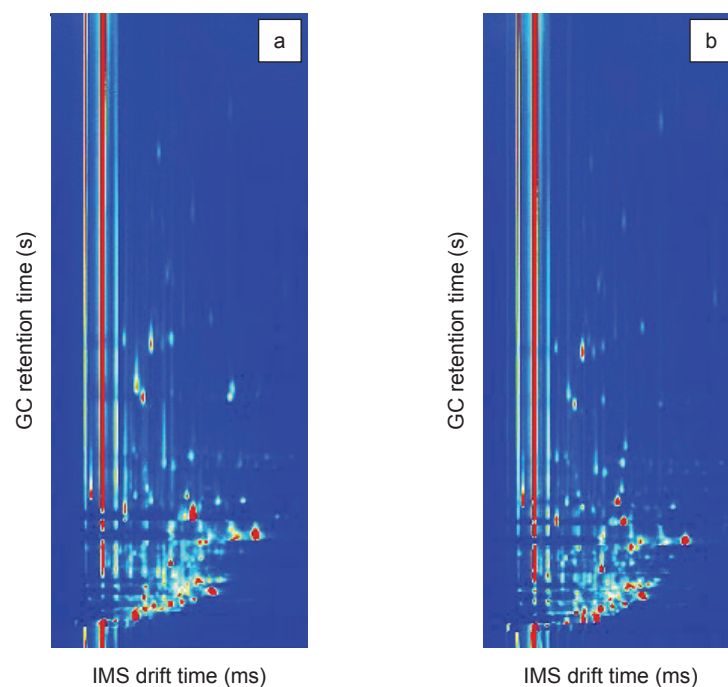


Figure 3. A side-by-side comparison of typical gas chromatography-ion mobility spectrometer (GC-IMS) spectra from Sabah (a) and Peninsular Malaysia (b) crude palm oil samples.

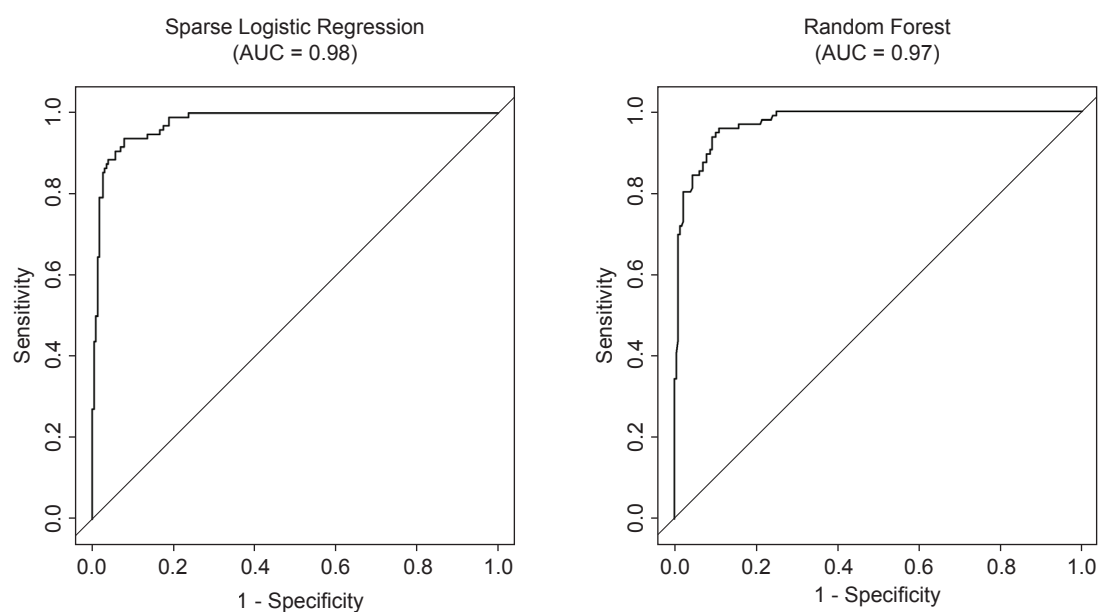


Figure 4. Examples of the receiver operating characteristics (ROC) curves, summarising the performance of each model used in study.

TABLE 2. MODEL PERFORMANCE COMPARISON FOR 100 FEATURES

Model	Area under curve (AUC)	Sensitivity	Specificity	<i>p</i> -value
Sparse Logistic Regression	0.98 (95% CI: 0.96-0.99)	0.94 (95% CI: 0.87-0.98)	0.92 (95% CI: 0.88-0.95)	<0.01
Random Forest	0.97 (95% CI: 0.96-0.99)	0.96 (95% CI: 0.90-0.99)	0.84 (95% CI: 0.84-0.93)	<0.01
Gaussian Process	0.96 (95% CI: 0.95-0.98)	0.91 (95% CI: 0.83-0.96)	0.91 (95% CI: 0.87-0.95)	<0.01
Support Vector Machine	0.96 (95% CI: 0.93-0.99)	0.95 (95% CI: 0.88-0.98)	0.95 (95% CI: 0.92-0.98)	<0.01
Artificial Neural Network	0.97 (95% CI: 0.96-0.99)	0.95 (95% CI: 0.88-0.98)	0.95 (CI: 0.92-0.98)	<0.01

origin and is the first to do so using a fingerprinting approach combined with chemometrics, on a much larger sample set. Nevertheless, even in this work, sample set is still a limitation because recommended minimum group size for chemometric analysis to be statistically significant is 30, which is close to that of the Peninsular Malaysia group (n=32). Furthermore, due to the availability of samples, group size in this study is not optimally balanced, meaning bias may be introduced. Any further work should be conducted on larger and better balanced groups. Nonetheless, this study has demonstrated promising results using the provided sample set and has shown that increased spatial specificity can be obtained. Further work should be conducted using CPO from the same mills/regions, but further studies should also ensure that samples are collected and analysed continuously over long-term periods of several months to years in order to capture as much variation as possible. In this way, it will be possible to successfully validate such approach and train predictive models more effectively.

Analytical methods for verification of geographical provenance of palm oils will have positive implications within the industry and will support and strengthen the current administrative controls in place. Whilst VOC fingerprinting is a well-studied approach and has been successfully used for other vegetable oils, further work should be undertaken annually, using as many authentic samples as possible, to assess the impact of seasonal variation, changes in fertilisation regime, changes in processing, *etc.*

CONCLUSION

Fingerprinting approaches combined with use of appropriate multivariate statistics (chemometrics) is common practice for authentication of foodstuffs. However, this is the first study of its kind that has shown that the application of chemometrics to raw chromatograms of GC-IMS data, is effective for discerning CPO samples by regional geographical provenance.

A single classification model was not selected for this study as the dataset is relatively small, alternatively five different models (linear and non-linear) were used and should continue to be used until a large enough dataset has been analysed. All models were successful in discerning CPO samples from Sabah and Peninsular Malaysia. Since GC-IMS is a rapid, sensitive and selective, cost-effective and non-destructive technique, which can be readily implemented on-site, it could be proposed as an initial screening technique for the geographical origin of CPO, prior to the utilisation of more costly and time-consuming targeted techniques.

Such analytical methods for verifying the geographical provenance of palm oils will have positive implications within the industry and will support and strengthen the administrative controls currently in place (Goggin and Murphy, 2018). This is only one of a few which have sought to distinguish crude palm oils by geographical origin and is only the second to do so on a regional level and the only one using GC-IMS. Whilst VOC fingerprinting is a well-studied approach and has been successfully used for other vegetable oils, further work should be undertaken annually to assess the impact of seasonal variation, changes in fertilisation regime, changes in processing, *etc.* A larger sample set should also be studied to determine whether further spatial specificity can be obtained (*i.e.* at mill or plantation level).

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ISOLATION AND CHARACTERISATION OF GIBBERELLIN 2-OXIDASE 6 GENE FROM OIL PALM (*Elaeis guineensis* Jacq.)

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ABSTRACT

To increase our understanding on the role of Gibberellin (GA) metabolism in oil palm (*Elaeis guineensis*), a gene encoding enzyme in GA catabolic pathways was identified and isolated. The oligonucleotide primers were constructed and used to amplify the EgGA2ox6 gene from oil palm. The sequence analysis of EgGA2ox6 gene provided a complete open reading frame (ORF) of 1014 bp which encodes 337 deduced amino acid residues. Homology sequence analysis has shown a 50%-70% sequence similarity to GA from other plant species. Based on this sequence motif analysis, the EgGA2ox6 is classified into C₁₉-GA2ox group. Expression studies on 13 different oil palm tissues showed that the highest expression profile of the gene was observed in the mesocarp tissue. Further expression analysis was conducted on the development stages of mesocarp using quantitative real-time polymerase chain reaction (qRT-PCR) showed that the gene was predominantly expressed in the early stages of mesocarp development at four weeks after anthesis (WAA). Then, the expression of EgGA2ox6 gene was gradually reduced as the fruit ripened. This preliminary study provides valuable resources to further elucidate the roles of GA2ox6 gene during oil palm fruit development.

Keywords: expression, GA 2-oxidase, isolation, mesocarp, oil palm.

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INTRODUCTION

Oil palm is a commercially important crop in the world with increasing production over the years. Malaysia is one of the palm oil producing countries that accounts for over one-third of world palm oil and fat production (Zulkifli *et al.*, 2017). Because of that, Malaysia plays a role in achieving the growing global need for oil and fat sustainably.

Gibberellin, also known as gibberellic acid (GA), is a phytohormone that plays essential roles in plant growth, in both vegetative and reproductive phases such as seed germination, stem elongation, leaf expansion, flowering and fruit development

(Hedden and Thomas, 2012; Daviere and Archard, 2013; Gupta and Chakrabarty, 2013; Rahman *et al.*, 2016). Accordingly, treatments with GA in agronomic sector have become a common practice for improvement of crop performance. Bioactive GA is among the most widely used as plant growth regulators (PGR) in order to 'fine-tuning' the crop plant to its particular growth condition (Rademacher, 2016). For example, in temperate fruit trees, PGR of GA₃ are used to stimulate the growth of parthenocarpic pears as well as for fruit set (Quinet and Jacquemart, 2015) and also improve fruit quality of cherries (Zhang and Whiting, 2013) and citrus species (Fidelibus *et al.*, 2002). Besides, bioactive GA_{4/7} is often used by apple growers to improve the fruit quality (Watanabe *et al.*, 2008). With the successive application of chemical regulator to the crop plantation, exogenous application of GA₃ to oil palm had also been studied and gave the effect

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to fruit width, fruit length, fruit weight and bunch weight (Sangwiroonthon *et al.*, 2017). However, the mechanism by which these effects are achieved has to be explored.

As alternative approach to the application of chemical regulator of GA would be genetic manipulation of their GA biosynthesis. The GA biosynthetic pathway has long been studied and most of the biosynthetic gene encoding enzymes have been identified and characterised in many plant species. Previous studies revealed that the level of bioactive GA is regulated by gene encoding enzymes from both biosynthetic and catabolic pathways (Olszewski *et al.*, 2002; Du *et al.*, 2015; Pan *et al.*, 2017). GA 20-oxidase and GA 3-oxidase are important regulating enzymes in the biosynthetic pathway (Yamaguchi, 2008; Hedden and Thomas, 2012; Salazar-Cerezo *et al.*, 2018). The regulation of this catabolic pathway involves GA 2-oxidase (GA2ox), a 2 β -hydroxylation enzyme that hydroxylates C-2 β of bioactive GA to produce a biologically inactive form (Martin *et al.*, 1999; Thomas *et al.*, 1999; Rieu *et al.*, 2008; Shan *et al.*, 2014).

Catabolism of GA maintains the level of endogenous GA through the reduction of bioactive GA, which is important in the regulation of plant growth. The genes encoding enzyme GA2ox were first identified by screening cDNA expression for 2 β -hydroxylase activity (Martin *et al.*, 1999; Thomas *et al.*, 1999). Early characterisation of GA2ox revealed that the enzyme hydroxylates the C-2 of active C₁₉-GA (GA₁ and GA₄) and its precursors (GA₉ and GA₂₀) to produce inactive forms of GA (GA₈, GA₃₄, GA₂₉, and GA₅₁) (Lange *et al.*, 2013; Chen *et al.*, 2016). Later, the gene encoding GA2oxs was able to catabolise the C₂₀-GA precursors (GA₁₂ and GA₅₃) and produce inactive GA (GA₁₁₀ and GA₉₇) (Schomburg *et al.*, 2003; Huang *et al.*, 2010; Chen *et al.*, 2016; Lange *et al.*, 2013).

GA2ox genes have been isolated from various plant species such as runner bean (*Phaeseolus coccineus*) (Dijkstra *et al.*, 2008), *Arabidopsis thaliana* (Thomas *et al.*, 1999), garden pea (*Pisum sativum*) (Lester *et al.*, 1999; Martin *et al.*, 1999) and rice (*Oryza sativa*) (Sakamoto *et al.*, 2001). The physiological functions of these genes have been determined, where overexpression of GA2ox genes in plants can cause dwarf phenotype (Lo *et al.*, 2008; Hu *et al.*, 2017) and in some cases, affect their reproductive development (Sakamoto *et al.*, 2001; Busov *et al.*, 2003; Zhou *et al.*, 2012; Huet *et al.*, 2017). Conversely, loss-of-function of these genes can result in a tall and slender phenotype (Martin *et al.*, 1999; Rieu *et al.*, 2008).

Considering the role of catabolic GA enzyme in response to the level of bioactive GA in plant biosynthetic pathways, we seek to identify gibberellin oxidase family in oil palm. In this study, we conducted the isolation and characterisation of

a GA2ox gene from oil palm (*EgGA2ox6*). We also examined the expression profile of the *EgGA2ox6* gene in vegetative and reproductive tissues of oil palm using reverse transcription-polymerase chain reaction (PCR) and real-time quantitative PCR (RT-PCR) and quantitative real-time (qRT-PCR). This result provides us some important information about the localisation function of *EgGA2ox6* gene in regulating GA catabolism pathway in oil palm and also gives an idea to further investigate the functional role of the gene.

METHODOLOGY

Plant Material

Oil palm tissues (*Elaeis guineensis*) were collected from several one-year-old of Deli *Dura* x AVROS *pisifera* (DxP) clonal oil palm seedlings. The oil palm seedlings were harvested and the tissues were categorised accordingly: shoot primordia, young leaf, young node, young internode, young root, mature leaf, mature node, mature internode, mature root, basal stem and cabbage. As for the sampling of the mesocarp tissue, oil palm fruits were collected at 4, 8, 12, 16, 20 and 22 weeks after anthesis (WAA). Kernel tissues were collected from fruits at 16 WAA. All tissues were cut into small sizes, flash frozen in liquid nitrogen and stored at -80°C.

Isolation of Total RNA from Oil Palm Tissues and cDNA Synthesis

To extract total ribonucleic acid (RNA) from oil palm, 100 mg of oil palm tissues was ground to powder in liquid nitrogen. Total RNA extraction was then carried out using innuPREP RNA mini kits (Analytik Jena, Germany) as per the manufacturer's protocol. The total RNA obtained was quantified by using NanoDrop spectrophotometer (ND-1000, ThermoScientific, USA). The quality and integrity of RNA were determined using Fragment Analyser (Advanced Analytical Technologies Inc., Ames, IA, USA). Then oligo (dT) primer and SuperscriptTM III Reverse Transcriptase (Invitrogen, USA) were used to reverse transcribe the total RNA, according to the manufacturer's protocol. After that, the synthesised cDNA was used as a template for RT-PCR amplification. For the amplification of 5'- and 3'- ends of cDNA, rapid amplification of cDNA ends (RACE) (Clontech, USA) were used to obtain full-length cDNA sequence.

EgGA2ox6 Gene Isolation

The sequence consensus available of GA2ox genes were used to search for putative oil palm GA-oxidase sequences available in Genomsawit

databases (<http://genomsawit.mpob.gov.my>). From the sequence obtained, a few sets of primers were designed using Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) that covered the GA-preferred motifs in the sequence. RT-PCR was performed using the Q5® High-Fidelity DNA Polymerase (NEB, USA) and specific primers (Table 1) according to the manufacturer's instructions. The cDNA generated from leaf tissue was used as the PCR template for preliminary screening of the target gene from oil palm. The RT-PCR reaction was carried out with initial denaturation step at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 56°C for 20 s and 72°C for 30 s and final extension at 72°C for 2 min. Beta-actin (β -actin) gene was used as an internal control that required 56°C of annealing temperature. The PCR fragment was observed on 1% (w/v) agarose. The gel was viewed using G: Box Syngene Images Analysers (Syngene, United Kingdom) and the targeted band was excised and purified using Gel Extraction Kit (QIAGEN, USA) and finally inserted into pMiniT vector (NEB, USA) prior to DNA sequencing. To obtain full-length cDNA sequence, amplification of 5' and 3'-ends of cDNA was performed using SMART™ RACE cDNA amplification Kit (Clontech, USA) together with the sense and anti-sense oligonucleotide primers (Table 1). PCR conditions for RACE were as follows: 40 cycles of 94°C for 30 s; 68°C for 30 s and 72°C for 3 min. The resulting PCR products were gel purified and cloned into a plasmid vector prior to sequencing.

Gene Analysis of *EgGA2ox6*

Analysis of *EgGA2ox6* gene sequence was carried out using *in-silico* approaches. The gene sequence obtained from the amplification of *EgGA2ox6* was

analysed and translated using ExPASy programme (<http://www.expasy.org/translate/>) to generate a deduced amino acid sequence. The amino acid sequence characterisation was predicted using the ProtParam tool (<https://web.expasy.org/protparam/>). Subcellular localisation of the protein was predicted using WoLF PSORT (<https://www.genscript.com/wolf-psort.html>) and TargetP tools (www.cbs.dtu.dk/services/TargetP/). The protein family of the gene was analysed using pfam database. The *EgGA2ox6* deduced amino acid residues were then submitted for NCBI BLASTP (www.ncbi.nlm.nih.gov) search to look for sequence similarities from other species. The genomic structure of *EgGA2ox6* was constructed using GeneWise (<https://www.ebi.ac.uk/Tools/psa/genewise/>). Multiple sequence alignment (MSA) was generated using Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) for selected GA2oxs. The motif sequences and protein domain identification were performed using MEME Suite tools (meme-suite.org). The phylogenetic tree of protein sequences was constructed using MEGA 6 (Tamura *et al.*, 2007) through Maximum Likelihood method with 1000 bootstrap replicates.

Expression Profile of *EgGA2ox6*

Expression analysis using semi-quantitative RT-PCR. For gene expression analysis, semi-quantitative PCR was carried out using synthesised cDNA of young and mature vegetative and also reproductive tissues. The tissues used were as follows: shoot primordia, young leaf, young node, young internode, young root, mature leaf, mature node, mature internode, mature root, basal stem, cabbage, mesocarp and kernel. Expression levels of

TABLE 1. LIST OF PRIMER SEQUENCES

Primer	Primer sequence
Gene amplification	
1. <i>EgGA2ox</i> (Forward)	5'-CATGGTGATCGCTTCAGGTCACTC-3'
<i>EgGA2ox</i> (Reverse)	5'-CATCTGCACGGAAGAGATCAAGG-3'
2. <i>EgGA2ox</i> (Forward)	5'-CATCATCCGTTCTCTCACTCTTTTCACCC-3'
<i>EgGA2ox</i> (Reverse)	5'-GCATGCTCTCCCTCTTTGTAATAACCCTG-3'
RACE	
5' <i>EgGA2ox</i>	5'-GCAGCGAGGCATTTGGGGTGTGGTC-3'
3' <i>EgGA2ox</i>	5'-GACCACACCCCAAATGCCTCGCTGC-3'
qRT-PCR	
<i>EgGA2ox</i> (Forward)	5'-GTCGACATCGTCTCACGGAT-3'
<i>EgGA2ox</i> (Reverse)	5'-ACACCAGCTTGTGGGAGAC-3'.
Control	
B-actin (Forward)	5'-TGAGAGGGAAATCGTGCCTG-3'
B-actin (Reverse)	5'-TGCTTGCTGATCCACATCTGC-3'

the gene in respective tissues were gauged by the intensity of the amplified products showed in the agarose gel. Then, the gene expression was further analysed by quantifying the expression in detail for each of the developmental stage.

Expression analysis using real-time quantitative (qRT-PCR). SYBR® Green Supermix (BioRad, USA) was used for qRT-PCR. PCR reactions were performed following this protocol: initial deactivation of 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. After amplification, the temperature starting from 65°C was gradually increased by 0.5°C every 5 s until 95°C. Each run was completed with a dissociation stage to generate melt curves for amplification specificity evaluation. The qPCR was conducted using CFX96™ RT-PCR Detection System (BioRad, USA). All qPCR data is a mean of three sample replicates. The cDNAs from mesocarp at 4, 8, 12, 16, 20 and 22 WAA were used as templates for qRT-PCR. Cyclophilin 2 (*Cyp2*) and Gibberellin-responsive protein 2 (*GRAS*) were used as reference genes to normalise the expression gene. Both of the reference genes were detected as suitable candidate genes for normalisation of the gene expression under oil palm seedlings and mesocarp tissue (Wong *et al.*, 2014; Yeap *et al.*, 2014; Halim *et al.*, 2019). The primers used for qRT-PCR are listed in Table 1. The relative normalised expression level was calculated according to the method of $2^{-\Delta\Delta C_q}$ (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

RNA Quality and Integrity Analysis

Prior to single-stranded cDNA synthesis, the purity and quality of intact total RNA are critical elements for the overall success of RNA-based analysis (Imbeaud *et al.*, 2005; Leige and Pfaffl, 2006; Azizi *et al.*, 2017). The extracted total RNA showed a purity level ranging from 1.8-2.0 ($A_{260/280}$ and $A_{260/230}$) with yield concentration of 100-500 ng μL^{-1} . The integrity of total RNA was checked using Fragment Analyser (Advanced Analytical Technologies, Inc., Ames, IA, USA) which resulted the RNA Quality Number (RQN) of the samples ranging between 8.0-9.2. A higher RQN value indicates a good quality of RNA sample. The RQN value of 1.0 is indicative of RNA degradation, whereas the value of up to 10.0 suggests that the RNA is fully intact (Wong and Pang, 2013). The RQN value is a proprietary algorithm linked to the Fragment Analyser system that has been shown to correlate with RNA Integrity Number (RIN), values as a quality metric indicator (Wong and Pang, 2013; Escobar and Hunt, 2017; Sanchez *et al.*, 2017). RNA quality values below 7.0 indicate high variation and

loss of statistical significance for gene expression when analysed by qRT-PCR (Jahn *et al.*, 2008). Furthermore, the RNA value of 7.0 or higher is a recommended range for experiment to proceed to cDNA synthesis and SMARTer RACE experiments. The digital gel image and electropherogram provided by PROsize (Data analysis software for Fragment Analyser) for the oil palm tissues showed clear bands of 25S and 18S rRNAs with no smearing along the migration of RNA sample (Figures 1a and 1b). Moreover, the measured electropherogram signals showed clear peaks of 25S and 18S rRNAs without any spike or abnormal fluctuation of the baseline. This indicated that the RNA sample was intact and in good quality.

Isolation and Structural Characterisation of *EgGA2ox6* cDNA

Oil palm orthologous gene analysis showed that there were several GA oxidase genes identified in oil palm genome. Out of many genes identified, one of them was selected to isolate and further analyse its expression. The putative gene was available in NCBI database with accession number of XM_010939891.3 and its LOC ID was LOC105057308. This gene shows similarity with two sets of gene prediction of *Pheonix dactylifera* 2-beta-dioxygenase 2-like, with LOC ID were LOC103709678 and LOC103701020 with 89.93% and 88.86% similarity, respectively (data not shown). Using the predicted GA2ox gene, the primers were generated covering the important motif sequence of the gene. Isolation of partial *EgGA2ox6* gene was performed using PCR amplification and the result showed the gene was of the expected size (Figure 2). Sequence analysis of the PCR product indicated that this fragment encodes a part of the GA2ox gene family. Results obtained from the RACE experiments presented a full-length cDNA including the 5' and 3'- untranslated regions (UTR) of the gene. The full-length cDNA sequence of *EgGA2ox6* was 1287 bp including a 5' and 3' UTR of 106 bp and 167 bp length, respectively. The open reading frame (ORF) of the gene was 1014 bp, encoding a deduced amino acid sequence of 337 residues. The predicted molecular mass of the protein was 37 kDa with a theoretical isoelectric point (pI) at 5.58. The *EgGA2ox6* gene was predicted to be located in the cytoplasm and nucleus, which is related to their functional location (Hedden and Thomas, 2000; Huang *et al.*, 2010).

Investigation of the structural features of *EgGA2ox6* gene was determined by using GeneWise tool where it presented that the *EgGA2ox6* gene consisted of two introns and three exons at chromosome 14. The sizes of exon I, followed by intron I, exon II, intron II and exon III were 382 bp, 1546 bp, 357 bp, 676 bp and 269 bp, respectively. The gene structure was constructed and illustrated as in

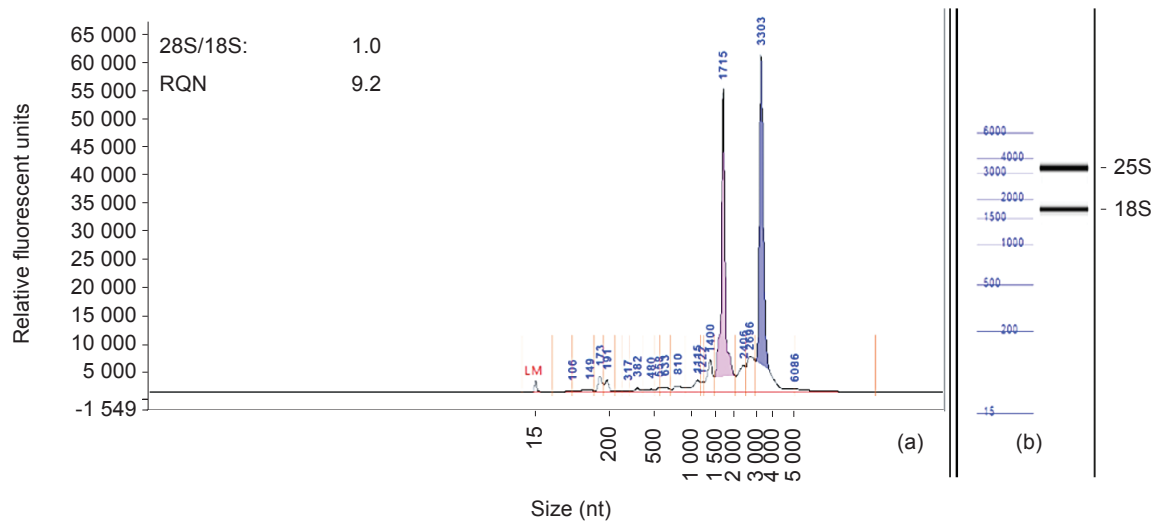


Figure 1. Electropherogram of total RNA generated from fragment analyser. (a) The blue peak corresponds to 25S rRNA and red peak corresponds to 18S rRNA. (b) The migration of 25S and 18S rRNA of total RNA by using fragment analyser.

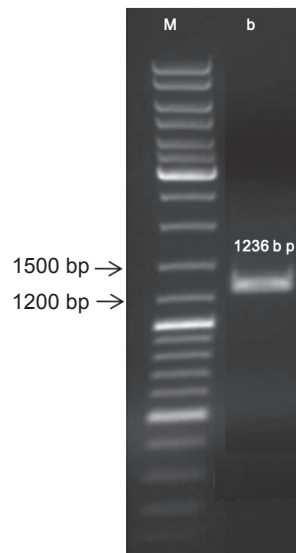


Figure 2. PCR amplification of EgGA2ox6. (M) GeneRuler™ Ladder Mix, (b) EgGA2ox amplified gene present in oil palm tissue. PCR amplification was performed using a specific primer to isolate GA2oxidase gene from oil palm leaf.

Figure 3. Comparative gene structure among GAox gene sequences showed that most of the GAox subfamilies have similar exon-intron numbers unless the gene undergo divergence evolutionary mechanism (Han and Zhu, 2011).

Analysis of the protein sequence of EgGA2ox6

demonstrated that the protein was categorised under the DIOX_N family which contains a conserved domain of 2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily (2-ODD). In general, this group of oxidative enzymes is part of the plant growth regulator

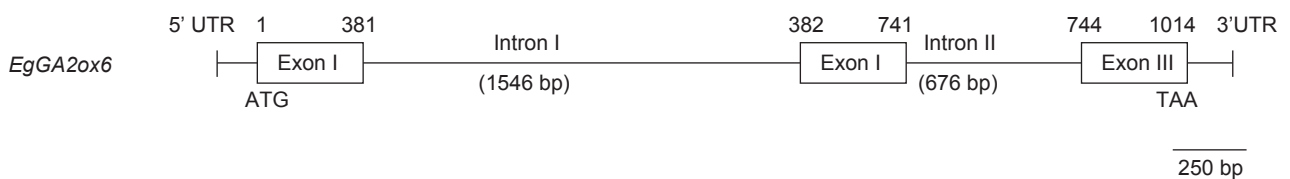


Figure 3. Schematic representation of the gene structure of EgGA2ox6. Numbers presented above the exon I, intron I, exon II, intron II and exon III indicate the position of bases in the coding sequence starting from ATG. Numbers below the exons and introns in parentheses indicate the size of each intron.

activation and catabolism as they are responsible for several structural modifications (Yamaguchi 2008; Pan *et al.*, 2017). The conserved domain of 2-ODDs in *EgGA2ox6* gene is likely to function similar to other GA2ox which is to catalyse the hydroxylation of active GA to inactive form (Lee and Zeevaart, 2005).

BLAST analysis of the deduced amino acid sequence with that of other available protein sequences in the database demonstrated that the deduced protein sequence shares sequence identity ranging from 50%-70% which is closely related to other GA2ox orthologues (Table 2). Alignment of the *EgGA2ox6* amino acid sequences with other GA2oxs highlighted a number of conserved motif and structural similarity among them. The genes shared common conserved motifs similar to other GA-oxidases that is a putative 2-oxoglutarate-binding (2-OG) and iron-binding sites (2-ODD). The site of 2-OG was located at Arg-269 and Ser-271 in *EgGA2ox6* sequence. For 2-ODD, the catalytic core consisted of iron atom chelated in an active site assisted by two His (His-198; His-259) and an Asp-200 in sequence composition of HXD/EXnH (Figure 4). The catalytic triad binds Fe(II) as a co-factor to assist in the catalysis of their substrates (Kawai *et al.*, 2014). In addition, the partial NXYPXC sequence of 2-ODD was also conserved in the sequence of *EgGA2ox6* as in other GA2oxs (Xu *et al.*, 1995; Xu *et al.*, 2002; Pan *et al.*, 2017). The sequence was located at Asn177-His177-Tyr178-Pro179-Pro180-Trp181 in the deduced protein sequence. This putative sequence was proposed to be involved in the binding of a common co-substrate (2-oxoglutaric acid) (Xu *et al.*, 1995; Xu *et al.*, 2002; Pan *et al.*, 2017). Furthermore, the sequence motif of GYGXnNGDXnEYL was also presented in the sequence, as it was highly conserved among C₁₉-GA2oxs gene. Thus, it proved that the *EgGA2ox6* is classified under C₁₉-GA2oxs group. This

conserved region was suspected to be involved in C-2 hydroxylation of active C₁₉-GA2oxs (Huang *et al.*, 2015; Chen *et al.*, 2016). Although the overall sequence similarity of the GA2ox genes is quite low, the amino acid sequence at its catalytic site is well conserved closely to the carboxyl-terminal of the sequence.

Phylogenetic Tree Analysis of *EgGA2ox6*

To classify the *EgGA2ox6* sequence among various GA2oxs, a phylogenetic tree was constructed and 15 discrete motifs were identified. From the phylogenetic analysis, the GA2oxs were divided into two main classes based on sequence conservation (Figure 5). The first main lineage consisted of GA2ox gene from six different plant species: *Manihot esculenta* (MeGA2ox), *Hevea brasiliensis* (HbGA2ox), *Jatropha curcas* (JcGA2ox), *Populus tomentosa* (PtGA2ox), *Prunus persica* (PpGA2ox) and *Durio persica* (DpGA2ox). Meanwhile, the second lineage was formed by GA2ox from *Ananas comosus* (AcGA2ox), *Elaeis guineensis* (EgGA2ox6), *Asparagus officinalis* (AoGA2ox), *Dendrium catenatum* (DcGA2ox) and *Phalaenopsis equestris* (PeGA2ox). *EgGA2ox6* was closely related to *AcGA2ox* (*Ananas comosus*) as they shared the same sub-lineage. Moreover, *AcGA2ox* shared a similar genomic structure as *EgGA2ox6* since the length and number of exons/introns were similar. The similarity of both genes might reflect by the shared functional conservative of the genes (Huang *et al.*, 2015).

Essentially, all GA2ox proteins shared similar conserved motifs except for motif 15. Motif 2 was located in the DIOX_N domain, while 2OG-FeII_Oxy domain included motif 1, motif 7 and motif 8. Motif 15 was shared only by four GA2ox – *Manihot esculenta* (MeGA2ox), *Hevea brasiliensis* (HbGA2ox), *Jatropha curcas* (JcGA2ox) and *Populus tomentosa* (PtGA2ox). Motif 15 may indicate a conserved functional role shared by these four

TABLE 2. BLAST RESULTS OF *EgGA2ox6*

No.	Species	Max. score	Total score	Query cover (%)	E-value	Identity (%)	Accession No.
1	<i>Asparagus officinalis</i>	441	441	99	2e-152	61	XP020254363.1
2	<i>Ananas comosus</i>	434	434	98	1e-149	66	ONK78771
3	<i>Durio zibethinus</i>	412	412	98	3e-141	59	OAY7773171
4	<i>Jatropha curcas</i>	409	409	96	3e-140	60	XP012082949.1
5	<i>Dendrobium catenatum</i>	410	410	98	6e-140	57	XP020701220.1
6	<i>Manihot esculenta</i>	405	405	96	1e-138	60	XP0216232581
7	<i>Phalaenopsis equestris</i>	403	403	93	9e-130	60	XP020582243.1
8	<i>Prunus persis</i>	394	394	97	6e-134	57	XP0072214871.1
9	<i>Populus tomentosa</i>	392	392	96	2e-132	57	AGW52151.1
10	<i>Hevea brasiliensis</i>	391	391	96	6e-133	59	XP021639858.1

[illegible]

Figure 4. The conserved residues of EgGA2ox with GA2ox from other plants include the catalytic site of 2-oxoglutarate-dependent dioxxygenase (pink) and iron-binding (red). The conserved general motifs in GA2ox and the putative C₁₉-GA2oxs sites are indicated in green and yellow, respectively. The asterisk (*) and period (.) symbols are indicated identical, conserved and semi-conserved substitutions, respectively.

plant species. It may be due to the evolutionary conservation of protein sequence among GA2ox family members.

Expression Profile of *EgGA2ox6* Genes in Different Oil Palm Tissues

The expression of *EgGA2ox6* gene was analysed in 13 different oil palm tissue samples which were spear leaf, matured leaf, young node, matured node, young internode, matured internode, white root, brown root, shoot primordia, cabbage, basal stem, mesocarp and kernel. The expression level of *EgGA2ox6* was indicated by the intensity of the amplified band in the agarose gel (Figure 6). *EgGA2ox6* transcript was strongly expressed in mesocarp tissue and scarcely expressed in mature leaf and mature internode. Other than this, no expression was observed in other tested tissues. From the expression results obtained, *EgGA2ox6* exhibited a tissue-specific expression in mesocarp, though there were traces of gene expression in mature leaf and internode. The expression of *EgGA2ox6* in mesocarp was analysed in detail, starting from early developmental stages of mesocarp development until the fruit ripened.

The Expression Pattern of *EgGA2ox6* Gene during Mesocarp Development

Melt curve was performed to examine the specificity of the primer to amplify the target gene. The melt curve profiles clearly demonstrated that the qPCR assays produced single and specific product by producing a single peak for each sample. Later, the relative normalised expression of *EgGA2ox6* in every stage of mesocarp tissues were conducted and analysed. The relative quantity of gene in each tissue was normalised to the relative quantity of the reference gene used. Throughout the developmental period of the mesocarp tissues, the *EgGA2ox6* transcription was predominantly expressed in 4 WAA of the mesocarp tissues. The expression was shown to decrease about two-thirds in Week 8, and slightly increased in Week 12 and then decreased until Week 22 (Figure 7). Oil palm fruit takes 20-22 weeks to ripen from the anthesis stage by completing its development, maturation and ripening stage. Initially, a fruit set represents a very early fruit development stage which undertakes cell division and expansion along with initial increase in fruit mass and size. These activities are coordinated by the concerted action of the many phytohormones including GA. High expression of *EgGA2ox6* gene indicates that the GA deactivating gene is important for establishing suitable GA levels during early fruit set development. There were reports where the levels

of bioactive GA₃ and GA₄ were intricated during oil palm mesocarp development (Teh *et al.*, 2014). Both bioactive hormones presented a clear opposite trend throughout fruit ripening as GA₃ appeared to be higher in Week 12 and decreased throughout the ripening process. Meanwhile, expression of GA₄ was said to increase throughout fruit ripening (Teh *et al.*, 2014; Transberger *et al.*, 2011). The expression pattern of *EgGA2ox6* throughout fruit development was thought to be coordinated with the expression level of bioactive GA₄ as the *EgGA2ox6* may be involved in its catabolic pathway. Throughout the maturation stages, the mesocarp fruit mass was increased and reached the competence to ripen about 16-18 WAA. As the ripening progressed, the expression level of *EgGA2ox6* was generally low possibly due to a major switch in relative hormone levels of the fruit ripening. Low expression of *EgGA2ox6* at stages of ripening was concomitant with the high accumulation of GA₄ at later stages of fruit ripening. In particular, GA₄ was said to be an important bioactive hormone in oil palm fruit ripening (Teh *et al.*, 2014) as well as in cucumber (Nakayama *et al.*, 1991) and *Arabidopsis* (Cowling *et al.*, 1998). Involvement of this catabolic enzyme was previously shown in plum fruit development (*Prunus salicina* L.) (El-Sharkawy *et al.*, 2012) where the *PslGA2ox* transcript was expressed mainly throughout immature stages before ripening. The same goes to tomato fruit, where the *SIGA2ox1* shows high expression in the fruit at the immature green stage but is barely expressed in the red ripening stage (Chen *et al.*, 2016; Serrani *et al.*, 2007).

Studies on the relationship between GA and fruit development was conducted by Chen *et al.* (2016) and Li *et al.* (2019). The expression of fruit-specific *SIGA2ox1* in tomato causes reduction of GA level in fruit tissues that leads to early ripening in transgenic tomato. The study also found out that the injection of GA biosynthesis inhibitor, prohexadione-Ca into mature-green fruits resulted in an early fruit ripening which is consistent with the previous observation in transgenic tomato fruit. This was a proof of the regulating role of the GA in fruit ripening. In addition, low level of GA concentration in fruit also accelerates the expression of ripening regulator, and finally triggers the expression of ethylene biosynthetic genes for early ripening compared to normal fruit ripening (Li *et al.*, 2019). There was also a report on the relationship between *AcGA2ox* and its physiological disorder of the harvested pineapple (*Ananas comosus*) which is a monocot plant. This study has shown that the expression of *AcGA2ox* resulted in reduced levels of bioactives GA, which triggered response in regulating of transcriptional of *AcPPO* (pineapple polyphenol oxidase) gene. *AcPPO* gene was upregulated in response to the level of GA_{4/7}. By increasing the expression of

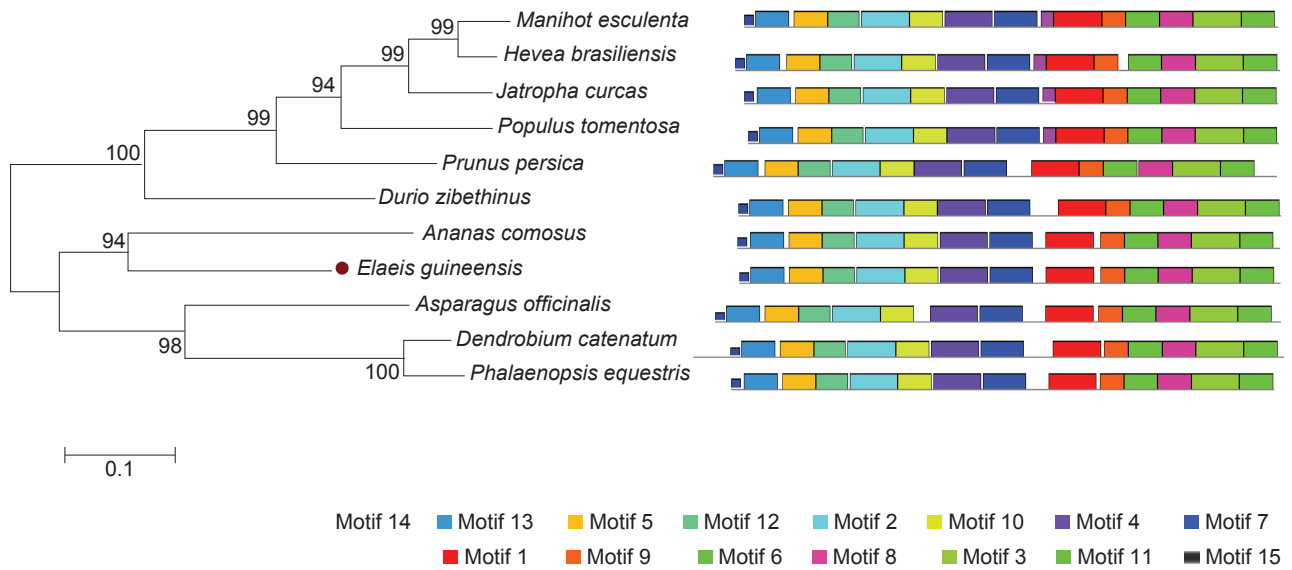


Figure 5. Phylogenetic tree and conserved motif analysis of EgGA2ox protein sequences among different plant species. The motifs were identified using MEME software and a phylogenetic tree was constructed using MEGA6 software. Fifteen motifs were identified and differentiated as shown by coloured boxes, including the distribution of each motif along the sequences corresponding to their positions.

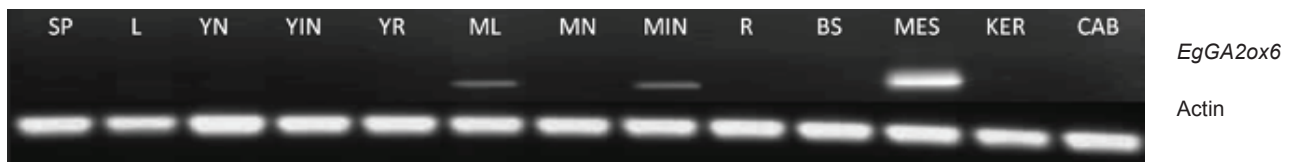


Figure 6. Expression profile of EgGA2ox6 in different tissues of oil palm. The oil palm tissue samples included: SP, shoot primordia; L, leaf; YN, young node; YIN, young internode; YR, young root; ML, mature leaf; MN, mature node; MIN, mature internode; R, root; BS, basal stem; MES, mesocarp; KER, kernel; CAB, cabbage. Actin represents control.

AcGA2ox, led to a decrease transcription level of *AcPPO*, which then resulted in a decreased oxidation of phenolic compound that caused internal browning of pineapple (Zhang *et al.*, 2016). Physical damages on fruits affect the quality and lead to a severe loss of their commercial value.

We envisage that *EgGA2ox6* as one of the GA catabolic enzymes that could metabolise the reduction of GA level in oil palm mesocarp. In

normal oil palm mesocarp development, different accumulation of *EgGA2ox6* throughout mesocarp development showed the importance of its catabolic enzyme in stabilising GA level in oil palm fruit. Modulating the expression of the enzyme could cause changes in phenological characteristic of reproductive growth, as well as substantial disorder that could give significant modification in fruit cycle.

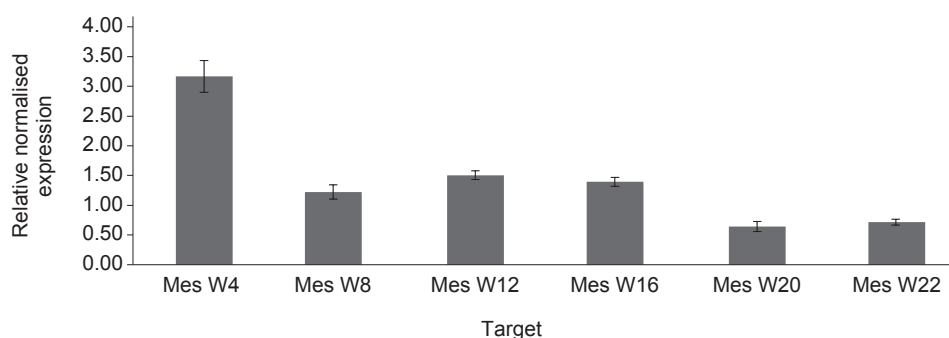


Figure 7. Expression pattern of EgGA2ox6 in different developmental stages of mesocarp tissues. The transcript levels were determined by qRT-PCR. Bars indicate standard error (SE).

CONCLUSION

In summary, *EgGA2ox6* gene was successfully identified and isolated in oil palm. Motif analysis and MSAs suggested that *EgGA2ox6* belongs to the 2-ODD C₁₉GA2ox class. Based on expression profile analysis, we can conclude that *EgGA2ox6* demonstrated a GA deactivating gene-specific that regulate GA metabolism in oil palm mesocarp. The identification and expression study of *EgGA2ox6* would provide fundamental molecular information to further analyse GA metabolic regulation in oil palm, particularly in mesocarp development.

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EXPRESSION OF CADHERIN GENE IN *Bacillus thuringiensis* Cry1A TOXIN RESISTANT AND SUSCEPTIBLE STRAINS OF *Tirathaba mundella*

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ABSTRACT

Bacillus thuringiensis (Bt.) insecticide has since been used as an essential insecticide in commercial oil palm plantations to manage insect pests. Resistance against Bt. toxins has been reported in many pest insects and threatening the effectiveness of Bt. insecticide. However, the insecticide resistance capability of *Tirathaba mundella* (T. mundella) was not well documented due to its finite molecular data. In this study, the potential of insecticide resistance development in T. mundella was examined through bioassay and cadherin gene expression analysis. Cadherin gene which often associated with the resistance against Bt. toxin was amplified and its relative expressions were studied using quantitative polymerase chain reactions. T. mundella collected from the plantations with frequent exposure to Bt. insecticide was found to develop resistance to Cry1A with a significantly lower cadherin gene transcript level and lower mortality compared to the susceptible strain. Besides, a relatively lower level of cadherin gene expression was found in the early development stages of the resistant strain. The adaptation of larvae to the Bt. insecticide may be caused by the irregular application of insecticide in the field.

Keywords: Bt. toxin, cadherin, expression profile, resistance, *Tirathaba mundella*.

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INTRODUCTION

Over the past decades, *Bacillus thuringiensis* (Bt.) which produce species-specific Cry toxin has been broadly applied to control pest outbreak and incorporated with plants to develop transgenic crops (Jouzani *et al.*, 2017). The Cry toxins are activated by the digestive protease in the insect midgut after ingested (Vachon *et al.*, 2012). The activated

toxins will then bind to the protein receptors which include cadherin, aminopeptidase-N and alkaline-phosphatase on the brush-border membrane (Pacheco *et al.*, 2009). The binding facilitates oligomer formation and integration of toxin into the midgut membrane. The insertion resulting in pore formation, cell lysis and cause the death of the insect (Sanahuja *et al.*, 2011).

The prevalence of Bt. insecticide to control agricultural pests has found to threaten its efficacy due to the development of resistance to Bt. toxins. The downregulation of cadherin gene has been discovered to contribute to the resistance mechanism (Tabashnik, 1994). Cadherin is a protein responsible for the calcium-dependent cell adhesion and cell sorting mechanism. It was reported as one of the binding receptors of Cry toxin in the midgut of Lepidopteran, Coleopteran and Dipteran insects. A mutation in the cadherin gene was found to be associated with the resistance to the Bt. toxin (Bel and Escriche, 2006). In the resistant strain of *Pectinophora*

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gossypiella, a deletion mutation of cadherin gene was discovered to confer resistance to Cry1Ac toxin (Wang *et al.*, 2018).

Bt. insecticide has since been adopted in the oil palm industry to control the infestation of *Tirathaba mundella* (*T. mundella*) (Lepidoptera: Pyralidae). *T. mundella* is a major pest that causes severe harms to oil palm, particularly those planted on peatland (Masijan *et al.*, 2015; Yaakop and Shafariza, 2015). The larval stage makes an opening on the immature fruit bunch and boring holes into the mesocarp. The infestation damaging the male and female inflorescence as well, resulting in the malformed or stunted developed bunches (Alouw *et al.*, 2005). The weight and quality of the bunches may greatly affect by the infestation and the damaged fruit bunches may fall prematurely (Yaakop and Shafariza, 2015). An uncontrolled infestation of *T. mundella* could cause 50% reduction in fresh fruit bunch (FFB) production (Su *et al.*, 2016). Saharul *et al.* (2017) reported an area of more than 47 700 ha of oil palm plantation in Malaysia was affected by the infestation. *Bt. var. kurstaki* has been reported as an effective bioinsecticide to control the infestation (Huan, 2012; Prasetyo *et al.*, 2018; Saharul *et al.*, 2017). Huan (2012) reported that six continuous fortnightly sprays of *Bt. var. kurstaki* over a three-month period were able to bring down the infestation to less than 15% in a plantation with 50% of infested bunches. Nevertheless, the potential of *T. mundella* to develop *Bt.* insecticide resistance under an inconsistent schedule of field spraying practices has not been well evaluated. Besides, the lack of molecular data for *T. mundella* has greatly constrained the study of its resistance mechanism.

In the current study, the cadherin gene of *T. mundella* was amplified and its expression was examined on the insects from plantations with different pest management practices. The objectives

were to study the development of resistant strains after a long-term of irregular insecticide exposure and evaluate its association with the gene transcript level at different developmental stages. The findings of this study could provide insight into the molecular mechanism of the resistance to Cry1A toxin.

MATERIALS AND METHODS

Sample Collection

Tirathaba mundella specimens were collected from two peatland oil palm estates with similar cultivation practices but different pest control management: a) Sabaju Estate (KM 31, Bakun Road, Sarawak, Malaysia) was frequently applied with Dipel, a commercial formulation of *Bt. var. kurstaki* with Cry1A toxin when the infestation of *T. mundella* was reported in the area (Figure 1). The insecticide was applied to the male and female inflorescences and fruit bunches of the infested palm using the manufacturer's recommended dosage. b) Tinbarap Estate (KM 22, Miri – Bintulu Road, Sarawak) was not exposed to any insecticide over a one-year period when the larvae were collected due to budget constraints (Figure 1). Although the two estates are maintained with different pest management methods, the land selection, harvesting and environmental management of both estates are carried out in accordance with the same protocol. Sabaju and Tinbarap Estates are established in mixed peat swamp forest areas and planted with oil palms for more than 10 years old. The soil series of Sabaju and Tinbarap estates are Sabaju and Gondang soil series respectively (Su, 2019).

Infested fruit bunches with fresh reddish-brown frass of *T. mundella* were carefully cut down and chopped into small pieces. A number of 200 larvae



Figure 1. Map of Sabaju Estate and Tinbarap Estate, Sarawak, Malaysia.

of different instars were screened from the chopped fruit bunches. Collected larvae were supplied with an artificial diet developed by Universiti Putra Malaysia Bintulu Campus (UPMKB) (Su, 2019). The diet contains essential nutrients required for the growth of larvae. Sucrose and casein were included in the diet ingredient as the source of carbohydrate and protein, respectively. Wesson salt was added as mineral sources while vitamin sources were provided by Vanderzant vitamin and ascorbic acid (Hervet *et al.*, 2016). Palm male inflorescence was added as phagostimulant to facilitate the adaptation of larvae toward the change of environment and food source (Morton, 1981). The insect rearing environment was maintained at 25°C-27°C and 12 hr light: 12 hr dark photoperiod (Su *et al.*, 2019).

Bioassay

For the bioassay, 157 mg ml⁻¹ of Cry1A toxin was prepared from the UPMKB insecticide collection following the suggested application amount. The toxin was incorporated in the artificial diet and sprayed on the diet surface. The sprayed diet was allowed to air dry before the placement into a rearing container. Cry1A toxin was replaced with sterile distilled water for the negative control. Healthy fourth instar larvae of *T. mundella* were released into the rearing container after starving for 12 hr to promote diet consumption. The bioassay was conducted in 15 replicates with one larva in each rearing container. Larval mortality was recorded after 96 hr of exposure to a toxin treated diet. The insects were identified as alive if they respond to touch (Yang *et al.*, 2009). The insects were transferred to a new rearing container with a normal diet after five days of toxin exposure to complete their normal development (Xu *et al.*, 2005).

Total Ribonucleic Acid (RNA) Isolation and Complementary DNA (cDNA) Synthesis

Two sets of insects were collected for ribonucleic acid (RNA) isolation: a) fourth instar *T. mundella* after exposing to Cry1A toxin (0, 2, 8, 24 and 48 hr), and b) different developmental stages of *T. mundella* under normal rearing conditions (fourth to seventh instar larvae and pupae) (Ayra-Pardo *et al.*, 2015). Whole bodies of insects were first chopped into smaller pieces with a scalpel to enhance the grinding process. Total RNA of *T. mundella* was extracted by using Tissue Total RNA Mini Kit (Favorgen, Ping Tung, Taiwan) according to the manufacturer's instructions. RNase Quiet (Nacalai Tesque, Kyoto, Japan) was treated on all apparatus to avoid RNase contamination. The extracted RNA was stored immediately at -80°C. Quantification of extracted RNA was performed using 1% TAE agarose gel electrophoresis. The extract was treated

with DNase (Toyobo, Tokyo, Japan) to avoid DNA contamination. First strand complementary DNA (cDNA) was synthesised using Revertra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) following manufacturer's protocols. Real time minus (RT-minus) control was reverse transcribed together with the specimens with the absence of RT enzymes as the negative control (Raymaekers *et al.*, 2009). The cDNA was kept at -20°C until further analysis.

Cadherin Gene Amplification

In the absence of gene sequence data for *T. mundella*, primers designed for the amplification of cadherin gene fragment of *Chilo suppressalis* (JQ747493) were selected to amplify the cadherin gene of *T. mundella* (Table 1). The polymerase chain reaction (PCR) reaction was performed using XP Cycloer (Bioer, Tokyo, Japan) under the condition of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 44°C for 2 min and elongation at 72°C for 1 min. The reaction ended with the final elongation at 72°C for 10 min.

The β -actin gene sequences of closely related Lepidoptera species (*Plutella xylostella*, NM_001309101.1; *Heliothis virescens*, AF368030.1; *Helicoverpa armigera*, HM629441.1; *Bombyx mori*, NM_001126254.1; *Manduca sexta*, L13764.1) were collected from National Center for Biotechnology Information (NCBI) database and aligned with Mega version 7. Gene specific primers were designed from the conserved region using PriFi (<https://services.birc.au.dk/prifi/>) (Table 1). The amplification of β -actin region was performed under the same PCR protocol with the annealing temperature of 54°C.

The PCR product was visualised using a 1% agarose gel electrophoresis. The amplified fragment was purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and sequenced by the Apical Scientific Sdn. Bhd. The amplified cadherin fragment of *T. mundella* was aligned with other Lepidopteran cadherin sequences to construct a maximum likelihood tree using Mega version 7 with 1000 replicates bootstrap support. Based on the sequences obtained, primers encoding for cadherin and β -actin gene with an amplicon length of 120 bp and 134 bp respectively were designed by using

TABLE 1. PRIMER SEQUENCES FOR THE AMPLIFICATION OF TARGETED GENES

Gene		Primer sequence
CadF	Sense	5'-GACGGAACCTCAGCAAACAAC-3'
CadR	Antisense	5'-CTCGGAATTATCAGTGGCAATG-3'
ActinF	Sense	5'-ATGTGCAAGGCCGTTTCGC-3'
ActinR	Antisense	5'-CTCGGTGAGGATCTTCATGAGG-3'

NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) for gene expression analysis (Table 2).

TABLE 2. PRIMER SEQUENCES FOR QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Gene	Primer sequence	
TmACTF	Sense	5'-GTCGTACCACCGGTATCG-3'
TmACTR	Antisense	5'-CATGAGGTAGTCGGTCAAGTC-3'
TmCADF	Sense	5'-GCCGTCAGGCGCTCTATTTA-3'
TmCADR	Antisense	5'-TCGCAGCAATACACACCACA-3'

Quantitative Reverse Transcription Polymerase Chain Reaction Analysis of Cadherin Gene

Quantitative polymerase chain reaction (qPCR) was performed using Bio-rad Mini Opticon PCR system (Bio-Rad, California, USA) to investigate the cadherin gene temporal expression pattern of fourth to seventh instar larvae and pupae of *T. mundella* as well as the relative gene transcript level of larvae after feeding on a toxin treated diet for 0, 2, 8, 24 and 48 hr. The qPCR reaction contained 4 µl of the ThunderBird SYBR qPCR Mix (Toyobo, Osaka, Japan), 10 pmol of forward and reverse primer, 1 µl of the first strand cDNA template (150 ng µl⁻¹) and double distilled water (ddH₂O) to the total reaction volume of 20 µl. The three steps qPCR program was performed as follows: 1 min of initial denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 15 s, annealing for 30 s and 1 min extension at 60°C. The annealing temperature for cadherin and β-actin primer were 53°C and 59°C, respectively. The samples were heated up to 95°C for melting curve analysis. Water and RT-minus control were served as a negative control to detect primer-dimers and genomic DNA contamination (Ren *et al.*, 2013). Only the amplification with single peak in melting curve analysis and amplification efficiency of 95%-100%

was selected for gene expression analysis (Guo *et al.*, 2015).

Data Analysis

The relative abundance of the cadherin gene was normalised to the reference gene β-actin and expressed using the relative expression method (2^{-ΔΔC_t}). Cadherin expression in each development stage was normalised against the expression of the pupae from Tinbarap Estate. To ensure the validity of the data, three technical replicates and three biological replicates were tested for each treatment (Yang *et al.*, 2012). Gene expression and bioassay data were statically analysed by using SAS software (SAS version 9.4). The mean difference of the gene expression at different development stages and during bioassay were determined using Tukey's HSD (p<0.05). The significant difference in gene expression, mortality and adult emergence rate between the sampling sites were analysed with independent-sample t test.

RESULTS

Resistance Gene

A fragment of 773 bp of gene sequence was amplified from *T. mundella* cDNA template. Identical sequence was amplified from the sample collected from Sabaju and Tinbarap Estates. The evolutionary of the cadherin gene was evaluated with seven Lepidoptera sequences from NCBI database. *T. mundella* gene sequence showed a genetic distance range of 0.722 to 0.864 compared to the other cadherin gene sequences (*Ostrinia furnacalis*, EU022587.1; *P. xylostella*, NM_001305494.1; *Pectinophora gossypiella*, KJ480752.1; *M. sexta*, AY094541.1; *B. mori*, AB026260.1; *Spodoptera exigua*, KC907716.1; *C. suppressalis*, JQ747493.2) (Table 3 and Figure 2).

TABLE 3. ESTIMATES OF EVOLUTIONARY DIVERGENCE BETWEEN CADHERIN SEQUENCES

No.	Sample	1	2	3	4	5	6	7	8
1	<i>Ostrinia furnacalis</i>								
2	<i>Plutella xylostella</i>	0.412							
3	<i>Pectinophora gossypiella</i>	0.493	0.517						
4	<i>Manduca sexta</i>	0.471	0.495	0.408					
5	<i>Bombyx mori</i>	0.472	0.496	0.497	0.475				
6	<i>Spodoptera exigua</i>	0.551	0.575	0.576	0.554	0.435			
7	<i>Tirathaba mundella</i>	0.839	0.863	0.864	0.842	0.723	0.722		
8	<i>Chilo suppressalis</i>	1.007	1.031	1.032	1.010	0.891	0.890	0.896	

Source: Kumar *et al.* (2016).

Note: The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Tamura-Nei model (Tamura and Nei, 1993). The analysis involved eight nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There was a total of 747 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

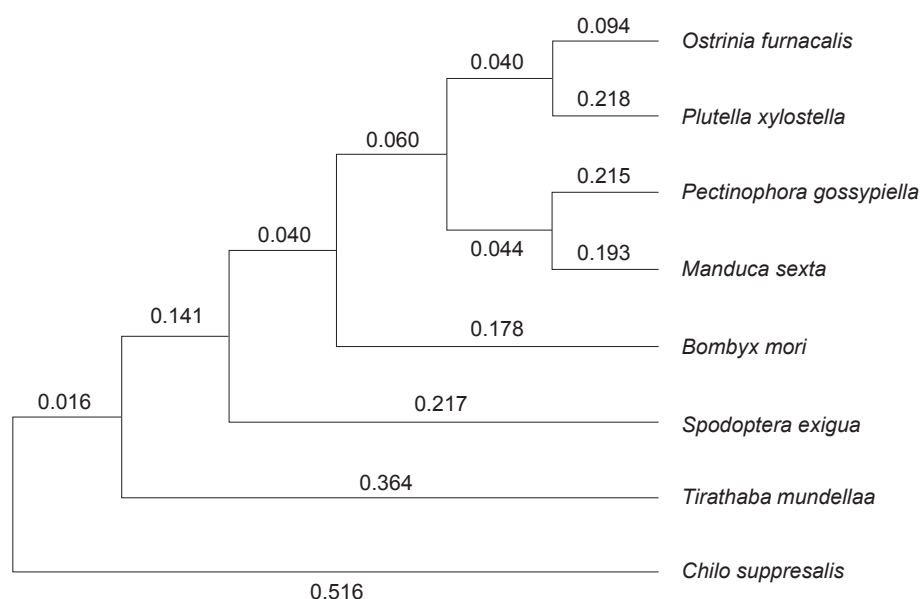


Figure 2. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-5854.7770) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

Mortality Assessment

Higher mortality was recorded on the sample collected from the Tinbarap Estate compared to the Sabaju Estate after the larvae were treated with the diet containing Cry1A toxin. An increment of 80.2% of mortality was found among the Tinbarap Estate samples after exposed to *Bt.* insecticide. On the other hand, larvae from Sabaju Estate showed a mortality rate of 66.8% lower compared to the larvae from Tinbarap Estate and its mortality rate was not affected by the toxin after 96 hr of exposure (Table 4). For the adult emergence rate, *T. mundella* from Sabaju Estate reduced significantly from $93.4 \pm 6.6\%$ to $53.4 \pm 8.3\%$ after toxin intake while all the larvae from Tinbarap Estate were failed to turn into moths. The insects were found dead in its larval stage or in the cocoon of its pupal stage (Table 5).

Cadherin Gene Expression of Development Stages

The expression of the cadherin gene was found to be significantly higher in the seventh instar of the Sabaju Estate sample and fourth instar of the Tinbarap Estate sample. The cadherin transcript level of Sabaju Estate sample increased from fourth to seventh instar larvae and reduced when it turns into pupae. Larvae of the seventh instar showed significantly higher cadherin expression level which increased from 0.2940 ± 0.0620 at sixth instar to 5.6839 ± 0.7847 at seventh instar. In contrast, significantly higher relative abundance was observed at the fourth instar of the Tinbarap Estate sample. The

relative cadherin abundance of 23.8145 ± 2.8313 was recorded at the fourth instar of the Tinbarap Estate sample. The expression level reduced significantly from fourth to the fifth instar and increased gradually until its pupal stage (Table 6).

Stage-specific expression analysis was performed between the sample from both estates. The cadherin expression level of the pupal stage of the Tinbarap Estate sample was set at 1 for the analysis. Significantly higher relative transcript level was found at the fourth, fifth and sixth instar larvae of the Tinbarap Estate sample. *T. mundella* of Sabaju Estate showed significantly higher cadherin expression at the seventh instar larvae and pupae stage compared to the specimens from the Tinbarap Estate (Table 6).

Cadherin Gene Expression Under *Bacillus thuringiensis* (*Bt.*) Insecticide Exposure

The transcript amount of cadherin of Sabaju Estate sample increased from the initial expression of 0.0016 ± 0.0002 to 0.3233 ± 0.0669 at the second hour after the intake of the toxin. The relative expression was reduced gradually to 0.0124 ± 0.0018 after 48 hr. An opposite trend occurred when no toxin was consumed, the relative expression of cadherin increased significantly only after 48 hr. The expression pattern of *T. mundella* of Tinbarap Estate was not altered by the consumption of Cry1A toxin. The relative abundance of cadherin reduced significantly from the beginning to the second hour (Table 7).

The PCR products were electrophoresed to ensure the validity of the result, a single band of DNA was observed on the agarose gel. Melting curve analysis verified that the amplification of non-specific products and primer-dimer were eliminated (Figure 3). This supports that the difference in gene transcript level is responsible for its abundance level at different conditions.

TABLE 4. MORTALITY RATE OF *T. mundella* FROM SABAJU AND TINBARAP ESTATES AFTER EXPOSED TO *Bacillus thuringiensis* (Bt.) INSECTICIDE FOR 96 HR

Bioassay	Mortality rate (%)*	
	Sabaju	Tinbarap
Control	6.6 ± 6.6 a,A	13.2 ± 8.1 b,A
Treatment	26.6 ± 12.5 a,B	93.4 ± 6.6 a,A

Note: *Each value represents the mean ± SE of 15 replicates of insects tested in each parameter. The significant difference between the mean values was analysed by independent-sample t test. Different lowercase letters denote a significant difference in the mortality rate between the control and treatment in bioassay. Different uppercase letters indicate significant differences of mortality rate between sampling locations.

TABLE 5. ADULT EMERGENCE RATE OF *T. mundella* FROM SABAJU AND TINBARAP ESTATES AFTER EXPOSED TO *Bacillus thuringiensis* (Bt.) INSECTICIDE

Bioassay	Adult emergence (%)*	
	Sabaju	Tinbarap
Control	93.4 ± 6.6 a,A	86.8 ± 8.1 a,A
Treatment	53.4 ± 8.3 b,A	0 ± 0 b,B

Note: *Each value represents the mean ± SE of 15 replicates of insects tested in each parameter. The significant difference between the mean values was analysed by independent-sample t test. Different lowercase letters denote a significant difference in the adult emergence rate between the control and treatment in bioassay. Different uppercase letters indicate significant differences of adult emergence rate between sampling locations.

TABLE 6. QUANTIFICATION OF CADHERIN GENE EXPRESSION IN DIFFERENT DEVELOPMENT STAGES OF *T. mundella* FROM SABAJU AND TINBARAP ESTATES

Development stage	Relatively transcript level*	
	Sabaju	Tinbarap
4 th instar	0.0202 ± 0.0053 c,B	23.8145 ± 2.8313 a,A
5 th instar	0.0268 ± 0.0050 c,B	0.3214 ± 0.0328 b,A
6 th instar	0.2940 ± 0.0620 c,B	0.6318 ± 0.1374 b,A
7 th instar	5.6839 ± 0.7847 a,A	0.6358 ± 0.0987 b,B
Pupa	3.6511 ± 0.2040 b,A	1 ± 0 b,B

Note: *Each value represents the mean ± SE of three technical replicates and three biological replicates of insects collected for each stage. The mean difference between the development stages was analysed by one-way ANOVA. Different lowercase letters denote a significant difference ($p < 0.05$, Tukey's HSD test) in the expression levels between the development stages. The mean difference between the sampling sites was analysed by independent-sample t test. Different uppercase letters denote a significant difference in the expression levels between the sampling sites. The expression level of pupa from Tinbarap Estate was set at 1.

DISCUSSION

A gene sequence of *T. mundella* was amplified with the primers encoding for the cadherin receptor of *C. suppressalis*, a destructive rice pest commonly found in East Asia. The insect was found to develop resistance against Cry1A toxin after a long-term of *Bt.* insecticide application in the field. The resistance was contributed by the mutations of the cadherin genes resulting in the downregulation and reduction in the toxin binding site (Zhang *et al.*, 2017). The amplified gene sequence of *T. mundella* demonstrated a relatively high similarity to the cadherin gene of other Lepidoptera species including *B. mori* (Yu *et al.*, 2018), *S. exigua* (Ren *et al.*, 2013) and *C. suppressalis* (Zhang *et al.*, 2017) which were reported to confer resistance against *Bt.* insecticide. This suggested that the amplified fragment of *T. mundella* may have common structures or functions with the cadherin protein of Lepidoptera.

Tirathaba mundella from Sabaju Estate showed no significant change in survival rate and 53.4% of the larvae were successfully turned into moth after toxin ingestion. Furthermore, lower cadherin transcript level was expressed at early developmental stages of the larvae. The resistance against *Bt.* insecticide was reported to associate with the low expression of the cadherin gene. Cadherin receptor which serves as the Cry toxin binding site was reduced by a decrease in the cadherin gene expression (Yu *et al.*, 2018). This allowed the larvae to remain unharmed after the ingestion of the fruit bunch sprayed with insecticide. The efficiency of insecticide application reduced as the larvae stay alive and continuously infest on the fruit bunch. The increment of cadherin expression at the seventh instar larvae and pupae stage explained the reduction in adult emergence (Table 5). The larvae were able to survive in the early developmental stages but may die during the metamorphosis process. The similar expression pattern was reported in the resistant strain of *M. sexta* (Midboe *et al.*, 2003), *P. xylostella* (Yang *et al.*, 2012) and *C. suppressalis* (Zhang *et al.*, 2017) where the expression increases with the development stages of larvae and dropped in the pupae. The pattern may be associated with the role of cadherin in cell proliferation and tissue morphology, more cadherin is expressed to generate tissue at the advanced instars (Bel and Escrache, 2006; Yu *et al.*, 2018).

In contrast, the percentage of mortality and adult emergence of *T. mundella* from Tinbarap Estate was greatly affected by the intake of the diet containing Cry1A toxin. The significantly higher cadherin expression at the fourth instar facilitates the binding of Cry toxin with cadherin receptor in insect midgut, resulting in the oligomerisation of toxin and pore formation on the midgut membrane. The pore formation leads to cell lysis and eventually cause the death of the insect (Marroquin *et al.*, 2000). The

expression pattern was similar to the third instar of the susceptible strains of *Diatraea saccharalis* which was reported to have higher expression compared to its fifth instar (Yang *et al.*, 2011). The significant lower expression of the cadherin gene from fifth to seventh instar in *T. mundella* from Tinbarap Estate suggests that the larvae may be able to survive if it is exposed to the toxin only after its fourth instar stage. This data supports the pest management practice reported by Huan (2012) which suggested that the application of insecticide every two weeks over a three months period is necessary to control the infestation by breaking its life cycle.

The distinct cadherin expression pattern and mortality of *T. mundella* from the two sampling locations suggest that the resistant strain may have developed in the Sabaju Estate after exposed to an inconsistent schedule of insecticide application. Besides, this study displayed a remarkable change

in cadherin expression pattern in the resistant strain of *T. mundella* when exposed to Cry1A toxin. The expression level dropped significantly after 2 hr of toxin exposure. The trend may associate with the resistance mechanism of larvae against the toxin. The affinity of the cadherin receptor towards the toxin protein was reduced with a decrease in gene transcript level. The risk of the midgut pore formation was minimised and helps the insect to survive after toxin ingestion (Lee *et al.*, 1995).

The current study demonstrated the connection of the cadherin gene expression of *T. mundella* with its resistance against Cry1A toxin. The data serves as a fundamental approach to understand the development of *Bt.* insecticide resistance over time. Nevertheless, further study on more population of *T. mundella* is necessary to give a better understanding of the insecticide resistance development mechanism.

TABLE 7. QUANTIFICATION OF CADHERIN GENE EXPRESSION OF *T. mundella* FROM SABAJU AND TINBARAP ESTATES AFTER EXPOSED TO *Bacillus thuringiensis* (*Bt.*) INSECTICIDE

Exposure duration	Relatively transcript level*			
	Sabaju		Tinbarap	
	Treatment	Control	Treatment	Control
0 hr	0.0016 ± 0.0002 b	0.0047 ± 0.0009 b	4.7682 ± 0.7848 a	2.4513 ± 0.3426 a
2 hr	0.3233 ± 0.0669 a	0.0055 ± 0.0008 b	1.0421 ± 0.1567 b	0.5739 ± 0.0983 b
8 hr	0.1007 ± 0.0170 b	0.0034 ± 0.0006 b	1.9774 ± 0.3769 b	0.0793 ± 0.0144 b
24 hr	0.0142 ± 0.0019 b	0.0066 ± 0.0013 b	0.5323 ± 0.0704 b	0.1796 ± 0.0398 b
48 hr	0.0124 ± 0.0018 b	0.0147 ± 0.0028 a	1.6938 ± 0.2634 b	0.7006 ± 0.1328 b

Note: *Each value represents the mean ± SE of three technical replicates and three biological replicates of insects tested in each parameter. The significant difference between the exposure durations was analysed using one-way ANOVA. Different letters denote a significant difference ($P < 0.05$, Tukey's HSD test) in the expression levels between the exposure durations. The expression level of pupa from Tinbarap Estate was set at 1.

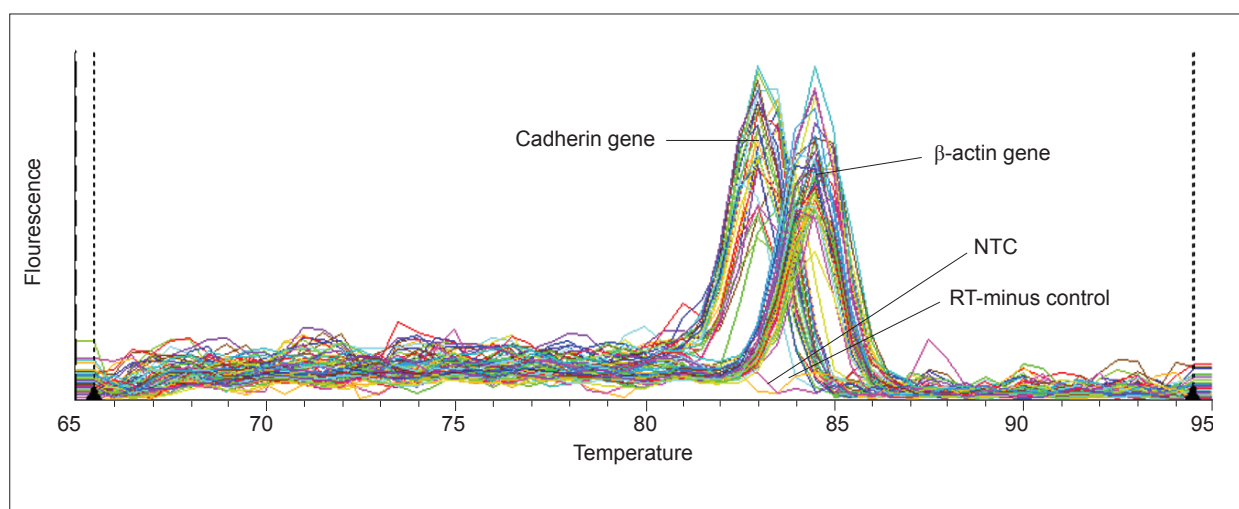


Figure 3. The melting curve of β -actin gene and cadherin gene by qRT-PCR. The melting curve of β -actin gene displayed a single peak at 84.5°C while cadherin gene displayed a single peak at 83.0°C. The RT-minus control and no template control (NTC) show no melting curve peak.

CONCLUSION

The findings of this study demonstrate the development of *T. mundella* resistance against *Bt.* insecticide at the molecular level. Lower expression of cadherin gene was identified in the resistance strain which contributes to the survival of insects under pest management practices. The data generated could assist in the enhancement of pest control strategies by serving as an indicator of the insect susceptibility against insecticide.

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SOIL CARBON DIOXIDE (CO₂) EFFLUX RATE AND OIL PALM YIELD FROM DIFFERENT PEAT TYPES IN SARAWAK, MALAYSIA

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ABSTRACT

Tropical peatlands have different characteristics as compared to temperate peatlands in terms of organic materials and topography. It is important to understand the soil characteristics for improving crop management practices. A study was conducted to investigate the effects of different types of peat on soil carbon dioxide (CO₂) emissions and oil palm yield in Sarawak. The study area was classified as Naman (Oa) and Kenyana (Oawu) series using the Malaysian Unified Classification of Organic Soils (MUCOS). Soil CO₂ efflux was determined by using a portable CO₂ analyser at monthly intervals from eight observational plots setup in each 10 ha study plot. The oil palm fresh fruit bunch (FFB) yield was recorded since the first year of harvest (i.e. after about 30 months field planting). Results showed that the average soil CO₂ efflux was the highest in Naman series plot (4.89±0.36 μmol CO₂ m⁻² s⁻¹) compared to Kenyana series plot (4.44±0.37 μmol CO₂ m⁻² s⁻¹). However, FFB yield was recorded 40% higher at Naman plot compared to Kenyana plot. Higher FFB yield in Naman plot was related to its soil consisting of sapric materials that have more nutrients available for the crop, while Kenyana plot consisted of sapric materials together with undecomposed wood that might hinder the palm growth. This study suggests that different types of peat have significant effects on oil palm yield and soil CO₂ emissions. The site-specific and peat soil management based on its characteristics is important for oil palm growth and performance especially for enhancing FFB yield and improving environmental management.

Keywords: FFB yield, peat type, soil CO₂ efflux, tropical peat.

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INTRODUCTION

Tropical peatland is a unique ecosystem due to its characteristics that support diverse biodiversity and store large amount of carbon and natural water. It is found mostly in Southeast Asia and other countries like Africa, Central and South America (Rieley, 1996). Most tropical peats developed from organic soil materials or the remains of woody materials and form distinct dome-shaped deposits, which can sometimes be more than 10 m deep (Paramanathan, 2010). Sarawak has the largest peatlands of 1 588 142 ha from the total about 2.43 million hectares

of tropical peatland in Malaysia (Wahid *et al.*, 2010). Agriculture development on lowland peat is more prominent in Sarawak due to scarcity of suitable land and the need to eradicate poverty among the indigenous communities living in the swamp area. This has led to the development of peatlands for agriculture especially oil palm cultivation. However, a lack of recognition of the different types of organic soils has resulted in low crop yields and often lead to other management problems due to poor soil management (Veloo *et al.*, 2015).

Previous peat classification was based on the surface vegetation of the tropical peatland forest in Sarawak (Anderson, 1958). There were six phasic peat of tropical peat forest community: Mixed peat swamp (PC1), Alan forest (PC2), Alan Bunga forest (PC3), Padang Alan forest (PC4), Padang Selunsur forest (PC5) and Padang Keruntum forest (PC6) as the centre of the peat dome based on Anderson (1958). Malaysian Unified Classification of Organic Soils (MUCOS) in Malaysian Soil Taxonomy (Paramanathan, 1998; 2010; 2012) was then developed by Paramanathan since 1984 for further efforts and improvement in classifying peat and mapping tropical peatland soil in Malaysia. This classification system considers depth, peat maturity, morphology, the presence or absence of wood, nature of wood (decomposed/ undecomposed) and underlying mineral substratum, which is more reflective of the characteristic of tropical peatland in Malaysia in complementing the international classification schemes.

Based on MUCOS, peat is defined as organic soil that consists of more than 50 cm of organic material thickness within the upper 100 cm soil layer with loss of ignition of more than 65%. It is necessary to classify the tropical peat soil by using a standard classification system in order to understand the soil for a better crop growth and performance as well as environment management. Paramanathan and Wahid (2014) had proposed the management practices needed based on soil management group, which is very useful for the successful cultivation of oil palm on peat in Malaysia.

Tropical peatlands are significant carbon sinks and store large amounts of carbon and support diverse ecosystems (Hooijer *et al.*, 2006; Page *et al.*, 2011). However, research that examines the rate of CO₂ flux from peatlands including oil palm plantation is relatively scarce (Page *et al.*, 2011). Peat carbon emissions had been a much-debated issue once the peat is drained. The magnitude of soil CO₂ emissions from oil palm plantation on peat still remains uncertain and more studies are needed to address this issue. The study of soil CO₂ respiration from tropical peatlands is an important aspect of environmental research due to its potential impact on future climate change. One aspect is the inevitable subsidence, which occurs following

drainage that is needed to provide suitable conditions for crop growth (Wösten *et al.*, 1997). In addition, the decline in peat depth imposes a limit to the peat productive life (Tie, 2004). Shallow peats may eventually disappear completely, exposing the underlying mineral soil, some of which may be acid sulphate and detrimental to crop growth. Therefore, minimising drainage is the key for sustaining the productive life span of peat soils and reducing green house gas (GHG) emissions (Tarmizi *et al.*, 2009; Hasnol *et al.*, 2010; Marwanto *et al.*, 2013).

Owing to several other issues of major concern regarding the use of peatland to grow oil palm, our aims were to report annual total soil CO₂ efflux (or soil respiration) and oil palm yield from oil palm plantation on a tropical peatland on different peat series (Naman and Kenyana) in Sarawak. This study is also relevant as Sarawak is the largest state in Malaysia that plant oil palm. For year 2017, out of 5.81 million hectares of oil palm planted in Malaysia, 1.56 million hectares or 26.8% is in Sarawak (Kushairi *et al.*, 2018). The study provides vital information that will be useful for a better understanding of carbon emissions and oil palm yield in tropical peatlands. A better understanding of the nature and characteristics of these peatlands may help in formulating and managing oil palm plantations based on different peat types or peat series. Therefore, this information will be useful for making rational decisions concerning the future use of peatlands for agriculture in Malaysia or elsewhere. The mapping of soil classification, optimum and best management practices are important to ensure successful planting, economic yield and better environmental management of oil palm cultivation on peat.

MATERIALS AND METHODS

Site Description and Soil Classification

The study site was established at a newly planted area in an oil palm plantation located in Sibu, Sarawak, Malaysia. The study area was situated between latitudes 2°33.5'N and 2°34'N and longitudes 111°58.5'E and 111°59.3'E. The study plot consisted of 10 ha each on different peat types; Naman series (Plot 1) and Kenyana series (Plot 2) and planted with oil palm in 2010. Oil palms were planted in triangular patterns at 8.6 m distance with planting density of 154 palm ha⁻¹. Fertilisers such as urea, rock phosphate, zinc sulphate, borate, copper sulphate and muriate of potash were applied based on the general fertiliser recommendation for immature palm (Haniff *et al.*, 2011). The estate has an annual rainfall between 2748-3072 mm with an occasional dry month (<100 mm). Land preparation was done according to the standard estate practices

consisting of compaction, stacking of woody biomass residues between planting rows and draining the water table to 50-60 cm below the ground surface (Gurmit, 2004). The water levels were maintained at the collection drains using weirs or stop-off to regulate the peat water in the plantation.

The study area was classified as Naman (Oa) series and Kenya (Oawu) series based on MUCOS (Paramanathan, 2011) (Table 1). Naman soil series is described as very deep (>300 cm) organic soil that is black in colour, non-woody and highly decomposed sapric organic soil material in the sub-surface tier (50-100 cm). It is often very poorly drained over non-sulphidic marine clay at more than 300 cm depth. The soil management group of Naman series is the Oa (organic and sapric) consisting of sapric materials, non-woody and low in fertility. Kenya soil series is very deep (>300 cm), dark brown to black sapric organic soil materials with undecomposed wood in the upper 100 cm and often undecomposed fibric organic soil materials with some pieces of wood below 100 cm depths. The underlying material of marine clay is at over 300 cm depth and it is very poorly drained. The soil management for Kenya series in this study was categorised as Oawu (organic, sapric and undecomposed wood) which was very deep (>300 cm) sapric material with undecomposed wood and low fertility (Table 1). The site was formerly Mixed Peat Swamp forest for Naman series and Alan forest for Kenya series as recorded by Soil Division, Department of Agriculture Sarawak. The peat depth ranged from 6-8 m and classified as Anderson 3 (Records of Soil Division, Department of Agriculture Sarawak).

Field Experimental Design

Eight subplots were established at each study plot for measuring soil respiration, soil temperature and soil moisture. One palm in the middle of each subplot was chosen as a reference palm in order to determine the placement of soil collars at different management zones. The soil collars for the soil CO₂ efflux measurements were located at three different management zones; inter-row, harvest-path and frond stacking area at the distances of 0.5, 1.75 and 3.5 m from the trunk of reference palm for each management zone (Figure 1). This is shown by Y shape of measurement points in the diagram. The soil collars were made from polyvinyl chloride (PVC) pipes with dimension of 10.5 cm diameter by 10 cm height and inserted into the soil with 8 cm below and 2 cm above the soil surface. Soil collars were installed three months prior to the initial measurement in order to avoid disturbance effects (Ventura and Baker, 2008; Grønlund *et al.*, 2008; Pumpanen *et al.*, 2004).

Measurement of Soil CO₂ Efflux, Soil Temperature and Soil Moisture Content

Soil CO₂ efflux rate was measured using a portable CO₂ analyser (EGM-4, PP-System, United Kingdom) based on the closed-static chamber approach (Livingston and Hutchinson, 1995). The EGM-4 consists of an infrared gas analyser (IRGA) connected to a cylindrical soil chamber SRC-1 (diameter 10 cm; height 15 cm). The rate of increase in CO₂ concentration with time within the chamber

TABLE 1. CLASSIFICATION, SOIL CHARACTERISTIC AND ORGANIC MANAGEMENT GROUP OF STUDY SITES

Soil series/ Depth phase	Classification of the soil (Phase level)	Main characteristic/ limitation	Management practices needed	Peak yield potential (t ha ⁻¹ yr ⁻¹)
Naman/ very deep (Oa)	Sapric ombrogambist, marine clayey, isohyperthermic, non-woody, autochthonous (Low ash, dysic, non-saline, sapric, drained, very deep)	• Very deep (>300 cm) • Sapric material • Non-woody to 100 cm • Poorly drained • Low fertility	• Compaction of planting rows • High planting density • Water control and management • Good fertiliser programme with Cu, B and Zn	26-28
Kenya/ very deep (Oawu)	Sapric topogambist, marine clayey, isohyperthermic, woody-undecomposed, autochthonous (Low ash, dysic, non-saline, sapric, drained, very deep)	• Very deep (>300 cm) • Sapric material with undecomposed wood • Poorly drained • Stunted growth common after five years • Termites • Low fertility • High cost of drain construction	• Compaction of planting rows • High planting density. • Water control and management • Good fertiliser programme with Cu, B and Zn • Thinning of stunted palms	22-24

Note: Oa - organic and sapric; Oawu - organic, sapric and undecomposed wood refers to soil management group.

Source: Paramanathan (2012); Paramanathan and Wahid (2014).

was then monitored over a 2 min enclosure period or until an increase of 100 ppm CO₂ had been detected. Soil CO₂ efflux rate was calculated by performing linear regression on the CO₂ concentration and time. The soil CO₂ emission rates were calculated based on CO₂ efflux calculations (Marthens *et al.*, 2014). The end results with a good linear fit ($R^2 \geq 0.8$ or $r = 0.89$) were used in the mean comparison analysis. The measurements were conducted at monthly intervals from 72 points with two replicates of measurement at each point in the study plot. The soil CO₂ efflux measurement was conducted since planting in 2010 until five years after planting. Soil temperature (STP-1 Soil Temperature Probe, PP-System, United Kingdom) and soil moisture (HydroSense CD620 and CS620, CSI, USA) were measured at 5-10 cm depth below the peat surface for each measurement point. All measurements were performed approximately from 8.00 am until noon as representative samples of the day and measurements were not taken immediately after rain to avoid effects of CO₂ diffusion caused by rain events.

Fresh Fruit Bunch Yield, Vegetative Measurement and Water Table

The fresh fruit bunch (FFB) yield was recorded to compare yield performance in tonnes (t) between different peat types since the first year of harvest in 2012 (after about 30 months field planting) until 2015. The vegetative measurement for morphological parameters such as standing biomass, trunk height and leaf area index (LAI) were based on the MPOB manual and report on oil palm measurements (Zuraidah *et al.*, 2017; Corley and Breure, 1981). The vegetative measurement was conducted since 2013 until 2015. The water table depth below the peat surface was monitored using three automatic water level data loggers (Cera-Diver, Schlumberger, Canada) in each study plot as well as manual checks. The automatic water level data loggers (diver) were installed in open-ended piezometer made from perforated 10 cm diameter PVC pipes. The field water table was recorded automatically at hourly intervals by divers.

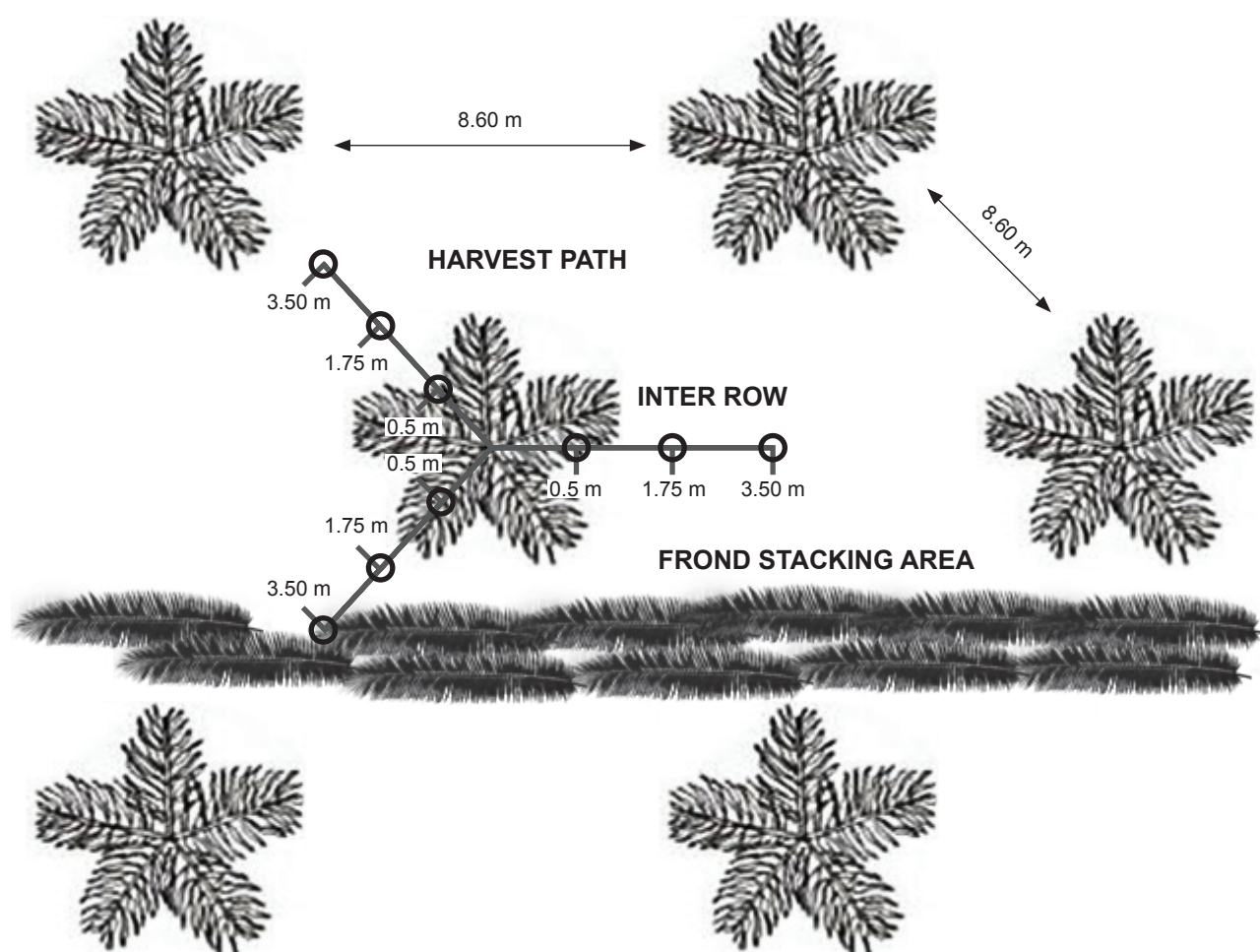


Figure 1. Location of soil carbon dioxide (CO₂) efflux sampling points at different management zones denoted by red circles.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20. The distribution of the variables was tested using the Shapiro-Wilk test. The independent samples *t* test was used to compare means between the study plots for each variable such as soil CO₂ efflux, field water table, soil moisture, soil temperature and FFB yield. Statistical significance was set at probability level of 0.05. Results were presented as mean with standard error of mean for each variable in their respective unit except for rainfall data (total).

RESULTS AND DISCUSSION

The annual mean total soil CO₂ efflux for six years (2010-2015) of measurements was analysed using independent *t* test. Soil CO₂ efflux was higher at the Naman plot at $4.89 \pm 0.36 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ($67.85 \text{ mg ha}^{-1} \text{ yr}^{-1}$) than the Kenyana plot at $4.44 \pm 0.37 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ($61.61 \text{ mg ha}^{-1} \text{ yr}^{-1}$) as shown in Table 3. There was a significant difference in soil CO₂ efflux between the plots at *p*-value < 0.001. The soil CO₂ efflux rates in this study were within the range of soil CO₂ emission reported by other studies of oil palm plantations on tropical peat swamp forest using the closed chamber technique (Ishikura *et al.*, 2018; Hergoualc'h *et al.*, 2017; Husnain *et al.*, 2014; Wigena *et al.*, 2014; Marwanto and Agus, 2014; Dariah *et al.*, 2013; Comeau *et al.*, 2013; IPB, 2011). Previous studies reported the total soil respiration of oil palm plantation on peat ranged between $38 \text{ mg ha}^{-1} \text{ yr}^{-1}$ (Ishikura *et al.*, 2018) to $104 \text{ mg ha}^{-1} \text{ yr}^{-1}$ (Comeau *et al.*, 2013). Studies reported by some researchers (Melling *et al.*, 2004; 2005; 2007; IPB, 2011) showed results of soil CO₂ emission measured at oil palm sites (4-5 years after planting) ranging from $56.5\text{--}85.0 \text{ mg ha}^{-1} \text{ yr}^{-1}$. Results of soil CO₂ efflux from this study were comparable with a soil CO₂ flux measurement using the closed-chamber method with IRGA (LICOR 820) conducted by Dariah *et al.* (2013) on 6-year-old oil palm plantation located at Jambi, Indonesia with the magnitude of $67.36 \text{ mg ha}^{-1} \text{ yr}^{-1}$ after one month of fertilisation. The average total soil respiration reported in their study ranged from $48.16\text{--}99.27 \text{ mg ha}^{-1} \text{ yr}^{-1}$ over a period of seven months of measurement. Husnain *et al.* (2014) and Wigena *et al.* (2014) also reported on the rate of soil CO₂ efflux measured using closed-chamber method with IRGA (LICOR 820) on 4-year-old oil palm on peat with 66.00 ± 25 and $66.17 \pm 25.54 \text{ mg ha}^{-1} \text{ yr}^{-1}$, respectively. Both studies were conducted between 6-13 months of measurement at the plantations in Riau and Jambi, Indonesia. Study conducted by Hergoualc'h *et al.* (2017) on 6-year-old and 1-year-old oil palm planted on peat in central Kalimantan, Indonesia showed soil CO₂ efflux

measured using the closed-chamber method (EGM-4) for about 13 months at $50.65 \pm 0.30 \text{ mg ha}^{-1} \text{ yr}^{-1}$ and $43 \pm 0.30 \text{ mg ha}^{-1} \text{ yr}^{-1}$, respectively.

Meaningful comparison is hard to achieve due to the method used, sampling frequency and the length of the soil CO₂ efflux measurement period that differ between studies. For example, the measurements for this study were taken from the first year until five years after planting at a newly cleared logged-over peat swamp forest. Other studies mostly reported for one year or less than one year of measurement in immature or matured oil palm plantations.

Our results indicated the mean annual total soil CO₂ efflux rates were decreasing over years (Table 2). Higher soil CO₂ efflux rate was recorded in the first year of measurement due to early disturbance of peatland. The microbial community changes and organic matter decomposition immediately following conversion possibly led to high soil temperature and subsequently increased in soil CO₂ emissions. However, soil CO₂ efflux decreased after four to five years after planting and expected to be lower when the palm and peat reach maturity. This might be due to decline in soil organic matter quality and nutrients which may decrease substrate-driven rates of CO₂ production from peat decomposition over time (Swails *et al.*, 2017).

In addition, the palm canopy provided shade as they grow and resulted in low soil temperature, which contributed to lower soil CO₂ emissions. Jauhainen *et al.* (2014) reported 90% of shaded area of agriculture land on tropical peatland resulted in a 33% lower CO₂ emission average on the unfertilised plots and a 66% lower emission average on the fertilised plots. As the palms matured, the soil temperature dropped, leading to lower CO₂ emission over time (Figure 2). The best management practices such as maintaining water table at 35-45 cm below the peat surface in oil palm plantation might contribute to reduced soil CO₂ emissions by minimising the peat oxidation layer and mineralisation rates (Tarmizi *et al.*, 2009; Hasnol *et al.*, 2011). In this study, the water table fluctuated between 30-43 cm (Table 2) below the peat surface which was regulated by weirs installed at collection drains and influenced by wet and dry seasons.

Field observation at the study sites showed that oil palm growth at Naman plot was healthier with larger canopies compared to Kenyana plot which showed poor growth. This was supported by the higher average standing biomass (Table 3) recorded in Naman plot ($91.85 \pm 1.57 \text{ kg palm}^{-1}$) compared to Kenyana plot ($83.34 \pm 2.02 \text{ kg palm}^{-1}$) and other morphological parameters such as trunk height; Naman plot ($84.28 \pm 2.51 \text{ cm}$), Kenyana plot ($70.26 \pm 2.08 \text{ cm}$) and LAI; Naman plot (3.23 ± 0.06) and Kenyana plot (2.73 ± 0.06). Hardon *et al.* (1969) reported a positive correlation was found between

the leaf area and yield of different palms within the same family. High biomass might contribute to higher soil CO₂ efflux due to soil respiration by roots and microbes utilising the roots exudates in the rhizosphere (Lohila *et al.*, 2003, Hirano *et al.*, 2007). This suggests that more oil palm roots density was found in Naman plot than Kenya plot which contributed to autotrophic respiration. Plant growth stage affects the root respiration that has a significant contribution to total soil respiration (Dariah *et al.*, 2013). Research conducted by Khalid *et al.* (1999) and Henson and Chai (1997) have shown that high root density was found closer to the oil palm trunk and thus resulted in higher total soil respiration than further away from palm trunks (Swails *et al.*, 2018; Manning *et al.*, 2017; Comeau *et al.*, 2016; Dariah *et al.*, 2014). Other parameters measured such as the average field water table at Naman plot was -34.52±1.81 cm and Kenya plot at -35.49±2.17 cm below the peat surface. Soil temperature and moisture content

were 27.57±0.04°C (Naman plot), 27.68±0.04°C (Kenya plot) and 43.77±0.75% (Naman plot) and 45.11±0.75% (Kenya plot), respectively.

The highest average FFB production was recorded in the research Naman plot (14.15±1.52 t ha⁻¹ yr⁻¹) and Kenya plot (8.43±0.98 t ha⁻¹ yr⁻¹) for the first four years of harvest (Table 3). There was a significant difference of FFB between the plots at *p*-value <0.05. The lower FFB yield recorded from both peat types (Naman; 9.68 t ha⁻¹ yr⁻¹ and Kenya; 6.04 t ha⁻¹ yr⁻¹) were recorded in the early 2012 since it was the first year of harvesting for this research plot and have declined slightly in 2014 (Figure 3) and this could be due to the dry spell. A study reported by Melling *et al.* (2007) on FFB yield planted for the first and second year of harvest on Mixed peat swamp ex-forest were 6.64 t ha⁻¹ yr⁻¹ and 9.88 t ha⁻¹ yr⁻¹ respectively. The FFB yield for oil palm planted on Alan ex-forest area was 2.12 t ha⁻¹ yr⁻¹ and 2.37 t ha⁻¹ yr⁻¹ for the first and second year of harvest. Gurmit (2004) also reported FFB

TABLE 2. AVERAGE SOIL CARBON DIOXIDE (CO₂) EFFLUX WITH STANDARD ERROR OF MEAN, FRESH FRUIT BUNCH (FFB) YIELD WATER TABLE AT DIFFERENT PLOT AND TOTAL ANNUAL RAINFALL (2010-2015); P1 IS PLOT 1 WHILE P2 IS PLOT 2

Parameter	Year					
	2010	2011	2012	2013	2014	2015
Soil CO ₂ efflux (μmol m ⁻² s ⁻¹)						
Naman (P1)	6.09±0.20	4.32±0.20	4.84±0.12	5.53±0.10	4.97±0.10	3.59±0.13
Kenya (P2)	5.90±0.20	4.20±0.19	4.26±0.12	5.07±0.11	3.79±0.09	3.39±0.14
FFB yield (t ha ⁻¹ yr ⁻¹)						
Naman (P1)	-	-	9.68±1.57	16.30±1.86	15.79 ± 2.51	14.83±1.02
Kenya (P2)	-	-	6.71±0.98	8.21±0.88	7.58±0.89	11.23±0.77
Water table (cm)						
Naman (P1)	-	-30.67±0.56	-36.68±0.50	-35.06±0.33	-39.85±0.52	-30.34±0.53
Kenya (P2)	-	-42.62±0.70	-33.12±0.50	-32.34±0.34	-38.33±0.41	-31.05±0.55
Annual rainfall (mm)	3 071.64	2 959.21	2 984.82	2 777.28	2 748.28	2 937.51

Note: The data represents the mean ± standard error of mean except for annual rainfall data (total).

TABLE 3. AVERAGE SOIL CARBON DIOXIDE (CO₂) EMISSION, FFB YIELD, SOIL AND MORPHOLOGICAL PARAMETERS

Item	Naman series	Kenya series
Total soil CO ₂ efflux (μmol m ⁻² s ⁻¹)	4.89±0.36 ^a	4.44±0.37 ^b
Field water table (cm)	-34.52±1.81 ^a	-35.49±2.17 ^b
Soil moisture content (%)	43.77±0.75 ^a	45.11±0.75 ^b
Soil temperature (°C)	27.57±0.04 ^a	27.68±0.04 ^b
Fresh fruit bunch (FFB) (t ha ⁻¹ yr ⁻¹)	14.15±1.52 ^a	8.43±0.98 ^b
Standing biomass (kg palm ⁻¹)	91.85±1.57 ^a	83.34±2.02 ^b
Trunk height (cm)	84.28±2.51 ^a	70.26±2.08 ^b
Leaf area index (LAI)	3.23±0.06 ^a	2.73±0.06 ^b

Note: The data represents the mean±standard error of mean. Plots with significantly different (*p*<0.05) parameters have been denoted by suffix a and b.

yield of the early oil palm plantings on deep peat in United Plantation Berhad was around 11.4–12.8 t ha⁻¹ for the fifth to sixth year after planting. The reduction in average FFB yield might be associated with the decreasing inorganic nutrient in peat surface especially phosphorus, calcium and iron towards the centre of the peat dome (Anderson, 1958; Muller, 1972). It was also stated that higher FFB yield was recorded from oil palm planted on Mixed peat swamp ex-forest than Alan ex-forest (Melling *et al.*, 2007). Veloo *et al.* (2015) also reported that Naman series with sapric materials gave significantly better yields ranging from 19.48–22.92 t ha⁻¹ for matured palms. Different types of peat have a significant effect on oil palm yield ranging from 9.47–22.9 t ha⁻¹ (Veloo *et al.*, 2015).

The higher soil CO₂ efflux rate might be attributed to low soil moisture and water table during the dry season (March until September) recorded in this study. There were significant differences ($p < 0.05$) in soil CO₂ efflux rate, field water table, soil moisture and FFB yield between the study plots. Results showed that different types of peat have significant effects on soil CO₂ emissions and oil palm yield. Annual variation of soil CO₂ efflux rates and FFB yield could be attributed to various factors such as seasonal variation, soil heterogeneity and plantation management. Therefore, further research needs to be explored to find the relationship of related variables that contribute to soil CO₂ emissions and oil palm yield from peatland.

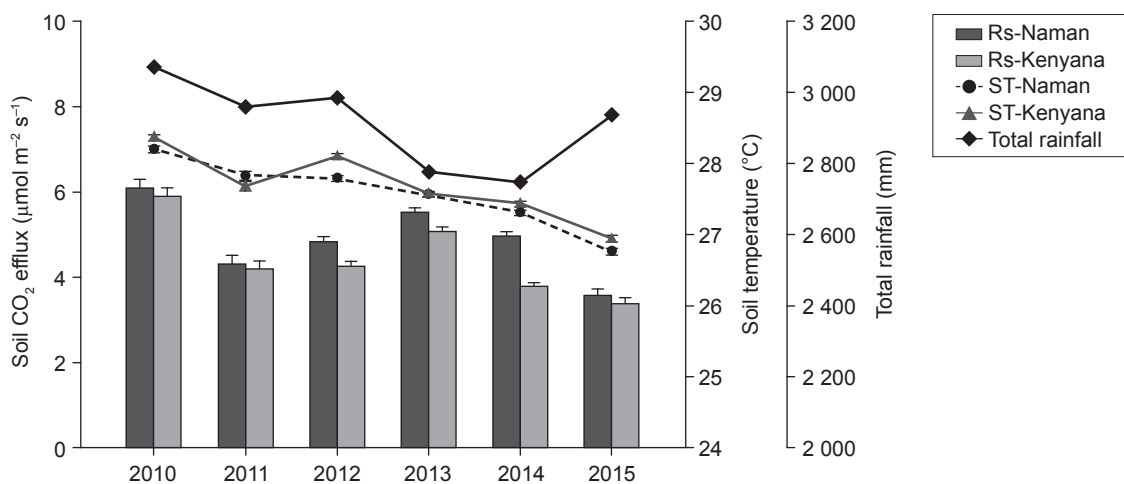


Figure 2. Average total soil respiration (Rs) rate and soil temperature (ST) with standard error of mean at different peat types and total rainfall.

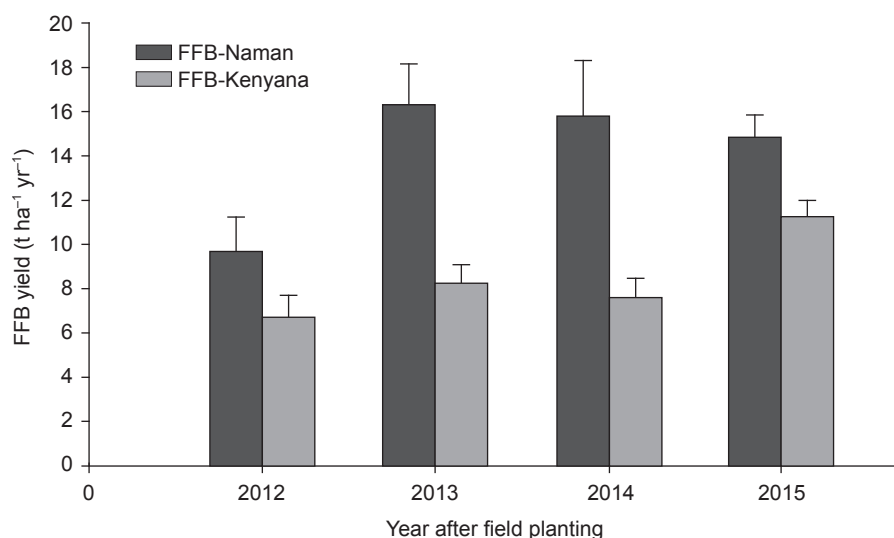


Figure 3. Average fresh fruit bunch (FFB) at different peat types.

CONCLUSION

There were significant differences in soil CO₂ efflux rate, field water table, soil moisture and FFB yield between the study plots. The average soil CO₂ efflux was highest in plot from Naman series compared to plot from Kenyana series. The highest FFB yield was recorded in plot from Naman compared to plot from Kenyana due to different characteristics of both peat soil series. Variation of CO₂ emission rates and FFB yield could be attributed by plantation management practices, interannual variation (wet and dry seasons throughout the year) and soil heterogeneity due to different peat types and properties in the study plots.

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THE DYNAMIC OF CARBON DIOXIDE (CO₂) EMISSION AND LAND COVERAGE ON INTERCROPPING SYSTEM ON OIL PALM REPLANTING AREA

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ABSTRACT

Oil palm replanting has faced environmental criticism as it is a source of carbon dioxide (CO₂) emission. The aim of this study was to determine the level of CO₂ emissions and the value of carbon stocks for oil palm smallholders replanting. The measurements were taken using the infrared gas analyser (IRGA) method for CO₂ emission. Non-destructive methods were used to measure carbon stocks for oil palm-stands while destructive methods were used for understorey and intercropped plants. This study shows that there was dynamic of CO₂ emission during the replanting averaging (t CO₂ ha⁻¹ yr⁻¹) 28.5 for 28-year-old oil palm, 59.0 for bare land after land clearing, 47.0 for intercropped plants in the vegetative phase, 51.6 for intercropped plants in the generative phase and 42.9 for one-year-old oil palm. It was found that CO₂ emissions were reduced due to the conditions of land coverage and that the reduction in CO₂ emissions occurred not only because of the absorption of CO₂ by plants for photosynthesis but also due to being stored away, as much of the carbon stock (t C ha⁻¹) was found in corn (10.2), legume cover crops (7.6), soyabeans (3.5) and natural vegetation (2.8). In 28-year-old oil palm it was found to be at 74.7.

Keywords: carbon stocks, corn, intercropped, smallholders, soyabeans.

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INTRODUCTION

In 2018, the total oil palm plantation area in Indonesia was 14.3 million hectares, of which 5.8 million

hectares belonged to smallholders (Direktorat Jenderal Perkebunan, 2017). Of the area held by smallholders, 2.7 million hectares require replanting as the plants are over 25 years old and unproductive. The significant increase in oil palm plantations in Indonesia has led to negative campaigns related to environmental issues, including deforestation, greenhouse gas (GHG) emissions, loss of biodiversity and forest and peatland fires.

The smallholders' oil palm replanting programme in Indonesia has the potential to generate a negative response from the international community due to the GHG emissions, especially carbon dioxide (CO₂) associated with the programme. Hence, information about the level of CO₂ emissions that will be generated during the oil palm replanting stage is needed.

Olivier *et al.* (2016) concluded that GHG emissions are a cause of global warming. Greenhouse gases include water vapour (H₂O),

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CO₂, nitrous oxide (N₂O), methane (CH₄), ozone (O₃) and chlorofluorocarbons (Zein and Chehayeb, 2015). Olivier *et al.* (2017) reported that most GHG emissions (about 72%) consist of CO₂, followed by CH₄ (19%), N₂O (6%) and fluorinated gases (3%). The agricultural sector plays a role in producing CO₂.

According to the Environmental Protection Agency (EPA) (2017), 9% of GHG emissions in 2017 resulted from agricultural activities. Henderson *et al.* (2018) found that several sectors contributed to the global greenhouse effect in 2010: 25% came from electricity and heat; 24% from agriculture, forestry and other land-use (AFOLU); 21% from industry; 14% from transportation; 6.4% from buildings; and 9.6% from other energy sources.

The formation of CO₂ occurs as a result of plant respiration, which includes root respiration and respiration of microorganisms. In photosynthesis, CO₂ is converted into organic matter with the help of sunlight. In turn, the dead organic matters are broken down into water vapour and CO₂, some of which is converted into C-organic in the soil. During the decomposition process, various products are released, including CO₂, energy, water, plant nutrients and resynthesised organic carbon compounds (Khatoon *et al.*, 2017). It is possible to reduce CO₂ emissions by improving the absorption capacity of plants, that is by increasing photosynthetic efficiency. Increasing plant photosynthesis can be done by increasing the number of plants including by planting bareland. Therefore, an intercropping system is a promising option for improving efficiency and optimising land use (Li-li *et al.*, 2015).

Intercropping is a farming system whereby the main commodity is planted in the same area as a second crop either at the same time or at a different time (Poppy *et al.*, 2017). This system has the advantages of optimising the use of light, water and nutrients; controlling weeds, pests and diseases; facilitating sustainable alternative agriculture (Sujayanand *et al.*, 2016; Zarekar *et al.*, 2018) and improving biomass yield and land-use efficiency (Jalal *et al.*, 2017). Legume cover crops are common in oil palm replanting areas as they can maintain moisture in the soil during the early replanting period.

However, legume cover crops are not economically viable for farmers as they do not provide an income for farmers for at least three years while the oil palms are still in the immature stage. Corn and soyabeans are more viable secondary crops for oil palm smallholders as they can provide additional income for farmers in the early stages of replanting.

It is not yet known whether corn and soyabeans can reduce CO₂ emissions more effectively than legume cover crops. The aim of this study was

to determine the level of CO₂ emissions and the value of carbon stocks at various stages of oil palm replanting on smallholders' land.

MATERIALS AND METHODS

Study Sites

The research was conducted on a smallholders' oil palm plantation at plasma PIR KUD Bina Usaha Baru, PT. Inti Indosawit Subur Ukui, Bukit Jaya Village, Ukui Subdistrict, Pelalawan District, Riau Province, Indonesia. The oil palms were planted in 1987 and 1988 (28 years old) and were due for replanting. The coordinate points were 00°09'35.2" SL and 103°06'23.5" EL, and the altitude was 44 m above sea level. The soil type was ultisol. The location is shown in Figure 1.

The CO₂ emissions were measured at five different stages of the replanting process: 1) before felling the 28-year-old palms (November 2016); 2) after land clearing (April 2017); 3) at the vegetative phase of the intercrops (September 2017); 4) at the generative phase of the intercrops (February 2018); and 5) when the replanted palms were approximately a year old (August 2018).

Measurements of CO₂ emissions at the vegetative and generative phases 45 and 75 days after planting (DAP), respectively-were taken for each planting season. Season 1 was from June 2017 to October 2017, season 2 from November 2017 to February 2018 and season 3 from March 2018 to July 2018.

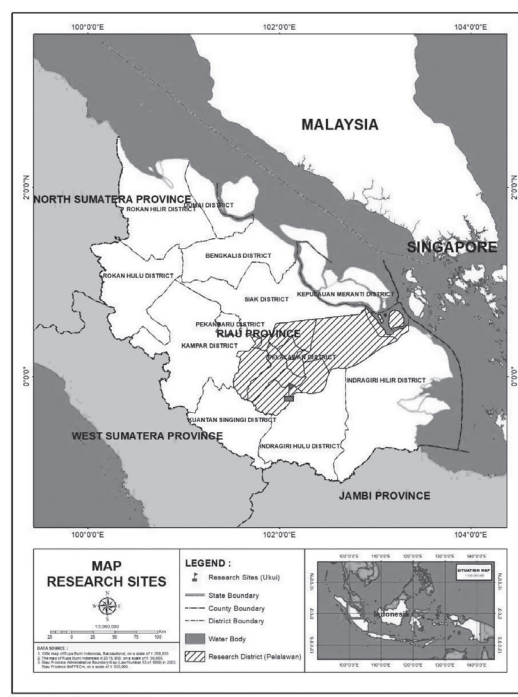


Figure 1. The research site location of Bukit Jaya Village, Ukui Subdistrict, Pelalawan District, Riau Province, Indonesia.

The carbon stocks of old oil palms and understorey vegetation biomass were measured in November 2016. The carbon stocks of the new oil palms planted with soyabeans were measured in May 2018 and those of the palms planted with corn in June 2018.

Materials and Equipment

The main crop was 28-year-old oil palm (*Elaeis guineensis* Jacq.) of the DxP Marihat variety, which was replaced with 12-month-old seedlings of the Topaz 3 variety. The intercropped plants were corn seed (*Zea mays* L.) of the BISI II variety, soyabean seeds [*Glycine max* (L.) Merrill] of Argomulyo variety, and legume cover crop seeds consisting of *Pueraria javanica*, *Calopogonium mucunoides* and *Centrosema pubescens*.

The young palms were planted in a triangular formation at a spacing of 9 m x 9 m x 9 m. The intercropped plants were grown at different planting distances: corn at 100 cm x 20 cm, soyabeans at 50 cm x 10 cm and legumes at 1 m x 1 m. For the natural vegetation treatment, the plants were allowed to grow freely. The distance between the intercropped plant row and the main crop row was 2 m. The harvesting ages of the soyabeans and corn were 85 DAP and 105 DAP, respectively.

An infrared gas analyser (IRGA) type LI-820, consisting of closed polyvinyl chloride (PVC) pipe chamber with a diameter of 25.5 cm and a height of 25 cm was used to measure CO₂ emissions. The IRGA was equipped with supporting tools, including a 12 V battery, a 12 V air pump, an air filter, a manometer, a data logger or laptop, a hose, an air flow meter, a fan, a thermometer, a machete and raffia. The carbon stocks of plant biomass were measured using a ruler, stationery (markers) and scales.

Measurement of CO₂ Emissions and Carbon Stocks

The sampling for measuring CO₂ emissions under 28-year-old oil palms before felling and after land clearing was carried out on three plots of 2 ha each. Each plot had five observation subplots, giving a total of 15 samples (Figure 2).

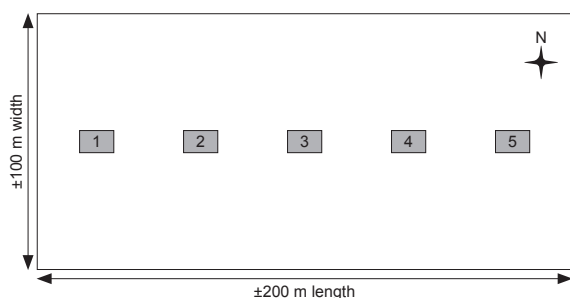


Figure 2. The infrared gas analyses (IRGA) placement for measurement of carbon dioxide (CO₂) emissions samples under 28-year-old oil palm and bare land in one plot.

The CO₂ emissions from the replanting area were measured using a split plot design. The main plot in this study was composed of land owned by three farmers (Farmer 1, Farmer 2 and Farmer 3), each of whom farmed 2 ha of land. The plot was divided into subplots for the intercrop plants (corn, soyabeans, legumes and natural vegetation) and repeated three times, giving 36 sample subplots.

The plant growth parameters were plant height and number of leaves for corn and plant height, number of branches and number of pods for soyabeans. Measurements were taken twice a month for each of the three planting seasons. The growth parameters for oil palms were plant height, length of fronds and number of fronds over an 11-month period.

The CO₂ emissions were measured using Husnain's method (Husnain *et al.*, 2014). Following Madsen *et al.* (2009), the linear relationship between measurement time and CO₂ concentration was used to calculate CO₂ flux. The closed chamber was placed above the ground for the following five conditions: 1) under the stand canopy of the 28-year-old oil palms; 2) above the ground after land clearing (bare land); 3) under vegetative phase of intercropped plants; 4) under generative phase of intercropped plant; and 5) under young oil palm plant aged 15 months (Figure 3). Measurements of CO₂ emissions were taken during the periods of 7.00-12.00 am and 14.00-17.00 pm.

The observation parameters for CO₂ emissions were based on those proposed by Husnain *et al.* (2014) and consisted of the CO₂ emissions in ppm, plant lid height after installation of above-ground lid (cm), air pressure inside lid (kPa), soil temperature (°C), plant lid temperature (°C) and air temperature (°C).

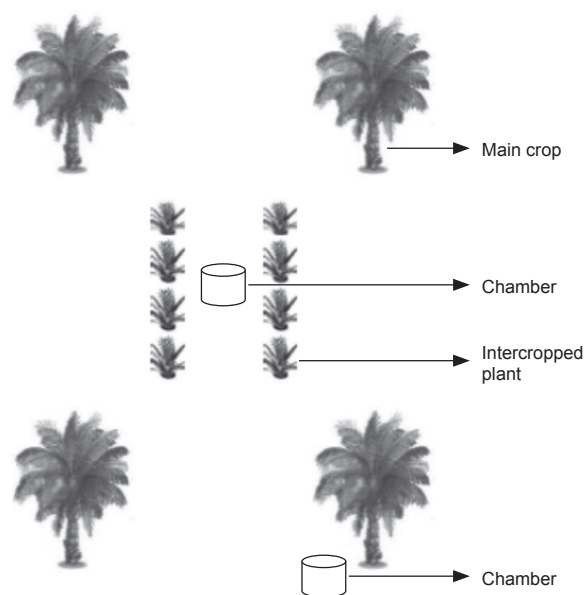


Figure 3. The location of chamber for measurement of carbon dioxide (CO₂) emissions.

A personal computer with an IRGA, the LI 820 CO₂ gas analyser, was used to calculate CO₂ emissions. Data were processed using Microsoft Excel 2013 software. Microsoft Excel was also used to calculate the linear regression equation between time (seconds) and the concentration of carbon in the lid. The Equation obtained was:

$$Y = ax + b \quad \text{Equation (1)}$$

where Y is the concentration of CO₂ in the chamber, a is the regression coefficient of change in CO₂ concentration of time and b is the initial concentration of CO₂.

The CO₂ flux was calculated using the following Equation (Madsen *et al.*, 2009):

$$F_c = \frac{ph}{R\tau} \times \frac{\partial C}{\partial t} \quad \text{Equation (2)}$$

where F_c is the CO₂ flux (mol m⁻² s⁻¹), h is the height of the chamber (cm), R is the ideal gas constant (8.314 Pa m³ °K⁻¹ mol⁻¹), τ is the air temperature of the chamber (°K) and $\frac{\partial C}{\partial t}$ is the change of CO₂ concentration every changing time, linear equation slope of concentration with time (ppm s⁻¹). Field measurement of soil and air temperatures use degrees Celcius, but calculation of the equation is converted to degrees Kelvin.

The technique of observing and measuring carbon stocks was proposed by Hairiah *et al.* (2010). The carbon stocks of the 28-year-old oil palms were measured using a non-destructive method, which involved measuring the plant height from ground level to the lowest fruit bunches. Each of the three plots (2 ha) consisted of 256 plants (±11.7% of the population), of which 30 were sampled, giving a total of 90 samples (Figure 4).

A destructive method was used to measure the carbon stocks of understory vegetation biomass. This involved taking out the plant samples. The size of the plot sample was 1 m x 1 m. The understory was all vegetation with a stem diameter of <5 cm, which was netted in wooden frame 1 m x 1 m in size (Figure 5).

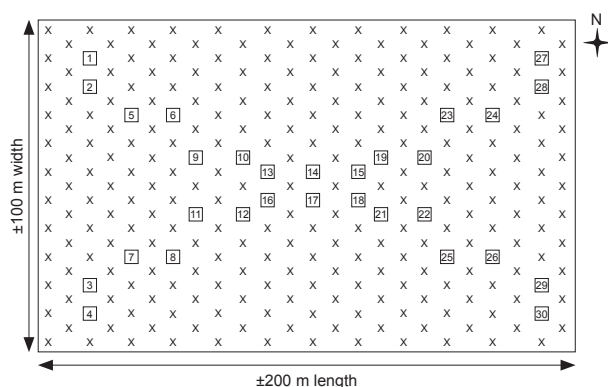


Figure 4. Sketch of stands height sample measurement at 28-year-old oil palms.



Figure 5. Understorey carbon stocks observation.

The carbon stocks of the intercropped plant (corn, soyabeans, legumes and natural vegetation) were observed for areas of 1 m x 1 m square. First, the plants were cut and weighed to obtain their wet weights. Then, 500 g of each sample was dried in an oven at 70°C for 48 hr to measure the dry weight of the biomass.

The dry weight (DW) of the oil palm biomass was calculated using the following Equation (Hairiah *et al.*, 2010):

$$DW = (0.0976 \times H) + 0.0706 \quad \text{Equation (3)}$$

where DW (t per palm) and H is the height of the plant (m).

The carbon stocks of the old oil palms were calculated using the following Equation:

$$\text{Plant carbon stock} = 0.46 \times \text{dry weight biomass} \quad \text{Equation (4)}$$

The conversion rate of 0.46 showed that the average C content in biomass was around 46% (Hairiah *et al.*, 2010).

RESULTS AND DISCUSSION

Environmental Conditions of Research Location

Air temperature and rainfall were the main climate elements to consider. The average temperature in the Pelalawan district while CO₂ emissions were being measured was 24°C-34°C. Rainfall from January to December 2017 ranged from 15-534 mm mth⁻¹ with an average of 270 mm mth⁻¹. The average humidity was 80%-85%. The soil pH at the location was 4.1-4.7. Field observations showed that there were 20 species of natural vegetation under the dense canopy of 28-year-old oil palms, including ferns, weeds, *borreria*, *Cromolaena odorata*, *laportea* and *Heliotropium indicum*. There was no vegetation cover at all on the bare land (after land clearing).

CO₂ Emissions

The CO₂ emissions for various cropping conditions are presented in Table 1. Table 1 shows the dynamic pattern of CO₂ emissions in the oil palm replanting area. The CO₂ emission in the 28-year-old oil palm area was 28.5±13.5 t CO₂ ha⁻¹ yr⁻¹. It increased when the land was bare (59.0±49.4 t CO₂ ha⁻¹ yr⁻¹) and then decreased in the vegetative phase of the intercropped plants (47.0±26.5 t CO₂ ha⁻¹ yr⁻¹). The CO₂ emissions in the generative phase of the intercropped plants and in 1-year-old oil palm were 51.6±28.8 t CO₂ ha⁻¹ yr⁻¹ and 42.9±23.3 t CO₂ ha⁻¹ yr⁻¹, respectively.

The lower CO₂ emissions from the oil palms at the age of 28 years (28.5±13.5 t CO₂ ha⁻¹ yr⁻¹) compared to those from bare land (59.0±49.4 t CO₂ ha⁻¹ yr⁻¹) was thought to be related to CO₂ uptake for the respiration of roots and microbes and the decomposition of organic materials. In the 28-year-old oil palm plantations, some of the released CO₂ would have been absorbed by the leaves for photosynthesis, so less would have been released into the air than from the bare land.

The level of CO₂ emissions decreased again (47.0±26.5 t CO₂ ha⁻¹ yr⁻¹) after the land was covered by intercropped plants (vegetative phase) due to the consumption of CO₂ by the plants. In the generative phase, the amount of released CO₂ increased to 51.6±28.8 t CO₂ ha⁻¹ yr⁻¹ because the leaves of the intercropped plants had aged and were less efficient

in carrying out photosynthesis. This meant that they absorbed less CO₂ than they did during the vegetative phase. According to Misbahuddin *et al.* (2018), soil respiration which produces CO₂ and causes it to be released into the air is determined by various factors, including temperature, soil moisture and soil pH (Pahlipi *et al.*, 2017).

After the new oil palms had grown (one year old) and needed CO₂ for photosynthesis, CO₂ emissions decreased to 42.9±23.3 t CO₂ ha⁻¹ yr⁻¹. Cetin and Sevik (2016) found that plants absorb CO₂ throughout the day, which indicates that oil palm replanting activities do not increase CO₂ emissions in the air. The CO₂ emissions from the old oil palm were 28.5±13.5 t ha⁻¹ yr⁻¹, which are lower than those reported in other studies (Table 2). The CO₂ emissions from 33-year-old oil palms at Rokan Hilir District Riau Province were 34.7 t CO₂ ha⁻¹ yr⁻¹ (Yahya *et al.*, 2017) and those from a logged-over forest were 32.62 t CO₂ ha⁻¹ yr⁻¹ (Khasanah and Nordwijk, 2019). In terms of CO₂ emissions, the old oil palm ecosystem is similar to a secondary forest ecosystem.

With corn, soyabeans, legumes and other vegetation as intercrop plants, CO₂ emissions from the oil palm replanting area decreased. The reduced emissions were due to the absorption of CO₂ by leaves during photosynthesis in planting seasons one to three for soyabeans and corn. Growth of the intercropped plants in the oil palm replanting areas of the three farmers during the three seasons are presented in Figures 6 and 7.

TABLE 1. EMISSION OF CARBON DIOXIDE (CO₂) AT DIFFERENT PHASES OF REPLANTING

Plant conditions	Mean±STD CO ₂ emissions (t CO ₂ ha ⁻¹ yr ⁻¹)		
	Morning	Afternoon	Average
28-year-old oil palm	30.3±14.7	26.7±11.8	28.5±13.5
Bare land (after land clearing)	39.9±29.7	78.1±57.8	59.0±49.4
Vegetative phase intercropping plant	46.9±27.3	47.0±25.6	47.0±26.5
Generative phase intercropping plant	63.4±34.2	39.8±14.9	51.6±28.8
1-year-old oil palm	49.9±31.5	35.9±16.4	42.9±23.3

TABLE 2. EMISSION OF CARBON DIOXIDE (CO₂) AT VARIOUS LAND USES

Land type	Vegetation	t CO ₂ ha ⁻¹ yr ⁻¹	Source
Peatland in Sarawak, Malaysia	Oil palm eight years	22.07±0.8	Manning <i>et al.</i> (2019)
Peatland in Jambi, Indonesia	Oil palm one year	96.13	Khasanah and Noordwijk (2019)
	Rubber > 30 years	75.17	
	Logged-over forest	32.63	
	Betel nut and coffee > 20 years	71.00	
	Coconut and coffee > 40 years	85.00	
Peatlands in Kalimantan, Indonesia	Oil palm	84.10	Rumbang (2015)
	Corn	69.20	
	Rubber plant	106.87	
Mineral land in Rokan Hilir, Riau, Indonesia	Oil palm 33 years	34.70	Yahya <i>et al.</i> (2017)
Mineral land in Kampar, Riau, Indonesia	Shrubs	98.40	Misbahuddin <i>et al.</i> (2018)

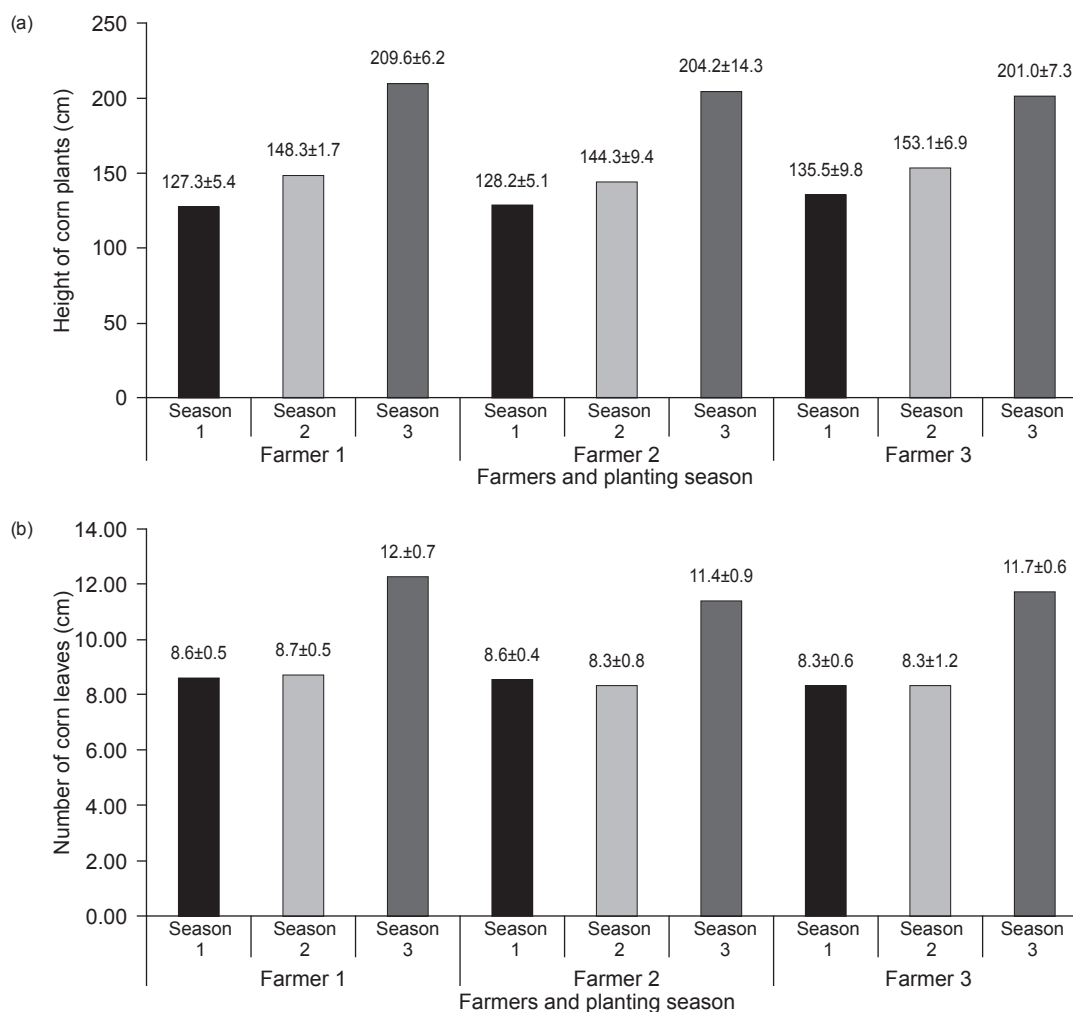
The CO₂ emissions from the oil palm replanting area decreased as a result of intercropping. The CO₂ emissions during the vegetative and generative phases of the intercropped plants are presented in Table 3.

Table 3 shows that the CO₂ emissions from intercropped plants during the vegetative phase were significantly different. CO₂ emission from corn was the highest at 67.2±31.1 t CO₂ ha⁻¹ yr⁻¹, followed by those from soyabeans, natural vegetation and legumes, at 48.7±20.7, 38.0±24.8 and 34.1±14.1 t CO₂ ha⁻¹ yr⁻¹, respectively. The average emission from intercrop plants was 47.0±26.5 t CO₂ ha⁻¹ yr⁻¹. The high levels of CO₂ emissions from corn can be attributed to the higher amount of inorganic fertiliser applied to corn than to soyabeans. Chemical fertiliser significantly increased CO₂ flux by 9% compared to solitary urea application (Salehi *et al.*, 2017). The application of inorganic fertiliser

over the long-term will increase the CO₂ flux (Dhadli and Brar, 2016).

The CO₂ emissions from the various intercropped plants during the generative phase were not significantly different. The average CO₂ emission from intercropped plants during the generative phase was higher (51.6±28.8 t CO₂ ha⁻¹ yr⁻¹) than that during the vegetative phase (47.0±26.5 t CO₂ ha⁻¹ yr⁻¹). This may be caused by the fact that microbial activity was still high, but the ability of the plants to absorb CO₂ for the metabolic activities in leaves had decreased. This meant that the amount of CO₂ absorbed by the leaves was low, resulting in higher CO₂ emissions in the generative phase.

The CO₂ emissions in the morning were higher than during the day (Table 3). Saleh *et al.* (2017) attributed a higher concentration of CO₂ in the morning to microorganism activity and a layer in the atmosphere that traps CO₂ between sunset and sunrise.

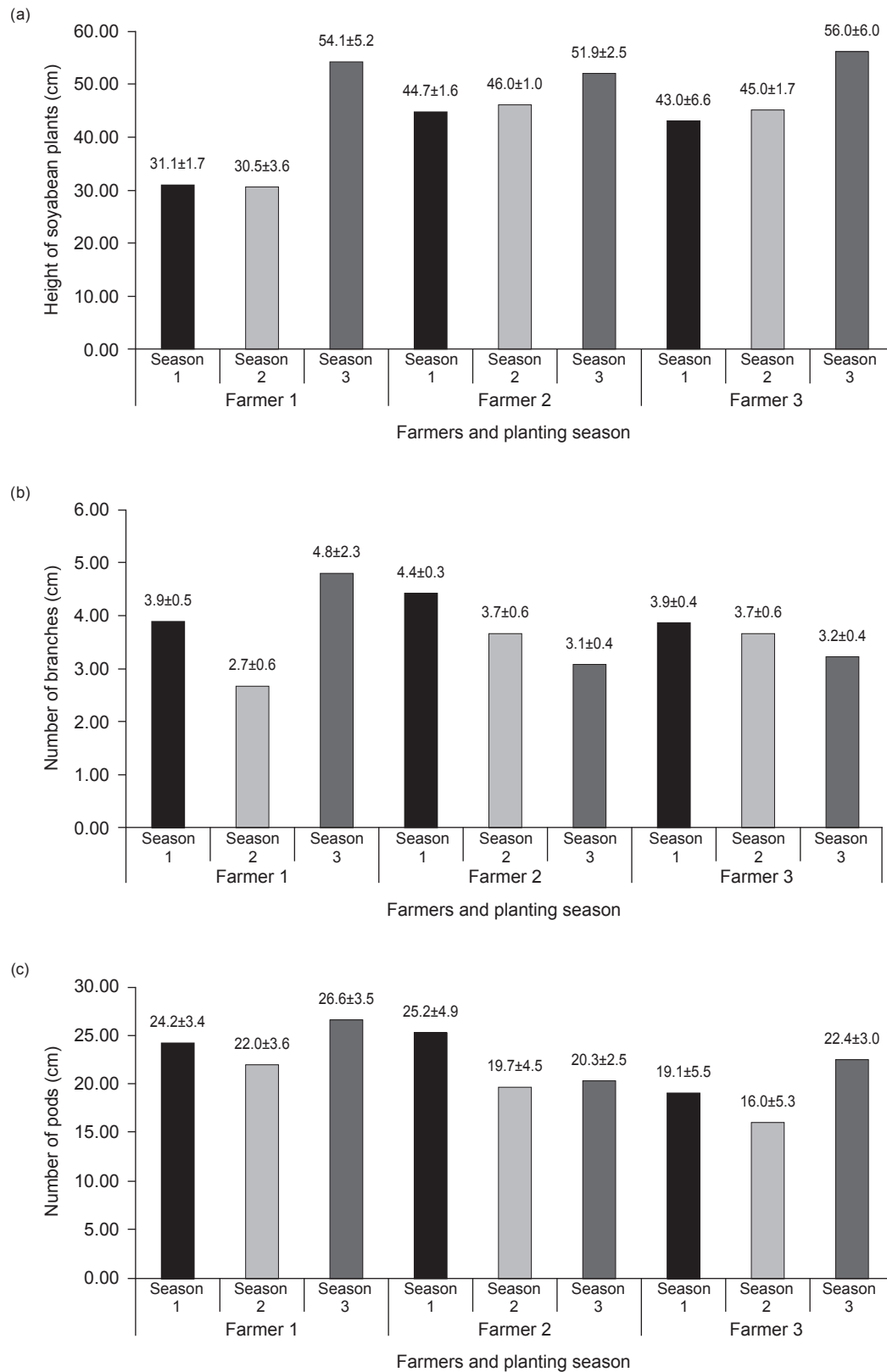


Note: Season 1 – June 2017 to October 2017.

Season 2 – November 2017 to February 2018.

Season 3 – March 2018 to July 2018.

Figure 6. Growth of corns plants. (a) height of plants, (b) number of leaves. The all groups were analysed by Duncan's Multiple Range Test (DMRT) at the level of 5%. The numbers followed by the same lowercase letter was not significantly different according to the DMRT at the level of 5%.



Note: Season 1 – June 2017 to October 2017.

Season 2 – November 2017 to February 2018.

Season 3 – March 2018 to July 2018.

Figure 7. The growth of soyabean plants. (a) height of plants, (b) number of branches, (c) number of pods. All groups were analysed by Duncan's Multiple Range Test (DMRT) at the level of 5%. The numbers followed by the same lowercase letter was not significantly different according to the DMRT at the level of 5%.

TABLE 3. CARBON DIOXIDE (CO₂) EMISSION DURING VEGETATIVE AND GENERATIVE PHASES OF INTERCROPPED PLANTS

Treatment (Intercropping plant)	Mean±STD CO ₂ emissions of vegetative phase (t CO ₂ ha ⁻¹ yr ⁻¹)			Mean±STD CO ₂ emissions of generative phase (t CO ₂ ha ⁻¹ yr ⁻¹)		
	Morning	Afternoon	Average	Morning	Afternoon	Average
Corn	67.2a±33.3	67.2a±29.7	67.2a±31.1	73.8a±46.1	37.9ab±19.2	55.9a±39.1
Soyabeans	50.1ab±21.7	47.2ab±20.5	48.7b±20.7	51.7a±18.5	39.1ab±13.8	45.4a±17.2
Natural vegetation	36.7b±24.5	39.2b±26.8	38.0bc±24.8	56.0a±24.0	34.5b±9.2	45.3a±20.9
Legume	33.9b±15.7	34.3±12.2	34.1c±14.1	72.0a±39.2	47.6a±14.1	59.8±31.4
Average	47.0±27.3	47.0±25.6	47.0±26.5	63.4±34.2	39.8±14.9	51.6±28.8

TABLE 4. LEVEL OF CARBON STOCKS IN VARIOUS CROPPING CONDITION

Plant conditions	Mean±STD carbon stocks (t C ha ⁻¹)
28-year-old oil palm	74.7±4.8
Understorey of 28-year-old oil palm	0.7±0.4
Corn	10.2±2.6
Legume	7.6±0.8
Soyabeans	3.5±0.6
Natural vegetation	2.8±0.8
1-year-oil palm*	43.5±0.4
2-year-oil palm*	45.4±1.3

Source: * Singh *et al.* (2018).

The lower CO₂ emissions in plantations with intercropping compared to bare land correlated with the carbon stocks presented in Table 4. The carbon reserve in the 28-year-old oil palms was 74.7±4.8 t C ha⁻¹, while that in the understorey of the 28-year-old palms was at 0.7±0.4 t C ha⁻¹. Corn had the highest carbon stocks of the intercropped plants at 10.2±2.6 t C ha⁻¹, followed by legumes, soyabeans and natural vegetation at 7.6±0.8, 3.5±0.6 and 2.8±0.8 t C ha⁻¹, respectively.

For comparison, the carbon stock of young oil palms at Mizoram, Northeast India was at 43.5±0.4 t C ha⁻¹ after one year and 45.4±1.3 t C ha⁻¹ after two years (Singh *et al.*, 2018). Carbon stocks of 5- to 6-year-old oil palms in Jambi and Riau were 10.7±0.4 t C ha⁻¹ and 9.5±0.2 t C ha⁻¹, respectively, while those of 33-year-old oil palms in Riau were 75.8 t C ha⁻¹ (Yahya, 2017). In Central Kalimantan, 7-year-old rubber plants had carbon stocks of 32.9±2.3 t C ha⁻¹ (Susanti and Dariah, 2014). In Northeast Brazil, the carbon stocks of 25-year-old oil palms average at 40 t C ha⁻¹ (Sanquetta *et al.*, 2015), corn at 2.36 t C ha⁻¹

(Ocampo and Oscar, 2016), soyabeans at 0.3 t C ha⁻¹ (Nagy *et al.*, 2017) and legumes at 14.6 t C ha⁻¹ (Guan *et al.*, 2016).

The vegetative growth of the oil palms over 11 months (August 2017 to June 2018) is presented in Figure 8.

Plant height, number of leaves and number of fronds indicate the carbon stocks of oil palm plants. Figures 8a-c shows that intercropping affected the vegetative growth of oil palms, although not significantly. The best plants in terms of plant height and length of fronds were found intercropped with legumes, while the highest number of fronds was found in palms intercropped with corn.

These results indicate that farmers should cultivate corn and soyabeans as intercrops during oil palm replanting. These plants reduce CO₂ emissions and increase carbon stocks in the replanting areas. In addition, the cultivation of corn and soyabeans is a source of additional income for smallholders.

CONCLUSION

The CO₂ emissions during the replanting of an oil palm area follow a dynamic pattern. The CO₂ emission from the area under the 28-year-old oil palm stands before the land clearing was 28.5±13.5 t CO₂ ha⁻¹ yr⁻¹. They were 59.0±49.4 t CO₂ ha⁻¹ yr⁻¹ from bare land, 47.0±26.5 t CO₂ ha⁻¹ yr⁻¹ in the vegetative phase of the intercropped plants, 51.6±28.8 t CO₂ ha⁻¹ yr⁻¹ in the generative phase of intercropped plants; and 42.9±23.3 t CO₂ ha⁻¹ yr⁻¹ when the new oil palms were a year old.

The carbon stocks of intercropped plants were 10.2±2.6 t C ha⁻¹ in corn; 7.6±0.8 t C ha⁻¹ in legumes; 3.5 ± 0.6 t C ha⁻¹ in soyabeans and 2.8 ± 0.8 t C ha⁻¹ in natural vegetation. The findings of this study show that oil palm replanting activities do not increase CO₂ emissions in the air but do increase carbon stocks.

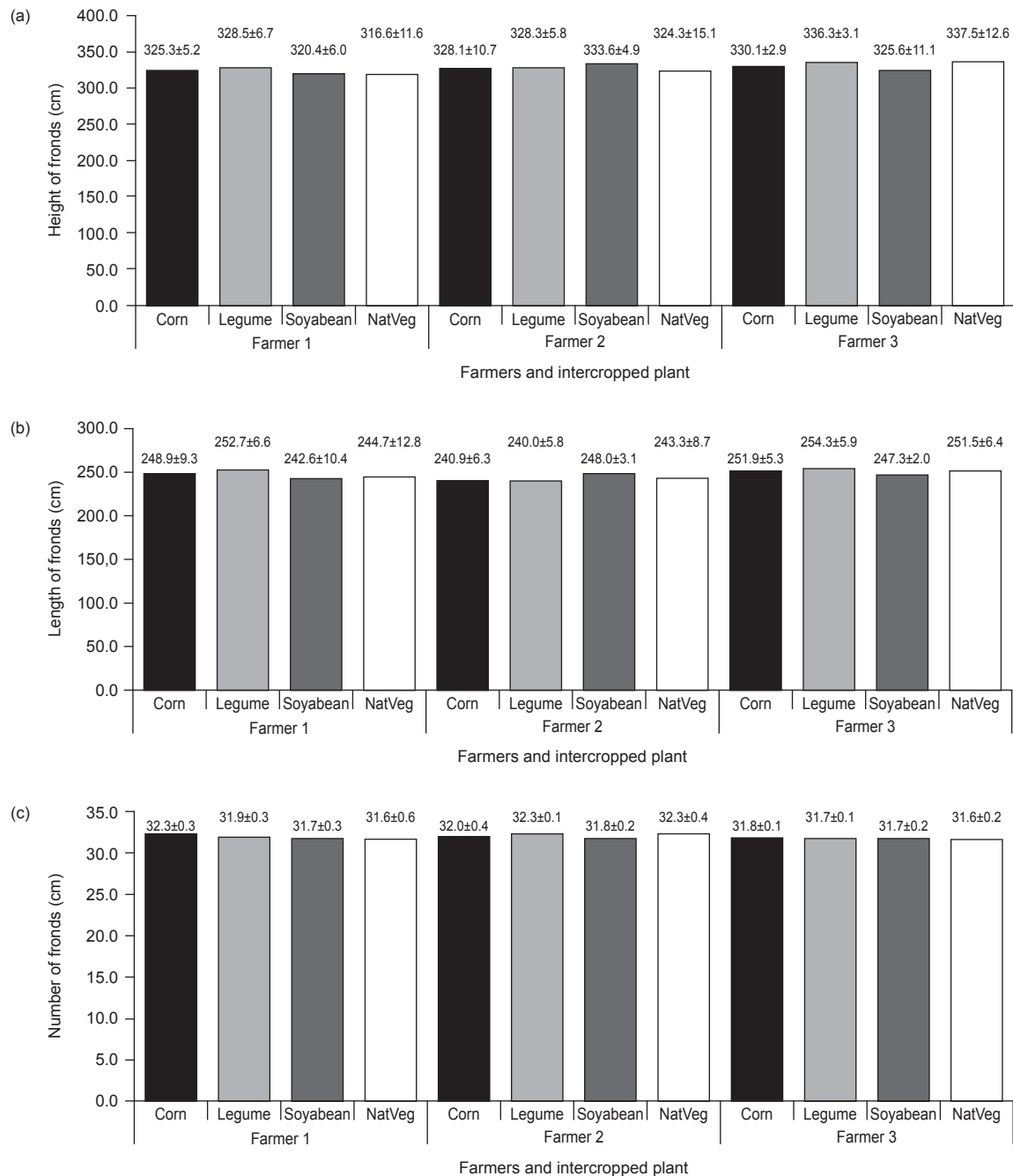


Figure 8. (a) Height of fronds, (b) length of fronds and (c) number of fronds of oil palm in different farmers' plantation and intercropping plants. All groups were analysed by Duncan's Multiple Range Test (DMRT) at the level of 5%. The numbers followed by the same lowercase letter was not significantly different according to the DMRT at the level of 5%.

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QUANTITATIVE APPROACH FOR IRRIGATION REQUIREMENT OF OIL PALM: CASE STUDY IN CHUPING, NORTHERN MALAYSIA

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ABSTRACT

The right decision is needed before the irrigation project starts because it is risky, costly and required a site-specific approach. The study aims to estimate oil palm irrigation water demand by using FAO-CROPWAT model. Study was conducted in Chuping Region, Northern Peninsular of Malaysia. Four points were selected to represent North, East, West and South for soil sampling. The samples were sent to a laboratory to measure the water content after pressure applied at 0, 1, 10, 33 and 1500 kPa. Total available water holding capacity was found at 105-227 mm for 100 cm soil depth and the lowest value was selected to be used in FAO-CROPWAT model, developed by Land and Water Development Division of Food and Agriculture Organisation of the United Nations (FAO). Prior to that, history of 14 years of monthly meteorological data were collected and serve as climatic data for potential evapotranspiration calculation. Based on the simulation, crop evapotranspiration (ET_c) and irrigation requirement (IR) was 1175 and 255.2 mm yr⁻¹ respectively. Total net irrigation was concluded at 132 mm yr⁻¹ with the assumption of 80% irrigation efficiency and 5.0 mm of irrigation input. Through this study, FAO-CROPWAT found to be a suitable approach to estimate crop water requirement (CWR) for oil palm and simulate irrigation scheduling for the entire year. It can help to strategise the management plan prior to any irrigation project design and increase potential for good economic return.

Keywords: available water content, FAO-CROPWAT, irrigation water supply, soil-water relation, water allocation budget.

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INTRODUCTION

Oil palm (*Elaeis guineensis*) is categorised as a commodity crop and it contributes a lot to the improvement of the living standard of Malaysian, especially in the rural area. It has been shown that oil palm's yield can reach up to 37 t ha⁻¹ yr⁻¹

(Goh, 1994). Results from other field trials has demonstrated better yield that can go up to 35-42 t ha⁻¹ yr⁻¹ during peak season (Kee *et al.*, 1998). However, the national average yield for Malaysia was still below 20 t ha⁻¹ yr⁻¹ for the last 15 years (Kushairi *et al.*, 2018).

Many factors contribute to this low yield, which includes plantation management, nutrient uptake, soil, topography, pest, disease and water. Water specifically in the form of moisture will maintain the functional growth of oil palm. This element plays important roles in photosynthesis and is also kept in the soil to make nutrient available for uptake by the palm. Water shortage will affect sex ratio index whereby, it is, lowering the number of female flowers at approximately 22-24 months before harvesting. During fruiting activity, any

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water shortage at 9-10 months or 4-5 months before harvest is damaging to the palm as it affects anthesis process and cause bunch failure.

Globally, industrial-scale oil palm is estimated at 18.7 million hectares as October 2017 (Meijaard *et al.*, 2018). Whereas in Malaysia, oil palm planted area further expanding up to 5.74 million hectares in 2016 (MPOB, 2017). Additional area means oil palm is going to be cultivated in marginal areas. In Peninsular Malaysia, land conversions to cultivate oil palm within marginal and moderate oil palm suitability classes have been increasing and about 30% of all conversions in 2012 were in areas with low oil palm suitability (Shevade and Loboda, 2019). Marginal soil classified as having at least one serious limitation to crop growth in the form of acute nutrient deficiencies, very poor drainage, steep slopes, massive laterites, acid peat, sandy texture, strong compaction, acid sulphate and saline conditions (Mohd Arif, 2005). Several of these limitations are highly related to the water deficit problem. Most oil palm plantations rely on the rainfall as the main water supply to oil palm. However, uneven rainfall distribution throughout the year and specific area (temporal and spatial distributions) has affected the yield. On top of that, *El-Nino* events sometimes occurs and worsen the situation (Amirul Hadi *et al.*, 2016; Nadia and Fatimah, 2016).

Irrigation is one of the ways to reduce the impact of water deficit and support oil palm functional growth. Irrigation was firstly demonstrated to increase yield of oil palm up to 225% as reported by Ochs and Daniels (1976). In Thailand, trials have been done which cover different type of irrigation and rate, and it has been shown that an irrigated plot gave extra yield up to 6 t ha⁻¹ yr⁻¹ (Palat, 2000). Whilst a study in different location has shown that drier area can give better palm response of up to 56% compared to non-irrigated palm (Lee and Izwanizam, 2013). Therefore, selection of an area for irrigation project is a very critical process in order to get high return on investment in the future.

FAO-CROPWAT 8.0 simulation model was developed by Food and Agriculture Organisation of the United Nations (FAO) to estimate Crop Water Requirement (CWR) especially for food crop. This model allows the development of irrigation schedules which cover different management options and also the calculation scheme of water supply for varying crop patterns. Studies were done on various crops such as maize and sorghum (Demba, 2014), rice, coconut, banana, areca nut, vegetables, pulses, rubber, tea, coffee and cotton (Surendran *et al.*, 2015). Nevertheless, this model has not been widely used for oil palm yet, and is now starting to draw some attention. A study done by Isa *et al.* (2016) has demonstrated the capability

of this model to estimate CWR and irrigation requirement (IR) for oil palm in South West Nigeria. Other than that, this model has been widely used to calculate water usage for water footprint evaluation in oil palm plantation (Mungkalasiri *et al.*, 2015; Zulkifli *et al.*, 2014; Muhammad Muaz and Marlia, 2014). Therefore, usage of FAO-CROPWAT into the oil palm plantation is justified. The objective of this study was to predict the crop and irrigation water requirement to help decision maker for irrigation planning in the oil palm plantation.

MATERIALS AND METHODS

Location and Soil Properties

An area located at FGV Agri Services Sdn. Bhd. (FGVAS) Chuping Estate (6°33'10.73"N, 100°18'19.15"E) was selected for this study with total of 28.8 ha. This place is located at a dry region and the highest maximum temperature ever recorded was 41°C. For the last 40 years, this area was cultivated with sugarcane and now being replanted with oil palm. Four points were selected for sampling purposes to represent North, East, South and West of the research plot. A semi-detailed soil survey of the area including the research plot was carried out by the relevant authorities. Basically, it consists of two main parent materials dominated by pediments and small part of sub-recent alluvium. Topography is undulating with slope class from 4%-12%. Soil developed over pediment parent material (reworked lateritic soil) was shallow and gravelled within 100 cm soil horizon. Topsoil was fine sandy loam and gravels consist of fine rounded petroplinthite. Particle class size ranging from loamy-skeletal to clayey skeletal with more than 35% gravels. Meanwhile, soil developed over sub-recent alluvium was deep, friable and fine sandy clay loam (Paramanathan, 2012; Jabatan Pertanian, 2008).

Total Available Water Holding Capacity (AWHC) Estimation

Soil samples were taken using bulk density ring at each layer. For each point, differences in soil layers were decided based on its physical and colour changes as shown in *Figure 1*. The thickness of each layer was recorded for Total AWHC calculation as in *Table 1*. These soil samples were sent to the laboratory for determination of water content at several pressure applications of 0, 1, 10, 33 and 1500 kPa using a pressure plate apparatus (Richards, 1947; Teh and Jamal, 2006). AWHC for each layer was calculated using the formula in Equation 1.

$$\frac{\text{Total AWHC}}{\text{of horizontal layer}} = \frac{\Sigma \text{AWHC for all depth}}{\text{of horizontal layer}} \quad \text{Equation (1)}$$

$$\text{AWHC} = \text{BD} \times \left(\frac{\text{FC} - \text{PWP}}{100} \right) \times \text{D}$$

where;

AWHC - available water holding capacity (mm)
 BD - bulk density (g cm⁻³)
 FC - field capacity (% in weight)
 PWP - permanent wilting point (% in weight)
 D - depth of horizontal layer (mm)

Examination of horizontal layer was done up to 1 m depth except for Point 1. It is because, high gravel proportion with dense horizontal layer beyond 60 cm become hindrance and making soil sampling by ring is nearly impossible. The value of each layer was added to get the total readings of Total AWHC (Equation 1). The lowest Total AWHC value was selected and used in FAO-CROPWAT software for simulation of crop water requirement and irrigation scheduling.

Collection of Historical Climatic Data

Weather data used in FAO-CROPWAT model was purchased from the Malaysian Meteorological Department. These data consists of 14 years collection from 2000-2013 (Table 2). Sunshine hours data was collected from Felda Chuping B Meteorological Station (06°30'13"N, 100°20'53"E) while the other data was from Chuping Meteorological Station (06°28'55"N, 100°14'4"E). The amount of rainfall, minimum temperature, maximum temperature, solar radiation and wind speed were used in the FAO-CROPWAT model to estimate the value of reference evapotranspiration (ET_o) in Equation 2 as described by Allen *et al.* (1998). Effective rainfall calculation was based on FAO method and the calculation is shown in Equation 3. ET_o and effective rainfall estimation were automatically calculated by the software.

$$\text{ET}_o = \frac{0.408 \Delta (\text{Rn} - \text{G}) + \frac{900}{\text{T} + 273} \text{U}_2 (\text{e}_s - \text{e}_a)}{\Delta + \gamma(1 + 0.34 \text{U}_2)} \quad \text{Equation (2)}$$

where;

ET_o - reference evapotranspiration (mm day⁻¹)
 Rn - net radiation at the crop surface (MJ m⁻² day⁻¹)
 G - soil heat flux density (MJ m⁻² day⁻¹)
 T - air temperature at 2 m height (°C)
 U₂ - wind speed at 2 m height (ms⁻¹)
 e_s - saturation vapour pressure (kPa)
 e_a - actual vapour pressure (kPa)
 e_s - e_a - saturation vapour pressure deficit (kPa)
 Δ - slope vapour pressure curve (kPa °C⁻¹)
 γ - psychrometric constant (kPa °C⁻¹)

$$\begin{aligned} P_{\text{eff}} &= (0.6 \times P_{\text{month}}) - 10 \quad \text{for } P_{\text{month}} \leq 70 \text{ mm} \\ P_{\text{eff}} &= (0.8 \times P_{\text{month}}) - 24 \quad \text{for } P_{\text{month}} > 70 \text{ mm} \end{aligned} \quad \text{Equation (3)}$$

where;

P_{eff} - effective rainfall

P_{month} - rainfall at particular month

FAO-CROPWAT Setting

In Crop Module, the crop coefficient (Kc) value of 0.80 was used as referred on the study conducted by Henson and Harun (2005). Suggestion by Tailiez (1971) stated the greatest quantity of rooting zone are within 20-60 cm from the ground level and has been used as rooting depth in the model. For the fourth module which is soil properties, two important inputs were the Total Available Moisture which is same meaning as Total AWHC and the other one is Maximum Rain Infiltration Rate (MRIR). In this study, MRIR was set to default value which is 30 mm day⁻¹ (Smith, 1992).



Figure 1. Different soil layers through soil horizon (Point 2).

Then, scheduling criteria such as irrigation timing and application depth were set accordingly in the 'Schedule' module. These inputs will have an impact on the determination of irrigation scheduling table and it is generally calculated by using soil water balance concepts. For this case, timing for irrigation was set when the deficits reached Critical Depletion (CD). The term CD refers to fraction of available moisture to be depleted before plant experience moisture stress affecting plant ET_o and plant production. The value for this fraction for broad range of crop has been presented by Allen *et*

TABLE 1. SOIL WATER RETENTION PROPERTIES AT DIFFERENT POINT OF SAMPLING ACCORDING TO EXAMINED DEPTH

Point	Depth (cm)	Bulk density (g cm ⁻³)	Gravimetric water content (%)					AWHC (mm)	
			0 kPa	1 kPa	10 kPa	33 kPa	1 500 kPa		
1	0-15	1.73	26.34	19.28	15.47	8.83	8.07	19.22	±3.51
	15-30	1.77	25.27	22.32	17.56	8.53	5.83	31.11	±0.49
	30-45	1.45	33.84	28.13	19.10	12.07	10.05	19.68	±1.21
	45-60	1.19	24.36	14.86	12.14	8.09	7.40	8.47	±0.62
2	0-25	1.61	27.37	17.67	14.01	6.84	5.74	33.35	±1.69
	25-60	1.20	15.81	12.65	9.59	8.07	7.28	9.66	±0.05
	60-100	1.22	43.88	31.32	29.18	22.76	16.43	62.34	±24.05
3	0-30	1.50	24.41	18.13	16.56	8.48	6.16	46.97	±1.27
	30-100	1.51	28.45	20.21	16.69	11.90	9.43	76.85	±39.10
4	0-100	1.70	24.01	20.15	17.17	4.57	3.81	227.17	±3.43
	Mean	1.49	27.37	20.47	16.75	10.01	8.02		

Note: AWHC - available water holding capacity.

TABLE 2. AVERAGE MONTHLY CLIMATIC DATA FROM NEAREST WEATHER STATION AT FGVS CHUPING ESTATE FOR 14 YEARS (2000-2013)

Month	Minimum temp. (°C)	Maximum temp. (°C)	Humidity (%)	Wind speed (m s ⁻¹)	Sunshine (hr)	Estimated by FAO-CROPWAT	
						Radiation (MJ m ⁻² day ⁻¹)	ET _o (mm day ⁻¹)
January	23.6	32.6	76	2.0	7.3	18.9	4.40
February	23.8	34.5	73	2.0	8.3	21.4	5.15
March	24.1	34.4	78	1.4	7.1	20.4	4.72
April	24.4	34.1	81	1.0	7.0	20.2	4.54
May	24.5	33.3	84	0.8	5.5	17.4	3.88
June	24.1	32.7	84	0.8	5.7	17.2	3.79
July	23.7	32.2	85	0.8	5.1	16.5	3.60
August	23.7	32.2	85	0.9	5.3	17.3	3.77
September	23.6	31.9	86	0.8	5.0	17.0	3.68
October	23.7	31.8	86	0.9	5.2	16.8	3.63
November	23.8	31.9	85	1.3	5.2	16.0	3.52
December	23.7	31.5	82	1.6	5.6	16.1	3.60
Average	23.9	32.8	82	1.2	6.0	17.9	4.02

Note: ET_o - reference evapotranspiration.

al. (1998). Unfortunately, there are no exact figures demonstrated for oil palm yet and in this case, 50% has been selected to be inserted in the model. Based on practical point of view, 4 mm was selected as an irrigation application amount. Irrigation efficiency was set at 80% based on the average percentage proposed by Pocaiades (2000) for micro irrigation method. Therefore, the depth of irrigation will be 5 mm considering losses up to 20%.

Evaluating Different Sources of ET_o Data Sources

Several studies by researchers have quantified the accuracy of FAO-CROPWAT model based on water deficit impact to the crop yield (Etissa *et al.*, 2016; Bekele and Tilahun, 2009) but this is not covered in this article due to several reasons including insufficient climatic data for 2014 and onward. In addition, the palms were replanted in March 2014 and started yielding two years later. Consequently, data sources as requisite input for ET_o calculation emphasised and put into evaluation.

Climatic data for years of 2013, 2012, 2011 and 2010 was served as 'observed' years. Then, climatic

data for previous years (PY), average of last five years (5Y), average of last 10 years (10Y) and data from FAO-CLIMWAT (Climwat) was served as 'predicted'. All the data was loaded into FAO-CROPWAT for determination of monthly ET_o value. Data from Climwat was downloaded by selecting nearest station with the research area namely as Kangar (6°25'48"N, 100°12'0"E). Comparison of ET_o value between 'observed' and 'predicted' was accessed by using mean absolute error (MAE) and root mean squared error (RMSE) based on 1:1 line (Equation 4).

$$MAE = \frac{1}{n} \sum_{i=1}^n |O_i - P_i|$$

$$RMSE = \sqrt{\frac{\sum |O_i - P_i|^2}{n}} \quad \text{Equation (4)}$$

where;

n - numbers of observation

O_i - 'Observed' data

P_i - 'Predicted' data

RESULTS

Soil-water Retention Properties

The results of soil water retention properties are shown in *Table 1*. Variations in soil layer can be observed for every sampling point. Point 1 consists of four main layers of up to 60 cm depth. Sampling process cannot go further beyond that level because of the existence of hard plinthite layer. Point 2 consists of three layers followed by Point 3 consists of two layers. There are no variations observed for Point 4 and the layers are in the same characteristic from ground level to 1 m depth.

The graph was plotted to represent average value of sampled layers and mirror S-curve shape was observed (*Figure 2*). Each area shows a saturated value ranged from 38.9%-42.1% and percentage of volumetric water content (VWC) reduce with the increasing of pressure applied. Points 1, 2 and 3 were located at the same parent material for soil origin, therefore the curve is likely to be the same trend and close to each other. Eventually, trend curve for Point 4 have a greater variation and much lower permanent wilting point (PWP) which is only 6.5%.

Using Equation 1, the value of Total AWHC according to the sample point is as shown in *Table 3*. The highest value was Point 4, which is 227 mm then followed by Point 3 and 2, which are 124 and 105 mm, respectively. Point 1 has a value of 78 mm but it is only represent for 60 cm depth.

TABLE 3. TOTAL AVAILABLE WATER HOLDING CAPACITY (AWHC) VALUE ACCORDING TO THE SAMPLING POINT

No.	mm m ⁻¹	cm cm ⁻¹
1	78.48*	0.131
2	105.35	0.105
3	123.82	0.124
4	227.17	0.227

Note: * only for 60 cm.

Crop Water Requirement (CWR)

Once the data is loaded into FAO-CROPWAT, the CWR properties could be generated and the result is shown in *Table 4*. A decade indicated 10-days value of CWR, and it was calculated using linear interpolation in FAO-CROPWAT (Smith, 1992). Total estimation of crop evapotranspiration (ET_c) is 1175.0 mm yr⁻¹ and effective rainfall is 1117.8 mm yr⁻¹. For each of IR calculations, the deficit will only be applied if the ET_c value exceeds the effective rainfall which total is 255.2 mm yr⁻¹.

Irrigation Scheduling

However, looking solely at *Table 4* does not reflect daily irrigation input. Therefore, 'Schedule' module calculates daily irrigation input based on soil water balance concept. In this case, value of 105 mm m⁻¹ was used besides the pre-defined

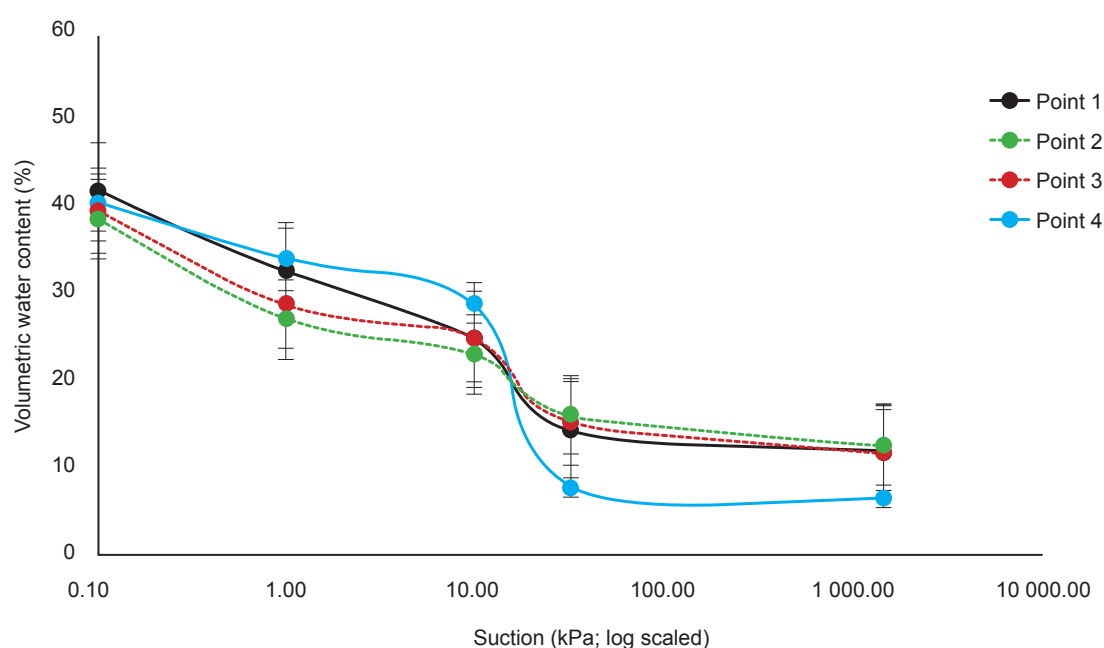


Figure 2. Soil water retention curves for each location.

requirement such as initial soil moisture, irrigation timing and irrigation depth as mentioned before. Simulation for daily irrigation schedule is shown in *Figure 3*. Two lines depicted by green and red colour indicate available water for 60 cm root zones and the latter is for water availability after considering root zone and CD (for this case is 50%). The results showed that Total Net Irrigation (TNI) was 132 mm for the entire year.

Evaluation of Various Dataset

It has been found that MAE value ranged from 0.10-0.32 (*Figure 4*). The lowest MAE value was represented by 2013-5Y data which is only 0.10. Surprisingly, 5Y generated lower MAE value compared to 10Y for all tested year. Moreover, PY also generates lower MAE than 10Y for years of 2013, 2012 and 2011. Overall, Climwat dataset has shown better MAE than 10Y.

TABLE 4. CROP WATER REQUIREMENT (CWR) FOR OIL PALM PLANTED AT FGVAS CHUPING ESTATE

Month	Decade	Kc	ETc (mm day ⁻¹)	ETc	Effective rainfall	IR
				(mm dec ⁻¹)	(mm dec ⁻¹)	(mm dec ⁻¹)
Jan	1	0.8	3.3	33.1	10.2	22.8
Jan	2	0.8	3.5	35.2	2.0	33.3
Jan	3	0.8	3.7	40.9	5.4	35.6
Feb	1	0.8	4.0	39.8	8.6	31.2
Feb	2	0.8	4.2	42.0	9.9	32.1
Feb	3	0.8	4.1	32.5	18.2	14.3
Mar	1	0.8	3.9	38.9	28.5	10.4
Mar	2	0.8	3.8	37.8	36.6	1.1
Mar	3	0.8	3.7	41.0	39.0	2.0
Apr	1	0.8	3.7	36.8	43.2	0.0
Apr	2	0.8	3.6	36.3	47.5	0.0
Apr	3	0.8	3.5	34.5	40.1	0.0
May	1	0.8	3.3	32.8	29.8	3.0
May	2	0.8	3.1	31.0	22.9	8.1
May	3	0.8	3.1	33.9	23.5	10.4
Jun	1	0.8	3.1	30.6	24.9	5.7
Jun	2	0.8	3.0	30.3	24.6	5.7
Jun	3	0.8	3.0	29.8	24.6	5.2
Jul	1	0.8	2.9	29.3	24.0	5.3
Jul	2	0.8	2.9	28.8	23.7	5.1
Jul	3	0.8	2.9	32.2	26.2	6.0
Aug	1	0.8	3.0	29.7	28.2	1.5
Aug	2	0.8	3.0	30.2	30.0	0.2
Aug	3	0.8	3.0	32.9	35.5	0.0
Sep	1	0.8	3.0	29.7	42.5	0.0
Sep	2	0.8	3.0	29.5	48.1	0.0
Sep	3	0.8	2.9	29.3	48.8	0.0
Oct	1	0.8	2.9	29.2	49.7	0.0
Oct	2	0.8	2.9	29.0	51.2	0.0
Oct	3	0.8	2.9	31.6	49.8	0.0
Nov	1	0.8	2.9	28.5	49.5	0.0
Nov	2	0.8	2.8	28.2	49.2	0.0
Nov	3	0.8	2.8	28.4	41.7	0.0
Dec	1	0.8	2.9	28.6	33.5	0.0
Dec	2	0.8	2.9	28.8	26.9	1.9
Dec	3	0.8	3.1	34.0	19.9	14.2
Total				1 175.0	1 117.8	255.2

Note: ETc – crop evapotranspiration; IR – irrigation requirement.

RMSE is likely to be same as MAE but is not identical since it will amplify large error to become more visible. As an example, MAE for 2013_10Y has relatively same value compared to 2012_Climwat data source. However, RMSE for former data source was higher, 0.452 compared to 0.360 as shown in Figure 5. It is likely due to two data which are far deviated from 1:1 line. Similarly for 2011-PY, the bar was increased and become the same level as 2011-5Y and 2011-10Y (Figure 4).

DISCUSSION

Total AWHC reflect the availability of water content between field capacity and PWP. PWP refers to the water content under high soil retention that affects the plants to withering and loose turgidity even in humid atmosphere (Briggs and Shantz, 1912). Normally, PWP is straight forward and directly represented by -1500 kPa pressure level. But it actually differ between crop species, developmental stage, soil condition and climate (Carlesso, 1995; Romano and Santini, 2002). In best situation, the field capacity needs to be measured in the field where the soil is undisturbed. But, this can be hampered by practical difficulties, laborious, time consuming and lateral losses through horizontal flow (Reichardt, 1988; Van Lier, 2002). Although 33 kPa is often used as estimation for field capacity, there are some arguments rised by Kirkham (2004). He stated that soil scientists realised that field capacity was an imprecise term and it was not a unique value, because equilibrium is never reached. Field capacity is usually determined by applying tension from 0.05-0.15 bar for sandy to loamy soils (McCarty *et al.*, 2016). Therefore, 10 kPa or 0.10 bar was selected as a field capacity point since the result was comparable to available water content suggested by Raveendra *et al.* (2017) (Table 5).

Crop water and irrigation demand are dependent on climate and location. There are no attempt being made in the Malaysian context to use FAO-CROPWAT for irrigation scheduling. As comparison, potential ET_0 ranged from 3.60-5.15 mm day⁻¹ (Table 1) is relatively high compared to study done by Isa *et al.* (2016). ET_0 for his study in South West Nigeria ranged from 2.92-4.49 mm day⁻¹ although similar annual rainfall amount was observed.

Generally, soil profiles in the study area are compacted and has high bulk density. This might be due to the previous sugarcane cultivation that lasted for almost 40 years. Long-terms sugarcane cultivation altered soil physical properties in term of higher bulk density, lower structural stability and an increased proportion of fine pores (Barzegar *et al.*, 2005). Variability among Total AWHC values shows there are need for variable rate of irrigation in the field but such irrigation concepts are still in an infancy level with inadequate trial results. Furthermore, sluggish price of crude palm oil for the last two years (MPOC, 2019) has also become major hindrance for planters to invest on huge capital cost project such as irrigation.

The others important information available shown in FAO-CROPWAT is peak demand for water sources and for this case concentrated in January to February. Normally, estates water source depends on stream or river for oil palm irrigation. For this example, high evaporation and low rainfall will greatly impact water level and irrigation attempt might be impossible. Irrigation input of 5.0 mm day⁻¹ was almost equivalent to a 1440 m³ of water day⁻¹ for a total of 28.8 ha area. Therefore, it is expected that the adjustment will be made to suit with water availability such as by reducing input of irrigation despite increasing irrigation interval. Another option is to have groundwater as an alternative water source, but this might

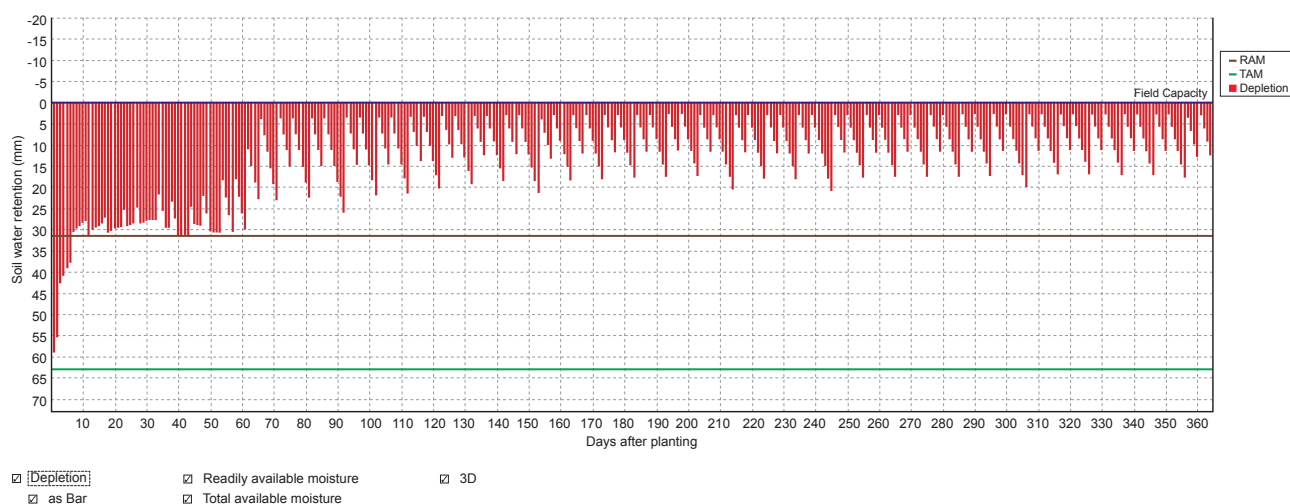
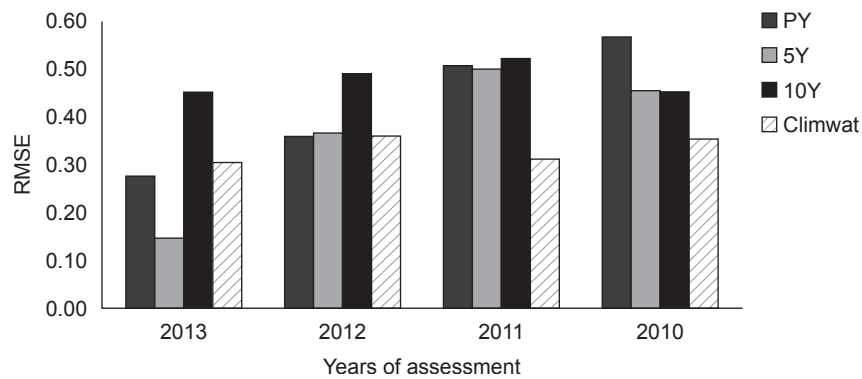
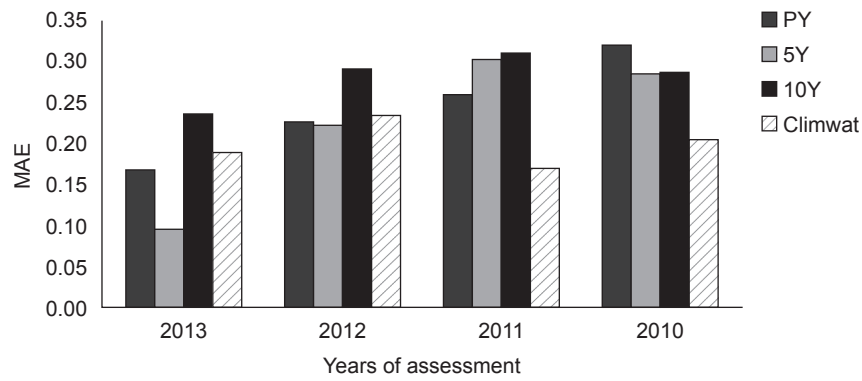
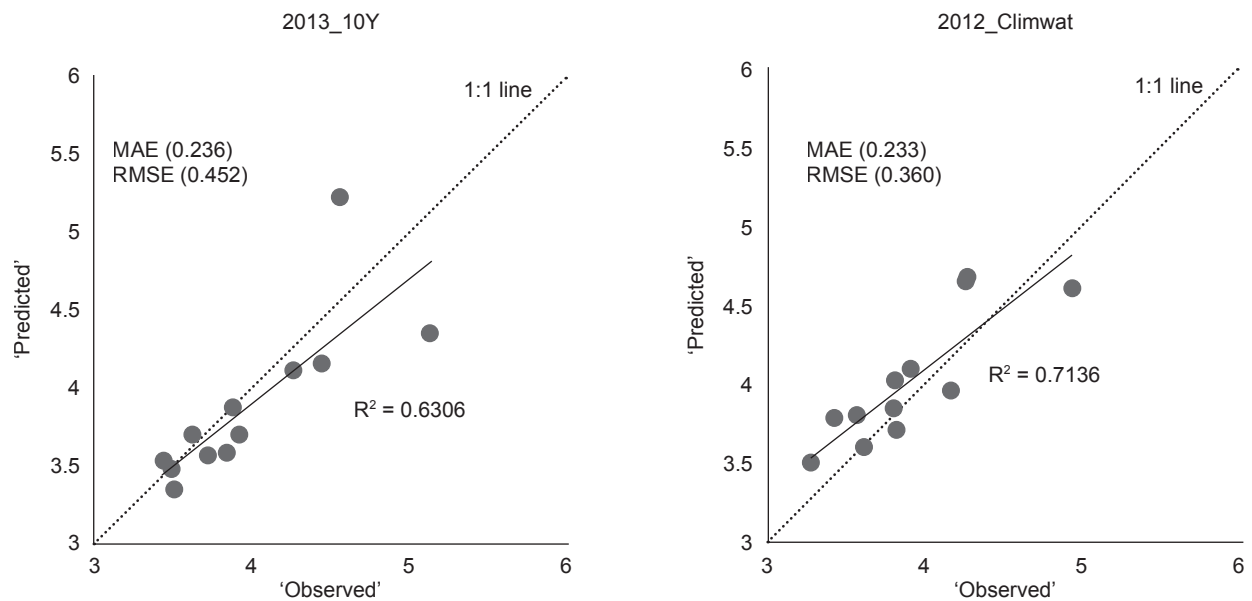


Figure 3. Irrigation schedule suggested by FAO-CROPWAT.



Note: MAE – mean absolute error; RMSE – root mean squared error.

Figure 4. Comparison between different datasets using MAE and RMSE statistical indices.



Note: MAE – mean absolute error; RMSE – root mean squared error.

Figure 5. Graphical representation of 'observed' (x-axis) and 'predicted' (y-axis) with their respective MAE and RMSE.

incur additional cost besides uncertainties of water quality and groundwater recharge rate.

In general, long-term historical data will be used to feed the module in FAO-CROPWAT model. Therefore, it makes sense to suggest that the longer data is collected or averaged, the closer it is to the expected value. However, the question that arises is how long the data should be required to obtain an accurate estimation of ET_o . Two statistical indices namely MAE and RMSE usually applied to make an assessment of modelling accuracy. Based on their objective, it is reasonable to use them and look into 'observed' - 'predicted' closeness.

ET_o generated from 5Y was found to be more close to the observed value in terms of MAE for 2012 and 2013. To certain extent, PY also gave lower value than 10Y for 2011, 2012 and 2013. This put into contexts that longer data period does not necessarily give better ET_o estimation. Result has shown that Climwat gave consistent results and sometimes better than the others. Thus, making Climwat dataset a good option to be used when local data are

partially invalid or not available. It is freely available with more than 5000 stations worldwide (43 stations for Malaysia) and 15-30 years compilation makes it very handy and reliable resources (FAO, 2020).

CONCLUSION

Chuping region is well known as a dry area and has a relatively higher temperature than other parts of Peninsular Malaysia. Soil formation was classified from two types which are pediments and sub-recent alluvium. However, Total AWHC varied greatly within examined soil layer. Based on climate, rainfall, crop and soil input the calculated IR is 255.2 mm yr⁻¹. However, TNI is only amounted to 132 mm yr⁻¹ and most irrigation events suggested are concentrated in January to February. This estimation will helps to design proper irrigation project and make a decision whether it could be profitable or not. MAE and RMSE showed climatic data from Climwat could be the good option if localise data

TABLE 5. SOIL MOISTURE AT FIELD CAPACITY (θ FC), PERMANENT WILTING POINT (θ PWP), AVAILABLE WATER CONTENT (AWC) AND BASIC INFILTRATION RATE (F)

Soil type	θ FC (% v)	θ PWP (% v)	F (mm day ⁻¹)	AWC (cm cm ⁻¹)
Sand	9 (6-12)	4 (2-6)	1 200 (600-6 000)	0.05
Coarse sand	3.2	1.2	11 200	0.02
Medium coarse sand	9.5	1.7	3 000	0.078
Medium fine sand	15.5	2.3	1 100	0.132
Fine sand	19.6	4.2	500	0.154
Sandy loam	14 (10-18)	6 (4-8)	600 (312-1 824)	0.08
Sandy loam	19.5	6.1	165	0.134
Light loamy medium (coarse sand)	24.2	10	23	0.142
Loamy medium coarse sand	18.1	2.1	3.6	0.16
Loamy fine sand	14.6	6	265	0.086
Fine sandy loam	27.3	8.7	120	0.186
Loam	22 (18-26)	13 (8-12)	192 (192-480)	0.09
Silt loam	33.8	9.2	6.5	0.246
Loam	29.3	9.8	50	0.195
Clay loam	27 (23-31)	13 (11-15)	192 (60-360)	0.14
Sandy clay loam	31.7	18	235	0.137
Silty clay loam	34.5	18.5	15	0.16
Clay loam	39.3	25.5	9.8	0.138
Silt clay	31 (27-35)	15 (13-17)	60 (7.2-120)	0.16
Clay	35 (31-39)	17 (15-19)	12 (2.4-120)	0.18
Light clay	34	21.5	35	0.125
Silty clay	44.7	25.7	13	0.19
Basin clay	49.8	32.1	2.2	0.177

Source: Raveendra *et al.* (2017).

are not available. Through this study, it has been found FAO-CROPWAT can be used as a tool to simulate crop water requirement which was previously developed for water management of food crops.

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ODOUR EXPOSURE LEVEL MEASUREMENT SURROUNDING PALM OIL MILL

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ABSTRACT

Odorous gases such as ammonia and hydrogen sulphide produced by anaerobic bacteria are emitted when palm oil mill effluent (POME) is treated via anaerobic digestion. Although odour is a nuisance rather than a hazardous nature most of the times, sensory annoyance complaints provoke local authority to seek for feasible mitigation. Malaysian Department of Environment (DOE) under the jurisdiction of the Air Division has proposed an odour emission limit of 12 000 OU m^{-3} for palm oil mills recently. The objective of this article is to establish the anaerobic pond odour concentration at palm oil mill using open ponding system for POME treatment, identify ammonia (NH $_3$) and hydrogen sulphide (H $_2$ S) gas content in odour sample collected from respective anaerobic pond, and establish the odour concentration (OU m^{-3}) at residential locations close to a palm oil mill using open ponding system, covered lagoons and tank digester for POME treatment. The odour sampling and assessment were performed according to MS 1963:2007 Air Quality – determination of odour concentration by dynamic olfactometry with enhanced VDI3940 Grid Method. Results showed that odour concentration at source of anaerobic pond ranged from 44 135-85 012 OU m^{-3} while H $_2$ S content ranged from 15.9-103.9 ppm and NH $_3$ content ranged from 4.1-16.6 ppm. Odour concentrations at residential locations are often dominated by local odour sources such as chicken farms. Odour emission levels at all surveyed residential areas identified as palm oil mills origin were well below 10 OU m^{-3} most of the times although high level of up to 108.4 OU m^{-3} has been observed occasionally. Thus, the proposed odour limit value needs to be reviewed and justified based on further study with focus on more data collection, odour control guidelines, community odour impact survey, inclusion limit values for odorous chemical substances, odour regulation framework, odour assessment methods and odour management plan guidelines.

Keywords: anaerobic digestion, European reference odour mass, odour nuisance, olfactometry, POME treatment.

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INTRODUCTION

For every tonne of fresh fruit bunches (FFB) processed at palm oil mills, 0.6 t of palm oil mill

effluent (POME) is generated to produce crude palm oil (CPO). This acidic effluent contains water, oil, protein and traces of minerals (Fatah *et al.*, 2012).

The average biochemical oxygen demand (BOD) of POME is about 25 000 ppm and needs to be treated in order to reduce its BOD to below 20 ppm before being discharged into water course. Conventionally, it is treated in a series of anaerobic and aerobic ponds that require long hydro retention time (Loh *et al.*, 2014). Open effluent pond digestion treatment is the most common treatment system practiced by Malaysian mills due to the

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low capital expenditure (CAPEX) and operation expenditure (OPEX) where the POME is digested by a consortium of the microorganisms commonly found in soil and air. Due to the recent biogas capture policy to further address sustainability issues, various treatment systems have been installed in palm oil mills, such as covered lagoon and digester tank systems. Practical data show that 1 m³ of POME could generate 25 m³ of biogas with average calorific value of 22 000 kJm⁻³ containing 62.5% methane, 37.0% carbon dioxide (CO₂) and traces of hydrogen sulphide (H₂S) and ammonia (NH₃) (Loh *et al.*, 2017).

Anaerobic digestion is a complex biochemical process with a consortium of micro-organisms converting organic compounds in the absence of oxygen (O₂) into methane and CO₂ in four stages, which are hydrolysis, acidogenesis, acetogenesis and methanogenesis (Loh *et al.*, 2014). Anaerobic POME treatment releases odorous gases such as NH₃ and H₂S that are known as air pollutants which have become a serious environmental concern for many years. Malaysian Department of Environment (DOE) is currently simulating the odour limit and sampling site to be gazetted in the new regulation. Environmental factors such as wind speed and direction, topography, atmospheric stability and pollutant concentration need to be considered during odour assessment.

Smell sensing is accomplished with two main nerves. Chemicals perception is processed by the olfactometry nerve whereas chemicals irritation or pungency is processed by the trigeminal nerve. All olfactory signals meet in the olfactory bulb where the information is distributed to two different parts of the brain. Limbic system pathway processes emotion and memory response of the body. The other information pathway is to the frontal cortex. From nostril to the brain signal, the process takes only about 500 milliseconds (Mauskar, 2008). Odour intensity is the perceived odour sensation strength which is related to the odorant concentration as shown in Equation (1) known as Stevens' Law or the power law where I is the intensity, k is a constant, C is the concentration, n is exponent ranges from about 0.2-0.8 depending on the odorant.

$$I = k(C)^n \quad \text{Equation (1)}$$

The odour concentrations mentioned as odour units per cubic meter is based on correlation between a physiological response when the nose detects odour, and exposure concentration of a particular sample. Odour unit is the amount of odorant(s) when evaporated into 1 m³ of neutral gas at standard conditions, elicits a physiological response equivalent to that elicited by one European Reference Odour Mass (EROM) which is

equivalent to 123 µg *n*-butanol evaporated in one cubic meter of neutral gas at standard conditions.

In the Draft Environmental Quality (Odour) Regulations 201X (unpublished data), a limit of 12 000 OUm⁻³ was proposed, meaning no part or activities in palm oil mills can emit odour beyond the 12 000 OUm⁻³ thresholds. Thus, Malaysian Palm Oil Board (MPOB) and Universiti Sains Malaysia (USM) started an odour investigation project in order to produce reliable odour emission data to validate Department of Environment's proposal. Three mills with different treatment systems: open lagoon, covered anaerobic lagoon and anaerobic tank digester, were studied. However, findings from the previous preliminary survey (Andrew *et al.*, 2019) demonstrated the inability of the mills to comply with the DOE proposed value. Thus, an alternative approach to the odour threshold limit is necessary to justify the odour pollution from palm oil mills.

Odour is a nuisance rather than a hazardous nature most of the times. Nuisance is defined as cumulative effect on human caused by repeated annoyance events over an extended period of time that leads to modified or altered behaviour (Van Harreveld, 2001). Thus, the odour exposure to the surrounding community is also a measurable data to validate odour emission issue from the palm oil mill. It seems probable that despite being non-compliant at source, odour may be minimal below the threshold at the boundary, which is less likely to be a nuisance to the surrounding community. The Nuisance Law approach will be implemented in Malaysia odour legislation.

The objectives of the survey are: (1) To establish the anaerobic pond odour concentration (OUm⁻³) at palm oil mill using open ponding system for POME treatment, (2) To identify NH₃ and H₂S gas concentration in odour sample collected from respective anaerobic ponds, and (3) To establish odour concentration (OUm⁻³) at residential locations close to palm oil mill using open ponding system, covered lagoons and tank digester for POME treatment. Wind speed and direction at every location was also recorded.

MATERIALS AND METHODS

Odour measurement method, project site and measuring instruments need to be duly determined prior to the survey execution. Odour measurements consist of in field odour measurement and emission point odour sampling measurement. The project schedule should cover normal and peak crop seasons in order to produce a comprehensive survey. Although weather conditions are uncontrollable, wind blow condition would be recorded.

Odour Measurement Methods

A defined odour measurement method is described in standard MS 1963:2007 (olfactometry) set out by the Department of Standards Malaysia (SIRIM) using *n*-butanol for equipment calibration and panel member selection. The odour assessment results are expressed in terms of OUm⁻³ EROM, where one EROM is equivalent to 123 µg *n*-butanol.

In-field Odour Measurement

Direct real time in-field odour measurement using olfactometer for ambient and boundary investigation was conducted. Enhanced VDI3940 Grid Method was adopted to execute in field odour survey which included the determination of odour intensity, odour concentration and odour characters for a 10 min observation period at each location.

The Scentroid SM100 in-field olfactometer incorporates a dilution device within a portable device, allowing direct odour concentration measurements to be carried out in real time in the field. The odour assessment method involved the determination of odour intensity, odour concentration and odour characterisation following that of Balch *et al.* (2015) and Bakhtari and Medina (2016). Three odour concentration (OUm⁻³) readings were recorded and further supported by odour intensity of 0 (no odour) to 6 (extremely strong). Odour characters such as sour vinegar, rotten egg or others were also noted during the field survey to better understand the odour impact to the surrounding communities. The odour survey form recorded the odour intensity and character, with a short description of the various scales and numbers described in the survey form for easy reference. The potential odour sources from on-site observation would also be recorded to further support the detection of odour and understanding possible nearby contributors.

Odour Sampling at Emission Point and Laboratory Analysis

Odour sampling at emission point and laboratory analysis in an odour free room was conducted for odour emission strength identification and odour control systems design. Samples were collected into 10 litre Nalophan bag and sent to the odour laboratory for olfactometry analysis using the Scentroid SS400 dynamic olfactometer to determine their concentration, expressed as OUm⁻³.

H₂S and NH₃ levels in the odour samples were analysed using a portable multi-sensor device, the OdoTracker TR8 (Scentroid, Canada).

Instruments Accuracy

The SM 100 Scentroid Olfactometer with detection range from 3.5-11 355 OUm⁻³ was used for in field measurement whereas the Scentroid SS400 dynamic olfactometer with detection range from 15 to 18 643 OUm⁻³ was used for source samples measurement within 30 hr of sample collection. Due to the predicted odour samples strength, pre-dilution was necessary prior to analysis. The Geometric Mean as shown in Equation (2) is used to compare and analyse the data properties.

$$\text{Geometric Mean, GM} = \sqrt[n]{a_1 \times a_2 \times \dots \times a_n} \quad \text{Equation (2)}$$

$$\Delta z_i = \frac{a_i}{GM} \text{ if } a_i \geq GM; \Delta z_i = \frac{GM}{a_i} \text{ if } a_i < GM; 1 \leq \Delta z_i \leq 1.5$$

OdoTracker TR8 (Scentroid, Canada) is equipped with electrochemical sensors for H₂S detection range from 1-2000 ppm with resolution of 1 ppm and NH₃ detection range from 0-100 ppm with resolution of 0.3 ppm.

Project Sites

Odour exposure measurements in the survey were carried out at three palm oil mills and selected boundaries as described in Table 1. The mills differed in terms of effluent treatment system which affected the odour emission from the anaerobic ponds. The survey boundaries for each location was selected within 2 km radius and determined based on the safe assessment into the established residential area. Thus, the boundaries distances were different for each study location.

TABLE 1. SURVEY LOCATIONS

POME treatment	Location	^A Boundary 1	^A Boundary 2
Open pond	Mill A, Kedah	0.8 km NE	2.0 km W
Covered lagoon	Mill B, Penang	0.7 km W	1.0 km E
Digester tank	Mill C, Perak	1.4 km N	1.9 km SW

Note: ^ABoundary 1: nearest residence, ^ABoundary 2: second-nearest residence to palm oil mill; POME - palm oil mill effluent.

The anaerobic pond odour complete with odorous gases contents assessments and in-field boundary odour assessments for the respective three selected palm oil mills were duly carried out according to the schedule as shown in Table 2.

During in-field odour concentration measurement, odour intensity was also recorded every 10 s for 10 min to a scale of 0-6 following UK Environment Agency guideline as shown in Table 3.

TABLE 2. PROJECT SCHEDULE IN YEAR 2019

Project assessment	Normal crop		Peak crop	
	Start	End	Start	End
Anaerobic Pond	28 January	30 January	19 August	21 August
Boundaries Mill A	19 February	21 February	13 August	15 August
Boundaries Mill B	25 February	27 February	29 July	31 July
Boundaries Mill C	25 March	27 March	8 July	10 July

TABLE 3. ODOUR INTENSITY DESCRIPTION

Intensity	Odour strength	Description
0	No odour	Odour detection threshold (ODT)
1	Very weak	Some doubt whether odour is actually present
2	Weak	Odour is present but cannot be described precisely
3	Distinct	The odour character is barely recognisable
4	Strong	The odour character is easily recognisable
5	Very strong	Odour is offensive and the exposure level is undesirable
6	Extremely strong	Odour is offensive and instinctive reaction would mitigate against further exposure

RESULTS AND DISCUSSION

Malaysia has tropical monsoon climate which is hot with normal ambient temperature ranging from 25°C-35°C, humidity up to 85% and rain throughout the year. The highest ambient temperature is usually recorded from 12:00-15:00 and the sun heating effect is negligible during 19:00-07:00. Thus, the microorganisms' activities within the POME treatment ponds vary from day to night but remained in similar trend throughout the year.

Odour intensity level 0-2 was recorded most of the time and intensity level 3 was occasionally recorded during in field assessments.

A bio-digester has been installed at Mill A since the first run during normal crop season as part of the palm oil mill effluent treatment system, which includes a covered anaerobic lagoon and a gas collection system. The bio-digester precedes five open anaerobic lagoons, a facultative pond, aeration pond, and two settling ponds before final discharge. At the time of the odour assessment in the second run during peak crop season, the same effluent treatment plant configuration was present, and although the bio-digester was completed, it was in the commissioning stage and yet to be fully operational. However, the odour concentration at source and in-field reduced significantly during the second assessment as shown in *Figure 4* compared to *Figure 3*. Similar observation was also found on

H₂S and NH₃ content in gas samples collected from anaerobic pond as shown in *Figure 2* compared to previous assessment as shown in *Figure 1*. Thus, biogas capture reduced odour emission from palm oil mill via quarantine approach.

Figures 5 and *6* show the boundary odour assessments for covered lagoon system during normal crop and peak crop season, respectively. *Figures 7* and *8* show the boundary odour assessments for digester tank system during normal crop and peak crop season, respectively. Results showed that the odour concentration at source was very much higher than in-field odour concentration. The odorous gases content varies with in-field odour concentration profile and is affected by environmental factors which influence microorganisms' activities such as temperature, humidity and air pressure due to wind blow.

Samples analysis showed that H₂S content was always higher than ammonia NH₃ content. Both odorous gases content is in ppm. Thus, the disappearance of the odour effect is mainly due to gases dilution with atmospheric air. NH₃ threshold is 500-1000 µgm⁻³ whereas H₂S threshold is 1.5-150 µgm⁻³ (Ding *et al.*, 2012). However, small portion of the odorous gases are decomposed via bacterial activities. Sulphur bacteria oxidise H₂S via pathway as shown in Equation (3) (Startsev, 2017).



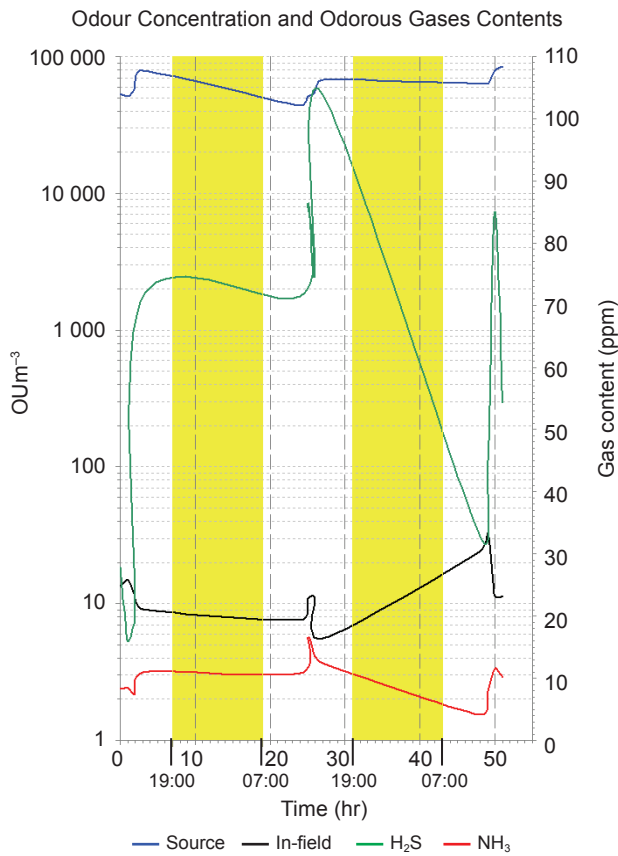


Figure 1. Normal crop anaerobic pond odour concentration and odorous gas content.

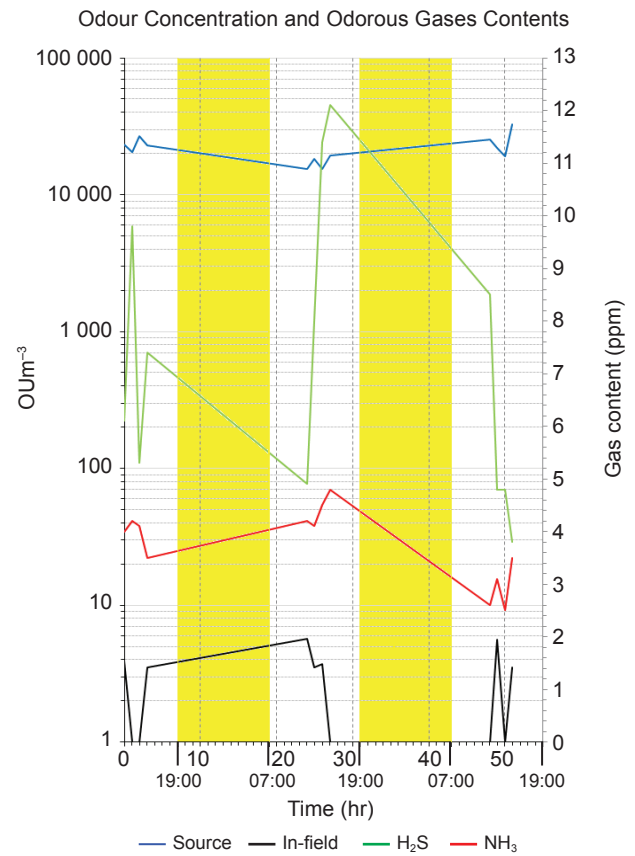


Figure 2. Peak crop anaerobic pond odour concentration and odorous gas content.

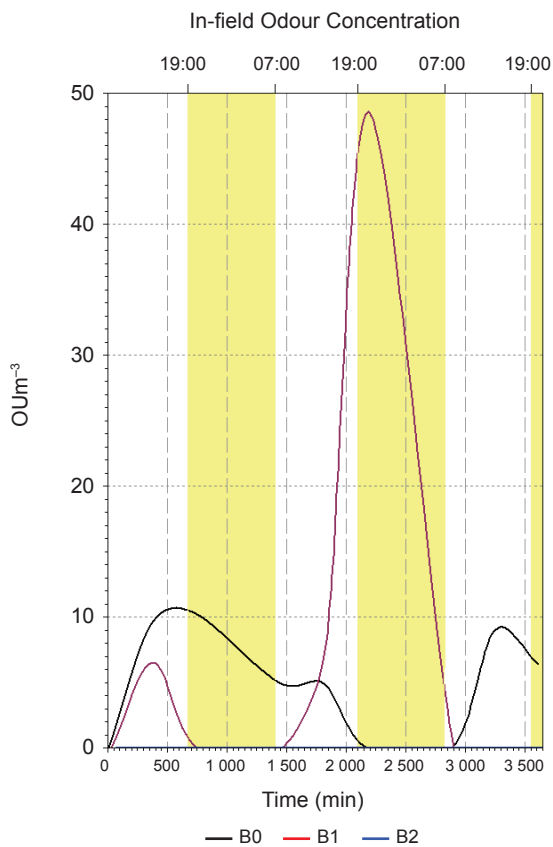


Figure 3. Boundary odour assessments for open pond system during normal crop.

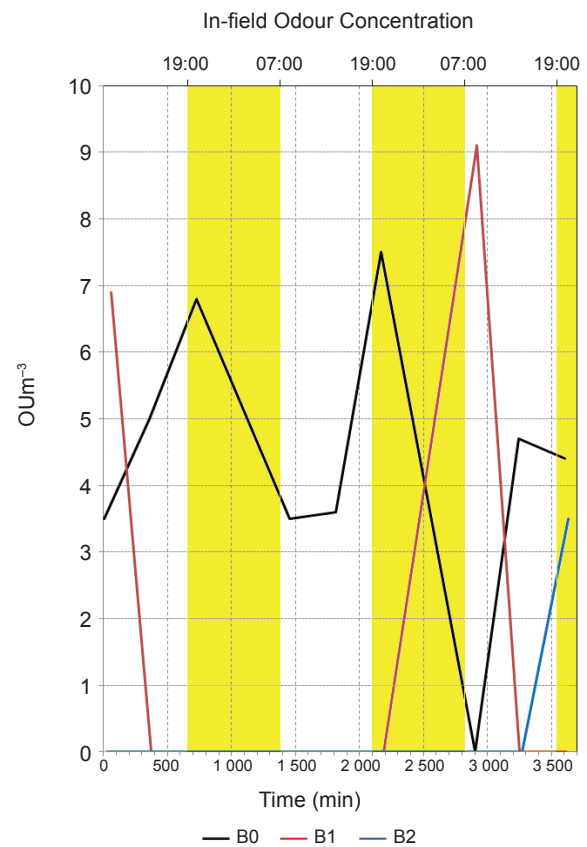


Figure 4. Boundary odour assessments for open pond system during peak crop.

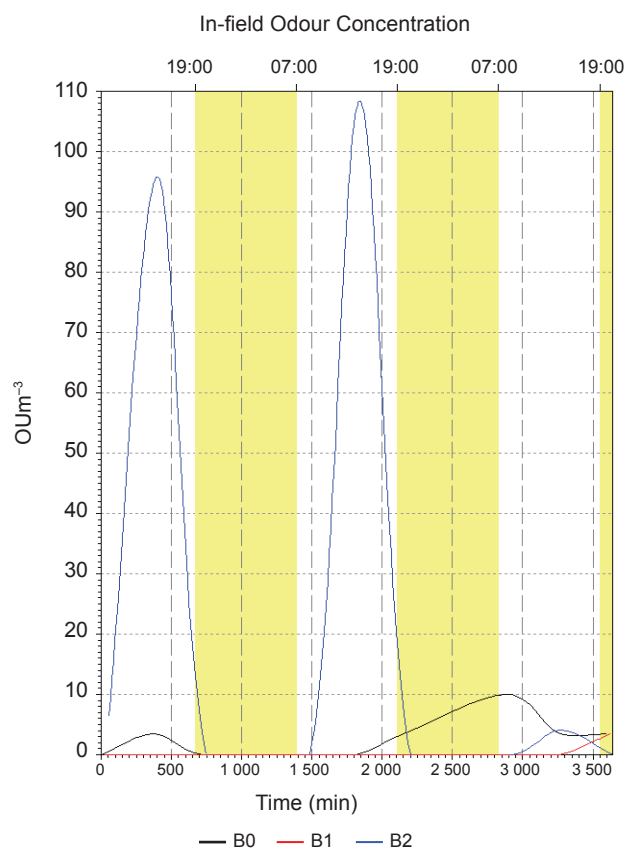


Figure 5. Boundary odour assessments for covered lagoon system during normal crop.

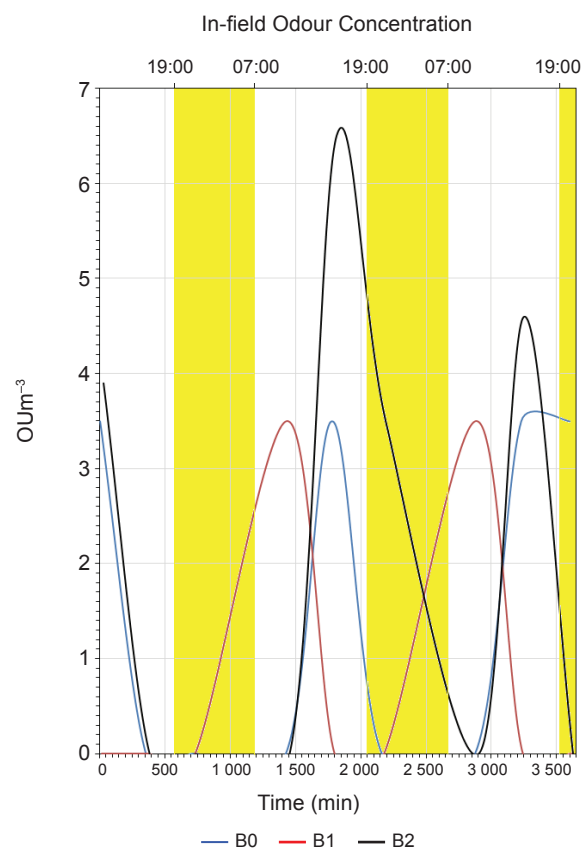


Figure 6. Boundary odour assessments for covered lagoon system during peak crop.

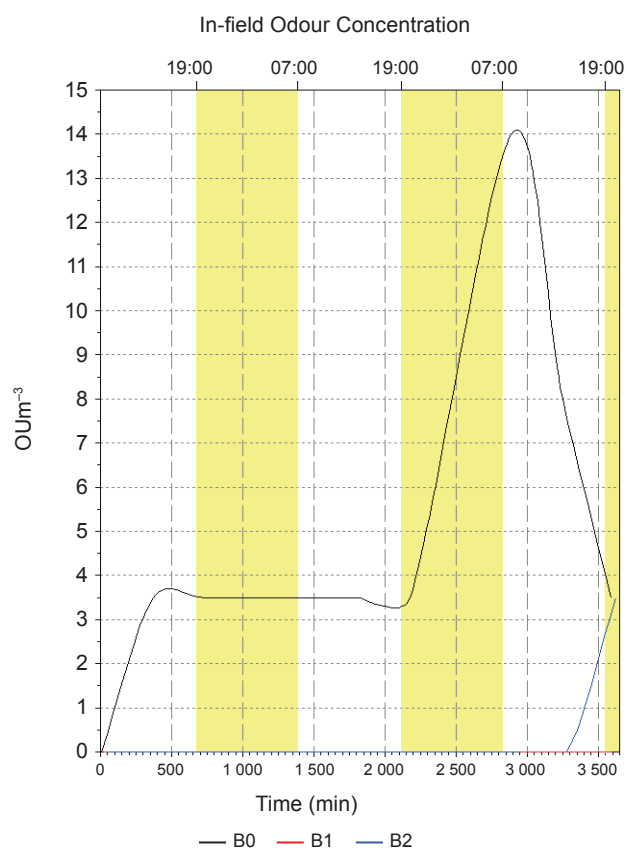


Figure 7. Boundary odour assessments for digester tank system during normal crop.

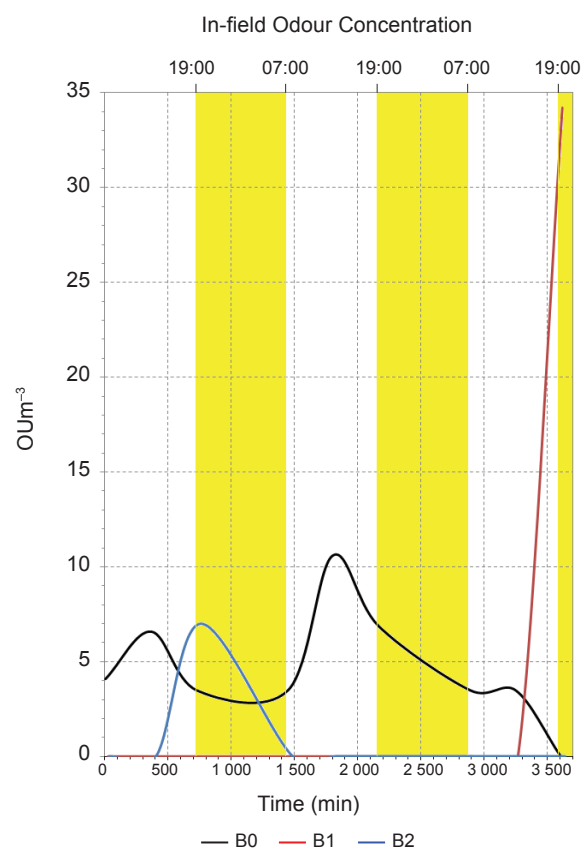
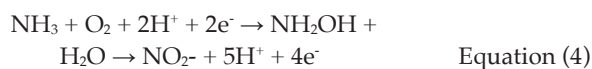


Figure 8. Boundary odour assessments for digester tank system during peak crop.

NH_3 is oxidised by nitrifying bacteria via complex autotrophic nitrification process that requires several enzymes, proteins and oxygen as shown in Equation (4).



Based on the empiric data and the threshold limits, the odour emission from palm oil mill is dominated by H_2S . Figure 9 shows the correlation between the odorous gas content and odour concentration of the gas samples collected from anaerobic pond. Low correlation between the respective odorous gas and odour concentration indicated that the odour of the gas samples collected from the anaerobic pond were not single odorant effect but rather a combination of various odorous gases generated from POME anaerobic fermentation. Although the amount of such odorous gases was regulated by the microbial activity, the odour concentration theoretically was proportional to the amount of odorant present in the gas sample. Thus, linear multivariate regression has been performed yielding empiric odour model as shown in Equation (5) where x_1 is H_2S , H_2S

content in ppm; x_2 is NH_3 , NH_3 content in ppm and y is odour concentration in OUm^{-3} .

$$y = 372.9286x_1 + 1718.5450x_2 + 17419.7682 \quad \text{Equation (5)}$$

Source	Degree of freedom	Sum of square	Mean square	F value
Regression	2	8551324254	4275662127	17.71520843
Residual	22	5309819930	241355451.4	
Total	24	13861144184		

The R^2 value increases to 0.7854 with estimation error 14573.7023 showed that besides H_2S and NH_3 , other unidentified odorous gases also existed and influenced the odour concentration.

Wind rose diagram for survey locations of Mill A, Mill B and Mill C is shown in Figures 10, 11 and 12, respectively. Diagrams analysis show that only Mill A Boundary 1 and Mill B Boundary 2 have potential of odour complaints due to wind blow. However, the wind speed at all survey sites was well below 5 mph most of the time even strong wind was recorded at Mill A; once 21.15 mph South and once 5.3 mph South to Southeast (SSE).

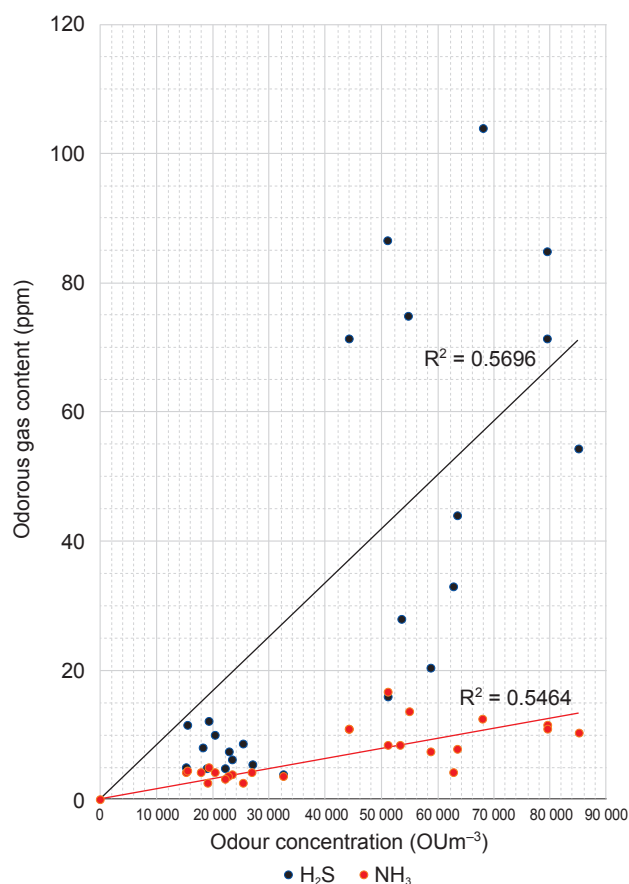


Figure 9. Correlation between odorous gas content and odour concentration.

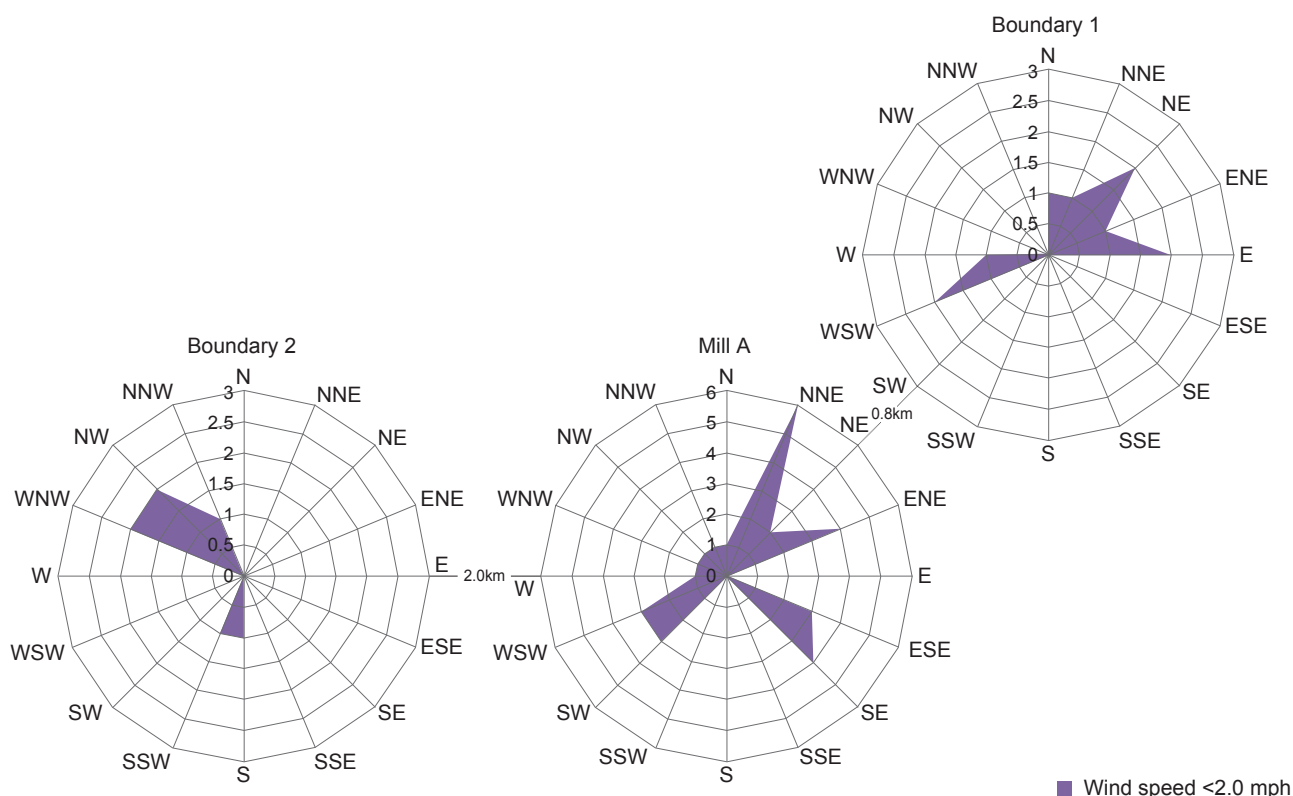


Figure 10. Wind Rose Diagram Analysis for Mill A and the survey boundary areas.

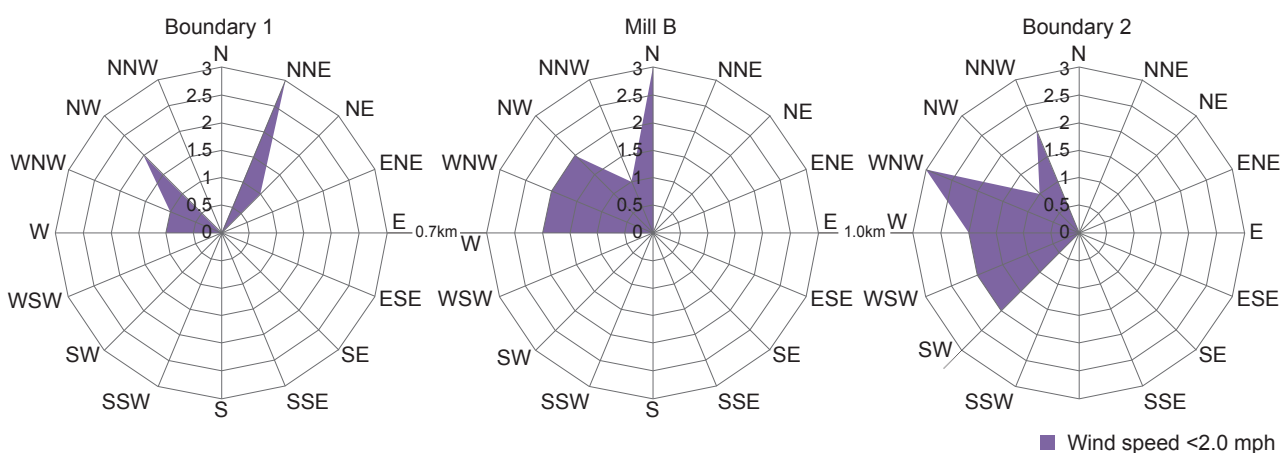


Figure 11. Wind Rose Diagram Analysis for Mill B and the survey boundary areas.

CONCLUSION

Experimental results showed that odour concentration at source of anaerobic pond ranged from 44-135-85-012 OUm^{-3} while H_2S content ranged from 15.9-103.9 ppm and NH_3 content ranged from 4.1-16.6 ppm. Odour concentrations at residential locations are often dominated by local odour sources such as chicken farms. Odour emission levels at all surveyed residential areas identified as palm oil mills origin were well below 10 OUm^{-3} most of the times although high level of up to 108.4 OUm^{-3} has been observed occasionally.

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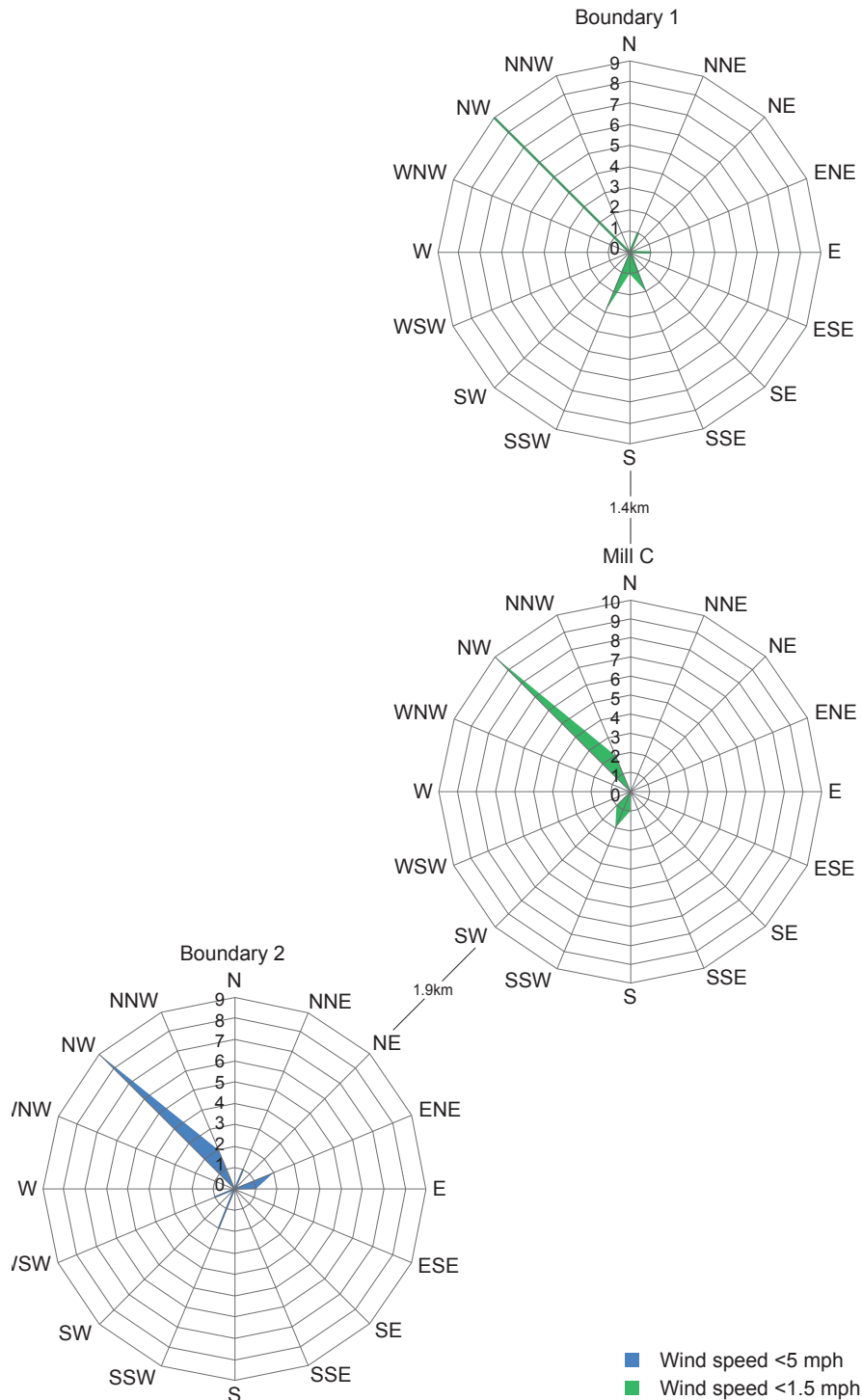


Figure 12. Wind Rose Diagram Analysis for Mill C and the survey boundary areas.

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INVESTIGATION ON THE EFFECT OF MONOGLYCERIDE CONCENTRATION ON PALM OIL-BASED ALKYD RESIN PREPARATION

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ABSTRACT

Alcoholysis and esterification are useful methods to prepare polyalkyds from vegetable oils. Reaction parameters and different concentration of raw materials have showed a significant role on the properties of the formulated polyalkyds. The formation of monoglyceride (MG) and diglyceride (DG) during the synthesis and their ratio may have a significant role on the properties of the polyalkyds. The goal of the present article was to investigate the effect of MG to DG ratio on alkyd resin synthesised from palm oil by alcoholysis-polyesterification reactions catalysed by homo-hetero catalytic system. Different ratios of MG:DG were obtained from alcoholysis reaction by taking samples at different reaction times. The formation of the alkyd resin was confirmed by Fourier transform infrared spectroscopy (FTIR) with the presence of ester group (C-O-C). The molecular weight (M_w) of alkyd resin was determined by gel permeation chromatography (GPC). The alkyd resin prepared at higher MG:DG ratio exhibited higher initial rate of polyesterification reaction with higher M_w . In addition, the film of alkyd resin prepared from higher MG:DG ratio presented better pencil hardness and chemical resistivity.

Keywords: alkyd resin, CuO nanoparticles, FTIR, monoglyceride, palm oil.

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INTRODUCTION

Alkyd resins are very important materials in coating industries. They have been used in varnishes,

composite coatings, lacquers and adhesives industries (Dolui, 2015; Meneghetti and Meneghetti, 2015). Triglyceride (TG) based vegetable oil can be used to synthesis alkyd resins. To synthesis alkyd resin in the laboratory, alcoholysis is the first step, which followed by esterification (Mutar and Hassan, 2017). For the alcoholysis process, the most common polyol used is glycerol, whereas, for esterification phthalic anhydride is widely used (Ong *et al.*, 2018). Different types of catalysts like lead(II) oxide (PbO), potassium hydroxide (KOH), zinc oxide (ZnO), lithium oxide (LiO), calcium oxide (CaO) and sodium hydroxide (NaOH) can be used for the reactions. The reported reaction temperature is around 240°C. The conversion in the first step, after the alcoholysis, the reaction products usually contain 40%-60% monoglyceride (MG) (Rarokar *et al.*, 2017). This MG is required to homogenise the reaction medium which is a favourable condition

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for the second step, polyesterification. MG and diglyceride (DG) reaction with polyacid can form the linear polymer. The chain length of the alkyd resin is related to MG:DG ratio, where DG acts as a chain stopper.

Several numbers of researches have investigated the polyalkyds preparation from vegetable oils using the two-step process, alcoholysis and esterification (Beg *et al.*, 2019). Optimisation of the reaction parameters and characterisation of the synthesised polyalkyds were the focus of these works. Some of the works highlighted about the drying and non-drying properties of the vegetable oils. In addition, further modification of the polyalkyds was also suggested. Based on the previous results, it was hypothesised that the esterification can be influenced by the factor of MG and DG ratio. Bearing this in mind, the current research work has been developed. Moreover, to the best of our knowledge, other researchers preferred the methanol solubility method during alcoholysis reaction, but this method does not quantify the MG concentration. Recently, our laboratory prepared MG from palm oil via copper(II) oxide - sodium hydroxide (CuO-NaOH) catalyst system and using high performance liquid chromatography (HPLC) method to quantify the MG concentration (Ong *et al.*, 2016a). The effect of MG:DG ratio on alkyd preparation has been undermined throughout the literature. In this context, the goal of this work is to investigate the effect of MG:DG ratio on polyesterification reaction. Moreover, the pencil hardness and chemical resistivity of the alkyd films were elucidated.

METHODOLOGY

Materials

The chemicals and reagents used in this study were ethanol (99.9%), copper (II) chloride salt ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), standard 1-oleoyl-rac-glycerol ($\geq 99\%$), NaOH, hydrazine monohydrate (64%), glycerol, cobalt octoate, KOH, 1,2-dioleoyl-sn-glycerol ($\geq 97\%$), methyl ethyl ketone peroxide (MEKP), styrene, and glyceryl trioleate ($\geq 99\%$) which were purchased from Sigma-Aldrich, Malaysia. Acetone and acetonitrile were procured from Merck (Malaysia) Sdn. Bhd. Refined palm oil was kindly supplied by Malaysian Palm Oil Board (MPOB), Malaysia. All chemicals were used in this research as they were received. Figure 1 depicts the flow diagram of methodology for this study.

Preparation of CuO Nanoparticles

The CuO nanoparticles were synthesised at ambient temperature by sol-gel method (Ong *et al.*, 2015a; 2015b). First of all, a fixed amount of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ was dissolved in 44 ml of glycerol. After that, 6 ml of hydrazine solution was added into the mixture dropwise. The concentration of copper was maintained from 100-300 mg litre⁻¹. Hydrazine solution (7.062 mM) was prepared earlier by diluting 64% of hydrazine monohydrate in ethanol.

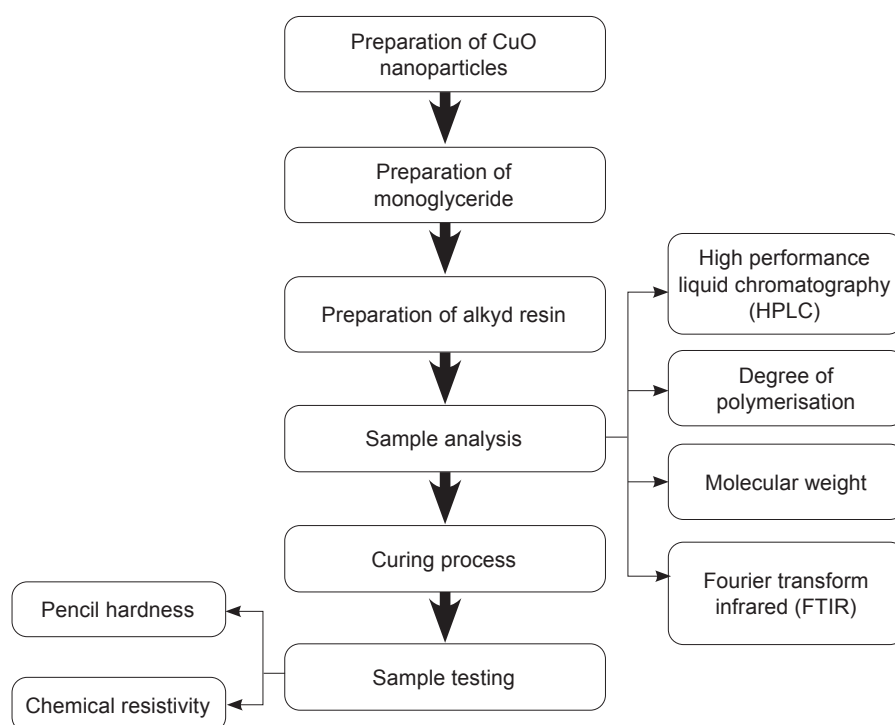


Figure 1. Flow diagram of methodology.

Preparation of Monoglyceride

Alcoholysis process was adopted to prepare the MG from palm oil and glycerine. In the process, nearly 100 g of palm oil was poured in a beaker with CuO nano particles (22.2 g). The mixture was stirred continuously and heated at 240°C. After that, additionally, a little amount of NaOH (0.3 g) was added. The reaction was conducted in a continuous flow of nitrogen gas into the reaction chamber.

Preparation of Alkyd Resin

After the completion of alcoholysis reaction, the temperature of the reactor was reduced to 140°C. After that, 38.7 g of phthalic anhydride was added into the reactor. Then, the temperature of the reactor was increased to 240°C. The acid value (AV) of the products was determined by ASTM D1639-90, at different times during the propagation of the reaction. When the AV reached to nearly 10 mg KOH g⁻¹, then the reactor was cooled down. To investigate the effect of MG:DG ratio on polyesterification reaction, the alcoholysis reaction was stopped at different times (30, 40 and 60 min) and the polyesterification reaction was conducted as mentioned above. The synthesis pathway of alkyd resin is presented in Figure 2.

High Performance Liquid Chromatography (HPLC)

The HPLC (Agilent 1200 Series) was used to quantify the reaction products. The machine was equipped with refractive index detector (RID)

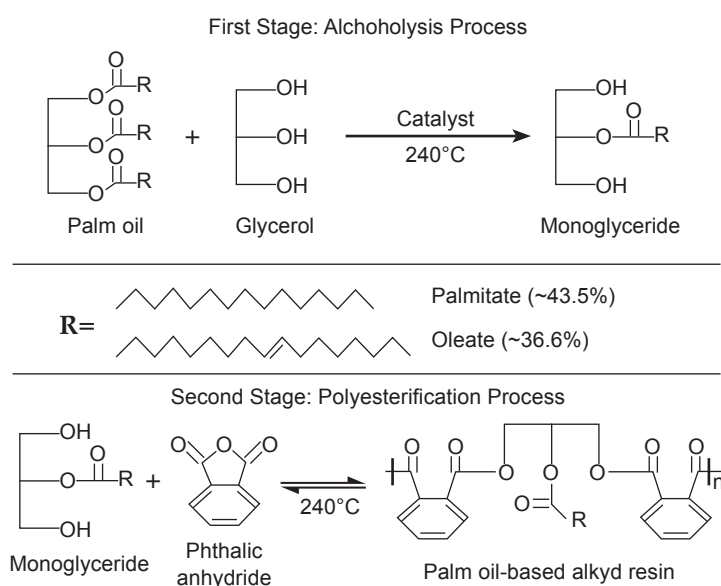
and diode-array detectors (DAD). A Zorbax C18 column with the specification of 4.6 m x 250 mm (5 µm) was used for the analysis. The flow rate was maintained at 1.0 ml min⁻¹. The detector and the column temperature were fixed to 40°C and 35°C, respectively. A mixture of acetone and acetonitrile (65:35, v/v) was used as a carrier of the sample through the column. Another composition of acetone and acetonitrile (1:1, v/v) was used to dissolve the sample. The volume of the injection was 10 µl. The standards of MG, DG and TG were used to quantify the reaction products. Different concentrations at 300, 800, 1000, 2000, 5000, 8000 and 10 000 ppm were used to prepare the calibration curves. At least three different runs were carried out for each analysis. A slow agitation and slightly warmed samples were used for the analysis to avoid the separation of the phases. The experimental deviations were considered less than 5% for the content of MG, DG and TG.

Degree of Polymerisation Determination

During the esterification reaction, the reaction products were collected for the AV testing. The titrimetric value was used for the determination of AV. The AV was used to calculate the extent of the reaction, P. The average degree of polymerisation (DP) was calculated using the value of P. The following equations were used for the calculation:

$$P = (C_0 - C_t) / C_0 \quad (1)$$

$$DP = (1 - P)^{-1} \quad (2)$$



Source: Ong *et al.* (2015d).

Figure 2. Synthesis pathway of palm oil-based alkyd resin (alcoholysis-polyesterification process).

where, C_0 = acid value at the beginning and C_t = acid value at the time t , t = reaction time.

Molecular Weight (M_w) Determination

A waters gel permeation chromatography (GPC) was used to determine the molecular weight (M_w) of the formulated alkyd resins. The testing was performed at room temperature using tetrahydrofuran as an eluent. Linear polystyrene was used for the calibration. Waters empower software was used for the data analysis.

FTIR Spectroscopy

The functional groups of palm oil and the resin (A-60) were observed by a Fourier transform infrared (FTIR) spectroscopy, model- Spectrum 100, Perkin Elmer. The scanning range was 600 to 4000 cm^{-1} with a resolution of 4 cm^{-1} .

Curing Process

MEKP was used for the curing process of the prepared alkyd resins. Exactly, 10 g of alkyd resin was poured in a beaker. After that, 4 phr of MEKP, 30 phr of styrene and 2 phr of cobalt oxalate was mixed with the resin and stirred for 10 min at 60°C. After that, the resin mixture was poured to a petri dish for the curing process. The samples were observed and fingertip was used to check whether the films had solidified or not. The spot less samples were accounted as the cured film eventually.

Pencil Hardness Testing

The hardness of the formulated alkyd films was determined by a set of pencils (6B to 6H) and pencil hardness tester, model- B-3084. To test the samples, different pencils were placed inside the tester at 45° angle and dragged onto the surface of the films. If the pencils were able to make a scratch onto the surface of the films, they are considered as 'fail'. The negative result of this are considered as 'pass'. The methods are stated in ASTM D3363.

Chemical Resistivity Test of Alkyd Film

For chemical resistivity testing, four different types of solution were prepared. The different types chemical solution were distilled water, aqueous Hydrogen chloride (HCL) (5%), brine solution (5%) and NaOH (5%). The prepared alkyd films which weighted nearly 0.5 g, were immersed into the solutions at ambient temperature for one day only. After that, the weights of the samples were measured. A qualitative performance according to their appearance after the immersion period was noted as the observations from the testing.

RESULTS AND DISCUSSION

Preparation of Alkyd Resin

The MG, DG and TG concentration at different time intervals were determined by HPLC and the data are presented in *Figure 3*. It can be seen that, TG concentration dropped sharply as the reaction progressed and reached after 30 min of reaction. The MG and DG concentrations had increased and reached plateau after 40 and 30 min, respectively. In order to investigate the effect of MG:DG ratio on polyesterification reaction, alcoholysis reaction was stopped at 30, 40 and 60 min and proceeded to polyesterification reaction. The samples at 30, 40 and 60 min were labeled as A-30, A-40 and A-60, respectively.

The AV of the reaction products was checked frequently to trace the progress of polyesterification. The unreacted carboxylic acid functional groups were estimated for this purpose. *Figure 4* presents the AV of the reaction products at different time intervals to show the effects of MG:DG ration on polyesterification. The primary and secondary hydroxyl group show different reactivity (Ong *et al.*, 2016b). Therefore, the AV of the reaction products changed as the reaction progressed. It was found that the AVs decreased as the reaction proceeded. The rate of decrease of AV was faster in the early period of the polyesterification compare to the later period. This is because of the superior reactivity of the primary hydroxyl group. The similar trends were also reported elsewhere. The viscosity of the reaction products increased due to the possible formation of a 3D crosslinked network structure at the advanced stages of the polyesterification.

The effects of the ratio of MG:DG are seen in the early period of the reaction in *Figure 4*. The three-point method was adopted to evaluate the initial rate of the reactions. The results are presented in *Table 3*. The MG:DG ratio for A-30, A-40 and A-60 was 1.91, 2.66 and 2.65, respectively. The initial reaction rates of A-30, A-40 and A-60 was 6.8169, 12.7860 and 14.3379 $\text{mg KOH g}^{-1} \text{s}^{-1}$, respectively. It can be seen that, the initial reaction rates for high MG:DG ratio (A-40 and A-60) were significantly higher than the lower MG:DG ratio (A-30). In addition, the initial reaction rate of A-40 was double than A-30. Comparing all ratios, A-40 and A-60 achieved the equilibrium after 1 hr. The DP of the reactions was determined using Equations 1 and 2.

The influences of MG:DG ratio on the DP are also illustrated in *Figure 4*. As expected, the DP had increased with the reaction propagation. Two separate stages were identified in the DP analysis. The first straight nature of the curve indicates the formation of bonds between PA and primary hydroxyl groups. The rate of the reaction was

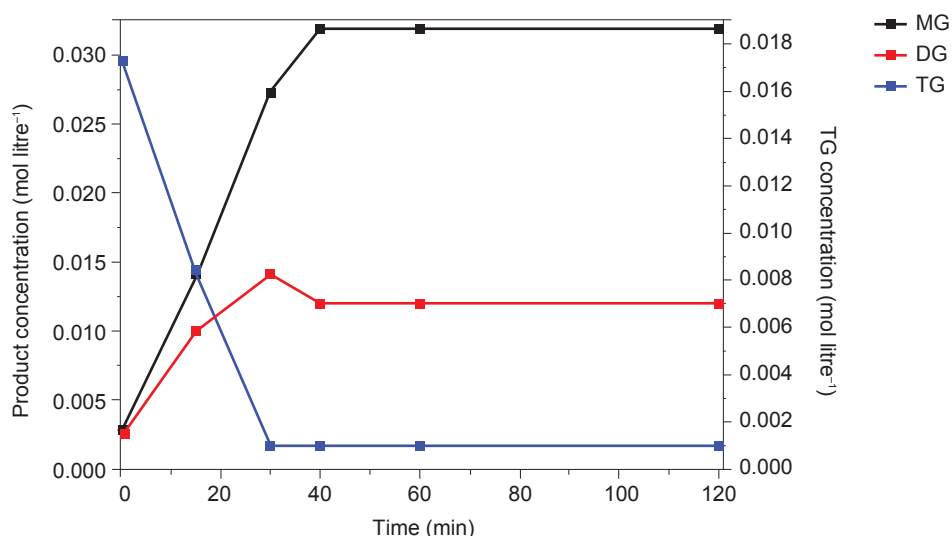


Figure 3. Plot of concentration of monoglyceride (MG), diglyceride (DG) and triglyceride (TG) vs. time over CuO-NaOH catalyst system.

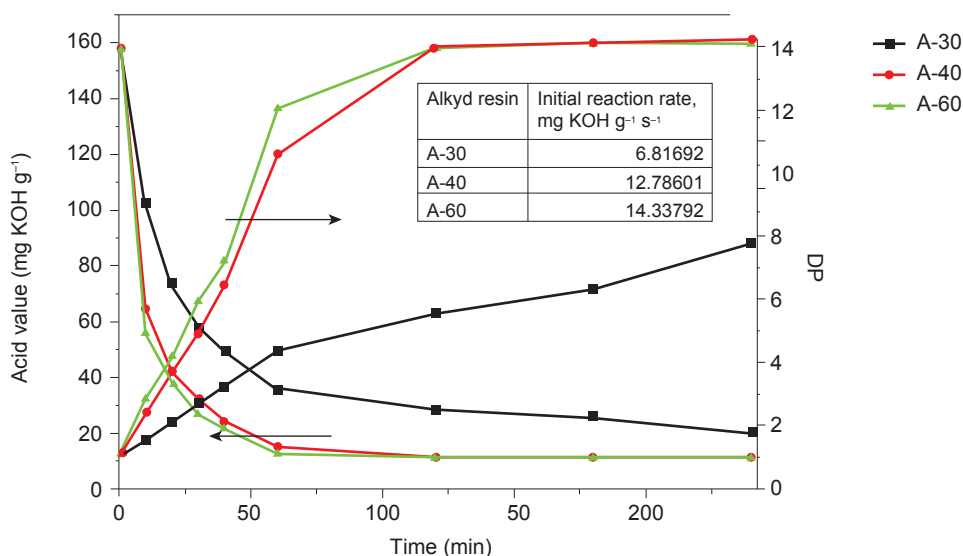


Figure 4. Plot of acid value and degree of polymerisation (DP) against reaction time for different monoglyceride/diglyceride ratio influenced polyesterification process (■ A-30, ● A-40, and ▲ A-60). Table of initial reaction rates is shown inside the figure.

substantially influenced by the MG:DG ratio. It can be inferred that high MG:DG ratio is responsible for high rate of reaction. On the other hand, the second stage indicates the reaction between the PA and secondary group of MG. In this stage, a high DP value was observed up to 2 hr of reaction time for the case of A-60. Therefore, A-60 was chosen as the optimal ratio.

Molecular Weight (M_w) Characterisation

Table 1 presents the weight molecular weight (M_w) of the alkyd resin at various reaction times. It can be seen that as the reaction preceded the increased M_w of the synthesised alkyd resin (A-30 and A-60). Initially, at the start of the polymerisation reaction, the rate of the polymerisation was low.

Therefore, the M_w of the formulated resin was also low and as the rate increased, the M_w of the polymer also increased. Previous researchers whom studied with vegetable oil-based alkyds recorded similar observations. It is noticed that, the M_w of A-60 was higher than A-30 at all time intervals. This phenomenon might be due to that the MG/DG ratio of A-60 (2.65) was higher than A-30 (1.91). It is well known that, MG and DG in the mixture could readily react with phthalic anhydride during polyesterification reaction and DG acted as a chain stopper. The chain length of the alkyd was significantly influenced by the ratio of MG to DG in the mixture, which directly affected the M_w of alkyd. Therefore, the A-60 sample with higher MG:DG ratio possessed higher M_w than A-30 sample.

TABLE 1. MOLECULAR WEIGHT (M_w) OF ALKYD RESIN, A-30 AND A-60 AT VARIOUS INTERVALS TIME

Time (min)	M_w (g mol ⁻¹)	
	A-30	A-60
0	740	740
30	1 202	1 406
60	1 373	1 655
120	1 988	2 108
180	2 374	2 505

FTIR Analysis

Figure 5 shows the FTIR spectra of A-60 and palm oil. The absorbance peak at 3650-3350 cm⁻¹ (Ong *et al.*, 2015c; Khan *et al.*, 2016; Ong *et al.*, 2016c) confirmed the presence of hydroxyl group. The strong absorbance peak in the range of 3050-2780 cm⁻¹ detected the C-H stretching. The stretching vibration of C-O-C and C=O functional group in ester of TG was confirmed by the peak at 1280-1100 cm⁻¹ and 1750-1700 cm⁻¹, respectively (Assanvo *et al.*, 2015). The absorption of C-H bending was at 1465-1445 cm⁻¹. The peak at 735 cm⁻¹, detected the unsaturated condition of the fatty acids. A new characteristic peak for the ester functional group appeared and confirmed by the peak at 1255-1225 cm⁻¹. The peak at 750 cm⁻¹, confirmed the C-H bending in the aromatic group. The peak can confirm the incorporation of phthalate group and the polyesterification.

Pencil Hardness Test

The pencil hardness testing was performed for the measurement of the hardness of the films. The results are presented in Table 2. The analysis showed

A-60 film has better hardness than A-30 film, as A-60 film passed pencil category B. This might be due to higher crosslinking in A-60 film compared to A-30 film.

TABLE 2. PENCIL HARDNESS OF A-30 AND A-60 CURED FILM

Pencil type	A-30 film	A-60 film
2B	Pass	Pass
B	Fail	Pass
HB	Fail	Fail

Chemical Resistivity

Table 3 presents the performance of chemical resistivity of A-30 and A-60. Results showed that both films were highly resistant to the chemicals (hydrochloric acid and aqueous sodium chloride) and distilled water, but the results were different for the case of NaOH. The resistivity was different for different samples. A-30 film showed poor resistivity to alkali solution, while A-60 film showed comparatively fair resistivity to the same solution. This is because of the hydrolysis process of the ester functional groups present in the alkyds (Essien *et al.*, 2015).

TABLE 3. CHEMICAL RESISTIVITY OF A-30 AND A-60 FILMS

Solution	A-30 film	A-60 film
Distilled water	Excellent	Excellent
5% of HCl (aq.)	Excellent	Excellent
5% NaOH (aq.)	Poor	Fair
5% NaCl (aq.)	Excellent	Excellent

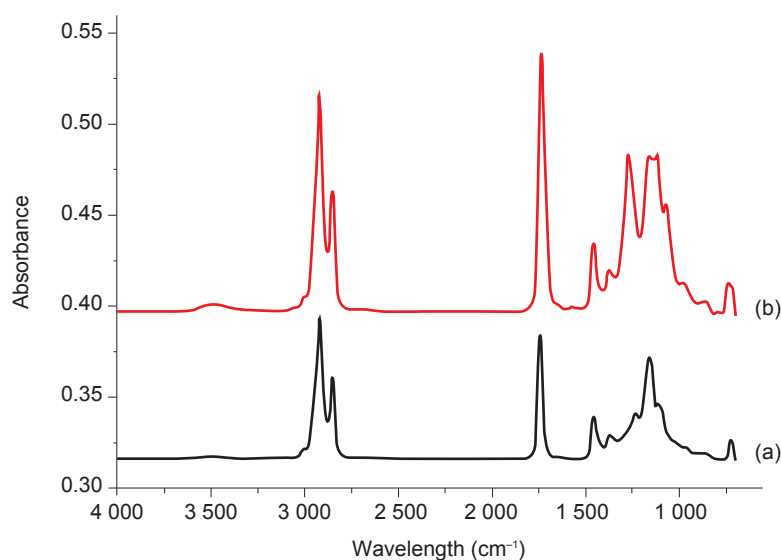


Figure 5. Infrared spectra of (a) palm oil, and (b) A-60 alkyd.

CONCLUSION

The scope of this work is to find the effects of the variation of MG/DG ratio on the properties of palm oil-based polyalkyds. The synthesis process includes alcoholysis followed by polyesterification. Different properties of polyalkyds like M_w , DP, structural, hardness and chemical resistivity were evaluated. The FTIR analysis confirmed the presence of ester functional groups in the prepared alkyd resins. It was found that, the reaction time had shortened due to the higher initial rate at higher MG:DG ratio. The initial rate for A-60 (MG:DG=2.65) was two times higher than the A-30 (MG:DG=1.91). In addition, higher MG/DG ratio in the polyesterification reactions produced the alkyd resin with higher M_w . Moreover, A-60 film possessed better pencil hardness and chemical resistivity than A-30 film. It can be concluded that a higher ratio of MG:DG resulted in a higher M_w polyalkyds, which can be suitable for coating application.

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XYLAN RECOVERY FROM DILUTE NITRIC ACID PRETREATED OIL PALM FROND BAGASSE USING FRACTIONAL FACTORIAL DESIGN

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ABSTRACT

Pretreatment enhances bioconversion of lignocellulosic biomass by disrupting and changing the properties of its material. This work is intended to examine the influence of several variables involved in the recovery of xylan from oil palm frond bagasse (OPFB) in the pretreatment of diluted nitric acid. Through a fractional factorial design of 2^{5-1} the temperature variables ranged from 37°C–90°C, time of reaction varied from 6–24 hr, solid loading ranged from 5%–20%, concentration of acid changed from 0.01%–1.0%, and agitation between yes or no were evaluated. As much as 27.63% of xylan successfully recovered at mild condition and it was completely hydrolysed as the pretreatment condition increased. This study found solid loading of 5% and acid concentration of 0.01% at 37°C for 24 hr pretreatment without agitation were best to maximise xylan recovery and minimise lignin content simultaneously. Temperature was the most important factor studied for the recovery of xylan with a contribution of 29.34%. The findings suggest that studying the condition influencing acid pretreatment is crucial towards improving pretreatment process.

Keywords: acid pretreatment, fractional factorial design, lignin removal, oil palm frond bagasse, xylan recovery.

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INTRODUCTION

Studies on alternative biochemical approaches have been given much attention recently due to the increasing awareness on the environmental degradation created by the use of traditional petrochemical processes in chemical production. Biorefinery is considered as one of the promising

processes in converting biomass or other renewable resources into fuel and chemicals which simultaneously can reduce carbon footprint. In several tropical countries especially in the Asian region, the oil palm industry produces a substantial amount of biomass that can be used for bio conversion. The industry generates several types of biomass and one of the most recently studied is the oil palm frond (OPF), due to its availability and valuable content. During the pruning operation, OPF can be obtained yearly up to about 44 million tonnes and 15 t ha⁻¹ during replanting (Awalludin *et al.*, 2015). These biomass if not utilised will contribute to the environmental degradation because it will only be left rotten or burned in landfills (Faizi *et al.*, 2017). Since 1991, OPF has been a subject of research as feed for farm animals and sorption agent. However, in 2010, OPF was identified as a potential source for fermentable sugar production (Goh *et al.*, 2010). Lignocellulosic biomass has recently received significant attention as a source for the processing

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of various chemicals in biorefineries (Asim *et al.*, 2019; Dai *et al.*, 2019; Noorshamsiana *et al.*, 2017; Solarte-Toro *et al.*, 2018). Lignocellulosic biomass is a plant dry matter of which cellulose, hemicellulose and lignin are the primary polymers that make up its complex biomatrix structure (Agbor *et al.*, 2011). Since OPF contains 44.8% glucan, 19.45% xylan and 19.53% lignin, it has become a source in many other products such as bioethanol (Soplah *et al.*, 2015), succinic acid (Tan *et al.*, 2016), glucose (Wan Omar and Amin, 2016), xylitol and furfural (Swain and Krishnan, 2015; Zhang *et al.*, 2017). However, the conversion may be restricted due to the resistance of the complex molecule structure of lignin to chemical and enzyme attacks. Hence, most of the chemical processes that use lignocellulosic biomass as a source should consider to remove lignin as much as possible for the process to be successful (Wan Omar and Amin, 2016).

Many processes have been implemented to convert lignocellulosic materials into valuable products. Usually, the process starts with pretreatment of the biomass aiming at the deconstruction of its plant cell wall. Two categories of pretreatment are identified, namely chemical and mechanical pretreatment. The initial step in pretreatment typically involves mechanical process where the biomass will be chipped, ground or milled to reduce its size (Kumari and Singh, 2018). Chemical treatment through acid, alkali and ionic liquid treatment is then used to break down the biomass chemical structure (Kumari and Singh, 2018). Alkaline pretreatment is the viable technique to break the components in biomass, especially if the process requires to isolate xylan (Carvalho *et al.*, 2013). Using the alkaline treatment, xylan was successfully extracted from corn stover (Lee *et al.*, 2015), oat straw (Romaní *et al.*, 2016), arecanut husk (Singh *et al.*, 2018) and hardwood (Rajagopalan *et al.*, 2017). Nevertheless, Kumar *et al.* (2009) mentioned that dilute acid pretreatment was also practical and this process is proven to be successful in altering and exposing the structure in biomass. Furthermore, dilute acid pretreatment is more economical. To date, the application of nitric acid in pretreatment processes has been far too little considered. Nitric acid pretreatment has been proven to shorten the overall treatment process and inhibit the formation of inhibitors thus, improving production yield (Zhang *et al.*, 2011). On top of that, Xiao and Clarkson (1997) reported that nitric acid pretreatment may contribute to solubilisation of huge amount of lignin from the substrate. The findings show that the method has potential in lignin removal which indirectly exposes hemicellulose surface to the enzyme attack in the enzymatic hydrolysis for xylan based products such as xylooligosaccharides. However, since several factors may influence the pretreatment process, screening the most influential

factor is a critical step that must be considered to improve the pretreatment process.

One of the best experimental design to quickly study the effects of different factors simultaneously is the use of response surface methodology (RSM) through factorial design (Abu Sepian *et al.*, 2019). In fact, factorial design, a tool provided by Design Expert software is also useable to evaluate the interaction between the factors involve in a process efficiently (Box and Wilson, 1951). Design Expert has been widely adopted to improve various pretreatment methods (Lyu *et al.*, 2018; Mesa *et al.*, 2017; Soares *et al.*, 2017) of numerous types of raw material by evaluating and selecting its best operational condition. However, limited studies on the use of factorial has been performed using acid pretreatment to retain xylan in solid waste for further enzymatic hydrolysis. Martín *et al.* (2019) demonstrated that acid concentration of 1% could lead to complete hydrolysis of xylan compared to a lower acid concentration. Hernández *et al.* (2013) claimed that xylan can be retained in solids only by implementing mild conditions pretreatment, since the product can be easily hydrolysed during pretreatment. However, it is still not clear whether the use of mild operational conditions may retain higher xylan since there are very few studies focussing on xylan recovery in solids. Therefore, this study was performed to address the research gaps by investigating the significance of several factors including temperature, reaction time, solid loading, acid concentration, and agitation on the pretreatment of dilute nitric acid using two-level factorial design in oil palm frond bagasse (OPFB). A 2^{5-1} fractional factorial design (FFD) was applied with 16 runs of experiment.

MATERIALS AND METHODS

Materials

This study used OPF from a nearby plantation in Kuantan, Pahang, Malaysia. To get the OPFB, the OPFB was squeezed to remove the OPF juice with a sugar cane pressing unit. The obtained bagasse was then sun-dried for three days until the moisture content of OPFB was less than 10%. After the drying process, the OPFB was ground to get OPFB fibre and then sieved to ensure that the thickness of the OPFB was less than 2 mm. The sieved OPFB was then stored in -20°C until further use.

Factorial Analysis for Acid Pretreatment

A factorial study was conducted with a Design Expert software, version 7.0 (Stat-Ease Inc., USA) to study the acid pretreatment process for the recovery of xylan. A 2^{5-1} fractional factorial design

was used to assess the statistical significance of five parameters including temperature, reaction time, solid loading, acid concentration and agitation. The raw OPFB fibre was soaked in diluted nitric acid at concentrations between 0.01% and 1.0%, while the solid loading ranged between 5% and 20%. The acid pretreatment of OPFB was conducted for 6–24 hr at 37°C–90°C in a water bath with (200 rpm) and without agitation. Once the pretreatment process was completed, the substrate was then washed thoroughly with tap water for neutralisation. The sample was then placed in a 60°C drying oven for one day before further analysis. Three components, which include glucan (%), xylan (%), and lignin (%), were assessed to improve xylan recovery. Sixteen assessments were undertaken in this work. Table 1 lists the coded values of various experimental variables.

TABLE 1. CODED VALUES AND ACTUAL VALUES OF VARIOUS PRETREATMENT FACTORS

Factors	Type of factor	Actual values		Units
		(–1)	(+1)	
A: Temperature	Numerical	37	90	°C
B: Reaction time	Numerical	6	24	hr
C: Solid loading	Numerical	5	20	% (w/v)
D: Acid concentration	Numerical	0.01	1.00	% (v/v)
E: Agitation	Categorical	No	Yes (200)	rpm

Determination of Lignocellulosic Biomass Composition

The analysis of glucan, xylan and lignin composition was performed following the National Renewable Energy Laboratory (NREL) procedure. The sample was first subjected to two-step extraction process with 8 hr water extraction in the first step, followed by 24 hr extraction using ethanol in the second step. The biomass was dried overnight in an oven at 60°C between the two extractions. The extraction process was done to remove extractives in the sample to prevent interference during analysis (Sluiter *et al.*, 2008).

The extracted sample was put through acid hydrolysis to extract structural carbohydrates and determine the amount of lignin in biomass using 72% sulphuric acid (H₂SO₄) (Sluiter *et al.*, 2012). In this process, 3 ml of the acid was first added into 0.3 g of the free extractive sample for incubation process with a temperature of 30°C. After 1 hr of incubation, the incubated mixture subsequently was mixed with distilled water up to 84 ml before being autoclaved at 121°C for 1 hr. The hydrolysate

containing carbohydrates (glucose as an acid hydrolysis product of glucan and xylose as an acid hydrolysis product of xylan) was analysed using Agilent 1200 high-performance liquid chromatography (HPLC). Glucan and xylan content in biomass were determined using Equation (1) and (2).

$$\text{Glucan (\%)} = \frac{0.9 \times V \times H_{\text{Glucan}}}{\text{SRS}_{\text{Glucan}} \times \text{HM}} \times 100\% \quad \text{Equation (1)}$$

$$\text{Xylan (\%)} = \frac{0.88 \times V \times H_{\text{Xylan}}}{\text{SRS}_{\text{Xylan}} \times \text{HM}} \times 100\% \quad \text{Equation (2)}$$

where V is defined as total volume of acid and ultrapure water in the vial (L), H is concentration of structural carbohydrate after acid hydrolysis (g litre^{–1}), SRS is sugar recovery standard and HM is the mass of biomass added to the hydrolysis vial on unextracted basis.

The autoclaved acid hydrolysis solution was vacuum filtered to obtain the remaining solid. Filter paper 0.22 µm, cellulose ester was used for the filtration and the weight of filter paper was recorded. Deionised water was used to quantitatively rinse solid particles from vial and inside filtration cup. Solid residue is considered as a remaining lignin in the biomass. Solid sample obtained was dried in an oven at 105°C until a constant weight is achieved, usually a minimum of 4 hr. The weight of solid residue with filter paper was recorded. The acid insoluble lignin content was calculated according to Equation (3).

$$\text{Lignin (\%)} = \frac{L_2 - L_1}{\text{HM}} \times 100\% \quad \text{Equation (3)}$$

where L_1 is filter paper (g) and L_2 is dried solid residue with filter paper (g).

Quantification of Structural Carbohydrates

Rezex RHM-Monosaccharide H+, 300 × 7.8 mm HPLC column (Phenomenex) was used together with a refractive index (RI) detector. Deionised water with 5 µl injection volume was used as the mobile phase at 0.4 ml min^{–1} flow rate with the column temperature was maintained at 60°C and 30°C for RI detector. Standard solutions were prepared with concentrations in the range of 0.5–10 g litre^{–1} to develop calibration curves. The standard curves obtained were used to measure concentrations of glucan (g litre^{–1}) and xylan (g litre^{–1}) in the samples.

RESULTS AND DISCUSSION

Characterisation of OPFB

It is necessary to carry out a characterisation analysis in this study in order to ensure that the significance of xylan could be retrieved later. Table 2 presents the chemical compositions of OPFB including several other raw lignocellulosic biomasses. OPFB consisted of 42.8% glucan, 20.9% xylan, 31.9% lignin, 0.8% ash and 3.6% extractives. Glucan content in OPFB was comparable with that in sugarcane bagasse (41.95%) but higher than those in rice straw, corn stover, and wheat straw. However, xylan composition in OPFB was higher than those in rice straw and corn stover. It is important to predetermine the composition of xylan from raw OPFB by comparing it with other raw lignocellulosic biomass to ensure that high xylan is retrieved through this pretreatment study. The selection of the OPFB as raw material is beneficial since the xylan observed in this study is worth and reasonable to be recovered. However, the lignin content was high in OPFB. High lignin content may hinder the extraction of xylan due to the formation of lignin-carbohydrate complex between lignin and hemicellulose (Syazana Mohtar *et al.*, 2017). Although OPFB contains a higher amount of lignin, ash was the lowest in the biomass. Low ash content could positively affect the pretreatment process. This is because the presence of high ash during pretreatment can cause nitric acid to neutralise alkaline ash during pretreatment rather than acid hydrolysis which may reduce the efficacy of pretreatment (He *et al.*, 2014). A low amount of extractives in OPFB is also preferable since a high content of extractives may result in the hydrolysis of extractives instead of lignin removal (Martínez-Patiño *et al.*, 2018). Therefore, low content of ash and extractives in OPFB are important to improve the recovery of xylan through acid pretreatment by an increase in the acid hydrolysis process.

Xylan Recovery from Pretreated OPFB

Diluted nitric acid pretreatment with milder conditions was performed with the intention to achieve higher xylan recovery through higher

lignin removal which occurred by disrupting the structure of OPFB. Delignification is expected to occur during pretreatment as the lignin component could become an enzyme inhibitor that prevents enzymatic hydrolysis in a further step towards the production of valuable products such as xylooligosaccharides (Wojtusik *et al.*, 2017). The primary goal of this research was to improve xylan recovery by controlling the pretreatment process condition with the assistance of Design Expert software. Maximising the xylan recovery by diluted nitric acid pretreatment was therefore assessed by simultaneously minimising lignin to ensure pretreatment effectiveness without considering the content of glucan. Glucan is supposed to be retained in this study as the mild condition used may protect the cellulose structure and thus, prevent its hydrolysis (Xu *et al.*, 2013).

Table 3 shows the components (glucan, xylan and lignin contents) obtained via 2^{5-1} fractional factorial design. The chemical compositions observed varied significantly between the experimental set of different processes with glucan, xylan and lignin values ranging from 47.06%–73.33%, 0.00%–27.63% and 15.03%–31.33%, respectively. The highest recovery of xylan (27.63%) was found at pretreatment condition of 37°C with solid loading and acid concentration of 5% and 0.01% for 24 hr without agitation (Standard Order 3). At the same condition, the lignin content of OPFB was hydrolysed to minimum (15.03%) which caused the increase of glucan content to 57.02%. The results show that the mild acid pretreatment condition was appropriate to remove lignin by producing phenolic compounds such as *p*-coumaric, ferulic and diferulic acids that contributed to crosslinking between lignin-hemicellulose binding. The cleavage of lignin from hemicellulose and cellulose by releasing the phenolic compounds indicated an increase in the efficiency of acid pretreatment which results in the exposure of the hemicellulose surface (Jönsson and Martín, 2016).

Results also showed that complete xylan removal occurred at standard order run 8 and 16 when zero percent of xylan was achieved at these two experimental runs. At Standard Order 8, the experiment was conducted using 20% solid loading

TABLE 2. CHEMICAL COMPOSITION OF VARIOUS RAW LIGNOCELLULOSIC BIOMASS

Source	Components					Reference
	Glucan	Xylan	Lignin	Ash	Extractives	
OPFB	42.8	20.9	31.9	0.8	3.6	This study
Sugarcane bagasse	42.0	21.6	24.7	3.5	3.5	Carvalho <i>et al.</i> (2018)
Rice straw	31.1	18.7	13.3	14.5	9.0	Chen <i>et al.</i> (2011)
Corn stover	31.5	15.4	14.1	7.9	25.9	Vergara <i>et al.</i> (2018)
Wheat straw	32.8	26.3	13.8	17.0	4.1	Vergara <i>et al.</i> (2018)

Note: OPFB - oil palm frond bagasse.

TABLE 3. EXPERIMENTAL DESIGN LAYOUT USING 2⁵⁻¹ FRACTIONAL FACTORIAL DESIGN FOR ACID PRETREATMENT WITH RESPONSES

Standard order	Variables					Responses (%)		
	A	B	C	D	E	Glucan	Xylan	Lignin
1	-1	-1	-1	-1	+1	51.39	23.64	17.58
2	+1	-1	-1	-1	-1	47.06	22.18	26.95
3	-1	+1	-1	-1	-1	57.02	27.63	15.03
4	+1	+1	-1	-1	+1	49.83	22.78	21.07
5	-1	-1	+1	-1	-1	53.06	23.80	22.53
6	+1	-1	+1	-1	+1	56.75	25.85	17.29
7	-1	+1	+1	-1	+1	52.73	23.25	23.76
8	+1	+1	+1	-1	-1	73.33	0.00	26.66
9	-1	-1	-1	+1	-1	54.50	25.43	18.95
10	+1	-1	-1	+1	+1	62.51	17.00	20.40
11	-1	+1	-1	+1	+1	50.56	22.46	21.53
12	+1	+1	-1	+1	-1	60.15	16.56	19.95
13	-1	-1	+1	+1	+1	49.32	21.08	19.11
14	+1	-1	+1	+1	-1	58.41	14.8	23.68
15	-1	+1	+1	+1	-1	49.01	21.03	26.41
16	+1	+1	+1	+1	+1	64.06	0.00	31.33

Note: A - temperature (°C); B - reaction time (hr); C - solid loading (%); D - acid concentration (%); E - agitation (rpm).

with 0.01% acid concentration at 90°C for 24 hr without agitation, resulting in the amount of glucan and lignin of 73.33% and 26.66%, respectively. Whereas, standard order run 16 which was incubated at 90°C for 24 hr involving mixing with 20% solid loading and 1.0% acid concentration had obtained 64.06% glucan and 31.33% lignin content. From the data, it can be identified that the complete removal of xylan happened when the pretreatment was performed at high temperature (90°C), while the maximum xylan recovery (Standard Order 3) was attained when low temperature (37°C) was applied even with the same pretreatment duration and acid concentration. The results indicate that temperature has very significant adverse impacts on the retention of xylan without considering changes in acid concentration and agitation conditions. The variation of xylan content at high and low operating temperature can also be confirmed from previous studies. When Hsu *et al.* (2010) performed sulphuric acid pretreatment on rice straw by varying the temperature between 160°C–190°C and acid concentration of 0.5%–1.0%, the preserved xylan in solid residue rice straw were between 0.7%–5.1% after the pretreatment. On the other hand, Gowdhaman and Ponnusami (2015) has recovered about 14.7% xylan in corncorb after the pretreatment which was performed using 0.1% H₂SO₄ at 60°C. This gives an early view on the factor that contributed in pretreatment process.

It also can be observed that overall, when xylan content was at its minimum, higher amount of glucan and lignin were attained. According to Chen *et al.* (2011), xylan removal could increase glucan and lignin content resulted from the acid-catalysed hydrolysis of xylan. In this study, the maximum xylan content (Standard Order 3) and zero percent of xylan (Standard Order 8 and 16) were achieved when the pretreatment was performed for 24 hr, when operated at 37°C and 90°C, respectively. Even with the same reaction time, opposite results were achieved. This happened because at certain condition when using acid as a pretreatment medium, xylan will be degraded into its monomeric sugar, xylose, which accumulated in the liquid fraction of hydrolysate. Besides, if the process period is prolonged, xylose in hydrolysate will further transform into inhibitors like furfural and acetic acid (Vergara *et al.*, 2018). Thus, the implementation of acid pretreatment very much controlled by the combined severity concept of factors involved in the process depending on the targeted material (Jung and Kim, 2015).

Statistical Analysis and Model Equation

The data obtained in Table 3 were analysed for the construction of glucan, xylan and lignin equation models by the Design Expert software. The final model equations generated explain the

relationship between the pretreatment condition with the amount of glucan, xylan and lignin are illustrated in Equation (4), Equation (5) and Equation (6), respectively.

$$\begin{aligned} \text{Glucan (\%)} = & 55.61 + 3.41A + 1.48B + \\ & 1.48C + 0.46D - 0.96E + 1.35AB + \\ & 2.65AC + 1.81AD + 1.22BC - 1.6BD \\ & - 1.83BE - 2.34CD + 1.51DE \end{aligned} \quad \text{Equation (4)}$$

$$\begin{aligned} \text{Xylan (\%)} = & 19.22 - 4.32A - 2.51B - \\ & 2.99C - 1.92D + 0.29E - 2.56AB - \\ & 1.74AC - 0.88AD + 1.22AE - 2.65BC \\ & + 1.03CE - 2.45DE \end{aligned} \quad \text{Equation (5)}$$

$$\begin{aligned} \text{Lignin (\%)} = & 22.01 + 1.4A + 1.2B + \\ & 1.83C + 0.66D - 0.51E - 1.51AC + \\ & 1.99BC + 0.93BD + 1.71BE - 0.63CD \\ & - 0.47CE + 0.93DE \end{aligned} \quad \text{Equation (6)}$$

where temperature, reaction time, solid loading, acid concentration and agitation are the main effects of pretreatment condition denoted by A, B, C, D and E, respectively. Meanwhile, the interaction effects are referred by AB, AC, AD, AE, BC, BD, BE, CD, CE and DE.

Statistical analysis of glucan, xylan and lignin responses were further analysed through analysis of variance (ANOVA) to assess the significance of each models (Table 4). The ANOVA results revealed that the models of glucan, xylan and lignin obtained are significant at F-value of 30.86, 230.47 and 19.05, respectively. In addition, the *p*-values for all models are less than 0.05, which are considered statistically

significant. Furthermore, the models are also significant, with R^2 values of 0.9950, 0.9989 and 0.9870, respectively. The R^2 should not be less than 0.80 for a good model (Lin *et al.*, 2017). The recorded values imply that only 0.5%, 0.11% and 1.3% of the models have the variability between experimental and predicted data.

TABLE 4. STATISTICAL ANALYSIS OF GLUCAN, XYLAN AND LIGNIN MODELS

Response	Glucan	Xylan	Lignin
Model	Significant	Significant	Significant
F-value	30.86	230.47	19.05
<i>p</i> -value	0.0318	0.0004	0.0166
R^2	0.9950	0.9989	0.9870
Adjusted R^2	0.9628	0.9946	0.9352

Further detailed analysis of variance for xylan recovery via diluted nitric acid pretreatment is displayed in Table 5. All factors, except for agitation, are significant in affecting xylan content. Temperature is the most significant factor affecting the pretreatment at probability of <0.0001. Solid loading, reaction time and acid concentration follow the significant ranks with *p*-values of 0.0003, 0.0005 and 0.0011, respectively. Agitation, by contrast, is shown to be statistically insignificant in improving xylan recovery, as its *p*-value exceeds 0.05 (0.1521). The result indicates that any changes in agitation with or without the condition will not affect xylan recovery. In terms of interaction, all interactions are considered statistically significant in determining xylan content with *p*-value below 0.05.

TABLE 5. ANALYSIS OF VARIANCE OF FACTORS AFFECTING ACID PRETREATMENT ON XYLAN RECOVERY

Source	Sum of squares	Df	Mean square	F-value	<i>p</i> -value
A-Temperature	298.93	1	298.93	812.2	< 0.0001
B-Reaction time	100.41	1	100.41	272.82	0.0005
C-Solid loading	143.12	1	143.12	388.86	0.0003
D-Acid concentration	59.18	1	59.18	160.8	0.0011
E-Agitation	1.34	1	1.34	3.65	0.1521
AB	104.57	1	104.57	284.11	0.0005
AC	48.58	1	48.58	132	0.0014
AD	12.49	1	12.49	33.93	0.0101
AE	23.89	1	23.89	64.9	0.004
BC	112.43	1	112.43	305.47	0.0004
CE	16.97	1	16.97	46.11	0.0065
DE	95.97	1	95.97	260.77	0.0005
Residual	1.10	3	0.37	-	-
Cor. total	1 018.98	15	-	-	-

Analysis the Main and Interaction Effects for Xylan Recovery

Pareto chart as seen in *Figure 1* describes the significant level of each main and interaction factor affecting acid pretreatment. The evaluation was only performed on xylan recovery, to study how the factors influencing its content. The varied range of t-values of effect observed for each factor are proportional to the height of the bars where the numerical effects is divided by its associated standard error to compute the values (Anderson and Whitcomb, 2007). It visualises the significance of factors using two limit values of the Bonferroni limit and the t-value limit as the reference point for defining the significant factors. The degree of significance of the effects decrease as the bars move from left to right with the reduction of the bars height (Wilkinson, 2006). In this case, factor A (temperature) is the most significant factor with the highest t-value while factor E (agitation) is least significant factor for the pretreatment process.

The limits are measured based on t-value of effects. Factors with t-value which exceeded the Bonferroni limit at 8.57517 is considered important and may considerably improve the xylan recovery (Abdulredha *et al.*, 2020). The chart provides a t-value of effects beyond the Bonferroni limit for the main factors A, B, C and D and interactions of BC, AB, DE and AC. These factors are important to influence the acid pretreatment process. The factors with an effect t-value between the limits of Bonferroni and t-value could be important for the pretreatment process (Alara *et al.*, 2017). The interactions of AE, CE and AD are significant for the process and might improve the diluted nitric

acid pretreatment process. When evaluating xylan content however, factors with t-value of effect underneath the t-value limit at 3.18245 are not considered significant.

The Pareto chart is also useful to demonstrate the positive and negative effects possess by each factor. Positive and negative effects are presented by orange and blue bars, respectively. Positive effects show that the xylan content increases as the factor level increases, while negative effects illustrate the opposite (Mah *et al.*, 2016). In this study, the main factor E has a positive effect in which the increasing factor value had increased the content of xylan. As for interaction, AE and CE have positive effects where agitation at higher temperatures and solid loading is necessary to increase the recovery of xylan compared to without agitation. However, the main factors of A, B, C and D have shown negative effects, whereby a decline of each value had increased the xylan content. Similarly, the interaction of AB, AC, BC and DE also resulted in negative effects, resulting in an increase of xylan content, as the interaction between temperature and reaction time, temperature and solid loading, reaction time and solid loading, and acid concentration and agitation decreased. These results indicated an advantage of high xylan recovery obtained at mild pretreatment conditions, therefore minimising the production cost.

The percentage of contribution is another method to represent the influence of factors toward acid pretreatment process. *Table 6* shows the contribution of each factor in affecting xylan recovery. Temperature showed the highest contribution at 29.34%. The percentage contribution corresponds to the t-value of effects (*Figure 1*). The

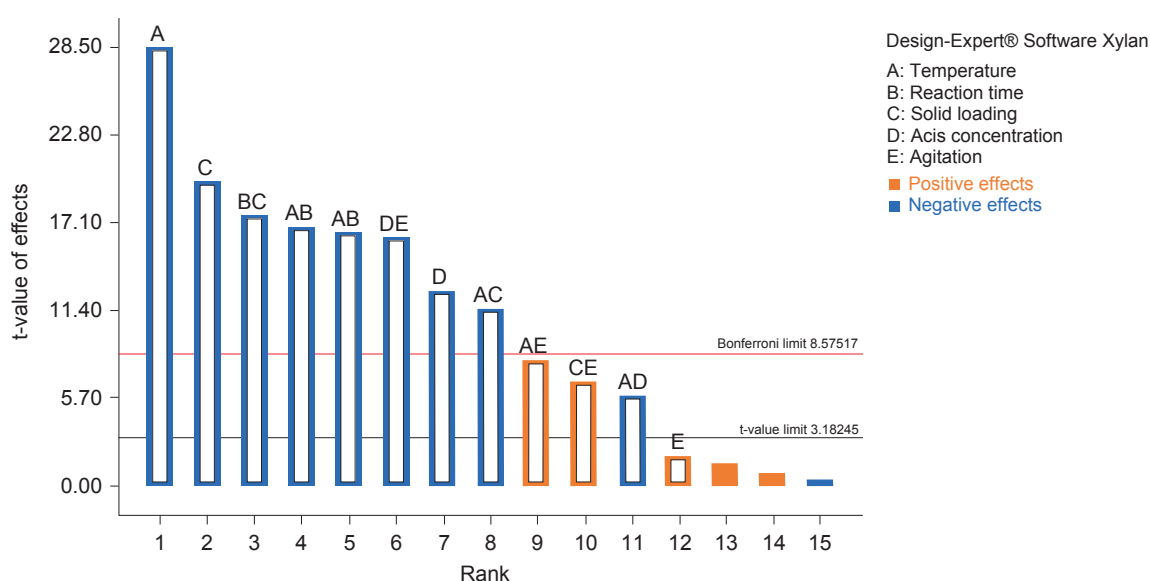


Figure 1. Pareto chart for factors affecting xylan content.

factors with a percentage contribution of less than 1% were excluded from the research in this report. The reduction of insignificant factors may improve the model constructed by the Design Expert software. However, the main effect of agitation (0.13) was kept in the analysis to support hierarchy.

TABLE 6. PERCENTAGE CONTRIBUTION OF FACTORS AFFECTING XYLAN CONTENT

Term	% contribution
A-Temperature	29.34
B-Reaction time	9.85
C-Solid loading	14.05
D-Acid concentration	5.81
E-Agitation	0.13
AB	10.26
AC	4.77
AD	1.23
AE	2.34
BC	11.03
BD	0.08
BE	0.02
CD	0.01
CE	1.67
DE	9.42

Effects of Different Variables on Xylan Recovery

Figure 2 displays the temperature plot as the most significant factor that affects the acid pretreatment process for xylan recovery. A high xylan content was observed at low temperature. High temperature could result in the degradation of hemicellulose (xylan) and conversion of xylan into sugar (xylose). In fact, excessive temperature may cause the formation of furfural from the xylose and increases the degradation of cellulose (glucan) (Ji *et al.*, 2017).

Figure 3 illustrates the interaction effects of several significant interaction factors involved in affecting xylan recovery. Figure 3a demonstrates the interaction between temperature and reaction time (AB). A high xylan content was observed at 37°C although the reaction time was increased. However, the xylan content was greatly reduced as reaction time was increased at 90°C. The results indicate that at high temperature (90°C) and longer reaction time (24 hr), most of xylan content might be converted into xylose. Likewise, a similar trend was observed in the interaction between temperature and solid loading (AC), where the xylan content was greatly increased at 37°C. In this case, the change of solid loading was less significant than

the change of temperature from high to low. The result shows that the xylan recovery increased at lower temperature for both high- and low-level solid loadings. However, it was observed that the xylan recovery was consistently higher at 5% solid loading compared to that at 20% solid loading. Less interaction between the factors can be shown by the two lines (almost parallel) which represent the degree of significance. Paralleled lines designate that there is no interaction between the factors and *vice versa* (Kim, 2016).

The interaction between reaction time and solid loading (BC) presented the highest impact in xylan content as reflected in Figure 3c. A substantial difference of xylan recovery was observed when different level of solid loading was provided at longer reaction time. At low level solid loading, higher lignin content could be removed since most lignocellulosic biomass surface may expose to be hydrolysed by dilute nitric acid, resulting in high xylan recovery. In fact, acid hydrolysis of lignin could be completed at a shorter reaction time as the xylan content was almost constant, even though the reaction time was extended by 6–24 hr. Meanwhile, the interaction between acid concentration and agitation (DE) is different from the interactions of AB, AC and BC. The two lines intersect which highlights the significant interaction between the factors. High xylan content was recovered at low acid concentration with agitation compared to that without agitation. Agitation could improve the pretreatment efficiency by increasing the acid hydrolysis accessibility to lignocellulosic biomass (Loow *et al.*, 2016). Several studies applied mild agitation to mix liquid and solid to improve pretreatment process (Rajagopalan *et al.*, 2017; Romero-Fernández *et al.*, 2018). However, the xylan

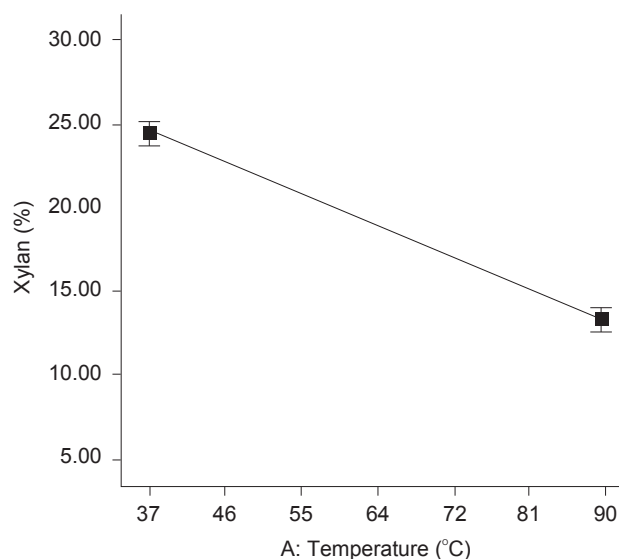


Figure 2. Plot of the temperature in affecting xylan recovery.

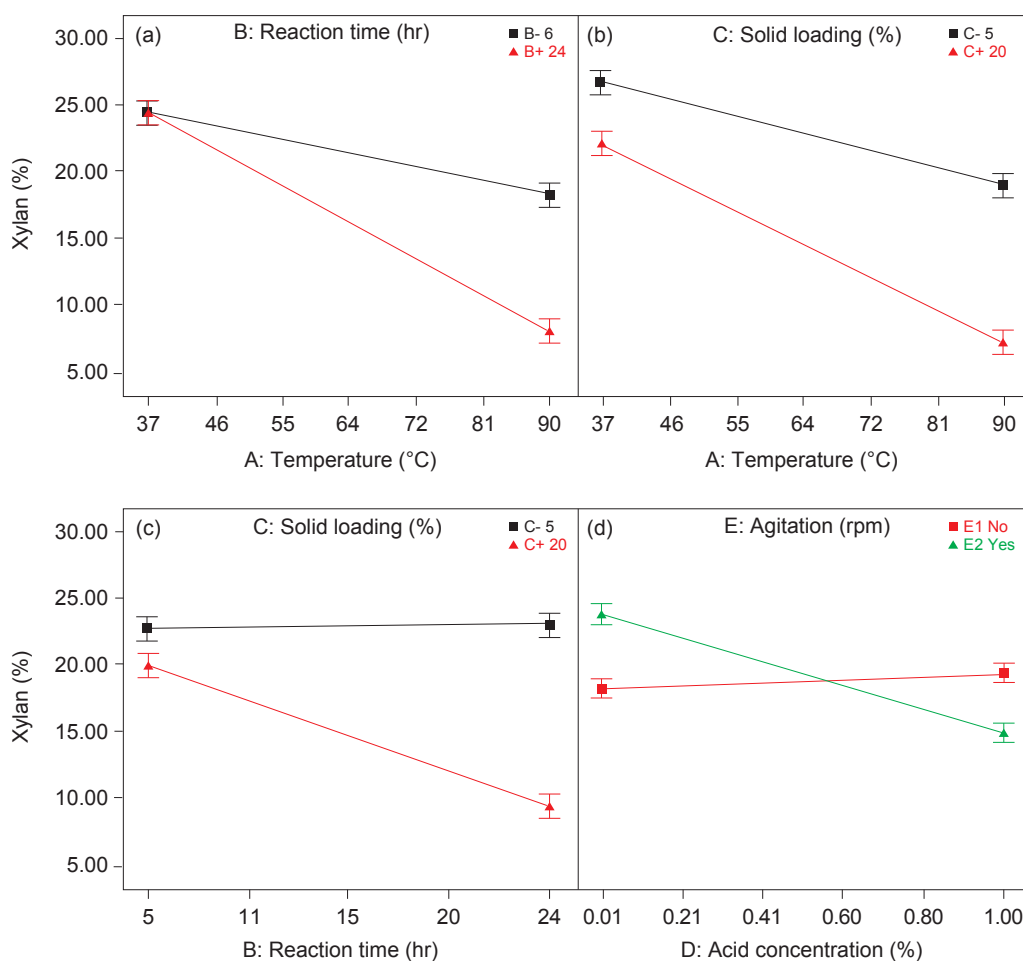


Figure 3. Plot of the significant interaction effects between (a) temperature and reaction time, (b) temperature and solid loading, (c) reaction time and solid loading, and (d) acid concentration and agitation.

content had reduced when the acid concentration was increased to 1%, which could be attributable to xylan hydrolysis at higher concentration and the deterioration was also further accelerated by the aid of agitation. This result can be further understood by comparing xylan recovery at similar acid concentration (1%) with and without agitation, where higher xylan recovery was obtained when the pretreatment was performed without agitation. This result is supported by the findings of other studies where no agitation was provided to enhance pretreatment (Palamae *et al.*, 2017; Sporck *et al.*, 2017; Vergara *et al.*, 2018).

Model Validation

Model validation was performed by pre-determining the preference of product recovery through the Design Expert setting. This study aims to increase xylan content and reduce the content of lignin simultaneously. Hence, higher yield of xylan and low lignin observed after pretreatment is considered successful and thus, could proceed to facilitate enzymatic hydrolysis in further study to

obtain the higher valuable product recovery such as production of xylooligosaccharides. As expected, glucan content was unaffected by the diluted nitric acid pretreatment process since disruption of glucan may only occur with the removal of xylan at high level condition (Xu *et al.*, 2013). In this case, preservation of glucan may indirectly improve xylan recovery by preventing acid hydrolysis of xylan. Triplicate validation tests were carried out to confirm the adequacy of the xylan recovery model obtained based on the conditions recommended by Design Expert software (Figure 4). By setting glucan to be in range, xylan to be maximum and lignin to be minimum, the best condition was proposed: 37°C, reaction time of 24 hr, solid loading of 5%, and acid concentration at 0.01% without agitation, with the predicted values for glucan, xylan, and lignin of 57.19%, 28.04% and 15.78%, respectively.

The experimental results of glucan, xylan, and lignin contents were $54.53 \pm 0.07\%$, $25.89 \pm 0.73\%$ and $16.48 \pm 0.18\%$, with the percentage errors of 4.43%, 7.69% and 4.43% respectively. The low percentage errors (less than 10%) observed in this study indicated that the experimental and predicted

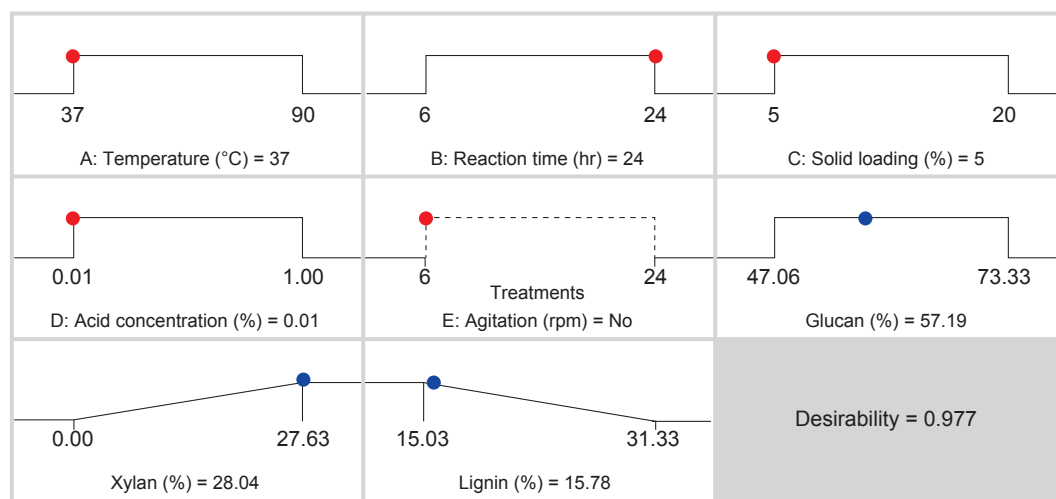


Figure 4. Best condition suggested by Design Expert software for maximum xylan recovery.

data are in close agreement thus, confirmed the suitability and adequacy of the model equations to predict the responses. Hence, a promising high xylan recovery at high lignin removal could be reproduced in the future using the verified model.

CONCLUSION

Studying on factors affecting acid pretreatment on xylan recovery have found the best condition points to prevent excessive acid hydrolysis of xylan from OPFB. These results found that temperature is most critical for the pretreatment process to be controlled. Diluted nitric acid pretreatment under mild conditions for 24 hr has resulted in a successful recovery of up to 27.63% of the xylan content. On the contrary, rising conditions resulted in the complete removal of xylan from the plant material due to xylan hydrolysis. Model equations for glucan, xylan and lignin produced are significant to illustrate the relationship between the factors and the response with validation percentage error between 4.43% and 7.69%. The study suggests that pretreatment with diluted nitric acid is one of the most advantageous one-step pretreatment processes to improve xylan recovery under mild pretreatment conditions.

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METHANOLIC EXTRACTION OF FREE FERULIC ACID FROM OIL PALM

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ABSTRACT

Ferulic acid, a hydroxycinnamic acid, is known to exhibit anti-microbial, anti-inflammatory and antioxidant properties, making it a valuable compound for various applications. Study was conducted to investigate the extraction of free ferulic acid (ferulic acid that is not linked to lignin or other biopolymers) in the oil palm fruits, pressed fibre, empty fruit bunches (EFB), as well as its leaves, termed as frond. The extraction of free ferulic acid from these palm components was carried out using an alcoholic solution at various sample to solvent ratios. With the exception of fresh sterilised fruits, varying the sample to solvent ratio did not seem to have significant effect on the amount of ferulic acid extracted. Similarly, except for EFB and sterilised fruits, the amount of ferulic acid extracted was not affected by the conditions of the samples (fresh or dried) prior to extraction. The free ferulic acid content in oil palm fruits, pressed fibre, EFB and oil palm frond ranged from 230-670 mg kg⁻¹.

Keywords: extraction, ferulic acid, palm.

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INTRODUCTION

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a hydroxycinnamic acid that exists naturally in almost all plant cell walls, ranging from vegetables, spices and medicinal plants to agricultural residues (Mussatto, *et al.*, 2007; Roby *et al.*, 2013; Zhao *et al.*, 2014). It has been known to exhibit anti-microbial, anti-inflammatory and antioxidant properties, making it a highly sought after compound in the medical, pharmaceutical and cosmetics industries (Bourne and Rice-Evans, 1998; Ou and Kwok, 2004; Mancuso *et al.*, 2007; Neo *et al.*, 2010; Aarabi *et al.*, 2016). In addition, ferulic acid also plays a role in the production of biovanillin (bio-flavour) (Torres-Mancera *et al.*, 2011). Food supplementation of ferulic acid with curcumin can be used for the management of Alzheimer's disease through its oxidative damage and amyloid pathology reduction properties (Bourne and Rice-Evans, 1998; Kikuzaki *et al.*, 2002; Ou and Kwok, 2004).

Concentrations of ferulic acid varies from 5 g kg⁻¹ in wheat bran to 9 g kg⁻¹ in sugar-beet pulp and 50 g kg⁻¹ in corn kernel. Free and linked ferulic acid mostly present in leaves and seeds of plants. It is commonly present in cell walls as cross-links, such as arabinoxylans, pectin and xyloglucans (Torre *et al.*, 2008; Aarabi *et al.*, 2016; Truong *et al.*, 2017).

The oil palm is a commodity plant widely cultivated in tropical countries such as Malaysia and Indonesia. It is mainly cultivated for its fruit bunches which yield oil. A number of beneficial phytonutrients have been identified to be present in palm oil, such as the carotenes, tocopherols (tocopherols and tocotrienols), as well as the coenzyme Q (Ng *et al.*, 2004; Ng and Choo, 2010; 2016). Besides the oil, the whole oil palm, including its by-products after oil extraction are utilised in various sectors, among others, renewable energy, cosmetics, animal feed, furniture and automobile. The presence of ferulic acid in oil palm/palm oil has been briefly investigated in the past, with attention given mostly to oil palm leaves and empty fruit bunches (EFB). As with other plants, the ferulic acid in oil palm consists of both free ferulic acid, and/or esters or linked to lignins

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and other biopolymers (Sun *et al.*, 2001; Neo *et al.*, 2008; 2010; Atawodi *et al.*, 2011; Aanifah *et al.*, 2014; Tang *et al.*, 2014; Stavova *et al.*, 2017).

Generally, oil palm milling starts with sterilisation of the oil palm bunches, followed by threshing of the bunch to strip the fruits, leaving behind the EFB. Thereafter, the fruits were pressed for its oil. Upon pressing, what remained of the fruit is called the palm pressed fibre. In other words, the EFB and pressed fibre are the leftovers or by-products from oil palm milling. The EFB, pressed fibre, as well as other parts of the oil palm, such as the frond, are potential sources of ferulic acid.

Studies on the extraction of ferulic acid from oil palm milling by-products, such as the pressed fibre, EFB, and other palm biomass such as the oil palm frond are quite limited. Aanifah *et al.* (2014) carried out a study on the hydrolysis of ferulic acid from oil palm EFB using alkali while Tang *et al.* (2015) reported on the extraction of ferulic acid from oil palm EFB lignin. While these studies focused on the extraction of the covalently linked ferulic acid, there is no mention on the extraction of ferulic acid that is present in free form. Free ferulic acid is not soluble in water at room temperature but is highly soluble in methanol which made it a good solvent for extraction. Theoretically, larger amount of solvent will allow more solutes to dissolved in it, hence higher amount of solutes of interest can be extracted. However, in economic point of view, the use of large amount of solvent will incur higher cost. It is also difficult to handle such large amount of solvent. As such, keeping the amount of solvent used to a minimum while achieving highest yield is the ideal extraction scenario. As ferulic acid is highly soluble in methanol, the objective of this study is to determine the optimum solvent to sample ratio needed for the extraction of free ferulic acid from various parts of the oil palm using methanol and its analyses by supercritical fluid chromatography (SFC). This is also the first report on the analysis of ferulic acid in palm using SFC.

MATERIALS AND METHODS

Materials

Fresh unsterilised and sterilised oil palm fruits, EFB and palm pressed fibre were obtained from a palm oil mill in Negeri Sembilan, Malaysia. Oil palm frond was obtained from an oil palm plantation in Negeri Sembilan, Malaysia.

All solvents used were of analytical or chromatography grades, purchased from Merck (Darmstadt, Germany). Carbon dioxide (CO₂) used for SFC was purchased from Malaysian Oxygen (MOX). Ferulic acid standard (99% purity) was purchased from Sigma-Aldrich (USA).

Sample Pre-treatment

Unsterilised oil palm fruits were peeled and the nuts were discarded, retaining the flesh (mesocarp). One gram of the peeled mesocarp was dried at 60°C overnight to remove moisture while the fresh mesocarp undergo extraction immediately. Palm pressed fibre, sterilised oil palm fruits, EFB and the oil palm fronds were weighed and dried in similar manner before extraction.

Extraction of Free Ferulic Acid by Methanol

One gram of fresh mesocarp (FM) were weighed in a round bottom flask. Methanol was added (ratio of FM to methanol, 1: 4, g ml⁻¹) and the mixture was refluxed in the dark at 60°C for 6 hr. Upon reflux, the mixture was cooled to room temperature. The mixture was then filtered to remove the solids. The filtrate were then rotavaped to near dryness. It was then centrifuged at 9000 rpm for 2 min. The precipitate was then collected while the supernatant was discarded. The precipitate were then made up to 5 ml with methanol for SFC analyses.

Similar extraction procedure were then repeated for dried mesocarp (DM), fresh sterilised fruits (FSF), dried sterilised fruits (DSF), fresh frond (FF), dried frond (DF), fresh empty fruit bunch (FEFB), dried empty fruit bunch (DEFB), fresh palm pressed fibre (FPPF) and dried palm pressed fibre (DPPF) and the sample to methanol ratio was 1:6 and 1:8.

SFC Analysis of Ferulic Acid Extracts

Isocratic SFC analyses of the ferulic acid was carried out using a JASCO SFC system coupled with a photodiode array detector. Column used was RP C18, 5 µm, 4.6 x 250 mm. The CO₂ flow rate was 2.0 ml min⁻¹ with 0.1 ml min⁻¹ methanol as modifier. The column temperature was set at 50°C and pressure 19 MPa. Injection volume was 20 µl. Identification of the ferulic acid peak was carried out with reference to authentic standard as well as comparing their respective UV spectra. Quantification of the ferulic acid was carried out by calibration with authentic standard.

All extraction and analyses were carried out in triplicates.

RESULTS AND DISCUSSION

The separation of ferulic acid standard and oil palm extracts by SFC is depicted in *Figures 1-3*. Due to the non-polar nature of supercritical carbon dioxide (SC-CO₂), SFC, coupled with CO₂ is rarely used for analyses of polar compounds. The separation of ferulic acid in the extracts by SC-CO₂ was made possible using a reversed stationary phase and

the use of up to 5% methanol as entrainer. The ferulic acid was eluted in less than 5 min. The calibration of ferulic acid in oil palm extracts were carried out with reference to authentic standard (Figure 3).

Alcohol extraction of free ferulic acid involved letting the free ferulic acid dissolved in the warm alcohol and concentrated it through removal of the alcohol thereafter where the ferulic acid, as well as other methanol soluble compounds, will crystallised in saturated solution. Under such circumstances, the amount of free ferulic acid extracted may be influenced by the condition of the samples (fresh or dried), as well as the amount of alcohol or solvent used for extraction. The amount of free ferulic acid in the extracts obtained from various parts of the oil palm under different sample to solvent (S/S) ratio are depicted in Table 1 and Figure 4. S/S ratio of less than 1:4 was not tested as the palm samples were not fully immersed in the small amount of solvent. With the exception of FSF and both FPPF and DPPF, the S/S ratio did not seem to have any significant effect on the extraction of ferulic acid. The amount of free ferulic acid extracted from FSF ($\sim 420 \text{ mg kg}^{-1}$), when S/S ratio was 1:8 was about 1.5 times more as compared to S/S ratio at 1:4 ($\sim 230 \text{ mg kg}^{-1}$) and 1:6 ($\sim 251 \text{ mg kg}^{-1}$). Extraction of free ferulic acid from palm pressed fibre, both FPPF and DPPF, was found to be optimum when the S/S ratio was 1:6. Although theoretically higher amount of extraction solvent will allow more ferulic acid to be dissolved and hence extracted, the results from S/S ratio of 1:8 for FPPF and DPPF appeared to be the opposite. In their study, Tang *et al.* (2015) encountered lower amount of ferulic acid than expected from chemical and enzymatic extraction

of ferulic acid from oil palm empty fruit bunch fibre lignin, but higher amount of vanillin. As reported by Tang *et al.* (2014), ferulic acid is a precursor to the bioconversion of vanillin. This could explain the lower amount of ferulic acid extracted in this study for FPPF and DPPF of S/S 1:8 whereby the ferulic acid was converted into vanillin. However, detailed study need to be further explored in this area to deduce such conclusion.

Higher concentrations of free ferulic acid are found in dried samples extracts, with the exception of dried unsterilised fruits (DUF) where slightly higher amounts of free ferulic acid was found in the fresh unsterilised fruits (FUF) extract compared to DUF. The moisture in fresh samples could have interfered with the solubility of free ferulic acid in methanol, hence, leading to lower amount of free ferulic acid extracted. During sterilisation, the oil palm fruits were heated with water to halt the enzyme activities which could deteriorate the quality of the extracted oil (Nokkaew and Punsuvon, 2014; Maya and Mohd Rozainee, 2013; Khanom *et al.*, 2009). Excess water was typically drained after the completion of sterilisation but the sterilised oil palm fruits still retained some of the moisture. The same goes with the FEFB and DEFB samples which saw the ferulic acid extracted from DEFB to be about twice the amount in the FEFB extract. It should be noted that the EFB was obtained after the sterilisation process. The EFB received similar treatment as sterilised fruits, resulting in the retention of some moisture after sterilisation. The amount of free ferulic acid extracted from DEFB ranged from $490\text{--}531 \text{ mg kg}^{-1}$ as compared to FEFB, $252\text{--}256 \text{ mg kg}^{-1}$, depending on the sample to solvent ratio.

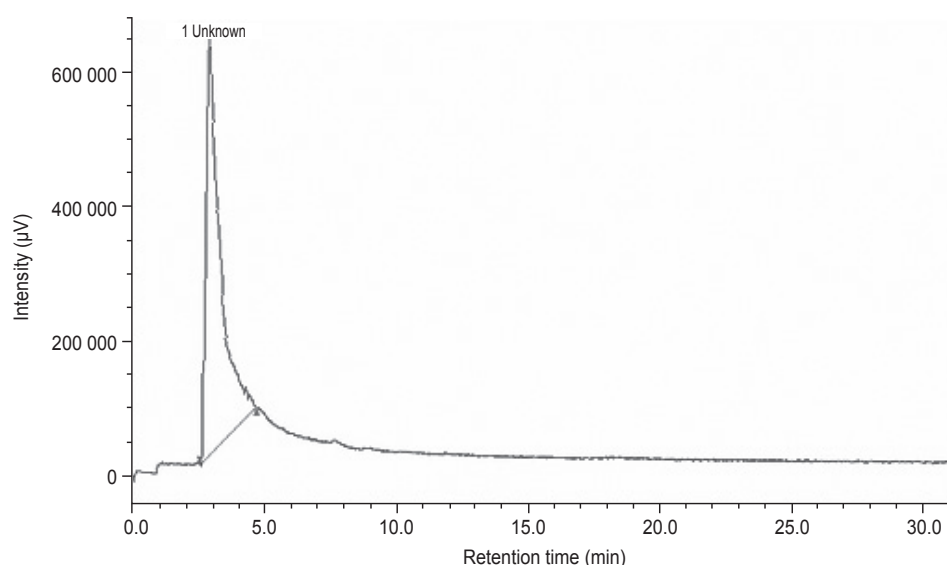


Figure 1. Supercritical fluid chromatogram of ferulic acid in dried palm pressed fibre (DPPF) extracted (sample to solvent, 1:6).

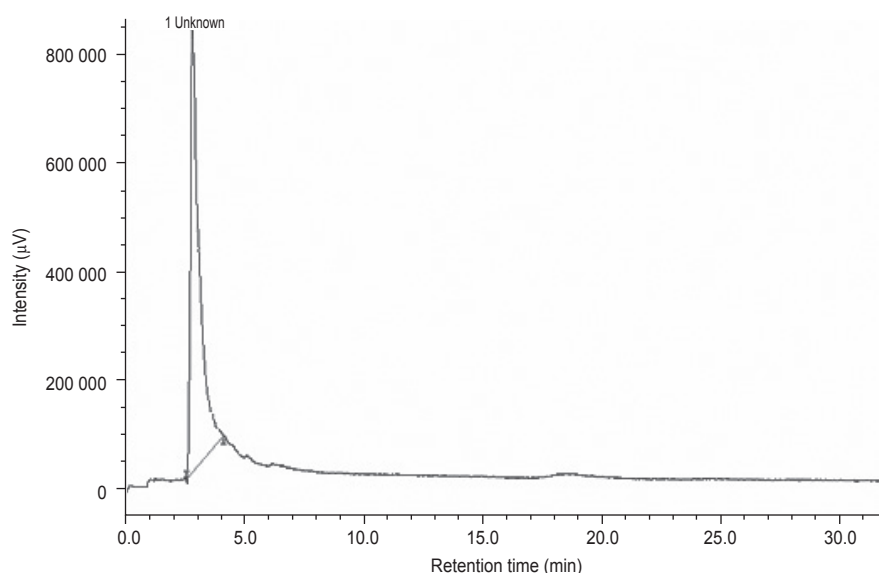


Figure 2. Supercritical fluid chromatogram of ferulic acid in fresh palm frond extracted (sample to solvent, 1:8).

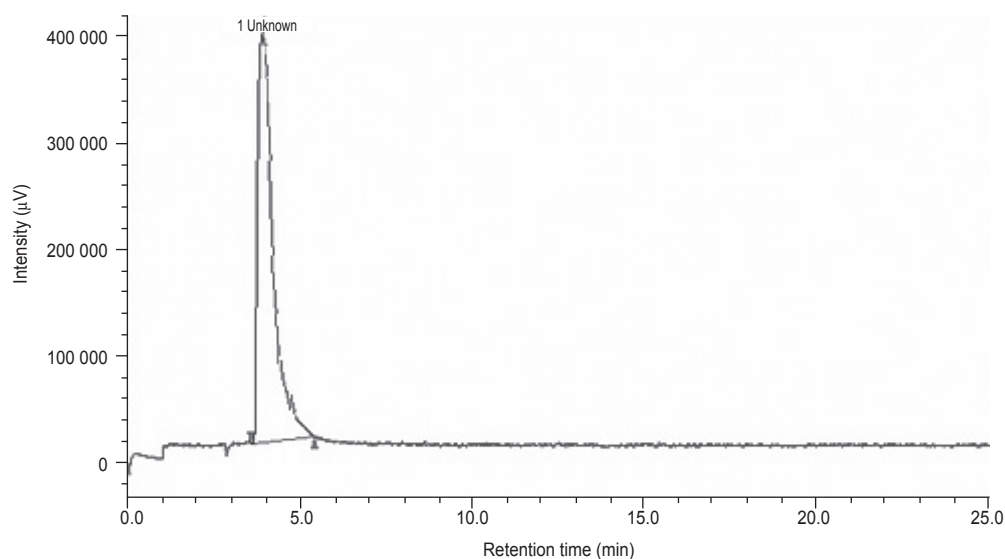
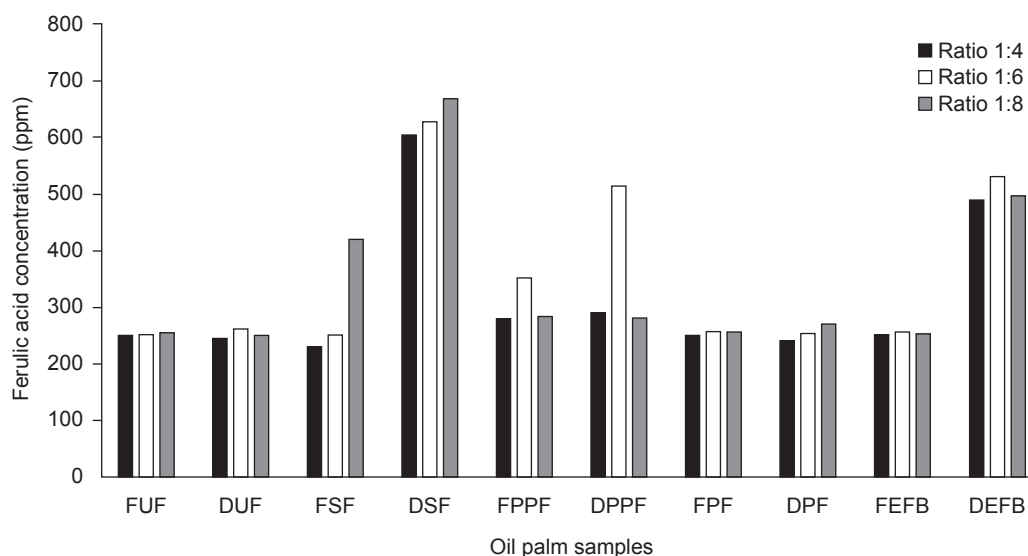


Figure 3. Supercritical fluid chromatogram of ferulic acid standard.

TABLE 1. FREE FERULIC ACID EXTRACTED FROM OIL PALM UNDER DIFFERENT SAMPLE TO SOLVENT RATIO

Sample		Ferulic acid concentration (mg kg ⁻¹) extracted under sample to solvent ratio		
		1:4	1:6	1:8
Unsterilised fruits	Fresh (FUF)	250 ± 3	252 ± 5	255 ± 8
	Dried (DUF)	245 ± 4	262 ± 1	250 ± 7
Sterilised fruits	Fresh (FSF)	230 ± 2	251 ± 6	420 ± 4
	Dried (DSF)	604 ± 8	628 ± 8	668 ± 12
Palm pressed fibre	Fresh (FPPF)	280 ± 6	352 ± 3	284 ± 4
	Dried (DPPF)	291 ± 6	514 ± 8	281 ± 9
Palm frond	Fresh (FPF)	250 ± 7	257 ± 3	256 ± 8
	Dried (DPF)	241 ± 8	254 ± 4	270 ± 10
Empty fruit bunch	Fresh (FEFB)	252 ± 4	256 ± 4	253 ± 6
	Dried (DEFB)	490 ± 8	531 ± 7	497 ± 2



Note: FUF - fresh unsterilised fruits; DUF - dried unsterilised fruits; FSF - fresh sterilised fruits; DSF - dried sterilised fruits; FPPF - fresh palm pressed fibre; DPPF - dried palm pressed fibre; FPF - fresh palm frond; DPF - dried palm frond; FEFB - fresh empty fruit bunch; DEFB - dried empty fruit bunch.

Figure 4. Composition of ferulic acid extracted from various oil palm biomass.

Besides the aforementioned scenarios, the S/S ratio as well as the conditions of the samples prior to extraction did not seem to have a significant effect on the extraction of free ferulic acid for the same type of sample. At 241-270 mg kg⁻¹, the amount of free ferulic acid in both dried and fresh palm frond (DPF and FPF) are comparable.

There was however, a significant difference in the amount of ferulic acid extracted from sterilised and unsterilised fruits for both types of sample conditions, fresh and dried. At S/S ratio of 1:8, the amount of free ferulic acid extracted from FSF at 420 mg kg⁻¹, was almost doubled that of FUF at 255 mg kg⁻¹. The difference was even more significant between DUF, 250 mg kg⁻¹ and DSF, 668 mg kg⁻¹. It is believed that the sterilisation process contributed to this difference. Besides halting the enzymatic activities, sterilisation also caused the oil palm fruits to be softened, hence, leading the mesocarp to be more readily penetrated by the extracting solvent, resulting in higher solubility of free ferulic acid, which then gave rise to higher yield (Nokkaew and Punsuvon, 2014; Maya and Mohd Rozainee, 2013; Khanom *et al.*, 2009).

This study focused on the extraction of ferulic acid in free form from various parts of the oil palm. Ferulic acid that is covalently linked to lignin and other biopolymers or as ester cross-links with polysaccharides in the cell wall cannot be extracted using the same method. Cleavages of the covalent bonds need to be first carried out before these ferulic acid can be obtained. Some methods reported that were able to free these covalently linked ferulic acid are alkaline hydrolysis, enzymatic extraction, and

more recently, the use of deep eutectic solvents and/or ionic liquids (Liu and Wang, 2011, 2012; Torre *et al.*, 2008; Truong *et al.*, 2017; Tang, *et al.*, 2015). As reported by Aanifah *et al.* (2014), as much as 3800-4200 mg g⁻¹ ferulic acid can be extracted from the palm EFB by way of alkaline hydrolysis, as compared to ~500 mg kg⁻¹ from methanol extraction in this study. Although the yield of ferulic acid extracted from alkaline hydrolysis is higher, nevertheless, alcohol extraction offers a simple and rapid method to extract the ferulic acid in its free form, where they can be readily used as raw material for production of further downstream products.

CONCLUSION

The oil palm biomass, especially the EFB, are good sources of free ferulic acid. The amount of free ferulic acid extracted from the palm pressed fibre and EFB are comparable to its amount in the oil palm fruits. This suggests that the milling process did not significantly affect the ferulic acid content in palm. This study showed that warm methanol is sufficient to extract free ferulic acid from the by-products of the oil palm milling as well as various parts of the oil palm.

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GROWTH PERFORMANCE AND CARCASS TRAITS OF GOATS FED WITH OIL PALM BY-PRODUCTS BASED FEED PELLET

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ABSTRACT

The study was conducted to develop a nutrient-balanced goat feed pellet from oil palm by-products and to assess the effects of the feed pellet on growth performance and carcass traits of Katjang goats. Treatment groups consisted of oil palm by-products based (OPB), control without addition of oil palm by-products control (CNT) and commercial (COM) pellets. Twelve 12-month-old Katjang goats were assigned to the three treatment groups and subjected to 14-weeks of feeding trial. Each group was offered 1.5% body weight (BW) of respective pellets and ad libitum supply of Napier grass. Feed intake (FI) was recorded daily and the BW of the goats was recorded weekly. At the end of the feeding trial, all goats were slaughtered for carcass traits study. All treatment groups showed no significant difference in BW increment ($p=0.51$) and average daily gain (ADG) ($p=0.94$). There were no significant differences ($p>0.05$) between the treatment groups for carcass traits parameters. As a conclusion, the OPB pellet has shown to contain complete nutrient for goat's diet and was comparable to the COM feed pellet in terms of growth performance of Katjang goat. The inclusion of oil palm by-products in the feed formulation did not adversely affect the carcass traits of the goats.

Keywords: carcass traits, goat feed pellet, growth performance, oil palm by-products.

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INTRODUCTION

Utilisation of local resources is one of the approaches to support the growing productivity and sustainability in agriculture industry (Rodriguez and Preston, 1997). Sontakke *et al.* (2014) described that the utilisation of the agricultural by-products not only could reduce the overall feed cost but could also decrease the competition for food between human and animals. Oil palm plantations in Malaysia cover a total of 5.81 million hectares in 2017 (MPOB, 2018). Crude palm oil (CPO) production for 2018 was 19.52 million tonnes (Kushairi *et al.*, 2018). The

huge oil palm plantation area and the volume of CPO production annually have contributed to the increase of waste and by-products. Palm oil industry produces approximately 21.625 t ha⁻¹ of solid biomass annually, which consists of oil palm fronds (OPF), 78%; empty fruit bunches (EFB), 9%; mesocarp fibre (PPF), 6%; oil palm trunks (OPT), 5%; and palm kernel shell, 2% (Hosseini *et al.*, 2015).

Most of the oil palm by-products have great potential to be utilised as ingredients in compound ruminant feeds. The proximate composition of the oil palm by-products is presented in Table 1. However, some of the by-products need further processing before they can be used effectively in livestock ration (Mohamed *et al.*, 2012). OPF can be utilised as a satisfactory alternative roughage source for ruminants. The OPF inclusion into the ruminant diet can be either as freshly chopped, silage or

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treated with other physical methods (Sudaryanto, 2017). EFB is a solid residue produced in abundance at oil palm mill after the oil extraction process. At present, EFB is widely used as organic mulch at the plantation, pulp for making paper, wood composite products and fibreboard (Abdullah and Sulaiman, 2013). Although these practices contribute to EFB disposal, there are still plenty of EFB available that can be used for other purposes, for instance as a raw material in ruminant ration. Yet, information on its utilisation as animal feed is very limited (Ginting *et al.*, 2018).

In crushing plant, palm kernels are crushed to yield crude palm kernel oil and the by-product is the palm kernel cake (PKC). The production of PKC from this process is around 50% of the palm kernel. As one of the major world's producer of palm oil, Malaysia produces more than 2 million tonnes of PKC annually, much of which is exported to Europe, New Zealand, Korea, China and other countries (MPOB Annual Report, 2016). The crude protein (CP) content in PKC ranges between 12%-18%, making it suitable to be utilised as a protein source for ruminant feed, mostly in beef and dairy cattle feed (Ferreira *et al.*, 2012; Tipu *et al.*, 2014; Pimentel *et al.*, 2015).

CPO refining process produces about 5% palm fatty acid distillates (PFAD), another by-product. PFAD is a readily available, relatively stable to oxidative rancidity and has considerable nutritive value, especially as fat supplement for livestock (Tomkins and Drackley, 2010). The primary market for PFAD in the livestock feed industry is as ruminant diets, either as hydrogenated fatty acids or as calcium soap (Palmquist, 2004). The use of PFAD in animal feed formulation is a practical, cost-effective and safe approach to produce good growth performance without any negative effects (Aliyu-Paiko and Hashim, 2012).

Goat and sheep farming industry in Malaysia is small. Thus, Malaysia still depends on the import of mutton and lamb from other countries such as

Australia, New Zealand and Indonesia (Meat and Livestock Australia, 2018). As compared to poultry (103.33%), eggs (119.06%) and beef cattle (23.42%), the goat's self-sufficiency level (SSL) in 2017 only reached 11.41% (DVS, 2018). The main factor contributing to the low goat's SSL is the lack of local quality feed, thus, most of the feed ingredients have to be imported at higher price, leading to the unsustainable production of meat throughout the year.

Malaysia imports more than 70% of raw materials annually to support the animal feed industry, including 2000 t and 1000 t of corn and soyabean meal, respectively. Ministry of Agriculture and Food Industries Malaysia (2016) reported that the value of imported animal feed in Malaysia was almost RM6.7 billion in 2016. To develop domestic ruminant industry, there is a need to improve and produce sufficient feed resources to support the production of these animals. By-products from the Malaysian oil palm industry are seen as promising alternative components for ruminant feed as they are cost-effective and are locally available throughout the year. It is hypothesised that inclusion of oil palm by-products in the feed formulation can provide adequate nutrients for goats to support their growth. Thus, the objectives of this study were to develop a nutrient-balanced goat feed formulation using oil palm by-products and to evaluate the effects of the formulated pellet on the growth performance and carcass traits of Katjang goats.

MATERIALS AND METHODS

Animals and Materials

Katjang goats, raw materials for feed production (corn, soyabean meal, rice bran, PKC, EFB, PFAD and others) and commercial feed were purchased from local suppliers. OPF was obtained from MPOB Keratong Oil Palm Plantation, Pahang, Malaysia.

TABLE 1. PROXIMATE COMPOSITION OF OIL PALM BY-PRODUCTS

Proximate analysis	OPF ^a	PKC ^b	EFB ^c
Dry matter (DM)	36.4	88.0-94.5	-
Ash (% DM)	6.6	3.0-12.0	-
Crude fat (% DM)	1.2	5.0-8.0	3.2
Crude protein (% DM)	5.8	14.5-19.6	3.7
Crude fibre (% DM)	44.8	13.0-20.0	48.8
Nitrogen free extract (NFE) (%)	43.3	46.7-58.8	-

Note: OPF - oil palm frond; PKC - palm kernel cake; EFB - empty fruit bunch.

^aAdapted from Zahari and Alimon (2005).

^bAdapted from Alimon (2004).

^cAdapted from Zahari *et al.* (2003).

Pellet Formulation

Two types of pellets were formulated to meet maintenance requirement for growing goats in accordance with the guidelines of nutrient requirements of small ruminants (NRC, 1981) as follows: control (CNT) pellet was formulated without using oil palm by-products based ingredients, and oil palm by-products based (OPB) pellet was formulated using OPF, PKC, EFB and PFAD. The other ingredients that were used in the pellet formulation are presented in Table 2. Another type of pellet was the commercial pellet (COM) purchased from a local supplier. Both CNT and OPB pellets were formulated to be isocaloric and isonitrogenic with COM pellet using *New Century*TM formulation software (FORMAT International, USA).

Proximate Analysis

The dry matter (DM), moisture, ash, CP, crude fat, crude fibre (CF) and gross energy (GE) of the pellet samples were analysed according to the Association of Official Analytical Chemist (AOAC, 1990) procedures. Organic matter was calculated based on ash content (100 - ash content). Nitrogen free extract (NFE) was calculated using the equation below, as described by McDonald *et al.* (2010):

$$\text{NFE (\%)} = 100\% - (\text{moisture \%} + \text{ash \%} + \text{crude fat \%} + \text{crude protein \%} + \text{crude fibre \%}).$$

Feeding Trial and Growth Performance

The experimental design was a completely randomised design with three treatment groups that corresponded to three different types of pellets; either CNT, COM and OPB with four goats per

treatment group. Twelve male Katjang goats of 12-month-old with initial body weight (BW) of about 14.7 ± 1.1 kg were randomly assigned to one of the treatment groups. The Katjang goats were kept in individual pens of 1.8×1.8 m with free access to water. Each group was fed twice daily with 1.5% BW of respective pellets and *ad libitum* supply of Napier grass. The goats were allowed for two weeks of adaptation period to adapt to the experimental conditions and diet prior to the 14-weeks of feeding trial.

The goats were weighed on the first day of the feeding trial and followed by weekly weighing before the morning feeding to determine the BW of the animals. Feed intake (FI) of each goat was recorded daily based on the amount of feed offered and refused. The amount of feed offered was adjusted weekly based on changes in BW of the goats. The average daily gain (ADG) was calculated by dividing the difference between initial and final BW by the period of feeding trial.

Slaughter and Sampling

At the end of the feeding trial, all goats were fasted for 24 hr with free access to water before slaughtering. The goats were weighed again before being slaughtered and recorded as slaughter weight. The slaughtering was done according to Halal method outlined in MS 1500:2009 (Department of Standard Malaysia, 2009). Each carcass was first weighed as hot carcass. Subsequently, the pelt, head, internal organs (lung and trachea, liver and kidney) and internal fats including peritoneal and mesenteric, kidney, channel, and heart fats were weighed separately. Each carcass was then chilled for 24 hr at 4°C. The next day, chilled carcasses were re-weighed and were split into two equal halves (right and left). The cuts were weighed and expressed

TABLE 2. INGREDIENTS OF OIL PALM BY-PRODUCTS BASED AND CONTROL PELLETS

Ingredient (%)	OPB	CNT
Corn	18.0	19.0
Soyabean meal	16.0	18.0
Rice bran	11.0	20.0
Oil palm by-products (consists of PKC, OPF, EFB and PFAD)	49.6	-
Rice straw	-	35.0
Soyabean oil	-	2.7
Molasses	3.50	4.00
Salt	0.50	0.50
Limestone	1.50	0.25
Dicalcium phosphate	-	0.25
Vitamin mineral premix	0.25	0.30

Note: OPB - oil palm by-products based formulated pellet; CNT - pellet without oil palm by-product inclusion; PKC - palm kernel cake; OPF - oil palm frond; EFB - empty fruit bunch; PFAD - palm fatty acid distillates.

as percentage of the total weight of the carcasses. Each cut was then further divided into meat, bone, and fat and the data was used to calculate carcass composition.

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System (SAS) statistical software package, version 9.1.3 (SAS Institute Inc., USA). Data obtained from all analytical parameters were analysed using one way analysis of variance (ANOVA), with significant differences determined by Duncan's new multiple range test. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Proximate Analysis of Feed Pellets

The proximate composition of all pellets is shown in *Table 3*. The oil palm by-products in OPB pellet was formulated to contain a similar level of CP (isonitrogenous) and GE (isocaloric) content to CNT and COM pellets. OPB has been shown to contain complete nutrients for goat diet in accordance with the goat's nutrient requirements and was comparable to the COM pellet.

Growth Performance

Growth performance of Katjang goats fed with different pellets is shown in *Table 4*. There was no significant difference ($p = 0.62$) in the initial BW between goat fed with OPB, CNT or COM diets, indicating homogeneity in the weight of animals used in this study. The feeding of OPB, CNT and COM on goats had no significant effect ($p = 0.93$) on the final BW of Katjang goat at the end of feeding trial. Overall, all treatment groups showed no significant difference ($p = 0.51$) in BW increment, which was from 14.1-20.9 kg (48.23%), 13.2-20.4 kg (54.54%) and 14.0-20.6 kg (47.14%) for OPB, CNT and COM, respectively. The ADG was also not significantly different ($p = 0.94$) between treatment groups. Although there was no significant difference, ADG of OPB group was numerically higher (68.88 g day⁻¹) than goats in the COM group (66.84 g day⁻¹), but lower than goats in the CNT group (72.96 g day⁻¹). The findings suggest that it is possible to incorporate OPB into goat's feed without negatively affecting the growth performance of the goats. Isocaloric and isonitrogenous content among OPB, CNT and COM treatments might be the reason for the acceptable growth performance of the Katjang goat. The result was in agreement with findings by Haryanto (2011), Rahman *et al.* (2013) and Ebrahimi *et al.* (2015). Haryanto (2011) reported that Ettawah crossbred

goats fed with molasses protected PKC based diet was able to gain up to 70.24 g day⁻¹ in BW. Rahman *et al.* (2013) observed Boer x local crossbred goats fed with 2% BW of PKC based concentrate diets gained 52.5 g day⁻¹ of BW. Meanwhile, study by Ebrahimi *et al.* (2015) showed that 25% of OPF inclusion in male Katjang crossbred goats diet resulted in ADG of 75.71 g day⁻¹.

No significant differences ($p > 0.05$) were observed for the dry matter intake (DMI) of the pellet, Napier grass and the diet as a whole (*Table 5*). However, OPB group had the highest total DMI with 572.54 g day⁻¹, followed by CNT and COM groups, with 572.33 and 548.41 g day⁻¹, respectively. The DMI (% BW) of goat fed with OPB pellet at 3.11% BW, which is within the requirement for goat in tropical regions, reported to range from 3.0%-3.66% BW (Devendra and McLeroy, 1982; Ashok and Wadhwani, 1992). The good DMI (% BW) indicates the inclusion of oil palm by-products into goat pellet did not give negative effect on the FI of the animals and the pellet was well accepted by the goats.

Yusuf *et al.* (2014) suggested that animals FI is influenced by several factors, such as diet composition, availability and palatability of the feed. Abubakr *et al.* (2013) reported that oil palm by-products such as PKC are usually considered as unpalatable feed. However, the current study has shown that inclusion of oil palm by-products up to 49.6% did not affect the animal FI, suggesting that the palatability might have been improved by processing the feed materials into pellet form. According to Leng (1990); Joo *et al.* (2012); Abubakr *et al.* (2013) and Karimizadeh *et al.* (2017), the FI can be improved through various processing techniques and supplementation. For example, Joo *et al.* (2012) suggested that feeding a combination of Napier and OPF to goats offer better results in certain aspects compared to the feeding of Napier grass or OPF alone, besides reducing feed cost and environmental waste. Thus, the results of this current study indicate that oil palm by-products based pellet provides adequate nutrients for goats to support their growth.

Carcass Traits and Composition

Carcass traits and composition of goats fed with different pellets are shown in *Table 6*. There were no significant differences ($p > 0.05$) in carcass traits between goats fed with OPB, CNT and COM. Overall, the slaughter weight of goat from all treatments ranged between 19.00-19.55 kg, whereas hot and cold carcass weights were between 8.10-9.13 kg and 8.00-8.98 kg, respectively. The lowest dressing percentage was observed in goat fed with CNT at 43.84%, which is slightly lower than general dressing percentage of 45%-50% for male goats as reported by Raghavan (1988). However, dressing

percentage result in this study was within the range (37%-55%) as reported by Ensminger (2002).

The goats fed with OPB had no significant differences ($p>0.05$) on carcass composition such as meat, bone and fat percentage when compared with that of CNT and COM. These results showed that feeding the goats with oil palm by-products did not negatively affect the carcass composition. One of the plausible reasons is because of the similar intake of isocaloric and isonitrogenous feed between OPB, COM and CNT. There was

no significant difference in growth performances thus, resulting in similar carcass weight at the end of the feeding period. This finding was consistent with the observation by Ebrahimi *et al.* (2013) who reported that feeding 25% oil palm by-products in the diet had no effect on the carcass yield when compared to the group fed with 100% commercial pellet. Abubakr *et al.* (2013) also reported that feeding goats with oil palm by-products did not significantly affect the dressing percentage and meat percentage of goats.

TABLE 3. PROXIMATE COMPOSITION OF OIL PALM BY-PRODUCTS BASED, CONTROL AND COMMERCIAL PELLETS

Proximate analysis	OPB	CNT	COM
Dry matter (DM)	90.58	87.89	87.37
Moisture content (% DM) ^a	9.42	12.11	12.63
Ash (% DM)	7.74	10.72	6.90
Organic matter (% DM) ^a	92.26	89.28	93.10
Crude fat (% DM)	4.36	5.59	4.00
Crude protein (% DM)	15.58	16.16	16.01
Crude fibre (% DM)	21.66	16.76	13.56
Nitrogen free extract (NFE) (%) ^a	41.25	38.65	46.91
Gross energy (cal g ⁻¹)	3954	3858	3933

Note: OPB - oil palm by-products based formulated pellet; CNT - pellet without oil palm by-product inclusion; COM - commercial pellet.

^aCalculated value.

Moisture content = 100 – dry matter.

Organic matter = 100 – ash.

Nitrogen free extract (%) = 100% – (moisture % + ash % + crude fat % + crude protein % + crude fibre %).

TABLE 4. GROWTH PERFORMANCE OF KATJANG GOATS FED WITH OIL PALM BY-PRODUCTS BASED, CONTROL AND COMMERCIAL PELLETS

Parameter	OPB	CNT	COM	<i>p</i> -value
Initial body weight (kg)	14.10 ± 1.27	13.20 ± 1.40	14.00 ± 0.75	0.62
Final body weight (kg)	20.90 ± 1.33	20.40 ± 2.18	20.60 ± 1.75	0.93
Total body weight gain (kg)	6.80 ± 1.28	7.20 ± 1.33	6.60 ± 1.37	0.51
Average daily gain (g day ⁻¹)	68.88 ± 13.05	72.96 ± 13.58	66.84 ± 13.98	0.94

Note: OPB - oil palm by-products based formulated pellet; CNT - pellet without oil palm by-product inclusion; COM - commercial pellet.

Values shown are means ± s.d. (n=4).

TABLE 5. FEED INTAKE OF KATJANG GOATS FED WITH OIL PALM BY-PRODUCTS BASED, CONTROL AND COMMERCIAL PELLETS

Parameter	OPB	CNT	COM	<i>p</i> -value
Pellet DMI (g day ⁻¹)	252.43 ± 0.00	252.43 ± 0.00	250.55 ± 2.17	0.46
Napier DMI (g day ⁻¹)	322.83 ± 0.29	319.90 ± 3.63	311.34 ± 13.43	0.17
Total DMI (g day ⁻¹)	572.54 ± 0.29	572.33 ± 3.63	548.41 ± 15.60	0.54
DMI (% BW)	3.11 ± 0.20	3.27 ± 0.31	3.13 ± 0.21	0.61

Note: OPB - oil palm by-products based formulated pellet; CNT - pellet without oil palm by-product inclusion; COM - commercial pellet; DMI - dry matter intake; BW - body weight.

Values shown are means ± s.d. (n=4).

TABLE 6. CARCASS TRAITS AND COMPOSITION IN KATJANG GOATS FED WITH OIL PALM BY-PRODUCTS BASED, CONTROL AND COMMERCIAL PELLETS

Parameter	OPB	CNT	COM	p-value
Carcass traits				
Slaughter weight (kg)	19.55 ± 1.24	19.00 ± 2.11	19.40 ± 1.66	0.90
Hot carcass weight (kg)	9.13 ± 1.11	8.10 ± 1.05	9.10 ± 0.80	0.37
Cold carcass weight (kg)	8.97 ± 1.06	8.00 ± 1.05	8.98 ± 0.81	0.39
Dressing percentage (%)	46.81 ± 2.06	43.84 ± 1.60	47.04 ± 4.12	0.38
Offal* (% of slaughter weight)	21.20 ± 1.10	20.50 ± 1.77	20.93 ± 1.58	0.85
Pluck* (% of hot carcass weight)	5.82 ± 0.46	6.52 ± 0.97	6.50 ± 0.78	0.46
Carcass composition (% of cold carcass weight)				
Meat	66.14 ± 1.13	66.84 ± 2.50	68.19 ± 4.10	0.68
Bone	22.94 ± 2.17	21.66 ± 1.66	22.07 ± 1.42	0.66
Fat	10.92 ± 3.28	11.50 ± 2.34	9.74 ± 2.86	0.76
Meat:bone	2.90 ± 0.23	3.10 ± 0.30	3.11 ± 0.37	0.65
Meat: fat	6.51 ± 2.28	6.03 ± 1.58	7.57 ± 2.59	0.66

Note: OPB - oil palm by-products based formulated pellet; CNT - pellet without oil palm by-product inclusion; COM - commercial pellet; Offal* - head, leg and skin; Pluck* - heart, liver, kidney, lungs and spleen. Values shown are means ± s.d. (n=4).

CONCLUSION

The growth performance and carcass quality of Katjang goats fed with oil palm by-products based pellet were comparable to goats fed with formulated control pellet, as well as those fed with the commercial pellet. The inclusion of oil palm by-products ingredients in goat pellets did not have any adverse effect on the palatability of the pellet. Therefore, oil palm by-products can be included in the feed formulation without compromising the growth performance and carcass composition of Katjang goats.

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EFFECT OF FEEDING PALM OIL SLUDGE AS PARTIAL REPLACEMENT OF MAIZE IN GROWING-FINISHING PIGS ON THE GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY AND BLOOD PROFILES

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ABSTRACT

The study was conducted to investigate the effect of dietary inclusion of palm oil sludge (POS) as partial replacement of maize in growing-finishing pigs on growth performance, nutrient digestibility and blood profiles. Twenty four crossbred (Large White Yorkshire x Hampshire) pigs of mixed sex (3-4 months of age) with initial body weight of 40.09 ± 1.01 kg were randomly allotted to four dietary treatments in a randomised block design. Palm oil sludge was included in the diets at 0% (Group-1, control), 10% (Group-2), 20% (Group-3) and 30% (Group-4) to replace maize as energy source. The body weight gain, average daily feed intake, average daily gain and feed conversion ratio were similar across the treatment groups. The inclusion of POS in the diets had no effect on the haematological parameters, serum metabolites and lipid profile. The digestibility coefficient adverse of ether extract (EE) and crude fibre (CF) were not affected by dietary POS supplementation but dry matter (DM), organic matter (OM), CF and nitrogen free extract (NFE) revealed significant ($p < 0.05$) difference among the various treatment groups. In conclusion, POS can replace maize up to 30% in the concentrate diets without any detrimental effects on the growth performance, nutrient utilisation and blood biochemical parameters of growing-finishing pigs.

Keywords: digestibility coefficient, growing-finishing pigs, growth performance, haemato-biochemical parameters, palm oil sludge.

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INTRODUCTION

Animal husbandry plays a vital role in the economic development of Mizoram, India and pig is by far the most populous which is reared in each and every house hold. However, the inadequate supply of

feed, both quantitatively and qualitatively and high cost of feed ingredients are the principal limiting factors for efficient pig production. Therefore, there is a continuous quest for other cheap and indigenous sources of alternative feedstuffs. Agro-industrial by-products have become important feed components in livestock and poultry diets mainly due to the increased competition for the conventional feed ingredients, especially cereals by humans and the food industries. Hence, a by-product of interest in palm oil production in terms of substituting energy in conventional monogastric diet is palm oil sludge (POS). Palm oil mill effluent (POME) is a general description for the discharge from palm oil extraction in the mill and is composed of various liquid, dirt, residual oil and

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suspended solids, mainly cellulosic material from the mesocarp of the fruits. POS or decanter cake is a by-product obtained after dehydration of POME. Hence, POS is a potential environmental pollutant and its utilisation as animal feed will minimise the environmental problem as well as provide energy for the animal (Webb *et al.*, 1977). The nutrient content of POS varies considerably depending on the variety (Bamikole and Ikatua, 2009) and the method of oil extraction (Seephueak *et al.*, 2011) but Devendra (1977) had reported the comparability of POS to maize in terms of chemical composition. Its composition is superior to that of maize, particularly with respect to protein and energy.

The use of POS as an unconventional feed in pigs has been substantiated by Vroom (1978); Hertrampf (1988); Perez (1997) and Ezekwe *et al.* (2011). Research on feeding of POS in pigs has so far not been carried out scientifically in India. There is a scope for utilisation of POS as a potential energy source for livestock as an alternative for maize in areas where oil palm industries are located. Therefore, this study was undertaken to evaluate the potential of POS as partial replacement of maize on the performance, nutrient utilisation and biochemical parameters of growing-finishing pigs.

MATERIALS AND METHODS

Study Location

The feeding trial was conducted at the Instructional Livestock Farm Complex (ILFC), College of Veterinary Science and Animal Husbandry, Selesih, Aizawl, Mizoram, India. Mizoram is a North Eastern state of India located at 92°15'E to 93°29'E longitude and 21°58'N to 24°35'N latitude with a total geographical area of 21 081 km². The state is rich in flora, which include tropical trees and plants. The people of the state are agrarian and oil palm cultivation is one of the major farming activities. Currently, Mizoram is producing about 6500 t of palm oil (MOFPI, 2017).

Experimental Animal and Their Management

Twenty four crossbred (Large White Yorkshire x Hampshire) pigs were taken from the Instructional Livestock farm of College of Veterinary Sciences and Animal Husbandry, Selesih. Transfer of animals to the experimental shed was done and care was taken to cause minimal stress during transportation. The pigs were housed individually under intensive system of management. The adaptation period was for 14 days during which the groups to be fed with experimental feeds were given an actual amount on

dry matter basis depending upon their group. All the animals were vaccinated against swine fever disease and dewormed before the commencement of the trial.

Experimental Design

Twenty four crossbred (Large White Yorkshire x Hampshire) growing pigs aged 3-4 months (40.0±91.01 kg) were assigned to four treatments in a completely randomised design with six pigs per treatment. Feeding programme was conducted in two phases *i.e.* Phase I (20-50 kg) and Phase II (50-80 kg) for total experimental period of 10 weeks, as per National Research Council (NRC) 1998. There were four dietary treatments; Group-1 (Control, 0% POS), Group-2 (10% of replacement of maize with POS), Group-3 (20% replacement of maize with POS) and Group-4 (30% replacement of maize with POS). The diets formulated were all isonitrogenous and isocaloric.

Feed Intake

Feeding was done individually and drinking water was made available at all times. The feeds were offered twice to the animals at 9.00 am in the morning and at 4.00 pm in the evening. The daily feed intake was recorded from the difference between the amount of feed offered and the residue left in the feeding throughout the day (24 hr).

Body Weight Changes and Feed Conversion Efficiency

Individual body weights of the pigs were recorded before the start of the feeding experiment and thereafter at fortnightly intervals before feeding and water provision in the morning, using an electronic scale to assess the body weight changes and growth rate. Feed efficiency during the respective as well as overall periods was calculated as the ratio between the feed intake per unit body weight gain.

Haemato-biochemical Analysis

Blood sample was collected aseptically from the anterior vena cava using 10 ml syringe at the onset and Day 50 of the feeding trial. Haematological parameters were estimated with the help of Automated Blood Cell Counter (Model: MS4E, The Netherlands) by following the standard procedures as per the manufacturers' protocol. Blood biochemical parameters were estimated with the help of UV Visible spectrophotometer (Serial No. A114518; Shimadzu Corp.) using commercially available diagnostic kits (Coral diagnostics).

Digestibility Trial

For the digestibility trial, four animals from each group were randomly selected. Three days adaptation period was given to animals prior to sampling and collection. During the digestibility trial, individual feed intake, residue left and faeces voided by the animals were recorded individually. Wholesome drinking water was made available to all animals *ad libitum*. The digestibility trial was conducted for four days after the end of the feeding trial and representative faecal sample and feed was collected and processed for chemical analysis.

Chemical Analysis

Proximate principles and fibre fractions. POS, feed and faeces were analysed (AOAC, 1995) for dry matter (DM; method 934.01), crude protein (CP; method 968.06; Kelplus, Pelican Equipments, Chennai, India), crude fibre (CF; Fibreplus, Pelican Equipments, Chennai, India) and ether extract (EE; method 920.39; Socsplus, Pelican Equipments, Chennai, India). The fibre fractions were determined as per the method described by Goering and Van Soest (1970). Calcium was estimated as per Talapatra *et al.* (1940), whereas phosphorus was estimated as per Association of Official Analytical Chemists (AOAC) (1995). All the samples for the analysis were weighed using an electronic balance (Model No. ABS 220-4, Kern and Sohn GmbH). The digestible energy was calculated using the prediction equation described by Noblet and Perez (1993).

Fatty Acid Analysis

Fatty acids content of POS was determined following the method of O'Fallon *et al.* (2007) with slight modification (Mandal *et al.*, 2014). In brief, 0.5 g sample was placed into a screw-cap pyrex culture tube to which 0.5 ml of the C13:0 internal standard (0.5 mg of C13:0 ml in methanol), 0.35 ml of 10 N KOH in water and 2.65 ml of methanol was added. The tube was incubated at 55°C for 1.5 hr with vigorous hand-shaking for 5 s every 20 min to properly permeate, dissolve and hydrolyse the sample. After cooling below room temperature in a cold tap water bath, 0.29 ml of 24 N H₂SO₄ in water was added. The tube was mixed thoroughly and incubated again at 55°C for 1.5 hr with hand-shaking for 5 s every 20 min. After fatty acid methyl ester (FAME) synthesis, the tube was cooled in a cold tap water bath, 1.5 ml of hexane was added, and the tube was mixed for 5 min on a multi-tube vortex. The tube was centrifuged for 5 min in a table top centrifuge at 2500 rpm and the hexane layer containing FAME was placed into a vial. The vial was capped and placed at -20°C until analysis. The prepared FAME was

determined by capillary gas chromatography (GC) on a HP-88, 100 m x 0.25 mm x 0.20 µm capillary column (Agilent Technologies Inc., Santa Clara, California, USA) with a flame ionisation detector. Identification and quantification of individual FA were performed using a purified standard (Supelco 37 component FAME mixture, Sigma-Aldrich Chemical Pvt. Ltd., Kolkata, India) and an internal standard (tridecanoic acid; Sigma-Aldrich chemical Pvt. Ltd. Kolkata, India). The fatty acid concentration of POS was expressed as g per 100 g of the sum of identified peaks measured in each sample.

Statistical Analysis

The data were analysed using general linear model procedure of statistical analysis system (SAS, 2003). The linear and quadratic effects of dietary increasing proportion of inclusion of POS against the control group on performance, haemato-biochemical and nutrient digestibility was determined by orthogonal polynomial contrasts. One-way analysis of variance (ANOVA) has been applied for comparison among treatments and paired t-test was performed for comparison between periods (Day 0 and Day 50) within group. Tukey's test was used to compare the means of the treatment with probability of 5% ($p < 0.05$).

The statistical model used for one-way ANOVA was:

$$Y_{ij} = \mu + \tau_{ij} + \varepsilon_{ij}$$

where,

Y_{ij} = response from the j^{th} unit receiving i^{th} treatment

μ = general mean effect

τ_{ij} = the effect due to i^{th} treatment

ε_{ij} = the random error.

RESULTS

Chemical Composition of POS and Experimental Diets

The result on the chemical composition, fibre fraction and fatty acid composition of POS is shown in Table 1. The proximate principles (g kg⁻¹ DM) *i.e.* moisture, crude protein (CP), ether extract (EE), crude fibre (CF), nitrogen free extract (NFE) and total ash (TA) in POS was 722.91, 138.70, 101.97, 214.75, 315.03 and 229.55, respectively. The POS contained on an average 72.80 (g kg⁻¹ DM) acid insoluble ash (AIA). The fibre fractions for neutral detergent fibre (NDF) and acid detergent fibre (ADF) was 568.00 and 547.00 (g kg⁻¹ DM), respectively.

The individual fatty acid composition of POS for C10:0, C12:0, C14:0, C14:1, C15:0, C16:0, C16:1, C17:0, C18:0, C18:1 cis, C18:2 cis, C18:3n6, C18:3n3, C20:1 and C24:0 is presented in *Table 1*. The sum of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and ratio of PUFA to SFA in POS was 62.84, 25.78, 10.75 (g per 100 g fatty acid) and 0.17, respectively.

The chemical composition of the experimental diets fed to the growing-finishing pigs during Phase I and Phase II stages were analysed and is presented in *Table 2*. The chemical compositions of the experimental diets were similar in the two phases and between the groups *i.e.* the diets were iso-nitrogenous and iso-caloric.

TABLE 1. PROXIMATE COMPOSITION, FIBRE FRACTION (g kg⁻¹ DM) AND FATTY ACID COMPOSITION (g per 100 g fatty acid) OF PALM OIL SLUDGE (POS)

Attributes	POS
Proximate composition (g kg⁻¹ DM)	
Moisture	722.91
Crude protein	138.70
Ether extract	101.97
Crude fibre	214.75
Nitrogen free extract	315.03
Total ash	229.55
Acid insoluble ash	72.80
Digestible energy (Kcal kg ⁻¹)	3 535.21
Fibre fractions (g kg⁻¹ DM)	
Neutral detergent fibre	568.00
Acid detergent fibre	547.00
Fatty acid composition (g per 100 g fatty acid)	
Capric acid (C10:0)	0.03
Lauric acid (C12:0)	0.08
Myristic acid (C14:0)	0.95
Myristoleic acid (C14:1)	0.35
Pentadecylic acid (C15:0)	0.09
Palmitic acid (C16:0)	53.38
Palmitoleic acid (C16:1)	0.13
Margaric acid (C17:0)	0.12
Stearic acid (C18:0)	8.06
Oleic acid (C18:1 cis)	25.23
Linoleic acid (C18:2 cis)	10.13
Gamma linolenic acid (C18:3n6)	0.40
Alpha linolenic acid (C18:3n3)	0.22
Gondoic acid (C20:1)	0.07
Lignoceric acid (C24:0)	0.13
Total	99.37
Σ SFA	62.84
Σ MUFA	25.78
Σ PUFA	10.75
PUFA:SFA	0.17

Note: POS - palm oil sludge; SFA - saturated fatty acid; MUFA - monounsaturated fatty acid; PUFA - polyunsaturated fatty acid.

Feed and Nutrient Intake

As shown in *Table 3*, the average daily DM intake was comparable throughout the experimental period.

Body weight changes and feed conversion ratio. The results pertaining to the performance of pigs fed increasing levels of POS are shown in *Table 3*. The average weight gain, average daily gain (ADG) and feed conversion ratio (FCR) were similar in all the groups in both Phase-I and Phase-II. Overall average for the above parameters was also similar in all the groups. Among the treatments, the ADG showed a marginal increase and a better FCR in Group-2 and Group-4 during the Phase-I feeding. In the Phase II, lesser ADG and higher FCR was observed in the POS fed animals when compared to the control. Group-3 recorded the lowest ADG and highest FCR in the overall of the experimental period. However, both ADG and FCR remained comparable throughout the experiment, irrespective of treatments.

Haemato-biochemical Profiles

Increasing levels of POS supplementation in the concentrate mixtures (*Table 4*) had no effect on the concentration of haematological parameters like haemoglobin (Hb), white blood cells (WBC), red blood cells (RBC), packed cell volume (PCV), mean corpuscular volume (MCV) and lymphocytes. However, the mean corpuscular haemoglobin (MCH) levels revealed a quadratic ($p < 0.05$) response and a linear ($p < 0.05$) effect in the monocyte levels among the treatment groups. A linear and quadratic increase ($p < 0.01$) in the level of mean corpuscular haemoglobin concentration (MCHC) was observed in Group-4 on the Day 50 of the experiment. Significant difference ($p < 0.05$) was observed in the level of Hb, RBC, MCV, mean corpuscular haemoglobin, lymphocytes and monocytes between two periods *i.e.* Day 0 and Day 50 in 30% POS supplemented group (Group-4). Level of MCV, MCH and lymphocytes were also significantly different ($p < 0.05$) between two periods within control and 10% POS supplemented group.

The results pertaining to serum metabolites are presented in *Table 5*. The concentration of serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), creatinine, urea, glucose, total protein, albumin, globulin, calcium (Ca) and phosphorus (P) in the serum was similar in the entire group throughout the experimental period. Similarly, the concentration of triglyceride, cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL) also showed no variation among the various treatment groups. There were no significant difference in the concentration of blood

TABLE 2. INGREDIENT AND CHEMICAL COMPOSITION (%) OF THE EXPERIMENTAL DIETS

Attributes	Phase I (20-50 kg)				Phase II (50-80 kg)			
	Group-1 (0% POS)	Group-2 (10% POS)	Group-3 (20% POS)	Group-4 (30% POS)	Group-1 (0% POS)	Group-2 (10% POS)	Group-3 (20% POS)	Group-4 (30% POS)
Maize	60	54	48	42	68	61.2	54.4	47.6
Palm oil sludge	-	6	12	18	-	6.8	13.6	20.4
Soyabean meal	19.44	19.44	19.44	19.45	13.97	13.97	14.15	14.14
Wheat bran	12.5	11	10.5	9.8	12	11	9.2	9.1
Palm oil	-	1.5	2	2.7	-	1	2	2.7
Fish meal	6	6	6	6	4	4	4	4
Lysine	0.06	0.06	0.06	0.05	0.03	0.03	0.05	0.06
Mineral mixture	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Salt	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100	100	100	100
Calculated								
Crude protein	18.12	17.99	17.99	17.97	15.57	15.50	15.51	15.48
Digestible energy (Kcal kg ⁻¹)	3 352.06	3 414.03	3 423.20	3 443.30	3 368.31	3 401.58	3 436.58	3 453.65
Estimated								
Crude protein	17.89	17.67	18.16	18.32	15.86	15.66	15.53	15.61
Ether extract	3.71	4.67	4.98	5.67	4.60	5.21	7.70	9.72
Crude fibre	3.12	3.56	4.01	4.67	3.32	5.24	5.92	6.34
Total ash	6.23	6.78	7.34	8.17	7.41	8.45	8.97	10.05
Acid insoluble ash	1.03	1.78	2.17	2.35	1.12	1.67	2.29	2.57
Digestible energy (Kcal kg ⁻¹)	3 445.94	3 413.80	3 405.52	3 384.48	3 380.89	3 420.73	3 346.41	3 255.26

TABLE 3. GROWTH PERFORMANCE OF GROWING-FINISHING PIGS FED PALM OIL SLUDGE (POS) BASED DIETS

Period	Group-1 (0% POS)	Group-2 (10% POS)	Group-3 (20% POS)	Group-4 (30% POS)	SEM	Contrast, p-value	
						Linear	Quadratic
Phase I							
Initial weight (kg)	40.04	40.13	41.44	40.16	1.000	0.860	0.754
Final weight (kg)	48.76	49.72	49.79	49.72	0.910	0.734	0.797
Ave. wt. gain (kg)	8.72	9.59	8.35	9.56	0.363	0.701	0.819
DMI (g/h/d)	2 160.60	2 186.19	1 995.71	2 216.07	52.06	0.959	0.324
ADG (g/h/d)	623.09	684.76	596.57	682.86	25.90	0.701	0.819
FCR	3.47	3.20	4.07	3.29	0.227	0.886	0.592
Phase II							
Initial weight (kg)	48.76	49.72	49.79	49.72	0.910	0.734	0.797
Final weight (kg)	82.80	82.01	81.68	82.98	0.880	0.981	0.586
Ave. wt. gain (kg)	34.04	32.30	32.89	33.26	0.710	0.667	0.308
DMI (g/h/d)	2 612.00	2 627.00	2 564.20	2 659.70	20.73	0.672	0.355
ADG (g/h/d)	607.92	576.79	569.50	593.93	12.68	0.677	0.308
FCR	4.32	4.58	4.56	4.56	0.101	0.464	0.540
Overall average							
Initial weight (kg)	40.04	40.13	41.44	40.16	1.000	0.860	0.754
Final weight (kg)	82.80	82.01	81.68	82.98	0.880	0.981	0.586
Ave. wt. gain (kg)	42.76	41.89	40.24	42.82	0.916	0.862	0.380
DMI (g/h/d)	2 521.70	2 539.50	2 450.50	2 571.00	24.99	0.793	0.320
ADG (g/h/d)	610.95	598.38	574.92	611.72	13.08	0.862	0.380
FCR	4.21	4.45	4.62	4.46	0.139	0.471	0.522

Note: DMI - dry matter intake; ADG - average daily gain; FCR - feed conversion ratio; Ave. wt. - average weight; SEM - standard error of mean.

biochemical parameters between the two periods *i.e.* Day 0 and Day 50 within groups except concentration of ALP, cholesterol, LDL and Ca and concentration of LDL and Ca, which were significantly different ($p < 0.05$) between the period within control group and 30% POS supplemented group, respectively.

Apparent Digestibility of DM and Other Nutrients

The effect of feeding graded levels of POS in the concentrate as partial replacement of maize on nutrient digestibility of the growing-finishing pigs is presented in *Table 6*. Feeding of increasing levels of POS so as to replace 0%, 10%, 20% and 30% of maize led to a linear decrease ($p < 0.01$) in the digestibility coefficient of DM, organic matter,

CP and nitrogen free extract. The digestibility coefficient of all the above components was higher in Group-1 and lowest in Group-4. However, the digestibility coefficient of ether extract and crude fibre was similar among the dietary treatments.

Economics

Feed economics of POS based concentrate fed to growing-finishing pigs is shown in *Table 6*. The cost of feed per weight gain in all the groups was similar. However, the average total feed cost had linearly decreased ($p < 0.01$) in the treatment groups as the level of POS was increased in the concentrate diets.

TABLE 4. EFFECT OF PALM OIL SLUDGE (POS) ON HAEMATOLOGICAL PARAMETERS OF GROWING-FINISHING PIGS

Attributes	Group-1 (0% POS)	Group-2 (10% POS)	Group-3 (20% POS)	Group-4 (30% POS)	SEM	Contrast, p-value	
						Linear	Quadratic
Haemoglobin (g/dl)							
Day 0	12.43	12.88	11.93	11.20	0.392	0.221	0.477
Day 50	12.45	13.03	11.39	13.67	0.320	0.410	0.134
p-value	0.986	0.877	0.504	0.014			
White blood corpuscle (m/mm ³)							
Day 0	15.15	18.94	15.39	16.77	0.600	0.777	0.261
Day 50	16.77	14.44	16.78	17.75	0.711	0.422	0.270
p-value	0.432	0.116	0.521	0.686			
Red blood cells (m/mm ³)							
Day 0	5.90	6.98	6.83	5.87	0.216	0.886	0.020
Day 50	6.35	7.03	5.51	7.24	0.280	0.610	0.302
p-value	0.589	0.922	0.202	0.041			
Packed cell volume (%)							
Day 0	36.20	41.85	39.28	36.13	1.174	0.784	0.073
Day 50	45.03	45.57	37.47	44.37	1.268	0.307	0.158
p-value	0.135	0.324	0.647	0.108			
Mean corpuscular volume (fl)							
Day 0	60.45	60.03	58.68	58.48	0.467	0.103	0.905
Day 50	60.55	61.70	60.40	61.30	0.337	0.763	0.859
p-value	0.025	0.025	0.130	0.004			
Mean corpuscular haemoglobin (pg)							
Day 0	17.80	18.00	17.68	17.68	0.095	0.446	0.624
Day 50	18.88 ^a	18.53 ^{ab}	17.60 ^b	18.83 ^a	0.192	0.647	0.008
p-value	0.109	0.710	0.794	0.006			
Mean corpuscular haemoglobin concentration (g/dl)							
Day 0	30.87	30.70	30.28	30.33	0.188	0.264	0.783
Day 50	29.75 ^a	29.57 ^a	29.47 ^a	30.80 ^b	0.165	0.006	0.003
p-value	0.030	0.028	0.167	0.273			
Lymphocytes (%)							
Day 0	45.90	44.70	44.15	45.35	0.740	0.765	0.471
Day 50	52.68	51.00	50.20	51.77	0.510	0.451	0.135
p-value	0.018	0.033	0.064	0.006			
Monocytes (%)							
Day 0	5.65	5.38	6.30	6.18	0.252	0.304	0.888
Day 50	6.18 ^a	8.07 ^b	7.67 ^b	8.20 ^b	0.261	0.004	0.079
p-value	0.345	0.010	0.217	0.001			

Note: ^{a,b} means with different superscripts in the same row differ significantly ($p < 0.01$); SEM - standard error of mean.

TABLE 5. EFFECT OF PALM OIL SLUDGE (POS) ON BLOOD BIOCHEMICAL PARAMETERS OF GROWING-FINISHING PIGS

Attributes	Group-1 (0% POS)	Group-2 (10% POS)	Group-3 (20% POS)	Group-4 (30% POS)	SEM	Contrast, p-value	
						Linear	Quadratic
SGPT (U/l)							
Day 0	45.82	42.42	40.64	41.72	0.962	0.112	0.246
Day 50	47.47	41.35	44.00	44.76	1.374	0.671	0.244
p-value	0.649	0.741	0.361	0.375			
SGOT (U/l)							
Day 0	46.37	52.34	54.03	52.87	2.177	0.319	0.449
Day 50	50.03	53.88	56.62	56.54	1.733	0.186	0.512
p-value	0.599	0.703	0.465	0.672			
ALP (U/l)							
Day 0	255.87	254.53	255.87	294.94	15.523	0.441	0.555
Day 50	195.29	181.65	143.15	203.85	11.589	0.899	0.119
p-value	0.018	0.274	0.079	0.120			
Creatinine (mg/dl)							
Day 0	1.78	1.56	1.72	1.71	0.079	0.937	0.549
Day 50	1.60	1.47	1.50	1.50	0.045	0.533	0.506
p-value	0.487	0.429	0.441	0.330			
Urea (mg/dl)							
Day 0	14.51	12.14	11.77	12.21	0.548	0.150	0.209
Day 50	14.78	13.78	15.43	12.30	0.633	0.316	0.407
p-value	0.904	0.486	0.077	0.960			
Glucose (mg/dl)							
Day 0	111.83	103.93	125.78	128.86	5.957	0.198	0.654
Day 50	106.89	110.44	90.43	103.83	4.259	0.455	0.571
p-value	0.833	0.520	0.141	0.094			
Total protein (g/dl)							
Day 0	7.04	7.57	7.32	7.81	0.143	0.118	0.950
Day 50	7.56	7.50	7.50	7.39	0.101	0.617	0.921
p-value	0.148	0.778	0.535	0.582			
Albumin (g/dl)							
Day 0	3.09	3.23	2.98	3.33	0.110	0.654	0.658
Day 50	3.50	3.15	3.18	3.19	0.102	0.366	0.413
p-value	0.104	0.882	0.712	0.351			
Globulin (g/dl)							
Day 0	3.96	4.34	4.34	4.49	0.171	0.350	0.745
Day 50	4.07	4.34	4.33	4.20	0.158	0.803	0.574
p-value	0.530	1.00	0.983	0.718			
Triglycerides (mg/dl)							
Day 0	28.68	32.91	32.73	35.00	1.136	0.077	0.659
Day 50	36.67	37.22	36.55	36.31	0.620	0.781	0.780
p-value	0.163	0.030	0.152	0.737			
Cholesterol (mg/dl)							
Day 0	81.68	94.48	87.41	96.68	2.684	0.111	0.726
Day 50	98.68	100.63	97.34	97.01	1.298	0.515	0.686
p-value	0.023	0.248	0.374	0.964			
HDL (mg/dl)							
Day 0	33.25	37.79	31.82	36.31	1.003	0.695	0.991
Day 50	38.13	42.18	32.94	33.23	1.703	0.111	0.557
p-value	0.126	0.542	0.716	0.403			

TABLE 5. EFFECT OF PALM OIL SLUDGE (POS) ON BLOOD BIOCHEMICAL PARAMETERS OF GROWING-FINISHING PIGS (continued)

Attributes	Group-1 (0% POS)	Group-2 (10% POS)	Group-3 (20% POS)	Group-4 (30% POS)	SEM	Contrast, p-value	
						Linear	Quadratic
LDL (mg/dl)							
Day 0	36.17	39.69	38.59	38.37	1.417	0.698	0.553
Day 50	43.55	41.45	47.41	46.78	1.116	0.116	0.728
p-value	0.026	0.473	0.303	0.023			
Calcium (mg/dl)							
Day 0	6.56	8.65	7.05	6.86	0.449	0.061	1.123
Day 50	10.50	8.52	10.44	10.15	0.412	0.809	0.310
p-value	0.019	0.931	0.069	0.015			
Phosphorus (mg/dl)							
Day 0	6.56	8.80	7.05	7.03	0.430	0.933	0.196
Day 50	8.44	7.94	8.02	7.54	0.357	0.349	0.987
p-value	0.300	0.421	0.512	0.525			

Note: SGPT - serum glutamic, pyruvic transaminase; SGOT - serum glutamic oxaloacetic transaminase; ALP - alkaline phosphatase; HDL - high density lipoprotein; LDL - low density lipoprotein; SEM - standard error of mean.

TABLE 6. DIGESTIBILITY COEFFICIENT OF NUTRIENTS AND ECONOMICS OF FEEDING PALM OIL SLUDGE (POS) BASED CONCENTRATE DIETS TO GROWING-FINISHING PIGS

Attributes	Group-1 (0% POS)	Group-2 (10% POS)	Group-3 (20% POS)	Group-4 (30% POS)	SEM	Contrast, p-value	
						Linear	Quadratic
Digestibility coefficients							
DM	84.13 ^b	79.85 ^{ab}	77.27 ^b	71.61 ^a	1.397	< 0.01**	0.695
OM	85.82 ^b	82.28 ^b	79.99 ^{ab}	75.74 ^a	1.153	< 0.01**	0.815
CP	85.39 ^b	84.61 ^b	76.95 ^{ab}	73.89 ^a	1.397	< 0.01**	0.584
EE	72.24	68.49	64.71	69.02	1.266	0.229	0.115
CF	59.99	65.88	65.59	67.50	1.462	0.105	0.496
NFE	88.21 ^b	84.12 ^{ab}	84.06 ^{ab}	78.26 ^a	1.166	< 0.01**	0.607
Economics							
Av. total feed intake (kg)	176.52	177.76	171.54	179.97	1.749	0.793	0.320
Av. total feed cost (Rs)	5 052.80 ^a	4 911.40 ^{ab}	4 561.80 ^b	4 588.10 ^b	63.55	< 0.01**	0.402
Av. total weight gain (kg)	40.04	40.13	41.44	40.16	1.000	0.860	0.754
Cost of feed/ kg weight gain (Rs)	118.28	117.66	116.17	108.69	2.799	0.247	0.559

Note: DM - dry matter; OM - organic matter; CP - crude protein; EE - ether extract; CF - crude fibre; NFE - nitrogen free extract; Av. - average; a,b means with different superscripts in the same row differ significantly (**p<0.01); SEM - standard error of mean.

DISCUSSION

Chemical Composition of POS and Experimental Feeds

The CP values obtained in the present study were similar to the observation made by Habib *et al.* (1997), Wong and Wan Zahari (1992) and Kekiepeu (2017). They found the CP content in POS was 12.75%, 12.50% and 14.30% respectively. However, the CP value in the present study contradicts with Devendra (1977); Bobadoye *et al.*

(2008) and Faradonbeh *et al.* (2011) who reported lower values in their works, which could be due to the processing methods of oil extraction. The ether extract content of POS in our study was similar to the values reported by Habib *et al.* (1997) and Kekiepeu (2017) but lesser than the values observed by Devendra (1977); Bobadoye *et al.* (2008) and Faradonbeh *et al.* (2011). The CF value was similar to the findings of Wong and Wan Zahari (1992) but higher than the values reported by Bobadoye *et al.* (2008) and Faradonbeh *et al.* (2011). The NFE value was similar to the values reported by Habib *et al.*

(1997); *et al.* (2008) and Faradonbeh *et al.* (2011). In the present study, the total ash in POS was similar to the findings of Wong and Wan Zahari (1992) and Kekiepeu (2017) but lesser than the observation made by Earp and Newal (1976); Abu *et al.* (1984) and Wan Zahari and Alimon (2003). The AIA content was found to be high (72.80 g kg⁻¹ DM) in POS. POS is the decanted form of POME, which consists of liquids, dirt, residual oil and suspended solids, mainly cellulosic material from the mesocarp of the fruits. Hence, the high ash content of the sludge may be due to the contamination by the soil or suspended solids from the mesocarp. The NDF and ADF values in this study were lower than the values reported by Bamikole and Babayemi (2008). The wide variability in the chemical composition of POS is due to the oil palm varieties (Bamikole and Ikatua, 2009) and the method of oil extraction (Seephueak *et al.*, 2011).

The major long chain fatty acids in POS were myristic, palmitic, stearic, oleic and linoleic acid. The palmitic acid (g per 100 g fatty acid) content was highest in POS (53.38 g). The SFA was found to be higher in comparison to MUFA and PUFA in POS. In this study, palmitic, oleic and linoleic acid were found to be higher than the findings of Habib *et al.* (1997) where the corresponding values of 22.45%, 15.54% and 9.53% was reported in raw POME. Myristic and stearic was higher in our study compared to their observation. Abubakr *et al.* (2015) reported that palmitic, stearic, oleic and linoleic acid in palm oil decanter cake were found to be 49.64%, 3.76%, 35.40% and 7.34%, respectively, where stearic acid level was comparable but oleic acid level was lower than the present study. Moreover, C18:0 (8.06 g) and C18:2cis (10.13 g) content was higher than the values observed in our study.

As shown in Table 2, the experimental pigs were fed in two Phases (I and II). The Phase I (20-50 kg) and Phase II (50-80 kg) diets for all the experimental groups was iso-nitrogenous and iso-caloric. However, the EE, CF and TA content increased with increasing level of POS in the diets of both the phases. This may be attributed to higher CF and ash content of POS and the incorporation of palm oil in the concentrate diets. Above all, the concentrate diets in Phase I and II feeding adhered with the specification of NRC 1998.

Feed and Nutrient Intake

There was no significant difference in DMI (g hr⁻¹ day⁻¹) in the whole experimental period. Thus, the performance of the pigs was not adversely affected by inclusion of graded levels of POS as a replacement of maize in both the phase of feeding (Phase I and II). However, DMI (g hr⁻¹ day⁻¹) partially tended to increase in the treatment groups except Group-3 which could be due to reduced dustiness and enhanced palatability of POS. The intake was

optimum in all the treatment groups which indicated that the experimental pigs had adapted to the POS based diets. Our finding were similar to the findings of Abu *et al.* (1984) who observed no changes in the feed intake of pigs when oil palm slurry was fed as a partial replacement for maize. Similarly, Fanimo and Fashina-Bombata (1998) also reported similar feed intake in weaner pigs fed iso-nitrogenous diets containing various levels (0%, 10%, 15%, 20% and 25%) of POS.

Body Weight Changes and Feed Conversion Ratio

The replacement of maize with POS had no effect on the body weight gain, ADG and FCR in both the feeding phases. Regardless of the inclusion levels of POS, the performance was not impaired and thus further establishes its use in concentrate diet as an energy source for growing-finishing pigs. This is due to enhanced palatability and the addition of crude palm oil had reduced the dustiness of the feed in the study. The weight gain and feed efficiency in the present observation is in concurrence with the finding of Abu *et al.* (1984) and Enemona (2008) who reported non-significant difference in pigs when maize was replaced up to 30% by POS in the basal diets. Similarly, feeding of palm kernel cake, another by-product of oil palm to pigs (up to 59.05 kg) at the level of 50% and 100% to replace maize did not influence the performance of the pigs (Adesehinwa, 2007).

Blood Profiles

The values were within the normal range for pigs described by Latimer *et al.* (2003) for MCH and Radostits *et al.* (2000) for MCHC and monocyte. There was no anaemic and immunosuppressive pigs in the herd during the whole experimental period and the reason for this may be due to individual variation within the groups. In contrast, Kekiepeu (2017) reported no variation ($p>0.05$) in the MCH, MCHC and monocyte concentrations in poultry when maize was replaced at various levels (0%, 5%, 10% and 15%) with POS in the concentrate diets.

All the serum biochemical parameters were within the physiological range for pigs. The present observation is in agreement with the findings of Kekiepeu (2017) who reported non-significant ($p>0.05$) difference in serum metabolites (SGPT, SGOT, total protein and glucose) of broiler poultry fed POS at 5%, 10% and 15% levels replacing maize. On the contrary, Onibi *et al.* (2011) observed that the serum cholesterol levels increased significantly ($p<0.05$) in chickens with increasing levels of POS in diets and further suggested that POS inclusion in the diets could lead to hypercholesterolaemic tendency in birds.

Apparent Digestibility of Nutrients

The digestibility indices decreased with the increase of POS level in all the test diets and the apparent digestibility of components were higher in Group-1 and lower in Group-4. The reason could be due to high content of ash and CF in the test ingredient. This is further supported by the fact that presence of CF affect the CP digestibility (Sauer *et al.*, 1980) and the excretion of fecal nitrogen compared to urinary nitrogen is increased when the total dietary fibre level is increased (Bindelle *et al.*, 2009). However, several reports suggest that when the source of fibre does not contribute significant amounts of protein to the diet, then an increase in the level of fibre to some extent does not affect protein digestibility significantly (Gouwens, 1966; Friend, 1970; Eggum, 1973; Kennelly and Aherne, 1980). Our findings are in conformity with Fanimo and Fashina-Bombata (1998) who observed decreased CP digestibility in the diet of weaner pigs fed POS based concentrate diets. The digestibility coefficient of EE and CF was found to be non-significant ($p>0.05$) among the treatments. The higher digestibility CF could be due to high intact or extracted fat (Kil *et al.*, 2010) for EE and more of soluble dietary fibre for CF in the diets. In our study, fibre rich POS was used with high energy feed ingredients *i.e.*, crude palm oil to maintain the DE content in the treatment diets. Dietary fat might have increased the retention time of digesta, which prolonged the digesta fermentation by the gut microbiota (Wilfart *et al.*, 2007). The finding is in agreement with Adesehinwa (2007) who reported that there was no significant difference ($p>0.05$) in apparent digestibility of crude fibre in growing pigs at 50% and 100% replacement of maize with palm kernel cake.

Economics

The feed cost was higher in Group-1 and lower in Group-4. This shows that it was far more economical to use POS to replace maize in the pig diets as it cost little when compared to maize. Although, there was no significant ($p>0.05$) difference in the feed cost per kg weight gain, the least cost per kg weight gain was observed for pigs on 30% replacement of maize by POS. Economically, incorporation of POS up to 30% level replacing maize was found to be cost effective and can be used as a feed for growing-finishing pigs in those areas where POS is readily available and affordable. Similar finding was observed by Hertrampf (1988) and Enamona (2008) in pigs. Likewise, Adesehinwa (2007) reported that feeding of palm kernel cake (PKC), which is also a by-product of oil palm revealed significantly ($p<0.05$) lower feed cost as the level of PKC inclusion increase. There was no

significant ($p>0.05$) difference in the feed cost per live weight gain between the various treatment groups. In contrast, Ezewke *et al.* (2011) reported that feed cost per kg gain was lower and better for pigs fed diets containing 15% and 30% POS diets in growing pigs.

CONCLUSION

On the basis of the above results, it can be concluded that feeding of POS as partial replacement of maize up to 30% in the basal diet had no adverse affect on the performance and the haemato-biochemical parameters of the growing finishing pigs. Hence, POS can be used as a substitute for costly feed ingredients like maize in the diet of pigs where it is easily available to minimise the cost of production. However, research can be carried out on supplementation of enzymes in POS based diets to further improve the digestibility of nutrients.

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PHASE BEHAVIOUR OF HIGH, MEDIUM AND LOW MELTING FRACTIONS IN LAURIC FAT BLENDS FORMULATED BASED ON MELTING TRIACYLGLYCEROL GROUP

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ABSTRACT

Lauric fat blends aimed for bakery shortening were prepared as a ternary mixture characterised by three distinct melting peaks on their differential scanning calorimeter (DSC) melting thermograms. Those melting peaks closely correlated to the high, medium, and low melting fractions (HMF, MMF and LMF) in the blends. In the simpler binary mixture, hard fats, (i.e. palm stearin and fully hydrogenated rapeseed oil) formed an ideal solubility in different liquid oils [i.e. medium chain triacylglycerol oil (MCT-oil) and high oleic sunflower oil (HOSO)]. However, slight deviations from ideality were observed when liquid oils were replaced by lauric oils (i.e. palm kernel oil and coconut oil). In the more complex ternary mixture, the HMF formed an ideal solubility in both the MMF and LMF within the range of given formulas. In addition, the interaction of solid crystals in lauric fat blends was of monotectic behaviour as shown in the isosolid diagram. Nevertheless, the influence of solubility seemed to override that of solid interaction. Both the solubility curves and isosolid lines can provide valuable information about the interaction of HMF, MMF and LMF in lauric fat blends. Therefore, they are useful for improving the formulation of plastic fats.

Keywords: fat blend, isosolid, lauric, phase behaviour, solubility, tag group.

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INTRODUCTION

Lauric fat blends in this research are a ternary mixture of a lauric oil [i.e. palm kernel oil (PKO) or coconut oil (CO)] with two other vegetable oils and fats prepared for the production of plastic fats (e.g. shortening, margarine and fat spread). Lauric fat blends can be formulated based on melting triacylglycerol (TAG) group (Nusantoro *et al.*, 2017),

enabling some adjustments on the solid fat content (SFC) at temperature intervals particularly from 0°C-20°C and above 20°C (Nusantoro *et al.*, 2013) to have the required SFC profiles for bakery shortening production. Lauric oils are mainly composed of fully saturated medium-chain fatty acids. The TAG derived from this type of fatty acids serve as the main source of medium melting TAG. These medium melting components play an important role in developing the key functionalities of plastic fats such as structure, mechanical performance and oral properties (Chai *et al.*, 2018; Liu *et al.*, 2018).

In order to get good plasticity, both solid and liquid phases should be present in a proper proportion in the fat blends (Nusantoro *et al.*, 2016; Yanty *et al.*, 2017). Liquid oils comprise entirely of low melting components and contribute to the

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major part of the blends as the liquid phase. In addition, some amount of hard fats is added as they are required in the blends to provide high melting components that give desired appearance, texture, and mouthfeel properties (O'Brien, 2009). Medium chain triacylglycerol oil (MCT-oil) and high oleic sunflower oil (HOSO) are examples of the liquid oils while palm stearin (PS) and fully hydrogenated rapeseed oil (FHRO) are those of the hard fats. Fat and oil sources for the fat blends can thus, come naturally or from fat modification.

The study of the phase behaviour of fat blends can lead to a better understanding of the interactions, occurring among the blend constituents and the various phase changes of their physical state. The knowledge of phase behaviour is of particular importance in the prediction of fat and fat blend properties, control of physical attributes, process optimisations and eventually product quality maintenance (Timms, 1984; Saberi *et al.*, 2011). The formulated lauric fat blends have three distinct fractions differentiated by the melting point of their TAG compositions. The mixing behaviour of higher melting fractions in lower melting fractions directly relates to crystallisation behaviour of the fat blends. Moreover, there is a softening effect of LMF due to the dissolution of high-melting TAG (solute) into low-melting TAG (solvent). A solubility curve of fat blends can then be constructed to show equilibrium relationships between the temperature and the composition of solid fat in liquid oil (Zhou and Hartel, 2006).

When lower melting components do not involve in crystallisation, fat blends form an ideal solution and the solubility of high-melting TAG in low melting TAG, which follows the Hildebrand equation (Equation 1).

$$\ln x_s = \frac{\Delta H_f}{R} \left(\frac{1}{T_m} - \frac{1}{T} \right) \quad \text{Equation (1)}$$

where x_s is the solubility of high-melting component (mole mole⁻¹), ΔH_f is the heat of fusion of high-melting component (J mol⁻¹), R is the universal gas constant (8.314 J mol⁻¹ K⁻¹), and T_m and T are melting temperatures of the high-melting component and the mixture (K), respectively. Although there are various methods to determine the melting temperature, the most preferred and widely used method is by using the differential scanning calorimeter (DSC). In the occurrence of some interactions among fat constituents, deviations from the ideal solution behaviour can be seen in a solubility curve (Walstra, 2003).

Lauric fat blends are a multicomponent system of TAG mixtures. In simpler binary TAG mixtures, phase behaviour of the blends can be either monotectic, eutectic or peritectic depending on their molecular interaction. Moreover, physical

classification of TAG can be utilised as a method to reduce the complexity of TAG mixtures. TAG species are sorted into TAG categories that are supposed to form distinct separate solid fractions in the crystallised fat blend. In lauric fat blends, classification of TAG is based on the melting point of which four categories are formed namely heated (H), body (B), room (R) and cool (C) melting TAG groups. These groups represent the high, medium and low melting components in the fat blends (Wesdorp *et al.*, 2005; O'Brien, 2009). Eventually, an isosolid diagram can then be constructed to investigate the type of solid solution behaviour of lauric fat blends. This pseudo-phase diagram shows lines of constant SFC at different temperatures (vertical axis) and composition (horizontal axis). For example, the isosolid line is commonly found to be a straight line parallel to the horizontal axis or to one of the lateral axes in the monotectic mixture. A eutectic mixture, on the other hand, exhibits a compression of the isosolid line which shows a constant SFC and a melting temperature below that of the individual fat. In the eutectic system, a considerable decrease of the SFC followed by softening of the fat blend is commonly observed (Timms, 1984; Zhou *et al.*, 2010).

In this study, the complex interaction of high, medium and low melting fractions (HMF, MMF and LMF) in lauric fat blends was investigated. The solubility of hard fats in either lauric or liquid oils was firstly observed by solubility diagram in a simpler system as binary mixtures. Then, the application of Hildebrand equation was extended to evaluate the solubility of both HMF in lower melting components and HMF/MMF in LMF as a ternary mixture of lauric fat blends. Accordingly, the isosolid diagram was constructed for lauric fat blend at different formulations. The melting group TAG formulation was employed to give different ratios of HMF to MMF as isosolid lines which can evaluate such interaction of both fractions. Any changes and deviations from ideality of both the solubility curves and isosolid lines were indicated in order to provide useful guidance for developing plastic fat products.

MATERIALS AND METHOD

Materials

All vegetable oils and fats are commercial products as follows: Neobee M5 as the MCT-oil (Stepan, USA), HOSO (Oleon, Belgium), PS and PKO (Loders Crocklaan, The Netherlands), coconut oil (Palko Sari Eka, Indonesia) and fully hydrogenated rapeseed oil (Palsgaard, Denmark). All other chemicals and solvents for analysis are those of the high purity grade.

The Binary Mixtures and Lauric Fat Blends

In the first part of the investigation, six binary blends were prepared from two types of hard fats (PS and FHRO) and three types of either lauric or liquid oils (PKO, HOSO and MCT-oil) at different molar ratios of the high melting fraction from 1 to 0 with an interval of 0.1 mole mole⁻¹. This simpler binary model of fat blends was used to study the ideal solubility of hard fats in lower melting components. The outcomes were then utilised to study the pseudo-phase behaviour of more complex ternary mixture of lauric fat blends.

Lauric fat blends were formulated based on the grouping of various TAG species according to their melting points (mp). The TAG were grouped into four namely chilled (C) (mp ≤ 5°C), room (R) (5°C < mp < 25°C), body (B) (25°C < mp < 45°C) and heated (H) (45°C ≤ mp) melting TAG groups. For example, a formulation of BH50H15 indicated that the sample consisted of 50% TAG from a combination of B and H groups and 15% TAG from H group (thus, B group had 35% TAG). The other 50% of TAG in the formulation then came from a combination of C and R groups (Nusantoro *et al.*, 2017).

In the second part of the study, two formulations of lauric fat blends were chosen. First, lauric fat blends were prepared from mixtures of FHRO, CO, and HOSO. The blends were formulated at constant BH50 and varied H from 2.5%-17.5% (seven blends, BH50 - H: 2.5/5/7.5/10/12.5/15/17.5). Second, lauric fat blends were prepared from FHRO, CO, and HOSO. The blends were formulated at constant H15 and varied BH from 50%-70% (five blends, H15 - BH: 50/55/60/65/70). Hildebrand plots and isosolid diagrams were then constructed from these formulated blends.

Fatty Acid Composition

The fatty acid composition of the samples was determined by gas chromatography (GC) after conversion to fatty acid methyl esters (FAME) using 2 N KOH in methanol solution. The FAME were analysed by a Thermo Focus GC (Interscience, Belgium) equipped with an RTX-2330 column (cyanopropyl polysiloxane, 60 m length, 0.25 mm internal diameter, 0.2 µm layer thickness) and a flame ionisation detector (FID). FAME were identified by comparing their retention time to authentic standards. Fatty acid levels were reported as relative proportions of the total composition.

TAG Distribution

The TAG distribution based on the equal carbon number was determined by non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC). The chromatographic system

consisted of a Shimadzu HPLC system (Shimadzu, Japan) fitted with a 150 x 3.0 mm Alltima HP C18-HL column (Grace Alltech, Belgium), and the detection was done by an Alltech 3300 ELSD (Grace Alltech, Belgium). An optimised method as previously described (Rombaut *et al.*, 2009) was used for the measurement. Elution was carried out using an acetonitrile/dichloromethane gradient at 0.72 ml min⁻¹. The result was processed by LC solution software (Shimadzu, Japan).

Melting Behaviour by DSC

Thermal analyses of the samples were performed on a TA Q1000 DSC (TA Instruments, USA) equipped with a refrigerated cooling system. Nitrogen was used to purge the system. The samples (5-15 mg) were hermetically sealed in aluminium pans. An empty pan was used as a reference during the measurements. The time-temperature program of the measurements was as follows: holding at 90°C for 10 min, cooling at a rate of 5°C min⁻¹ to a temperature of -70°C, holding for 10 min at that temperature to allow complete crystallisation, and then heating at a rate of 5°C min⁻¹ to 90°C. The obtained data were processed by the TA Universal Analysis 2000 software (TA Instruments, USA).

SFC by pNMR

SFC was measured according to the official IUPAC method by a Maran Ultra NMR (Oxford Instruments, United Kingdom) equipped with R4 cryostat cooler (Grant Instruments, United Kingdom). NMR tubes (Bruker, Germany) were used for the direct SFC measurement. The samples were prepared by the stabilising fat procedure where a tempering stage was included at 26°C for 40 hr and subsequently at 0°C for 90 min. The SFC values were then recorded at 5°C intervals from 0°C-60°C. Before each reading, the samples were conditioned for 60 min at that chosen measurement temperature.

Determination of Solubility and Isosolid Diagrams

The solubility of the fat blends was depicted by solubility curves showing the equilibrium relationship between temperature and composition of higher melting components in lower melting components. The Hildebrand equation (Equation 1) was used in the calculation to construct the solubility diagram of which the melting point of samples was measured by DSC. Meanwhile, the SFC data (by p-NMR measurement) was used to construct the isosolid diagram following a procedure as previously reported (Timms, 1979). The solid interaction of the fat blends is then described by isosolid lines that connect points with a common SFC value.

Texture Analysis

Texture analysis was carried out using a universal testing machine Instron 5942 (Instron, USA). A constant speed penetration was used to measure the samples as previously described (Braipson-Danthine and Deroanne, 2004). Prior to analysis, approx. A 50 g lauric fat blends were completely melted at 90°C for 10 min and then stored in a thermostatic cabinet at 15±1°C for 24 hr. Four penetration tests were run on each fat blend and the data were processed by the Bluehill 3 software (Instron).

RESULTS AND DISCUSSION

Chemical Composition of Starting Oils and Fats

The fatty acid composition of starting oils and fats is presented in *Table 1*. Lauric oils, such as coconut oil and palm kernel oil, characterised by a high content of medium-chain saturated fatty acids namely lauric (48.90%-50.49%) and myristic (15.46%-17.70%) acids. High oleic sunflower oil is composed mainly of long-chain monounsaturated fatty acid, namely oleic acid (85.13%). Meanwhile, palm stearin and fully hydrogenated rapeseed oil consisted of long-chain saturated fatty acids with palmitic (82.19%) and stearic (89.77%) acids as their major constituents, respectively. The TAG distribution of starting oils and fats was also found to be close to the expected proportions of the relevant fatty acids as indicated in *Table 2*. When TAG were sorted based on their melting points, a handful of variables with easier physical interpretation can be obtained. It was shown that high, medium, and low melting fractions were closely related to the categorisation of TAG in the fat blends without neglecting the nature of starting oils and fats. The LMF was associated with both C and R groups while the MMF and HMF were associated with B and H groups, respectively.

Solubility of the Simpler Binary System

Melting properties of hard fats in the binary mixtures. A pure hard-melting fat which is composed of only a few TAG species usually melts within a very narrow temperature range. Moreover, the hard fat with longer fatty acid chain-length has a higher melting point and molar enthalpy of fusion compared to the one with shorter fatty acid chain length (Zhou and Hartel, 2006). *Figures 1 and 2* showed the DSC melting thermograms of binary mixtures of hard fats (PS or FHRO) in medium melting fat (PKO) or low melting oils (HOSO or MCT-oil) at different molar ratios of the high melting fraction. Palm stearin melted completely at a lower temperature compared to FHRO in different types of solvents (PKO, HOSO, or MCT-oil) at all molar ratios. The softening effect of the solvents was also observed on which the melting point of the hard fats was depressed followed their molar ratios in the binary mixtures. For example, the peak maximum of PS and FHRO in HOSO was decreased from 61.08°C and 68.26°C to 48.93°C and 57.42°C, at 0.7 and 0.1 molar of high melting fraction, respectively. This depressed melting point of hard fats plays an important role in the formulation of plastic fats as their high melting components can result in unwanted pasty and waxy mouthfeel. The HMF is known to have a tendency to adhere to palate, teeth and tongue (Drewnowski and Almiron-Roig, 2010; Podchong *et al.*, 2020). Higher SFC of fat blends is commonly achieved by the addition of hard fats but higher molar ratios of the high melting fraction can significantly increase the melting point of the products. Therefore, the amount of hard fat should be carefully calculated to avoid undesired textural and sensorial properties, yet still giving appropriate functional attributes to the system such as structure, stabiliser, and moisture barrier (O'Brien, 2009; Danthine, 2012; Nisantoro *et al.*, 2016).

TABLE 1. FATTY ACID COMPOSITION OF STARTING OILS AND FATS

Fatty acid	Samples				
	HOSO (%)	CO (%)	PKO (%)	PS (%)	FHRO (%)
Caprylic (C8:0)	-	8.59	4.07	-	-
Capric (C10:0)	-	6.62	4.06	-	-
Lauric (C12:0)	-	48.90	50.49	-	-
Myristic (C14:0)	-	17.70	15.46	1.31	-
Palmitic (C16:0)	3.95	8.26	7.81	82.19	6.97
Stearic (C18:0)	3.17	2.44	2.13	4.73	89.77
Oleic (C18:1)	85.13	5.51	13.75	9.57	0.40
Linoleic (C18:2)	7.75	1.27	2.23	1.92	-
Arachidic (C20:0)	-	-	-	0.28	1.96
Behenic (C22:0)	-	-	-	-	0.90

Note: HOSO - high oleic sunflower oil; CO - coconut oil; PKO - palm kernel oil; PS - palm stearin; FHRO - fully hydrogenated rapeseed oil. Each value in the table represents the means of triplicate.

TABLE 2. TRIACYLGLYCEROL (TAG) DISTRIBUTION OF STARTING OILS AND FATS

Group	TAG*	Samples				
		HOSO (%)	CO (%)	PKO (%)	PS (%)	FHRO (%)
Chilled	OLL	0.54	-	-	-	-
Chilled	OOL	2.55	-	-	-	-
Chilled	OOO	87.99	-	-	1.15	-
Chilled	PLO	-	-	-	0.27	-
Room	CaCC	-	0.24	-	-	-
Room	CaCLa	-	2.22	-	-	-
Room	LaOO	-	0.43	1.37	-	-
Room	POO	5.24	-	-	1.50	-
Room	LaLaO	-	0.71	3.12	-	-
Room	SOO	3.00	-	-	0.43	-
Room	LaMO	-	0.60	2.78	-	-
Room	MLP	0.68	-	-	-	-
Room	PPL	-	-	-	0.50	-
Body	CCLa	-	17.20	7.70	-	-
Body	MPO	-	-	0.44	-	-
Body	LaPO	-	-	2.25	-	-
Body	CLaLa	-	22.41	9.78	-	-
Body	POP	-	-	-	9.38	-
Body	LaLaLa	-	25.18	37.34	-	-
Body	LaLaM	-	17.44	21.91	-	-
Body	POS	-	-	-	6.37	-
Body	LaMM	-	8.98	8.91	-	-
Heated	LaMP	-	3.67	2.68	-	-
Heated	LaPP	-	0.93	0.93	-	-
Heated	PPP	-	-	-	80.41	-
Heated	SSP	-	-	-	-	18.12
Heated	SSS	-	-	-	-	81.87
[chilled]	(Total)	91.08	-	-	1.42	-
[room]		8.92	4.20	7.27	2.42	-
[body]		-	91.21	88.63	15.75	-
[heated]		-	4.59	3.61	80.41	99.99

Note: HOSO - high oleic sunflower oil; CO - coconut oil; PKO - palm kernel oil; PS - palm stearin; FHRO - fully hydrogenated rapeseed oil. Each value in the table represents the means of triplicate.

*Arranged fatty acid Ca - caprylic; C - capric; M - myristic; La - lauric; P - palmitic; S - stearic; O - oleic and L - linoleic.

In an ideal solubility mixture, the type of solvents (*i.e.* medium melting fat and liquid oils) does not give any different influence as long as those solvents do not interact with the high melting fraction during solidification. In this investigation, however, the melting thermogram showed some deviations on the onset of both binary mixtures at melting peaks of the high melting fraction. When the interval of melting peaks between the solute and the solvent was getting narrow such as in PKO mixtures (Figures 1c and 2c), the deviations of the onset on the baseline were getting apparent. Some exothermic peaks, albeit few and small, were also observed on those deviated baselines. The presence of exothermic peak on the melting thermogram might indicate recrystallisation or polymorphic changes of the fat crystals. When molecular volume, shape

and polymorph of the components in the mixture differ considerably yet those components have a comparable melting point, the eutectic system tends to take place (Timms, 1984).

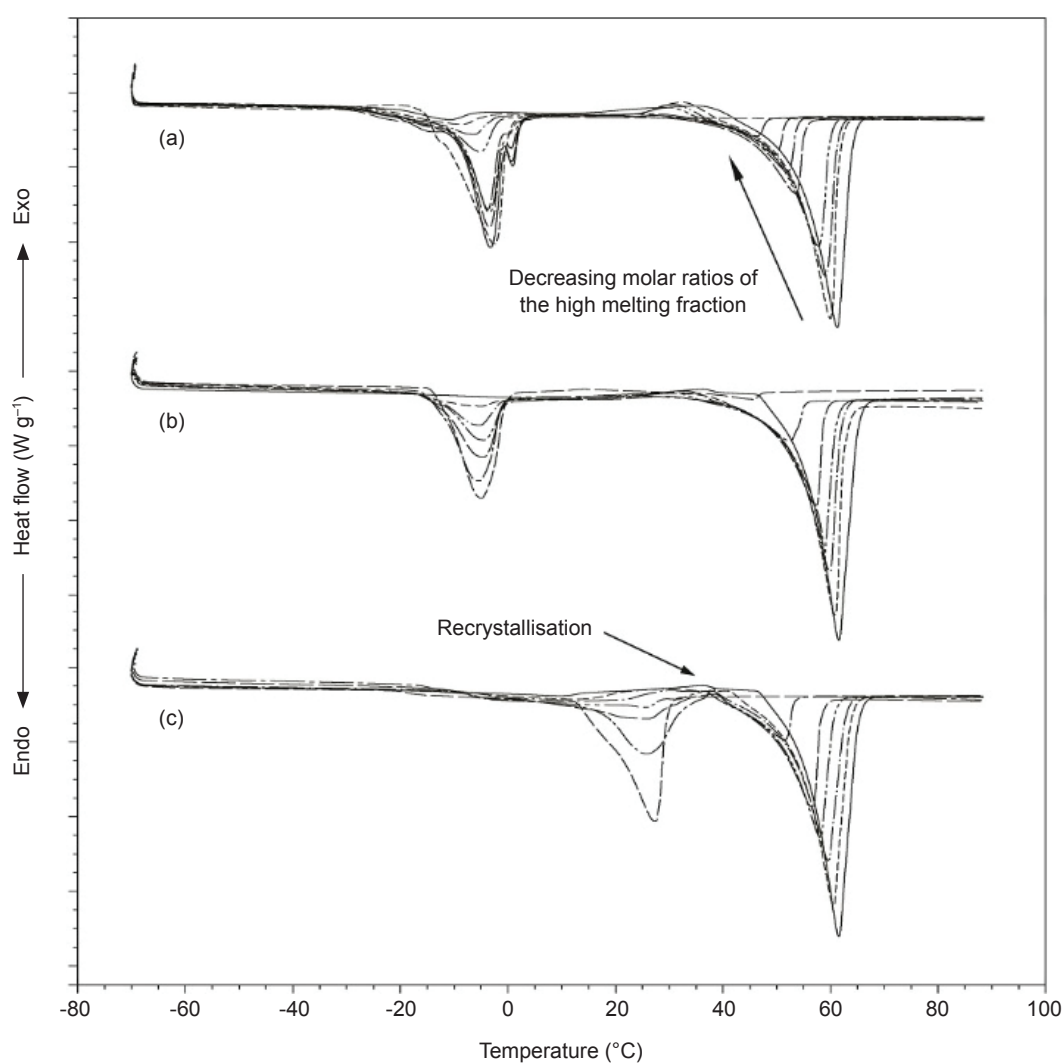
Meanwhile, the shape of melting peaks of the high melting fractions at different molar ratios was generally comparable for both PS and FHRO binary mixtures. Lower molar ratios of hard fats gave smaller peaks with shifts of peak maximum to the lower temperatures. Table 3 showed that the type of solvents weakly influenced the experimental melting point (T_m) of the hard fats. PS had lower experimental T_m than FHRO at the interval temperature range from 63.12°C-63.68°C and from 70.12°C-70.30°C respectively, as influenced by the different type of solvents. Those values were comparable and in agreement with the melting point of pure systems.

TABLE 3. THE EXPERIMENTAL MELTING TEMPERATURE (T_m) AND HEAT OF FUSION (ΔH_f) OF HARD FATS IN DIFFERENT LOWER MELTING OILS

Fat blend	Melting properties	
	Melting temperature ($^{\circ}\text{C}$)	Heat of fusion (kJ mol^{-1})
PS in MCT-oil	63.12 ± 0.07	155.80 ± 1.87
PS in HOSO	63.30 ± 0.00	145.41 ± 0.03
PS in PKO	63.68 ± 0.13	115.44 ± 2.81
FHRO in MCT-oil	70.13 ± 0.03	187.91 ± 1.26
FHRO in HOSO	70.12 ± 0.05	174.51 ± 1.49
FHRO in PKO	70.30 ± 0.02	146.09 ± 0.71

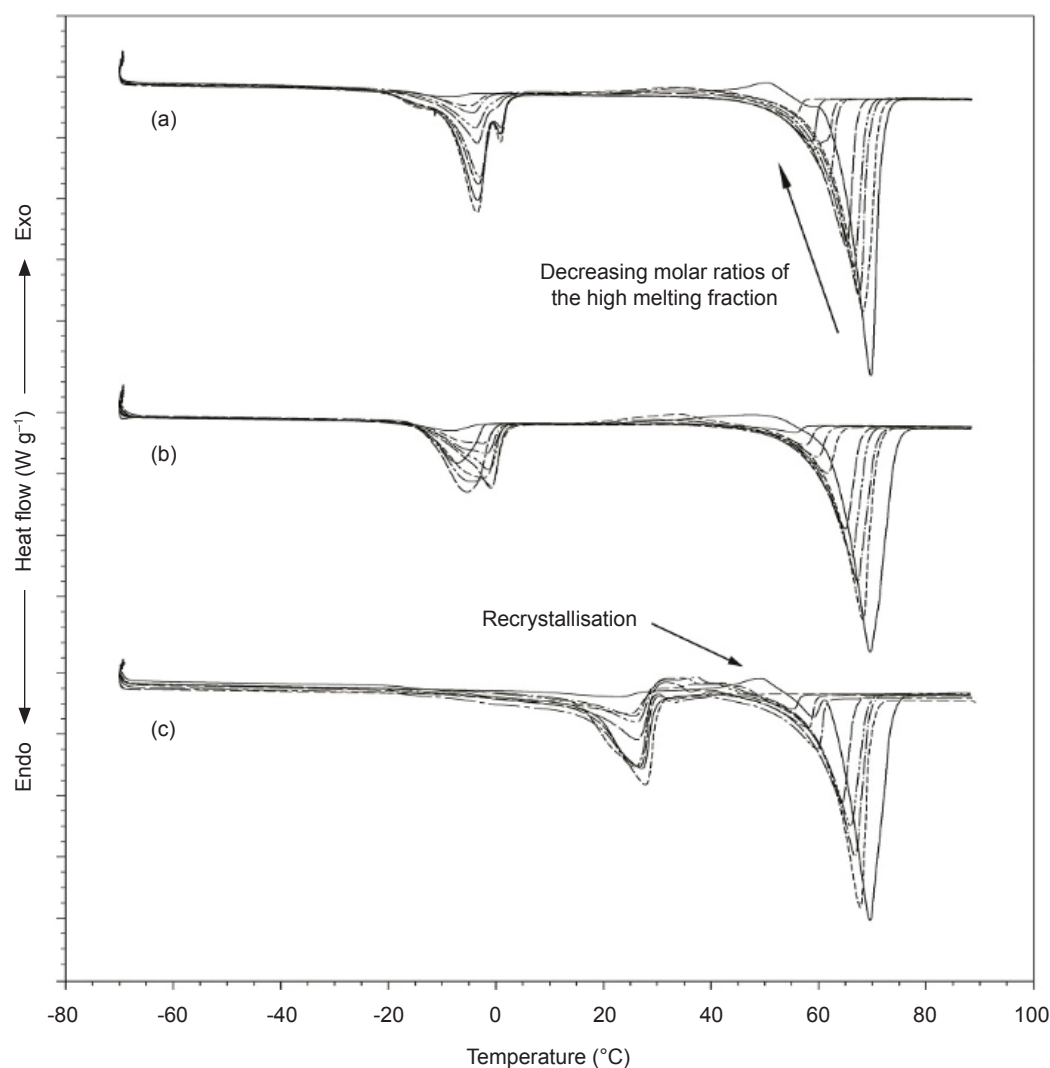
Note: MCT-oil - medium chain triglycerides oil; HOSO - high oleic sunflower oil; PKO - palm kernel oil; PS - palm stearin; FHRO - fully hydrogenated rapeseed oil.

Hildebrand equation is used in the calculation using data from differential scanning calorimeter (DSC) measurements. Each value in the table represents the means of triplicate.



Note: DSC - differential scanning calorimeter; PS - palm stearin; MCT-oil - medium chain triacylglycerol oil; HOSO - high oleic sunflower oil; PKO - palm kernel oil.

Figure 1. DSC-melting behaviour of binary mixtures of PS in (a) MCT-oil, (b) HOSO, and (c) PKO.



Note: DSC - differential scanning calorimeter; FHRO - fully hydrogenated rapeseed oil; MCT-oil - medium chain triacylglycerol oil; HOSO - high oleic sunflower oil; PKO - palm kernel oil.

Figure 2. DSC-melting behaviour of binary mixtures of FHRO in (a) MCT-oil, (b) HOSO, and (c) PKO.

On the other hand, the experimental heat of fusion (ΔH_f) of the hard fats was altered considerably depending on the type of solvents used in the binary mixtures. The liquid oils composed only of low melting TAG (*i.e.* MCT-oil and HOSO) slightly affected and resulted in comparable values of the experimental heat of fusion. The experimental ΔH_f of PS in MCT-oil and HOSO were 155.80 and 145.41 kJ mol⁻¹, respectively while those of FHRO were 187.91 and 174.51 kJ mol⁻¹, respectively. Nevertheless, PKO as the solvent in the binary mixtures caused considerably lower values of the experimental heat of fusions. The experimental ΔH_f of PS and FHRO with PKO as the solvent were 115.44 and 146.09 kJ mol⁻¹, respectively. These lower values of experimental ΔH_f also coincided with stronger deviations on the onset of melting peaks of the hard fats. It should be taken into account that PKO contains some amount of high melting TAG such as 1-lauroyl-2-myristoyl-3-palmitoyl-*sn*-glycerol (LaMP) and 1-lauroyl-2,3-

dipalmitoyl-*sn*-glycerol LaPP (Table 2). These TAG could solidify together with the HMF of PS or FHRO thus modified its HMF composition, especially at lower molar ratios of the hard fats (meaning higher ratios of PKO, thus, more LaMP and LaPP in the blends). As a result, different crystallisation behaviour and compensation of smaller ΔH_f of LaMP/LaPP brought the experimental ΔH_f of PS and FHRO lower.

In general, even though there were some indications of deviations from ideal solubility, the experimental T_m values were still comparable and in agreement with the values of pure systems. Meanwhile, the values of experimental ΔH_f were also found to be comparable to those of pure systems when the solvents composed only of low melting TAG (*e.g.* MCT-oil and HOSO). As a comparison, the melting point and heat of fusion of tripalmitin (purity >85%) are 63.4°C and 150.5 kJ mol⁻¹, respectively (Zhou and Hartel, 2006).

Solubility of hard fats in the binary mixtures. The texture, mouthfeel, and melting point of plastic fats are influenced by the solubility of high-melting components in LMF of the fat blends. The melting and dissolution processes are characterised by melting enthalpy and enthalpy of dissolution of the mixtures. Hildebrand solubility equation (Equation 1) is theoretically valid when there is no enthalpy of dissolution during the process. However, it is acknowledged that fats and fat blends are a multicomponent TAG system of which some deviation from ideal solubility is commonly observed at some degrees (Humphrey and Narine, 2005).

In this investigation, the temperature of peak maximum on melting thermogram was chosen as the melting temperature (T) of the blends for Hildebrand equation calculation. Meanwhile, the molecular weight of the samples was estimated from the average molecular weight of their fatty acid composition and it was used to convert the unit from gram to mole. The Hildebrand plot for the binary mixtures of two hard fats in three lower melting oils was then depicted in *Figure 3*. It was shown that the log value of the solubility [$\ln(1/x_s)$] generally had a linear relationship with the temperature ($1/T$), with a high coefficient of determination (R^2) values. These linear relationships indicated that hard fats (PS or FHRO) might form an ideal solution with each of the lower melting oils. However, it was again noticed that hard fats diluted in PKO had solubility lines with stronger deviation from ideality compared to those of diluted in MCT-oil and HOSO, especially on the lower molar ratios. These deviations could be attributed to the changing in HMF composition of the hard fat and thus, their crystallisation behaviours. In general, the explanations were similar to the DSC melting behaviour as already discussed above.

Solubility of High, Medium and Low Melting Fractions in Lauric Fat Blends

Lauric fat blends were formulated as a ternary mixture composed of HMF, MMF and LMF. It was demonstrated in the simpler binary mixtures that hard fats were dissolved in either lauric or liquid oils. At the same time, the lauric oil was also shown to be dissolved in the liquid oils. Therefore, three distinct endothermal peaks were then observed on the DSC melting thermogram of lauric fat blends referring to those three fractions. Using the parameter of melting peaks of both HMF (H-group) and MMF (B-group), Hildebrand plot of FHRO lauric fat blends was constructed where the relationship between the solubility of both groups and temperature was illustrated by solubility curves as shown in *Figure 4*. We did not further elaborate the solubility of lauric fat blends with PS

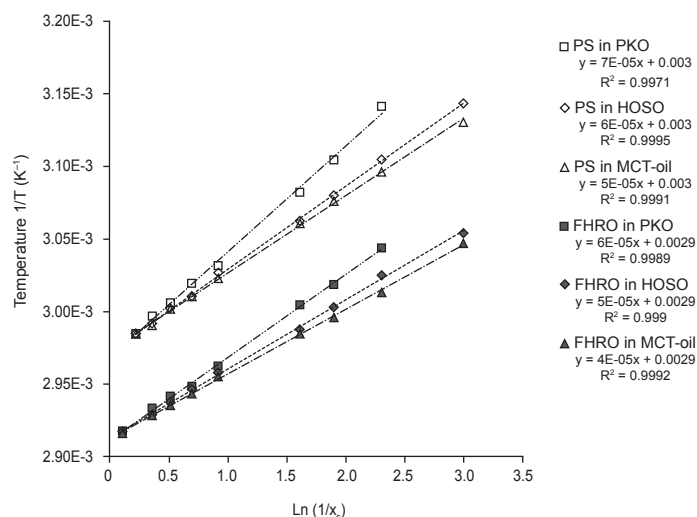
as the hard fat in this study as they had a similar pattern with that of FHRO lauric fat blends.

Lauric fat blends with FHRO as the hard fat formulated at constant BH50 with varied H from 2.5%-17.5% had two solubility curves representing the H and B groups. When the amount of H-group was increased, the amount of B-group was decreasing in order to compensate for the final sum of both groups at 50% in the blends. The changing of their ratios can be considered as the solubility of H and B groups in the LMF (C and R groups, fixed at 50%). In the case of H-group, this HMF was dissolved both in MMF (B-group) and LMF (CR-group). As a result, the solubility of H-group was similar to that of the simpler binary mixture between HMF and lower melting components. It was found from the solubility curve that H group had a good linear relationship with R^2 value of 0.9782 thus showed an ideal solubility. For B-group, this MMF dissolved in the LMF at the presence of H-group fat crystals. The lower amount of B-group resulted in more solid fat of H-group in the blends. The presence of solid fat might alter the crystallisation behaviour of B-group especially when the amount of both H and B groups was always changed following different formulation of the blends. The solidification of fats in the presence of other fat crystals could change the crystallisation behaviour of fat blends from homogenous to heterogeneous nucleation. It was eventually shown that stronger deviation from ideality was indicated on the solubility curve of MMF (B-group) at which the linear relationship between the composition [$\ln(1/x_s)$] and temperature ($1/T$) had a lower R^2 value of 0.9401.

On the other hand, FHRO lauric fat blends formulated at constant H15 with varied BH from 50%-70% had only one solubility curve in terms of H-group solubility in both MMF and LMF. Because the HMF was kept at 15% for all formulation, the solubility curve of H-group could not be drawn instead there was an average value of the correlation between the composition [$\ln(1/x_s)$] and temperature ($1/T$). Meanwhile, the solubility of B-group in the LMF was influenced by the presence of H-group fat crystals, now at a similar amount (15% H-group) for all blends. Since the amount of fat crystal was set constant, they affected in the same way and gave comparable influences on the solubility of B-group in all blends. As a consequence, the solubility of B-group in lauric fat blends formulated at H15 was closer to ideality than that of blends formulated at BH50. Better linearity on the solubility curve of lauric fat blends formulated at H15 was shown by a higher R^2 value of 0.9954.

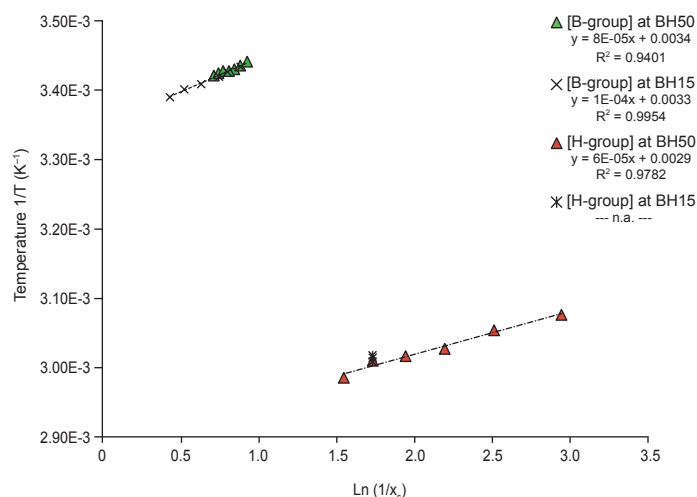
Solid Interaction in Lauric Fat Blends

The interaction of solid fractions in the fat blends can be described by an isosolid diagram.



Note: PS - palm stearin; FHRO - fully hydrogenated rapeseed oil; PKO - palm kernel oil; HOSO - high oleic sunflower oil; MCT-oil - medium chain triacylglycerol oil.

Figure 3. Hildebrand plot of binary mixtures of high melting fractions in lower melting oils.



Note: B-group and H-group indicate the amount of medium melting fractions and high melting fractions in lauric fat blends, respectively. A formulation at BH50 or H15 refers to the fat blend composed of 50% TAG from a combination of B and H groups or the fat blend composed of 15% TAG from H groups, respectively. Fully hydrogenated rapeseed oil (FHRO) was the hard fat added to lauric fat blends.

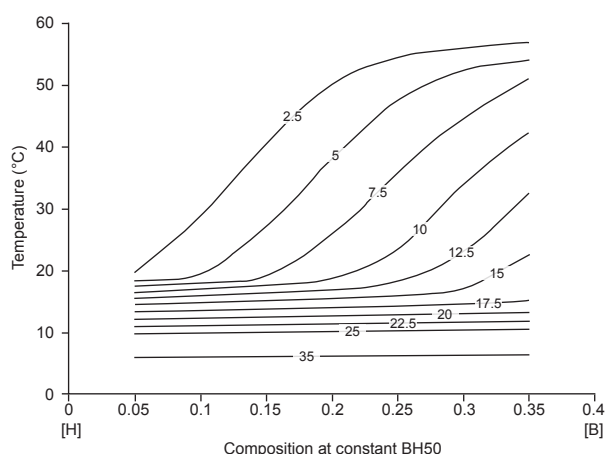
Figure 4. Hildebrand plot of FHRO lauric fat blends at different formulations.

This diagram contains isosolid lines representing the correlation between temperature and composition at constant SFC values. An isosolid line showed monotectic interaction as a straight horizontal line connecting two starting fats in fat blends. Meanwhile, eutectic interaction is indicated by compressed isosolid lines with melting temperatures below than those of the starting fats. Eutectic blends are usually undesirable as they had lower SFC than those of starting fats, showing that starting fats are not compatible (Timms, 1984; Lida *et al.*, 2002). For solid interaction, we also discussed only FHRO lauric fat blends in this study as lauric fat blends with PS as the hard fat showed a comparable result.

The isosolid diagram of FHRO lauric fat blends formulated at constant BH50 was shown in Figure 5. In this formulation, all blends had a similar amount of LMF (C and R groups) at 50%. Therefore, different H values indicated different ratios of both H-group and B-group in the constant amount of LMF (CR50). No depression was observed on the isosolid lines of lauric fat blends at this range of formulation (BH50 with varied H from 2.5%-17.5%) although a monotectic interaction was evident. Taking into account the previous binary mixture at which the solubility of hard fats in medium melting components had a slight deviation from ideality, this monotectic interaction seemed to coincide with its solubility curve. Thus, the effect

of solid interaction was considered to be less pronounced than that of solubility. Moreover, the MMF formulated at BH50 was completely melted at approximately 20°C as indicated in the isosolid diagram. At any temperature higher than 20°C, the SFC of the blends was apparently the function of H-group ratios. It was shown that the higher ratios of H-group yielded higher SFC for isosolid lines, yet, with constant SFC less than 17.5%.

Figure 6 showed the isosolid diagram of FHRO lauric fat blends formulated at constant H15. In this formulation, the amount of HMF in the blends was set constant at 15%. Meanwhile, the amount of MMF (B-group) was varied against that of LMF (CR-group) in order to fix the amount of both fractions at 85% in the blends. As the amount of HMF was fixed at 15%, the isosolid lines at constant SFC up to 10% displayed straight horizontal lines. These lines indicated the amount of fat crystal from H-group present in the blends at the temperature above the melting temperatures of both B-group and CR-group. Further, the isosolid lines at constant SFC values higher than 15% showed a monotectic interaction. The presence of solid fat (H-group) during B-group solidification seemed only imparting small influence as no depressions were noticed on those isosolid lines. The more closely spaced isosolid lines were also observed with the increment of B-group ratios indicating the dilution effect of B-group in CR-group (Ribeiro *et al.*, 2009). The effect of solid interaction between HMF and MMF was thus considered to be less pronounced than the solubility effect of HMF/MMF in LMF similar to the previously formulated blends (BH50).



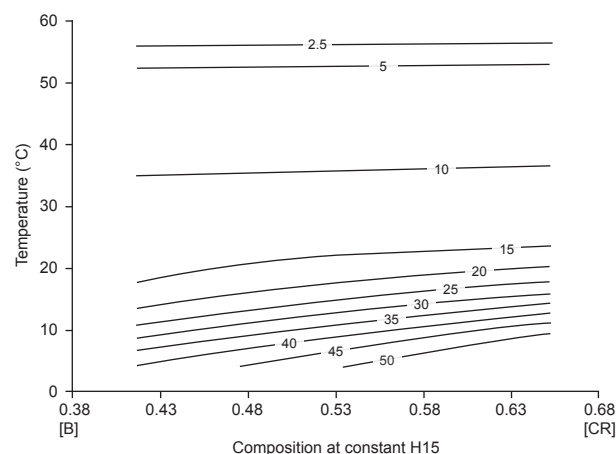
Note: A formulation at BH50 refers to the fat blend composed of 50% triacylglycerol (TAG) from a combination of B and H groups. Fully hydrogenated rapeseed oil (FHRO) was the hard fat added to lauric fat blends.

Figure 5. Isosolid diagram of FHRO lauric fat blends formulated at constant BH50.

Hardness of Lauric Fat Blends

The viscoelastic behaviour of plastic fats is mainly dictated by the structure of fat crystal network where the amount of solid fat, polymorphism and microstructure are known as the main influencing factors (Braipson-Danthine and Deroanne, 2004; Narine and Humphrey, 2004; Yanty *et al.*, 2017). Hardness is commonly used to correlate that behaviour to the perceived texture of a plastic fat. It was shown that hardness of lauric fat blends was influenced by the amount of solid fat from both H and BH group. When the amount of TAG from BH-group was fixed at 50% (BH50) but that of H-group was varied (differ on the HMF ratio, H2.5-H17.5), hardness was generally found to be similar in the range 141-239 g and 164-308 g for PS and FHRO lauric fat blends, respectively (Figure 7). The different type of hard fats (PS, FHRO) also gave approximately comparable hardness. Nevertheless, an obvious pattern was observed at which hardness of FHRO lauric fat blends had higher values than that of PS lauric fat blends. The effect of both different ratios of HMF and hard fat types on hardness seemed to be small as the measurement was done at 15°C at which the amount of solidified fractions, both HMF and MMF, was thought to be similar (BH50).

Higher hardness values were found when the amount of TAG from BH-group was increased from BH50 to BH70 in lauric fat blends (Figure 8). At those blends, the HMF was set constant at 15% (H15). Hardness corresponded well with the total amount of both HMF and MMF although the correlation was not linear. The variability in hardness could still arise from different crystal polymorph and network

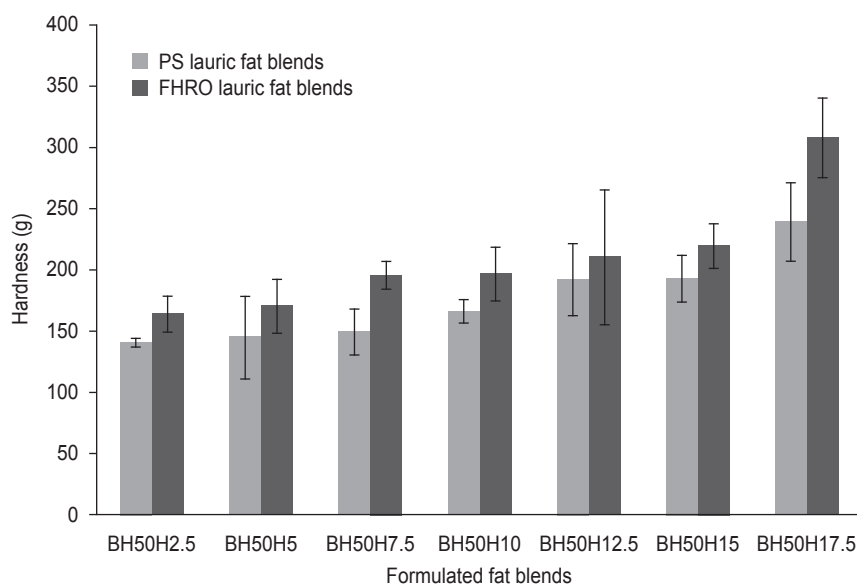


Note: A formulation at H15 refers to the fat blend composed of 15% triacylglycerol (TAG) from H group. Fully hydrogenated rapeseed oil (FHRO) was the hard fat added to lauric fat blends.

Figure 6. Isosolid diagram of FHRO lauric fat blends formulated at constant H15.

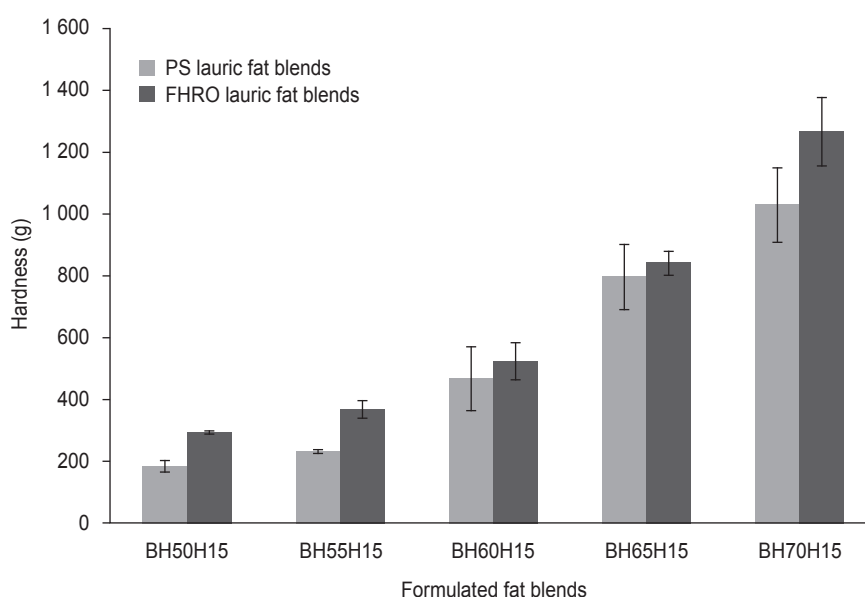
structures (Braipson-Danthine and Deroanne, 2004) even when the system was revealed to have a monotectic interaction. In addition, higher hardness was also evident for all FHRO lauric fat blends compared to that of PS blends even so the values

were mostly comparable. The higher concentration of the hard fats and longer carbon chain length of fatty acids on the TAG can result in fat blends with firmer texture (Zárubová *et al.*, 2010; Manaf *et al.*, 2019).



Note: Either palm stearin (PS) or fully hydrogenated rapeseed oil (FHRO) was the hard fat added to lauric fat blends. Meanwhile, a formulation at BH50 refers to the fat blend composed of 50% triacylglycerol (TAG) from a combination of B and H groups. Lauric fat blends were formulated at constant BH50 and varied H from 2.5%-17.5% (BH50H2.5, BH50H5, BH50H7.5, BH50H10, BH50H12.5, BH50H15 and BH50H17.5).

Figure 7. Hardness of lauric fat blends formulated at constant BH50.



Note: Either palm stearin (PS) or fully hydrogenated rapeseed oil (FHRO) was the hard fat added to lauric fat blends. Meanwhile, a formulation at H15 refers to the fat blend composed of 15% triacylglycerol (TAG) from H group. Lauric fat blends were formulated at constant H15 and varied BH from 50%-70% (BH50H15, BH55H15, BH60H15, BH65H15 and BH70H15).

Figure 8. Hardness of lauric fat blends formulated at constant H15.

CONCLUSION

This study demonstrated that the simpler binary mixtures could provide some basic information in describing the phase behaviour of more complex ternary lauric fat blends. It was shown in the binary mixture that hard fats (PS or FHRO) formed an ideal solubility in different liquid oils (MCT-oil or HOSO). However, the solubility of hard fats in the lauric oil was indicated by some deviation from ideality as PKO contained some amount of high melting TAG. They might, therefore, affect the composition of high melting fraction and its crystallisation behaviour of the hard fats. The experimental melting point (T_m) and heat of fusion (ΔH_f) of the hard fats were comparable to those of pure system as ideal solubility was evidence. In the ternary lauric fat blends, the HMF formed an ideal solubility in the both MMF and LMF within the range of given formulations (at constant BH50 and H15). Meanwhile, the MMF also had a linear relationship on their solubility curves but with stronger deviation from ideality as affected by the presence of fat crystals from HMF. Further, the isosolid diagram of lauric fat blends generally exhibited monotectic behaviour. The influence of solubility, nevertheless, seemed to override the influence of solid interaction. In addition, the hardness of lauric fat blends was influenced by the total amount of HMF and MMF. Different type of hard fats gave small variability, yet FHRO lauric fat blends gave higher hardness values than those of PS blends. The solubility curves and isosolid lines, in general, provided valuable information about the interaction of high, medium and low melting fractions in the lauric fat blends. The pseudo-phase diagrams presented here could then be useful for improving the formulation of plastic fats, enriching the previous formulation based on the melting TAG group.

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BLENDING PALM FRACTIONS AS CONFECTIONERY FATS: A PRELIMINARY STUDY

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ABSTRACT

The study looked into the mouldability of cocoa butter alternative (CBA) from blended palm fractions namely palm mid fraction (PMF) IV 45, palm kernel oil (PKO) and palm stearin (POs) IV 33 and IV 14 for the production of chocolate bar. PMF, PKO and POs IV 33 and IV 14 were blended in eight different optimised ratios based on solid fat content (SFC) response of more or close to 50% at 20°C, SFC of less than 10% at 37°C and SFC of less than 5% at 40°C to produce a melt in the mouth and mouldable plain chocolate bar. Mouldability and snappability of the chocolate bar were the main parameters of the study. Physicochemical properties of the fat blends namely the fatty acid composition (FAC), triacylglycerol composition (TAG), solid fat content (SFC), crystallisation rate at isothermal temperature of 5°C, 10°C and 20°C, thermal behaviour, polymorphism, crystal morphology and compatibility test with cocoa butter (CB) for selected fat were also determined. Fat F blend with 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14 showed the highest SFC (47%) at 20°C and was able to be moulded and demoulded at 10°C by 45 min. It has the highest rupture tension ($105.28 \text{ g}_f \text{ cm}^{-2}$) and breaking force (5501 g_f) at 10°C. The composition of oleic acid, linoleic acid, total monounsaturated fatty acid, crystal morphology (density) as well as SFC profile at 20°C and crystallisation rate profile at 5°C, 10°C (after 40th min) and 20°C (before 35th min) of fat F were comparable with CB while other FAC, TAG, thermal behaviour and polymorphism were dissimilar to CB. Fat F, 10%-30% was observed to be compatible (no eutectic) with CB and therefore, has the potential as fat for chocolate bar. The addition of 10%-30% of fat F to CB increased the SFC to 57% at 20°C. Fat F (30%) with CB could be demoulded by 30 min and produced snappability (breaking force) of $5032.71 \pm 85.91 \text{ g}_f$ at 20°C after one day stabilisation period. Palm-based fat blend having 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14 and CB has the potential to be used as alternative fat for chocolate bar which can eventually benefit the confectionery industry.

Keywords: blended palm fractions, chocolate bar, confectionery fats, mouldability, rupture tension.

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INTRODUCTION

Fat is an important ingredient for chocolate and other confectionery products as it has a major influence on their organoleptic and physical

properties. It is responsible for the snap, appearance, mouthfeel and flavour release of chocolate confectionery (Rios *et al.*, 2014). Cocoa butter (CB) is the traditional fat for chocolate confectionery. It contains three main fatty acids which are palmitic, stearic and oleic. The primary triacylglycerols (TAG) composition of CB are the 1,3-dipalmitoyl-2-oleoyl glycerol (POP), 1(3)-stearoyl-2-oleoyl-3(1)-palmitoyl glycerol (POS) and disaturated 1,3-distearoyl-2-

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oleoyl glycerol (SOS) (Mohamed, 2013). This TAG composition is responsible for the sharp melting of CB between 30°C and 35°C but maintains a solid consistency at 20°C which is desirable for chocolate confectionery (Timms, 2003). Due to difficulty in cultivation of cocoa plants, low productivity and pest attacks, the production of CB is hampered although its demand is high. These scenarios lead to the production of CB alternatives (CBA) and are classified into three groups namely CB equivalent (CBE) having similar physicochemical properties as CB, non-lauric CB replacer (CBR) and lauric-based CB substitutes (CBS) having similar physical properties but different chemical properties from CB (Norazura Aila and Noor Lida, 2017).

There are various methods in producing confectionery fats which include blending, fractionation, hydrogenation and interesterification. Based on various studies, currently, interesterification, supercritical extraction and enhanced fractionation are the focus of CBA research. Palm mid fraction (PMF) has been the focus for interesterification studies, particularly enzymatic, for the production of CBE and has been discussed in many published articles (Wu and Deng, 2005; Borhan *et al.*, 2011; Soekopitojo *et al.*, 2009; Soekopitojo, 2011; Gibon, 2011; Mohamed, 2013; Mutia *et al.*, 2016; Biswas *et al.*, 2018). Supercritical carbon dioxide process has been employed by Nik Norulaini *et al.* (2004) and Zaidul *et al.* (2006; 2007) to further process palm kernel oil (PKO) to obtain CBE and CBR, respectively. Timms (2003) reported that the simplest CBS was produced through hydrogenation of PKO. Calliauw *et al.* (2005) published article describing the process of obtaining high-quality CBS from PKO through a two-stage static fractionation process. Kang *et al.* (2003; 2013) demonstrated the production of CBE with palm stearin (POs) through additional acetone fractionation process. However, interesterification, supercritical extraction, hydrogenation and enhanced fractionation are highly processed methods. We are of the opinion that blending on the other hand is simple and straight forward compared to the above processes.

PMF (IV 45) that is obtained through second stage dry fractionation of palm oil (De Clercq *et al.*, 2012) exhibiting melting points between those of POs and palm olein (POo) (Sakamotoa *et al.*, 2004) has been vastly utilised for blending with other vegetable fats in the production of CBE. These vegetable fats include exotic fats (Talbot, 2009; Elham *et al.*, 2013; Jun *et al.*, 2016) as well as hard stearin and high oleic-high stearic sunflower hard stearins (Bootello *et al.*, 2012; 2013). Having high level of POP makes PMF the fat of choice for blending with other sources of fats containing high POS and SOS TAG composition to mimic TAG composition of CB

(Sonwai *et al.*, 2014). In addition, PMF offers unique melting profile similar to CB. To-date, no study has been found for blending of PKO with other fats for the hard-confectionery application. However, blend of PKO with other fats is found to be suitable for confectionery filling by Biswas *et al.* (2017). As PKO (lauric fat) is rich in lauric and myristic fatty acids, eutectic might be a challenge when it is blended with non-lauric fats. POs has been used extensively as natural alternative to eliminate *trans*-fat especially in formulating hardstock such as margarine and shortening. Few blending studies of POs and other fats for confectionery application have been investigated earlier. These include studies by Jahurul *et al.* (2014b) that blended POs and mango seed fat as a CBR and Cain *et al.* (1995) that blended PKO and palm kernel stearin (PKS) for hard coating application.

Hard PMF having IV 35 has been acknowledged to be useful for hard-confectionery (Braipson-Danthine and Gibon, 2007). However, blended soft PMF (IV 45) with other palm fractions has yet to be proven for hard-confectionery applications. Therefore, in this study, blended PMF IV 45, POs (IV 33 and/or IV 14) and PKO at optimised ratios based on solid fat content (SFC) were investigated in term of physicochemical properties including their mouldability for the hard-confectionery application. POs might be able to increase SFC of the blended fats and enhance crystallisation rate while PKO could minimise tailing of the blended fats.

MATERIALS AND METHODS

Materials

PMF IV 45 and refined, bleached and deodourised (RBD) PKO were obtained from Cargill Palm Product Sdn. Bhd., Malaysia while RBD POs IV 33 was obtained from Sawit Raya Sdn. Bhd., Malaysia. RBD POs IV 14 was provided by Mewah Oils Sdn. Bhd., Malaysia. In this article, RBD PKO, RBD POs IV33 and RBD POs IV14 were simplified as PKO, POs IV33 and POs IV14, respectively.

Experimental Design and Preparation of Fat

Interaction of blended fats namely PMF/ PKO/ POs IV 33/ POs IV 14 were studied using simplex-lattice design. SFC was used as the response parameter. Twenty randomised trials including five replicates were used as inputs before optimisation. Experimental error and fit of the model was determined from the replications. Scheffe model was used to fit a polynomial equation. Analysis of variance (ANOVA) was used to check quality of the model. Optimisation of the blended fats was conducted based on SFC parameter. Targeted SFC

values were more than 45% SFC at 20°C, less than 10% SFC at 37°C, and less than 5% SFC at 40°C. These were carried out using point prediction method.

The PMF, PKO, POs IV 33 and POs IV 14 were then mixed at different ratios as shown in *Table 1* according to the optimised ternary diagram when POs IV 14 was set to zero and optimised ternary diagram when POs IV 14 was set to 2% and 3%. The fats were melted at 80°C for at least 1 hr prior to mixing.

TABLE 1. FAT BLENDS OF PMF/PKO/POs IV 33/POs IV 14 PRODUCED THROUGH MIXTURE DESIGN

Fat blend	PMF (%)	PKO (%)	POs IV 33 (%)	POs IV 14 (%)
A	88	1	11	0
B	89	1	10	0
C	91	1	8	0
D	92	1	7	0
E	96	1	3	0
F	91	1	5	3
G	93	1	3	3
H	95	1	2	2

Note: PMF - palm mid fraction; PKO - palm kernel oil; POs - palm stearin.

Solid Fat Content

SFC of the blended fats was measured according to American Oil Chemists' Society (AOCS) Official Method Cd 16b-93 (AOCS, 2012) using pulsed nuclear magnetic resonance (NMS Minispec from Bruker, Germany). Samples were melted at 80°C for 1 hr and filled into NMR tubes of 0.8 cm (diameter) and 3 cm (height). For this procedure, samples were chilled for 90 min at 0°C, followed by tempering for 40 hr at 26°C, 90 min at 0°C and finally maintained for 60 min at desired test temperatures of 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 37°C and 40°C prior to each measurement.

Fatty Acid Composition (FAC)

FAC of the blended fat was analysed using gas chromatography (GC) based on fatty acid methyl esters (FAME) according to MPOB Test Methods p3.5 (MPOB, 2005). Samples of 0.1 g were weighed and dissolved in 1.8 ml hexane. The sample's solution was then mixed with a vortex mixer. Sodium methoxide solution (100 µl) was added to the samples for methylation process followed by water addition. Mixing was performed in between each process. The sample's solution was left for 1 hr to separate. The clear supernatant was transferred to another vial added with sodium sulphate anhydrous and kept for 15 min before complete clear supernatant (1.5 ml) was injected into GC (Agilent Technologies, Model 7890B). The GC was equipped with a flame ionisation detector (FID).

The injector and detector temperatures were both set at 240°C while the column temperature was set at 185°C. Helium gas (carrier) with flow rate of 1 ml min⁻¹ was used. The peaks were identified by comparing retention times with FAME standards and quantified using peak area normalisation method.

Triacylglycerol Composition (TAG)

TAG composition was performed using ultra high-performance liquid chromatography (U-HPLC) system (1290 Infinity LC System, Agilent Technologies, USA) and Agilent 1260 RI detector (Waters Corp., USA) according to AOCS Official Method Ce 5b-89 (AOCS, 2012). A Cortecs UPLC C18 column (2.1 mm x 150 mm length i.d; 1.6 µm particle size) (Waters Corp., Milford, Massachusetts, USA) maintained at 30°C was used. The mobile phase used was a mixture of acetone-acetonitrile with the ratio of 63.5:36.5 (v/v) at a flow rate of 0.25 ml min⁻¹. Samples (0.1 g) was solubilised in 1 ml of acetone. The chemicals and reagents used were HPLC grade. HPLC analysis was conducted in duplicate and data was recorded as percent areas.

Crystallisation Rate at Isothermal Temperature

Crystallisation rate was measured using pulsed nuclear magnetic resonance (NMS Minispec from Bruker, Germany) according to Chen *et al.* (2015) with modification. Samples were melted at 80°C for 1 hr. NMR tubes of 0.8 cm (diameter) and 3 cm (height) were used in this analysis. Tubes with samples were held for 1 hr at 30°C. The tubes were then placed at 5°C, 10°C and 20°C. SFC readings were taken every 1-5 min.

Thermal Behaviour

Thermal properties of the oil samples were measured using a differential scanning calorimetry (DSC)-7 (Perkin Elmer, Norwalk, CT). Calibration was performed using an indium standard and n-decane. Oil sample of 3-5 mg was hermetically sealed in an aluminium pan. The following temperature programme was used to perform the melting and cooling measurements on each sample: cooled to -40°C at 5°C min⁻¹, held at -40°C for 5 min and then heated to 80°C at 5°C min⁻¹ following temperature programme from Biswas *et al.* (2017) with modification.

Polymorphism

Polymorphism was determined at room temperature using coupled X-ray Diffraction (XRD)-DSC Rigaku TTRAX III (Tokyo, Japan).

The sample holders were flat stainless-steel plates with rectangular holes. The X-ray incident-beam 2 θ range was from 1°-30° with scanning rate of 5°C min⁻¹ according to Biswas *et al.* (2017) with modification. Samples were melted at 80°C and tempered at 10°C for 30, 45 and 60 min. α , β' and β polymorphs were determined using short spacing characterisations.

Crystal Morphology

Crystal microstructure of the fats were determined using Polarised Light Microscope (PLM) following the method as described by Narine and Marangoni (1999). A small droplet (about 15 μ l) of melted fat was placed on a glass slide and covered with a glass slip. The sample was first heated to 80°C for 20 min to erase crystal memory. The sample was tempered at 10°C, 20°C and 25°C for 48 hr prior to measurement. A Leica DMLP polarised light microscope (Wetzlar, Germany) equipped with a Linkam THMS 600 temperature controller stage and a JVC 3-CCD colour video camera was used. Temperature was thermostatically controlled by a Linkam TP 94 multiramp temperature programme conching machine and LNP automatic cooling system (Linkam, Tadworth, Surrey, United Kingdom). Liquid nitrogen was used as the coolant. The photomicrograph of the crystal was taken at 50X magnification.

Mouldability

Chocolate bars were produced from the blended fats according to plain chocolate formulation by Timms (2003) as per Table 2. The amount of cocoa powder, sugar, blended fat and lecithin were made constant in order to produce the chocolate bars. These raw materials were then refined using 3-mill refiner and conched using chocolate conching machine. The chocolate bars were cooled at 10°C (Keijbets *et al.*, 2010) for 30, 45 and 60 min. The bars were not subjected to further solidification period. Mouldability of the chocolate bars were determined based on observation of the moulding and demoulding activity (easy demoulding or otherwise). Triplicates of chocolate bars were produced for each blended fat.

TABLE 2. FORMULATION OF PLAIN CHOCOLATE

Ingredients	Weight (%)	Weight (g)
Cocoa powder	20.00	200.00
Blended fat	33.00	330.00
Sugar	46.60	466.00
Lecithin	0.40	4.00
Total	100.00	1 000.00

Source: Timms (2003).

Mechanical Resistance Test (Snappability)

Snappability of the chocolate bars at 10°C was conducted using a Texture Analyser (TA-XTplus, Stable Micro Systems) with a Three Bend Rig probe according to Stroppa *et al.* (2014) and Jorge *et al.* (1999). The setting for pre-test velocity was 3 mm s⁻¹, test velocity was 1.7 mm s⁻¹ and post-test velocity of 10 mm s⁻¹. Rupture force was adjusted to the center of the chocolate bars. Three repetitions were conducted for each chocolate having different fat blends and CB. The values of the force obtained were divided by the cross-section area of each bar and the unit for the rupture tension was reported in g_f cm⁻² in order to eliminate the effect of thickness variations of the bars.

Compatibility Test

The results obtained from our preliminary analysis were utilised to select the best blended palm fractions. The selected fat was blended with CB at different ratio (0%-100%). The SFC at 20°C for all ratios were determined according to AOCS Official Method Cd 16b-93 (AOCS, 2012). Mouldability at 10°C and mechanical resistance test at 20°C were conducted for blend of selected fats and CB.

Statistical Analysis

Results were analysed with Minitab version 16.0 (Pennsylvania, USA) using one-way ANOVA. Significance differences ($p \leq 0.05$) among the samples were analysed using the Tukey's *post-hoc* test. All tests were carried out in duplicate or triplicate.

RESULTS AND DISCUSSION

Solid Fat Content

SFC profiles of the fats are shown in Figure 1. CB had a high SFC at low temperature, the SFC then decreased sharply from 20°C-35°C with 0% SFC at 35°C. Similar finding was reported by Biswas *et al.* (2017) and Kadivar *et al.* (2016) in which some finding showed 0% SFC at 37°C based on its varieties. The sharp melting profile is due to its significant level of SOS and POS TAG (Biswas *et al.*, 2017; Kadivar *et al.*, 2016). All of the eight blended fats PMF/PKO/POs (IV 33 and/or IV 14) showed marginal difference in SFC profiles at all temperatures tested. This was explained by the minor deviation in TAG value of all the fats (Table 4). This trend was also reported by Jahurul *et al.* (2014a) when blending PMF with mango seed fat to produce CBE. Blended PMF/PKO/POs (IV 33 and/or IV 14) had 76%-79% SFC at 5°C.

A steep decrease of SFC in all the fats occurred between 15°C-25°C with zero solid at 40°C. Fat F had the highest SFC at 20°C having value of 47% followed by fats G and H. Higher percentage of trisaturated TAG led to higher SFC (Zhou *et al.*, 2010). Overall, all of the blended fats (PMF/PKO/POs (IV 33 and/or IV 14)) exhibited similar SFC trend as compared to CB with high SFC at low temperature, nearly zero SFC at 35°C-40°C and high melting profile in between. Hence, these blended fats have the potential to be used as confectionery fat.

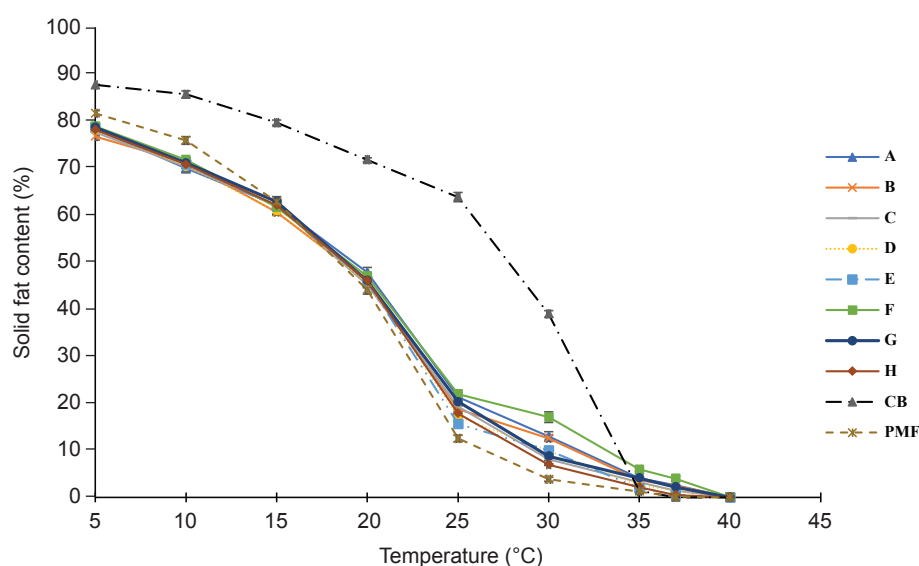
Fatty Acid Composition

FAC of the eight blended fats are tabulated in Table 3. The main fatty acids in CB are palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1). This has been described in numerous earlier studies, among others were by Żyżelewics *et al.* (2014), Jahurul *et al.* (2013) and Compos and Marangoni (2010). Fatty acid profile of the eight blended fats was more diverse with major fatty acids being palmitic and oleic acids, followed by linoleic acid (C18:2). Other fatty acids were present in small quantity in the fats. Lauric acid (C12:0) and myristic acid (C14:0) values in all blended fats were low (0.70%-1.10% and 1.41%-1.89%, respectively) as only 1% PKO was recommended

after optimisation. Palmitic acid in all fats was significantly different ($p < 0.05$) and observed to be higher than in CB. Stearic acid in all the blended fats (A-H) was not significantly different ($p > 0.05$). It was observed that stearic acid in all fats was significantly lower than in CB. Although the values of oleic acid for all the blended fats were found to be close to one another and in CB, composition of oleic acid in all the fats was examined to be significantly different ($p < 0.05$) compared to each other and CB. Polyunsaturated fatty acid (PUFA) of all blended fats (A-H) was observed to be higher than in CB. The trait of unsaturated fatty acids was that it has lower melting points due to the existence of double bonds (Larsen *et al.*, 2014). In this study, the amounts of oleic and linoleic as well as monounsaturated (MUFA) fatty acids (palmitoleic acid and oleic acid) were comparable to CB.

Triacylglycerol Composition

The TAG composition of all fats is shown in Table 4. TAG (18 types) were found in CB. Saturated-unsaturated-saturated (SUS) TAG namely POS, SOS and POP dominated TAG of CB having 39.26%, 31.11% and 16.18%, respectively. Both PMF and POs were hard fractions of dry fractionation. TAG (16 types) were identified in all fat blends. Major TAG in all fat blends were POP, followed by PLP.



Note: Fat A - 88% PMF, 1% PKO, 11% POs IV 33.
 Fat B - 89% PMF, 1% PKO, 10% POs IV 33.
 Fat C - 91% PMF, 1% PKO, 8% POs IV 33.
 Fat D - 92% PMF, 1% PKO, 7% POs IV 33.
 Fat E - 96% PMF, 1% PKO, 3% POs IV 33.
 Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.
 Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.
 Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.
 CB - cocoa butter; PMF - palm mid fraction; PKO - palm kernel oil; POs - palm stearin.

Figure 1. Solid fat content of blended fats, cocoa butter and palm mid fraction.

High content of POP TAG is responsible for the β' crystal formation (Toro-Vazquez *et al.*, 2004). As percentage of PKO was very minor in all fats, LLL, LLM and LMM TAG were not present/detected. The values of POP TAG among the blended fats were comparable to each other having values ranging from 48.52%-50.28%. However, the POP TAG of these blends was significantly different ($p < 0.05$) from CB. The POS and SOS TAG of the blended fats did not differ much from one another having the range of 9.16%-9.58% and 1.08%-1.38%, respectively. However, both of the TAG were significantly different with CB ($p < 0.05$). All of the blended fats had higher unsaturated-unsaturated-unsaturated (UUU) TAG (1.12%-1.34%) compared to CB (0.17%). Saturated-unsaturated-unsaturated (SUU) TAG of the blended fats was higher than CB ranging from 14.52%-15.42%. Despite of this, SUS TAG for all blended fats was found to be higher in CB (88.79%) compared to the blended fats (59.07%-60.84%). Saturated-saturated-saturated (SSS) TAG of the blended fats ranged from 17.06%-18.97% while similar TAG in CB showed a value of 5.51%. Fat F exhibited the highest SSS TAG of 18.45% among all the blended fats. Majority of the fats contained insignificant TAG ($p > 0.05$) which included PLL, OLO, OOO, POO, SOO, POS and SOS.

In the present study, the most significantly different ($p < 0.05$) TAG was PPP. PPP increased progressively with the addition of POs (POs IV 33 and POs IV 14). The addition of POs to PMF improved the SFC due to the presence of high melting fraction (Jahurul *et al.*, 2014a) such as PPP. Further addition of POs IV 14 increased the level of PPP significantly ($p < 0.05$). However, PPP is an undesirable TAG as it provides waxiness to the end product (Cleenewerck, 2010). The use of POs IV 14 in the formulation was low in percentage for fats F, G and H with values of 3%, 3% and 2%, respectively. However, fats F, G and H also consisted of 5%, 3% and 2% of POs IV 33, respectively. The value of PPP for fats F, G and H increased tremendously compared to fats A-E that have no POs IV 14 in the formulation and only comprised of POs IV 33 of 11%, 10%, 8%, 7% and 3%, respectively. There is potential of using single POs namely POs IV 14 as substitute to POs IV 33 if PPP could be of an assistance for the chocolate mouldability. All of the TAG of the blended fats were not comparable to CB. However, the increased level of PPP in the formulation facilitates crystallisation (Basso *et al.*, 2010) and eventually the mouldability property.

Crystallisation Rate at Isothermal Temperature

Crystallisation rates of all the fats at 5°C, 10°C and 20°C (isothermal temperature) are shown

in Figure 2. At 5°C, CB reached 80% SFC within 10 min and began to plateau at 90% SFC by 20th min. The eight fat blends, on the other hand, did not reach 50% SFC at 10th min crystallisation time. The SFC of all fat blends at 5°C were close to each other. Overlapping of SFC of all fats started to be observed by 35th-40th min of crystallisation time. PMF followed by fat E illustrated the slowest crystallisation rate at 5°C. At 10°C, CB reached 80% SFC by the 15th-20th min crystallisation time. By the 30th-35th min, the SFC of CB begun to plateau. Fats A, B, C, D, F, G and H demonstrated overlapping SFC by the 40th-45th min of crystallisation rate. POs which was part of the formulation played important role in this crystallisation pattern. PPP which is the highest melting fraction in POs influenced the fat crystallisation (Basso *et al.*, 2010; Vereecken *et al.*, 2009) as agent for seeding and crystallisation booster particularly for POP-containing system (Vereecken *et al.*, 2009). PMF and fat E still exhibited the lowest crystallisation rate at 10°C.

At 10th min, CB had the highest SFC compared to other fats at isothermal temperature of 20°C. Fat F showed the highest crystallisation rate from 1st-20th min compared to CB. Fats A, B, C, F, G and H demonstrated higher crystallisation rate than CB from 25th-35th min. At 40th min and above, CB had the highest SFC at 20°C imposing fastest crystallisation compared to other fats. Foubert *et al.* (2006) suggested that the shift of slope in CB may be due to the development of distinct polymorphic form or occurrence of various fractions crystallisation. Unblended PMF fat showed the poorest crystallisation performance at this isothermal temperature with only very minor increase of SFC towards 60th min. Similar result was obtained by Peyronel *et al.* (2016) when rapid crystallisation of PMF started to take place only after 1.5 hr. POP and PPP played important role in determining the rate of crystallisation at isothermal condition of 20°C of all the fats. Higher content of POP in PMF made the crystallisation rate slower. Fat E, having the highest POP content and lowest PPP content, resulted to the lowest crystallisation rate among all of the fats. This result was aligned with the study by Sonwai *et al.* (2014) who reported higher crystallisation rate of PMF when the POP level was low.

In the present study, crystallisation rate of all blended fats was found to have less effect at low temperature of 5°C. All of the blends have close crystallisation rate profile with CB. However, at 10°C and 20°C, crystallisation rate of all the blended fats varied from other another as well as with CB. At 10°C, the crystallisation rate of all the fat blends had similar plateau profile as CB after 40th min. Notwithstanding this, similar crystallisation rate profile was found for all of the fat blends with CB before 35th min at 20°C.

TABLE 3. FATTY ACID COMPOSITION OF VARIOUS FAT BLENDS

Fatty acid	Cocoa butter	Fat blends							
		A	B	C	D	E	F	G	H
Caprylic acid (C8:0)	-	0.12±0.04 ^a	0.14±0.00 ^a	0.16±0.01 ^a	0.17±0.01 ^a	0.11±0.04 ^a	0.17±0.03 ^a	0.17±0.02 ^a	0.15±0.02 ^a
Capric acid (C10:0)	-	0.07±0.01 ^b	0.11±0.00 ^a	0.11±0.02 ^a	0.11±0.01 ^a	0.07±0.01 ^b	0.1±0.00 ^a	0.11±0.00 ^a	0.09±0.00 ^{ab}
Lauric acid (C12:0)	-	0.70±0.05 ^c	1.06±0.05 ^a	1.09±0.05 ^a	1.09±0.08 ^a	0.72±0.08 ^{bc}	1.02±0.02 ^a	1.1±0.00 ^a	0.84±0.01 ^b
Myristic acid (C14:0)	0.10±0.00 ^b	1.63±0.26 ^a	1.80±0.12 ^a	1.57±0.19 ^a	1.74±0.05 ^a	1.8±0.30 ^a	1.89±0.57 ^a	1.59±0.00 ^a	1.41±0.04 ^a
Palmitic acid (C16:0)	25.97±0.00 ^e	49.35±0.10 ^{ab}	49.02±0.07 ^{bc}	49.53±0.30 ^c	48.48±0.20 ^c	48.54±0.13 ^c	46.92±0.48 ^d	49.92±0.02 ^a	49.64±0.08 ^a
Palmitoleic acid (C16:1)	0.70±0.00 ^a	0.17±0.01 ^b	0.15±0.02 ^b	0.19±0.01 ^b	0.16±0.00 ^b	0.17±0.02 ^b	0.17±0.05 ^b	0.17±0.01 ^b	0.17±0.02 ^b
Stearic acid (C18:0)	33.25±0.00 ^a	5.66±0.13 ^b	5.61±0.03 ^b	4.92±0.08 ^b	5.71±0.18 ^b	5.66±0.25 ^b	4.81±0.39 ^b	4.9±0.00 ^b	4.93±0.02 ^b
Oleic acid (C18:1)	32.05±0.00 ^f	34.48±0.08 ^e	34.46±0.09 ^e	34.66±0.06 ^{de}	34.83±0.07 ^{cd}	35.04±0.21 ^{bc}	38.01±0.20 ^a	34.51±0.02 ^{de}	35.2±0.11 ^b
Linoleic acid (C18:2)	3.56±0.00 ^c	6.84±0.09 ^a	6.73±0.01 ^a	6.89±0.20 ^a	6.84±0.07 ^a	6.87±0.01 ^a	6.14±0.09 ^b	6.67±0.01 ^a	6.77±0.04 ^a
Linolenic acid (C18:3)	0.42±0.00 ^a	0.32±0.03 ^b	0.26±0.02 ^{bc}	0.19±0.02 ^{cd}	0.28±0.00 ^b	0.32±0.02 ^b	0.18±0.07 ^d	0.17±0.02 ^d	0.16±0.01 ^d
Arachidic acid (C20:0)	1.23±0.00 ^a	0.54±0.06 ^{bc}	0.51±0.04 ^c	0.53±0.09 ^{bc}	0.47±0.02 ^c	0.65±0.05 ^b	0.44±0.05 ^c	0.54±0.01 ^{bc}	0.5±0.05 ^c
Others	0.32±0.00 ^a	0.14±0.01 ^b	0.14±0.00 ^b	0.16±0.03 ^b	0.14±0.01 ^b	0.14±0.01 ^b	0.17±0.04 ^b	0.15±0.00 ^b	0.14±0.01 ^b
SAFA	62.99±0.00 ^a	58.07±0.14 ^{ab}	58.26±0.10 ^{ab}	57.91±0.32 ^{ab}	57.77±0.13 ^{ab}	57.49±0.23 ^{ab}	55.34±0.12 ^{ab}	51.65±11.55 ^b	57.56±0.13 ^{ab}
MUFA	33.07±0.00 ^a	34.65±0.09 ^a	34.61±0.10 ^a	34.85±0.06 ^a	44.99±7.38 ^a	35.21±0.19 ^a	38.51±0.43 ^a	34.68±0.02 ^a	35.37±0.10 ^a
PUFA	3.46±0.00 ^c	7.15±0.07 ^a	6.70±0.02 ^a	7.08±0.22 ^a	7.12±0.07 ^a	7.19±0.01 ^a	6.32±0.06 ^b	6.84±0.02 ^a	6.92±0.04 ^a

Note: Each value in the table is the mean ± standard deviation of three measurement.

Means within each row bearing different superscript letters are significantly different ($p < 0.05$).

Fat A - 88% PMF, 1% PKO, 11% POs IV 33.

Fat B - 89% PMF, 1% PKO, 10% POs IV 33.

Fat C - 91% PMF, 1% PKO, 8% POs IV 33.

Fat D - 92% PMF, 1% PKO, 7% POs IV 33.

Fat E - 96% PMF, 1% PKO, 3% POs IV 33.

Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.

Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.

Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.

SAFA - saturated fatty acid; MUFA - monounsaturated fatty acid; PUFA - polyunsaturated fatty acid;

PMF - palm mid fraction; PKO - palm kernel oil; POs - palm stearin.

TABLE 4. TRIACYLGLYCEROL (TAG) COMPOSITION OF VARIOUS FAT BLENDS

TAG (%)	Fats								
	Cocoa butter	A	B	C	D	E	F	G	H
OLL	-	0.19±0.00 ^{ab}	0.17±0.03 ^b	0.14±0.01 ^b	0.25±0.01 ^a	0.19±0.00 ^{ab}	0.15±0.02 ^b	0.14±0.00 ^b	0.17±0.03 ^b
PLL	-	1.48±0.03 ^a	1.26±0.23 ^a	1.22±0.07 ^a	1.53±0.02 ^a	1.50±0.00 ^a	1.20±0.22 ^a	1.14±0.17 ^a	1.23±0.29 ^a
MLP	-	0.68±0.02 ^a	0.31±0.11 ^{abc}	0.36±0.01 ^{abc}	0.58±0.15 ^{ab}	0.69±0.00 ^a	0.22±0.00 ^{bc}	0.30±0.12 ^{abc}	0.44±0.27 ^{abc}
OLO	-	0.55±0.00 ^a	0.60±0.00 ^a	0.59±0.01 ^a	0.60±0.00 ^a	0.55±0.01 ^a	0.58±0.00 ^a	0.57±0.02 ^a	0.55±0.03 ^a
PLO	0.37±0.02 ^e	5.20±0.06 ^{bcd}	5.36±0.01 ^a	5.27±0.00 ^{ab}	5.21±0.02 ^{bcd}	5.25±0.04 ^{bc}	5.19±0.00 ^{bcd}	5.13±0.02 ^d	5.16±0.00 ^{cd}
PLP	1.61±0.17 ^e	10.88±0.07 ^{ab}	10.38±0.05 ^{bcd}	10.34±0.01 ^{cd}	10.74±0.09 ^{abc}	11.06±0.14 ^a	10.10±0.19 ^d	10.30±0.08 ^{cd}	10.10±0.24 ^d
MOP	0.22±0.04 ^a	-	-	-	-	-	-	-	-
MPP	-	0.65±0.01 ^{bc}	0.64±0.01 ^{bc}	0.65±0.01 ^{bc}	0.62±0.00 ^{bc}	0.54±0.00 ^d	0.73±0.02 ^a	0.66±0.00 ^b	0.61±0.02 ^c
OOO	0.17±0.00 ^b	1.12±0.18 ^a	1.34±0.01 ^a	1.32±0.00 ^a	1.34±0.04 ^a	1.19±0.03 ^a	1.29±0.03 ^a	1.29±0.00 ^a	1.29±0.01 ^a
SLO	0.18±0.02 ^a	-	-	-	-	-	-	-	-
POO	1.40±0.01 ^b	12.70±0.76 ^a	13.49±0.02 ^a	13.34±0.10 ^a	13.35±0.18 ^a	13.04±0.06 ^a	13.17±0.08 ^a	13.19±0.10 ^a	13.32±0.12 ^a
POP	16.18±0.21 ^c	48.97±0.33 ^b	48.58±0.06 ^b	49.20±0.33 ^{ab}	48.92±0.32 ^b	50.28±0.56 ^a	48.76±0.23 ^b	49.35±0.03 ^{ab}	49.61±0.32 ^{ab}
PLS	2.78±0.01 ^a	-	-	-	-	-	-	-	-
PPP	1.57±0.00 [*]	4.09±0.03 ^c	4.18±0.00 ^b	3.83±0.03 ^d	3.23±0.00 ^f	2.35±0.02 ^g	4.79±0.03 ^a	4.26±0.02 ^b	3.44±0.02 ^e
SOO	-	1.82±0.04 ^a	1.92±0.01 ^a	1.91±0.05 ^a	1.85±0.01 ^a	1.87±0.05 ^a	1.94±0.02 ^a	1.85±0.01 ^a	1.94±0.11 ^a
POS	39.26±0.01 ^b	9.16±0.12 ^a	9.39±0.24 ^a	9.32±0.02 ^a	9.29±0.08 ^a	9.47±0.16 ^a	9.37±0.02 ^a	9.37±0.06 ^a	9.58±0.22 ^a
PPS	0.53±0.02 ^d	0.99±0.03 ^{abc}	1.05±0.01 ^{abc}	0.96±0.01 ^{abc}	0.82±0.01 ^{bcd}	0.75±0.17 ^{cd}	1.18±0.08 ^a	1.12±0.14 ^{ab}	0.88±0.04 ^{abc}
SOS	31.11±0.33 ^a	1.32±0.43 ^b	1.10±0.01 ^b	1.27±0.23 ^b	1.38±0.11 ^b	1.09±0.05 ^b	1.11±0.05 ^b	1.08±0.02 ^b	1.31±0.34 ^b
SSP	0.80±0.01 ^a	0.20±0.02 ^{bc}	0.22±0.01 ^{bc}	0.28±0.04 ^{bc}	0.28±0.03 ^{bc}	0.18±0.05 ^c	0.23±0.01 ^{bc}	0.25±0.06 ^{bc}	0.36±0.09 ^b
SLS	0.91±0.11 ^a	-	-	-	-	-	-	-	-
AOO	0.42±0.01 ^a	-	-	-	-	-	-	-	-
SOA	2.02±0.03 ^a	-	-	-	-	-	-	-	-
SSS	0.49±0.01 ^a	-	-	-	-	-	-	-	-
UUU	0.17±0.00 ^b	1.12±0.18 ^a	1.34±0.01 ^a	1.32±0.00 ^a	1.34±0.04 ^a	1.19±0.03 ^a	1.29±0.03 ^a	1.29±0.00 ^a	1.29±0.01 ^a
SUU	1.81±0.02 ^b	14.52±0.72 ^a	15.42±0.02 ^a	15.25±0.06 ^a	15.20±0.17 ^a	14.91±0.11 ^a	15.11±0.10 ^a	15.04±0.09 ^a	15.26±0.22 ^a
SUS	88.78±0.09 ^a	59.45±0.89 ^{bc}	59.07±0.28 ^c	59.79±0.08 ^{bc}	59.60±0.28 ^{bc}	60.84±0.35 ^{bc}	59.23±0.26 ^c	59.79±0.05 ^{bc}	60.50±0.44 ^{bc}
SSS**	7.12±0.08 ^e	18.97±0.08 ^a	18.05±0.28 ^{bc}	17.64±0.12 ^{cd}	17.80±0.07 ^{bc}	17.06±0.25 ^d	18.44±0.15 ^{ab}	18.03±0.00 ^{bc}	17.06±0.23 ^d

Note: M - myristic; P - palmitic; S - stearic, O - oleic; L - linoleic; UUU - triunsaturated; SUS - disaturated; SSS** - trisaturated.

* The value is shared with PPP and SOO.

Each value in the table is the mean ± standard deviation of two measurements.

Means within each row bearing different superscript letters are significantly different (p<0.05).

Fat A - 88% PMF, 1% PKO, 11% POs IV 33.

Fat B - 89% PMF, 1% PKO, 10% POs IV 33.

Fat C - 91% PMF, 1% PKO, 8% POs IV 33.

Fat D - 92% PMF, 1% PKO, 7% POs IV 33.

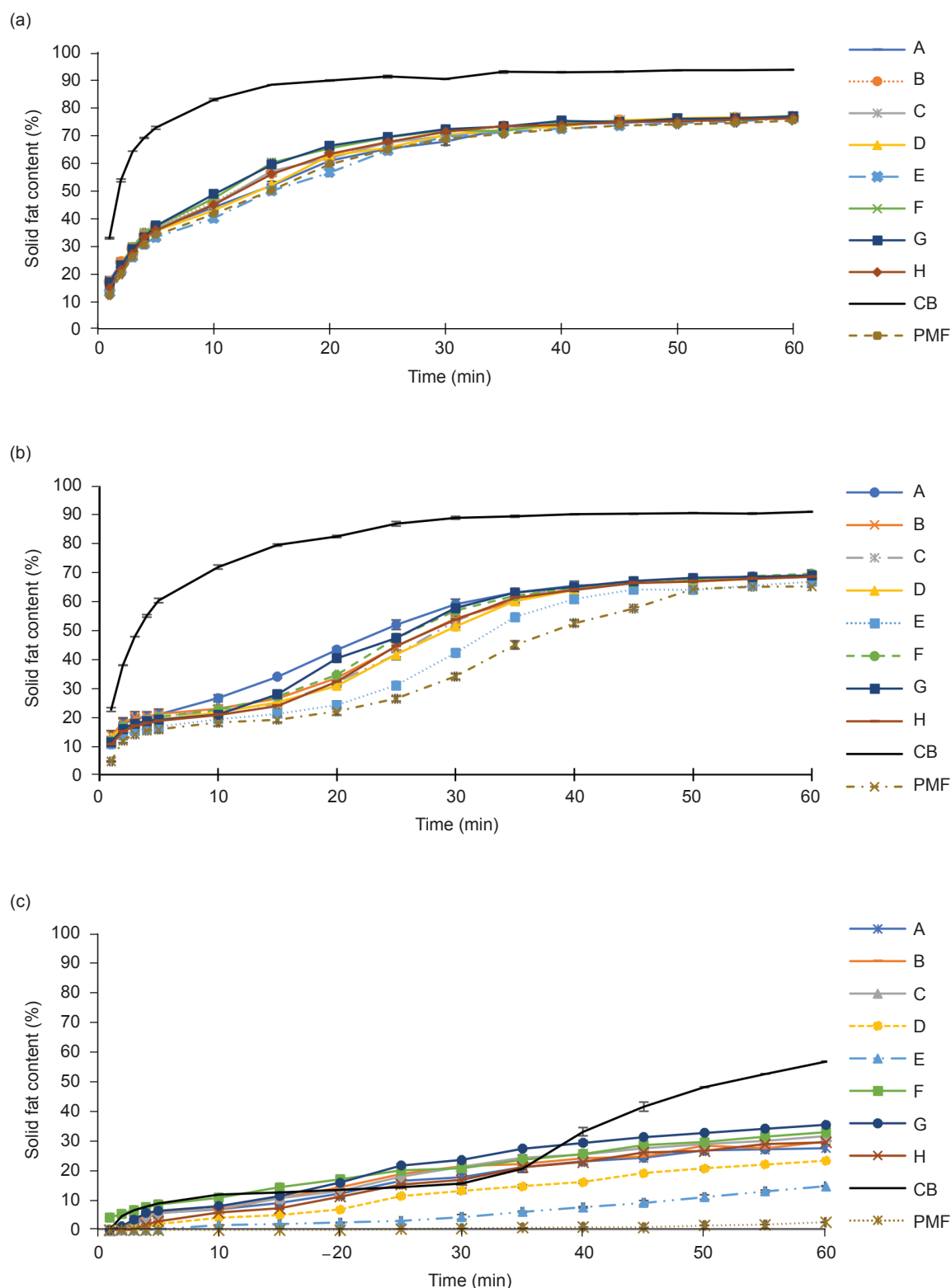
Fat E - 96% PMF, 1% PKO, 3% POs IV 33.

Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.

Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.

Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.

PMF - palm mid fraction; PKO - palm kernel oil; POs - palm stearin.



Note: Fat A - 88% PMF, 1% PKO, 11% POs IV 33.
 Fat B - 89% PMF, 1% PKO, 10% POs IV 33.
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 Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.
 CB - cocoa butter; PMF - palm mid fraction; PKO - palm kernel oil; POs - palm stearin.

Figure 2. Crystallisation rate of all fats at isothermal temperature of (a) 5°C, (b) 10°C, and (c) 20°C.

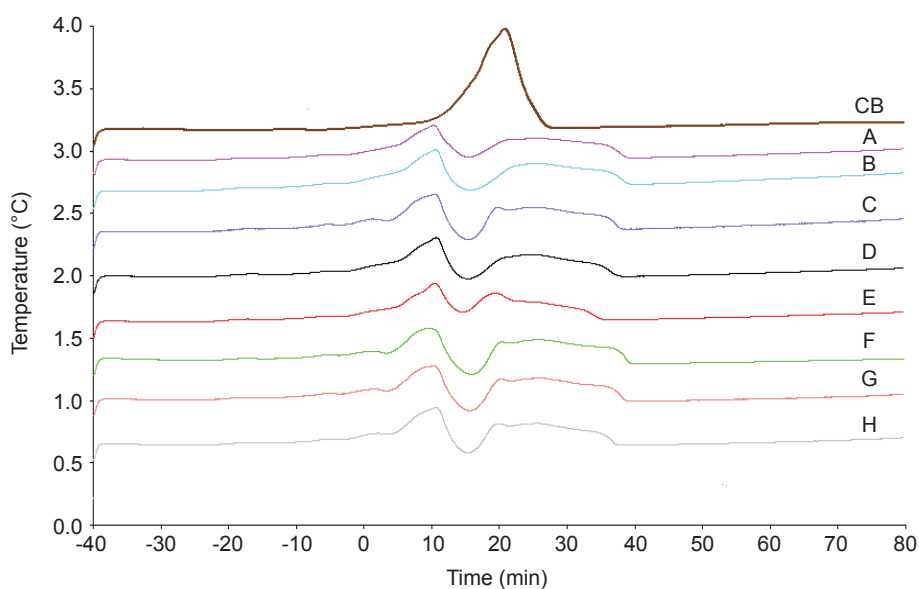
Thermal Behaviour

Thermal melting profiles of all fats are shown in Figure 3. CB produced one sharp thermal melting peak at 22°C which is similar to the finding by Sonwai *et al.* (2014). Two endothermic peaks were observed; low and high melting peaks having sharp and broad peaks, respectively were observed for all the fats. The range of the sharp peaks was from 9.26°C-10.64°C while for the broad peak was from 25.38°C-27.89°C. As all of the fats were PMF dominant, the result was in accordance with the study of PMF by Sonwai *et al.* (2014) in which PMF produced two distinguished peaks. These fats were observed to have small difference in melting temperatures and were mostly influenced by FAC and TAG compositions (Tables 3 and 4). The low melting peaks may consist of UUU and SUU TAG such as OOO, POO and SOO while the high melting peaks may be an overlapping of saturated-saturated-unsaturated (SSU), SUS and SSS TAG such as PPO and POP for the SSU and SUS TAG and PPP for the SSS TAG. It was observed that the higher the level of PMF in the fats (indicated by fats A-H in Figure 3 having PMF in increasing trend), the low melting peaks shifted more to the left producing slightly lower melting temperature. In addition, lesser POs made the melting curves moved to the left having slightly lower melting temperature too. The broad melting peak may suggest tailing or waxiness to the end product as it may consist of PPP TAG. In

general, thermal behaviour of all the fat blends was different from CB. However, they had broad high melting peaks that may assist crystallisation rate and consequently the mouldability property.

Polymorphism

Crystallisation of a molecule in distinctive configuration of crystal packing is defined as polymorphism. The static crystallisation of CB, PMF, PKO, POs IV 33 and IV 14 and fats A-H at 10°C for 30, 45 and 60 min is demonstrated in Figure 4. At 10°C, CB displayed β' polymorphism having two spacings at 4.2 Å and 3.9 Å. Similar findings were reported by Marangoni and McGauley (2003) in which they observed β' polymorphism for CB for temperature between -15°C and 20°C. Three peaks at spacings of 3.8 Å, 4.2 Å and 4.3 Å were detected for individual PMF having high percentage of POP. The spacings of 3.8 Å and 4.2 Å demonstrated β' polymorphism and the spacing of 4.3 Å was approximate of that pseudo- β' configuration (Sonwai *et al.*, 2014). POs IV 33 showed spacings of 4.6 Å, 4.3 Å, 4.2 Å and 3.9 Å indicating β' and β polymorphism. POs IV 14 (high in PPP) showed spacing at 4.5 Å, 4.1/4.2 Å and 3.9 Å indicating β' and β polymorphism. PKO (high in LaLaLa) had β' polymorphism from the spacings of 3.8 Å, 4.2 Å and 4.4 Å which was similar to the finding by Zhou *et al.* (2010).



Note: Fat A - 88% PMF, 1% PKO, 11% POs IV 33.
 Fat B - 89% PMF, 1% PKO, 10% POs IV 33.
 Fat C - 91% PMF, 1% PKO, 8% POs IV 33.
 Fat D - 92% PMF, 1% PKO, 7% POs IV 33.
 Fat E - 96% PMF, 1% PKO, 3% POs IV 33.
 Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.
 Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.
 Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.
 CB - cocoa butter; PMF - palm mid fraction; PKO - palm kernel oil; POs - palm stearin.

Figure 3. Melting profiles of blended fats and cocoa butter.

β polymorphism is promoted by trisaturated TAG such as PPP (Nusantoro, 2009; Basso *et al.*, 2010) and SSS (Oleivera *et al.*, 2015). Sato (2001b) and Biswas *et al.* (2017) reported that POP/POS were accountable for β' polymorphism.

Fats A-H (high in POP) tempered at 10°C for 30, 45 and 60 min (Figures 4a, b and c) showed β' polymorphism with spacings of 3.8 Å, 4.1/4.2 Å and 4.3 Å were similar to PMF as PMF was the main fat used in the formulation. Spacing between 3.8-4.3 Å was referred to as having β' polymorphism (Biswas *et al.*, 2017). The amounts of POs IV 33 and 14 were too small to be detected at spacing of 4.5/4.6 Å that represented β polymorphism. The physical structure of fats may also change with time at constant temperature because of polymorphic transformations (Metin and Hartel, 2005). However, the experimental time difference in this study may be too short for the polymorphic transformation. In this study, all blended fats were comparable with CB (β' dominance) in terms of polymorphism at 10°C.

Crystal Morphology

By using PLM, crystal morphology of individual CB, PMF, POs IV 33, POs IV 14, PKO as well as ternary and quaternary fats of PMF, POs IV 33, POs IV 14 and PKO at 10°C, 20°C and 25°C were observed (Figures 5-7). Crystallisation of fat is crucial for fat-based product developments particularly in chocolate confectionery. Crystallisation of fat consists of several steps which include primary crystallisation, development of microstructure, formation of continuous 3-dimensional crystal network and finally formation of solid bridge called sintering between aggregated crystals and aggregates (Delbaere *et al.*, 2016).

At 10°C, all fats including CB, PMF, POs IV 33, POs IV 14 and PKO showed dense crystal structures. CB and PKO had small granular and well dispersed crystal structures. POs IV 33, on the other hand, had rougher but well dispersed crystal structure. PMF showed unclear spherulitic needle-like crystal structure radiating and branching out from the central nucleus due to low temperature. It could be mistaken with granular crystal structure. All of the blended fats did not show much variation in crystal structure and reflected crystal structure of unclear spherulitic needle-like crystal structure radiating and branching out from the central nucleus and granular. Among the blended fats, fat F had the densest crystal structure due to the addition of POs IV 14. There was a declining trend of crystal packing from fat A to fat E as well as from fat F to fat H indicating decreasing amounts of POs in the fat blends.

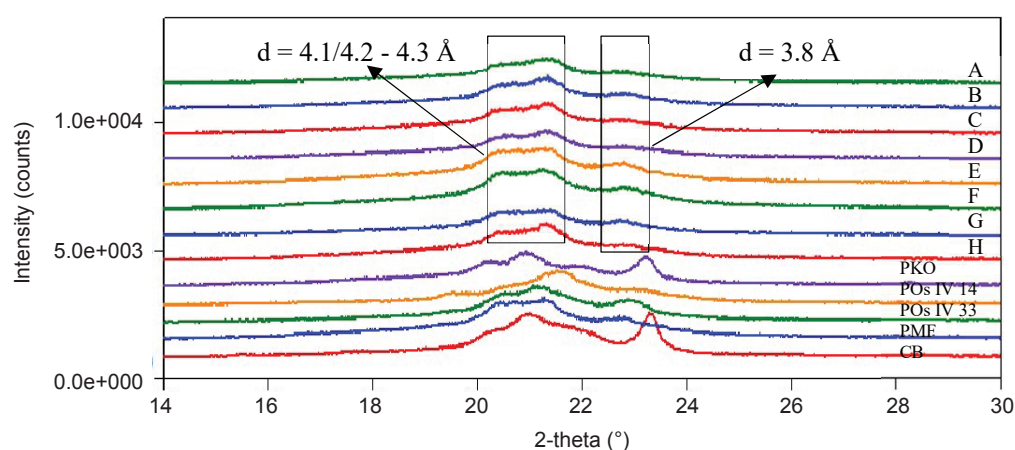
At 20°C, all fats including CB, PMF, POs IV 33, POs IV 14 and PKO had slightly lost their crystal packing compared to fats at 10°C. Fats A, B, C,

D and E showed spherulitic needle-like crystal structure radiating and branching out from the central nucleus at 20°C. This structure became clearer when the level of POs lessened. These fats showed a good trend of decreasing POs IV 33 when these crystals became less dense in packing when only 3% of POs IV 33 was added to fat E. Similar scenario was observed by Norizzah *et al.* (2004) when palm kernel olein was added to POs. Structures of evenly distributed small granular crystal were observed in fats F and G due to the addition of POs IV 14. These crystals might be more stable (especially in terms of heat) than other fats as they have denser packing and evenly distributed small crystal structure. Small and dense crystal microstructure might implicate the macroscopic and mechanical attributes of the fat (*i.e.* hardness) as reported by Bootello *et al.* (2018) for their sunflower stearin-based CBE. Fat H having lesser POs IV 14 (2%) was less dense in packing compared to fats F and G.

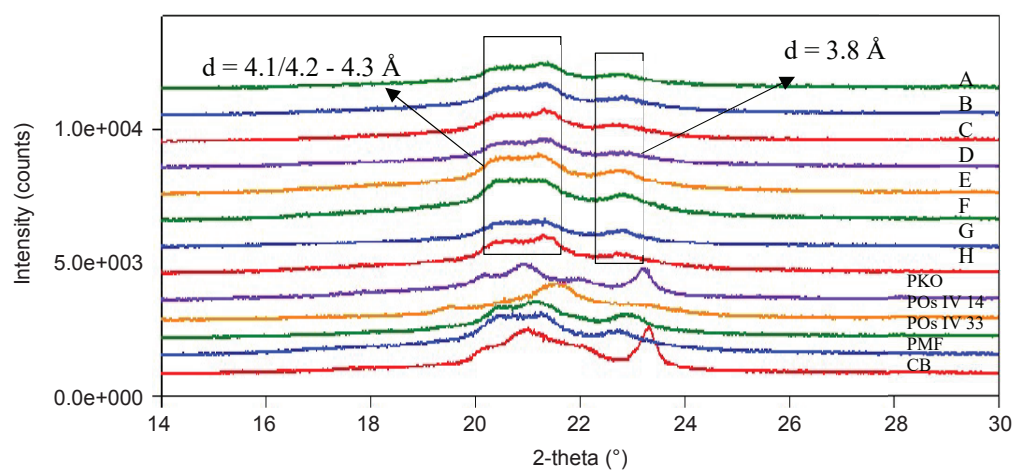
Spherulitic needle-like crystals structure was observed in commercial CB at 25°C. The structure was similar to the findings by Sonwai *et al.* (2014). Bigger and clearer spherulitic needle-like crystals structure was observed in PMF. Granular crystals were observed for POs IV 33 and POs IV 14. Granular crystals of POs IV 14 were observed to have denser packing. The morphology of PKO displayed feather-like crystals. It was observed that the crystal structure of fats A, B, C, D and E were quite similar to one another having spherulitic needle-like crystal structure radiating and branching out from the central nucleus. However, they were different in terms of crystal packing depending on the amounts of POs IV 33. Notwithstanding this, similar crystal structures were found at 20°C with less dense packing. Fats F, G and H also showed spherulitic needle-like crystal structure radiating and branching out from the central nucleus. However, the nucleus of the structure had become larger. The decremental addition of POs IV 33 and IV 14 to PMF led to the development of large and loosely packed crystals. Fat F showed the densest crystal packing compared to other fats. According to Delbaere *et al.* (2016), a denser crystal packing in chocolate reduced overall mobility of fat resulting to the decreased rate of oil migration.

It can be concluded that crystal structure for all fats at temperatures of 10°C, 20°C and 25°C varied from one to another. The disparity might be due to different TAG (Table 4) composition and distinct textural properties of PMF, PKO and POs IV 33 and IV 44 (Çiftçi *et al.*, 2009) which relates to the chemical composition of the fat as well as temperature. In addition, crystal size is dependent on temperature history. The packing (density) of fat F was comparable with CB and therefore, might impose good mouldability property.

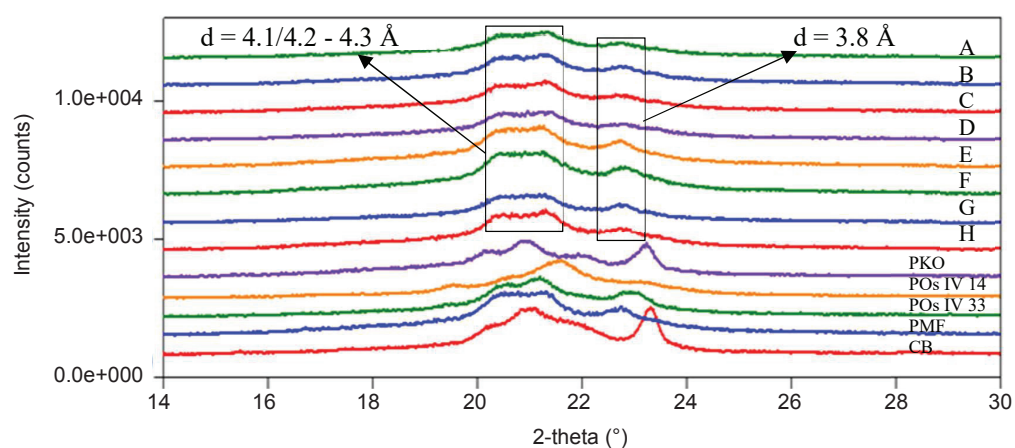
(a)



(b)



(c)



Note: Fat A - 88% PMF, 1% PKO, 11% POs IV 33.

Fat B - 89% PMF, 1% PKO, 10% POs IV 33.

Fat C - 91% PMF, 1% PKO, 8% POs IV 33.

Fat D - 92% PMF, 1% PKO, 7% POs IV 33.

Fat E - 96% PMF, 1% PKO, 3% POs IV 33.

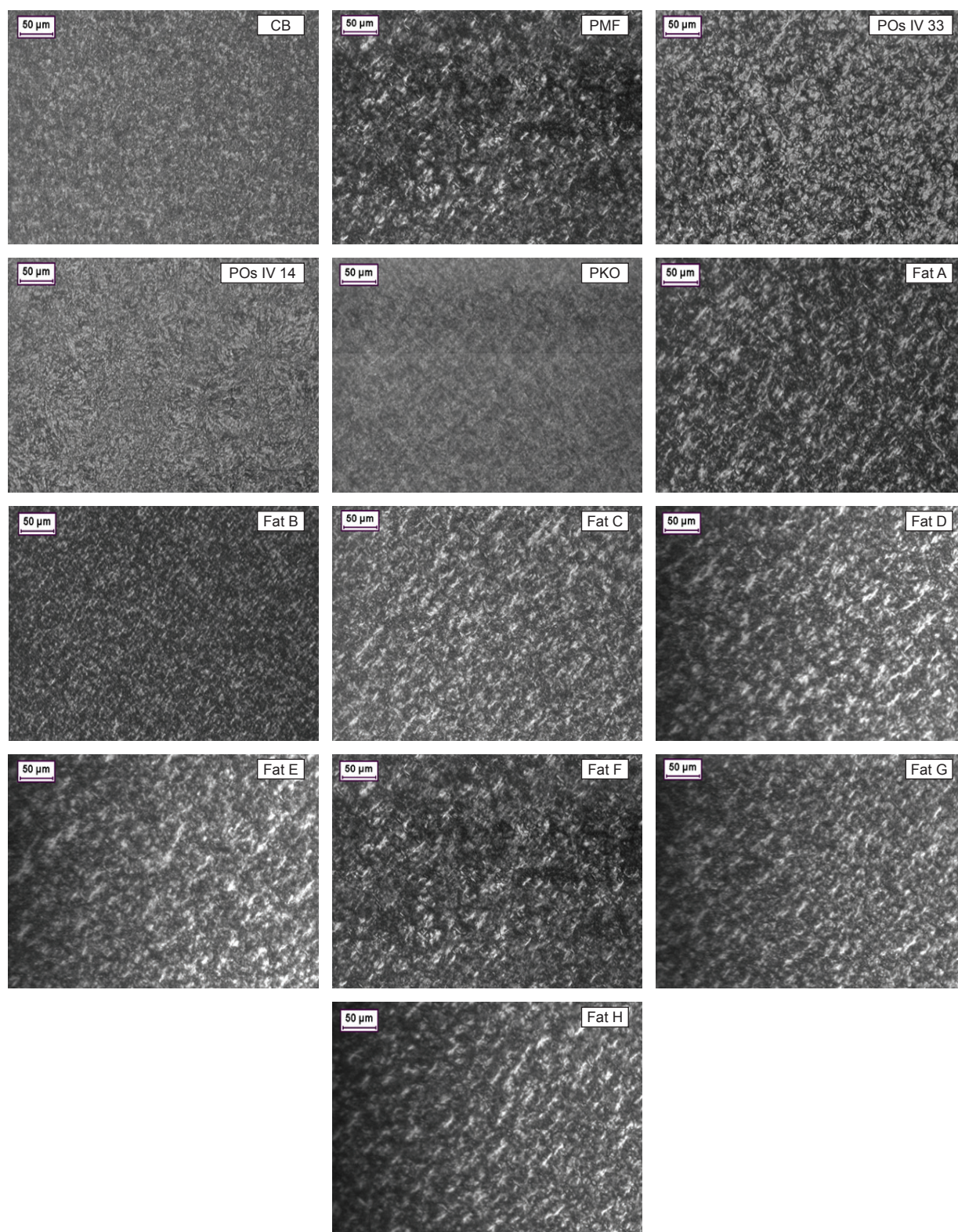
Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.

Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.

Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.

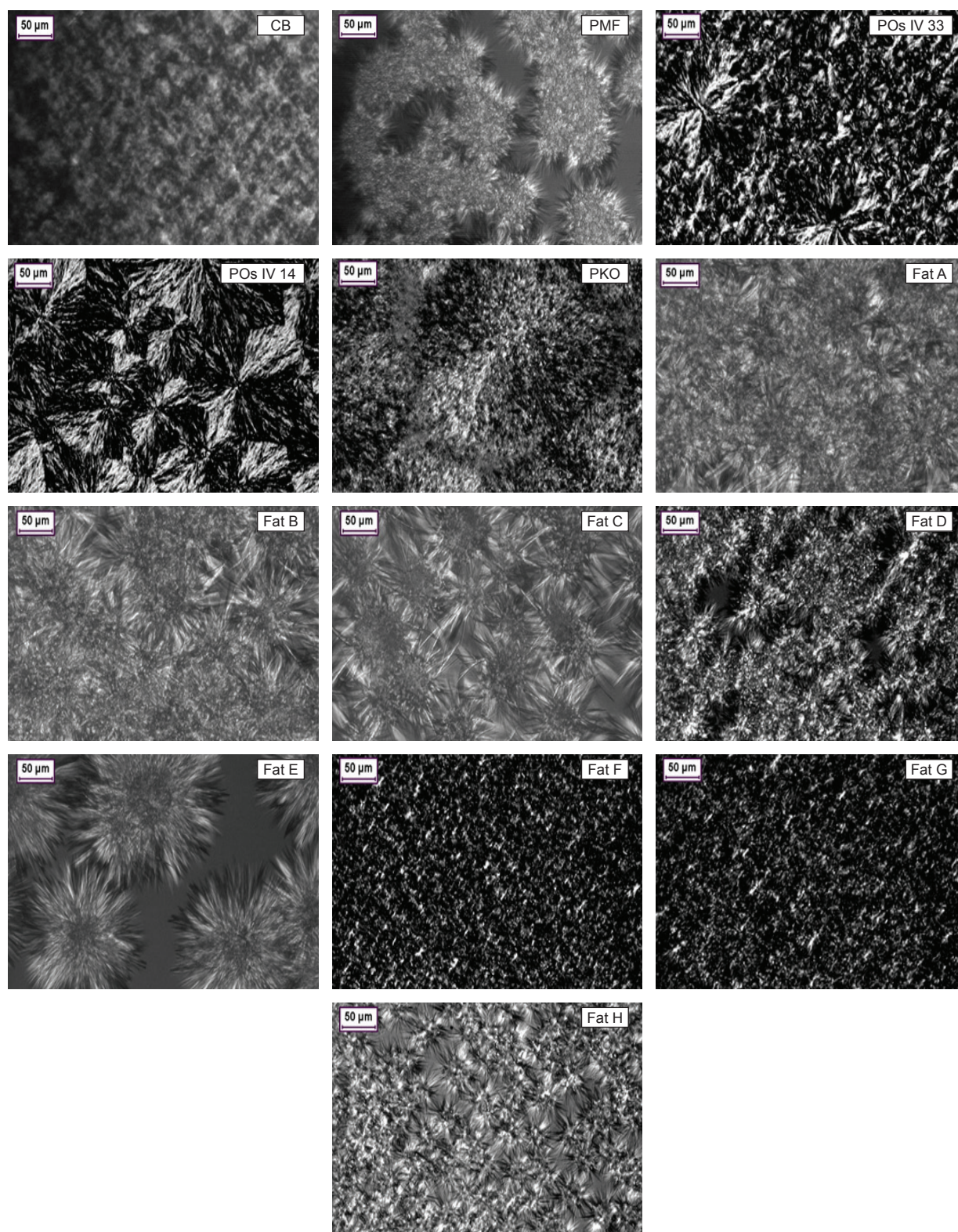
PKO - palm kernel oil; POs - palm stearin; PMF - palm mid fraction; CB - cocoa butter.

Figure 4. Polymorphism of all fats at (a) 30 min, (b) 45 min, and (c) 60 min.



Note: Fat A - 88% PMF, 1% PKO, 11% POs IV 33.
 Fat B - 89% PMF, 1% PKO, 10% POs IV 33.
 Fat C - 91% PMF, 1% PKO, 8% POs IV 33.
 Fat D - 92% PMF, 1% PKO, 7% POs IV 33.
 Fat E - 96% PMF, 1% PKO, 3% POs IV 33.
 Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.
 Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.
 Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.
 PMF - palm mid fraction; POs - palm stearin; PKO - palm kernel oil; CB - cocoa butter.

Figure 5. Polarised light micrographs (x20 lens) of cocoa butter, PMF, POs IV 33, POs IV 14, PKO and fats A-H at 10°C.



Note: Fat A - 88% PMF, 1% PKO, 11% POs IV 33.

Fat B - 89% PMF, 1% PKO, 10% POs IV 33.

Fat C - 91% PMF, 1% PKO, 8% POs IV 33.

Fat D - 92% PMF, 1% PKO, 7% POs IV 33.

Fat E - 96% PMF, 1% PKO, 3% POs IV 33.

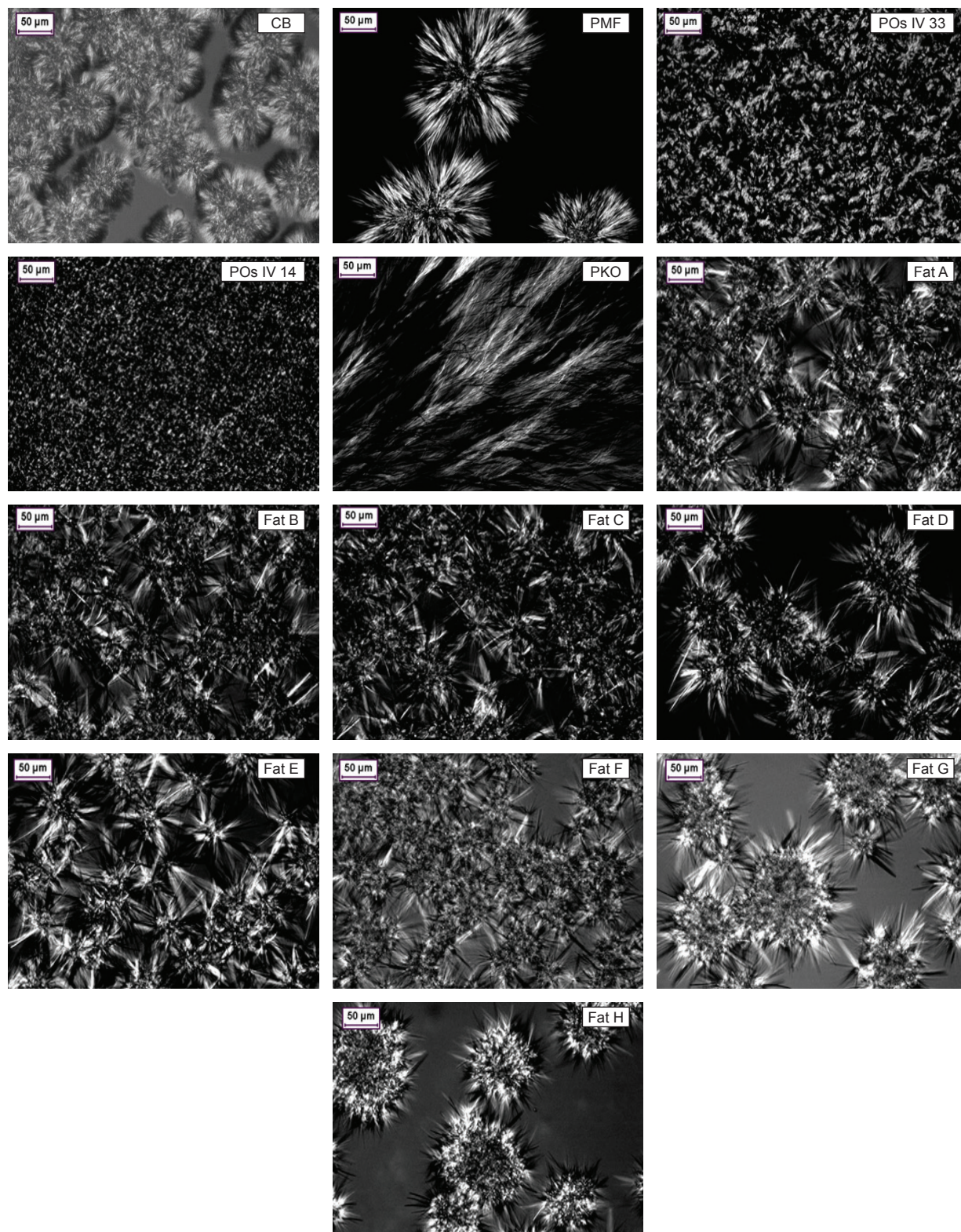
Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.

Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.

Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.

PMF - palm mid fraction; POs - palm stearin; PKO - palm kernel oil; CB - cocoa butter.

Figure 6. Polarised light micrographs (x20 lens) of cocoa butter, PMF, POs IV 33, POs IV 14, PKO and fats A-H at 20°C.



Note: Fat A - 88% PMF, 1% PKO, 11% POs IV 33.
 Fat B - 89% PMF, 1% PKO, 10% POs IV 33.
 Fat C - 91% PMF, 1% PKO, 8% POs IV 33.
 Fat D - 92% PMF, 1% PKO, 7% POs IV 33.
 Fat E - 96% PMF, 1% PKO, 3% POs IV 33.
 Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.
 Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.
 Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.
 PMF - palm mid fraction; POs - palm stearin; PKO - palm kernel oil; CB - cocoa butter.

Figure 7. Polarised light micrographs (x20 lens) of cocoa butter, PMF, POs IV 33, POs IV 14, PKO and fats A-H at 25°C.

Mouldability

Table 5 shows the mouldability of chocolate bars from all blended fats at cooling time of 30, 45 and 60 min. Cooling temperature of 10°C-15°C is typically used by commercial chocolate manufacturers (CB-based) but cooling temperature of 10°C is normally required to eliminate the sensible and latent heat from the liquid chocolate for easy demoulding in order to produce solid chocolate product. The time needed for cooling and solidification relies upon the temperature of the cooling air which translates as the rate of heat transfer from chocolate product to air (Keijbets *et al.*, 2010). The solidification of chocolate that relates to crystal structure renders the hardness of chocolate (Jahurul *et al.*, 2013) and subsequently influences its mouldability.

Chocolate bars prepared from CB and palm-based CBA are able to be moulded and demoulded between 15-20 min (Keijbets *et al.*, 2010; MPOC, 2006; Bolliger *et al.*, 1998). However, chocolate bars from the blended fats were unable to be demoulded even at 30 min of cooling time. Despite of this, chocolate bars from all blended fats were able to be demoulded at 45 and 60 min of cooling time without fracture. Although polymorphism of all fats observed using XRD that were conditioned for 30, 45 and 60 min at 10°C showed β' dominance, the mouldability outcome varied in which chocolate bars from all of the blended fats were mouldable at 45 and 60 min but was not mouldable at 30 min. Solidification time which relates to crystallisation rate plays important role in this scenario. Figure 2 demonstrates that at 40-45 min of crystallisation at isothermal temperature of 10°C, all fats showed overlapping SFC (approximate value of 65%-70% SFC) and began to plateau indicating equilibrium phase had been reached. Therefore, they may be able to be moulded and demoulded easily. Optimised cooling time for all the fats was at 45 min at isothermal temperature of 10°C due to complete crystallisation. Prolonging the crystallisation time gave similar mouldability result to 45 min crystallisation time. In addition, all of the eight fat blends regardless of formulations were able to produce similar mouldability result. However, crystal morphology among all fats were dissimilar which may influence the snappability. In addition, Wainwright (1996) reported that CBS that has higher crystallisation rate compared to CBR imposes rapid crystallisation and has better manufacturing advantage than CBR.

Mechanical Resistance (Snappability)

Table 6 shows the snappability value (rupture tension and breaking force) of chocolate bars from various blended fats at cooling time of 45 and 60 min at 10°C right after demoulding as the chocolate bars at 45 and 60 min were able to be demoulded without

being fractured. Chocolate bars prepared at cooling time of 30 min were omitted as they fractured during demoulding process at 10°C. The snappability of chocolate bars at 45 and 60 min was significantly different from each other ($p < 0.05$). However, fats A, B, C, D, G and H were insignificantly different from each other ($p > 0.05$) at 60 min. Fat F produced the highest rupture tension and breaking force both at 45 and 60 min of chocolate bars at 10°C while fat E showed the lowest values for both parameters. The rupture tension of chocolate bars of fat F at 45 and 60 min were 105.28 $\text{g}_f \text{cm}^{-2}$ and 107.92 $\text{g}_f \text{cm}^{-2}$, respectively while the breaking force of fat F at 45 and 60 min were 5501 g_f and 5639 g_f , respectively. Fat F had the densest crystal structure compared to other fats at 10°C and had influenced on the snappability value.

The addition, POs IV 33 and POs IV 14 significantly influenced the snappability values when compared to 100% PMF (having the lowest snappability value). Therefore, the addition of POs IV 33 and POs IV 14 influenced the snappability value of the chocolate bar. Increasing trend of snappability values were observed with increasing amount of added POs IV 33 and POs IV 14. However, the increase in snappability value became more prominent when POs IV 14 was added. This also showed that POs IV 33 and POs IV 14 are chemically compatible with PMF. Snappability of chocolate is associated to ingredients in the formulation (Quast *et al.*, 2011) which consequently relates to the mechanical strength of network of fat crystal (Smith, 2009; Marangoni, 2002). Minor changes in the TAG composition can give huge impact on its crystallisation and further development of the fat structure (De Graef *et al.*, 2012; Himawan *et al.*, 2006 and Sato, 2001a). Incompatible fats such as blend of CB with lauric-based CBS showed decreased snappability value due to incompatibility between TAG as has been reported in many articles including Quast *et al.* (2013). Right crystal formation and its homogeneity also influenced snappability as reported by Rowat *et al.* (2011).

According to Timms (2003), chocolate should be crystalline at 20°C and therefore will impart good snappability. However, all of the chocolate using fats A-H had difficulties to retain their shape at 20°C and therefore, the best fat having highest rupture tension and breaking force at 10°C (fat F) was blended with CB.

Compatibility with Cocoa Butter

The compatibility of fat F and CB at 20°C is illustrated in Figure 8. Compatibility of fats refers to eutecticity (softening) when different types of molecular volume, shape and polymorph are found for each fat (Gold *et al.*, 2011). Fat F was found to be fully miscible with CB with no

significant eutecticity observed except for dilution and dissolution effect. However, 70% and 80% addition of fat F to CB showed minor lowering of SFC. Addition of 10%-30% of fat F to CB provided the best mixtures as there were no lowering of SFC observed and they had high SFC as well. Sonwai *et al.* (2014) reported that their blended CBE from mango kernel fat and PMF with CB having a decreasing trend of SFC with different CBE/CB ratio as being fully compatible and does not show

any sign of eutecticity. Jeyarani and Reddy (1999) also reported that their mixtures of kokum fat and mahua stearin which showed an increasing trend of SFC as fully compatible with each other with no major changes in solid phase or melting range other than changes in dilution and dissolution. Fat F (30%) and CB (70%) was able to be demoulded by 30 min and produced snappability (breaking force) of 5032.71 ± 85.91 g_f at 20°C after one day stabilisation period.

TABLE 5. MOULDABILITY OF CHOCOLATE BARS AT COOLING TIME OF 30 MIN, 45 MIN AND 60 MIN AT 10°C

Fat	Cooling time 30 min	Cooling time 45 min	Cooling time 60 min
A	Not mouldable	Mouldable	Mouldable
B	Not mouldable	Mouldable	Mouldable
C	Not mouldable	Mouldable	Mouldable
D	Not mouldable	Mouldable	Mouldable
E	Not mouldable	Mouldable	Mouldable
F	Not mouldable	Mouldable	Mouldable
G	Not mouldable	Mouldable	Mouldable
H	Not mouldable	Mouldable	Mouldable

Note: Score reading is average of three replicates.

Fat A - 88% PMF, 1% PKO, 11% POs IV 33.

Fat B - 89% PMF, 1% PKO, 10% POs IV 33.

Fat C - 91% PMF, 1% PKO, 8% POs IV 33.

Fat D - 92% PMF, 1% PKO, 7% POs IV 33.

Fat E - 96% PMF, 1% PKO, 3% POs IV 33.

Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.

Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.

Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.

PMF - palm mid fraction; CB - cocoa butter; PKO - palm kernel oil; POs - palm stearin.

TABLE 6. RUPTURE TENSION AND BREAKING FORCE OF CHOCOLATE BARS AT COOLING TIME OF 45 MIN AND 60 MIN AT 10°C

Fat	45 min		60 min	
	Rupture tension (g _f cm ⁻²)	Breaking force (g _f)	Rupture tension (g _f cm ⁻²)	Breaking force (g _f)
A	86.81±11.22 ^{bcd}	4536±586 ^{bcd}	93.57±2.56 ^{bc}	4889±134 ^{bc}
B	85.45±7.81 ^{cde}	4465±408 ^{cde}	88.11±6.26 ^{bc}	4604±327 ^{bc}
C	74.91±1.61 ^{def}	3914±84 ^{def}	84.25±4.82 ^{bc}	4402±252 ^{bc}
D	72.63±1.59 ^{ef}	3795±83 ^{ef}	83.23±6.72 ^{bc}	4349±351 ^{bc}
E	67.37±3.69 ^f	3520±193 ^f	81.97±7.58 ^c	4283±396 ^c
F	105.28±6.51 ^{ab}	5501±340 ^{ab}	107.92±4.42 ^b	5639±231 ^b
G	92.06±1.00 ^{bcd}	4810±52 ^{bcd}	95.08±0.80 ^{bc}	4968±42 ^{bc}
H	86.33±3.37 ^{bc}	4511±176 ^{bc}	92.27±7.77 ^{bc}	4821±406 ^{bc}
PMF	66.26±4.34 ^f	3462±227 ^f	91.41±4.00 ^{bc}	4776±209 ^{bc}
CB	121.44±8.56 ^a	6345±447 ^a	162.39±20.84 ^a	8485±1089 ^a

Note: Score reading is average of three replicates.

Means within each row bearing different superscript letters are significantly different (p<0.05).

Fat A - 88% PMF, 1% PKO, 11% POs IV 33.

Fat B - 89% PMF, 1% PKO, 10% POs IV 33.

Fat C - 91% PMF, 1% PKO, 8% POs IV 33.

Fat D - 92% PMF, 1% PKO, 7% POs IV 33.

Fat E - 96% PMF, 1% PKO, 3% POs IV 33.

Fat F - 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14.

Fat G - 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14.

Fat H - 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14.

PMF - palm mid fraction; CB - cocoa butter; PKO - palm kernel oil; POs - palm stearin.

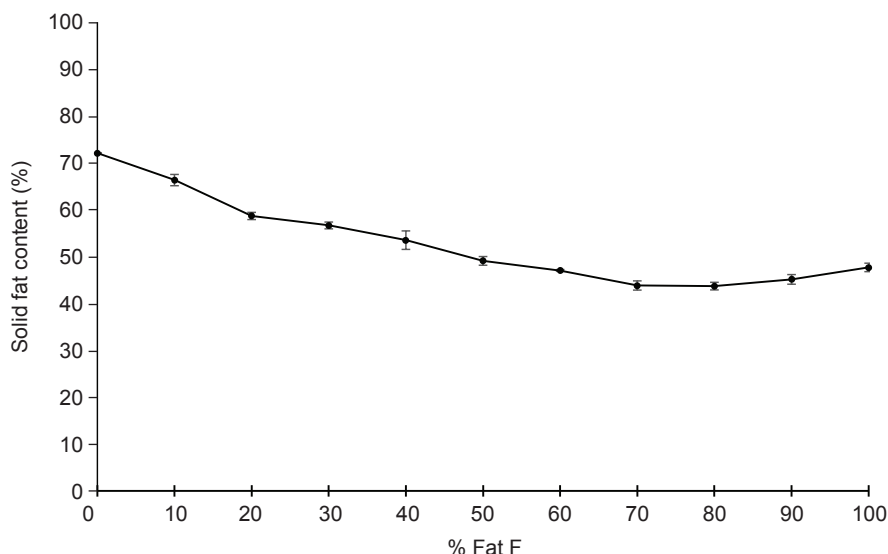


Figure 8. Compatibility of fat F (91% palm mid fraction: 1% palm kernel oil: 5% palm stearin IV 33: 3% palm stearin IV 14) with cocoa butter at 20°C.

CONCLUSION

Eight fat blends namely A) 88% PMF, 1% PKO, 11% POs IV 33, B) 89% PMF, 1% PKO, 10% POs IV 33, C) 91% PMF, 1% PKO, 8% POs IV 33, D) 92% PMF, 1% PKO, 7% POs IV 33, E) 96% PMF, 1% PKO, 3% POs IV 33, F) 91% PMF, 1% PKO, 5% POs IV 33 and 3% POs IV 14 G) 93% PMF, 1% PKO, 3% POs IV 33 and 3% POs IV 14, H) 95% PMF, 1% PKO, 2% POs IV 33 and 2% POs IV 14 of CBA from palm-based fractions (PMF, PKO, POs IV 33 and 14) have been identified through optimised ternary blends. Chocolate bar produced from these blended palm fractions were able to be moulded and demoulded at 45 and 60 min at cooling temperature of 10°C with optimised time of 45 min. The addition of POs IV 33 and IV 14 increased the snappability (rupture tension and breaking force) of all fat blends progressively. Blend of 91% PMF: 1% PKO: 5% POs IV 33: 3% POs IV 14 (Fat F) was observed to have the highest snap value at 10°C. It has comparable oleic acid content, linoleic acid content, monounsaturated fatty acid content, crystal morphology (density) as well as SFC profile at 20°C and crystallisation rate profile at 5°C, 10°C (after 40th min) and 20°C (before 35th min) with CB with dissimilarity in other FAC, TAG, melting behaviour and polymorphism. Therefore, this blend is the most potential CBA to be used as fat for chocolate bar. However, its SFC at 20°C was less than 50% producing a soft chocolate bar. Mixture of 10%-30% of fat blend consisting of 91% PMF: 1% PKO: 5% POs IV 33: 3% POs IV 14 with CB improved the SFC at 20°C and produced SFC of 57%-72% with no eutecticity. Fat blend having 91% PMF: 1% PKO: 5% POs IV

33: 3% POs IV 14 with amount of 30% and CB with amount of 70% was able to be demoulded by 30 min and produce snappability (breaking force) of 5032.71±85.91 g_f at 20°C after one day stabilisation period.

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POTENTIAL OF *Syncephalastrum racemosum* AND *Rhizopus arrhizus* ISOLATED FROM OIL PALM TRUNKS PRODUCE CHITOSANS THAT INHIBIT THE GROWTH OF *Ganoderma boninense*

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ABSTRACT

Chitosan has been proposed as a treatment to suppress *Ganoderma* infection on oil palm. *Syncephalastrum racemosum* and *Rhizopus arrhizus*, growing on oil palm trunk, were examined on their ability to produce chitosan. Chitinous materials from both fungi were deproteinised and deacetylated using different concentrations of sodium hydroxide (NaOH). Chitosans from *R. arrhizus* (FuCsRa), *S. racemosum* (FuCsSr) were compared with crab shells' chitosan (CrCs) through the Fourier-transform infrared spectroscopy (FTIR) analysis. The deacetylation degrees of different chitosan sources were also determined. FTIR analysis showed that chitosans produced by these fungi and crab shell's chitosan had a similar pattern of FTIR spectrum, but differed in their deacetylation degrees. The next experiment examined the ability of these chitosans to suppress the growth of *Ganoderma boninense* (G. *boninense*) in vitro. The experiment consisted of 11 treatments: controls, media with CrCs (at 5000, 7500, 10 000 ppm), media with FuCsRa (at 5000, 7500, 10 000 ppm), media with FuCsSr (at 5000, 7500, 10 000 ppm) and antagonistic test (*S. racemosum*; *R. arrhizus*). The results showed that CrCs at 5000, 7500 and 10 000 ppm enhanced the growth of G. *boninense*. A concentration of 10 000 ppm of CrCs nearly doubled the growth of *Ganoderma* compared to control. FuCsSc inhibited the growth of G. *boninense* at all concentrations from -28% to 31% compared to control. The in vitro antagonistic effect of *S. racemosum* showed a high effect at inhibiting the growth of G. *boninense* (-43% compared to control). This in vitro study demonstrated the ability of chitosan extracted from fungi growing on oil palm trunks to suppress *Ganoderma* growth.

Keywords: chitosan, crab shells, *Ganoderma boninense*, oil palm trunk fungus.

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INTRODUCTION

Ganoderma boninense (G. *boninense*) is a lethal disease that affects the growth of oil palm trees. It is a severe disease in oil palm plantations in North Sumatra, Indonesia, as *Ganoderma* attacks can be severe and harmful. When *Ganoderma* infects a young oil palm, its growth may be severely affected and may lead to its death. Oil palm farmers have tried various products to control the problem. Many

commercial products were offered with the promise of controlling *Ganoderma*. However, there is no scientific study on the efficacy of such products. One belief among oil palm growers is that chitosan can suppress *Ganoderma* attacks.

Chitosan is a biopolymer that has a similar structure to cellulose and can form a membrane. Chitosan has a hydroxyl and amino reactive group which allows it to be chemically modified, such as membrane. Chitosan can be found in nature, at the exoskeleton of crustaceans, mollusks, annelids, coelenterates and insects. Some fungi have the potential to produce chitosan. The fungus *Syncephalastrum racemosum* was found to produce

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the highest concentration of chitosan (152 mg g⁻¹ mycelium) compared to nine other strains (Amorim *et al.*, 2003). *Rhizopus arrhizus* SIS 30 can produce chitosan as much as 12.56 mg g⁻¹ mycelium, which is below the production of chitosan from *Cunninghamella elegans* SIS 41 (15.63 mg g⁻¹ mycelium) (Berger *et al.*, 2018). A study has found that the addition of chitosan to artificial media could inhibit the growth of phytopathogenic *Colletotrichum* species (Berger *et al.*, 2018).

Sabrina *et al.* (2018) found that *S. racemosum* and *R. arrhizus* can be isolated from chipped oil palm trunks. This study aimed to measure the ability of these two fungi to produce chitosan and investigate if they can be used to inhibit the growth of *Ganoderma*.

MATERIALS AND METHODS

Experimental Site and Treatment Application

This study was conducted at the University of Sumatera Utara from March to November 2018. *Rhizopus arrhizus* strains ATCC 111450, and *Syncephalastrum racemosum* isolates VPCI 1857/11 (identified using DNA marker) were isolated from pieces of oil palm trunk from Labuhan Batu district in North Sumatera Province, Indonesia. The trunk has C= 29.64%, N = 0.53% and C/N = 55.9 (Sabrina *et al.*, 2018). Crab shells were obtained from a crab farm in Langkat district. *G. boninense* was from the plant protection laboratory collection of the Faculty of Agriculture, University of Sumatera Utara.

Extraction of Chitosan and Analysis

The preparation began with washing of the raw crab shell waste with water repeatedly. The shell was dried under the sun until it was completely dry. The dried shell was ground with an electric blender and passed through 50 mesh sieves. The crab shell powder that passed through the 50 mesh sieve was dried in an oven at 60°C for 6 hr. Extraction of chitosan from the crab shells included deproteinisation with 4% (w/v) sodium hydroxide (NaOH) (1:10, 80°C 1 hr), demineralisation with 150 ml 1 M hydrochloric acid (HCl) (1:15), depigmentation with 4% (w/v) sodium chloride (NaCl), deacetylation 40% (w/v) NaOH (1:15, 80°C 1 hr).

Isolated *R. arrhizus* and *S. racemosum* were cultivated on Potato Dextrose Agar (PDA) at 28°C for seven days. A suspension of each strain was prepared and adjusted to contain 10⁷ sporangioles ml⁻¹ using a hemocytometer. A 1 ml suspension was inoculated to sterile petri dishes containing PDA and maintained for 24 hr at 28°C. Cultures were sub-cultured on PDA plates, incubated at 28°C for seven days, then cultivated in liquid PDA separately. Mycelia from both fungi were harvested

by filtration, washed with distilled water and freeze-dried. The dry weight obtained for *S. racemosum* was at 26 g 900 ml⁻¹ and *R. arrhizus* at 28 g 900 ml⁻¹.

The chitosan from 3-10 g lyophilised mycelia was isolated following the procedures of Synowiecki and Al-Khateeb (1997). The overall process involved: deproteinisation with 2% w/v NaOH solution (30:lv/w; 90°C, 2 hr), separation of the alkali-insoluble fraction (AIF) by centrifugation (4000 x g, 15 min), extraction of chitosan from AIF under reflux (10% v/v acetic acid, 40: lv/w, 60°C, 6 hr), and precipitation of chitosan from the extract at pH 9.0, adjusted with 4 M NaOH solution. Chitosan was washed on a coarse sintered glass funnel (G-4) with water, ethanol, and acetone and air-dried at 20°C.

The chitosans obtained from the crab shells and the fungi were characterised using fourier-transform infrared spectroscopy (FTIR) a Shimadzu IR-Prestige 21. The materials were made into pellets with 1% KBr (Kaban, 2007).

The degree of deacetylation (DD) was calculated from FTIR spectra using the baseline method of Domszy and Rovert (Khan *et al.*, 2002). The absorbance value was calculated using the formula:

$$A = \log (P_0/P)$$

P₀:% transmittance at a minimum peak. P:% transmittance on the baseline. A comparison between absorbance at A = 1655 cm⁻¹ (amide band absorption 1) with absorbance at A = 3450 cm⁻¹ (hydroxyl group uptake) was calculated. The chitin N-deacetylation (100%) obtained from absorbance value at 1655 cm⁻¹ was 1.33. The degree of N-deacetylation can be calculated by:

$$\%N\text{-deacetylation} = 1 - \frac{AA_{1655}}{AA_{3450} \times 1.33} \times 100$$

A1655: Absorbance at a wavelength of 1588 cm⁻¹ amide/acetamide for group absorption. A3450: Absorbance at 3410 cm⁻¹ hydroxyl (OH) wavelength.

The chitosans were also characterised in terms of colour, particle-size, odour and pH. Colour was determined visually, particle-size was based on feel, while odour was based on smell. The pH was determined in a 1:1 soil-to-water suspension.

Inhibition Test of Growth of *G. boninense* by *S. racemosum*'s Chitosan (FuCsSr), *R. arrhizus*'s Chitosan (FuCsRa) and Crab Shell's Chitosan and Antagonistic Test with *S. racemosum* and *R. arrhizus* Fungi

The inhibitory test had 11 treatments in a complete randomised design with three replications. The treatments were: Control (without treatment); media containing chitosan crab shell (CrCs) at 5000,

7500 and 10 000 ppm; media containing chitosan *R. arrhizus* (FuCsRa) at 5000, 7500, 10 000 ppm; media containing chitosan *S. racemosum* (FuCsSr) at 5000, 7500, 10 000 ppm and antagonist test with *S. racemosum* and *R. arrhizus*.

Dextrose agar media with FuCsSr, FuCsRa and CrCs were made by dissolving FuCs and CrCs. The FuCS solution was made in 1% (v/v) acetic acid and added to the PDA (pH adjusted to 5.6 with NaOH 30% (b/v) to obtain a final FuCsSr and FuCsRa concentration of 5000, 7500 and 10 000 ppm (Berger *et al.*, 2018).

Inhibitory tests were carried out by growing *G. boninense* on chitosan-treated media, and also on petri dishes with *S. racemosum* and *R. arrhizus* isolates. Isolated *S. racemosum* and *R. arrhizus* were placed at a distance of 1 cm from the edge of the petri dish the *G. boninense* colony was cultured on the opposite side with a distance of 1 cm from the edge of the petri dish. All isolates were obtained from their respective cultures using cork borer with a diameter of 4.3 mm. The plates were incubated at room temperature. The antagonistic ability calculation used the inhibition zone formula. Observations were made at 1 day, 2 days, and 3 days after inoculation (DAI) on the inhibitory ability by measuring the areas of *Ganoderma* colonies using the formula $A = 2\pi r^2$, where A = area; r = radius of *Ganoderma* colony.

RESULTS AND DISCUSSION

Chitosan Production from Crab Shells, *S. racemosum* and *R. arrhizus*

The characterisation of chitosan extracted from crab shells, *S. racemosum*, and *R. arrhizus* using FTIR is shown in Figures 1-3. Most amide groups were converted into amine groups through the

deacetylation process. The specific wavenumber at 1647.21 cm^{-1} in CrCs and both FuCs was the absorption of the C=O amide group left from chitin, indicating the presence of an acetyl group, which was reduced due to the process. The uptake of the methyl group ($-\text{CH}_3$) at wavenumber 1435.04 cm^{-1} confirmed the presence of an amide group from chitin (Puspawati and Simpen, 2010). Wavenumber 3437.15 cm^{-1} indicated that the absorption of O-H was overlapping with $-\text{NH}_2$ from amines. Flap $-\text{NH}_2$ absorption band and twist at 894.97 cm^{-1} confirmed that chitosan had been formed (Figures 1-3).

The chitosan FTIR characterisation showed mixed results in all aspects of character testing. The chitosan was extracted from various sources showed diverse results. The best quality chitosan was extracted from *S. racemosum* with 52.50% deacetylation value and the lowest was from *R. arrhizus* which was 29.10% (Table 1).

Characterisation of chitosan was carried out including colour, particle-size, aroma, pH and deacetylation degree. The results showed that chitosan with chitin deacetylation had a similar physical appearance. The differences were only apparent in the number of parameters. The extracted chitosans had a fine powder texture and coarse powder texture. The chitosan extracted from the crab shells gave out odour while chitosans extracted from the fungi were odourless. The pH level of all chitosans was alkaline with a value between 11.7 to 12.7 and had a degree of deacetylation of 29.10% up to 52.50% (Table 2).

Chitosans extracted from the crab shell, *S. racemosum* and *R. arrhizus* had similar characteristics (Kumar, 2000). The readings using FTIR showed the results of different deacetylation degrees of each chitosan. The results showed that the chitosan from crustacean and chitosans from fungi were very different. The deacetylation process causes chitosan to contain

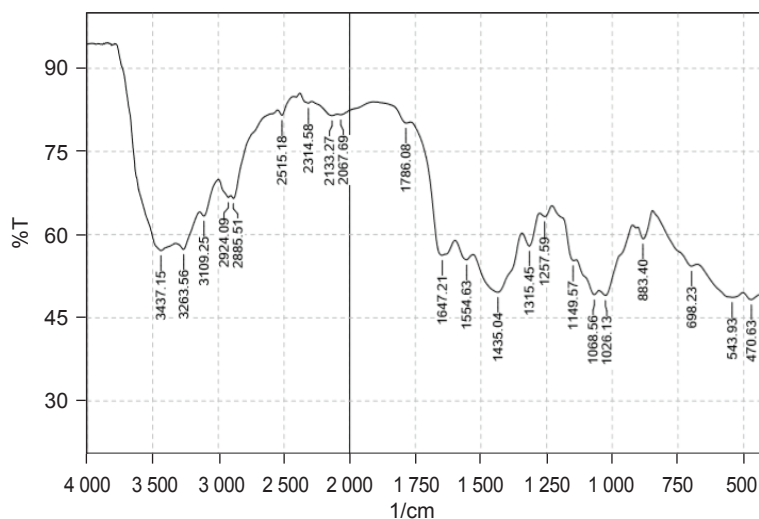
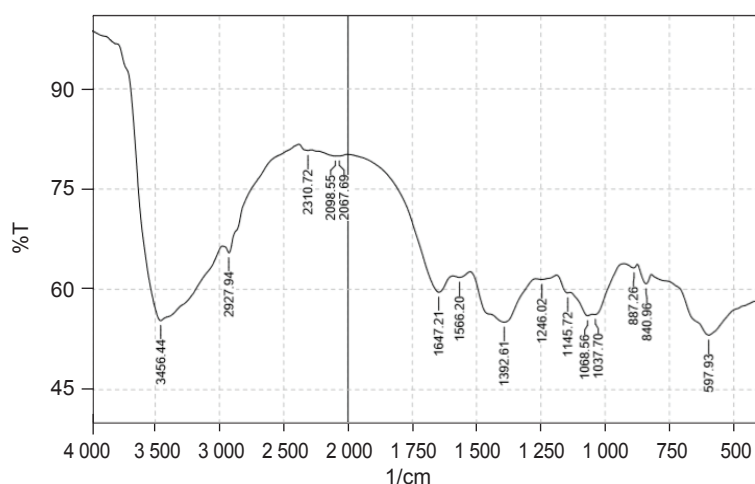
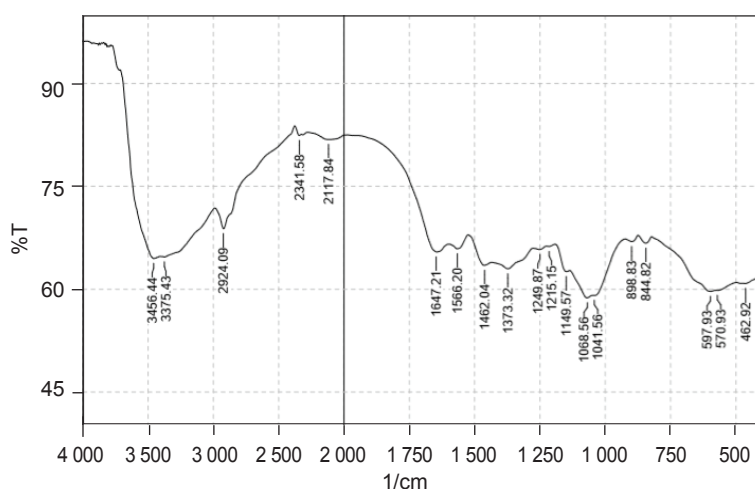


Figure 1. Chitosan spectrum origin of a crab shell.

Figure 2. Chitosan spectrum origin of *S. racemosum*.Figure 3. Chitosan spectrum origin of *R. arrhizus*.TABLE 1. CHARACTERISATION OF CHITOSAN FTIR SPECTRUM OF CRAB SHELL, *S. rasemosum* AND *R. arrhizus*

No.	Vibration modes	Wavelength (v cm ⁻¹)			
		Standard chitosan*	Crab shell	<i>S. rasemosum</i>	<i>R. arrhizus</i>
1	v(NH ₂) assoc. in primary amines v(OH) assoc. in pyranose ring	3 377.95	3 437.15	3 456.44	3 456.44
2	(vb) C-H Aliphatic	2 922.85	2 924.09	2 927.94	3 375.43
3	(vs) C-H Aliphatic	2 922.80	2 885.51	2 310.72	2 924.09
4	(vs) C-H Aromatic	2 361.41	2 314.58	2 098.56	2 431.58
5	(v) C=H Amide I band	1 660.55	1 647.21	1 647.21	1 647.21
6	(v) C=H) Amide II band amide II band	1 587.94	1 554.63	1 566.20	1 566.20
7	(v) C-H	1 422.73	1 435.04	1 392.61	1 462.04
8	(vs) C-O	1 259.54	1 257.59	1 246.02	1 249.87
9	(vs) C-O	1 154.64	1 149.57	1 145.72	1 149.57
10	v(C-O-C)	1 077.93	1 068.56	1 068.56	1 068.56
11	v(C-O-C)	1 026.63	1 026.13	1 037.70	1 041.56
12	ω β -1,4-glikosidik	897.41	883.40	887.26	898.83

Source: Dompeipen (2017).

amine and hydroxyl groups, which have high chemical reactivity (Adriana *et al.*, 2001). Amine groups can be used immediately in chemical reactions to form salts and acids, for example,

with calcium (Ca²⁺), phosphate (PO₄³⁻), sodium (Na⁺) and several other elements (Sanford, 1989). Stretching vibration at wave number 1647.21 cm⁻¹ is an absorption band of C=O bond group, which

shows the presence of a secondary amine group, while the absorption band of 3437.15 and 3456.44 cm^{-1} is a vibration of the OH and NH_2 groups. This absorption band pattern was the same as that of the standard chitosan spectrum, namely at wavenumbers 1660.55 and 2922.85 cm^{-1} . One characteristic absorption for chitosan compounds is the presence of weak stretch absorption in the area of 1650 cm^{-1} which indicates the presence of a C=O group on the bond ($-\text{NHCOCH}_3$) (Silverstein *et al.*, 1981). The absorption bands at 2885.51, 2927.94 and 2924.09 cm^{-1} in the extracted chitosans and 2922.85 cm^{-1} in standard chitosan showed symmetrical stretching vibration of C-H bonds. The absorption bands at wavenumber 1554.63, 1566.20, and 1566.20 cm^{-1} showed that there was a stretching vibration of the C=O bond, which indicated the presence of carbonyl groups. The standard chitosan spectrum also shows absorption in the same wavenumber area of 1587.94 cm^{-1} (Dompeipen, 2017).

TABLE 2. CHARACTERISATION OF CHITOSANS

Characteristics	Source of chitosan		
	Crab shells	<i>R. arrhizus</i>	<i>S. racemosum</i>
Colour	White	Black	Dark green
Texture	Smooth	Coarse powder	Coarse powder
Aroma	Odour	Odour less	Odour less
pH	12, 7	11, 7	11, 7
DD	43.00%	29.10%	52.50%

Note: DD - degree of deacetylation.

Based on the analysis of the infrared spectrum characteristics compared to standard chitosan, there was no significant difference between the two spectra. The presence of the main spectrum in certain wavelength regions indicates the presence of a major functional group, which indicates that the compound from the deacetylation reaction is

chitosan (Dompeipen, 2017). The characteristic that strengthens the formation of chitosan was characterised by the appearance of wave absorption bands, which were formed at a vibration stretching wave between 3550 and 1655 cm^{-1} . The length of absorption of the waveband indicated the presence of secondary amide groups and OH and NH groups. The loss of the methyl (CH_3) group bound to amide (NHCOCH_3) could be seen from the loss of absorption at wavenumber 2982.65 cm^{-1} and the loss of group C=O an amide (NHCO) known from the loss of absorption band at wavenumber 1670.35 cm^{-1} . The typical absorption of chitosan at wavenumber 1666.30 cm^{-1} shows the vibration stretching of N-H from amide (Silverstein *et al.*, 1981).

Ganoderma Growth Inhibition Test

The *in vitro* inhibitory test on 1, 2 and 3 DAI showed different results in the suppression of *Ganoderma* growth. The incubation test at 1 and 2 DAI showed that *R. arrhizus* was the best treatment in suppressing the growth of *Ganoderma*. Incubation test in 3 DAI showed different results, namely, the treatment with *S. racemosum* inoculation was the best treatment. The incubation results in 3 DAI were not significantly different from the direct treatment of *R. arrhizus* inoculation (Table 3).

The addition of chitosan from crab shells increased the development of *G. boninense*. CrCs concentration of 10 000 ppm in the media resulted in increased growth of *G. boninense* 1.57 times compared to control. This result warrants careful consideration on the source of chitosan for use in *G. boninense* treatment.

Chitosan from *S. racemosum* suppressed *G. boninense* development, but there was no difference in the effect of different concentrations. Chitosan as an antifungal material produced by *R. arrhizus* and *S. racemosum* could affect the growth

TABLE 3. TEST FOR INHIBITION ON GROWTH OF *G. boninense*

Treatments	Development of <i>G. boninense</i> colony area (mm^2)					
	1 day		2 days		3 days	
Control	4.97	ab	10.20	ab	16.20	cd
CrCs 5 000 ppm	4.83	abc	9.63	bc	12.53	de
CrCs 7 500 ppm	5.03	a	10.57	ab	21.47	ab
CrCs 10 000 ppm	4.90	abc	13.13	a	25.47	a
FuCsRa 5 000 ppm	4.53	cd	7.70	bc	12.43	de
FuCsRa 7 500 ppm	4.43	d	10.07	b	16.17	cd
FuCsRa 10 000 ppm	4.63	bcd	9.53	bc	19.73	bc
FuCsSr 5 000 ppm	5.07	a	8.40	bc	11.50	de
FuCsSr 7 500 ppm	5.03	a	7.90	bc	11.13	de
FuCsSr 10 000 ppm	4.63	bcd	9.07	bc	11.40	de
Antagonist test <i>S. racemosum</i>	4.73	abcd	7.73	bc	9.23	e
Antagonist test <i>R. arrhizus</i>	4.43	d	6.37	c	10.13	e

Note: The number followed by the same letter in the same column is not significantly different according to the Duncan test at a 5%.

of *Ganoderma*. This is in line with the research of Berger *et al.* (2018), which stated that Mucolares fungi naturally produce chitin and FuCS on their cell walls during their growth through the action of chitin deacetylase enzymes, which catalyse chitin deacetylation to form chitosan.

Basically, *Ganoderma* does have slightly slower growth. But in this experiment, the growth of the treated *Ganoderma* had slower growth than control. The results of secondary metabolism produced from the fungi *R. arrhizus* and *S. racemosum* affected the growth system of *Ganoderma*. El Ghaouth *et al.* (1997) reported that chitosan inhibits the growth of *in vitro* of most postharvest pathogens. Chitosan was also reported to inhibit the proliferation of *Botrytis cinerea*, causal fungus that damages and stimulates cellular changes in *Rhizopus stolonifer* and *B. cinerea*. The treatment of chitosan was also reported to cause pathogenic hyphae to undergo cellular disorganisation that ranges from the decomposition of cell walls to cytoplasmic disintegration.

CONCLUSION

Chitosan has a fine to a coarse powder particle-size with white, black, dark green colour with a pH ranging from 11.7-12.7. All types of chitosan from this experiment have a purity level (degree of deacetylation) of 29.10%-52.50%. Chitosan can be extracted from fungi found in chipped oil palm trunks.

Chitosan from *S. racemosum* at concentrations of 5000, 7500 and 10 000 ppm were able to inhibit the growth of *Ganoderma* (-28% to -31%), similar to the ability of well-known antagonists *R. arrhizus* (-37%) and *S. racemosum* (-43%). The results of this study indicated that the application of FuCsSr and *S. racemosum* had a significant effect in inhibiting the growth of *Ganoderma in vitro*, therefore it is recommended to carry out a test in the field.

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Contents of the Coming Issue

Journal of Oil Palm Research

Vol. 33 (3) September 2021*

- Potential of Functionalised Cellulose from Oil Palm Biomass as Nitrogen and Phosphorus Based Nutrient Adsorbent – A Review
Mei Mei Chong; Lian See Tan; Nurfatehah Wahyuni Che Jusoh; Masafumi Goto and Sumathi Sethupathi
- Neural Networks Method in Predicting Oil Palm FFB Yields for the Peninsular States of Malaysia
Yousif Y Hilal; Yahya, A; Ismail, W I W and Asha'ari, Z H
- Quality Improvement of Palm Kernel Cake as Broiler Feed using Pre-Cleaning System
Rohaya Mohamed Halim; Ridzuan Ramli; Che Rahmat Che Mat; Nu'man Abdul Hadi; Mohd Firdaus Othman and Astimar Abd Aziz
- Development of Palm-based Vanaspati (Vegetable Ghee): Effects of Crystallisation Procedures on the Product Texture
Rafidah Abd Hamid; Sivaruby Kanagaratnam; Norazura Aila Mohd Hassim; Nur Haqim Ismail; Wan Rosnani Awg Isa and Noor Lida Habi Mat Dian
- Identification and Determination of the Spectral Reflectance Properties of Live and Dead Bagworms, *Metisa plana* Walker (Lepidoptera: Psychidae) using VIS/ NIR Spectroscopy
Mohd Najib Ahmad; Abdul Rashid Mohamed Shariff; Ishak Aris; Izhal Abdul Halin and Ramle Moslim
- Prediction of Acid, Peroxide and TBA Values of Heat-Treated Palm Oil using a Partial Least Squares–Ordinary Least Squares Model Based on Fourier Transform Infrared Spectroscopy
Kusnandar, F; Lioe, H N; Faridah, D N; Anggraeni, R and Pricilia
- Principal Component Analysis (PCA) Evaluation of Liquid Chromatography-mass Spectrometry (LC-MS) Datasets of *Ganoderma boninense* Intracellular Metabolites
Zain Nurazah; Abrizah Othman and Umi Salamah Ramli

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