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Original Article

Dual role of biosurfactants in bioremediating crude oil hydrocarbons co-contaminated by cadmium

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ABSTRACT

The use of biosurfactants produced from bacteria is an alternative approach to bioremediate the sites contaminated by crude oil which often contains heavy metals. However, there are limited studies on the production of biosurfactants in the presence of both hydrocarbons and heavy metals. Hence, this study aims to investigate the effect of cadmium (Cd) on biosurfactant production by *Exiguobacterium profundum*, previously isolated from a hydrocarbon refinery and characterised. *E. profundum* was able to grow in culture media containing crude oil as the sole carbon source and in the presence of 0.1 mg/L Cd, demonstrating its resistance to Cd. The harvested bacterial cells showed 58.4% cell adherence to crude oil using bacterial adhesion to hydrocarbons (BATH) assay, suggesting hydrophobic cell surfaces due to potential secretion of biosurfactants. In the presence of 0.1 mg/L Cd, biosurfactant dry mass was significantly increased ($p<0.05$) by 29.6% when compared to the control without Cd. At room temperature, the biosurfactant was able to form emulsion layers with all three tested hydrocarbons, with the calculated emulsification index of 35.8 ± 1.43% in benzene ($p<0.05$) followed by 5.0 ± 0.0% in hexane and 8.3 ± 3.36% in crude oil. The biosurfactants tested positive for the presence of sugar moiety but negative for protein. This suggested that the biosurfactants produced by *E. profundum* are from the glycolipid family. The presence of Cd significantly increased the secretion of biosurfactants suggesting that *E. profundum* employed the biosynthesis and secretion of glycolipid biosurfactants as mechanisms to counter the toxicity due to Cd.

KEYWORDS: emulsification, defence mechanism, *Exiguobacterium profundum*, glycolipid.

INTRODUCTION

One of the most important biological variables that can limit the biodegradation of hydrocarbon compounds by microbes is the bioavailability of the hydrophobic components (substrates) to bacterial cells. This specifically refers to the ability of hydrophobic hydrocarbons to be solubilised and transported into the immediate vicinity of the cells [1]. The low water solubility of the majority of crude oil hydrocarbon compounds limits the capacity of microbes, which generally exist in aqueous phases, to have access to and degrade these substrates. This 'bioavailability' is the rate-limiting step of metabolizing hydrophobic hydrocarbons during bioremediation [2].

The microbial community that produces biosurfactants has the distinct advantage of increasing the emulsification of poorly water-soluble hydrocarbons and the bioavailability of such compounds for biodegradation [2]. Biosurfactants are usually found as extracellular products or as part of the cell membrane component [3]. A variety of bacteria produce biosurfactants, which are amphiphilic in nature

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and consist of hydrophobic and hydrophilic groups [4, 5]. The hydrophilic group consists of sugar or proteins whereas the hydrophobic group consists of fatty acids [5]. As biosurfactants possess amphiphilic properties, it can change the surface tension between oil and water and allows the two phases to mix together [1].

However, there are limited studies about the production of biosurfactants in the presence of both hydrocarbons and heavy metals. This is important because most sites contaminated with crude oil often contain heavy metals, that can decrease the efficiency of the microorganisms to biodegrade the hydrocarbons [6]. According to United States Environmental Protection Agency (USEPA) [7], different hydrocarbon-based industries dealing with natural gas petrochemical processing, and petroleum refining discharge were found to produce wastes contaminated by Cadmium (Cd). Due to the complex nature of wastewater that contains both hydrocarbons and Cd, a treatment process that is able to eliminate both types of pollutants is most desirable to minimise environmental contamination [8]. Although one of the current techniques adopted to treat mixed effluents is bioremediation [9], whether the presence of heavy metals will enhance or reduce biosurfactant production, is yet to be verified.

Hence, the objective of this experiment is to study the effect of Cd on biosurfactant production by bacteria exposed to crude oil hydrocarbons and to characterise the biosurfactants in order to better understand its application in bioremediation of Cd-contaminated wastes.

METHODS

Preparation of standardized inoculum

The bacterium *Exiguobacterium profundum* was previously isolated from a crude oil refinery, in Kuala Terengganu, Malaysia [10], and characterised to be a hydrocarbon degrader. The stock bacteria kept in glycerol was revived in a nutrient broth (Oxoid, UK) and incubated at room temperature on 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 OD 600 nm ≈ 0.5 (× 10^8 CFU/mL) [11]. This served as the starting inoculum in the subsequent experiment.

Incubation of *E. profundum* in a culture mixture of crude oil and Cd

The starting inoculum (1% v/v) and *Tapis* crude oil (1% (v/v) were inoculated in Bushnell-Haas (BH) containing 0.1 mg/L Cd. Similarly, a control without Cd was also prepared. All cultures were incubated for 120 hours, at 30 °C and 100 rpm. At the end of the incubation period, growth of the bacteria was measured using spectrophotometer at OD 600 nm. The bacterial cultures were then centrifuged (3000 rpm, 15 min) and the bacterial cells collected were then used to determine the surface hydrophobicity *via* BATH assay. The resulting supernatant collected was used to precipitate biosurfactants in later experiments. The experiments were carried out in triplicates.

Bacterial adhesion to hydrocarbons (BATH) assay

BATH assay was performed to estimate the hydrophobicity of the bacterial cells according to the method described by Rosenberg *et al*. [12]. Briefly, the bacteria cells harvested were washed twice and resuspended using 0.85% of NaCl to give a concentration of 1.0 × 10^8 cfu/mL of bacterial cells. The bacterial cell suspension was then aliquoted into glass test tubes containing *Tapis* crude oil in the ratio 1:1 (v/v). Tubes were then vortexed for 5 mins and left to stand for 60 mins to allow the cells to settle. At the end of the 60 mins, the aqueous phase was collected and the absorbance was read at OD 600 nm to enumerate the bacterial concentration. The hydrophobicity of the bacterial cells is expressed as the percentage of cells adhering to crude oil and calculated as follows:

1 - (OD of the aqueous phase/OD of initial cell suspension) × 100

Production and characterisation of biosurfactants

The supernatant collected was added with 95% cold ethanol (ratio 1:3) and stored at 4 ºC overnight [13] before being centrifuged at 10,000 rpm, at 4 ºC for 5 min to precipitate the biosurfactants.

The pelleted biosurfactants were then re-dissolved in deionized water and were used to determine the emulsification index (EX24) and the biosurfactants' chemical composition.

For the calculation of EX24, the biosurfactants were mixed with three types of hydrocarbons in equal volume (v/v). Each of the tested hydrocarbons represents aliphatic (hexane), monoaromatic (benzene) and polycyclic aromatic (benzopyrene) hydrocarbons. Two sets of control using commercial surfactant (SDS, Triton X-100) were also prepared and mixed with three types of hydrocarbons as well. The mixture was prepared in a glass test tube (125 mm × 15 mm) and vortexed for 2 min and left to stand for 24 hours [14]. 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 was quantified by the method by Dubois [15] using phenol solution and sulphuric acid and its absorbance was measured at OD 490 nm. The reading was compared to a standard curve prepared using glucose (0.1-1.0 mg/L).

As to the detection of proteins, a Biuret test protocol by Feigner & Michel [16] was employed using NaOH solution and $CuSO_4$. A positive result was indicated by the formation of violet colour, due to the reaction of peptide bond with Cu ions.

RESULTS

The effect of Cadmium on the growth of bacteria

The growth of *E. profundum* inoculated in the BH medium supplemented with 1% (v/v) *Tapis* crude oil and 0.10 mg/L of Cd, and the control without the addition of Cd is shown in Figure 1. At the end of the 120-hour incubation period, both sets showed significant growth compared to time 0 hours ($p<0.05$). This showed *E. profundum* was able to degrade the crude oil and use it as the sole carbon source for growth. There was no significant difference in the growth patterns between the tests and controls ($p>0.05$). This suggests that Cd at 0.10 mg/L has no inhibitory effect on *E. profundum*.

BATH assay and biosurfactant production

The BATH assay showed *E. profundum* gave a reading of 58.4% ± 0.001 of cell adherence to crude oil. This shows the cell surface of *E. profundum* is hydrophobic and might be attributed to the secretion of biosurfactants covering the cell surface.

At the end of 120-hour incubation, the supernatants from the culture with and without the addition of Cd, were used to precipitate biosurfactants using cold acetone. Table 1 shows the amount of biosurfactants obtained (gram dry weight). The culture with Cd showed significantly higher biosurfactant production compared to the control ($p<0.05$). The addition of 0.1 mg/L Cd increased the production of biosurfactants to almost two times in *E. profundum*, compared to culture without Cd.

Characterization of biosurfactants

The ability of the biosurfactant to emulsify hexane, benzene and crude oil, expressed as the emulsifying index 24 (EX24), is shown in Table 2. Biosurfactants obtained from both sets of cultures emulsified hydrocarbons in the order hexane< crude oil<benzene. The biosurfactants collected from the cultures with and without Cd showed the highest EX24 value in benzene ($p<0.05$), followed by both hexane and crude oil. However, the biosurfactants collected from the cultures with and without Cd did not show any significant differences in the EX24 reading for the same hydrocarbons. This suggested biosurfactants synthesized by *E. profundum* was substrate-specific, solubilizing or emulsifying different hydrocarbons at different rates. The result also suggests that the presence of Cd did not induce

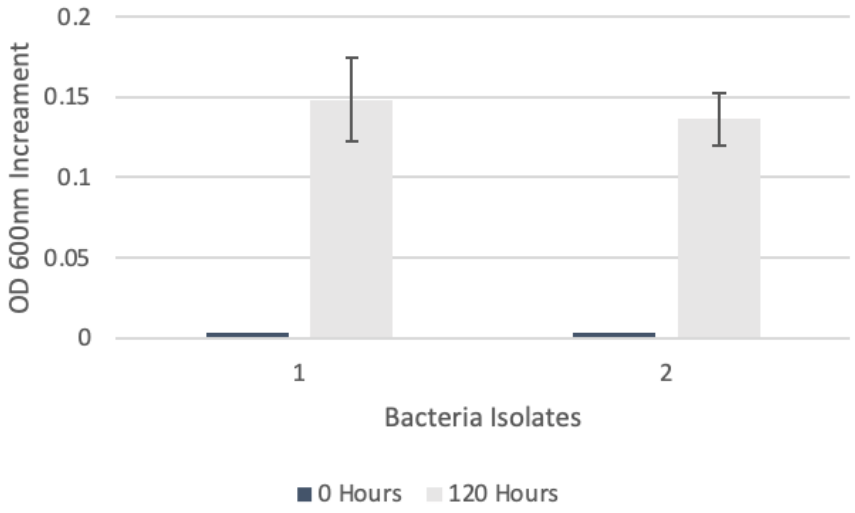

Figure 1. Growth of *E. profundum* (OD 600 nm) in BH media spiked with 1% (v/v) Tapis crude oil with 0.1 mg/L Cd (1) and the control set without Cd (2).

Table 1. Production of biosurfactant obtained from *E. profundum* after 120 h of incubation.

Culture	Cd (mg/L)	Biosurfactant (g dry weight)
With Cd	0.1	0.42 ± 0.06
Control	-	0.28 ± 0.04

Table 2. Emulsifying index values of biosurfactants on hexane, benzene and crude oil.

Type of hydrocarbon	EX24 (%)	
	Control culture without Cd	Culture exposed to Cd
Hexane	5.83 ± 3.82	6.33 ± 1.60
Benzene	60.76 ± 6.25	70.00 ± 1.43
Crude oil	6.67 ± 2.89	7.62 ± 3.36

different types of biosurfactants, since EX24 for both sets of biosurfactants remained the same for all three types of hydrocarbons tested.

The semi-purified biosurfactants showed presence of sugar moiety (47.30 ± 3.8 µg/mL) using Dubois assay. However, no protein was detected in the biosurfactants, since no colour changes were observed when the biosurfactants were tested using NaOH solution and $CuSO_4$. Taken together, these two results suggested the biosurfactants secreted by *E. profundum* is most probably from the glycolipid family.

DISCUSSION

The growth of *E. profundum* in BH medium supplemented with 1% (v/v) crude oil showed that the bacteria can degrade crude oil and use it as the sole carbon source to support its growth. A similar growth pattern was observed in the control without Cd, indicating that the bacteria is resistant to Cd toxicity at 0.1 mg/L. This Cd concentration is ten times higher than the permissible level of Cd in industrial effluents (0.01 mg/L) according to Malaysia Environmental Quality (Sewage and Industrial Effluents) Regulations [17]. This suggested

that *E. profundum* possesses a mechanism which allows the bacteria to reduce the toxicity of Cd, as well as to degrade crude oil hydrocarbons.

The BATH assay result (58.4%) indicated that the cell surface of *E. profundum* was hydrophobic. The most likely underlying mechanism is the ability of the bacteria to biosynthesize and secrete biosurfactants to envelope the cell to give it a hydrophobic nature in the presence of crude oil. Microbes are known to adopt the strategy of secreting biosurfactants to increase the solubility of hydrophobic components within the crude oil hydrocarbons [18, 19]. Various research also reported that the bacteria can secrete biosurfactants to increase the bioavailability of hydrocarbons by emulsifying them into small droplets before absorbing them into the cells [4, 5, 10]. Biosurfactants can solubilise and emulsify the crude oil hydrocarbons by reducing the surface tension due to the repulsive forces between crude oil and the aqueous media, allowing these two phases to mix more easily [20]. The formation of oil-water emulsion is important for the biodegradation of crude oil hydrocarbons because it increases the contact surface between hydrophobic hydrocarbons and the bacterial membrane-bound enzymes [4]. This action accelerates the biodegradation process [18].

The *E. profundum* culture spiked with 0.1 mg/L Cd produced twice the amount of biosurfactants (g dry weight) than those harvested from the culture without Cd. Since both cultures contained 1% (v/v) of crude oil, this result suggests it was the presence of Cd that plays a role in increasing the biosurfactant production and secretion. It seems that secretion of biosurfactants is not merely to facilitate the degradation of crude oil but also acts as a defensive mechanism by *E. profundum* to resist Cd toxicity in the media.

In the report done by Wong *et al*. [11], Cd was observed to be bound to the outer membrane of bacteria *via* the production of biosurfactants which served as a metal-chelating agent. Under transmission electron microscope (TEM), a dense area was observed on the bacterial cell surfaces, indicating Cd was absorbed on the outer surface of the cells coated by biosurfactants. The secretion of biosurfactants was suggested as a mechanism employed by the bacterial cells to reduce the toxicity of the metals by preventing the Cd from penetrating into the cell [11].

Biosurfactants are reported to play a crucial role in heavy metal bioremediation because the charged surfaces found on the hydrophilic group can bind to metal ions to form complexes [21, 22]. Biosurfactants secreted by the culture of *Bacillus circulans* exhibited significantly higher conductivity compared to commercial surfactant sodium dodecyl sulphate (SDS), demonstrating more charged surfaces can be found in the biosurfactants [23]. In another biosurfactant analysis using the Fourier transform infrared spectroscopy (FTIR), the results showed a strong vibrational shift, which further confirmed ionic bonding occurred between metal ions and the biosurfactants [23], and thus the ability of biosurfactants to be used to chelate metal ions from the solution.

CONCLUSION

Results from BATH assays indicate that hydrophobic cell surfaces are due to the secretion of biosurfactants. The biosurfactants isolated from *E. profundum* culture possessed good emulsifying activities when tested on benzene, hexane and crude oil at room temperature. The biosurfactants showed positive sugar content when tested using Dubois assay whilst protein assay was negative using the Biuret test. This suggested that the biosurfactants were from the glycolipid family. *E. profundum* culture spiked with 0.10 mg/L Cd showed significantly higher ($p<0.05$) biosurfactant production compared to the control (without Cd). This demonstrated the biosurfactant production increased in the presence of Cd, in order to emulsify crude oil and also to chelate Cd to reduce the latter's toxicity.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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