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EVALUATION OF OIL PALM FROND FOR BIOBUTANOL PRODUCTION BY DIFFERENT *Clostridia* SPECIES

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ABSTRACT

Ideal substrates and biobutanol producers are the key and most important ingredients for efficient biobutanol production. In this research, we evaluated the composition of oil palm fronds based on the different ages of the plant. Fresh oil palm frond (OPF) juice was found to be the most potent OPF component for biobutanol production and was pressed from 5, 10, 15 and 20 yr old trees using a sugarcane pressing machine. The total sugar concentrations were estimated by the use of a DNS and spectrophotometer and were found to be decreasing with the increasing age of the palm oil tree, with the highest sugar obtained at OPF ages 5 and 10. HPLC analysis revealed that glucose was the highest fraction in the juice, accounting for 57% to 66%, while fructose (12%-17%) and sucrose (19%-25%) made up the remaining percentage. Fermentation inhibitors like gallic and ferulic acids were also detected in the juice using the HPLC technique, and their concentrations also increased with age. Therefore, the research screened for a robust bacterial strain that can tolerate these phenolics in the hydrolysate for biobutanol production. Out of the 11 strains of Clostridium *screened, strain A1 was found to successfully utilise the hydrolysate and yield the highest concentration of biobutanol at 2.32 g/L with productivity of 0.064 g/L/hr. The strain was identified as* Clostridium *strain A1 based on 16S rRNA gene sequencing techniques.*

Keywords: biobutanol, *Clostridia* sp, OPF age, phenolic compound, sugar.

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INTRODUCTION

The reliance of most countries in the world on the existing energy source (fossil fuels) as their main supply has resulted in several detrimental environmental consequences, including global warming and air pollution, which create a lot of health issues as well as social and economic concerns (Jacobson *et al*., 2022). Moreover, beside its price fluctuation and hiking, there is a depletion of non-renewable resources, which may be scarce or non-existent in future generations. As a result, the energy source is shifting from non-renewables like fossil fuels (coal, gas and oil) to renewable energy sources like bioethanol and biobutanol. Consequently, the world's attention is increasingly focused on developing and using chemicals and fuels from renewable resources. Among the alternative sources of energy, biobutanol is considered the most attractive biofuel. It undoubtedly possesses superior properties relative to ethanol and others, owing to its higher energy density, low volatility, hygroscopicity and low greenhouse gas emissions (Grande *et al*., 2021). Apart from being a solvent for various industrial applications, biobutanol is an essential chemical precursor for producing paints, polymers and plastics (Zhen *et al*., 2020).

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Although biobutanol can be produced by fermenting several carbohydrate derivatives derived from agricultural and forest residues, substrate sustainability is one of the most difficult aspects of biofuel development in general, including biobutanol production (Rafieisakhaei and Barazandeh, 2017). Resources with high carbohydrate content, an effective conversion method, and an available and low-cost source are required. Therefore, the efficiency, economy, and sustainability of biobutanol production are primarily influenced by the availability of raw materials. Many substrates have been described as feedstock for biobutanol production, and few studies in recent years have reported the laboratory-scale production of biobutanol from oil palm frond (OPF) juice, yet those studies mostly focused on the characterisation of the OPF juice as fermentation substrate for biobutanol production (Asri *et al*., 2019). It is reported that OPF juice meets all the conditions for a useful fermentation substrate due to its renewability, accessibility, and constant availability (Rafieisakhaei and Barazandeh, 2017). According to a report by the Malaysian Palm Oil Board (MPOB), approximately 51.80 million tonnes of biomass are generated annually from oil palm industries, with OPF biomass accounting for the majority; only a small portion of this is used as animal feed, with the remainder simply left in the plantation sites (Abubakar *et al*., 2022). Moreover, juice extracted from OPF is less toxic to microbial growth and product formation since it contains low impurities. A study conducted on the production of biobutanol from oil palm frond (OPF) juice by *Clostridium acetobutylicum* ATCC 824 in a 2 L batch culture using 50 g/L OPF juice yielded as high as 9.3 g/L of butanol (Huzir *et al*., 2019), but the research did not indicate the age of the oil palm frond juice used for the production. However, accumulations of both primary and secondary metabolites in plants are affected by several factors, including their developmental stage (age), this scenario has been neglected by most scientists while considering substrates as feedstock for biobutanol production. Therefore, the current study focused on the analysis and utilisation of different ages of OPF juice as a fermentation substrate for biobutanol production in batch fermentation mode.

Besides, different microorganisms participate in converting sugar-rich hydrolysate to biobutanol by acetone-butanol-ethanol (ABE) fermentation. A variety of *Clostridia* species were reported to have been used to produce solvent mixtures, usually in the ratio of 3:6:1 acetone-butanol-ethanol, respectively (Jiang *et al*., 2022). However, the capacity of the fermentation microbe to achieve a high yield of fermentation products despite the presence of phenolic inhibitors and the fermentation products as well as the ability to utilise both hexose and pentose is one of the significant factors leading to the production economy and sustainability. Currently, the main natural butanol producers are the *Clostridia* species, but due to their poor tolerance to butanol, a very low titre values are usually obtained in ABE fermentation broth. It is reported that, the maximum butanol that can be tolerated by *Clostridia acetobutylicum* is 2% and only less than 1.8% can be tolerated by *C. beijirankii* (Russmayer *et al*., 2019).

Genetic engineering to enhance the butanol tolerance in those species proved to be very complicated step, since the butanol tolerance is control by the action of many genes. Therefore, the other option like searching for robust and tolerant strains became a good alternative for the enhanced microbial butanol production. However, this remains an obstacle to realising sufficiently high butanol fermentation and product recovery levels (Liu *et al*., 2017). So, to progress in the scale-up of the production of biobutanol, some of the significant factors are that toxicity tolerance needs to be demonstrated in the fermentative strain and a considerable enhancement in the development of strains with the capability to ferment the variety of carbohydrate fractions of biomass hydrolysate to produce biobutanol. Therefore, the current research initially analysed the chemical composition of different segments of OPF and selected the best portion for biobutanol production, and finally screened and identified *Clostridium* species that can efficiently convert the substrate to biobutanol.

MATERIALS AND METHODS

Oil Palm Frond Collection and Preparation

Fresh and basal parts of OPF petioles with an average length of 1.0-1.5 m were collected during the monsoon period from Kampung Terjun Rimba Pontian, Johor Bahru, Malaysia, located at latitude 1° 27′ 59.20″ N and longitude 103° 27′ 20.50″ E. The location was chosen because of its abundance of different ages of palm trees and because it is also close to a juice extraction site. To avoid the influence of external factors, the samples were collected in duplicate from palm trees (*Elaeis guineensis*) of varying ages (5, 10, 15 and 20 years). To minimise the intrusion of microbes and dirt, freshly harvested petioles were cut at both edges and spread with 40% (v/v) of ethanol just before being put in sealed plastic bags and immediately transported to the juice processing site for juice extraction. The leaves from the fronds were also collected for elemental compositional analysis using plastic bags. To obtain the juice, a sugarcane juicing machine (Hisaki, TFS3777) was used to press the OPF petiole repeatedly until the juice content was completely removed. To collect the juice from different plant

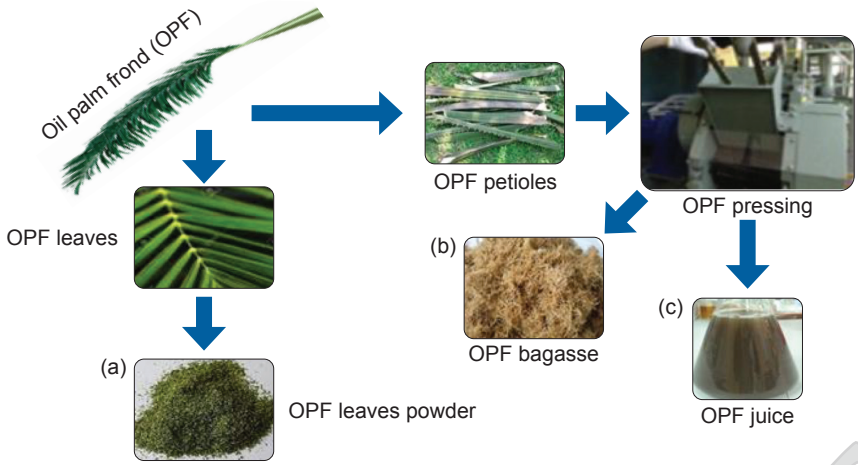

Figure 1. Sample collection and preparation.

ages, 1 L sterilised Scott bottles were used. However, the juice-free bagasse and the leaflets from the various oil palm fronds of varying ages were processed by oven-drying at a temperature of 70°C until the moisture content was less than 10% for subsequent analysis (Mat Rozi *et al*., 2021). *Figure 1* illustrates the sample collection and preparation.

Strain and Inoculum Development

The solvent-producing bacteria such as *Clostridium acetobutylicum* SR1 and other strains that include A1 and A2 (UPM), A1 and A2 (LM), A1 and A2 (June, 2011), L1, L2, WK and S1, which were previously isolated by Ramanathan *et al.* (2018), were screened for fermentable sugar utilisation for the production of biobutanol and tolerance towards phenolic compounds. Prior to the development of the inoculums, Reinforced Clostridial Medium (RCM) was prepared by adding g/L of meat extract (10 g), peptone (10 g), yeast extract (3 g), glucose (5 g), soluble starch (1 g), NaCl (5 g), and sodium acetate (3 g). The contents were sterilised by autoclaving at 121°C for 15 min. After cooling off the contents at room temperature, L-cysteine HCL (0.5 g), para-aminobenzoic acid (0.001 g), and biotin (0.008 g) were added separately via filter sterilisation using PTFE (0.2 m) (Fonseca *et al*., 2020). The stock cultures of the various species that were maintained in RCM were first heat-shocked at 90°C for 90 s (Wani *et al*., 2018). 1 mL of the respective heat-shocked culture for each strain was transferred separately into freshly prepared modified RCM and incubated at 37°C without agitation. The growth of the bacteria was monitored using optical density (OD) at 600 nm after every 4 hr of incubation until the cells reached a stable exponential phase (24 hr).

ABE Fermentation

To ferment the OPF juice for the production of biobutanol, a batch culture mode was employed. Initially, 135 mL of sterilised P2 medium were transferred into separate 150 mL serum bottles, leaving a space of 15 mL to aseptically sparge the medium with oxygen-free nitrogen gas using the modified Hungate technique. This was done to remove oxygen from the medium for strict anaerobic metabolism. The production medium (P2) is a stock solution containing KH_2PO_4 (0.75 g/L), K_2HPO_4 (0.75 g/L), yeast extract (5.0 g/L), NH_4NO3 (2.0 g/L), $MgSO_4.7H_2O$ (0.4 g/L), $MnSO_4.H_2O$ (0.01 g/L), $FeSO_4.H_2O$ (0.01 g/L), NaCl (0.5 g/L), and 50 g/L reducing sugar from the OPF juice (Gomez-Flores *et al*., 2018). After autoclaving the media, a solution containing 0.001 g/L of para-aminobenzoic acid, thiamin, and biotin was filter-sterilised into the sample mixture, and the pH was adjusted to 6 by the addition of 1M NaOH. To inoculate the media, 10% (v/v) of the well-grown cells of the respective strains were first heat-shocked at 90°C for 90 s and inoculated separately into fermentation media using sterilised syringes and needles. Finally, the inoculated media were incubated at 37°C without agitation for 96 hr. Samples were taken at 8 hr intervals for ABE, acid formation, biomass formation, and residual sugar analysis. The data reported in the figure and tables are the mean values of duplicate analysis and the standard error was always lower than 5%.

Identification of the Isolate

The 16S rRNA partial gene sequence was used to identify bacteria. To conduct this experiment, genomic DNA (gDNA) was extracted from actively growing cells of the selected strain during its exponential phase using the basic steps and protocol described by Guerrero *et al*. (2021). The extracted DNA was sent to Apical Scientific Malaysia for purification and sequencing services. The nucleotide sequences of the gene were determined using the genetic analyser by Homology Searching Tools (BLASTN) with the standard programme default

(http://www.ncbi.nlm.nih.gov/BLASTN/, NCBI, Bethesda, Md., USA), which was conducted based on the previous method (Hirakawa *et al*., 2019). The unknown bacterium was identified based on the highest percentage of sequence similarity with the strains in GenBank. MEGA 11 software was used for the construction of a phylogenetic tree using the neighbour-joining method. The evolutionary relationship of the bacterial isolate strain A1 and other related bacteria obtained from the Gene Bank was analysed by the distance matrix calculated using neighbour-joining using an out-group with an accession number of NR 113246.1. A bootstrap value of 1000 datasets was used by the software to assess the stability of the relationship (Traverso *et al*., 2020).

Analytical Methods

Determination of sugar and phenolic compounds. The total reducing sugar concentration was determined via the dinitrosalicyclic (DNS) method (Deshavath *et al*., 2020). Glucose monohydrate was used as a standard solution, and the absorbance of both the samples and the standards was read at 540 nm. On the other hand, the Folin-Ciocalteu reagent was used to determine total phenolic compounds with an absorbance read at 765 nm by a spectrophotometer, and here, gallic acid was utilised as the benchmark. However, the concentration of individual sugars and phenolic compounds in the juice was determined using an Agilent 1100 series HPLC technique with Rezex RPM-monosaccharide Pb^{2+} columns and a lead ion (Pb^{2+}) as the stationary phase for sugar determination and a C18 column for phenolic compound determination. All the data generated were analysed by use of SPSS v.10 software (SPSS Inc, Chicago, IL). One-way analysis of variance between ages of OPF for each composition was determined, and $P<0.05$ was considered significant.

Determination of lignocellulose composition. The determination of oil palm frond leaves, and bagasse structural components were conducted following the standard technique proposed by Lin *et al*. (2010) by considering the OPF structure to consist majorly of extractives, lignin, hemicellulose, and cellulose (Ang *et al*., 2015). Quantification of the extractives was performed by taking the weight loss of the sample contents; after that, the dried OPF leaflet and bagasse were treated with 99.5% (v/v) acetone. The weight loss of the 99.5% (v/v) acetone-treated samples after incubation for 3.5 hr at 80°C was the amount of hemicellulose (Ang *et al*., 2015; Lin *et al*., 2010). The percentage of lignin was recorded as the amount of weight lost after treating the extractive-free sample with 98% (v/v) H_2SO_4. Lastly, the content of cellulose was measured by taking the differences between the weighted total of the other products (extractives, hemicellulose and lignin) and the initial weight of the OPF leaves and bagasse powder.

Elemental analysis using X-ray fluorescence (XRF). XRF is an analytical technique used to determine the elemental composition of materials. This technique determines the chemistry of a sample by measuring the fluorescent (or secondary) X-ray emitted from a sample when it is excited by a primary X-ray source (Marguí *et al*., 2009). To conduct this analysis, leaflets from the various ages of oil palm fronds were processed by oven-drying at a temperature of 70°C until the moisture content was less than 10%. The dried leaves were ground into a powder with the help of a blending machine. The powdered samples were then placed on an X-ray beam for elemental composition determination.

Determination of acids and solvents using Gas Chromatography (GC). The concentration of acids such as acetic acid and butyric acid and solvents such as acetone, butanol and ethanol was determined using gas chromatography equipped with a DB-WAX column and a Flame Ionised Detector (FID). The carrier gas was helium, with a flow rate of 25 mL/min. The temperatures of the oven was 200°C while for both column inlet and detector was set at 300°C (Abe *et al*., 2020). Prior to the GC analysis, solvent-solvent extraction was done to separate the products from the broth. At first, a drop of 5% HCl was used to acidify the broth to separate the culture from the sample, and the acidified sample was extracted with an equivalent amount of dichloromethane (DCM). The mixture was then centrifuged at 10 000 rpm for 30 min; the top layer was discarded and the bottom layer was kept, which was expected to contain the acids and solvents, including the DCM. Finally, 20 µL of the samples were injected into the column using auto-injection. Acids and solvents of analytical grade (Sigma Aldrich, USA) were used as standards. The concentrations of acids and solvents in the sample were determined by comparing the area peaks of each sample with the area of the reference sample (the standards for acids and solvents). Prior to the analysis, calibration curves for the ABE and acids were constructed by plotting the peak area ratios against concentrations. Excellent linearity of the acids and solvents with correlation coefficients in the range of 0.991-0.996 was obtained, and the precision of the method was determined from duplicate analyses of the same with relative standard deviations (RSDs) of less than 5%.

RESULTS AND DISCUSSION

Selection of Oil Palm Frond Component Based on Composition and Age of the Tree

One of the ultimate aims of this study was to select the best material from the OPF biomass that could be used as an economical and efficient substrate for biobutanol production. Two parameters were taken into consideration, which include segment composition and OPF age. The initial phase of the research, therefore, analysed the major segments of the oil palm frond for their lignocellulosic composition. This was very necessary because, to fulfil the demand as a promising substrate for bioenergy, it is very important that the feedstock contain a reasonable percentage of its primary metabolites (carbohydrate (simple sugar, cellulose, hemicelluloses, starch, *etc*.) in high volume in the biomass, which later can be converted by the solvent producers to biofuels. The OPF is generally a highly fibrous lignocellulosic material that contains two major segments: Leaflets (tips) at the top and the basal portion (petioles). Both of the segments were analysed for the lignocellulose fraction, and the results are provided in this section based on the different ages of the palm tree.

Effect of Frond Age on the Lignocellulose and Elemental Composition

Since one of the aims of this study was to investigate the effect of ageing on economically important biobutanol traits, the profile of chemical composition of OPF leaflet ages was studied, and the results showed considerable differences at the 5% level of significance. Additionally, the environmental influences from chemical nutrients and soil type were minimised by sampling trees within a single plantation trial site. *Table 1* summarises the lignocellulose and elemental compositions of the different samples of OPF leaflets. The results indicated that the cellulose and hemicellulose fractions are generally low in all the OPF leaflet ages and frequently decrease with an increase in the age of the frond. The highest cellulose and hemicellulose percentages were 11.4 ± 2.04 and 13.7 ± 1.54% found in a 5 year OPF leaflet, while the lowest percentages were detected in a 20 year OPF leaflet, amounting to 10.2 ± 2.14 and 8.4 ± 1.61% for cellulose and hemicellulose, respectively. However, the percentage of cellulose and hemicellulose found in the OPF leaflet is far too low for the economical production of biobutanol. This is because, to date, no research has demonstrated a good butanol recovery from a substrate containing less than 30% holocellulose as a carbohydrate usable for butanol production. Moreover, the lignin fractions were found to be very high in all the OPF leaflet samples, which ranged from 52.4 ± 4.11 to 60.9 ± 3.08% (*Table 1*). The high amount of lignin in the OPF leaflets justifies the presence of abundant metals, which might be usable for many other industrial applications. Hence, the research went further to determine the elemental composition of the OPF leaflets.

Silicon was found to be the most abundant element in the entire powdered OPF leaflet samples, with relatively high levels compared to the other chemical elements present, such as calcium, potassium, and molybdenum, as determined by XRF analysis of their elemental composition. Even though some researchers reported a higher abundance of potassium in OPF leaves relative to silicon, this might be a result of the chemical constituents of the soil type in which the oil palm trees were grown. Other elements were found in trace quantities in OPF leaves, which include titanium, zinc, manganese, magnesium, aluminium, calcium and chlorine. Studies revealed that oil palm trees are good sources of silicon, potassium, and calcium, a fair source of microelements such as chlorine, magnesium, and sulphur, and a minor source of phosphorus (Alotaibi *et al*., 2019).

Another study reported that oil palm contains considerable amounts of calcium, magnesium, phosphorous, potassium, iron and zirconium (Mansir *et al*., 2017). From the results in *Table 1*, age was found to play a significant role in determining the amount of element present in the OPF leaflets, as it can be seen that the amount of silicon in the OPF leaflet samples is increasing with the increase in the age of the plant. The same trends were observed for manganese, aluminium, and chlorine, while for magnesium, zinc, and copper the reverse is the case. Based on our search, no reports are available in the literature for comparing the elemental composition of oil palm according to age, as determined in this study.

Effect of Frond Age on Lignocellulose Composition of OPF Bagasse

OPF petioles are reported as a good source of abundant unutilised carbohydrates, which consist of about 30%-50% cellulose, 20%-30% hemicellulose, and 20%-30% lignin and other extractive materials (Tnah *et al*., 2022). From the freshly harvested OPF, after the juice extraction, the composition of different ages of OPF bagasse was analysed for fractions of cellulose, hemicelluloses, lignin, and extractives. The weight percentages of each component per gramme of dry biomass are presented in *Table 2*. The highest fraction was found to be cellulose in 5, 10, 15 and 20 years of OPF bagasse, with the highest cellulose in the 5 year sample accounting for about 40.1 ± 2.13% of the total and the lowest percentage obtained in the 20 year sample (21.3 ± 2.35%).

TABLE 1. LIGNOCELLULOSE AND ELEMENTAL COMPOSITION OF OPF LEAFLET

Composition 5 years		Samples of OPF leaflet (ppm)				*P*-value
		10 years	15 years	20 years		
Lignocellulose composition (%)	Cellulose	11.4 ± 2.04	10.9 ± 1.67	11.0 ± 0.84	10.2 ± 2.14	0.0447
	Hemicellulose	13.7 ± 1.54	11.5 ± 1.22	12.6 ± 2.00	8.4 ± 1.61	0.0034
	Lignin	52.4 ± 4.11	52.6 ± 2.34	60.9 ± 3.08	59.3 ± 2.44	0.0452
	Extractives	22.4 ± 1.56	24.6 ± 2.15	15.5 ± 2.64	22.1 ± 2.02	0.9621
Elemental analysis (ppm)	Mn	993 ± 21.4	1 790 ± 26.0	4 230 ± 43.5	3 190 ± 35	<0.0001
	Mg	1 300 ± 138	1 160 ± 138	1 200 ± 135	961 ± 128	<0.0001
	Al	1 050 ± 21.5	17 800 ± 22.8	21 700 ± 25.3	24 000 ± 19.6	<0.0001
	Si	836 000 ± 421	864 000 ± 660	754 000 ± 340	854 000 ± 504	0.0466
	Zn	232 ± 6.36	243 ± 4.64	206 ± 4.65	118 ± 3.15	0.0525
	Cu	302 ± 6.04	253 ± 7.83	228 ± 5.73	69 ± 3.08	0.0312
	Cl	2 800 ± 9.04	4 900 ± 28.0	5 750 ± 26.7	29 200 ± 30.5	<0.0001
	K	57 200 ± 298	26 500 ± 215	40 900 ± 280	34 800 ± 231	<0.0001
	Ca	8 560 ± 371	35 100 ± 201	39 500 ± 216	41 000 ± 212	<0.0001
	Mo	5 700 ± 16.9	ND	35 300 ± 83.1	ND	<0.0001
	Ru	9 510 ± 43.4	ND	89 300 ± 136	ND	<0.0001

Note: ND - not determined, while the number before " ± " denotes mean values of three replicate and the number after " ± " denotes standard deviation. *P*-value less than 0.05 indicate significant difference, while *p*-value of 0.05 and above indicates that there is no significant difference in the composition across the different ages of palm tree.

The second most abundant fraction in the lignocellulose composition of OPF bagasse was hemicellulose, which amounts to 18% to 28% of the lignocellulose content. The percentages of cellulose and hemicelluloses in OPF bagasse in this study were quite comparable with the previously reported biomass material, including OPF itself (*Table 2*). However, this result shows slightly lower holocellulose content in comparison with 56.0% and 16.7% for cellulose and hemicelluloses, respectively (Khalil *et al*., 2012). Additionally, the percentage of holocellulose (cellulose and hemicellulose) in this study showed a decreasing trend as the oil palm plants grew older. The differences observed in the percentage of cellulose, hemicellulose, and lignin across the different ages of OPF (with a *p*-value of <0.0001, which indicated the difference between the four ages used at a 5% level of significance) might not be only due to the differences in the source used but also in the different maturity levels of the plants. According to Perlo *et al.* (2020), the chemical composition of lignocellulose biomasses may vary with plant age, plant height, and anatomical structure, which can limit or increase the cellulose, hemicellulose and lignin ratios.

The breakdown of lignocellulose material into reducing sugar as a product can still be maximised by effective pretreatment, even though OPF has a lower cellulose and hemicellulose content than other reported biomass materials. This is so because the effectiveness of enzymatic hydrolysis is heavily dependent on the biomass's digestibility. There are three major types of pretreatment methods in use today; physical, chemical, and biological methods, all aimed at breaking down the naturally resistant carbohydrate-lignin barrier that prevents enzymes from accessing holocellulose by disrupting the internal structure of holocellulose and increasing the reaction surface and porosity of biomass (Dutra *et al*., 2018; Rafieisakhaei and Barazandeh, 2017). However, pretreatment is the most critical need that must be addressed for this lignocellulosic biomass to become a commercially viable and cost-effective material for bio-based productions, such as biobutanol. It is reported that, out of the total cost of the bioconversion of biomass to biofuels, nearly 40% of the projected cost is connected with the release of sugars from cellulose and hemicellulose through the pretreatment operations (Zhang, 2019). Therefore, there is a need to continue research and development for an efficient pretreatment technology to produce low-cost fermentable sugar from the processing of OPF bagasse.

Effect of Frond Age on Total and Individual Fermentable Sugar of OPF Juice

The juices extracted from the oil palm frond samples were investigated for their total reducing sugar content via the DNS method, and the results are presented in *Table 3*. The results indicated a decreasing order of total fermentable sugar in OPF juices as the plant increases in age, with 69.04 ± 1.14 g/L, 65.44 ± 0.55 g/L, 48.22 ± 2.46 g/L, and 43.59 ± 1.52 g/L for 5, 10, 15 and 20 years of OPF juice, respectively. However, all of the studied OPF juice samples contained total fermentable sugar contents higher than the minimum recommended level for

TABLE 2. LIGNOCELLULOSE COMPOSITION OF OPF BAGASSE

Age of palm oil tree	Pretreatment type	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractive (%)	References
5 years	Physical (Pressing)	38.7 ± 2.25	27.4 ± 1.84	24.8 ± 0.88	09.5 ± 1.55	
10 years	Physical (Pressing)	40.1 ± 2.13	24.5 ± 3.04	22.7 ± 2.41	12.7 ± 2.14	
15 years	Physical (Pressing)	30.3 ± 2.66	26.4 ± 2.30	31.4 ± 3.06	11.9 ± 1.41	This study
20 years	Physical (Pressing)	21.3 ± 2.35	18.4 ± 1.08	52.9 ± 1.20	07.5 ± 4.20	
p-value		<0.0001	<0.0001	<0.0001	<0.0001	
Report from other researches						
NA	Physical (Pressing)	43.6	8.22	10.4	NA	(Rusli *et al*., 2016)
NA	Physical (Ball milling)	32.5	22.5	15.2	16.4	(Zakaria *et al*., 2014)
NA	Physico-chemical	44.0	30.4	15.4	04.1	(Sabiha-Hanim *et al*., 2011)
NA	Chemical	28.7	26.0	37.6	07.6	(Sukri *et al*., 2014)
NA	Chemical	56.0	16.7	28.5	04.4	(Khalil *et al*., 2012)
NA	Chemical	45.0	32.0	16.9	02.3	(Megashah *et al*., 2018)
NA	Chemical	42.8	20.9	32.0	04.4	(Dávila *et al*., 2016)

Note: The number before "±" denotes mean values of three replicate and the number after "±" denotes standard deviation. *P*-value less than 0.05 indicate significant difference exist in the lignocellulose composition across the different ages of palm tree.

a substrate intended for the production of biofuels. The relatively lower fermentable sugar found in the 15 and 20 year samples can be attributed to the negative correlation that exists between holocellulose and lignin. Plants usually become lignified as they grow older in defense against external factors, which invariably lower the level of cellulose and hemicellulose. Thus, the results of the current research indicated a significant relationship between the total amount of fermentable sugar and the age of the plant (with a *p*-value less than α, which indicated a significant interaction of the age and sugar contents), with the older plant having a lower sugar content compared with the younger plant (*Table 3*). The trend of the results is in agreement with the previously reported study, where they found that the content of sugar in OPT at 19 years is 6.6 kg/350 kg of dried OPT, which is significantly higher than the OPT at 31 years with only 4.6 kg/350 kg (Murai and Kondo, 2011).

To utilise the best oil palm frond juice as feedstocks for the bioenergy conversion process, knowledge on the variation of sugar types between the different ages of oil palm fronds is considered very important, as the compositional data of a feedstock is crucial to gaining an understanding of feedstock properties prior to selecting a processing or conversion technology. This is because not all types of sugars can be readily fermented by some microorganisms. According to Zwetsloot *et al*. (2020), specific sugar mixtures such as glucose, fructose, arabinose, galactose, mannose, xylose, *etc*., in the substrate determine the activity of the microbial communities and their utilisation. It is observed that the specific sugars (*e.g*., glucose and fructose) in sugar substrates are shown to positively associate with microbial growth and fermentation. For example, the presence of glucose in a substrate influences the growth of the abundant genera *Lactobacillus* and *Saccharomyces*, which are the most utilised microbial communities related to butanol fermentation (Wei *et al*., 2021). Meanwhile, glucose and fructose are the preferred substrates for anaerobic strains to produce acetone, butanol and ethanol in fermentation (Ezeji *et al*., 2007). Even though the proper combination of sugar mixture in a substrate and its ratio has a significant influence on microbial growth and fermentation, there is little or no information on the profile of sugar in OPF juice at the different maturity stages. Hence, the present study analyses the profiles of individual sugars in the juice samples extracted from OPF ages, and the results are shown in *Table 3*.

As can be observed in *Table 3*, differences exist in the concentration of individual sugars present in the various OPF juices extracted from different ages of oil palm trees. It is found that the fermentable sugars in the juices contain two monosaccharides (glucose and fructose) and one disaccharide (sucrose) in various proportions and concentrations. Among the individual sugars, glucose was found to have the highest percentage, ranging from 55% to 65% of the total sugar in all the OPF juice samples tested (63.17, 60.43, 56.95 and 64.46% for ages 5, 10, 15 and 20, respectively). The remaining percentage of the total fermentable sugar was made up of the two sugar types detected in the samples, which were detected in relatively lower concentrations, with fructose ranging from 19% to 26% and sucrose from 12% to 20%. Even though the total fermentable sugar of all the OPF juice samples analysed in this study has significant differences across the

TABLE 3. TOTAL FERMENTABLE SUGAR CONTENT IN OPF JUICE SAMPLES

	Total fermentable sugar	Glucose		Sucrose		Fructose		References
		g/L	%	g/L	%	g/L	%	
5 years	69.03 ± 1.14 g/L	43.61 ± 1.6	63.17	16.53 ± 0.4	23.90	8.19 ± 2.1	12.0	
10 years	65.44 ± 0.55 g/L	39. 55 ± 0.5	60.43	12.64 ± 1.3	19.32	8.02 ± 1.5	12.25	
15 **years**	48.22 ± 2.46 g/L	27.46 ± 1.1	56.95	12.14 ± 1.5	25.18	9.56 ± 0.2	19.8	This study
20 **years**	43.53 ± 3.52 g/L	28.06 ± 2.2	64.46	9. 69 ± 0.6	22.23	7.43 ± 2.6	17.06	
p-value		<0.0001		0.0086		<0.0357		
				Reports from other studies				
NA	44.2 g/L	24.80	56.11	15.2	34.39	3.10	7.01	(Tan *et al*., 2016)
NA	65.0 g/L	42.00	64.62	22.0	33.85	1.00	1.54	(Zahari *et al*., 2014)
NA	76.1 g/L	53.95	70.00	20.46	27.00	1.68	3.00	(Zahari *et al*., 2012)
NA	116.4 g/L	89.83	77.17	19.6	16.84	6.93	5.95	(Abdullah *et al*., 2021)

Note: The number before " ± " denotes mean values of three replicate and the number after " ± " denotes standard deviation. *P*-value less than 0.05 indicate significant difference exist between the sugar compositions across the different ages of palm tree.

various ages of the palm plants, fluctuations in the concentrations of individual sugars were also observed as the plant's age increased. It is noted that the sucrose percentage in the OPF juices increases as the plant's age increases, *i.e*., from a younger sample to the most matured sample tested, where glucose and fructose showed reverse gradients, with the younger plant sample having the higher percentage. These inherent fluctuations in the sugar composition of the oil palm plant with age have yet to be fully cleared. Some researchers hypothesised a similar situation on temperate fruit-bearing species such as the oil palm tree. They suggested that the variation in chemical composition with age and storage compartment could be as a result of the need for the plant to balance the unsatisfactory photosynthesis to sustain organ demands (Suryawan and Heru, 2019).

Effect of Frond Age on Total and Individual Phenolic Compounds of OPF Juice

The total phenolic content of the oil palm frond juice samples investigated was found to vary among the different ages of the juice samples. The concentrations of each of the total phenolic contents measured at the different ages of the studied samples are presented in *Table 4*. The result indicated a decreasing order of total phenolic compound concentration in the samples, *i.e*., younger oil palm fronds contained juice with higher phenolic compounds relative to the older. The juice from 5 years of OPF contains a total phenolic compound of 0.332 ± 0.01 g/L, which is higher than the concentration of phenolic compounds found in the 10, 15 and 20 years, with level of 0.317 ± 0.00, 0.282 ± 0.02 and 0.260 ± 0.01 g/L, respectively. This means that more phenolic compounds are accumulated in younger OPFs than in older ones. However, two previous studies suggested the reverse, such that phenolic contents in plants increase with plant age. For example, the research conducted on *Mentha* L. species on the variation of secondary metabolites, especially the polyphenols and tannins, based on the 1-4 years has indicated an improvement in the quantity of polyphenols and tannins as the plant gets older, with the highest polyphenols of 12.74% obtained in the 4 years, compared to only 11.98% and 9.44% obtained from the 3 and 2 years, respectively (Aleksic and Knezevic, 2014; Muyny and Chantarapanont, 2022).

Moreover, research conducted to compare the levels of secondary metabolites (phenols and flavonoids) and their distribution shows that the content of phenols is not only affected by the differences between the plant organs but also by the stage of plant growth (Fialová *et al*., 2015; Saffaryazdi *et al*., 2020). Their results showed significant differences in the flavonoid content between leaf and stem as well as with the change in growth stage. At the flowering stage, the flavonoid content of the leaves was five times higher than the value measured in the stems. However, during the vegetative stage, the measured flavonoid levels in the leaves were 1.3 times higher than their content in the stems. The research finally suggested that the changes in flavonoid content were also affected by both physiological and growth stages (Saffaryazdi *et al*., 2020). Therefore, the present study suggested that the differences in phenolic compound levels could be attributed to the influence of external factors (long-term weather before harvesting time, intensity of sunlight, pathogens, and time of harvesting).

Table 4 presents the differences that exist in the concentration and profile of the various OPF juices extracted from different ages of oil palm trees. The results indicated that phenolic compounds such as gallic acid, ferulic acid, and vanillic acid were detected in the 15 and 20 year old juice samples,

TABLE 4. TOTAL AND INDIVIDUAL PHENOLIC CONTENT IN OPF JUICE SAMPLES

Phenolic compounds	Concentrations of phenolic compounds (mg/L)				
	5 years	10 years	15 years	20 years	*P*-value
Total phenolic content (g/L)	0.332 ± 0.01	0.317 ± 0.00	0.282 ± 0.02	0.260 ± 0.01	<0.0001
Gallic acids (g/L)	0.264 ± 0.02	0.251 ± 0.01	0.207 ± 0.00	0.194 ± 0.03	<0.0001
p-coumaric acids (g/L)	ND	ND	ND	ND	ND
Vanillic acids (g/L)	ND	ND	0.013 ± 0.04	0.017 ± 0.01	<0.0001
Ferulic acids (g/L)	0.061 ± 0.00	0.050 ± 0.01	0.064 ± 0.02	0.079 ± 0.02	<0.0001

Note: ND - Not detected, while The number before " ± " denotes mean values of three replicate and the number after " ± " denotes standard deviation. *P*-value less than 0.0001 indicate significant difference between the profiles of phenolic compound across the different ages of palm tree.

while only gallic acid and ferulic acid were detected in the 5 and 10 year old juice samples. Earlier, it was proposed that variations in the types and distribution of secondary metabolites across plant organs, maturity stage, and nutrient presence in the soil might account for the observed differences in phenolic compound types. However, beside the differences in the number of phenolic compounds detected, the concentrations of individual phenols differ. Gallic was discovered to be the most abundant individual phenol, accounting for approximately 80% of the total phenolic compound in the juices. However, the concentration of gallic acid decreases with an increase in the age of the palm tree. The second most abundant phenolic compound type is ferulic acid, which consists of about 18% of the total phenolic compound, and its concentration fluctuates across the ages of the palm tree. The other phenolic compound detected was found only in 15 and 20 years, with 0.01 3± 0.04 and 0.079 ± 0.01 g/L, respectively.

Depending on the type of phenolic compound, its concentration, and other physicochemical parameters of the culture medium, such as temperature and pH, the effects of a variety of phenolic compounds usually vary among the different organisms used in the fermentation process. For example, *C. beijerinckii* BA101 was less affected by acetates, furfural, and HMF at concentrations less than 1.9 g/L (Ezeji *et al*., 2007), while solvent production halted in *C. acetobutylicum*, especially when the cells were in a medium where acetic and butyric acids accumulated quickly and caused the pH to drop (Suryawan and Heru, 2019). Another study on the toxicity of various phenolic compounds found that phenolic compounds (coumaric acid, ferulic acid, and 4-hydroxybenzoic acid) at 1 g/L can inhibit the growth of most microorganisms by up to 70%, effectively inhibiting butanol production (Muyny and Chantarapanont, 2022). However, syringaldehyde and vanillin were reported to be less toxic to cell multiplication, even though butanol production can be highly inhibited (Aleksic and Knezevic, 2014). In general, phenolic compounds like ferulic acid were found to be more toxic to most *Clostridia* sp., followed by coumaric acid, especially at concentrations above 0.5 g/L (Fialová *et al*., 2015).

A correlation was established between the age of the OPF samples and their fermentable sugar levels and phenolic compounds. The fermentable sugars in the OPF juice tend to decrease as the oil palm tree gets older. The same trend was seen in the relationship between the age of oil palm trees and the level of fermentable sugar. Higher phenolic compounds in the OPF juices also show a decreasing pattern with the increase in the age of oil palm trees, as in the case of the concentration of fermentable sugar. Another correlation was observed between the total fermentable sugar and the lignocellulose composition of the OPF bagasse, such that the higher the sugar levels, the lower the level of cellulose and hemicellulose. For example, 5 year old OPF, which had the highest fermentable sugar, showed a decrease in the percentage of holocellulose, suggesting that the fermentable sugars are generated from the holocellulose. It is observed that there is a specific relationship between potassium availability and sugar composition and concentration; research shows that, in rachis, the sugar composition changed a lot under low potassium availability, irrespective of the age of the rachis, with a 60% increase in glucose (Cui *et al*., 2020). However, in this study also, the concentration of potassium detected in 5 year old OPF leaves is higher than what is observed in 10, 15 and 20 year old OPF leaves, showing strong agreement with the previously mentioned report because both potassium and total sugar are higher in 5 year old OPF leaves than they are in the older OPF leaves.

Screening and Identification of Potential Bacteria for ABE Fermentation Using OPF Juice

In an effort to improve the performance of biobutanol production via ABE fermentation using OPF juice, a strain screening process was employed

to assist in the selection of the best and most robust strain of *Clostridium* for biobutanol production. Eleven potential bacteria were screened, including *Clostridium acetobutylicum* SR1, which was previously isolated from cow dung, L1 and L2 WK1, WK 2, LM 1, LM2, A1, A2 and S1, previously isolated and preserved in a -4°C refrigerator in the Biorefinery Research Laboratory, UTM. All the isolates were studied for tolerance towards phenolic compounds as well as their ability to utilise hydrolysate from OPF juice for ABE fermentation. Ten years old oil palm frond juice was used as the fermentation feedstocks, which contain 50 g/L reducing sugar (37 g/L glucose, 6 g/L fructose, and 7 g/L sucrose) as the sole carbon and energy source from the P2 medium.

Table 5 presented the results of bacterial screening for the biobutanol production using OPF juice. The results indicated that, eight out of the total of 11 strains screened have successfully thrived in the presence of phenolic compounds and were able to utilise the hydrolysate from oil palm frond juice for biobutanol production via ABE fermentation. Strains SR1, L2, A1, WK1, WK2, LM1, LM2 and S1 were able to produce certain amounts of butanol, while on the other hand, strains L1, A2 and A1 (UPM) were unable to ferment the reducing sugars in the hydrolysate to produce a significant amount of biobutanol.

The general fermentation kinetics of the strains screened for the production of butanol via ABE fermentation are presented in *Table 5*. It can be seen that the different strains have shown different abilities for both acid and solvent production. The strains also demonstrated dissimilar potentials during the fermentation process, as different end product concentrations were obtained. Strain A1 resulted in the highest production with a butanol concentration of 2.32 g/L and a productivity of 6.4×10^{-2} g/L/hr. This was followed by strains SR1 and L2, with butanol production of 2.04 g/L and 1.96 g/L, while other strains showed poor utilisation ability of the hydrolysate for the production of butanol, with the least production of butanol of 0.03 g/L by L1 and zero production from A2. However, other solvents, such as acetone and ethanol production, were also fairly high in the culture with the A1 strain. Acetone and ethanol production observed were 2.59 g/L and 1.53 g/L, respectively, with total solvent production of 6.44 g/L. Strain A1 also showed a considerably high acid production during ABE fermentation, with acetic acid and butyric acid concentrations of 0.185 g/L and 1.234 g/L, respectively, and a total acid production of 1.419 g/L produced. The higher solvent produced by A1 indicated that the strain can tolerate the highly acidic conditions caused by the production of organic acids during the acidogenic phase, and the nature of the waste, which contains high levels of undissociated organic acids and phenolic inhibitors.

During the 96 hr of batch culture fermentation, it was observed that the acidogenic phase of most of the strains was between 16 and 24 hr of the fermentation, where acetic acid and butyric acid were the main products produced, except for strains L2, WK1 and WK2, which could not produce the acids until after 30 hr of the fermentation. However, the production of total acids after 96 hr of fermentation by different strains was observed within the range of 0.001 to 2.781 g/L, with the highest being the S1. Unfortunately, this high production of acetic and butyric acid within 48 to 96 hr of ABE fermentation by strains S1 and LAMA1 could possibly be due to the incomplete phase transition from the acidogenic phase to the solventogenic phase, which later hinders solvent production. This is because the presence of organic acids at high concentrations that accumulated during fermentation decreased the capability of the microorganisms for uptake and recycling of both butyric and acetic acids, resulting in a very low ABE concentration.

After 96 hr of fermentation, A1 had used about 70% of the fermentable, leaving 13.4 g/L as the main residual sugars in the medium (*Figure 2*). This indicated that the isolate could utilise all the sugar types in the hydrolysate. The higher solvent production by strain A1 (*Table 5*) and good substrate utilisation ability of the strain can be directly related to the good biomass production. Efficient substrate utilisation indicates that the strain is able to use the carbon source provided, which results in an increase in microbial growth. As can be seen from *Table 5*, a total of 0.372 g/L of biomass was obtained in the culture of A1 at the end of the ABE fermentation with a doubling time of 1.61/hr. Other isolates, like LAMA1, displayed higher substrate utilisation relative to A1. However, this isolate could only produce a high amount of acids with a relatively lower solvent concentration, which might be because of the lower pH and other phenolic compounds and product inhibition (Capilla *et al*., 2021). The isolate LAMA2 was unable to use the substrate provided for new cell propagation, which could have aided in increased solvent production. The inability of the isolates to utilise the hydrolysate is proven based on the higher residual reducing sugar left in the culture at the end of the fermentation.

Identification of the Strains

The 16S rRNA gene has been identified as roughly 1.55 kb in length and is composed of both variable and conserved regions with sufficient interspecific polymorphisms that can also be responsible for distinct and statistically accurate

TABLE 5. FERMENTATION KINETICS OF THE STRAINS SCREENED FOR THE PRODUCTION OF BUTANOL VIA ABE FERMENTATION

Parameters	Strain SR1	Strain L2	L1	Strain A1	A2	WK 1	WK 2	LAMA 1	LAMA 2	S1	A1 (UPM)
Max. acetone (g/L)	0.38 ± 0.02	0.92 ± 0.04	0.11 ± 0.01	2.59 ± 0.03	0.00	0.07 ± 0.01	0.09 ± 0.02	0.26 ± 0.01	0.00	0.00	0.11 ± 0.05
Max. butanol (g/L)	1.96 ± 0.04	2.04 ± 0.01	0.03 ± 0.02	2.32 ± 0.05	0.00	0.35 ± 0.05	0.15 ± 0.01	0.40 ± 0.02	0.03 ± 0.05	0.04 ± 0.02	0.03 ± 0.01
Max. ethanol (g/L)	2.30 ± 0.06	1.67 ± 0.03	0.01 ± 0.00	1.53 ± 0.05	0.00	0.04 ± 0.03	0.00	0.43 ± 0.07	0.00	0.00	0.04 ± 0.08
Max. solvent (ABE) (g/L)	4.58 ± 0.11	4.63 ± 0.08	0.16 ± 0.04	6.44 ± 0.12	0.00	0.45 ± 0.06	0.24 ± 0.05	0.97 ± 0.06	0.03 ± 0.02	0.04 ± 0.02	0.16 ± 0.14
Max. acetic acid (g/L)	1.35 ± 0.04	0.25 ± 0.02	0.03 ± 0.01	2.19 ± 0.06	0.86 ± 0.00	0.02 ± 0.01	0.01 ± 0.02	0.22 ± 0.05	0.27 ± 0.03	1.14 ± 0.22	0.02 ± 0.01
Max. butyric acid (g/L)	2.90 ± 0.03	0.90 ± 0.06	0.07 ± 0.02	2.36 ± 0.04	0.13 ± 0.02	0.13 ± 0.03	0.01 ± 0.05	1.64 ± 0.11	0.16 ± 0.08	1.64 ± 0.24	0.06 ± 0.02
Max. acid (g/L)	4.25 ± 0.07	1.05 ± 0.08	0.09 ± 0.04	4.55 ± 0.10	0.99 ± 0.02	0.16 ± 0.03	0.02 ± 0.02	1.86 ± 0.16	0.43 ± 0.04	2.78 ± 0.41	0.09 ± 0.04
Acetone productivity (g/L/hr)	1.1×10^{-2}	1.9×10^{-2}	0.3×10^{-2}	5.3×10^{-2}	0.00	0.3×10^{-2}	03×10^{-2}	1.0×10^{-2}	0.00	0.00	0.2×10^{-2}
Butanol productivity (g/L/hr)	8.1×10^{-2}	5.6×10^{-2}	0.2×10^{-2}	6.4×10^{-2}	0.00	0.7×10^{-2}	0.6×10^{-2}	1.6×10^{-2}	0.1×10^{-2}	0.1×10^{-2}	0.1×10^{-2}
Ethanol productivity (g/L/hr)	9.5×10^{-2}	6.9×10^{-2}	0.1×10^{-2}	4.2×10^{-2}	0.00	0.1×10^{-2}	0.00	1.7×10^{-2}	0.00	0.00	0.1×10^{-2}
Acetic acid productivity (g/L/hr)	5.6×10^{-2}	0.9×10^{-2}	0.1×10^{-2}	6.0×10^{-2}	0.00	0.1×10^{-2}	0.04×10^{-2}	0.9×10^{-2}	0.7×10^{-2}	4.7×10^{-2}	0.05×10^{-2}
Butyric acid productivity (g/L/hr)	8.0×10^{-2}	1.8×10^{-2}	0.3×10^{-2}	4.9×10^{-2}	3.5×10^{-2}	0.5×10^{-2}	0.04×10^{-2}	1.0×10^{-2}	0.6×10^{-2}	6.8×10^{-2}	0.1×10^{-2}
Overall acetone productivity (g/L/hr)	0.5×10^{-2}	1.4×10^{-2}	0.2×10^{-2}	4.7×10^{-2}	1.0×10^{-2}	0.1×10^{-2}	0.1×10^{-2}	0.5×10^{-2}	0.00	0.00	0.2×10^{-2}
Overall butanol productivity (g/L/hr)	4.0×10^{-2}	4.2×10^{-2}	0.1×10^{-2}	4.4×10^{-2}	0.00	0.7×10^{-2}	0.3×10^{-2}	0.6×10^{-2}	0.00	0.00	0.06×10^{-2}
Overall ethanol productivity (g/L/hr)	3.9×10^{-2}	3.4×10^{-2}	0.02×10^{-2}	3.1×10^{-2}	0.00	0.1×10^{-2}	0.00	0.6×10^{-2}	0.00	0.00	0.05×10^{-2}
Overall total solvent productivity (g/L/hr)	7.1×10^{-2}	9.6×10^{-2}	0.4×10^{-2}	12×10^{-2}	0.00	1.3×10^{-2}	0.5×10^{-2}	2.0×10^{-2}	0.1×10^{-2}	0.00	0.3×10^{-2}
Overall acetic acid productivity (g/L/hr)	2.8×10^{-2}	0.5×10^{-2}	0.1×10^{-2}	4.5×10^{-2}	2.3×10^{-2}	0.08×10^{-2}	0.02×10^{-2}	0.4×10^{-2}	0.05×10^{-2}	1.0×10^{-2}	0.04×10^{-2}
Overall butyric acid productivity (g/L/hr)	6.0×10^{-2}	1.0×10^{-2}	0.15×10^{-2}	4.9×10^{-2}	0.4×10^{-2}	0.5×10^{-2}	0.02×10^{-2}	3.4×10^{-2}	0.3×10^{-2}	5.0×10^{-2}	0.07×10^{-2}
Overall total acids productivity (g/L/hr)	5.9×10^{-2}	2.1×10^{-2}	0.2×10^{-2}	9.4×10^{-2}	4.1×10^{-2}	0.3×10^{-2}	0.03×10^{-2}	3.9×10^{-2}	0.69×10^{-2}	5.1×10^{-2}	1.8×10^{-2}
Initial total fermentable sugar (g/L)	47.50	47.50	47.50	47.50	47.50	47.50	47.50	47.50	47.50	47.50	47.50
Final total fermentable sugar (g/L)	18.2	19.5	26.7	13.4	22.5	31.7	20.6	12.2	36.8	17.0	29.8
Percentage of sugar consumed (%)	66.7	59.1	45.3	69.1	52.6	33.2	56.6	74.3	22.5	64.2	37.3
Sugar utilisation rate (g/L/hr)	0.33	0.31	0.217	0.35	0.260	0.165	0.280	0.368	0.111	0.318	0.184
Xmax (g/L)	0.317	1.051	0.283	0.372	0.187	0.123	0.265	0.520	0.161	0.420	0.247
Doubling time td (hr^{-})	1.395	0.659	1.610	1.863	0.609	0.374	0.741	1.261	0.322	0.951	0.410

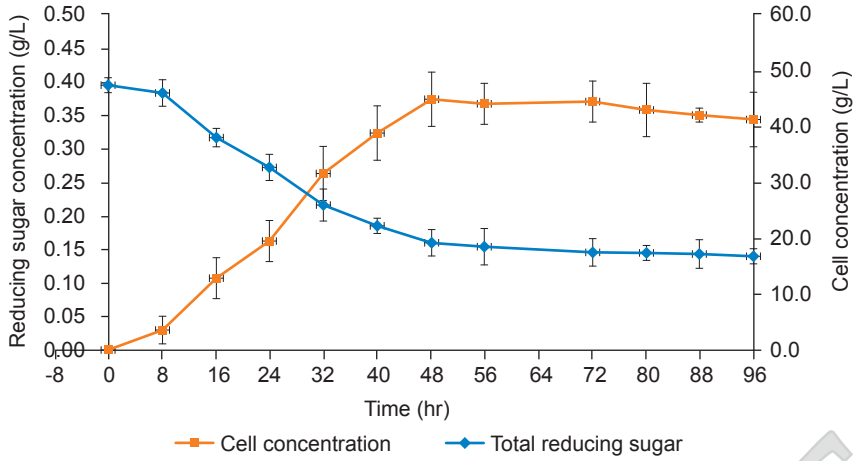

Figure 2. Time course for bacterial growth and sugar utilisation by Clostridium *strain A1 in P2 medium containing 70% of OPF hydrolysate.*

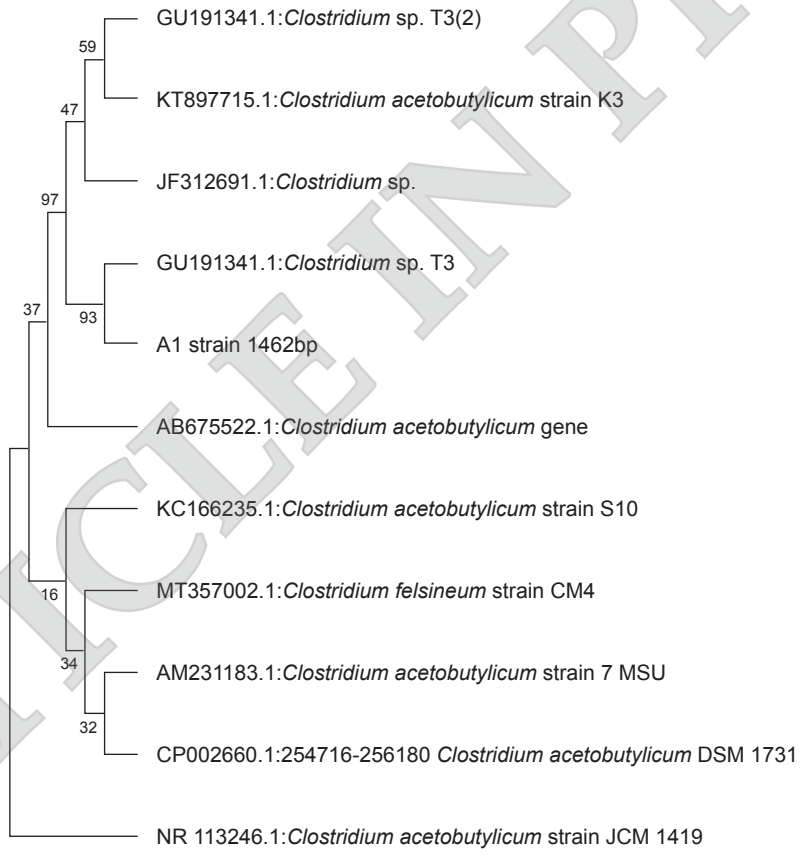

Figure 3. Phylogenetic tree based on 16S rRNA gene sequence link showing the relationship between bacteria of the genus Clostridium *sp. and the strain A1.*

measurements (Al-Haideri, 2019). Apart from being the most conserved region among the three rRNA genes (16S, 23S and 5S), the 16S rRNA gene is highly conserved in bacteria, making it the best gene for comparing and identifying newly isolated bacteria. The 16S rRNA gene sequence analysis of the pure bacterium in this study revealed 1427 base pairs in the gene sequence. The bacteria's evolutionary history was investigated by constructing a phylogenetic tree using the neighbor-joining technique (Hong *et al.*, 2021). The P-distance method was used to calculate the

evolutionary distance (Criscuolo, 2020). The 16S rRNA analysis with BLASTN indicated that the bacterial strain A1 belongs to the *Clostridium* species and is 99% identical to *Clostridium acetobutylicum* GXO1. As a result, the strain was given the name *Clostridium* strain A1. The phylogenetic tree in *Figure 3* depicts the relationship between closely related isolates with a higher similarity index.

The present research highlighted new developments in feedstock selection criteria and utilisation, microbial strain development, and fermentation process development, all of which significantly impact the production economy. This technology is hoped to offer a synergistic relationship for industry to capitalise on underutilised organic wastes such as OPF biomass for biobutanol production. Considering that the investigation of changes in biobutanol economic traits as trees age will inform optimal harvesting ages and biomass requiring fewer pretreatments to deconstruct biomass.

CONCLUSION

The current study evaluated the chemical composition of OPF leaflets, bagasse, and juice at different ages for its potential application for biobutanol production. Relative to leaflets and bagasse, pressed juices from different ages of OPF are indicated to contain a higher amount of renewable sugars, including glucose, sucrose, and fructose. However, an increase in palm age was seen to significantly decrease the concentration of both fermentable sugars and total phenolic compounds in the juice. Thus, juice from 10-year-old OPF containing 65.44 g/L of total fermentable sugar and 0.317 g/L of phenolic compounds was selected and used for biobutanol production using 11 strains of *Clostridia* species. Despite the presence of phenolic compound inhibitors, strain A1 was found to be the most successful strain that tolerated the inhibitors and utilised the sugars in the hydrolysate to produce 2.32 g/L of biobutanol. The strain was identified as *Clostridium* strain A1 based on 16S rRNA gene sequencing. Therefore, the research recommends optimising biobutanol production from 10-year-old OPF juice using *Clostridium* strain A1 as the fermentation microorganism.

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