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BACTERIAL DEGRADATION OF PALM OLEIN IN SEAWATER AND IDENTIFICATION OF SOME CULTIVABLE STRAINS

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ABSTRACT

Palm oil transported in bulk through ocean can increase pollution risks due to accidental spillage or ship collision, especially the refined, bleached and deodorized (RBD) palm olein. Bacterial degradation of RBD palm olein in seawater was investigated as a preliminary finding on palm oil degradation by marine bacteria. The degradation of RBD palm olein was evaluated in seawater in shaken-flask cultures with different oil concentrations. Biochemical oxygen demand (BOD) in the seawater was determined based on changes in dissolved oxygen (DO) values before and at end of 5-day incubation. The concentrations of major fatty acid components in RBD palm olein before and after degradation were determined using gas chromatography-flame ionization detector (GC-FID). Isolated bacteria were screened for lipolytic activity using Spirit Blue Agar before molecular identification. The DO content reduced 20% over a 5-day incubation period and BOD value was determined to be 1.24 mg O_2/L based on DO values. The concentrations of fatty acids, namely palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1) decreased by 53%, 31% and 37%, respectively. The bacterial count increased from 980 CFU/mL during inoculation to 1.8 x 10^4 CFU/mL on day 5. Five phenotypically different bacterial strains (*Pseudoalteromonas gelatinolytica*, *Staphylococcus haemolyticus*, *Vibrio harveyi* and *Vibrio alginolyticus*) showed lipolytic activity. This study indicates that marine bacteria utilizes RBD palm olein as substrate, thus degrading it over time.

Key words: Palm oil, degradation, seawater, marine bacteria, South China Sea

INTRODUCTION

Palm oil industry is a socio-economically important sector in Malaysia, where 39% of global palm oil is cultivated and accounts for 44% of global exports (Malaysian Palm Oil Board [MPOB], 2017). Two types of oils are obtained from palm tree (*Elaeis guineensis*), crude palm oil (CPO) from the mesocarp and crude palm kernel oil (CPKO) produced from the kernel of the fruit (Gourichon, 2013). Various palm oil derivatives are produced after further refining process such as palm olein (PO), palm stearin (PS), kernel olein (KO), and kernel stearin (KS) (Mba *et al*., 2015). Palm oil are exported in bulk to key importing countries such as People's Republic of China, the European Union, Pakistan, India, and the United States (Bucas & Saliot, 2002). One of the most exported palm oil product is RBD palm olein (May, 2012).

Palm oil industry has contributed to pollution of marine environment in the form of accidental spill or oil spillage due to vessel accidents. Several cases of oil spills have been recorded and the recent incidents occurred at Chinese waters (South China Morning Post, 2017). Hong Kong beaches were shut after a while and oily substances floated and covered the water surface. Almost 9000 tonnes of palm oil stearin was spilled and reports show that only 90 tonnes was collected (The Straits Times, 2017).

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Biodegradation is the removal of organic component by microbial community via metabolic processes at a given environment (Al-Darbi *et al*., 2005). End products of the biodegradation pathway are energy, carbon dioxide and water (Koshimizu *et al*., 1997; Al-Darbi *et al*., 2005). Microorganisms capable of degrading oil have adapted to oil-contaminated environments and play a vital role in the bioremediation process (Margesin & Schinner, 2001). These microbes exist readily in seawater and are able to carry out partial or complete oil degradation and formation of water-soluble compounds or in some cases until the formation of carbon dioxide and water (Dean-Raymond & Bartha, 1975; Tango & Islam, 2002). Meanwhile, rate of biodegradation changes depending on the amount of nutrients available, presence of oxygen, pH, and temperature, composition of the vegetable oil, bacterial population and concentration of oil spilled (Al-Darbi *et al*., 2005). Degradation is also influenced by lipases produced by bacteria. Lipases are known to degrade triglycerides into glycerol and fatty acids (Salihu & Alam, 2012). Hence, the occurrence of vegetable oil degradation is highly possible in lipase-producing bacteria (Cornish *et al*., 1993). Previously, bacteria isolated from wastewater treatment system were found to degrade plant oil and fats, and reported to produce lipase (Bhumibhamon *et al.,* 2002).

Biodegradation of non-petroleum hydrocarbons such as vegetable oils and fish oils has been studied by Al-Darbi and colleagues (2005). They found that the degradation of oil is dependent on the structure of fatty acid including the length of carbon chain, number of double bonds and the biological reactivity of fatty acid molecules. However, there are no concrete studies on the microbial population that plays important role in palm oil biodegradation in marine environment. Investigation on this aspect is important as Malaysia exports large quantity of palm oil via ports and palm oil-degrading microbial population can be integrated as part of pollution or palm oil spill mitigation measure.

MATERIALS AND METHODS

Study Area

A total of 2 L of seawater sample was collected from Dapo Pata Uptown Kontena Beach, Tok Jembal, Kuala Nerus, Terengganu (Figure 1) at a depth of 15 cm from the surface in sterile 1-L Schott bottles (Hassanshahian *et al.,* 2014).

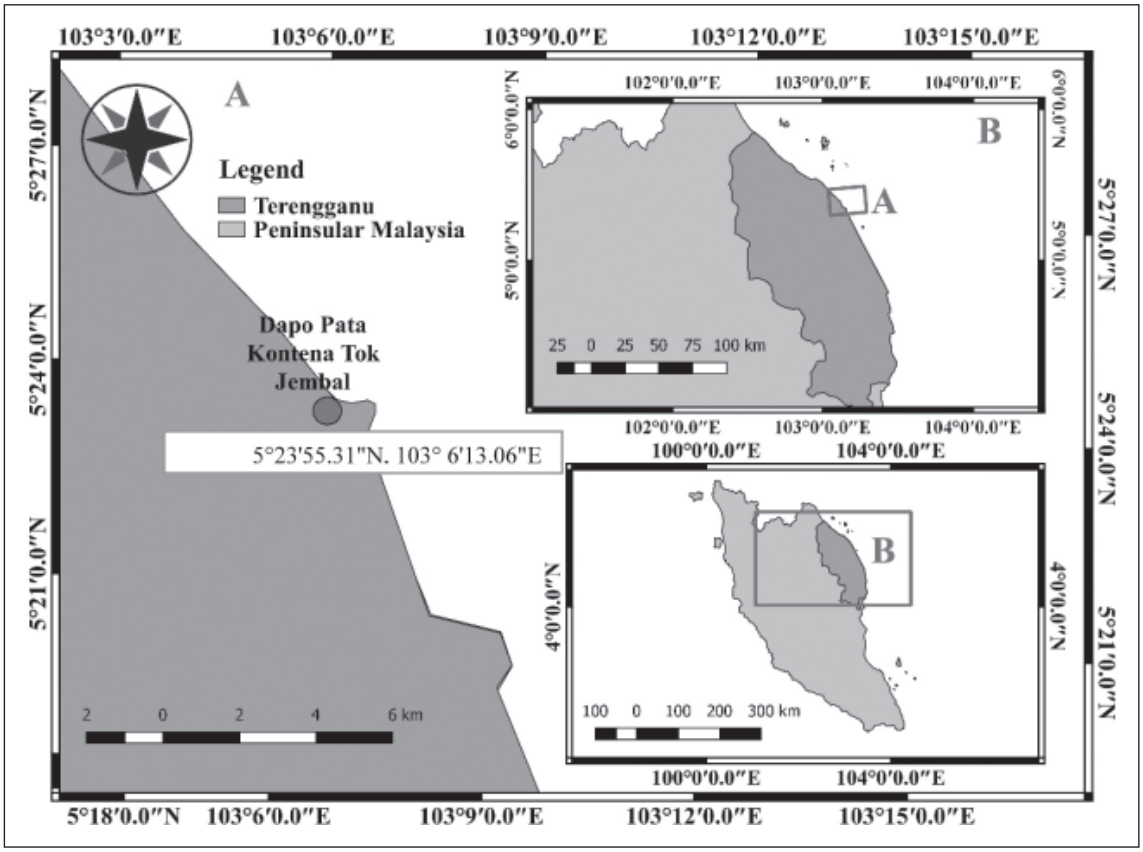

Fig. 1. The sampling location, Dapo Pata Uptown Kontena, Pantai Tok Jembal, Kuala Nerus (A), Terengganu (B).

Biochemical Oxygen Demand (BOD)

The pH of freshly collected water samples was adjusted to pH 7.0 in a BOD bottle and it was then stored overnight covered with stopper. The incubation bottle was then wrapped with aluminium foil and was incubated for 20 min at 20°C ± 0.1. Initial reading of dissolved oxygen (DO) at day-0 and final reading at day-5 of incubation were measured using BOD meter. BOD was calculated with the following formula from (Siang *et al*., 2012):

$$BOD = DF \times (DO_1 - DO_2) mg\ O_2/L$$

Where;
DO_1 = Initial DO reading at day 0
DO_2 = Final DO reading at day 5
DF = Dilution factor

Palm Oil Degradation and Bacterial Cultivation

A total of four concentrations (1 g/L, 3 g/L, 5 g/L and 7 g/L) of RBD palm olein-seawater culture were prepared in triplicates using 50 mL of filtered seawater, each of which was placed in 250 mL Erlenmeyer flasks (Bhubalan *et al*., 2010). A total of 1 mL/L of each nutrient stock solution was added into each sample flask as stated in Organization for Economic Cooperation and Development Guidelines for Testing Chemicals, OECD TG 306 with some modifications (OECD, 1992). The flasks were then placed in Protech Orbital Shaker *721* and shaken at 28°C, 150 rpm for 7 days. A 10-fold dilution series was performed on day 0, 3 and 5. Bacteria were cultured on *Zobell* marine agar and Colony Forming Unit (CFU) count was determined.

Determination of Fatty Acid by GC-FID

The direct ethylation of free fatty acid in RBD-Palm olein was derived using an acid-catalysed method following the procedure described by Anuar *et al.* (2015). Firstly, 1 mg of each RBD palm olein was mixed with 1% sulphuric acid in a test tube. Then 2 mL methanol was added to the mixture followed by 1 mL toluene. The mixtures were left overnight at 50°C in water bath. Next day, the samples were washed with 5 mL of 5% sodium chloride in distilled water using pipette before adding another 5 mL hexane to extract the fatty acid methyl esters (FAMEs). The extraction with hexane was performed twice to ensure that all the FAMEs were extracted. Hexane layer was washed with 2% $NaHCO_3$ solution (2mL) and dried with 2 g of anhydrous sodium sulphate (Na_2SO_4). Prior to GC analysis, samples were weighed and diluted with hexane. In the fatty acid analysis, Supelco 37 Components FAME 37 mix was used as standard reference material. The samples and standards were analyzed using Agilent 7890 GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector (FID) and are run on BPX-70 column 110 m x 0.25 mm x 0.25 mm (Agilent Technologies, Santa Clara, CA, USA) (Anuar *et al*., 2015). The GC parameters were set based on methods described by Anuar *et al*., 2015 with slight modification. The parameters were as follows: volume of injection: 1 µL, FID carrier gas: H_2, make up gas: compressed air, flow rate: 35mL min^{-1}, inlet temperature: 250°C, detector temperature 250°C. The temperature program was as follows: 35°C (hold 5 min); 25°C min^{-1} to 195°C (0 min); 3°C min^{-1} to 205°C (0 min); 8°C min^{-1} to 230°C (hold 6.64 min).

Isolation and Identification of Palm Olein-Utilizing Bacteria

After 5 days, a 1:10 serial dilution of up to a concentration of 10^{-3} was performed for each palm olein-seawater culture and spread plated on *Zobell* marine agar. The cultures were incubated for 24 hours at 30°C. Phenotypically different colonies were isolated and presence of lipase was determined using Difco™ Spirit Blue Agar, prepared using 1% palm olein. The isolated colonies were streaked on Spirit Blue Agar and incubated at 30°C for 24 h. Isolated colonies that were found positive for the presence of lipase were identified by sequencing 16S rRNA genes amplified in *Bio-Rad S1000 Thermal Cycler* using direct colony-based PCR technique with primers 63F (5'-CAGGCCTAACA CATGCAAGTC -3) and 1389R (5'-ACGGGCGGT GTGTACAAG-3'). The PCR protocols were set based on methods described by Hongoh *et al.* (2003) with some modifications. The protocol was as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 51°C for 1 min, extension at 72°C for 2 min and the steps from denaturation to extension was repeated for 34 cycles with a final extension at 72°C for 5 min. The PCR product was incubated at 10°C prior to purification. The PCR products were purified using Promega™ Wizard® SV Gel and PCR Clean-up System based on manufacturer's protocols. The purified products were sent to 1st BASE Apical Scientific for sequencing and identified by comparing the sequences to Basic Local Alignment Search Tool (BLAST) databases using Standard Nucleotide BLAST (BLASTn).

RESULTS

Biochemical Oxygen Demand (BOD) and Bacterial Growth

Table 1 shows the DO (mg/L) values of the water sample at day 0 and 5. Results showed that there is slight decrease of DO in seawater. Based on the DO readings, the BOD value calculated was 1.24 mg

Table 1. Dissolved oxygen reading of seawater and bacteria growth analysis at day 0 and 5

Day	Dissolved oxygen* (mg/L)	Bacterial growth (CFU/mL)
0	6.76	9.8×10^1
3	N. A[b]	1.2×10^3
5	5.42	1.8×10^4

* Samples kept in dark condition at room temperature.
[b] No measurement was taken for DO at day 3.

O_2/L. The CFU count of bacteria cultured from water sample on selected interval showed that there is an increase in microbial count over the 5 day period of study as the DO level decreases. The bacterial count on day 5 was 1.8×10^4 CFU/mL which is higher than 9.8×10^1 CFU/mL count on day 0.

Fatty Acid Composition in Residual Oil

Figure 2 shows the concentration of fatty acids in RBD palm olein before and after degradation. The concentration of palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1) decreased by about 53%, 31% and 37%, respectively. However, the concentration of myristic acid (C14:0) was not detected on sample from day 5.

Isolation and Identification of Palm Olein Utilizing Bacteria

A total of five phenotypically different bacterial colonies were isolated. All five isolates were tested positive for the lipolytic activity (Figure 3(a)-3(f)). Table 2 showed the nucleotide BLAST results of these five isolates. The bacteria identified were

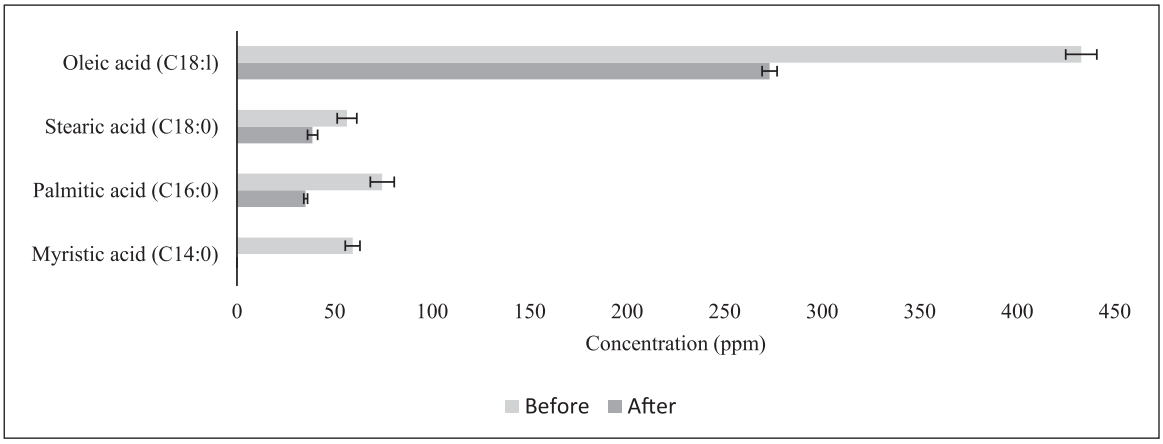

Fig. 2. Fatty acid composition of RBD palm olein before and after incubation in seawater.
*Note: Myristic acid (C14:0) was not detected in residual oil after incubation period.

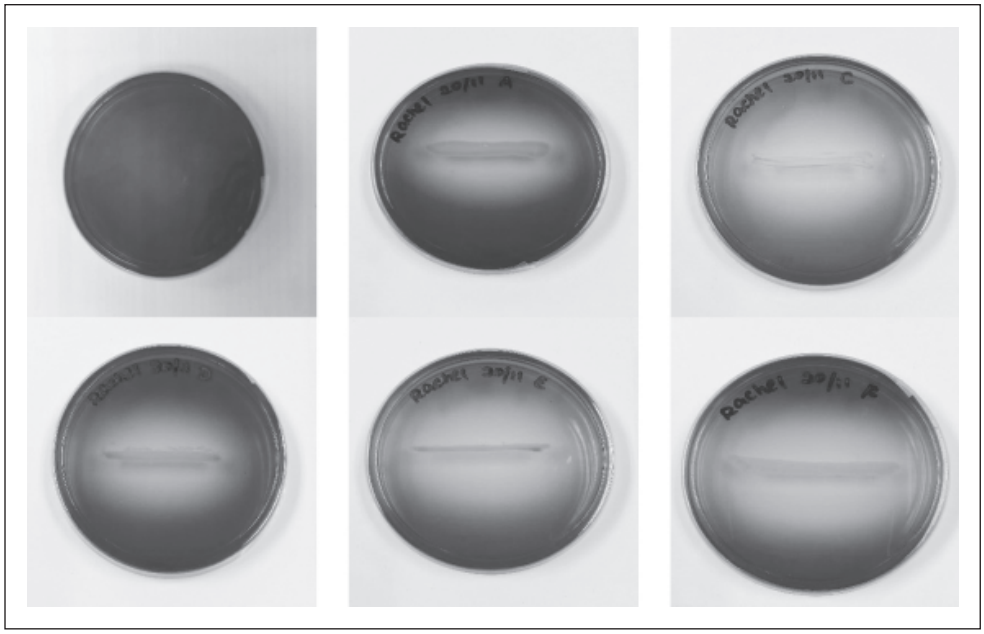

Fig. 3. Spirit Blue Agar after 24 hrs incubation with a) negative control (bacteria absent) b) bacteria A c) bacteria B d) bacteria C e) bacteria D and f) bacteria E; b-f showed lipolytic activity for the presence of lipase.

Table 2. Closest BLAST matches against NCBI-database of 16S rDNA sequences recovered from bacterial isolates

Bacterial Isolate	Accession No.	Identification result	Similarity (%)	Query Cover (%)
A	NR_152003.1	*Pseudoalteromonas gelatinolytica* (NH153)	98	87
B	NR_113345.1	*Staphylococcus haemolyticus* (JCM 2146)	98	99
C	NR_113781.1	*Vibrio alginolyticus* (NBRC 15630)	95	93
D	NR_113781.1	*Vibrio alginolyticus* (NBRC 15630)	94	96
E	NR_043165.1	*Vibrio harveyi* (NCIMB1280)	97	97

Pseudoalteromonas gelatinolytica, *Staphylococcus haemolyticus*, *Vibrio harveyi* and *Vibrio alginolyticus.*

DISCUSSION

During the degradation experiment, presence of white particles of varying sizes and shapes were noticed in the palm olein-seawater culture. The white particles were oils that underwent saponification process where palm oil reacted with sodium, potassium or calcium in seawater to produce fatty acid salts (Al-Darbi *et al*., 2005). This corresponds with few other vegetable oils whereby when introduced into a seawater culture, both canola and sunflower oil forms white particles or solid polymers in seawater (Mudge *et al*., 1993; Al-Darbi *et al*., 2005). Polymerization of canola oil was reported in a study by Campo *et al.* (2012) with existence of solid flakes insoluble in dichloromethane (DCM).

BOD is defined as amount of oxygen required by microbes to degrade organic matter (Obahiagbon *et al*., 2009). In a five-day period of incubation, slight decrease in DO level was observed. The BOD value utilized was 1.24 mg O_2/L which reflects the growth of bacteria by utilizing oxygen. Seawater contains microbes that partially or completely degrade organic matter into carbon dioxide and water (Tango & Islam, 2002). Furthermore, the growth of microbes was clearly observed in increasing CFU counts in *Zobell* marine agar. The availability of nutrients in the form of RBD palm olein in seawater culture enabled the growth of microbes which is translated in the increasing CFU counts. A study by Bucas and Saliot in 2002 reported that bacteria grows rapidly around oil slick layer or solidified balls in event of vegetable oil spill in seawater. Similar trend in terms of bacterial growth was reported by Al-Darbi *et al.* (2005) where microbial count increases along with incubation period which reflects the oil degradation in seawater.

In the fatty acid analysis, samples were converted to FAME by using direct acid-catalyzed ethylation using H_2SO_4 because free fatty acids are difficult to be analyzed in underived form (Anuar, 2015). The oleic acid concentration was the highest in RBD palm olein and similar reports were made in previous studies where oleic acid is about 39.8-43.6% of fatty acid composition (weight % as methyl esters) (Standard Malaysia, 2007). Furthermore, the composition of stearic acid, palmitic acid and myristic acid are reported to be in the range of 3.7-4.8%, 38.2-42.9% and 0.9-1.2% respectively (Standard Malaysia, 2007). The comparison of fatty acid concentration (ppm) between unutilized RBD palm olein and residual RBD showed a decrease in concentration. This decrease in fatty acid concentration can be attributed to degradation of palm oil in seawater and subsequent consumption by microbes. The reduction in fatty acid concentration was observed in previous study of canola oil degradation (Campo *et al.,* 2012). Concentrations of palmitic acid, stearic acid, oleic acid, linoleic acid were reduced in the 30 days period of experiment and this reduction was further substantiated by 85.7% of oil biodegradation of tested canola oil (Campo *et al.,* 2012).

Five phenotypically different bacteria were isolated from incubation medium and all the isolates showed positive results towards screening for lipolytic activity. According to Broekhuizen and coworkers (2003), the first step in vegetable oil degradation is cleavage of ester bonds to fatty acid which is catalyzed by enzymes esterase and lipases that are synthesized by microorganisms. The presence of lipolytic activity in the isolated bacteria proves that these bacteria can break-down and utilize the free fatty acid of RBD palm olein. Previous study on vegetable contaminated soil sample also recorded the presence of bacteria from genus *Staphylococcus* and *Bacillus* which exhibited lipase activity (Kamble *et al.,* 2011). Marine bacteria from genus *Bacillus* was isolated and found to exhibit lipolytic activities (Baharum *et al.,* 2009). However, there were no vegetable oil degradation tests that were carried out in this study.

CONCLUSION

RBD palm olein was degradable in seawater and this was evident in the observed BOD value and CFU counts and reducing fatty acid concentration in

recovered oil. The isolated microbes were found to thrive under palm olein enrichment. Future studies on the palm oil-degradation rate by these strains is recommended and the toxicity of palm oil-degraded by-products should also be investigated to aid in employing appropriate bioremediation methods.

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