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IDENTIFICATION AND SCREENING CHARACTERISATION OF POTENTIAL BIOSURFACTANT PRODUCING BACTERIA ISOLATED FROM PALM OIL MILL EFFLUENT

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Abstract

Biosurfactant-producing bacteria isolated from palm oil mill effluent (POME) were screened for biosurfactant production using qualitative and quantitative screening test. The presence of biosurfactants evaluated using drop collapse, hemolysis and oil spreading tests were compared for their reliability in relation to the ability of cultures to reduce surface tension. The screening methods were used to detect biosurfactant production in 18 purified strains isolated from POME. From the qualitative screening results, 6 strains exhibited high surface activity using the qualitative drop collapse test, 2 strains gave the diameter of clear zone between 2cm to 3cm obtained with the oil spreading test and 4 strains lysed blood agar which indicates biosurfactant production. Quantitative screening test was performed using the Du Nouy ring test to measure the surface tension activity. Of 18 strains, 2 strains showed the ability of reducing surface tension of pure water from 72 to 29 mNm^{-1}. These 2 isolates namely PUT-R4 and PUT-R10 were selected and identified using 16S rDNA gene sequence analysis. The results suggested that the isolates belong to the genus *Bacillus*. Biosurfactant-producing bacteria isolated from palm oil mill effluent can be applied for bioremediation process particularly on improving biovailability of non-aqueous phase liquids.

Keywords: Biosurfactant, Drop collapse test, Oil spreading test, Hemolysis test, Surface tension

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Abbreviations

DCT	Drop collapse test
HM	Hemolysis test
NAPLs	Non-aqueous phase liquids
OST	Oil spreading test
PAHs	Polycyclic aromatic hydrocarbon
PCBs	Polychlorinated biphenyls
POME	Palm oil mill effluent
ST	Surface tension

1. Introduction

Contamination of soils by non-aqueous phase liquids (NAPLs) pollutants is one of the major problems occurring in many places of the world. NAPLs contaminated sites are mainly polluted with polychlorinated biphenyls (PCBs), pesticides, phenols and polycyclic aromatic hydrocarbon (PAHs). Though current bioremediation technologies are being an inexpensive and effective tool for treating sites contaminated with NAPLs, these technologies are often unsuccessful due to the slow degradation process of NAPLs [1, 2].

The efficiency of biodegradation of NAPLs often restricted by their solubility and recalcitrant towards degradation makes it unavailable for microorganism [3-5]. These conditions can substantially affect the microbial growth and the biodegradation capabilities. This phenomenon is referred to as limited bioavailability. Limited bioavailability is often defined when the uptake rate by organisms is limited by physicochemical barrier between pollutant and the organism [6, 7].

An alternative that has been considered to improve the bioavailability of NAPLs is the application of surface-active agents. Surfactants, of both biological and chemical origin, are amphipathic molecules that accumulate at interfaces, decrease interfacial tensions, and forming aggregate structures that allow hydrocarbon solubilisation [8, 9]. Due to these properties, surfactants modify interfacial behaviour and impact on the way other molecules behave at interfaces and in solution.

Biosurfactants are natural surface-active products from bacteria, yeast and fungi. Besides being less toxic than chemical surfactants, biosurfactants are better environmental compatibility, higher biodegradability and higher specific activity at extreme temperatures [3, 7, 8, 10]. Most biosurfactant-producing microorganisms reported are from the genus *Pseudomonas, Candida* and *Bacillus* is known to produce rhamnolipids, glycolipids and lipopeptide surfactants [3, 11].

In light of this, the work embarked with an attempt to isolate potential biosurfactant-producing bacteria from various processes involved in the milling of palm oil. Next qualitative assessments on the isolated strains were performed to evaluate the surface activity and hemolytic activity. Prior to the selection of the potential biosurfactant producers, quantitative assessment was carried out using the Du Nouy ring method on the ability to reduce the surface tension below 40 mNm^{-1} [11-13]. The selected biosurfactant-producing bacteria were identified using 16S rDNA gene sequencing.

2. Method

2.1. Isolation of biosurfactant-producing bacteria

Palm oil mill effluent (POME) samples were collected from the Tennamaram Oil Mill (Selangor, Malaysia). The samples were taken before and after the treatment process of POME. Direct isolation from mixed cultures of potential strains for biosurfactant production was performed by serial dilution of POME samples in sterile peptone water. Aliquots were spread on Trypticase Soy II Agar with 5% of sheep blood (Isolab, Malaysia) and incubated at 37 °C for 24 hours. Potential isolates were identified when colonies showing the presence of clear zone on the blood agar. Selected colonies were sub-cultured by repeated plating on fresh nutrient agar (Oxoid, USA) at least three times to obtain pure culture.

2.2. Screening of biosurfactant-producing bacteria

2.2.1. Qualitative screening tests

Screening of biosurfactant production was performed using the drop collapse test modified by Bodour and Miller-Maier [14]. Strains were inoculated in 3 ml of nutrient broth and incubated for 24 to 72 hours at 37°C. After incubation, cultures were aseptically placed in 25 ml sterile centrifuge tubes and centrifuge for 20 minutes at 20,000 rpm to remove the cells. The supernatant of each strain was harvested for use in the drop collapse test. The drop collapse test was conducted by using 96-well micro plate. Wells was coated with 2µl of SAE HD30 Pennzoil. The coated wells were equilibrated for 1 hour to ensure uniform oil coating. 5µl of 24 hours culture was transfer into the centre of the well. Each experiment was repeated three times.

For oil spreading test, 20 µl of synthetic motor oil 10W50 (Perodua, Malaysia) was added to the surface of 50 ml of distilled water in a 150mm diameter petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant was gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity [15].

Hemolysis test was developed by Mulligan et al. [16]. Cultures were grown in nutrient broth for 24 hours at 37°C. The cultures were inoculated on Trypticase Soy II Agar with 5% of sheep blood (Isolab, Malaysia) using sterilized toothpicks. The inoculated blood agar plates were incubated for 24 hours at 37°C. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies. Hemolysis can also be shown with purified biosurfactant or chemical surfactant.

2.2.2. Quantitative screening tests

Surface tension reduction was measured using a digital surface tensiometer (SEO DST- 60 Surface Tension Analyser). The tensiometer using the principle of Du Nouy ring method [14] were used in this experiment. A platinum wire ring was submerged in the cell free culture broth and pulled slowly through the liquid-air-

interface, where the tensiometer platform automatically moved by the equipment. The calibration was done using water (surface tension = 72.0 mNm^{-1} ±0.5) and medium broth was used as a negative control and Tween 80 (Sigma Aldrich, USA) was used as a positive control. Between each measurement the platinum ring was rinsed 3 times with distilled water and solvent and was allowed to dry. The surface tension results were taken three times from the same culture. The criterion used for selecting biosurfactant-producing bacteria was the ability of the isolates to reduce the surface tension below 40 mNm^{-1}. The results were compared to qualitative screening measurement for further analysis.

2.3. Identification of biosurfactant-producing bacteria

The identification of the biosurfactant-producing bacteria was performed according to Ramasamy et al. [17]. Two biosurfactant-producing bacteria that displayed high biosurfactant production were selected and identified by 16S rDNA gene sequencing. The biosurfactant-producing bacteria (1%) was cultured in 3 ml of nutrient broth and incubated for 24 hours at 37°C.

Aliquots of culture were centrifuged (13000 g at 4°C for 5 minutes), and supernatant was discarded. The DNA was extracted using MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). The 16S rDNAgene was amplified [18] with universal primers 27f (5'–AGAGTTTGATCATGCCTCAG-3') and 1429r (5'-TACGGTTACCTTAC CTTGTTACGACTT -3'). The amplification was performed in a total reaction volume of 50µl containing 20 µl of Tag DNA Polymerase Kit (Qiagen, USA), 5 µl of each of the primer, 4 µl of DNA template and 16 µl of nuclease free water using PCR System 2700, GeneAmp®, Applied Biosystems.

PCR condition consisted of an initial denaturing step of 5 min at 95°C followed by 30 cycles of 94°C for 45s, annealing at 55°C for 45s and 72°C for 45s. The reaction was completed with final extension at 72°C for 7 min and 25°C for 30s. The amplified fragments were harvested by electrophoresis in 1% agarose gels and visualised by ethidium bromide staining. The PCR products were purified using Wizard® SV Gel and PCR Clean-up System (Promega Corp, Madison, WI, USA), ligated and cloned into pGEM®-T Vector System II (Promega Corp, Madison, WI, USA) and used to transformed *Escherichia coli* JM109 as the host strain. Plasmid DNA was extracted from clones by using Wizard® Plus Minipreps DNA Purification System (Promega Corp, Madison, WI, USA).

The samples were then sent for sequencing. Sequences were manually edited and aligned using BioEdit v7.0.5 and matched with DNA sequences from GenBank using BLAST software (BLASTN) at the National Center for Biotechnology Information, (NCBI) [19]. The sequences were deposited in the GenBank for accession numbers. Sequence alignment was performed using CLUSTAL W, and phylogenetic analysis was conducted by neighbour joining (NJ) method using Molecular Evolutionary Genetic Analysis 4 (MEGA4; The Biodesign Institute, Tempe, AZ, USA) software. A bootstrap analysis was performed using 1000 resamples of data. *Escherichia coli* was used as the outgroup.

4. Results and Discussion

4.1. Isolation of biosurfactant-producing bacteria

Eighteen bacterial isolates (Table 1) were isolated from POME collected at Tennamaram Oil Mill plant of which 7 isolates were from untreated POME (influent and sludge) and 11 isolates from treated POME (effluent). These isolates exhibited clear zones on the blood agar, suggesting the potential of the isolates for biosurfactant production.

4.2. Screening chraracterisation of biosurfactant-producing bacteria

All 18 strains isolated from POME were examined for a variety of biosurfactant producing activities. Results from the qualitative and quantitative screening protocols are listed in Table 1. The four methods that were selected to detect biosurfactant production by different isolates were chosen due to their strong advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible [10, 13].

Six isolates (PUT-R1, PUT-R2, PUT-R3, PUT-R4, PUT-R5 and PUT-R10) were positive (response level '+++') for biosurfactant production by drop collapse test. Eleven of the strains (PUT-R9, PT-E1, PT-E2, PT-E3, PT-E4, PT-E5, PT-E6, PT-E7, PT-E8, PT-E9, PT-E10, PT-E11) that were also tested negative (response level '++', '+' and '-') for biosurfactant production by drop collapse test showed the same negative indication (response level '++', '+' and '-') by oil spreading test and hemolytic activity. High surface tension (> 50 mNm^{-1}) was also observed in these cultures suggesting that the strains were not potential biosurfactant producers. Of the 16 isolates (PUT-R1, PUT-R2, PUT-R3, PUT-R5, PUT-R9, PT-E1, PT-E2, PT-E3, PT-E4, PT-E5, PT-E6, PT-E7, PT-E8, PT-E9, PT-E10, PT-E11) that gave negative detection of biosurfactant by oil spreading test, 4 strains (PUT-R1, PUT-R2, PUT-R3, PUT-R5) were able to produce biosurfactant by drop collapse test, but the results obtained with Du Nouy ring method for the same 4 strains had surface tensions above 50 mNm^{-1}, indicating negative for biosurfactant production.

Oil spreading test demonstrated that 2 strains (PUT-R4 and PUT-R10) with diameters of spreading on the oil surface ranging from 2 to 4 cm, which suggests the presence of biosurfactant. The same strains gave positive biosurfactant production for hemolytic activity. From the surface tension results, some of the strains were quite promising compared to most biosurfactant-producing bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa* that are known to reduce surface tension of water from 72 mNm^{-1} to 27 mNm^{-1} [20]. Apparently, strain PUT-R4 and PUT-R10 isolated from untreated POME successfully reduced surface tension of water (72 mNm^{-1}) to 29 mNm^{-1} which suggest that the strains have excellent surface active properties. It should be noted that the criterion used for selecting biosurfactant-producing bacteria is the ability to lower the surface tension of water to below 40 mNm^{-1} [3].

Most researchers have used maximum two to three screening methods before selecting biosurfactant producers. It is suggested that a single method is not suitable to identify all types of biosurfactants [13]. Therefore, a combination of various methods is required for effective screening. Thus by examining the

qualitative and quantitative screening results, it is possible to decide a potent biosurfactant producer.

Table 1. Results for Qualitative and Quantitative Screening Test for Biosurfactant Production.

Source	Strain	[a]DCT	[b]OST	[c]HM	[d]ST [mNm^{-1}]
Untreated POME	PUT-R1	+++	+	-	55.3
	PUT-R2	+++	+	-	55.6
	PUT-R3	+++	+	-	54.8
	PUT-R4	+++	+++	+++	29.2
	PUT-R5	+++	+	++	57.3
	PUT-R9	++	+	++	58.5
	PUT-R10	+++	+++	+++	29.0
Treated POME	PT-E1	++	-	-	58.4
	PT-E2	++	-	-	59.6
	PT-E3	+	+	-	59.2
	PT-E4	++	-	-	58.3
	PT-E5	++	+	-	59.2
	PT-E6	-	+	-	58.4
	PT-E7	++	++	-	59.4
	PT-E8	+	+	-	57.2
	PT-E9	+	+	-	59.4
	PT-E10	+	-	-	60.3
	PT-E11	++	-	-	59.2
Positive Control	Tween 80	+++	++++	+++	36.2
Negative Control	Distilled water	-	-	-	72.0

[a] Drop Collapse Test (DCT): flat drops with scoring system ranging from ' + ' to '+++' corresponding to partial to complete spreading on the oil surface. Rounded drops were scored as negative '-' indicative of the lack of biosurfactant**.**

[b] Oil Spreading Test (OST): '-', no spreading < 0.5 cm; '+', incomplete spreading with diameter of clear zone < 1 cm; '++'; partial spreading with diameter of clear zone < 2 cm; '+++', complete spreading with diameter of clear zone > 2 cm but < 4 cm; and '++++', complete spreading with a diameter of clear zone > 5 cm.

[c] Hemolysis Test (HM): '-', no hemolysis; '+', incomplete hemolysis; '++', complete hemolysis with diameter of lysis< 1 cm; '+++', complete hemolysis with diameter of lysis> 1 cm but < 3 cm; and '++++', complete hemolysis with a diameter of lysis> 3 cm and green colonies.

[d]Surface Tension (ST)

4.3. Identification of biosurfactant -producing bacteria

The identity and diversity of the potential biosurfactant-producing bacteria based on 16S rDNA gene sequences of the selected biosurfactant-producing bacteria are shown in Fig. 1. Based on the phylogenetic analysis, PUT-R10 were positioned in the first clade of the phylogenetic tree, clustered together with *Bacillus subtilis*. Isolates PUT-R4 identified as *Bacillus tequilensis*, as they formed monophyletic clade with this bacterium. Hence, 16S rDNA sequence analysis revealed that *Bacillus subtilis* H10-5-5 had 99.2% homology with PUT-R10 and *Bacillus*

tequilensis M55 had 99% homology with PUT-R4. Thus, PUT-R10 was identified as *Bacillus subtilis* and PUT-R4 as *Bacillus tequilensis*. The 16srDNA sequences of strain *Bacillus subtilis* H10-5-5 and *Bacillus tequilensis* M55 were determined and deposited in the GenBank database under accession number KG281748 and KF281752.

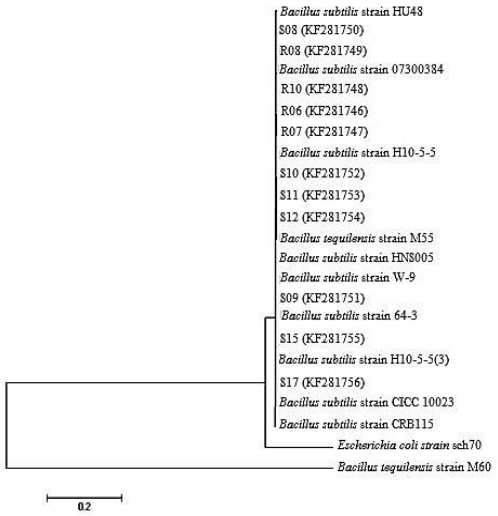

Fig. 1. Phylogenetic tree based upon the neighbour joining method. References sequences are included, which were found to be closest relative to retrieved sequences, as well as the accession numbers for all the 16S rDNA sequences (between brackets). The bar indicates 5% sequence divergence.

5. Conclusions

In the present study, 18 strains were isolated from palm oil mill effluent. Their ability to reduce surface tension and capability to lyse blood agar were among properties that evaluated by qualitative and quantitative screening method. Out of 18 strains, 2 strains namely PUT-R4 and PUT-R10 showed biosurfactant producing ability through qualitative assessment. These two strains also showed reduction of surface tension below 40 mNm^{-1}. PUT-R4 and PUT-R10 were selected and identified using 16S rDNA sequence analysis. Phylogenetic analysis revealed that all the isolates were closely related to *Bacillus* species. Further studies have been initiated to determine their potential if their bioremediation application in improving the bioavailability of non-aqueous phase liquids.

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