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Short Communication

Characterisation and toxicity evaluation of a biosurfactant produced from *Pseudomonas* sp.

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ABSTRACT

Biosurfactants are amphiphilic molecules produced by microorganisms, particularly bacteria. Biosurfactants have many applications including the bioremediation of hydrocarbon pollution in the environment. Hence, the toxicity of biosurfactants produced from bacteria should be evaluated. In this report, a biosurfactant harvested from the *Pseudomonas* sp. was characterized in terms of its ability to form an emulsion layer with hydrocarbons, its biochemical content and its toxicity towards germinating seeds. The biosurfactant produced was characterized as a glycolipid since a positive result was obtained in the sugar assay and a negative result was obtained in the protein assay. The ability of the biosurfactant to emulsify engine oil was found to be as strong as that of Triton X. Seeds of mung beans were germinated using the biosurfactant to assess the phytotoxicity of the biosurfactant. The biosurfactant was found not to have toxic effects on the development of roots and shoots of mung bean. The calculated germination index (GI) value of the mung bean exposed to the biosurfactant was significantly higher than that of the mung bean exposed to the chemical surfactant Triton. Hence, it can be concluded that the biosurfactant is non-toxic and exhibits non-inhibitory effects on the growth of mung bean and fenugreek and can be applied in the environmental bioremediation process.

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INTRODUCTION

Biosurfactants are known to be secreted by various bacteria [1]. Different microorganisms produce different types of biosurfactants that can be categorized according to their microbial origin and chemical properties. Most of the bacterial biosurfactants can be categorized into the groups: glycolipids, lipopeptides and surfactins. The polar head of biosurfactants may consist of phosphate, carbohydrate or amino acid while the nonpolar tail is a hydrocarbon chain [2]. Due to this amphiphilic structure, the interfacial and surface tensions between solids, gases and liquids can be reduced and this allows the aquas and organic phases to disperse and mix easily [3].

The majority of currently used synthetic surfactants in industries are produced by chemical means and are more toxic than biosurfactants. The synthetic surfactants are non-biodegradable and end up in the environment after use, whereas biosurfactants are biodegradable and will not have an adverse impact on the environment [4]. However, some biosurfactants have been reported to be toxic, as demonstrated by a biosurfactant produced by *L. mesenteroides* which exerts cytotoxicity against mammary cells [5]. Hence, if biosurfactants are to replace synthetic surfactants in environmental bioremediation application, the toxicity of biosurfactants needs to be evaluated. Thus, the objective of this report is to characterize a biosurfactant produced from *Pseudomonas* sp.

and to assess the toxicity of the biosurfactant using phytotoxicity assay.

MATERIALS AND METHODS

Preparation of standardized inoculum

The bacterium *Pseudomonas* sp. was previously isolated from a crude oil refinery, in Kuala Terengganu, Malaysia [6]. The stock bacteria kept in glycerol was revived in a nutrient broth (Oxoid, UK) and incubated at room temperature in a rotary shaker at 100 rpm for 24 hours. The bacterial cells were then harvested *via* centrifugation and washed twice before being resuspended in 0.85% NaCl to give an OD600nm ≈ 0.5 ($\times 10^8$ CFU/mL) [7]. This served as the starting inoculum in the subsequent experiment.

Production and isolation of biosurfactant

A flask that contains 98 mL of Bushnell-Haas media (BH) was inoculated with 1 mL of starting inoculum and the contents were added with 1 mL of hexadecane. The culture was incubated at room temperature in an orbital shaker that was set at 120 rpm, for 7 days [8].

After 7 days, the culture was filtered using Whatmann filter paper to absorb the excess hexadecane. The bacterial cell concentration was measured using a spectrophotometer at OD600 nm, and then the filtrate was centrifuged at 5000 rpm for 20 mins to remove the bacterial cells. The supernatant obtained was mixed with cold acetone at the ratio of 3:1 and kept at 4 °C for 48 hours to allow the biosurfactant to precipitate. The supernatant was centrifuged at 5000 rpm for 10 mins at 4 °C. The pelleted biosurfactant was air-dried and weighed [9].

Characterisation of the biosurfactant

The supernatant collected was added with 95% cold ethanol (ratio 1:3) and stored at 4 ºC overnight [8] before being centrifuged at 10,000 rpm, at 4 ºC for 5 min to precipitate the biosurfactant. The pelleted biosurfactant was then re-dissolved in deionized water and used to determine the emulsification index (EX24) and the biosurfactant's chemical composition.

The biosurfactant was mixed with engine oil in equal volume (v/v), in a glass test tube (125 mm × 15 mm) and vortexed for 2 min and left to stand for 24 hours [6]. A control using synthetic surfactant, Triton X instead of the biosurfactant was also prepared. After 24 hours, the height of the emulsion layer formed was measured. The emulsifying index (EX24) is calculated as follow:

$$\frac{\text{Height of the emulsified layer (mm)}}{\text{Total height of the liquid in the glass test tube (mm)}} \times 100$$

The presence of sugar in the biosurfactant was quantified by the method of Dubois [10] using phenol solution and sulphuric acid and its absorbance was measured at OD490 nm. The reading was compared to a standard curve prepared using glucose (0.1-1.0 mg/L) [8].

The presence of proteins in the biosurfactant was detected using the Biuret protocol by Feigner & Michel [11]. A positive result was indicated by the formation of violet colour, due to the reaction of peptide bond with Cu ions in the Biuret solution.

Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated using seeds of mung bean. 10 seeds were rinsed with 0.5% sodium hypochlorite (NaOCl) solution [12] and placed onto a piece of Whatman filter paper pre-soaked with 5 mL of the biosurfactant solution. The seeds were incubated at room temperature for 4 days in the dark. A negative control was prepared by replacing the biosurfactant solution with distilled water. A second control was prepared by replacing the biosurfactant solution Triton. After 4 days, the germination index (GI) was calculated by using the following formulas:

Relative seed germination (%) = (number of seeds germinated in test solution/number of seeds germinated in the control) × 100

Relative root elongation (%) = (mean root length in test solution/mean root length in the control) × 100

Germination Index = (% of seed germination) × (% of root growth)/100

Statistical analysis

Data obtained from the phytotoxicity assay was analyzed using *T*-test with 95% confidence level by using IBM SPSS software (Version 20). Results were reported as ± standard deviation ($n = 3$).

RESULTS

Pseudomonas sp. inoculated in the BH media with hexadecane turned cloudy indicating growth at the end of the incubation period. Approximately 15.3 g biosurfactant/g cell biomass was successfully precipitated from the culture filtrate using cold acetone.

The result from the Dubois method shows a positive reading of 0.14 ± 0.001, indicating the presence of glucose in the biosurfactant. However, the result from Biuret assay showed protein was absent from the biosurfactant. Hence, the biosurfactant sample was most probably a glycolipid because it contained the sugar moiety in its biochemical structure and not from the family of lipoprotein since protein was not detected.

The ability of the biosurfactant and Triton X to emulsify engine oil was quantitatively in terms of emulsifying index (EX24) as shown in Table 1. Both the biosurfactant and Triton X were able to emulsify engine oil. Statistical analysis performed on the data of EX24(%) of both biosurfactant and Triton-X showed no significant differences ($p > 0.05$). This suggested that the biosurfactant produced from the *Pseudomonas* sp. has the same emulsifying ability as the synthetic Triton X.

The toxicity of the biosurfactant produced from the *Pseudomonas* sp. was assessed using mung beans' seeds in the phytotoxicity assay. Seeds of mung bean were observed to germinate rapidly, with elongated roots and shoots in both deionized water and biosurfactant plates. However, the germination of seeds of mung bean in Triton X was observed to be stunted, as demonstrated by the minimal formation of roots and non-detection of shoots (Table 2). The length of the roots and shoots of the germinated seeds in biosurfactant showed no significant differences ($p > 0.05$) from that of seeds germinated using deionized water. However, seeds germinated in the biosurfactant developed roots that were 6-times longer than the seeds germinated in Triton-X. This showed the biosurfactant isolated from *Pseudomonas* sp. did not have any inhibitory effect on seed germination. Furthermore, the GI value of biosurfactant was 6 times higher than the GI value of Triton X (Table 3). This suggested that the biosurfactant was

Table 1. EX24 values of the biosurfactant and Triton X mixed with engine oil.

Type of substances tested	Emulsifying index 24 (%)
Biosurfactant	46.21 ± 4.49
Triton X	45.39 ± 5.04

Table 2. The lengths of roots and shoots of mung bean seeds germinated in the deionized water, biosurfactant and Triton X.

Mung bean	Length of root	Length of shoot
Deionized water	2.15 ± 0.945	1.83 ± 0.497
Biosurfactant	2.16 ± 0.443	1.84 ± 0.344
Triton X	0.34 ± 0.135	0

Table 3. The germination index (GI) of mung bean seeds soaked in the biosurfactant and Triton X.

	GI value (%)
Biosurfactant	100.46
Triton X	15.81

relatively non-toxic compared to the synthetic biosurfactant Triton X.

DISCUSSION

In this study, the biosurfactant obtained from the *Pseudomonas* sp. grown on hexadecane was able to emulsify engine oil as efficiently as the industrial synthetic surfactant Triton X. The EX24 index of the biosurfactant reported in this study was lower than the EX24 index (86%) of a biosurfactant produced from another *Pseudomonas* sp. [13]. In the study by Kaustuvmani *et al*. [13], the *Pseudomonas* sp. was cultured using the highly hydrophobic crude oil, instead of hexadecane. Thus, the biosurfactant produced in their study most probably targets these highly hydrophobic compounds. Wong *et al*. [7] reported that bacteria including *Pseudomonas* sp. secreted large amounts of biosurfactant when initially exposed to crude oil, and the emulsification activity progressively decreased when the percentages of the hydrophobic polycyclic aromatic hydrocarbons (PAHs) decreased. This suggested that in this study, the biosurfactant secretion induced by the less-hydrophobic aliphatic hexadecane might not produce the biosurfactant efficiently to emulsify hydrophobic PAHs in the engine oil. From this observation, it can be deduced that the quality of biosurfactant secreted by the same organism, in this case *Pseudomonas* sp., highly depends on the source of the hydrocarbon.

The biosurfactant produced from the *Pseudomonas* sp. in this study was made of glycolipids. Within the glycolipid biosurfactant family, they varied structurally due to the existence of different congeners. The biosurfactant that demonstrated a higher EX24 index as reported by Kaustuvmani *et al*. [13] was found to be constructed from two different congeners, the mono-rhamnolipid congener and di-rhamnolipid congener, whereas a biosurfactant produced from the *Pseudomonas* sp. using hexadecane was reported to exhibit a relatively lower EX24 index because the biosurfactant contains only di-rhamnolipid [14]. These circumstantial observations suggest that the biosurfactant produced by *Pseudomonas* sp. using aliphatic hexadecane in this study, possibly contains a single congener of di-rhamnolipid as well, which explains the lower EX24 index.

Phytotoxicity assay shows that the biosurfactant isolated from the *Pseudomonas* sp. in this study was not toxic to mung bean. The biosurfactant did not inhibit the growth of the seeds of mung bean. The germination index (GI) calculated was 100.46%, which exceeded the recommended 80% that denotes a non-toxic substance [15]. It can be summarised that the biosurfactant secreted by *Pseudomonas* sp. in this study was not toxic to seed germination and did not show any inhibitory effect on the elongation of the roots and shoots of the mung bean. Thus, the biosurfactant is safe to be used to bioremediate hydrocarbon pollution in the environment.

CONCLUSION

In this study, the *Pseudomonas sp.* was able to produce 15.3 g/g of biosurfactant, consisting of glycolipid. The biosurfactant was able to emulsify engine oil, with the calculated EX24 index of 46.21 ± 4.49, which is similar to the emulsification activity of the synthetic surfactant Triton X. The biosurfactant has no toxic effect on the seeds of mung bean, whereby the seeds were able to germinate and develop roots and shoots normally. The calculated GI value of the biosurfactant was 100.46%, which is far above the safe GI value recommended for environmental applications.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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