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THE EFFECTS OF FERMENTATION USING CELLULOLYTIC MICROBES ON PALM OIL FRUIT DETACHMENT AND CHEMICAL PROPERTIES OF CRUDE PALM OIL

ELISA JULIANTI[1]*; ZULKIFLI LUBIS[1]; HOTNIDA SINAGA[1], SENTOSA GINTING[1], and PRETTY PERONIKA SINAGA[1]

ABSTRACT

*This study investigated the effects of cellulolytic microbes (*Bacillus subtilis, Aspergillus niger, Trichoderma harzianum *and their combinations) and fermentation periods (20, 40 and 60 hr) on the detachment efficiency of palm oil fruits and the chemical properties of crude palm oil (CPO). Fermentation treatments significantly enhanced detachment efficiency by 5-22 times, increasing the number of detached fruits and empty bunches. The combination of* B. subtilis *and* A. niger *improved oil yield, while* T. harzianum *preserved β-carotene content. Both combinations also contributed to lowering free fatty acids (FFA). The highest oil yield (16.40%-28.60%) was achieved with a 20-hr fermentation, whereas longer durations (40 and 60 hr) resulted in lower yields of 13.90%-19.70% and 10.00%-14.40%, respectively. Compared to the control, fermentation treatments reduced moisture from 2.80% to 0.17%-2.46%, FFA from 3.43% to 0.45%-1.55% and β-carotene from 423.45 ppm to 135.2-373.9 ppm, while increasing the deterioration of bleaching index (DOBI) value. However, longer fermentation periods significantly (*$p<0.05$*) increased FFA, moisture and peroxide values while decreasing β-carotene and DOBI. In conclusion, combining* B. subtilis *with* A. niger *or* T. harzianum *for 20 hr of fermentation effectively enhances fruit detachment and improves CPO stability against oxidation.*

Keywords: *Aspergillus niger*, *Bacillus subtilis*, detachment efficiency, fruit detachment, *Trichoderma harzianum*.

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INTRODUCTION

The worldwide consumption of palm oil has increased significantly from 59.38 x 1,000 t in 2015/2016 to 76.04 x 1,000 t in 2022/2023 (Shahbandeh, 2023a). Indonesia contributes as the highest producer of crude palm oil worldwide followed by Malaysia which produces around 54.5 x 1,000 and 18.8 x 1,000 t, respectively (Shahbandeh, 2023b). Palm oil is an edible oil which is highly saturated and rich in carotenoid content. The majority (90%) of palm oil is generated from the fruit pulp and a small fraction (10%) is obtained from the palm kernel oil (Chew et al., 2022; Vincent et al., 2014). It has been highlighted by excellent studies that one of the most important factors which significantly affects the quality of palm oil is the detachment of palm oil fruits (POF) from bunches, called threshing. This process started with sterilisation as a pretreatment followed by mechanical forces to be further processed for oil recovery (Chew et al., 2022; Vincent et al., 2014). Pretreatment plays an important role in affecting the efficiency of fruit detachment as well as modifying the quality of generated palm oil. Pretreatment aims to increase the solubilisation and separation of biomass-hemicellulose, cellulose, lignin and extractives thus allowing the release of the oil fraction.

The sterilisation process is crucial because it will soften the fruits thus enhancing the loosening and the detachment of palm fruits from the bunch; however, it alters the amount of free fatty acids

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[1] Department of Food Technology,
Faculty of Agriculture,
Universitas Sumatera Utara,
Padang Bulan, Medan 20155, Indonesia.

* *Corresponding author e-mail: elisa1@usu.ac.id*

(FFA) (Chew et al., 2022; Vincent et al., 2014). Moreover, the presence of contaminants which are carcinogens in palm oil has gained attention such as glycidyl esters (GE) and 3-monochloropropane-1,2 diol (3-MCPD). Therefore, development in controlling the quality of refined palm oil during the process is worth investigating (Chew et al., 2022). Conventional sterilisation inactivates the generation of lipase enzymes which induces the formation of FFA and breaks the oil-bearing cells for easier extraction. However, it reduces the production efficiency due to the production interruption (Hock et al., 2020). Conventional sterilisation requires more energy to generate steam (Vincent et al., 2014), risks the loss of fruits and thus oil yield as well as a higher FFA (Balakrishnan et al., 2021). Therefore, several methods have been highlighted for reducing energy consumption, improving the quantity and the quality of the generated oil, including continuous sterilisers, dry-heating and microwave heating (Chew et al., 2022; Vincent et al., 2014).

Aimed to minimise the oil loss and quality deterioration of palm oil, boiling pressure and time in conventional sterilisation have been reported (Febrina et al., 2019). Mild thermal processing generates a higher level of carotene and squalene as well as galactolipids, polyketides and sphingolipid derivatives (Kua et al., 2022). To this day, microwave treatment is one of the most commonly evaluated methods (Chow & Ngan, 2005; Hock et al., 2020; Sarah et al., 2023; Sukaribin & Khalid, 2009). Steam-microwave at atmospheric pressure avoids the interruption of the treatment thus saving more energy (Hock et al., 2020); continuous microwave sterilisation generates FFA with less than 5% (Chow & Ngan, 2005; Sarah et al., 2023). The efficiency of microwaves in POF detachment has been investigated which highlights higher efficiency and lower damage to the POF surface (Sukaribin & Khalid, 2009). The utilisation of ethylene has also been reported separately for improving the POF detachment (Balakrishnan et al., 2021; Chew et al., 2021; Zakaria et al., 2022) and ethephon (Balakrishnan et al., 2021).

As emphasised previously, these treatments are expected to modify the solubilisation of biomass, including hemicellulose, cellulose, lignin and extractives (Chew et al., 2022; Vincent et al., 2014). Those methods are mainly done by evolving physical and chemical treatments. To the best of our knowledge, the incorporation of biotechprocessing which includes fermentation in modifying the POF detachment has never been reported. Fermentation offers the advantage of utilising microbial enzymatic activity to break down the cellulose structure within POF, potentially enhancing fibre loosening and facilitating oil release. Although fermentation as a pre-treatment is not yet mainstream in the palm oil industry due to concerns over time efficiency and practicality in large-scale operations, it presents a valuable opportunity for decentralised, smallholder-based processing systems, especially in remote areas where access to advanced machinery is limited. Moreover, microbial-assisted processes align with sustainable development goals by promoting low-energy, environmentally friendly technologies. Therefore, exploring fermentation as a pretreatment strategy opens up a biotechnological pathway for improving fruit detachment while potentially enhancing oil quality parameters when optimised appropriately.

This study aimed to evaluate the effects of cellulolytic microorganisms-*Bacillus subtilis*, *Aspergillus niger* and *Trichoderma harzianum*-both individually and in combination, on the detachment of POF. These three microbes were selected due to their complementary enzymatic profiles. *Bacillus subtilis* is recognised for its strong production of cellulases and hemicellulases, along with its adaptability to a wide range of pH and temperature conditions, which makes it highly suitable for industrial bioprocesses (Kim et al., 2012). Meanwhile, filamentous fungi such as *Aspergillus*, *Trichoderma* and related genera are widely known for secreting a variety of hydrolytic enzymes-including lipases, cellulases and hemicellulases-that contribute to plant cell wall degradation and facilitate oil release during extraction (Barbieri et al., 2025). The assessment focused on their influence on detachment efficiency, oil yield and key chemical properties of the extracted oil, including FFA content, deterioration of bleachability index (DOBI), β-carotene levels, moisture content (MC) and peroxide value.

MATERIALS AND METHODS

The POF bunches of *Tenera* varieties at optimum maturity level (12.5%-25.0% of POF detached naturally from its bunches), were collected from the plantation field of the College of Agricultural Sciences, Plantation and Agribusiness (Sekolah Tinggi Ilmu Pertanian Agrobisnis Perkebunan), Medan, North Sumatera, Indonesia. The average weight of the POF ranged from 7-20 kg/bunch.

Potato dextrose broth (PDB) was purchased from Merck (Jakarta, Indonesia). *Bacillus subtilis* and *A. niger* were supplied by the Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara, Medan, Indonesia. *Trichoderma harzianum* was provided by the Indonesian Institute of Sciences (LIPI), Jakarta, Indonesia. All chemicals used were of analytical grade.

Preparation of Microbe Inoculums.

Potato dextrose broth (PDB) was prepared by dissolving 7.2 g of PDB in 300 mL of distilled water and autoclaved at 121°C for 20 min.

Bacillus subtilis culture was inoculated in nutrient agar media and then aseptically taken by using an ose needle into 9 mL of nutrient broth aseptically. It was incubated for 24 hr at 27°C ± 2°C. After that, 1 mL of the starter was added into 9 mL of nutrient broth aseptically and incubated again for 24 hr at 27°C ± 2°C.

Aspergillus niger and *T. harzianum* were inoculated in potato dextrose agar. The culture was taken aseptically by using an ose needle and dipped into 9 mL of PDB followed by a 48 hr incubation at 27°C ± 2°C (room temperature). After that, 1 mL of the starter was added to 9 mL of PDB and incubated for another 48 hr at 27°C ± 2°C. The microbial starter was subjectively tested using Mcfarland 0.5 solution, and a Total plate count (TPC) test was done to determine the number of colonies of 10^8 CFU/mL.

Experimental Design.

The POF bunches were inoculated with five different microbes: Single cultures (*A. niger, T. harzianum and B.subtilis*) and mixture cultures (mixture of *B. subtilis* and *A. niger* [1:1]; *B. subtilis* and *T. harzianum* [1:1]). The inoculated microbes (5%) were applied to a bunch (10-12 kg) by spraying. The fermentation was done in a wooden box at room temperature at 3 different times including 20, 40 and 60 hr. A control was prepared without the fermentation process with a 20 hr of storage. Therefore, 16 treatments of POF were obtained.

The treatment was carried out in triplicate. After the fermentation, the number and weight of fruits that naturally detach due to fermentation are counted as detached POF. The remaining fruits that do not detach naturally are manually detached using a knife. The detachment efficiency was determined by assessing the ratio between detached POF, empty bunches and attached POF with the number of bunches.

The detached treated POF samples, except the control, were then treated with 1 atm autoclave treatment at 121°C for 20 min for sterilisation. The autoclaved samples were dried in an oven (Memmert, USA) at 105°C for 24 hr before oil extraction.

Oil Extraction

The oil extraction was carried out following the procedures described by the Malaysian Palm Oil Board (MPOB) Test Method p.2.5: 2004 – as described previously (Chew et al., 2021). The dried POFs were cut and sliced to obtain POF mesocarp. The mesocarp (20 g) was placed in the Soxhlet Extractor's thimble. Hexane 300 mL was used as a solvent, and it was heated to a temperature of 60°C. The oil extraction process was run for 4 hr until the yellow colour disappeared. The hexane was removed using a rotary evaporator (Model Heidolph, USA) and was left in the oven (Model Memmert, USA) at 105°C for 2 hr to remove any moisture. The final weight of obtained crude palm oil (CPO) was determined to calculate the percentage of oil yield extraction. The CPO was investigated for its MC, FFA, DOBI, β-carotene and peroxide value.

Assessment of Threshing Efficiency

The detachment efficiency was determined by assessing the ratio between detached POF, empty bunches and attached POF with the number of bunches, as the Equations (1)-(3) below.

$$\text{DBR: } \frac{\text{weight of detached POF (kg)}}{\text{initial bunches weight (kg)}} \tag{1}$$

$$\text{EBR: } \frac{\text{weight of empty bunches (kg)}}{\text{initial bunches weight (kg)}} \tag{2}$$

$$\text{ABR: } \frac{\text{weight of attached POF (kg)}}{\text{initial bunches weight (kg)}} \tag{3}$$

where, DBR is the POF detached and bunches ratio, EBR is the empty bunches and bunches ratio and ABR is the POF attached and bunches ratio.

Evaluation of Chemical Quality Parameters

Moisture content (MC). The MC of CPO was determined by the oven drying method for 2 hr until it reached the stable weight as described in a previous study (Sarah et al., 2023). The CPO (10 g) was weighed into a known-weight of aluminum Petri dish. The Petri dish was heated in an oven at 103°C for 2.0-2.5 hr to obtain a stable weight. After that, it was cooled to room temperature in a desiccator. The percentage of mass loss was calculated as MC.

Free fatty acids (FFA). The determination of FFA was conducted following the procedures as described by Sarah et al. (2023) using the AOCS official methods Ca 5a-40. The CPO (7.05 ± 0.05 g) was weighed into Erlenmeyer and 75 mL of ethanol (95%) was added. After that, the mixture was shaken vigorously followed by addtion of 2 mL of phenolphthalein indicator. The mixture was then titrated with NaOH (0.25 M) until a rose-red colour stable for 30 s was obtained. FFA was calculated with Equation (4):

$$\text{FFA} = \frac{25.6 \times M \times V}{m} \qquad (4)$$

where, M is the molarity of NAOH solution (M), V is the volume of NaOH used during the titration and m is the mass of CPO used (g).

β-carotene and deterioration of bleaching index (DOBI). The β-carotene content was determined using a UV-Vis spectrophotometer following the procedures MPOB Test Method p.2.6: 2004 as described by previous study (Sarah et al., 2023). An amount of 0.04 g of CPO was weighed into a 10 mL measuring flask, and the volume was made up with n-hexane as solvent. The absorbance of a homogeneous and diluted sample of the CPO was done at 446 nm wavelength. The content of β-carotene was calculated with Equation (5).

$$\beta\text{-carotene} = \frac{V \times 383 \times Asb}{100 \times W} \qquad (5)$$

where, V is the solvent volume (mL), Asb is the absorbance of the sample and W is the weight of palm oil (g).

The DOBI was determined by calculating the numerical ratio between the absorbance of the homogenous sample at 446 nm and 269 nm.

Peroxide value. Peroxide value was measured to evaluate the oil oxidation using American Oil Chemists' Society (AOCS) official method Cd 8-53, AOCS, 2017 as previously described (Chew et al., 2021).

Statistical analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) using the F-test, and the least significant differences were computed at 5% probability for comparing the means between treatments. All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software, version 25.

RESULTS AND DISCUSSION

Threshing Efficiency

The efficiency of the threshing process on POFs detachment from bunches was evaluated based on the ratio between the amount of detached POF, remaining attached POF and empty bunches compared to the initial amount of bunches before treatment. The results are presented in *Table 1*. All treatments increased the detached and bunches ratio (DBR) which describes the ratio between detached fruits and initial weight of the bunches. The higher level of DBR depicted the increase in the amount of detached fruits from their bunches. The longer the fermentation time, the higher the DBR which ranged between 0.05-0.10, 0.07-0.13 and 0.14-0.22 for 20, 40 and 60 hr fermentation, respectively. Meanwhile, the control had a DBR level of 0.01. By this, the utilisation of cellulolytic microbes in the fermentation has increased the detachment of POFs from the bunches between 5-22 times higher than that in control. The DBR in the current study is lower compared to that in ethylene-treated bunches which generated up to 0.26-0.30 DBR (Balakrishnan et al., 2021; Zakaria et al., 2022).

The ratio between the remaining attached fruits and the initial weight of bunches, attached and bunches ratio (ABR), showed that the treatment decreased the ABR. The control had ABR at 0.85 while the treated samples had ABR at a range of 0.58-0.79. The lower the ABR describes the lower the amount of remaining attached fruits. This phenomenon is aligned with the higher level of DBR as mentioned previously. The higher level of detachment in the current study can also be explained by the level of empty bunches ratio (EBR). The higher EBR shows a higher amount of empty bunches, as the current study presented. Most of the treatments generated a higher EBR (0.14-0.29) compared to that in control (0.13). The improvement in the level of fruit detachment shows that the presence of cellulolytic microbes might have degraded the hemicellulose and cellulose matrix of the bunches, thus facilitating the loosening of the POFs from the bunches (Zhang & Zhang, 2010).

The utilisation of *B. subtilis*, *A. niger* and its combination increased the level of DBR as the fermentation period increased. However, *T. harzianum* and its combination with *B. subtilis* had a stable DBR level up to 40 hr fermentation followed by an increase at 60 hr fermentation. This phenomenon might show the mechanism of specific microbes in affecting the detachment of the POFs. The activity of *B. subtilis* and *A. niger* might have enhanced significantly throughout the fermentation while the activity of *T. harzianum* was stable during the 40 hr fermentation. The capability of *B. subtilis* to degrade and modify the structure of lignocellulosic biomass has been excellently reported (Kuhad et al., 2023; Huang et al., 2014; Malik & Javed, 2021; Park et al., 2021; Zhang & Zhang, 2010). *Aspergillus niger* is reported for its capability to facilitate the saccharification of lignocellulosic biomass (Siqueira et al., 2020) thus degradation of lignocellulose structure.

TABLE 1. THE IMPACT OF CELLULOLYTIC MICROBES ON THE THRESHING EFFICIENCY OF PALM OIL FRUITS (POF) AND ITS OIL YIELD EXTRACTION

Fermentation treatment	Fermentation period (hr)		
	20	40	60
Ratio between POF detached and initial bunches (RPD)			
Control		0.050 ± 0.031	
B. subtilis	0.111 ± 0.001^{d}	0.117 ± 0.004^{d}	0.175 ± 0.001^{b}
A. niger	0.156 ± 0.025^{c}	0.181 ± 0.036^{b}	0.186 ± 0.009^{a}
T. harzianum	0.111 ± 0.005^{d}	0.114 ± 0.004^{d}	0.112 ± 0.002^{c}
B. subtilis + A. niger	0.106 ± 0.006^{d}	0.110 ± 0.002^{d}	0.114 ± 0.002^{d}
B. subtilis + T. harzianum	0.112 ± 0.017^{d}	0.114 ± 0.001^{d}	0.156 ± 0.012^{c}
Ratio between empty bunches and initial bunches (REB)			
Control		0.134 ± 0.006	
B. subtilis	0.181 ± 0.012	0.170 ± 0.008	0.174 ± 0.016
A. niger	0.178 ± 0.002	0.170 ± 0.011	0.1930 ± 0.005
T. harzianum	0.171 ± 0.010	0.183 ± 0.012	0.186 ± 0.004
B. subtilis + A. niger	0.157 ± 0.011	0.170 ± 0.011	0.174 ± 0.018
B. subtilis + T. harzianum	0.152 ± 0.014	0.162 ± 0.011	0.205 ± 0.009
Ratio between POF remains attached and initial bunches (RPRA)			
Control		0.082 ± 0.032	
B. subtilis	0.738 ± 0.011^{a}	0.712 ± 0.009^{ab}	0.652 ± 0.016^{c}
A. niger	0.666 ± 0.005^{c}	0.648 ± 0.007^{c}	0.588 ± 0.012^{d}
T. harzianum	0.718 ± 0.013^{ab}	0.703 ± 0.014^{b}	0.655 ± 0.009^{c}
B. subtilis + A. niger	0.737 ± 0.013^{a}	0.717 ± 0.016^{ab}	0.716 ± 0.034^{ab}
B. subtilis + T. harzianum	0.735 ± 0.014^{a}	0.724 ± 0.012	0.639 ± 0.017
Crude oil yield extraction (%)			
Control		24.333 ± 0.357	
B. subtilis	20.975 ± 0.995^{c}	13.878 ± 1.453^{fg}	10.661 ± 0.993^{hi}
A. niger	24.463 ± 0.896^{b}	18.460 ± 1.936^{cde}	12.882 ± 2.416^{gh}
T. harzianum	25.626 ± 1.640^{b}	17.067 ± 2.689^{de}	14.369 ± 1.431^{fg}
B. subtilis + A. niger	28.609 ± 0.786^{a}	19.680 ± 0.139^{cd}	10.665 ± 1.809^{hi}
B. subtilis + T. harzianum	16.393 ± 1.168^{ef}	13.954 ± 1.587^{fg}	10.028 ± 1.403^{i}

Note: The data is shown as mean ± standard deviation from triplicate treatments. Letters show the significant differences from other treatments in the same column ($p<0.05$).

Oil Yield Extraction

The impact of microbes and the fermentation period significantly ($p<0.05$) affected the yield extraction. As presented in *Table 1*, the longer the fermentation period generated a lower level of yield extract. Initially, different microbes significantly fluctuated the amount of generated oil ranging between 16.4%-28.6%. The highest yield extract was obtained in the combination of *B. subtilis* and *A. niger* while the lowest was in the combination of *B. subtilis* and *T. harzianum*. After 40 and 60 hr fermentation, the yield extraction declined to a range of 13.9%-19.7% and 10.0%-14.4%, respectively. Compared to control, fermentation at 40 and 60 hr generated a lower level of oil yield extraction. The results showed that the treatments with *B. subtilis* and its combination with *T. harzianum* had a lower amount of rendement, regardless of the fermentation period, compared to other treatments. These results might have shown the capability of those specific microbes to facilitate the release of oil fraction from the mesocarp. *Aspergillus niger* and *T. harzianum* as well as coupled microbes (*B. subtilis* and *A. niger*) generated a higher level of oil fraction, particularly in 20 hr fermentation, it was higher than that in control. The combination of *B. subtilis* and *A. niger* had a higher yield extract showing that there might be synergistic effects between these two species in improving the release of oil fraction from mesocarp.

Meanwhile, the combination of *B. subtilis* and *T. harzianum* might have had antagonistic effects. *Aspergillus niger* has been highlighted as having high capability in lipid extraction from biomass in solid-state fermentation (Dulf et al., 2018; Hui et al., 2010). It was observed that the growth of *B. subtilis* can be exhibited by the presence of certain compounds in lignocellulosic biomass such as phenolic groups (Van Der Maas et al., 2021) and lignocellulosic-derived (Pereira et al., 2016); thus, generating a lower amount of yield oil extracts as discovered in the current study. The potential of cellulolytic microbes in oil recovery from biomass has been reported (Cheirsilp & Kitcha, 2015; Wei et al., 2013).

As described in the previous section, the longer fermentation period increased the amount of detached fruits showing a higher degradation occurred in a higher fermentation period. This phenomenon could lead to a higher yield extraction. After autoclave treatment, the lower yield was obtained in a higher fermentation period. This could explain the impact of autoclave treatment as the sterilisation process has exhibited the oil release from the mesocarp. The sterilisation process is an important step in order to inactivate the lipase activity, thus prohibiting the production of FFA. Therefore, finding a proper sterilisation method that allows higher release of yield extract is seemingly important for future investigation. As has been highlighted previously, the improvement of yield extract by finding the proper sterilisation method has been proposed in a previous study (Chew et al., 2022).

Moisture Content (MC)

The MC of the CPO (*Table 2*) showed that all the fermentations with cellulolytic microbes treatment dramatically declined the amount of MC, compared to that in control (2.90%). The longer the fermentation period generated a higher MC. The MC increased from 0.17%-0.37% at 20 hr fermentation to 0.96%-2.90% and 1.73%-2.46% at 40 and 60 hr fermentation, respectively. This phenomenon might occur due to the longer exposure of oil fraction released with water during fermentation thus forming a stronger bond interaction formed between water and the oil fraction. Consequently, the water might have been entrapped in oil fractions thus lowering the free water content. In most of the treatments, microbes has no significant (p>0.05) impact on the MC of the CPO. The fermentation process with cellulolytic microbes had a lower MC compared to that in the control. This phenomenon can be attributed to the higher free water available after fermentation thus easily evaporating during the autoclave treatment. In the control, the MC might still remain with the oil fraction with a stronger bond thus remaining in the oil fraction after autoclave treatment.

A new standard of MC of CPO has been proposed to 0.25% as the maximum level in order to maintain the level of FFA remaining at 5.00% maximum (Chew et al., 2022). Maintaining the MC of CPO as low as possible is important to maintain the hydrolytic and oxidative stability of CPO (Chew et al., 2022; Sarah et al., 2023). The low MC in the current study is due to the drying process after autoclave treatment which allows the remaining water to evaporate. Conventional sterilisation (wet process) might generate CPO with a higher level of FFA as an impact of water hydrolysis oil into FFA while the current study presented a dry sterilisation process which therefore generated a very low level of MC. The importance of maintaining a low MC of CPO has also been emphasised in a previous study (Sarah et al., 2023).

FFA Content

The results (*Table 2*) showed that all the treatments declined the amount of FFA compared to the control. The control and the treated samples met the FFA standard for CPO, which is below 5.00%. Within the fermentation treatments, the longer fermentation period generated a higher level of FFA. Regardless of the cellulolytic microbes, fermentation at 20 hr ranged the FFA at 0.45%-0.67% while fermentation at 40 and 60 hr resulted in the FFA at a range of 0.67%-0.99% and 0.78%-1.55%, respectively. The increase in FFA due to the longer incubation period and mechanical oil separation has also been observed with ethylene treatment (Balakrishnan et al., 2021). Compared to control, the fermentation on POFs bunches lowered the FFA by around 54.8%-86.8%. It is highlighted that maintaining a low level of FFA is recommended as it will improve the stability of the oil and reduce the oil rancidity (Chew et al., 2022). Therefore, the fermentation pre-treatment before sterilisation will offer a higher stability of generated CPO. The combination of *B. subtilis* with the other two cellulolytic microbes generated a lower level of FFA regardless of the fermentation period. The lower level of FFA might have shown that the fermentation treatment has increased the stability of the fatty acids or its resistance to lipid hydrolysis reaction and or fermentation might have highly inactivated the lipase enzyme presence in the mesocarp. Ebongue et al. (2006) found that the lipase enzyme derived from oil palm mesocarp exhibits its highest activity at a temperature of 35°C and pH 9. The fermentation process creates acidic conditions, which leads to a decrease in lipase activity and consequently results in lower

TABLE 2. CHEMICAL PROPERTIES OF CRUDE PALM OIL GENERATED BY FERMENTATION USING DIFFERENT CELLULOLYTIC MICROBES

Fermentation treatment	Fermentation period (hr)		
	20	40	60
Moisture content (%)			
Control		2.890 ± 0.186	
B. subtilis	0.266 ± 0.018^{fg}	0.960 ± 0.109^{e}	1.752 ± 0.106^{b}
A. niger	0.245 ± 0.003^{fg}	1.226 ± 0.096^{d}	1.847 ± 0.130^{b}
T. harzianum	0.172 ± 0.003^{g}	1.168 ± 0.142^{d}	1.729 ± 0.179^{b}
B. subtilis + *A. niger*	0.318 ± 0.012^{fg}	0.992 ± 0.048^{e}	1.826 ± 0.115^{b}
B. subtilis + *T. harzianum*	0.366 ± 0.029^{f}	1.436 ± 0.091^{c}	2.463 ± 0.122^{a}
Free fatty acids (%)			
Control		3.432 ± 0.191	
B. subtilis	0.668 ± 0.061^{fg}	0.811 ± 0.065^{e}	1.350 ± 0.059^{b}
A. niger	0.628 ± 0.000^{g}	0.942 ± 0.000^{d}	1.129 ± 0.124^{c}
T. harzianum	0.597 ± 0.120^{gh}	0.662 ± 0.060^{fg}	0.776 ± 0.060^{ef}
B. subtilis + *A. niger*	0.453 ± 0.059^{i}	0.669 ± 0.123^{fg}	0.813 ± 0.059^{e}
B. subtilis + *T. harzianum*	0.492 ± 0.0059^{hi}	0.993 ± 0.057^{d}	1.553 ± 0.064^{a}
β-carotene (ppm)			
Control		423.446 ± 1.330	
B. subtilis	317.354 ± 2.032^{b}	181.058 ± 1.570^{i}	167.109 ± 1.965^{j}
A. niger	274.989 ± 2.221^{d}	202.058 ± 2.114^{g}	182.726 ± 7.058^{i}
T. harzianum	308.241 ± 2.025^{c}	242.281 ± 1.387^{f}	191.053 ± 3.883^{h}
B. subtilis + *A. niger*	373.929 ±6.471^{a}	254.435 ± 1.248^{e}	245.756 ± 5.441^{f}
B. subtilis + *T. harzianum*	194.323 ± 10.151^{h}	176.562 ± 2.001^{i}	135.202 ± 7.052^{k}
Deterioration of bleaching index (DOBI)			
Control		0.800 ± 0.179	
B. subtilis	1.257 ± 0.125^{d}	0.935 ± 0.068^{b}	0.576 ± 0.051^{e}
A. niger	0.832 ± 0.045^{bc}	0.627 ± 0.022^{de}	0.481 ± 0.005^{f}
T. harzianum	1.458 ± 0.091^{a}	0.845 ± 0.023^{bc}	0.738 ± 0.049^{d}
B. subtilis + *A. niger*	1.356 ± 0.071^{a}	1.055 ± 0.147^{b}	0.706 ± 0.019^{d}
B. subtilis + *T. harzianum*	0.791 ± 0.047^{c}	0.583 ± 0.019^{e}	0.402 ± 0.026^{g}
Peroxide value (meq/kg)			
Control		9.172 ± 2.301	
B. subtilis	12.996 ± 2.235^{g}	26.216 ± 2.386^{de}	52.085 ± 4.157^{a}
A. niger	28.584 ± 2.197^{d}	41.938 ± 2.378^{b}	53.664 ± 1.865^{a}
T. harzianum	18.221 ± 2.271^{f}	28.553 ± 2.253^{d}	55.774 ± 2.047^{a}
B. subtilis + *A. niger*	12.968 ± 2.233^{g}	20.727 ± 2.285^{f}	32.579 ± 2.262^{c}
B. subtilis + *T. harzianum*	16.983 ± 2.129^{f}	25.943 ± 2.354^{e}	36.226 ± 2.370^{c}

Note: The data is shown as mean ± standard deviation from triplicate treatments. Letters show the significant differences from other treatments in the same column ($p<0.05$).

FFA production. The low level of FFA is aligned with the lower level of MC in the treated samples as described in the previous section. Control sample had a higher MC thus higher FFA content. The MC in oil will increase the hydrolysis which leads to high production of FFA (Chew et al., 2022). Therefore, the low level of FFA in the current study is aligned with the capability of the treatment in facilitating moisture release from mesocarp.

FFA is formed due to the lipid hydrolysis by the presence of lipase enzyme thus deteriorating the CPO (Chew et al., 2022). Lipase enzymes occur naturally in the POFs matrix. A higher activity of lipase could occur if higher lipase is released from the mesocarp due to cell wall rupture. Cell wall rupture releases the lipase enzymes, causing the hydrolysis of triacylglycerol (the main compound of CPO) converted sequentially to diacylglycerol, monoacylglycerol and FFA (Wong et al., 2016). Therefore, the lower level of CPO might have shown the capability of the fermentation to reduce the cell damage of POFs. The same phenomenon has also been observed due to the ethylene treatment in maintaining the FFA content of CPO (Chew et al., 2021). Conventionally, heat treatment is used to inactivate the lipase enzyme (Chew et al., 2022). However, suggestions for maintaining the FFA value to be low include minimising bruises of fruits during harvesting and carriage, reducing time between harvesting and sterilisation and modifying the sterilisation with pre-treatment (Chew et al., 2022; Vincent et al., 2014). Mechanical treatment increased the FFA up to nearly 4.0% while without mechanical treatment maintained the FFA at 0.6%-0.8% as the maximum (Balakrishnan et al., 2021). Ethylene treatment has also succeeded in decreasing the FFA from 3.7% in untreated CPO to 1.7% in ethylene-treated CPO (Chew et al., 2021); and dry-heating treatment declined the FFA to a range of 1.0%-2.2% (Hadi et al., 2009).

β-carotene

As presented in *Table 2*, the results showed that different cellulolytic microbes significantly ($p<0.05$) fluctuated the β-carotene content of CPOs. Compared to the control, all the treatments reduced the β-carotene. Within the treatments, the highest β-carotene was obtained in the combination treatment of *B. subtilis*: *A. niger*, followed by single treatment *B. subtilis*. This phenomenon might demonstrate the synergistic effect of *T. harzianum* and *B. subtilis* in preserving the level of β-carotene. The longer incubation period generated a lower level of β-carotene. The presence of β-carotene is responsible for the dark red colour in CPO. Therefore, a lower level of β-carotene resulted in a lighter colour of CPO. Carotene is a colour pigment in CPO naturally present in the mesocarp. The lower level of carotene due to the fermentation demonstrated the possibility of the carotene being entrapped in the mesocarp matrix. It was mentioned earlier that the fermentation process might not damage the cell wall thus declining the release of carotene. The fluctuation of carotene due to several treatments has also been reported; most of the studies highlighted the sensitivity of carotene in CPO to heat and light.

Continuous sterilisation decreased the carotene content to 546 ppm from 598 ppm in batch sterilisation (Kandiah et al., 2006). The decrease of carotene was also observed due to the higher power of microwave irradiation (Sarah et al., 2023). Moreover, the longer the microwave irradiation, the lower the carotene content from 614-439 ppm (Chow & Ngan, 2005). In contrast, microwave irradiation was able to enhance β-carotene in CPO (Chew et al., 2022) and ethylene increased the carotene content from 591-635 ppm (Chew et al., 2021). The alteration in carotene content might be influenced by the extraction methods which can cause the carotene to remain unextracted in the mesocarp (Sarah et al., 2023). Carotene is an antioxidant that prevents the CPO from oxidation. However, the lower level of carotene in CPO does not always depict its stability against oxidation. DOBI number represents the amount of carotene and secondary oxidation products present in the CPO. This value is often used to evaluate the quality of CPO which will be discussed further.

DOBI

The results (*Table 2*) showed that the fermentation pretreatment increased the DOBI value from 0.8 in control to a range of 0.8-1.5 in 20 hr fermentation and 0.6-1.1 and 0.4-0.7 in 40 and 60 hr fermentation respectively. The DOBI value declined as the fermentation period was longer. A higher DOBI value demonstrated a higher stability of the CPO against oxidation. Therefore, a lower DOBI shows a higher amount of oxidised carotenes. By this, the fermentation pretreatment was able to enhance the oil stability against oxidation which might occur during the sterilisation and oil extraction process. This result is aligned with the higher MC as the incubation period was higher. A higher MC in CPO will facilitate the oil hydrolysis thus further oxidation during the sterilisation and mechanical pressing during extraction. DOBI value describes the refining efficiency that could be done. All CPO is required to be refined and bleached. A higher level of DOBI shows an easier refining process can be expected. In contrast, CPO with a lower DOBI might be more difficult to refine as the oxidised carotenes are difficult to remove. In order to achieve an efficient refining and bleaching process, a minimum of 2.3 DOBI value is required (Chew et al., 2022).

Although the DOBI value in the current study is much lower than the proposed standard, it is worth noting that the applied fermentation pretreatment showed a capability in maintaining the DOBI value to be higher than without fermentation pretreatment. Utilisation of ethylene has also been able to increase the DOBI value (Chew et al., 2021). The low level of DOBI in the current study might be due to the sterilisation process, correlation between sterilisation effectiveness and pressure applied during the oil extraction in affecting the carotene content, thus the DOBI value (Vincent et al., 2014). Lower temperature processing can be expected to increase the DOBI value as proposed in an excellent review (Chew et al., 2022).

Peroxide value

The impact of fermentation pretreatment and autoclave treatments on the peroxide value of the obtained CPO is presented in *Table 2*. In general, the majority of the fermentation pretreatments generated a higher amount of peroxide value, compared to the control. A higher fermentation period had a higher level of peroxide value. The single use of the cellulolytic microbes generally increased the peroxide value at a higher rate after 40 and 60 hr fermentation. However, the combination of *B. subtilis* with *A. niger* or *T. harzianum* showed a lower rate of increasing the peroxide value. This phenomenon demonstrated the capability of those combined microorganisms to preserve the stability of the CPO against oxidation. This protective effect can be attributed to the reduction in FFA levels during fermentation, which decreases the availability of substrates for oxidation reactions, as FFAs are more prone to oxidative degradation than intact triglycerides (Lin et al., 2012). A higher level of peroxide value might show a higher level of metal contents, which was not investigated in the current study. It was observed that the peroxide level is aligned with the amount of iron and copper in CPO (Chew et al., 2021). The presence of iron and copper could have induced the lipid peroxidation thus increasing the peroxide value. The peroxide level is also influenced by the pre-existing peroxide content. The control contained 9.2 meq/Kg which can be categorised as pre-existing peroxide content. This initial amount could be broken down by autoclave treatment into free radicals, thus inducing excessive lipid peroxidation. Another reason for the higher amount of peroxide could be due to the higher level of MC as identified previously. The presence of hydrophilic groups in CPO could decrease the oil surface tension and increase the oxygen diffusion. This then leads to acceleration in lipid oxidation (Chew et al., 2021).

CONCLUSION

The study is the very first time revealing the enhancement of efficiency of POFs detachment by fermentation process as pretreatment incorporating cellulolytic microbes such as *B. subtilis, A. niger* and *T. harzianum* as well as combination of *B. subtilis* with *A. niger* or *T. harzianum* at 1:1 ratio. After sterilisation with autoclave treatment, the obtained CPO contained a lower MC and FFA and higher DOBI value compared to that in CPO without fermentation pretreatment. This phenomenon revealed the capability of those cellulolytic microbes in preserving the stability of CPO against the hydrolysis and oxidation process. Evaluating the fermentation period, a longer fermentation period increased the MC, FFA and peroxide value as well as declined yield recovery, carotene and DOBI, suggesting that a shorter fermentation period could be expected. The study demonstrated a synergistic effect by combining two cellulolytic microbes. Combining *B. subtilis* and *A. niger* resulted in a higher yield recovery. *B. subtilis* combined with *A. niger* or *T. harzianum* lowered FFA concentration and peroxidation rate. Although microbial fermentation is not proposed as a direct replacement for current industrial-scale sterilisation and stripping processes due to time and scalability constraints, it holds promising potential as a sustainable and low-cost pre-treatment approach especially suitable for small-scale or decentralised processing systems. It may also serve as a proof-of-concept for exploring biological innovations in palm oil processing with further optimisation to reduce fermentation time and enhance microbial performance. Further investigation into the sterilisation methods and shelf-life stability of the CPO is seemingly needed.

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