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Molecular typing of crude-oil-degrading bacterial strains from Riau, Indonesia

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Abstract

Petroleum contamination is a global concern in microbial enhanced oil recovery (MEOR) procedure, including in Riau, Indonesia, being one of the provinces with petroleum activities. These contaminants could be removed through bioremediation using crude-oil-degrading microorganisms or hence called hydrocarbonoclastic bacteria, especially the well-adapted indigenous bacterial strains from the contaminated sites. In this study, three indigenous isolates, *Bacillus cereus* IMB-11, *Lysinibacillus fusiformis* IMB-12, and *Pseudomonas stutzerii* IMB-15, were successfully recovered and identified from a petroleum-containing site of Chevron Pacific Indonesia (CPI) Ltd., Petapahan, Riau, Indonesia. These hydrocarbonoclastic isolates were strain-typed based on 16S rDNA and the results showed that *B. cereus* IMB-11 was closely related to the USA strain, *L. fusiformis* IMB-12 was closely related to the China and Indian strains, while *P. stutzeri* IMB-15 was related to strains from Africa, Asia and Europe. Also, the bioremediation assay of petroleum in a 72-h incubation experiment resulted in the removal of total petroleum hydrocarbon(s) (TPH) and chemical oxygen demand (COD) reduction by these bacterial strains during fermentation in crude oil-supplemented media (10%, v/v). Results showed that the highest TPHs removal was achieved by both *B. cereus* IMB-11 and *P. stutzeri* IMB-15 at 76.64% while *L. fusiformis* IMB-12 was the lowest at 62.62%. In COD analysis, the initial concentration was 15 mg/mL, also with the control flask, however, the highest COD removal was achieved by *B. cereus* IMB-11 at 88.55%, followed by *L. fusiformis* IMB-12 at 82.01%, and then *P. stutzeri* IMB-15 at 42.05%. Based on these results, the hydrocarbonoclastic bacterial strains have the potential to be used as bioremediation agents.

Keywords: Batch fermentation, Hydrocarbonoclastic, Petroleum

1. Introduction

The impacts of petroleum-based commodities are very significant in the production of energy for both industrial and human daily activities. During petroleum exploration which involves refining, and transportation, accidental leaks and spills could occur, thereby causing serious damage to the environment, including land and aquatic habitats [1]. In cases of large petroleum discharges, it is always difficult to be recovered, thereby leading to persistent pollutants in the environment, which are continuously accumulated by the organisms [2]. Accumulation of these pollutants or xenobiotics in animal and plant tissues could lead to the occurrence of disruptive genetic mutations and even death [3]. Therefore, the application of remediation technology and technique is needed to reduce the impact of environmental damage caused by these hydrocarbons contamination.

Microbial bioremediation is a technique which involves the integration of microorganisms to detoxify and degrade target pollutants, and converting them into less harmful or safer products. It is considered as an eco-friendly and cost-effective method due to its reusability. The methods might vary based on the indigenous microorganisms (natural attenuation) to the exogenous application of large-inoculum biodegradative microbes (bioaugmentation), to accelerate the elimination of these petroleum products and other contaminants [4]. Biodegradation by the indigenous microorganisms is an indication of natural conversion of the petroleum contaminants into harmless compounds [5].

Crude-oil-degrading or hydrocarbonoclastic bacteria in petroleum-contaminated environment, are well adapted to the stress of contaminants due to its compatible enzymatic and genetic elements. Also, these indigenous hydrocarbonoclastic bacteria are regarded as potential bioremediation agents in the treatment of oil pollutants. Research are still very much in progress on the effective application technique of this group of bacteria and the biological characteristics. In addition, the development of bacterial remediation technology has attracted considerable attentions from researchers in the natural or oil-leakage sites [6].

Petroleum-contaminated sites have been found as potential sources of novel indigenous hydrocarbon-degrading microorganisms. Also, a diverse group of bacteria, within the genera of newly reported strains, *Bacillus, Pseudomonas,* and *Serratia,* have been evaluated to be an effective hydrocarbon bioremediation agents and regarded as natural producers of biosurfactant which could be applied *in situ* or *ex situ* [7-10]. Additionally, there are limited studies on indigenous hydrocarbon-degrading bacteria from Riau Province, Indonesia, despite being one of regions rich in petroleum and natural gas industries.

This study reported an assemblage of culturable hydrocarbonoclastic bacteria recovered from a petroleum-containing site of Chevron Pacific Indonesia, Ltd., as one of the intensive petroleum industry located in Riau, Indonesia, identified as members of *Bacillus, Lysinibacillus,* and *Pseudomonas*. In addition, the molecular strain information of these isolates were conducted based on bioinformatics analyses followed with the initial testing of its hydrocarbon-degrading performances in batch fermentation. However, it expected that the results are subjected to further investigation regarding its biological phenomenon and optimization in the laboratory.

2. Materials and methods

2.1 Isolation of hydrocarbonoclastic bacteria

The hydrocarbon-degrading bacteria were isolated from enriched petroleum samples from the waste tanks of Chevron Pacific Indonesia, Ltd., Riau, Indonesia. The petroleum samples (2%, v/v) were enriched with Basal Mineral Salt Medium containing (w/v): 5 g $CaCO_3$, 2.5 g NH_4NO_3, 1 g Na_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.2 g $MnCl_2.7H_2O$ in 1,000 mL of distilled water. The samples were stirred at 120 rpm for 7 d at 25-28 ˚C. Then, the aliquot of samples were diluted at concentration of 10^6 and spread on top of Stone Mineral Salt Agar (SMSA) medium with same composition of minerals with additional 10% (v/v) crude oil and 15 g agar (w/v) in 1,000 mL of distilled water. All these are incubated at 25-28 ˚C for 5 d, after which three bacterial isolates with distinct morphologies were purified into Nutrient Agar (NA) as stock cultures. These were designated as isolates IMB-11, IMB-12, and IMB-15 and later identified on the basis of 16S rDNA.

2.2 Identification of hydrocarbonoclastic bacteria

The Wizard® Genomic DNA Purification Kit Protocol and GoTaq® Green Master Mix Protocol from Promega Corp., USA, were used for the genomic extraction of bacterial isolates and 16S rDNA amplification. The PCR reaction was performed in a 50 µL solution with 10 pmol of 63f (5'-AGAGTTTGATC(A/C)TGGCT CAG-3') and 1387r (5' GG(C/T)TACCTTGTTACGACTT-3') solution, 25 µL GoTaq® Green solution, 3 µL DNA template (10 ng/µL) solution, and 20 µL nuclease free water (NFW). The PCR program of 35 cycles was specified as: 94 ˚C (3 min), 55 ˚C (30 sec), 72 ˚C (30 sec), and 72 ˚C (5 sec). Also, the DNA amplicons were visualzed in 0.75% agarose (w/v) gel electrophoresis in 50 mL Tris/Borate/EDTA (TBE) buffer under UV illumination. Finally, the PCR products were subjected to commercial sequencing by Macrogen, Inc (Singapore).

2.3 Bioinformatics analysis

The unassembled 16S rDNA sequence sizes from Sanger dideoxy readings by Macrogen, Inc were 1,369 (IM B-11), 1,189 (IMB-12), and 1,061 bp (IMB-15). These sequences were subjected to database search and alignment using the Basic Local Alignment Search Tool (BLAST) following the standard nucleotide search on https://blast.ncbi.nlm.nih.gov/Blast.cgi. The *Standard Database* and *Nucleotide Collection (nr/nt)*by excluding *Models (XM/XP)* and *Uncultured/Environmental Sample Sequences* were the database used in this search, and then optimized for BLASTn. The BLAST results were retrieved and downloaded as sequence databases (.fas)

for further offline multiple sequence alignment using MEGA X software [11]. This was performed to assemble the sequences using MUSCLE featured in the software [12].

The phylogenetic tree of the 16S rDNA in the database strains was constructed for each isolate based on evolutionary inference or distance-based method using the UPGMA method. The percentage of replicate trees was estimated for its robustness as denoted in bootstrap value (BV) using the bootstrap test for 1000× [13]. Then, ambiguous positions were removed by selecting the *pairwise deletion* option after which the phylogenetic trees were analyzed descriptively with reference to the GenBank.

The three isolates designated as *Bacillus cereus* IMB-11, *Lysinibacillus fusiformis* IMB-12, and *Pseudomonas stutzeri* IMB-15 were submitted to GenBank as through the portal https://submit.ncbi.nlm.nih.gov/subs/genbank/ [14]. These sequences were deposited and given the accession codes MG675633.1 for *Bacillus cereus* IMB-11, MG675634.1 for *Lysinibacillus fusiformis* IMB-12, and MG675635.1 for *Pseudomonas stutzeri* IMB-15.

2.4 Batch fermentation of petroleum degradation

The three bacterial isolates were nourished into new Stone Mineral Salt Broth medium. Then, the pre-grown isolates 10% (v/v) OD_{600} : 0.1 were inoculated into 250-mL flasks, each containing 50 mL of SMSS broth and replacing the glucose as carbon source into 10% (v/v) crude oil. The control flasks were prepared without adding the inoculum and all procedures were conducted in duplicate. Finally, all the culture flasks were incubated at 28 ˚C for 72 h and stirred at 120 rpm.

2.5 Bacterial growth measurement

The growth measurement involved the sampling of 0.1 mL aliquot periodically in an interval of 6 h, and serially diluted prior to spreading on SMSS agar in order to count its colony forming unit (CFU/mL). Following the total plate count, 0.1 mL of aliquot was measured at an optical density of OD_{600} using nanophotometer. A calibration curve was then generated for each isolate by plotting the linear regression analysis between CFU/mL unit and OD_{600} value with the final expression as log CFU/mL.

2.6 Total petroleum hydrocarbon (TPH) estimation

The TPHs in the fermentation medium were measured at the end of incubation period, lasting 72 h. Approximately 40 mL of the fermentation medium was extracted in the liquid-liquid partitioning using 150 mL of acetone/hexane (1:1, v/v). The extracted oil in solvent system was concentrated in a rotary-evaporator using BUCHI Rotavapor® R-300, England while the residual TPHs were measured gravimetrically with the use of the formula [15]:

$$Percentage\ of\ TPH\ removal = \frac{(C_1 - C_2)}{C_1} x\ 100\%$$

Where C_1 is the concentration (mg/mL) of the TPHs in the early fermentation period, and C_2 is the concentration (mg/mL) of the TPHs at the end.

2.7 Chemical oxygen demand (COD) estimation

The CODs in the fermentation medium were measured at the end of the incubation period using the procedure of dichromate colorimetry method [16]. This principle was based on the number of oxygen equivalent to the organic compounds being oxidized by potassium dichromate in a 50% sulfuric acid solution within COD digestion reactor. The oxidized solution was measured by spectrophotometer on the wavelength of A_{365} or A_{420} and A_{620} nm for low and high range, respectively, in the presence of Cr^{3+}/Cr^{6+}. The COD reduction percentage was calculated similarly to TPHs removal.

3. Results

3.1 Genetic characterization of the strains

The species on the BLASTn list showed that the genetic sequences of the three strains were considerably similar to some of the strains in the database. The isolate *Bacillus cereus* IMB-11 was similar to the strain of USA with the score of 2159 as shown in Table 1. The isolate *Lysinibacillus fusiformis* IMB-12 was similar to the strain of China and India with total score of 2012 as shown in Table 2. Then, the isolate *Pseudomonas*

stutzeri IMB-15 had similar score to different strains from Africa, Asia and Europe. In addition, a phylogenetic tree was constructed for each strain in order to determine the statistical relationship among database strains. The isolate *Bacillus cereus* IMB-11 was placed in the same clade with *B. cereus* strain IHB B 1647 (KF475840.1) from India with the bootstrap value (BV) of 65% as shown in Figure 1. Both *Lysinibacillus fusiformis* IMB-12 and *Pseudomonas stutzeri* IMB-15 were placed in separate clades indicating high distinctions in their genetic sequences, as shown in figures 3 and 4. The sequences of both isolates could be considered unique despite originating from Indonesia or Southeast Asia region.

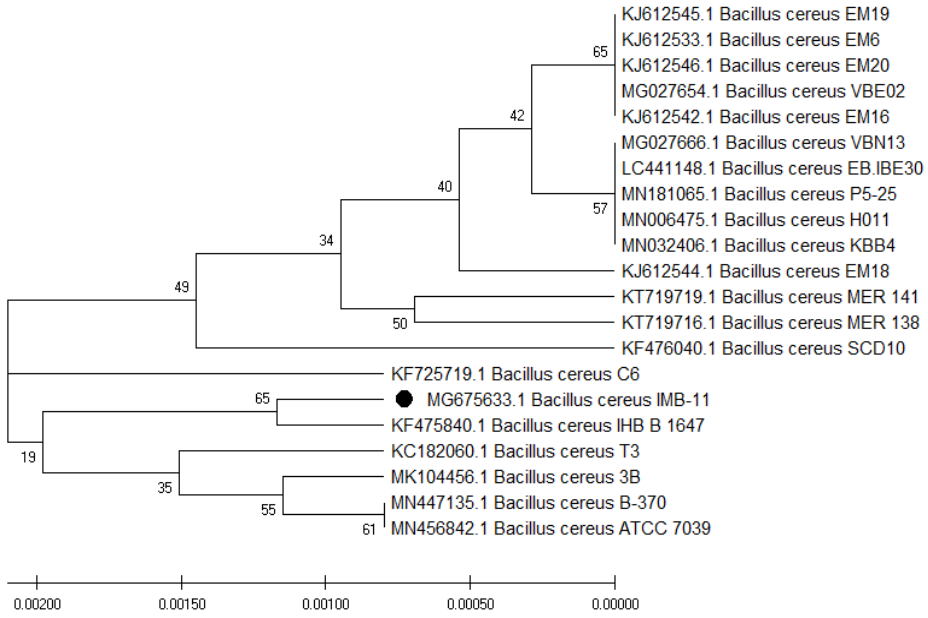

Figure 1 Phylogenetic inference of *Bacillus cereus* IMB-11 among the 20 database strains.

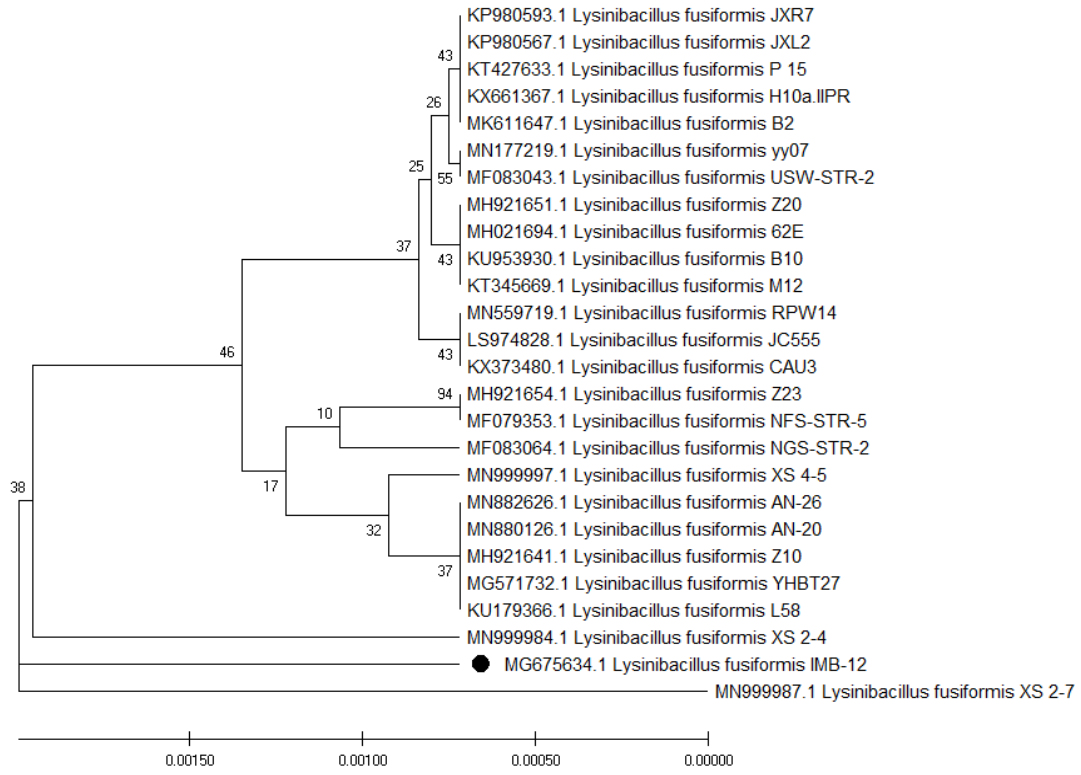

Figure 2 Phylogenetic inference of *Lysinibacillus fusiformis* strain IMB-12 among database strains.

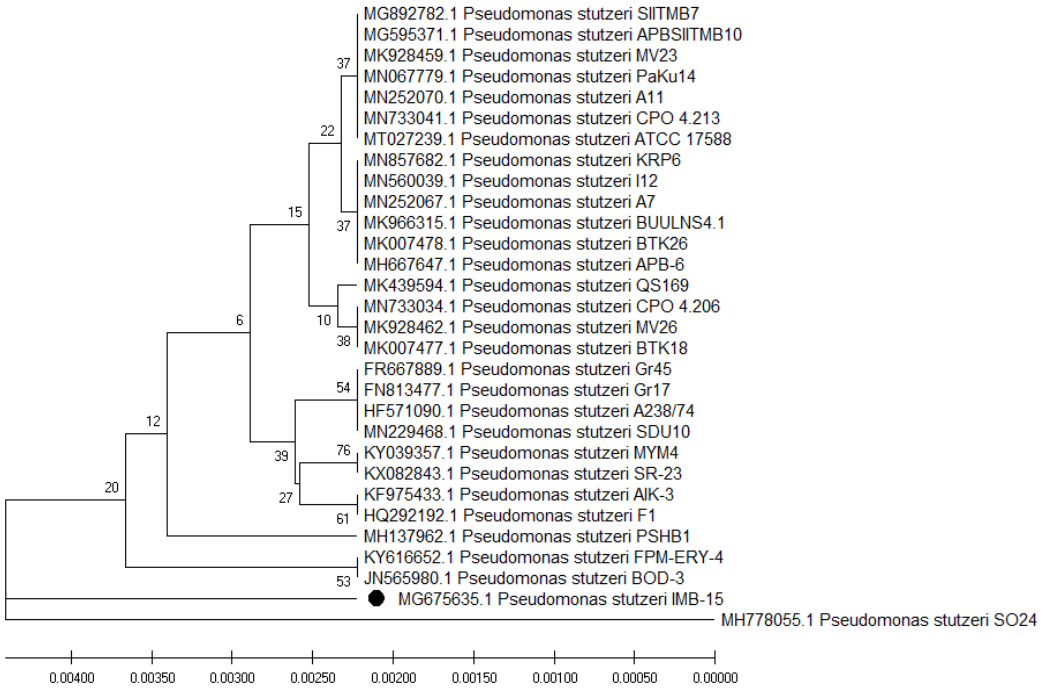

Figure 3 Phylogenetic inference of *Pseudomonas stutzeri* strain IMB-15 among database strains.

3.2 Bacterial growth in petroleum-based medium

The growth performances of the hydrocarbonoclastic bacteria are a way of monitoring the petroleum degradation or utilization process. This was calculated through direct and indirect measurements, by simply correlating the number of bacterial colonies growing in plate (CFU/mL) and bacterial suspension or turbidity (OD_{600}) to generate a calibration curve, although data not shown, from two replicates. Based on the growth performances, the *B. cereus* IMB-11 showed the highest population at log 9.5 CFU/ mL, followed by *L. fusiformis* IMB-12 at log 7 CFU/mL and then *P. stutzeri* IMB-15 at log 6 CFU/mL, as shown in Figure 4. Also, both *B. cereus* IMB-11 and *L. fusiformis* IMB-12 showed their population peaks at 18-h of incubation while *P. stutzeri* IMB-15 showed a double peak in growth at 18 and 54-h of incubation. However, a sharp reduction was observed in the population growth of *B. cereus* IMB-11 at 60-h before regaining its normal population afterwards. Based on the trendline, each strain developed a distinct and fluctuative growth during the fermentation process indicating a dynamic utilization of substrate and cell viabilities.

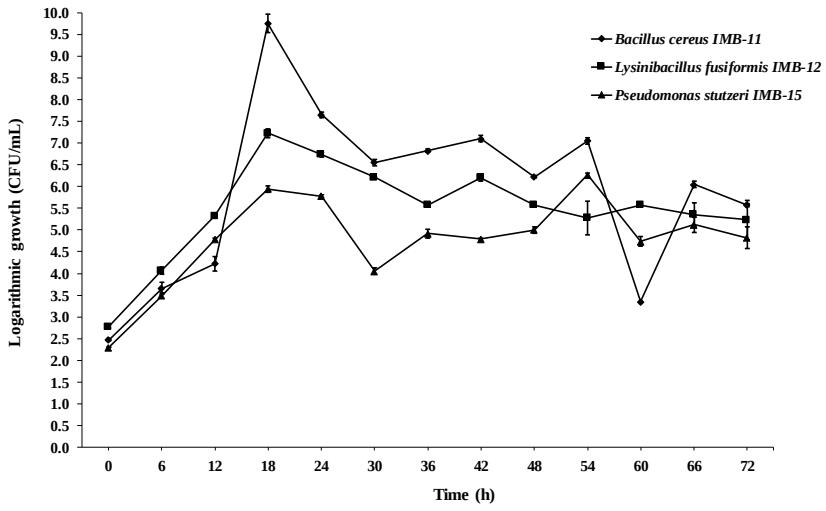

Figure 4 Logarithmic growth performance of hydrocarbonoclastic bacterial isolates grown in petroleum medium. Results are presented in mean and S.D (error bars).

3.3 Petroleum degradation efficiency

The petroleum bioremediation assay in a 72-h incubation experiment resulted in the removal of TPHs and reduction of COD by the hydrocarbonoclastic bacterial strains. The highest removal of TPHs were achieved by both *B. cereus* IMB-11 and *P. stutzeri* IMB-15 at 76.64% while *L. fusiformis* IMB-12 was 62.62% . In COD analysis, the initial concentration was 15 mg/mL as also in the control flask. However, the highest COD removal was achieved by *B. cereus* IMB-11 at 88.55%, followed by *L. fusiformis* IMB-12 at 82.01%, and then *P. stutzeri* IMB-15 at 42.05% . Based on these, the hydrocarbonoclastic bacterial strains were considered bioremediation agents.

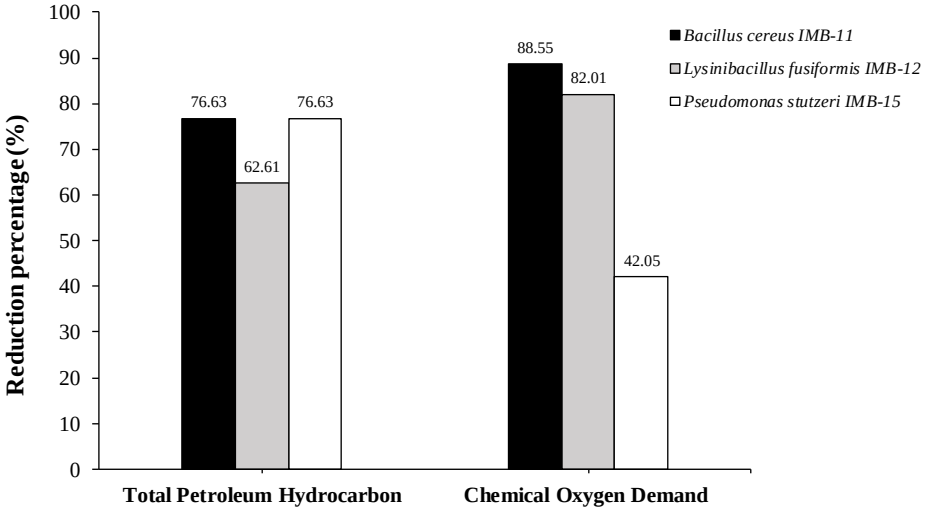

Figure 5 Reduction percentage (%) of Total Petroleum Hydrocarbon (TPH) and Chemical Oxygen Demand (COD) by three hydrocarbonoclastic bacterial isolates in petroleum-containing medium after 72 h of incubation.

4. Discussion

The biodegradation of petroleum compounds is a multiplex process carried by the indigenous microorganisms with limited access to the pollutants. Achieving this process through bioremediation agents like bacteria is still gaining attention due to its low production cost and eco-friendly nature. The principle of using salt as basal growth medium enriched with crude oil have proven to be an effective screening method for hydrocarbon or oil-degrading microorganisms. It improves their biodegradation capacity and utilization of substrate through the available cofactors. This culture-based technique is proven to be effective in recovering hydrocarbonoclastic bacterial strains from the natural and petroleum-contaminated sites in addition to the current molecular technique [17].

The three isolates have been identified to the species level based on 16S rDNA sequence and BLAST result from NCBI GenBank database. However, the high scores in BLAST result might not solely show the accurate relationship or genetic distance in sequence alignment analysis, especially under strain level [18]. The distance-based method of phylogenetic tree construction could then be use to infer the sequence similarity and relationship among database strains retrieved from BLASTn results. Based on strain typing analysis, the indigenous hydrocarbonoclastic strains might be different to the sequences deposited in GenBank, especially *L. fusiformis* IMB-12 and *P. stutzeri* IMB-15 showing the lowest genetic distances in both trees. Although the 16S rDNA-based strain typing is possible, the results only give an estimate which still needs another supportive feature such as the multilocus sequence typing (MLST) from other housekeeping genes within a species group [19,20].

Based on previous studies, the indigenous bacterial species or at least the same genera identified in this study has been recovered from some oil-contaminated sites. The *Bacillus cereus* DRDU1 identified using 16S rDNA sequence analysis, was isolated from an automobile engine which displayed a tolerant growth in the presence of diesel, crude oil, and even used engine oil, which also produced a biosurfactant capable of degrading 97% of kerosene and crude oil in laboratory experimentation [9,10]. Furthermore, *Bacillus cereus* DRDU1 was reported to produce biosurfactant as expressed from *sfp* gene while showing tolerant growth under stressful condition such as limited supply of N and P, indicating its promising use for future studies of microbial enhanced oil recovery (MEOR) [8]. In another report, *Lysinibacillus fusiformis* 15-4 recovered from an oil-free soil in Tibetan region using 2% petroleum as carbon source, showed its ability to grow within petroleum-containing

environments [21]. Additionally, *Pseudomonas stutzeri* AG11 was isolated from oil-contaminated soil and it was found to degrade diesel oil when supplemented with Triton X-100 and Tween-80 [22].

Different hydrocarbonoclastic species have various capacity in the removal of hydrocarbons compounds, explaining the importance of knowing their biological characteristics. All the bacterial isolates grew in the fermentation medium containing 10% (v/v) crude oil, indicating that each isolate utilized the petroleum as carbon source for growth. Also, the mechanism of petroleum degradation and utilization is facilitated by the production of biosurfactant and hydrolytic enzymes under optimum condition by the hydrcarbonoclastic bacteria [23,24]. However, there is need for further investigation with regards to the enzymatic performance of each isolate and the profile of remaining hydrocarbons detected using a more sensitive analytical instrument such as Gas Chromatography. Moreover, the population of viable cell from each bacterial isolate was not regularly maintained as shown by their irregular growth in the medium. Naturally, petroleum or crude oil is a complex mixture of hydrocarbons made up of saturated, aromatics, asphaltenes, and resins [25]. During the degradation process, some resultant products and other derivatives might be more readily to be utilized as carbon source by the microbes. Meanwhile, the other products might become dynamic limiting factors delaying the growth of bacteria during the fermentation process.

Regarding the taxonomical groupings, several reports have shown some genera or species of hydrocarbonoclastic bacteria commonly used as bioremediation agents, such as the *Bacillus* and *Pseudomonas* species. The study on the experimental biodegradation of Egyptian crude oil using *B. subtilis* and *P. aeruginosa*, showed removal percentages of 76.7 and 88.5% during fermentation in 28 d [26]. Also, a hydrocarbonoclastic isolate, *Bacillus pumilus* MVSV3 from petroleum effluent site in India was reported to degrade BTEX hydrocarbons with the removal percentage ≥50% within 2 d [27]. Additionally, an assemblage of marine species of the *Bacillus* group, isolated from Indonesian seawater, showed 12 culturable strains with *B. flexus* showing the highest degrading capacity of crude oil of ≥70% within 14 d [28]. Three hydrocarbonoclastic bacterial isolates were also recovered from crude oil in Mangalore, India, in which *P. aeruginosa* WD23 showed the highest percentage of TPH reduction at 27.25% within 15 d [29]. A newly reported strain, *Serratia* sp. KDS isolated from oil-spillage site of Assam, India, showed maximum degradation capacities to about 87.5 and 84.5% in 28 d towards diesel and kerosene, producing biosurfactant with promising application in field trial in improving plant growth [7]. Based on these studies, the more adapted microbial strains recovered from contaminated sites could produce better tolerance and biodegradation capacity to crude oils in the laboratory. Moreover, microbial hydrocarbon degradation during experiments might vary in terms of effectiveness and efficiency, depending the experimental conditions, strains used, type of pollutant/substrates used, composition of fermentation medium and technique [30]. Petroleum contamination in natural sites of Indonesia is considered a serious problem which needs special attention in terms of selecting the suitable oil-degrading microbes, especially those adapted to the environment. Oil recovery in the country could be enhanced by the introduction of indigenous microorganisms, although this is still limited in some aspects, especially with the availability of indigenous isolates. The reported isolates in this study, *Bacillus cereus* IMB-11, *Lysinibacillus fusiformis* IMB-12, and *Pseudomonas stutzeri* IMB-15 are new strains from Riau, Indonesia with a promising biodegradation capacity in the laboratory for only 3 d of batch fermentation. Although these isolates produced considerably high and active performances in petroleum degradation, there is need to thoroughly study the mechanisms and other biological characteristics for a better understanding of each isolate before future application. Furthermore, there is need to characterize the biosurfactant properties of these indigenous isolates. This is to obtain evidences upon their potential use as bioremediation agents, especially when applied in some oil-spillage and other natural sites contaminated by petroleum products in Riau.

5. Conclusion

The strain typing of 16S rDNA sequence from the three isolates using distance-based method or UPGMA showed that *Bacillus cereus* IMB-11 was closely related to the USA strain, *Lysinibacillus fusiformis* IMB-12 was closely related to the China and Indian strains, while *Pseudomonas stutzeri* IMB-15 was related to strains from Africa, Asia and Europe. Also, the highest population in SMSS medium supplemented with 10% crude oil during 72 h batch fermentation was observed from *B. cereus* IMB-11, followed by *L. fusiformis* IMB-12 and then *P. stutzeri* IMB-15. Furthermore, the highest TPHs removal were observed, at the end of the incubation period, from both *B. cereus* IMB-11 and *P. stutzeri* IMB-15 at 76.64%, while the lowest was observed with *L. fusiformis* IMB-12 at 62.62% . Based on the COD analysis, highest removal was observed from *B. cereus* IMB-11 at 88.55%, followed by *L. fusiformis* IMB-12 at 82.01%, and then *P. stutzeri* IMB-15 at 42.05%. Therefore, these results showed that the hydrocarbonoclastic bacterial strains have the potential to be used as bioremediation agents based on laboratory experimentation.

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