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Research paper



Isolation and Molecular Identification of Biosurfactant Producing Soil Bacteria

N A N Zamani[1], T E Tengku Zainal Mulok[2*], R Mat Nor[3]

School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
**Corresponding author E-mail: tetzm@salam.uitm.edu.my*

Abstract

Biosurfactants are amphiphilic compound, having hydrophilic and hydrophobic moieties enabling them to reduce surface and interfacial tension at the surface. Their unique properties are applied in various industries such as foaming and wetting agents, emulsifiers, detergents and bioremediation. A total of 98 isolates showed biosurfactant activity using hemolytic activity, drop collapse test and oil spreading assay. All isolates were rod-shaped, Gram positive and majority of them were non-endospore former. Only the isolates showing the highest percentage of emulsification index (E_{24}) and ability to reduce tension were used for species identification using *16S rDNA* gene sequencing which were isolates A1(6) and A2(1). Both isolates were identified as *Bacillus* sp. cp-h50 and *Bacillus* sp. XT-24 respectively, rod-shaped, endospore former and Gram positive. The biosurfactant produced by both species showed high emulsification index (E_{24}) (A1(6), 63.3% and A2(1), 46.7%) and good surfactant capacity. The size of amplified gene of *16S rDNA* gene was approximately 1.5 kb. These features provide evidence that both species could be a potential biosurfactant producer with proper optimization for the production of biosurfactant. The biosurfactant produced by both bacterial species were identified as surfactin using Fourier Transform Infrared Spectroscopy (FTIR).

Keywords: *Biosurfactant; FTIR; screening methods;surfactin; 16S rDNA gene sequencing*

1. Introduction

Bacteria can produce a wide range of extracellular products having many properties with many applications. One of them is biosurfactant in which the extracellular amphiphilic compounds produced by bacteria, fungi and yeast (Thavasi *et al*., 2011)[22]. Biosurfactants are naturally occurring surface-active compounds that are derived from microorganisms. They are complex molecule that covers a wide range of chemical types which include glycolipid, fatty acids, peptides, antibiotics, lipopeptides and others. Biosurfactant producing microorganisms can be found naturally in the oil contaminated soil, rhizosphere soil and aquatic environment. Microorganisms exhibit the activity of emulsifying by producing biosurfactants. They also utilize the hydrocarbons as a substrate by mineralizing or converting them into harmless products. Genera *Pseudomonas, Flavobacterium, Achromobacter, Bacillus, Micrococcus, Klebsilla, Arthrobacter, Streptococcus* sp., *Moraxella* sp., *Corynobacterium* sp. and *Proteobacteria* are the most prevalent bacterial surfactant producers and hydrocarbon degraders. Surfactin and rhamnolipid are the best studied among the different classes of biosurfactants. *Pseudomonas aeruginosa* mostly produced rhamnolipids (Santhini, 2014) [17]. Nowadays, many products are improperly accumulated in the environment causing contamination of soil and groundwater. This is due to the growth of industrial production (Rufino *et al*., 2012) [15]. The natural environment usually gets exacerbated by the harmful effects of industrial pollution. The industrial activities that utilize the heavy metals can severely affect the micro and macro biota inhabiting the water bodies when they were released into the environment. Therefore, the entire ecosystem will be disturbed (Sriram *et al*., 2011) [21]. Biosurfactants have received attention in the environmental remediation field of processes due to their efficacy as remediation agents, dispersion and their environmentally friendly characteristics such as high biodegradibility and low toxicity (Thavasi *et al*., 2011) [22]. The objectives of the study were to screen and isolate biosurfactant producing bacteria from soil in mangroves, dumping site and automobile workshop, to characterize and identify the type of biosurfactant produced and lastly to identify the best biosurfactant producing bacterial isolates using *16S rDNA* gene sequencing.

2. Materials and Method

2.1. Chemicals and raw materials

The chemical used were Blood Agar, ethanol, crude oil, palm oil, cycloheximide, crystal violet, iodine solution, acetone, safranin, malachite green, TopTaq™ Master Mix Kit, Purelink Genomic DNA Kits, forward primer (27F), reverse primer (1492R), sodium dedocyl sulphate (SDS), chloroform, methanol, hydrochloric acids (HCl) and potassium bromide.

2.2. Sample collection

The soil samples were collected from mangroves swamp, automobile workshop and dumping site. The pH and temperature of the soil where the samples were collected from all sites were recorded.

2.3 Hemolytic activity

The aliquots of 0.1 mL from 10^{-4}, 10^{-5} and 10^{-6} were transferred onto Blood Agar plates and incubated at 37°C for 24 to 48 hours.

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The presence of a clear zone around a colony appearing on the Blood Agar indicated the presence of lytic activity. However, there was no lytic activity when there was no clear zone observed [1].

2.4 Isolation and purification of bacterial strains

Each colony with clear zone on Blood Agar was later transferred and streaked onto a fresh Nutrient Agar plates and incubated overnight at 37°C. The streaking method was repeated for at least four times until a single isolated pure culture was obtained.

2.5 Drop collapse test

The glass slide was initially rinsed with hot water followed by ethanol and finally with distilled water. The dried slides were coated with crude oil and equilibrated for 24 hours to ensure a uniform oil coating. The culture supernatant of 1µL volume was applied to the centre of the oil drops by using a micropipette. The results were observed after 1 hour. The result was positive if the drop collapsed indicating the presence of surfactant but negative if the drop remained beaded indicating the absence of surfactant [13].

2.6 Oil spreading assay

Distilled water of 15 mL was added to a plastic petri dish and 2 µL of crude oil was pipetted to the surface of water to form a thin layer of oil. Culture of 10 µL volume was then added to the centre of oil surface. If there was presence of biosurfactant in the aliquots, the oil was displaced with an oil free clearing zone. If there was no oil displacement or clearing zone observed, it was an indication of negative displacement activity [13].

2.7 Inoculum preparation and medium composition

A few colonies from pure culture agar plate were selected randomly and sub-cultured into 100 mL growth medium. It was later incubated for 18 to 24 hours at 37°C to obtain the initial cell concentration. By using a spectrophotometer, the optical density was measured at 600nm wavelength. Once the OD achieved a reading of 0.8 to 1.0, an indication of cell concentration of 10^7 cells/mL, 10% (v/v) of the culture was later transferred into the fermentation medium.

2.8 Production of biosurfactant using shake flasks fermentation

The 10% (v/v) inoculum was transferred into a 100 mL volume of Nutrient Broth. It was later incubated for 24 to 72 hours at 37°C and agitated at 250 rpm. The bacterium was cultivated in a submerged culture with shaking (Rufino *et al*., 2016) [14]. The fermentation medium contained Bushnell Haas Broth and 2% (w/v) of frying oil (Peter *et al*., 2014) [11]. The initial pH of the medium was 7.0 was then sterilized by autoclaving at 120°C for 15 minutes. The fermentation medium was then cultivated at 37°C with shaking at 250 rpm for 120 hours (Rufino *et al.*, 2016) [14].

2.9 Emulsification index (E_{24}%)

Both the sample and crude oil at 2 mL volume each were added into a test tube. The mixture was later mixed by vortexing for two minutes and incubated for 24 hours [9]. The E_{24} index was determined based on equation 1 which was expressed as:

$$\frac{\text{Height of emulsion formed (cm)}}{\text{Total height of the solution (cm)}} \quad (1)$$

2.10 Biosurfactant extraction

After hours of incubation, the culture was harvested at 7,900 rpm for 15 minutes. Concentrated HCl was later added into the supernatant to achieve pH 2.0 and was stored overnight at 4°C. It was then centrifuged again at 7,900 rpm for 15 minutes and the grey precipitate was collected. Chloroform and methanol (2:1 v/v) were added and incubated in a rotary shaker at 30°C for 15 minutes at 250 rpm agitation for further extraction. It was centrifuged at 7,900 rpm for 15 minutes. The supernatant was then evaporated by air drying.

2.11 Determination of dry weight for biosurfactant

The sterile falcon tube was weighed prior to addition of sediment. The sediment was then poured into the falcon tube. They were dried and their weight was measured [20]. The dry weight was calculated by using the equation 2

$$\text{Dry weight of biosurfactant} = \text{weight of the falcon tube after drying} - \text{weight of empty falcon tube}$$

$$E = \qquad \times 100 \quad (2)$$

2.12 Surface tension

The surface tension measurement was done by using pendant drop shape technique [16]. The experimental apparatus needed for pendant drop method were a needle, a light source and a camera that was connected to a computer [2].

2.13 FTIR analysis

FTIR analysis was used to characterize the biosurfactant. The Infra-Red absorption spectra were obtained by using Perkin-Elmer grating 1430 IR in dry atmosphere. The absorption spectra were plotted by using a built-in plotter. IR spectra were collected from 400 to 4000 wavenumbers per wave number. The samples were prepared by dispersing solid uniformly in potassium bromide matrix [18].

2.14 Genomic extraction

The DNA from selected bacterial isolates was extracted by using DNA extraction Kit (PureLink® Genomic DNA Kits, USA).

2.15 Polymerase chain reaction

The PCR was run using a T100™ Thermal Cycler from Bio-Rad (United States). The composition of the master mix was 5x Green (5 µL), $MgCl_2$ (2 µL), 27F primer (1 µL), 1492R primer (1 µL), dNTP Mix (0.5 µL), DNA polymerase (0.25 µL), DNA template (2.5 µL) and dH_2O (12.75 µL). The primers used were 5'-AGAGTTTGATCCTGGCTCAG-3' (forward primer) and 5'-GGTTACCTTGTTACGACTT-3' (reverse primer). The PCR was conducted using the following program with 35 cycles, denaturation (95 °C) for 30 seconds, annealing (55 °C) for 30 seconds and elongation (72 °C) for 60 seconds.

2.16 DNA sequencing and analysis

The purified PCR products were later sent to the third party for DNA sequencing. The gene sequence was analyzed using the molecular biology database called ENTREZ and the retrieval system which was developed by National Center for Biotechnology Information by using BLAST/FASTA (http://www.ncbi.nlm.nih.gov).

3. Results and Discussion

The total number of isolates showing hemolytic activity from three different places was 98, 22 isolates (automobile workshop), 51 isolates (mangroves) and 25 isolates (dumping site). However, this method has several limitations therefore other screening methods should be included in order to confirm the biosurfactant is indeed being produced. The other screening methods were drop collapse test, oil spreading assay and emulsification index (Table 1). From a total of 98 isolates, only 21 isolates were chosen due to time constraint.

Table 1: Results obtained from hemolytic activity, drop collapse test, oil spreading assay and emulsification index using isolates from automobile workshop, mangroves and landfill

Isolates	Hemolytic activity	Drop collapse test	Oil spreading (mm)	Emulsification index (%)
A1(1)	+	+	4.0	36.7
A1(2)	+	+	4.0	10.0
A1(3)	+	+	4.0	29.6
A1(4)	+	+	4.0	36.7
A1(6)	+	+	6.0	63.3
A2(1)	+	+	5.0	46.7
A2(2)	+	+	4.0	16.7
M1(1)	+	+	4.0	39.1
M2(4)	+	+	6.0	23.3
M3(1)	+	+	10.0	20.0
M3(4)	+	+	20.0	20.0
M3(5)	+	+	4.0	13.3
M3(6)	+	+	5.0	33.3
L1(1)	+	+	4.0	26.7
L1(2)	+	+	4.0	24.1
L1(3)	+	+	4.0	30.0
L1(4)	+	+	4.0	25.0
L1(6)	+	+	4.0	10.0
L1(13)	+	+	4.0	9.4
L1(14)	+	+	4.0	13.3
L3(1)	+	+	4.0	30.0

Note: A1: Sample 1 from automobile workshop, A2: Sample 2 from automobile workshop, M1: Sample 1 from mangroves, M2: Sample 2 from mangroves, M3: Sample 3 from mangroves, L1: Sample 1 from dumping site and L3: Sample 3 from dumping site.

All 21 isolates showed positive results in the drop collapse test. Drop collapse test was reported to be not as sensitive as the oil spreading assay in detecting low levels of biosurfactant production while the oil spreading technique has been demonstrated as a reliable method for the detection of biosurfactant production by diverse microorganisms [12]. Other than that, there was a linear correlation between the diameter of clear zone and surfactant concentration [24, 25]. The largest clearing zone was observed at 20.0 mm produced by culture of isolates M3(4) from mangroves and this could be a potential strong biosurfactant producer since large diameter of spread indicated high biosurfactant production [25]. In addition, drop collapse test, surface tension and oil spreading assays have been reported to be having a direct correlation between them [22].

In emulsification index, the results that showed the highest percentage of emulsification index was obtained by isolate A1(6) (63.3%) followed by isolate A2(1) (46.7%) collected from automobile workshop. Lopes *et al*. (2014) [10] stated that emulsification index values are significant when they are above 50%. Isolate A1(6) showed E_{24}% = 63.3%, which was obviously higher than 50 % suggesting this isolate was a good biosurfactant producer. Isolate A2(1) could also be a good biosurfactant producer since the percentage of emulsification index was close to 50%. Surprisingly, isolate M3(4) with large diameter of oil spread did not show high percentage of emulsification index (E_{24}%) and similar result was observed by Ibrahim *et al.* (2013) [7]. On the contrary, study by Hassanshahian (2014) [6], the largest diameter of oil spread showed highest percentage of emulsification index. This showed that there is no correlation between oil spreading assay and emulsification index. Two isolates that showed the highest percentage were chosen, A1(6) and A2(1) and later analyzed for biosurfactant characterization. In the surface tension measurements, both isolates displayed values less than 40 mN/m (Table 2).

Table 2: Surface tension measurement using biosurfactant produced by isolates A1(6) and A2(1)

Sample	Surface Tension (mN/m)		
	Replication 1	Replication 2	Replication 3
A1(6)	33.29	33.93	32.87
A2(1)	30.74	31.23	32.86
SDS (positive control)	29.69	28.57	27.60
BHS (negative control)	74.17	72.95	72.67

These results confirmed with the study done by Dadrasnia *et al*. (2015) and Gudina *et al*. (2012) [3, 5] stated that a bacteria is considered as a biosurfactant producer if the bacteria can reduce surface tension to ≤ 40 mN/m. The dry weight of both isolates was determined as shown in Table 3.

Table 3: Dry weight of biosurfactant

Sample	Dry Weight of Biosurfactants (g)		
	Replication 1	Replication 2	Replication 3
Biosurfactant produced by A1(6)	0.1288	0.1237	0.1255
Biosurfactant produced by A2(1)	0.1336	0.1318	0.1310

The t-test was then performed on the dry weight of biosurfactant and surface tension activity between both isolates as shown in Table 4.

Table 4: The P-value for dry weight of biosurfactant produced by both isolates and surface tension measurement

	P-Value
Dry Weight of Biosurfactant	0.0355
Surface Tension	0.0907

Based on the result, there was a significant difference between two isolates in dry weight of biosurfactant because P-value was lower than 0.05. On the other hand, the P-value of surface tension was 0.0907 which was greater than 0.05. Therefore, it showed that there was no significant difference between these two isolates. This gave an indication that isolates A1(6) and A2(1) might belong to the same Genus which was a biosurfactant producing bacteria.

The FTIR absorbance bands were observed at absorbance peaks at 3292.30 cm^{-1}, 2920.09 cm^{-1}, 1659.43 cm^{-1}, 1533.18 cm^{-1} and 1384.70 cm^{-1} from biosurfactant produced by isolate A1(6). On the other hand, FTIR absorbance bands were observed at absorbance peaks at 3422.33 cm^{-1}, 2918.01 cm^{-1}, 2849.97 cm^{-1}, 1705.35 cm^{-1} and 1384.80 cm^{-1} from biosurfactant belonging to isolate A2(1). The spectra of both biosurfactant samples from the two isolates (A2(1) and A1(6)) were later compared with that of a standard surfactin. According to Shao *et al*. (2015) [19], absorbance ranging from 3500 cm^{-1} to 3200 cm^{-1} is a result of typical feature of N-H stretching vibrations in the peptide. Other than that, Varadavenkatesan *et al*. (2013) [23] indicated that the absorbance ranging from 3600 cm^{-1} to 3100 cm^{-1} showed a typical carbon containing compounds with amino group. Therefore, absorbance peaks at 3292.30 cm^{-1} and 3422.33 cm^{-1} observed from biosurfactant from isolate A1(6) and biosurfactant from isolate A2(1) respectively were typically associated with the stretching vibrations of N-H stretch. Absorbance peaks at 3292.30 cm^{-1} and

3422.33 cm^{-1} were also indicative of the stretching mode of O-H bond.

Shao *et al*. (2015) [19] also stated that the absorbance peaks between 3000 cm^{-1} to 2800 cm^{-1} were indicative of aliphatic chain. de Faria *et al*. (2011) [4] also observed that the absorbance peaks from 2970 cm^{-1} to 2850 cm^{-1} and 1450 cm^{-1} to 1380 cm^{-1} indicating the presence of aliphatic chain featured by the C-H modes. The absorbance peaks observed at 2920.09 cm^{-1} and 1384.70 cm^{-1} from biosurfactant from isolate A1(6) and at 2918.01 cm^{-1}, 2849.97 cm^{-1} and 1384.80 cm^{-1} from biosurfactant from isolate A2(1) exhibited the stretching frequencies belonging to an aliphatic chain. Other than that, absorbance peaks at 2920.09 cm^{-1}, 2918.01 cm^{-1} and 2849.97 cm^{-1} also combined with the O-H bonds known as carboxylic acid.

The frequency band at 1533.18 cm^{-1} observed for biosurfactant from isolate A1(6) (Figure 4.4) was assigned to C=N stretching mode. Sharp absorbance peak was observed at 1705.48 cm^{-1} for biosurfactant from isolate A2(1). The presence of carbonyl group was presented by the absorbance peak from 1800 cm^{-1} to 1700 cm^{-1}. The presence of C=O bonds lead to C=O stretching vibrations producing the absorbance peaks in these regions (Shao *et al*., 2015) [19].

Based on the characteristic frequencies of absorption and the bonds that they are associated with, the type of biosurfactant for both isolates A1(6) and A2(1) presumably belong to lipopeptide called surfactin. Similar FTIR spectrum belonging to surfactin was observed by Varadavenkatesan *et al*. (2013) [23]. It was stated that the FTIR spectrum indicated the production of lipopeptide biosurfactant.

In another study done by de Faria *et al*. (2011) [4] also showed similar curves and absorbance peaks in FTIR spectrum as observed in FTIR spectrum for biosurfactant belonging to A1(6) and A2(1). de Faria *et al*. (2011) [4] confirmed that the biosurfactant produced by *Bacillus subtilis* was a lipopeptide (surfactin). A study done by Shao *et al*. (2015) [19] further confirmed that the absorbance peaks and curves of the FTIR spectrum indeed belong to a lipopeptide biosurfactant called surfactin.

Based on the similarity in the absorption bands with presence of characteristic frequencies of absorption and the bands that they are associated with, it can be concluded that the type of biosurfactant produced was surfactin which was a lipopeptide. A large number of compounds can be estimated by identifying the functional groups. However, looking into fingerprint region, peaks at 1384.70 cm^{-1} and 1384.80 cm^{-1} from biosurfactant from isolate A1(6) and biosurfactant from isolate A2(1) respectively showed quite similar with absorbance peaks of FTIR spectra belonging to Sigma-Aldrich standard surfactin which was at 1388 cm^{-1}.

In 0.8% (w/v) agarose gel electrophoresis, the genomic DNA migrated mainly as a single band at a size of 23 kb (Lanes 2 and 3, Figure 1).

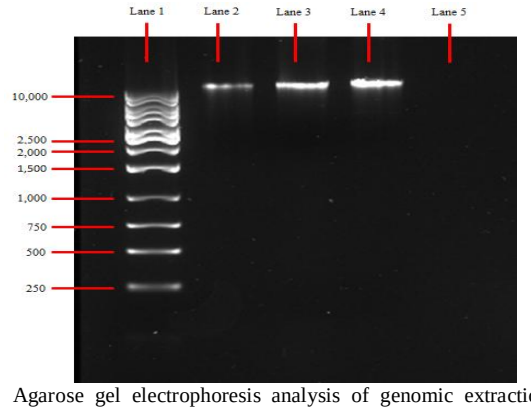

Fig. 1: Agarose gel electrophoresis analysis of genomic extraction of isolates A1(6) and A2(1)

Note: Lane 1: 1kb DNA ladder ; lane 2: Genomic DNA (Sample A1(6)) ; lane 3 : Genomic DNA (Sample A2(1)) ; lane 4: Positive Control (*Bacillus subtilis*) and lane 5: Negative Control (Distilled water)

The expected size of the amplified gene was approximately 1.5 kb long and the amplified PCR product was confirmed by electrophoresis of 5µL PCR mixture reaction on a 1% (w/v) agarose gel (Figure 2). Gene of almost same size was also reported for *16S rDNA* gene of *Bacillus subtilis* which was 1.55 kb (Kannan *et al*., 2008) [8].

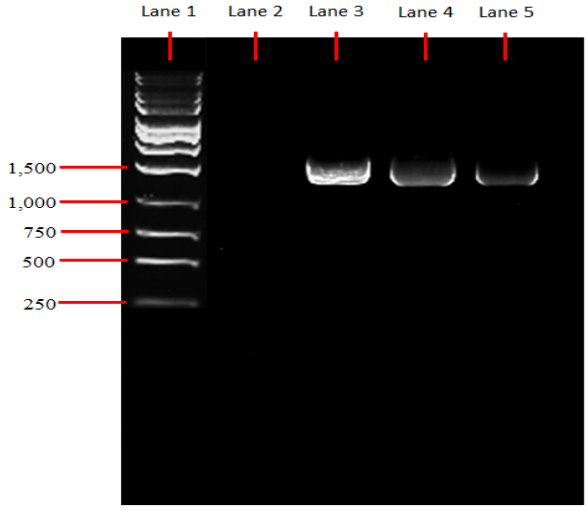

Fig. 2: Agarose gel electrophoresis analysis of PCR amplification of *16S rDNA* gene of isolates A1(6) and A2(1)

Note: Lane 1: 1kb DNA ladder ; lane 2: Genomic DNA (Sample A1(6)) ; lane 3 : Genomic DNA (Sample A2(1)) ; lane 4: Positive Control (*Bacillus subtilis*) and lane 5: Negative Control (Distilled water). The size of amplicon gene was approximately 1.5kb.

The purified genomic DNA was later sent to the third party for sequencing (MyTACG Bioscience Enterprise). The gene amplified for isolate A1(6) shared 100% similarity with *Bacillus* sp. cp-h50 while isolate A291) showed 98% similarity with *Bacillus* sp. XT-24.

4. Conclusion

Forty five isolates were found to be potential biosurfactant producers. Based on results from emulsification index, only two isolates were found to be a promising biosurfactant producer, namely isolates A1(6) and A2(1). The type of biosurfactant for both isolates was found to be a lipopeptide called surfactin. Based on the gene sequencing, the isolates A1(6) and A2(1) were both identified as *Bacillus* sp. cp-h50 and *Bacillus* sp. XT-24 respectively. The morphological characteristics of the bacteria were Gram positive, rod-shaped and endospore former. Both isolates showed positive results in all biosurfactant production screening tests and showed high percentage of emulsification index (E_{24}). Other than that, these two bacteria were able to reduce the surface tension to below 40 mN/m.

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