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REVIEW ARTICLE

ISOLATION AND SCREENING OF BIOSURFACTANT-PRODUCING MARINE BACTERIA FROM KUANTAN PORT, PAHANG, MALAYSIA

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ABSTRACT

Biosurfactants play an important role in bioremediation of organic pollutants such as petroleum hydrocarbon. The unique properties of biosurfactants make them possible to be used in the remediation of hydrocarbon contaminated sites. Therefore, the existence of indigenous microorganisms that have the ability to consume petroleum hydrocarbon as carbon source and simultaneously produce biosurfactants in order to facilitate the hydrocarbon metabolism can be manipulated for bioremediation purposes. In this study, isolation and screening of potential biosurfactant-producing bacteria from two sampling points in Kuantan Port seawater were successfully done. Amongst the isolates, 4 out of 7 isolates from Point A were Gram negative bacteria and 2 out 5 isolates from Point B were Gram negative bacteria. The positive oxidase test resulted for all isolates from Point A and only B5 from Point B produced negative result. Catalase test conducted produced positive results on isolates from Point A (A3, A5, A6& A7) and Point B (B1, B2, B4 & B5).The highest percentage emulsification index measured belonged to isolate B4 and B5 which are 67%, thus make these isolates to be the most promising biosurfactant producers. Further identification by 16S rRNA gene found that isolates were closely related to *Rhodococcus erythropolis* (A1), *Psedomonas stutzeri* (A2), *Pseudoalteromonas lipolytica* (A3, A6 and B4), *Vibrio brasiliensis* (A4 and B2), *Vibrio tubiashii* (B1), *Marinobacter salsuginis* (A5), *Labrenzia aggregate* (A7), *Marinococcus halophilus* (B3) and *Thalassospira xianmenensis*(B5). Hence, through biosurfactant activities exhibited by isolates, B4 and B5 were the most potential isolates to produce biosurfactant. Therefore, these isolates can potentially be exploited to aid in bioremediation of petroleum hydrocarbon contaminated sites and would also be useful to enhance oil recovery in petroleum industry.

KEYWORDS

Biosurfactant, biosurfactant-producing bacteria, marine bacteria, emulsification index, petroleum hydrocarbon.

1. INTRODUCTION

Hydrocarbons are widespread environmental pollutants; particularly the higher molecular weight compounds polycyclic aromatic hydrocarbons (PAHs) which are potent carcinogens and recognized as priority pollutants by both the EU and the US EPA [1,2]. Contamination of hydrocarbons in water resulted from anthropogenic activities such as petroleum drilling and transportation of petroleum crude by pipelines and ships across the oceans. Marine environment is affected by this petroleum hydrocarbon pollution through spills from pipeline failures, shipping accidents and also pollution from ports, oil terminals, oil rigs and sewage treatment systems [3]. Apart from that, petroleum hydrocarbons originated from anthropogenic activities also carried into the sea in the form of solutions with small portions remains as sediment [4]. Contamination of hydrocarbons in water will lead to bioaccumulation within seafood and food webs that can severely affect the marine ecosystems [5].

Biosurfactant-producing microorganisms can be isolated from sites contaminated by petroleum or other substances containing hydrocarbons; where they are indigenous population of those sites [6]. They can simply thrive in these polluted environments and at the same time degrade and metabolize the hydrocarbons which they use as their carbon source. In order to facilitate the metabolism of these insoluble hydrocarbons, microorganisms can produce a variety of substances. These substances are amphiphilic surface-active compounds produced as part of the microorganisms' cell membrane or excreted extracellularly. The structure of these compounds consists of two parts, a polar (hydrophilic) moiety and a non-polar (hydrophobic) group. The hydrophilic group consists of mono-, oligo-, or polysaccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols [7]. These compounds can be generally divided into two main classes; low-molecular-weight compounds called biosurfactants and high-molecular-weight polymers called bioemulsifiers [8].

Biosurfactants play a number of roles including increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, binding of heavy metals, quorum sensing and biofilm formation [9]. Compared with synthetic surfactants, biosurfactants have higher surface activity, lower toxicity, higher biodegradability and better environmental compatibility [10]. Although remediation of hydrocarbons contamination commonly involved the usage of synthetic surfactants and chemical dispersants, however, this remediation procedure usually can lead to environmental

destruction for long term used [11]. Therefore, the existence of indigenous microorganisms that have the ability to consume hydrocarbons as carbon source potentially would facilitate the degradation of hydrocarbons naturally. This would make bioremediation of hydrocarbon contaminated sites using biosurfactant-producing microorganisms more promising to be implemented.

In this study, isolation and screening of potential biosurfactant-producing bacteria from seawater of Kuantan Port, Malaysia were conducted. Several screening methods were employed to qualitatively measure biosurfactant activity produced by isolates including oil displacement test, drop-collapse test, emulsification test and microplate assay.

2. MATERIALS AND METHODS

2.1 Sampling

Seawater samples were collected from two sampling points which are Point A (3°58'13.9"N 103°25'41.4"E) that was located near the ships area and Point B (3°58'49.0"N 103°24'46.4"E) that was located a little bit far from ships area at Kuantan Port, Kuantan, Pahang. Samples were stored at 4°C for preservation until used.

2.2 Enrichment and isolation of bacteria

Bacteria were isolated based on their ability to grow on minimal media with petroleum crude as sole carbon source. Enrichment of these bacteria was done by adding 5 ml of water samples into 50 ml of Bushnell Haas broth supplemented with 1% (v/v) petroleum crude and incubated at 150 rpm for 7 days at 30°C. Next, serial dilution was performed using Ringer's solution for 3rd, 5th and 7th day of incubation and100 µl of each serial dilution and undiluted samples were spread on Bushnell Haas agar supplemented with 1%(v/v) petroleum crude. The plates were incubated for 48 hours at 30°C. Colonies obtained were selected based on different morphological characteristics. Then, the colonies were subcultured on Marine agar to obtain pure culture of bacteria. Several different isolates having distinct morphological characteristics were chosen for further screening and characterization.

2.3 Characterization of isolates

The bacteria were differentiated by Gram staining procedure and biochemical test, including oxidase and catalase test to further examine physiological characteristics of the bacteria. Oxidase test were carried out by soaking a filter paper in Kovac's reagent. Fresh growth of bacteria colony was scraped using loops and smeared on the soaked filter paper. Colour changes can be observed within 10 seconds where deep purple-blue colour indicated positive oxidase test and no colour changes indicated negative results. Catalase test was done by transferring a small amount of colony to a surface of a glass slide by the sterile wooden slick. A drop of 3 % (v/v) H_2O_2 was placed on the slide and mixed. Quick bubbling formation within 5 to 10 seconds was observed.

2.4 Qualitative screenings of biosurfactant activity

Qualitative screening methods used in this study, including oil displacement test, drop-collapse test, emulsification index and microplate assay. Twelve isolated bacteria were cultured into 50 ml of Marine broth and incubated at 150 rpm for 5 days at 30°C. Then, the cultures were centrifuged at 15000 rpm for 30 minutes at 4°C to harvest the supernatant produced and stored at 4°C for further screening purposes. Positive control used was 1% (v/v) SDS solution meanwhile distilled water acted as negative control.

The oil displacement test was carried out by adding 10 µl of crude oil to the surface of 20 ml distilled water. Then, 10 µl of culture supernatant was gently positioned on the centre of oil layer. Oil displacement and clearing zone formation will indicate the presence of biosurfactant. A drop-collapse test was conducted using 96-wells microplate. The wells were coated with 2 µl of crude oil in triplicate for each culture supernatant. The coated wells were equilibrated for 24 hours to ensure uniform oil coating. About 5 µl of culture supernatant was added into the middle of the well for 1 minute. Using a magnifying glass, the drop was observed where flat droplet is considered as positive biosurfactant production and rounded droplet suggest lack of biosurfactant production.

Emulsification index, E_{24} was done by adding 2 ml of petroleum crude to 2 ml of culture supernatant into a clean test tube with cap. The mixture was vortexed for 2 minutes and let stand for 24 hours. The E_{24} was defined as the percentage of the height of emulsified layer divided by the total height of the liquid column. Next, 100 µl of culture supernatant was added to 96-wells microplate for microplate assay. Optical distortion of the supernatant was viewed using graph paper placed underneath of the microplate. The distortion indicates the presence of biosurfactant activity.

2.5 Identification of bacterial isolates by PCR amplification

The bacteria were further identified using 16S rRNA gene identification. The DNA extraction of bacteria was conducted using Vivantis GF-1 Nucleic Acid Extraction Kits according to the manual given by the manufacturer.
Amplification of 16S rRNA gene was done by using universal forward primer F27 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer1492R 5'-TACGGYTACCTTGTTACGAC-3' [12]. Polymerase chain reaction (PCR) was carried out in 25 µl of the 2X PCR master mix, 2.5 µl of each of the forward and reverse primer, 0.2 µl ultrapure BSA and 6.3 µl of nuclease-free water. PCR conditions were set to have initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 1 minute (25 cycles), annealing at 57°C for 1 minute, extension at 72°C for 2 minutes and final extension at 72°C for 7 minutes. The PCR products were visualized by electrophoresis on 1.2% (w/v) agarose gel. Lastly, the PCR products were purified using Vivantis GF-1 Clean-up Kit according to the manufacturer's manual. The purified products were sent to 1st Base Laboratory for sequencing. Sequences were manually edited and aligned using BioEdit v7.2.6 and matched with DNA sequences from GenBank using BLASTN from NCBI. Sequence alignment was performed using CLUSTAL W, and phylogenetic analysis was conducted by a neighbour joining method using Molecular Evolutionary Genetic Analysis 7 (MEGA7; The Biodesign Institute, Tempe, AZ, USA) software.

3. RESULTS

A total of 12 bacteria was successfully isolated from seawater samples of Kuantan Port using Bushnell Haas supplemented with 1% (v/v) petroleum crude as sole carbon source. Table 1 described the characteristics of each isolated bacteria based on their morphology. Isolates from Point A and Point B were denoted as A1-A7 and B1-B5 respectively.

Based on the Gram staining procedure for isolates from Point A, 4 isolates were Gram negative bacteria (A2, A4, A6 & A7) and 3 isolates were Gram positive bacteria (A1, A3 & A5). On the other hand, 2 isolates from Point B were Gram negative (B2 & B5) and 3 isolates were Gram positive bacteria (B1, B3 & B4). For biochemical test conducted, oxidase test showed that all isolates from Point A and B except B5 turned the filter paper soaked with Kovac's reagent into deep purple-blue colour that indicated positive results for the presence of oxidase. Meanwhile, catalase test conducted on isolates from Point A (A3, A5, A6 & A7) and Point B (B1, B2, B4 & B5) resulted in quick bubbling formation which indicated positive results. The absent of bubbling formation by A1, A2, A4 and B3 were considered as negative results. Table 1 showed the overall results for Gram staining and biochemical tests conducted.

Table 1: Isolation and identification of bacteria through morphology, Gram staining and biochemical tests.

Isolate	Description of colony	Colour	Gram staining	Oxidase test	Catalase test
A1	Irregular and wrinkled	Pale orange	+	+	-
A2	Disc-shaped with ridges radiating from the center	Reddish-brown	-	+	-
A3	Smooth and circular colonies	Not determined	+	+	+
A4	Smooth, convex and circular colonies	Cream-coloured	+	+	-
A5	Round in shape	Whitish	-	+	+
A6	Smooth and circular colonies	Not determined	-	+	+
A7	Transparent and smooth colonies	Slightly pink	-	+	+
B1	Irregular and slightly convex colonies	Yellow	+	+	+
B2	Smooth, convex and circular shaped	Cream-coloured	-	+	+
B3	Smooth and convex colonies	Yellow-orange	+	+	-
B4	Smooth and circular shaped	Not determined	+	+	+
B5	Circular and convex colonies	Cream-yellow	-	-	+

Gram staining: + = Gram positive bacteria; - = Gram negative bacteria
Biochemical tests: + = Positive results; - = Negative results

Screening of biosurfactant activities by oil displacement test produced positive results for all isolates from Point A and Point B. The isolates were able to produce clearing zones as a result of oil being displaced when supernatants from each isolates were placed on the centre of the oil. In addition, drop-collapse test also resulted in flat droplet for all 12 isolates, thus determined the activity exhibited by potential biosurfactants present within the isolates. On the other hand, highest emulsification activity was demonstrated by isolates B4 and B5, which were 67% for both and A6 produced the lowest emulsification activity which was only 30%. Meanwhile, microplate assay recorded positive results for all 12 isolates when optical distortions were observed using the graph paper. The results of all biosurfactant activity screenings were simplified in Table 2.

Table 2: Screening of biosurfactant activity for isolated bacteria

Isolate	Oil Displacement Test	Drop Collapse Test	Emulsification Index, E_{24} (%)	Microplate Assay
A1	+	+	63	+
A2	+	+	40	+
A3	+	+	43	+
A4	+	+	37	+
A5	+	+	30	+
A6	+	+	47	+
A7	+	+	40	+
B1	+	+	50	+
B2	+	+	53	+
B3	+	+	33	+
B4	+	+	67	+
B5	+	+	67	+

+ = Positive results; - = Negative results

Finally, molecular identification using 16S rRNA gene found that all 12 isolates were closely related to several bacteria deposited in GenBank. Isolates from Point A were closely related to *Rhodococcus erythropolis* (A1), *Psedomonas stutzeri* (A2), *Pseudoalteromonas lipolytica* (A3 & A6), *Vibrio brasiliensis* (A4), *Marinobacter salsuginis* (A5) and *Labrenzia aggregate* (A7). Meanwhile, closely related bacteria for Point B were *Vibrio tubiashii* (B1), *Vibrio brasiliensis* (B2), *Marinococcus halophilus* (B3), *Pseudoalteromonas lipolytica* (B4) and *Thalassospira xianmensis* (B5). Results obtained from BLAST NCBI for all 12 isolates were summarized in Table 3. Phylogenetic tree analysis displayed that all isolates were grouped together with their closely related species (Figure 1).

Table 3: BLAST results obtained from GenBank NCBI

ISOLATES	CLOSELY RELATED SPECIES (ACCESSION NUMBER)	SOURCES	PERCENT IDENTITY (%)
A1	*Rhodococcus erythropolis* strain SBUG 2052 K3 16S rRNA gene (KU663053)	Oil-polluted soil from the Kumkol deposit, Kyzylorda region, Kazakhstan [26]	99

A2	*Pseudmonas stutzeri* strain KG-2 16S rRNA gene (KX580703)	Oilfield	100
A3	*Pseudomonas lypolytica* strain LMEB 39 16S rRNA gene (NR_116629)	East China Sea sediment [27]	100
A4	*Vibrio brasiliensis* strain 0017KARWAR 16S rRNA (KC178717)	Open sea sediment	100
A5	*Marinobacter salsuginis* strain Xmb040 16S rRNA gene (KT986168)	Ocean water	92
A6	*Pseudoalteromonas lipolytica* strain K-W45 16S rRNA gene (JQ799095)	Tropical marine sediment [28]	100
A7	*Labrenzia aggregate* strain 0163 16S rRNA gene (KP236294)	Coastal area	80
B1	*Vibrio tubiashii* strain M14-00202-2F 16S rRNA gene (KY229818)	Tillegerry Creek, Port Stephens [29]	99
B2	*Vibrio brasiliensis* strain 0017KARWAR 16S rRNA gene (KC178717)	Open sea sediment	100
B3	*Marinococcus halophilus* strain SDT3S7 16S rRNA gene (JQ045798)	Bay of Bengal sediment sample	100
B4	*Psedoalteromonas lipolytica* strain LMEB 39 16S rRNA gene (NR_116629)	East China sea sediment	100
B5	*Thalassospira xiamenensis* strain PM01 16S rRNA gene (HM587995)	Maroon Oilfield, Khuzestan [30]	95

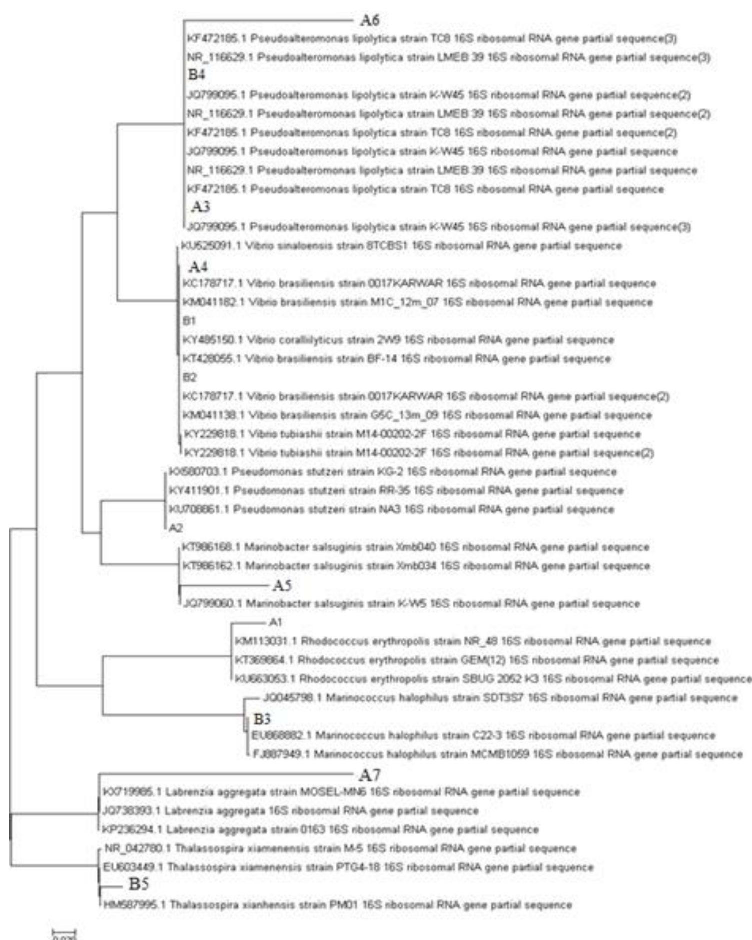

Figure 1: Phylogenetic tree based upon the neighbor joining method constructed using MEGA 7.

4. DISCUSSION

By using enrichment media where Bushnell Haas media were supplemented by 1% (v/v) petroleum crude, bacteria from seawater of Kuantan Port were able to be isolated and screened. This finding suggested that enrichment technique is a promising way to grow selected criteria of bacteria as it provides favourable condition for the desired bacteria [13]. Amongst the isolates, 4 out of 7 isolates from Point A were Gram negative bacteria and 2 out 5 isolates from Point B were Gram negative bacteria. Since Point A was nearer the ships areas are compared to Point B, this point of sampling was suspected to have high concentrations of hydrocarbon contamination. A study done by Ramankutty and co-workers resulted that Gram negative bacteria were abundant and was said to be more tolerant to hydrocarbon contamination [14]. Contrary, a study done by Bodour and co-workers discovered opposite finding where Gram positive bacteria were more abundant isolated from contaminated site [15]. Hence, both studies proved that both types of bacteria may have equal potential to co-exist within hydrocarbon contaminated sites.

Generally, oxidase enzyme is present when tetramethyl-p-phenylenediamine reagent was oxidized into iodophenols that gave purple colour as end product. If the end product was colourless, the enzyme is absent. On the other hand, catalase reaction is determined by the decomposition of hydrogen peroxide within living tissue to produce oxygen gas and water. The rapid formation of bubbles indicated the presence of oxygen facilitated by catalase reaction. Both tests were done to identify the potential bacteria that possess degrading ability. Based on the results obtained, only B5 from Point B isolates were negative for oxidase test. In addition, catalase was absent in isolates A1, A2 and A4 from Point A and B3 from Point B.

Biosurfactants produced by the bacteria isolates were determined by a screening of its activity based on the physical effects that can be easily observed. Different screening methods were required to select potential bacteria that able to produce biosurfactant since the compounds were heterogeneous in nature. Four tests comprised of oil displacement test, drop collapse test, emulsification index and microplate assay were chosen for biosurfactant screening due to its advantages of low cost, high clarity and use of common equipment that is easily attainable [16].

Oil displacement test was developed where clearing zone was formed as oil being displaced by the presence of biosurfactant [17,18]. Besides, a study by Hamzah and co-workers using oil displacement test were able to screen nine potential biosurfactant-producing isolates instead of only one isolate by using the drop-collapse test [19]. There were several other studies that also supported that oil displacement test as a reliable and sensitive test [18-20]. In addition, Khopade and co-workers also able to screen *Streptomyces* sp. B3 as a potent biosurfactant producer through oil displacement test and drop collapse [21]. In this study, oil displacement and drop collapse test for all 12 isolates were positive which roughly indicated the presence of biosurfactants. Formation of oil displacement, the clearing zone and flat droplet when compared to 1% (v/v) SDS as a positive control showed similar results, thus enhanced the reliability of the finding.

According to Satpute et al., (2008), biosurfactant production was determined by good emulsification activity exhibit by bacteria which is measured to be more than 30% in E_{24} [22, 23]. The best biosurfactant producer was defined to be able to maintain at least 50% of its original emulsion after 24 hours of emulsification formation [24]. Purified glycolipid biosurfactant done recorded an increased in E_{24} values from 77.5% to 82% when compared to the crude biosurfactant [25]. This finding showed that emulsification test is one of the strong screening methods in the determination of biosurfactant producer. Based on the results obtained in Table 2, at least five isolates which are A1 (63%), B1 (50%), B2 (53%), B4 (67%) and B5 (67%) have the potential to be good biosurfactant producers according to the percentage E_{24} measured.

All isolated bacteria showed positive results for microplate assay (Table 2). The theory of this assay suggested that the presence of biosurfactant was proven if the solution used appeared to be concave with diverging lens-like shape [13]. If the biosurfactant was absent, the solution will remain flat. However, based on Youssef *et al*., negative results may be exhibited if the biosurfactant produced by bacteria was too low [18].

Among all 12 isolated biosurfactant-producing bacteria in this study, two of them belonged to *Pseudomonas* genera. Several studies done by a number of researchers agreed that *Pseudomonas* sp. was a common hydrocarbon degrading bacteria and able to degrade xenobiotic and more than 100 organic compounds due to the wide diversity of complex enzymatic system present within this genus [7,19,26]. The bacteria obtained from NCBI were isolated from the sea, sea sediment and water environment which made the finding to have a strong basis. For overall finding based on screening test, especially E_{24}, the most potent isolates to produce bacteria were isolates B4 and B5 [27]. Phylogeny analysis demonstrated that isolate B4 belong the group that was closely related to *Pseudoalteromonas lipolytica* species, meanwhile isolate B5 was grouped together with *Thalososspira* genera [28-30].

5. CONCLUSION

The 12 bacteria obtained from seawater of Kuantan Port were successfully identified. The qualitative screening tests were able to determine the potential bacteria that are able to produce biosurfactants. There were isolates from the genera of *Rhodococcus, Pseudomonas, Vibrio, Marinobacter, Pseudoalteromonas, Labrenzia, Marinococcus* and *Thalassospira* based on the molecular identification of 16S rRNA gene. Isolates of B4 and B5 that were closely related to *Pseudoalteromonas lipolytica* and *Thalassospira xianmenensis* respectively were found to be the most promising biosurfactant producer based on their ability to grow on minimal media supplemented with petroleum crude as sole carbon source, produce a high emulsification index and yield positive results for oil displacement test, drop collapse test and microplate assay. Further study on maximizing the biosurfactant production by these potentially biosurfactant-producing bacteria should be conducted to enhance the chance of biosurfactant usage as remediation strategy.

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