Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

MICROBIAL DEGRADATION OF PALM OIL IN NATURAL SEAWATER AND IDENTIFICATION OF OIL DEGRADING BACTERIAL CONSORTIUM

(Degradasi Mikrob Minyak Sawit dalam Air Laut Semula Jadi dan Pengenalpastian Konsortium Bakteria Pendegradasi Minyak)

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Received: 15 March 2022; Accepted: 3 July 2022; Published: 30 October 2022

Abstract

Palm oil industry is among the most important commodities industry in Malaysia where Malaysia dominates 39% and 44% of global palm oil production and exports respectively. Most of the palm oil exports to various countries are done via sea-shipping which increases the risk towards marine pollution in form of oil spillage from vessels. Microbial degradation studies are important in establishing baseline data which is instrumental for mitigation planning and policy making. Degradation of palm oil derivatives was investigated using natural seawater collected from Klang Port and isolated bacteria *Pseudomonas aeruginosa* UMTKB-5 based on modified shake flask method as described by OECD Guidelines for Testing Chemicals, OECD TG 306 (Biodegradability in Seawater). Analytical method for determination of CPO and CPKO degradation comprises of measurement of dissolved organic carbon (DOC), colony forming unit (CFU), bacterial diversity using 16S rDNA gene based metagenomic analysis, and fatty acid measurements. Degradation of CPO and CPKO in seawater collected from Klang Port show increase in bacterial population which peaked in day 7 and 21 before declining indicating that palm oil is being used as substrate for bacterial growth in tandem with degradation which is aided by lipase enzyme produced by selected bacteria. Similar growth pattern observed in *P. aeruginosa* UKTKB-5 cultivated sample. DOC removal in sample showed negative value showing that carbon input from CPO and CPKO degradation is higher than consumption by bacteria. Fatty acid measurement shows changes in composition where bacterial degradation and utilisation of oil. The metagenomic analysis revealed diverse bacteria population in the different sampling locations and four lipase-producing bacterial strains were isolated at the end of the biodegradation experiment. The study has shown the biodegradability of palm oil in seawater and able to provide baseline data to understand and formulate the action plan in the event of spill in marine environment.

Keywords: palm oil, bacteria diversity, microbial degradation, fatty acids

Abstrak

Industri minyak sawit adalah antara industri komoditi terpenting di Malaysia di mana Malaysia menguasai 39% dan 44% pengeluaran dan eksport minyak sawit secara global masing-masing. Kebanyakan eksport minyak sawit ke pelbagai negara dilakukan melalui perkapalan laut yang meningkatkan risiko pencemaran marin dalam bentuk tumpahan minyak dari kapal. Kajian degradasi mikrob yang mampan adalah penting dalam mengesahkan data asas yang memainkan peranan penting untuk perancangan mitigasi dan pembuatan dasar dan polisi. Degradasi minyak sawit tidak ditapis (CPO) dan minyak isirong sawit tidak ditapis (CPKO) dalam air laut semula jadi yang dikumpul dari Pelabuhan Klang and bakteria *Pseudomonas aeruginosa* UMTKB-5 telah dikaji menggunakan kaedah kelalang goncang yang diubah suai seperti yang diterangkan oleh garis panduan OECD untuk pengujian bahan kimia, OECD TG 306 (kebolehbiodegradan dalam air laut). Analisa process degradasi CPO and CPKO melibatkan pengukuran karbon organik terlarut (DOC), kiraan unit pembentuk koloni (CFU), analisis metagenomik berasaskan gen 16S rDNA untuk kepelbagaian bakteria, dan perubahan acid lemak bebas. Keputusan menunjukkan peningkatan dalam kiraan CFU apabila bilangan hari meningkat dan kiraan CFU berada pada tahap tertinggi pada hari ke-7 dan 21 sebelum menurun. Situasi ini menunjukkan penggunaan minyak sawit sebagai substrat oleh bakteria. Peningkatan populasi bakteria yang sama ditunjukkan oleh bakteri *P. aeruginosa* UMTKB-5. Perubahan nilai penyingkiran DOC kepada nilai negatif mendedahkan kemasukan karbon melalui process degradasi CPO and CPKO pada tahap yang melebihi penggunaan oleh bakteria. Perubahan komposisi asid lemak penggunaan substrat oleh bakteria. Analisis metagenomik mendedahkan populasi bakteria yang pelbagai di lokasi persampelan yang berbeza dan lebih daripada empat strain bakteria telah diasingkan pada akhir eksperimen biodegradasi. Strain positif juga diuji untuk aktiviti lipolitik. Kajian ini telah mendedahkan kebolehbiodegradasian minyak sawit dalam air laut dan dijangka dapat membekalkan maklumat asas kepada pihak berkuasa berkaitan minyak sawit, pengangkutan dan agensi alam sekitar untuk memahami dan merangka pelan tindakan sekiranya berlaku tumpahan dalam persekitaran marin.

Kata kunci: minyak sawit, kepelbagaian bakteria, bakteria pendegradasi, asid lemak

Introduction

The demand for vegetable oil for food and as alternative fuel has fuelled the rapid production of oilseed [1]. The vegetable oil production is estimated to be around 187 million tonnes for 2016/2017 and is expected to grow to 195 million tonnes in the following year [2]. Four types of vegetable oil namely palm oil, soyabean oil, rapeseed oil and sunflower oil dominated the global vegetable oil market [1,3]. The major vegetable oil produced are palm oil (PO) and palm kernel oil (PKO) which accounts for 70.3 million tonnes (37.6%) of global production and followed by soybean oil where 55 million tonnes (30%) are produced [1].

The major producer of PO and PKO are Indonesia and Malaysia where nearly 86.5% of oil is produced. Malaysia is the second largest producer of palm oil where it accounts for 39% of global palm oil production and 44% of exports [4]. The oil palm tree *Elaeis guineensis* of tenera (thin shelled) variety is commonly planted in Malaysia due to its high oil yield [5]. Oil palm of tenera variety has higher palm oil content where it yields about four to five tonnes of crude palm oil (CPO) and one ton of crude palm kernel oil (CPKO) per hectare per year which made it as suitable planting material for commercialisation approach.

Significant portions of export and import activities of palm oil and its derivatives are conducted via sea shipping [6]. Malaysia exports palm oil to countries such as People's Republic of China, European Union, India, and United States via sea-shipping through major ports such as Johor Port, Klang Port, and Sandakan Port. This mode of transportation has led to pollution of marine environment where spillage of palm oil and its derivatives has occurred. There are several reported incidents of palm oil, and the most recent incident is the oil spill that occurred in Chinese waters where 9000 tonnes of palm stearin spilled from vessel collision [7] as well as dumping of palm oil by ships at the North Sea, Great Britain [8]. Vegetable oil spill incidents can negatively impact the exposed ecosystem where exposed organisms suffer oxygen depletion, toxicity effects, and smothering of surface-dwelling organisms causing fatal effects.

The United States Environmental Protection Agency (USEPA) has classified environmental impact of

vegetable oil spill as similar to hydrocarbon oil spills [9]. However, there are limited studies on the degradation of vegetable oil especially palm oil in marine environment. A study into natural palm oil degradation process and the role microbes play in the degradation process is necessary to understand the potential short and long-term impact of vegetable oil spills. This study aims to investigate the utilisation of different palm oil derivates by naturally occurring bacteria and cultivated marine bacteria to assess the degradability of oil via assessment of bacterial population, carbon removal, and fatty acid profile.

Materials and Methods

Degradation of crude palm oil and crude palm kernel oil using natural seawater: Study area

The sampling site chosen for natural seawater collection was Klang Port where the sample was collected at the coordinates for Site A: 2.999119°N, 101.379022°E and Site B: 3.004477°N, 101.354947°E (Figure 1). The seawater was collected based on the methods by American Public Health Association (APHA) [10]. Furthermore, the physical parameters such were measured and recorded *in situ* using portable Hydrolab Quanta Multiprobe meter. The sample was stored in ice until further analysis.

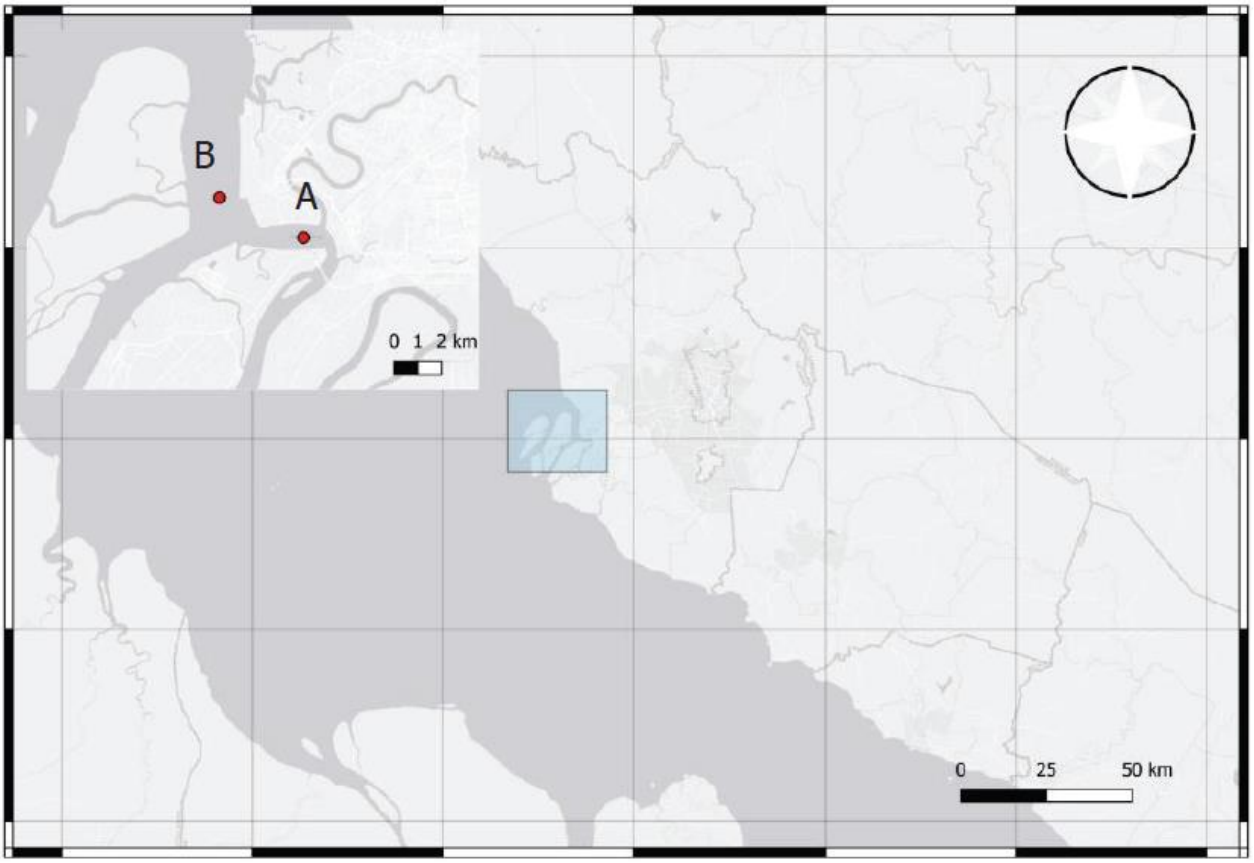

Figure 1. Map indicating the seawater sampling location at Klang Port, Selangor

Degradation of palm oil

The degradation of crude palm oil (CPO) and crude palm kernel oil (CPKO) that were obtained courtesy of MPOB were investigated using OECD Guidelines for Testing Chemicals, OECD TG 306: Biodegradability in Seawater with some modifications [11]. Shaken flask culture method was used in this study. The seawater was enriched with 1 mL litre^{-1} of each mineral nutrients that were prepared according to OECD guideline. The degradation study included blank, which is enriched

seawater without any test substance, test substance at two loading rate (1 g L^{-1} and 10 g L^{-1}) and aniline as reference substance. The degradation study was carried out for a period of 42 days at room temperature and was incubated at 150 rpm using Protech Orbital Shaker 721 ambient shaker. Samples were taken at selected interval for colony forming unit (CFU) determination using Zobell Marine Agar 2216 (HiMedia, India) and dissolved organic carbon (DOC) measurement. Furthermore, formation of floating and precipitate particles, oil droplets and dispersion were observed. Experiment was conducted in triplicates.

Determination of DOC

The DOC levels in culture medium were determined on samples from day 0, 7, 14, 21 and 28. The collected water samples were filtered using 0.45-µm polyether sulfone syringe filter (Sartorius, Germany) [12]. The DOC was measured using high-temperature catalytic oxidation (HTCO) method with a Shimadzu TOC-L & SSM 5000A (Shimadzu, Japan) total organic carbon equipped with non-dispersive infrared gas analyser (NDIR) detector [13]. The sample were acidified to pH then the samples were then sparged for 5 min before being analysed. Calibration of the instrument was done using freshly prepared standards [14]. Based on the DOC data obtained, degradation value was calculated based on formula provided in the guidelines [11].

Identification of bacterial consortium

The bacterial population of water sample before and after cultivation period were identified. The water sample collected during sampling was outsourced to Apical Scientific Sdn. Bhd. immediately after sample for metagenomic assessment of bacterial population. Meanwhile, phenotypically different bacterial colonies obtained at the end of degradation experiment was identified using 16S rDNA sequencing. Polymerase chain reaction (PCR) amplification was performed under the conditions: 3 min initial denaturation at 95°C; 34 cycles of denaturation (30 s at 95°C), annealing (45 s at 55°C) and extension (90 s at 72°C); and final extension at 72°C for 5 min [15,16]. The primer sets used were 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') [15,16]. The PCR products were sent to Apical Scientific Sdn. Bhd. for purification and sequencing. The obtained sequences obtained were compared with Basic Local Alignment Search Tool (BLAST) databases using Standard Nucleotide BLAST (BLASTn) to identify the bacteria.

Degradation of crude palm oil and crude palm kernel oil using natural seawater: Bacterial strain and utilisation test

P. aeruginosa UMTKB-5 (GenBank accession number: KT194193.1) was obtained from the culture collection of Institute of Marine Biotechnology, Universiti Malaysia Terengganu (Terengganu, Malaysia) [17]. The strain was isolated from marine sediment of Pulau Bidong. The strain was maintained on Zobell Marine Agar 2216 (HiMedia, India). The utilisation test was conducted using modified OECD TG 306 method based on previous study [15]. Test substance which are aniline, CPO, CPKO refined bleached and deodorised palm oil (RBDPO) and refined, bleached and deodorised palm olein (RBDPL) were added at concentration of 1 g L^{-1} into 100 mL working volume of artificial seawater. The utilisation study was carried out for a period of 8 days at room temperature and was incubated at 150 rpm using Protech Orbital Shaker 721 ambient shaker. The utilisation of different palm oil derivatives by *P. aeruginosa* UMTKB-5 was determined by CFU count determination, residual oil measurement, emulsification test [18] and free fatty acid determination.

Residual oil measurement

The residual oil was extracted using 30 mL of hexane on the culture broth [10,19]. The mixture was shaken vigorously, and 5 mL of hexane layer is transferred onto pre-weighted container and air-dried until complete evaporation of hexane before weighing.

Fatty acid determination via gas chromatography-flame ionisation detector (GC-FID)

The acid-catalysed ethylation of sample was done according to a methodology described by Anuar et al. [20] and some modifications based on Bhubalan et al. [15]. Total 1 mg of sample was mixed with 1% H2SO4 (1 mL) in a test tube. The mixture was then added into 2 mL of methanol followed by 1 mL of toluene (C_8H_8) before leaving the mixtures overnight at 50°C. Then the

samples were washed with 5 mL of 5% sodium chloride (NaCl) in distilled water using pipette. After washing, 5 mL of hexane was used to extract the fatty acid methyl ester (FAME). The extraction was performed twice to ensure all the FAMEs were extracted. The hexane layer was washed using 2 mL of 2% NaHCO3 solution and dried with anhydrous sodium sulphate (Na_2SO_4). Prior to GC analysis, samples were weighed and diluted with hexane. The GC-FID parameter setting is done according to previous study as reported by Bhubalan et al [15].

Results and Discussion

Degradation of CPO and CPKO using natural seawater collected from Klang Port

Based on Figure 2, it can be noted that, the bacterial counts were increasing over the 42 day of experiment excluding the sudden drop at day 14. The colony count with aniline (AR, BR) as test substance dropped sharply from day 28 compared to other test substances. Table 1 shows the degradation of test substances in percentage of DOC removal where 70% DOC removal is needed for a substance to be concluded to have potential biodegradation ability in marine environment. Based on the results obtained, only aniline (1 g L^{-1}) [AR, BR] achieved more than 70% DOC removal. The CPO (1 g L^{-1}) [A1C1 B1C1] CPO (10 g L^{-1}) [A1C2, B1C2], CPKO (1 g L^{-1}) [A2C1, B2C1] and CPKO (10 g L^{-1}) [A2C2, B2C2] showed a negative trajectory on regards of DOC removal which is an interesting observation.

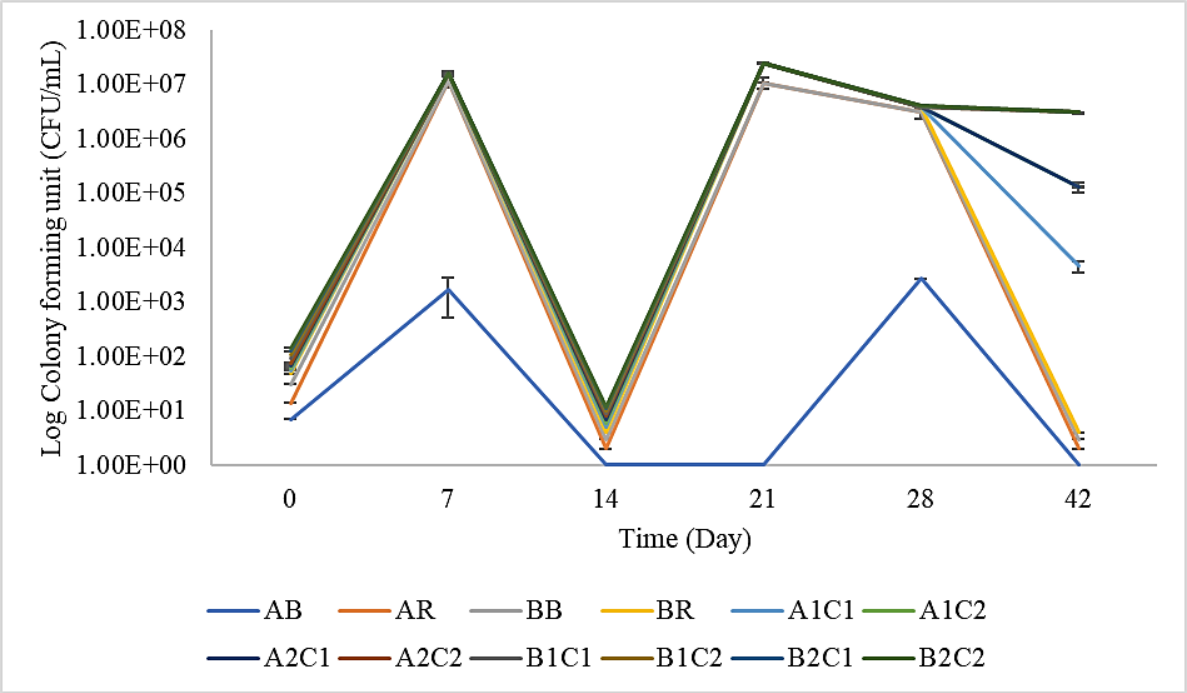

Figure 2. CFU (log CFU/mL) count of bacteria in test substance over 42-day period

Table 1. Degradation in percentage DOC removal of test substance over test period

Sample ID	7	14	21	28	42
AR	98.086	99.322	97.881	97.820	100.335
A1C1	-5510.538	-1874.647	-1999.754	-1170.653	-310.645
A1C2	-4821.903	-981.477	-2801.420	-3299.845	-3794.894
A2C1	-1529.398	-1008.931	-2196.576	-2628.693	-8420.959
A2C2	-218.753	-926.742	-751.751	-1828.224	-1877.354
BR	99.446	98.960	98.054	98.886	99.154
B1C1	-168.624	-1010.523	-579.506	-1026.525	-1960.295
B1C2	-32.329	-7303.336	-12856.814	-4824.614	-6293.079
B2C1	-929.503	-5407.807	-2223.564	-3737.025	-2781.695
B2C2	-41.934	-988.270	-810.753	-1815.315	-3089.373

The metagenomic results of bacterial population at the start of the experiment shows a diverse bacterial population compared to the bacteria isolated at the end of 42 days of experiment (Figure 3). The genus of isolated bacteria was present in the metagenomic results indicating their presence in the beginning of the experiment. A total of four phenotypically different bacteria were isolated. Table 2 shows the nucleotide BLAST results of these bacteria. The isolated bacteria were *Microbacterium* sp. 1204, *Vibrio fluvialis* NCTC 11327, *Oceanobacter* sp., and *Thalassospira profundimaris* WP0211.

Table 2. BLAST matches against NCBI-database of 16S rRNA sequences obtained from bacterial sequences

Bacterial Isolate	Accession No.	Identification Result	Similarity (%)	Query Cover (%)
N	NR_149816.1	*Microbacterium* sp. 1204	97.85%	92%
J	NR_118443.1	*Vibrio fluvialis* NCTC 11327	98.70%	100%
P	NR_113758.1	*Oceanobacter* sp.	92.78%	92%
O	NR_042766.1	*Thalassospira profundimaris* WP0211	97.36%	95%

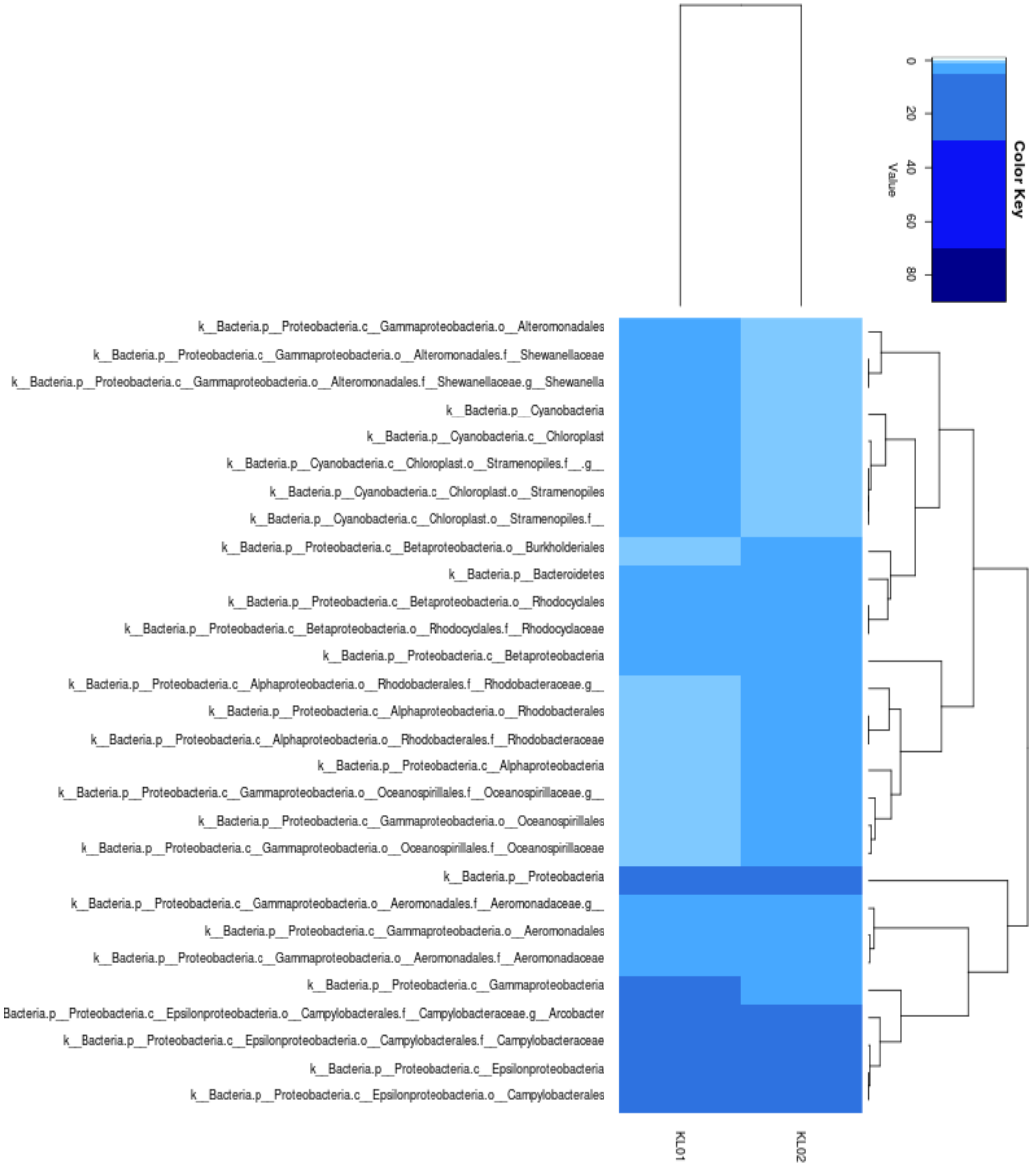

Figure 3. Metagenomic result of bacterial population at genus level of water sample collected at Klang Port

Degradation of palm oil and its derivatives using cultivated marine bacteria *P. aeruginosa* UMTKB-5

Figure 4 shows the CFU (log CFU/mL) count of *P. aeruginosa* UMTKB-5 supplemented with different substrate. The bacterial growth generally increased from day 0 to day 4 but decreased at day 8. The CPO and CPKO were the only two carbon substrates showing highest log CFU/mL on Day 4 (9.426 ± 0.6703 and 9.434 ± 0.7358, respectively) but by comparing Day 0 log CFU/mL between CPO and CPKO, it was clear that bacteria showed more growth in CPO to achieve similar log CFU/mL to CPKO at Day 4. The RBDPO and RBDPL had very little bacterial growth where log CFU/mL at Day 4 was 8.236 ± 0.2386 and 8.297 ± 0.9197 respectively.

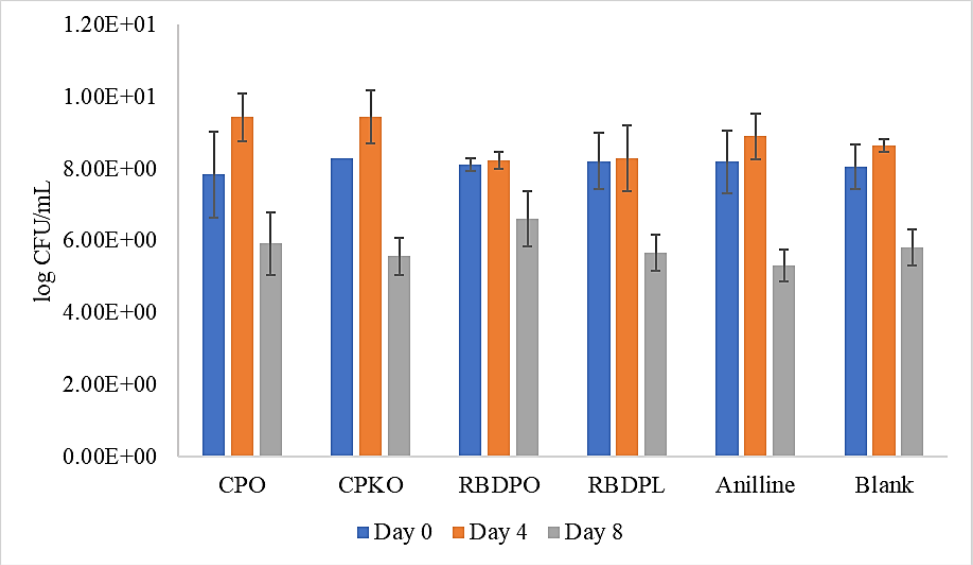

Figure 4. CFU count of *P. aeruginosa* UMTKB-5 cultured in test substances for Day 0, 4 and 8

Based on Figure 5, it can be noted that all the palm oil substrates tested were utilised. However, among all the substrate CPO was utilised the most where 83.37% of CPO initial weight added is lost. CPKO had the most of amount of residual oil remaining (69.3%) indicating lesser utilisation by *P. aeruginosa* UMTKB-5.

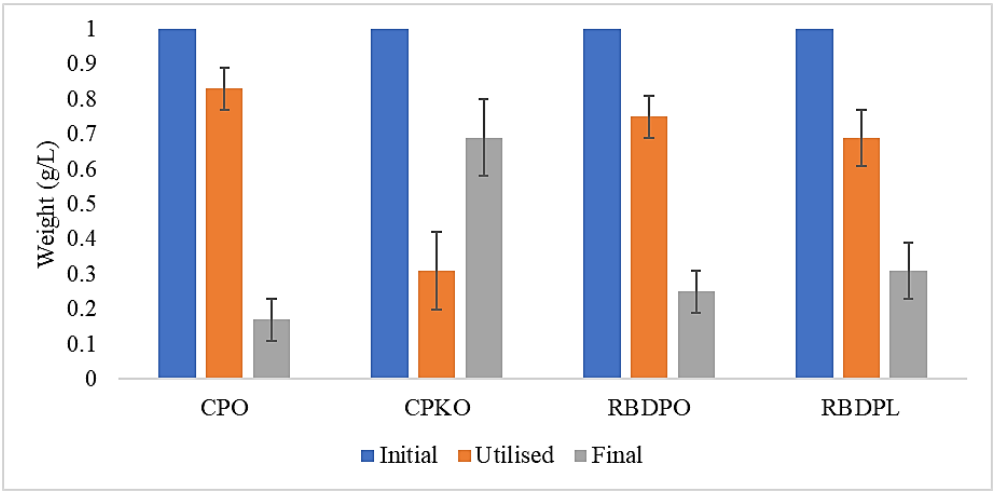

Figure 5. Residual oil measurement of carbon substrate left after the eight-day utilisation

Palmitic acid (C16:0) was detected in all the carbon substrate (Figure 6). Meanwhile, capric acid (C10:0), caprylic acid (C8:0) and caproic acid (C6:0) was found exclusively in CPKO. Palmitic acid in RBDPO and RBDPL were not detected after the utilisation. Concentration of palmitic acid in CPO decreased by 21.6%. Capric acid in CPKO was observed only before utilisation. After the 8-day incubation, both caprylic acid and caproic acid showed a decrease in concentration (37.1% and 6.98%, respectively).

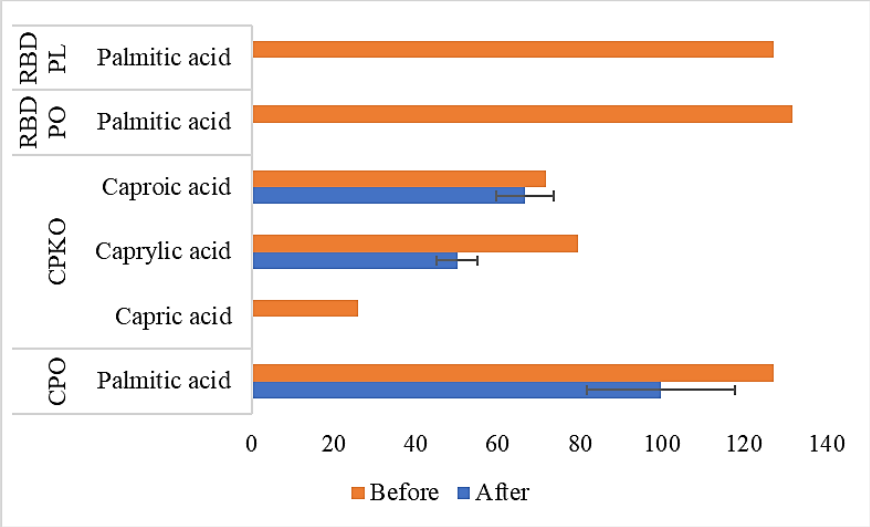

Figure 6. Fatty acid composition of all carbon substrates before and after utilisation by *P. aeruginosa* UMTKB-5

All the culture broth where palm oil derivatives supplemented as carbon substrate to *P. aeruginosa* UMTKB-5 able to exhibit emulsification activity (Figure 7). The CPO containing culture broth was able to exhibit emulsification activity at day 1,4 and 8. However, CPKO, RBDPL and RBDPO containing culture broth was able to exhibit emulsification activity only once. Furthermore, at day 4, RBDPL exhibited the highest emulsification index (E24) [12.28 ± 1.75%] compared to other palm oil derivatives tested.

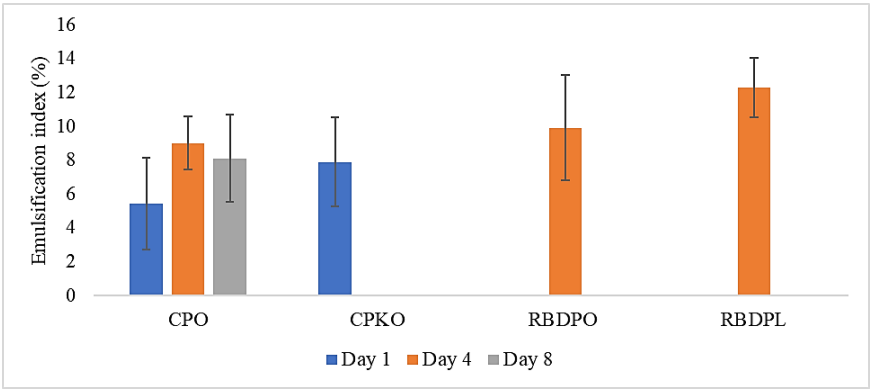

Figure 7. Emulsification index (E24) of selected carbon substrates test substances for Day 1, 4 and 8

Vegetable oil spill in the marine environment has been reported as early as 1975 where palm oil and coconut spilled near coral reef area of Fanning Atoll, Kiribati [21]. Since then, more incidents of vegetable oil spill have been reported and their environmental impacts were investigated. Mudge reported that vegetable oil spilled in the marine environment could pose lethal and sub-lethal effects towards organisms [22]. However, Mudge further reported that naturally occurring marine bacteria can degrade the vegetable oil whilst being in an unpolymerized state [22].

The assessment of ex-situ degradation of CPO and CPKO using Klang Port water showed some interesting

results where the bacterial count increased over the 42 days of experiment but the DOC removal (%) showing a negative trend. This modified experimental design based on OECD TG 306 method was intended to observe the degradation pattern in cases of large amount of oil spill where 1 g L^{-1} and 10 g L^{-1} of test substance was supplemented instead of 5 to 40 mg L^{-1} range proposed by the OECD method [11]. However, the fluctuations in DOC removal rate (%) along with bacterial counts indicates that there is utilisation of CPO and CPKO as carbon substrate and degradation of those palm oil is occurring. Despite not achieving 70% of DOC removal as set by OECD guideline, the negative result does not indicate that the substance is non-biodegradable but a more sophisticated confirmatory is needed [23].

The research by Al-Darbi et al. studied the biodegradation of natural oils namely canola oil, mustard oil, and olive oil in seawater [24]. In regard to the bacterial counts, they reported similar observation where bacterial counted increased over time in oil contaminated samples. However, the bacterial counts differed based on the type of oil tested. Canola oil had the highest bacterial count (500 000 CFU/mL) in fortified seawater at day 15 compared to other oil type. Thus, they concluded that canola oil degraded easily followed by mustard, cod and olive oil. The structure of oil in terms of length of carbon chain and number and location of double bond influences the degradation process [24].

Formation of white and yellow particles were noticed during the degradation study. According to Al-Darbi and colleagues [24], the white particles are saponified oil that reacted with sodium, potassium and calcium that are present in seawater to produce fatty acid salts. Similar observations are made by previous studies when seawater interacted with sunflower oil, canola oil and palm olein to form solid white polymer [15,24,25]. Furthermore, Mudge added that polymerised oils will reduce the natural biodegradation rate [22].

The presence of the isolated bacteria before and after the 42 days of experimentation shows their ability of survive, utilise CPO and CPKO as carbon substrate and to degrade them. Different bacteria species of the same genus were obtained in this study as from study conducted by Bhubalan et al., where *Vibrio fluvialis* was isolated in this study while *V. harveyi* and *V. alginolyticus* was isolated by Bhubalan et al. [15]. Variation and concentration of microbial communities due to origin of water sample influences the biodegradation process as presence of competent degrader is essential [23,26].

P. aeruginosa UMTKB-5 is a rhamnolipid biosurfactant-producing bacteria that was isolated from marine sediment. Previously, different *P. aeruginosa* strains were reported being able to utilise palm oil as substrate for growth and to produce biosurfactant. Palm oil were supplemented as carbon source for *P. aeruginosa* IFO3924 and *P. aeruginosa* A41 to produce rhamnolipid [27,28]. The ability of biosurfactant-producing bacteria is crucial is bioremediation effort of oil spill. Rhamnolipid application can be useful in degradation of oil due to their emulsifying property. In the presence of biosurfactant the rate of biodegradation of organic compounds is increased due to increased solubility [29]. The increased solubility enables microorganisms to be more accessible to the compounds. In the utilisation study of different palm oil derivatives by *P. aeruginosa* UMTKB-5, the increased bacterial counts were reflected by the reduced residual oil obtained at the end of day 8 of experiment as well as the emulsification index (E_{24}) value obtained. Furthermore, the changes in the concentration of fatty acid (ppm) composition indicates the utilisation of palm oil by *P. aeruginosa* UMTKB-5.

The utilisation of palm oil derivatives by bacteria as carbon substrate fuelled the population growth. Studies by Bhubalan et al. [15] along with Al-Darbi et al. [24] reported the similar trends where the bacterial counts increased over time when supplemented with RDB palm olein and canola oil respectively. However, the bioremediation of oil spill using biosurfactant producing bacteria is more focused on hydrocarbon-based oil spill [29]. The summary of findings of this study and other comparable literature is summarised in Table 3 below.

Table 3. Summary of research findings and comparison with other literature

Oil Type	Findings	Reference
CPO and CPKO	• Bacterial population increased over 42 days with population number peaking on day 7 and 21. • Increase in DOC level thorough input of carbon from bacterial degradation of oil.	This study
CPO, CPKO, RBDPO, RBDPL	• Degradation by rhamnolipid-producing bacteria increased the bacterial population. • Presence of rhamnolipid increased the degradation process and decrease in fatty acid concentration indicted the utilisation of palm oil as substrate by bacteria.	
Canola Oil, Mustard Oil, Olive Oil	• Increase in bacterial population in oil exposed seawater with population count highest on day 15. • Chemical structure of oil influences the degradability of oil with shorter chain oil more susceptible to degradation. • Formation of white and yellow particles that are saponified oil due to polymerisation.	[24]
RBD Palm Olein	• Growth of bacterial population in oil exposed sample. • Decreased in fatty acid composition of palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1)	[15]
Palm oil (commercial product)	• *P. aeruginosa* IFO3924 utilised palm oil for bacterial growth and synthesis of rhamnolipid.	[27]
Palm oil	• Rhamnolipid production by *P. aeruginosa* A41 by utilisation of palm oil as carbon feedstock.	[28]

This study serves as preliminary study into the potential application of biosurfactant in bioremediation of vegetable oil thus more detailed study in needed.

Conclusion

As the increasing demand for palm oil increases the potential risk of spillage occurring in the marine environment, there is a need to understand the degradation potential of the palm oil. This study shows that naturally occurring marine bacteria can utilise different palm oil derivatives as substrates for growth and subsequently degrade them. Furthermore, the isolated bacteria were able to survive for an extended period in palm oil enriched environment indicating their potential as degrader. *P. aeruginosa* UMTKB-5 a biosurfactant-producing oil-degrading bacteria is able to exhibit emulsifying activity in palm oil-exposed water as well as using palm oil as carbon substrate. The emulsifying property could be helpful in bioremediation of oily contamination. However, it is important to investigate the optimum method of deployment.

Acknowledgement

This study was supported by Fundamental Research Grant Scheme [FRGS] [FRGS/1/2017/WAB09/UMT/02/2] awarded by Ministry of Higher Education, Malaysia. The authors would like to acknowledge MPOB for providing the palm oil that was used in this study. Furthermore, we would like to thank Jabatan Laut for providing us support in accessing the port area. The authors thank Tan Suet May Amelia for proofreading and formatting the manuscript.

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