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Chapter 4

BIOSURFACTANTS FROM ACINETOBACTER CALCOACETICUS BU03 ENHANCE THE BIOAVAILABILITY AND BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

Biosurfactants produced by an isolated thermophilic strain Acinetobacter calcoaceticus BU03 were demonstrated to be effective in enhancing the solubility of polycyclic aromatic hydrocarbons (PAHs) and the present study aimed at investigating its effectiveness in increasing bioavailability of PAHs in soil for biodegradation under thermophilic composting condition. At 25 times of its critical micelle concentration (CMC), biosurfactants by BU03 significantly increased the apparent aqueous solubility of phenanthrene (PHE) and benzo[a]pyrene (B[a]P) to 54.3 and 2.08 mg L⁻¹, respectively. After confirmation of its ability in enhancing the solubility of PAHs, the isolated biosurfactants were applied to a thermophilic soil composting system. Within 42 days of composting period, the degradation of PHE and B[a]P in the absence of the biosurfactants was 71.2 and 16.4%, respectively. Inoculation of A. calcoaceticus BU03 or biosurfactants produced by this strain significantly increased the emulsifying capacity of soil, and therefore enhanced the desorption of PAHs from soil to aqueous phase in which they can be degraded by an inoculated degradative strain Bacillus subtilis B-UM. Therefore inoculation of A. calcoaceticus BU03 or biosurfactants from BU03 together with inoculation of B. subtilis B-UM increased the degradation of B[a]P to 83.8 and 65.1%, respectively, while PHE was almost completely removed with these two treatments. The results indicate that the application of biosurfactants produced by A. calcoaceticus is an effective means to enhance the biodegradation of PAHs in thermophilic composting, while inoculation of biosurfactants producing strains in PAHs contaminated soil is a more practical and cost-effective approach than direct addition of biosurfactants.

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Keywords: biosurfactant, polycyclic aromatic hydrocarbons, *Acinetobacter calcoaceticus* BU03, biodegradation, phenanthrene, benzo[a]pyrene

1. INTRODUCTION

The remediation of soil contaminated by polycyclic aromatic hydrocarbons (PAHs) is of major importance because most PAH compounds are known as carcinogens and mutagens (Wilson and Jones, 1993). Owing to the serious health risks associated with even extremely low levels of PAHs, increasing effort has been devoted recently to develop innovative technology to remove PAHs from soil. Since PAHs are biodegradable in the presence of suitable microorganisms (Guerin, 1999; Sepic et al., 2003), thermophilic composting provides a cost-effective technology for the clean up of PAHs (Wong et al., 2002; Wan et al., 2003; Atagana, 2004). It exploits the degradative potential of indigenous or inoculated microorganisms or their extracts, to dissolve and biologically convert contaminants into less toxic compounds. Adenuga et al. (1992) showed that pyrene could be degraded during the composting of soil/sludge mixtures but the rate and extent were not mentioned in that study. PAHs with 2 or 3 rings, i.e., naphthalene, anthracene and phenanthrene (PHE) were removed to the remediation target of 1 mg kg\(^{-1}\) in three to four months of composting. However, one major constraint of this approach is the low bioavailability of PAHs especially those with high molecular weight. The fate of benzo[a]pyrene (B[a]P) during composting was investigated in a number of studies and the results showed that 29.9% and 65.6% of B[a]P was removed after 54 and 95 days, respectively and still quite substantial amounts remained in soil (Sasek et al., 2003; McFarland and Qiu, 1995). The low biodegradability of high molecular weight PAHs during composting is due to their low aqueous solubility and high sorption to soil particles (Kim and Weber, 2005; Doong and Lei, 2003). A promising means to enhance the bioavailability of PAHs is the application of surfactants. Addition of surfactant increased the solubilization and biodegradation of PAHs (Boonchan et al., 1998; Kim and Weber, 2005). Addition of nonionic surfactants has been shown to enhance the solubility, desorption and bioavailability of PAHs (Bernal-Martinez et al., 2005). However, in our previous studies the addition of Tween 80 or Triton X100 caused inhibition on the biodegradation of PAHs due to the toxic effects of surfactants on a PAHs degrading bacterium *Bacillus subtilis* B-UM (Wong et al., 2004). Similar to synthetic surfactants, biosurfactants produced by microorganisms can enhance the solubilization and desorption of PAHs. Two kinds of rhamnolipids, i.e. dirhamnolipid and monorhamnolipid enhanced the biodegradation of PAHs in aqueous systems (Zhang et al., 1997). Biosurfactants from *Acinetobacter* sp. increased the apparent solubility and biodegradation of PAHs (Barkay et al., 1999), and they are more biodegradable and less toxic and...
expensive as compared to chemical surfactants (Rosenberg and Ron, 1997; Zhao and Wong, 2009).

Although the use of biosurfactants for bioremediation of PAHs looks promising, the cost of biosurfactant production is about 3 to 10 times higher than that of the synthetic surfactants (Mulligan and Gibbs, 1993). To date, all of the studies of surfactant-aid bioremediation of soil were conducted under mesophilic conditions. Thermophilic condition may be more effective for the biodegradation of PAHs since elevated temperature is expected to increase solubility, and mass transfer rates of PAHs (Cheng et al., 2004). Under thermophilic condition, a high removal rate of PAH compounds from contaminated soil may be achieved and substrate utilization rates of thermophilic bacteria have been reported to be 3-10 times greater than that of mesophilic bacteria (Goswami et al., 1983).

In our pervious study a thermophilic bacterium, *Acinetobacter calcoaceticus* BU03, was isolated from petroleum contaminated soil collected from Dagang Oil Field, China (Zhao and Wong, 2009). Therefore in the present study, the potential of the biosurfactants produced by *A. calcoaceticus* BU03 in enhancing the solubilization and biodegradation of PAHs was investigated under thermophilic condition. Bench-scale thermophilic composting was performed to investigate the effects of addition of the biosurfactants and inoculation of *A. calcoaceticus* BU03 on the bioremediation of PAHs contaminated soil.

2. MATERIALS AND METHODS

2.1. PAHs

B[a]P and PHE (analytical grade of 96% in purity, Sigma Chemical Co. St Louis, MO, USA) were used in this study as model compounds of PAHs.

2.2. PAHs degradative bacterium

A PAHs degradative bacterium *Bacillus subtilis* B-UM, which was enriched and isolated from PAH-contaminated soil and compost in our research group (Wong et al., 2002), was used in the present study.

2.3. Preparation of biosurfactants

Cells of *A. calcoaceticus* BU03 were cultured in medium containing 10 g glucose, 10 g peptone, 4 g NaH₂PO₄, 0.01 g FeCl₃ and 0.025 g MgCl₂ per liter, pH 6.5 on a gyratory shaker (150 rpm) at 55°C. After 36 h, bacterial cells were removed by centrifugation at 6000 × g for 20 min. The supernatant was subjected to extraction
by adding 100 mL n-hexane to 300 mL supernatant and the extraction was repeated two more times. The emulsified phase was collected, washed twice with double distilled water (DDW) and rotary evaporated at 55°C. Residues were subjected to freeze-drying and dissolved in DDW. Undissolved material was removed by filtration through 0.45-µm cellulose acetate membrane.

Biosurfactants produced by *Pseudomonas aeruginosa* ATCC9027 were used in this study for comparison. *Pseudomonas aeruginosa* ATCC9027 was cultured in PPGAS medium (1.07 g NH₄Cl, 1.49 g KCl, 18.90 g Tris-HCl, 10.0 g glucose, 10.0 g peptone and 0.19 g MgSO₄ per liter) in a shaker set at 150 rpm and 37°C. After 72 h of incubation, bacterial cells were removed, by centrifugation and the supernatant was subjected to acid precipitation by adjusting the pH to 2.0 with 5N HCl. The precipitate was centrifuged at 8000 × g for 20 min and freeze-dried. The dried biosurfactants were extracted with n-hexane three times at room temperature. After evaporating the organic solvent on a rotary evaporator at 55°C, the biosurfactants were dissolved in DDW and filtered through 0.45-µm cellulose acetate membrane. The concentration of biosurfactants at 55°C was determined following the method of critical micelle dilution (CMD) method (Philp et al., 2002).

2.4. Effect of biosurfactants produced by BU03 on the solubility of PAHs

Five milligrams of PHE or B[a]P dissolved in dichloromethane (DCM) was carefully added to the bottom of a 20 mL glass vial. The amount of added PHE or B[a]P was well in excess of its aqueous saturation. After the DCM was evaporated, 10 mL Bushnell-Haas medium containing various concentrations of surfactants, i.e., 0, 0.5, 1, 3, 10 and 25 × CMC was added to the tubes. The vials were covered with aluminum foil and capped, and then shaken in a rotary shaker at 150 rpm and 55°C for an equilibrium period of 48 h determined in a previous study (Cheng et al., 2004). After reaching equilibrium, 2 mL sample was removed from each vial and filtered through a 10 mL glass syringe packed with glass wool to remove any undissolved PAHs particles. The solubilized PAH in aqueous phase was extracted three times with n-hexane. The extracts were combined and concentrated to appropriate volume for quantification of PAHs concentrations using a high-performance liquid chromatography (HPLC) equipped with a fluorescence detector (FLD). A sample volume of 15-μL was separated on a Reverse Phase C18 column (5 μm, 3.6 × 25 cm, Ultrasphere, Beckman) with 100% acetonitrile as mobile phase with a flow rate of 1.5 mL min⁻¹.
2.5. Effect of BU03 produced biosurfactants on the biodegradation of PAHs during thermophilic composting

Soil collected from abandoned shipyards at North Tsing Yi, Hong Kong SAR, China was air-dried at room temperature, sieved to < 2 mm and spiked with PHE and B[a]P dissolved in DCM to a final concentration of 250 mg kg⁻¹ each. The DCM in soil was allowed to evaporate in a fume hood for one day and the spiked soil was stored at room temperature for an aging process of 6 months before use.

Table 1 shows the 10 treatments of the composting study with a combination of contaminated soil, pig manure, chemical surfactant or biosurfactants produced by BU03, degradative microorganisms, as well as biosurfactants producing microorganisms. The bench-scale composting experiment was carried out in 1 L composting tanks and each treatment was performed in triplicate. Pig manure was mixed with soil at a ratio of 3:1 (w/w dry weight) as co-composting material to provide nutrient for microbial growth. About 600 g of soil-pig manure mixture were added to the composting tank. The moisture content of the composting material was adjusted to about 70% of its water-holding capacity with DDW. For the control treatment (Control), no pig manure was added to soil. The flasks were aerated by negative air pump conditioned at 350 mL min⁻¹, which should provide sufficient oxygen for the decomposition of organic matter. A condenser was connected to the outlet of each flask to reduce moisture loss from the system. All composting flasks were incubated at 55°C for 42 days in order to achieve a thermophilic condition. Periodically, samples of composting material were collected from the composting flasks for the analysis of carbon dioxide (CO₂) evolution, total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) using the methods of TMECC (2002). PAHs degradative and total heterotrophic populations, as well as PAHs in composting mass were determined as described elsewhere (Wong et al., 2002), while soil emulsifying activity followed that described by Zhao and Wong (2009).

2.6. Statistical analyses

Analyses were performed in triplicate samples and the mean values with standard error were presented. The data were subjected to one way analysis of variance (ANOVA) and Duncan’s multiple range test using SPSS ver.11.5 software.
Table 1. Description of treatments employed in the thermophilic composting

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Degradative cells B-UM (CFU g⁻¹)</th>
<th>Surfactants concentrations (× CMC)</th>
<th>Biosurfactants producing cells BU03 (CFU g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>PM a</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>I b</td>
<td>10⁻⁷</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>I T c</td>
<td>10⁻⁷</td>
<td>10 (Tween 80)</td>
<td>×</td>
</tr>
<tr>
<td>B10 d</td>
<td>×</td>
<td>10 (Biosurfactant)</td>
<td>×</td>
</tr>
<tr>
<td>I B1</td>
<td>10⁻⁷</td>
<td>1 (Biosurfactant)</td>
<td>×</td>
</tr>
<tr>
<td>I B10</td>
<td>10⁻⁷</td>
<td>10 (Biosurfactant)</td>
<td>×</td>
</tr>
<tr>
<td>I C e</td>
<td>10⁻¹</td>
<td>×</td>
<td>10⁻¹</td>
</tr>
</tbody>
</table>

a: PM indicates soil amended with pig manure only; b: I indicates the inoculation of degradative cells of B-UM; c: T indicates the addition of Tween 80; d: B indicates the addition of BU03 produced biosurfactants followed by a number which indicates the concentration (CMC); and e: C indicates the addition of biosurfactant producing cells of BU03.

3. RESULTS AND DISCUSSION

3.1. Effect of synthetic surfactants and biosurfactants on solubility of PAHs

PAHs solubility was plotted as a function of aqueous surfactant concentrations in the range of 0 to 25 × CMC (Figure 1). Aqueous equilibrium concentration of PHE without surfactants was measured to be 1.82 mg L⁻¹ at 55°C in DDW, while that of B[a]P was undetectable. PAHs solubility was significantly enhanced at surfactant concentrations above their respective CMCs because of the formation of micelles. Among the tested surfactants, biosurfactants produced by BU03 were the most effective in enhancing the solubility of PHE and B[a]P. In the presence of biosurfactants from BU03 at 25 CMC, the aqueous solubility of PHE and B[a]P was increased to 54.3 and 2.08 mg L⁻¹, respectively.

3.2. Effect of biosurfactants produced by A. calcoaceticus BU03 on bioremediation of PAHs contaminated soil with thermophilic composting

3.2.1. Changes in nutrient contents

In general, there was a sharp drop in TOC in the first 7 days for the treatments amended with pig manure (Figure 2). During the initial period of composting, the rapid growth of microorganisms quickly reduced the TOC contents from 13.6 to...
about 6% and after 42 days, the TOC contents for all treatments were lower than 2%. Either addition of surfactants or inoculation of selected microorganisms did not affect the TOC contents significantly. In the Control without pig manure addition, the TOC content decreased from 3.4 to 0.44% within 42 days. The slow reduction in TOC content in the Control may be due to the low microbial activity and lack of readily metabolizable carbon sources in soil (Viel et al., 1987).

The concentrations of TKN decreased from 0.67 and 0.10% to about 0.3 and 0.06% over the composting period, for those treatments amended with pig manure and the Control, respectively (Figure 4). Similar to TOC content, the initial concentrations of TKN increased following the addition of pig manure while the addition of surfactants or inoculation of microorganisms did not cause any significant additional change.

Although nitrogen is a critical nutrient which usually limits PAHs biodegradation in soil (Ritter and Scarborough, 1995), addition of a single limiting nutrient may not benefit the growth of heterogeneous microorganisms (Breedveld and Sparrevik, 2000). In the bioremediation process, different microbial species may be required to degrade PAHs sequentially, and each species has its own nutrient requirements. Therefore, pig manure used in the present experiment served not only as a source of microbial population, but also as an organic material to provide nutrients for the growth of PAHs degradative microorganisms.
Figure 2. Changes in TOC during the thermophilic composting of PAHs contaminated soil. (Control = control soil only; PM = addition of pig manure only; I = inoculation of B-UM; T = addition of Tween 80; B = addition of biosurfactants produced by BU03; 1 = 1 × CMC; 10 = 10 × CMC; and C = inoculation of BU03)

Figure 3. Changes in TKN during thermophilic composting of PAHs contaminated soil. Refer to Figure 2 for the legends explanation.
3.1.2. Carbon dioxide (CO₂) evolution

As an indicator to the microbial activities, the generation of CO₂ during the composting period is presented in Figure 4. The addition of pig manure significantly enhanced the CO₂ generation due to the increase in organic matter and also the high microbial activity in pig manure. In the first three days, the evolution of CO₂ for the treatments amended with pig manure increased to about 1.5 mM day⁻¹ g⁻¹, and then decreased from day 3 to day 21 gradually followed by a levelling-off phase from day 21 to day 42. There was no significant difference in the generation of CO₂ caused by the inoculation of microorganisms and addition of surfactants. This might be possibly due to the large amount of CO₂ generated from the utilization of organic matter in pig manure that masked the effects of inoculated microorganisms and surfactants.

A close relationship between the generation of CO₂ and the utilization of TOC was observed in the present study since the CO₂ was the metabolic by-products of organic carbon produced by heterotrophic microorganisms during their respiration. Higher organic carbon contents would produce more CO₂ and thus the generation of CO₂ was proportional to the decrease in the organic carbon contents.

3.2.3. Heterotrophic and PAHs degradative bacterial population during thermophilic composting

The population of total heterotrophic microorganisms is shown in Figure 5. In the Control, the population of total heterotrophic microorganisms in the composting mass was significantly lower than other treatments with pig manure and microbial
inoculation during the composting period. The addition of organic amendment, i.e. pig manure, not only obviously increased the initial total heterotrophic bacterial population but also promoted the microbial growth, which indicates that the organic amendment might serve as both the source of and nutrients for microorganisms. In the treatments amended with pig manure, the heterotrophic microorganisms reached their maximum concentrations at day 7 and ranged from $3.49 \times 10^8$ to $4.52 \times 10^8$ CFU g$^{-1}$. No significant effect of the inoculation of microorganisms and addition of biosurfactants was observed on the total population of heterotrophic microorganisms, while Tween 80 slightly inhibited the growth of microorganisms.

![Figure 5](image1.png)

*Figure 5. Changes in total heterotrophic bacterial population during the thermophilic composting of PAHs contaminated soil. Refer to Figure 2 for the legends explanation.*

The growth of PAHs degradative microorganisms is shown in Figure 6. In the Control, the PAHs degradative populations in soil were quite small, and only increased slightly to $4.68 \times 10^6$ CFU g$^{-1}$ at the end of the composting period. The addition of organic amendment, i.e. pig manure, obviously promoted the growth of PAHs degradative populations, which indicates that the organic amendment may serve as nutrients for PAHs degradative microorganisms. The inoculation of B-UM further increased the degradative populations to $1.13 \times 10^8$ g$^{-1}$. However, the addition of chemical surfactant, i.e., Tween 80 slightly inhibited the growth of degradative populations. The addition of biosurfactants produced by BU03 or inoculation of BU03 slightly increased the degradative populations but the difference was not significant.
3.2.4. PAHs degradation during thermophilic composting

The removal of PHE and B[a]P during thermophilic composting is plotted with time in Figures 7 and 8, respectively. Within the experimental period of 42 days, the removal of PHE and B[a]P in the Control were 71.2% and 16.4%, respectively. More than 98% of PHE was removed from treatments with pig manure amendment and no significant difference was noted among these treatments. However, the removal of B[a]P differed significantly among the various treatments. In the treatment amended with pig manure alone, about 33.7% of B[a]P was removed. Addition of biosurfactants produced by \textit{A. calcoaceticus} BU03 or inoculation of PAHs degradative strain B-UM increased the removal of B[a]P to 41.5 and 56.8%, respectively. The combined addition of B-UM together with \textit{A. calcoaceticus} BU03 or biosurfactants produced by BU03 significantly increased the degradation of B[a]P to 83.8% and 65.1%, and the average removal rate of B[a]P was calculated as 4.95 and 3.85 mg kg\(^{-1}\) day\(^{-1}\), respectively.

Few reports that documented the biodegradation of B[a]P reported that the resting cells of \textit{Sphingomonas paucimobilis} EPA505 could degrade 33% of 10 mg L\(^{-1}\) B[a]P in aqueous systems (Ye et al., 1996). A bacterial consortium including members of the genera \textit{Mycobacterium} and \textit{Sphingobacterium} rapidly mineralized B[a]P in the presence of diesel fuel and the degradation rate was 1.08 mg L\(^{-1}\) d\(^{-1}\) (Kanaly et al., 2000). Besides, a litter-decomposing basidiomycete \textit{Stropharia rugosoannulata} almost completely removed or transformed 10 mg L\(^{-1}\) B[a]P.
Biosurfactants from Acinetobacter calcoaceticus BU03

Figure 7. Removal of PHE during the thermophilic composting of PAHs contaminated soil. Refer to Figure 2 for the legends explanation.

Figure 8. Removal of B[a]P during the thermophilic composting of PAHs contaminated soil. Refer to Figure 2 for the legends explanation.

within 6 weeks (Steffen et al., 2002). Another fungus, Stropharia coronilla degraded B[a]P in Mn²⁺-supplemented cultures and the degradation rate was about 1.43 mg L⁻¹ d⁻¹ (Steffen et al., 2003). However, degradation of B[a]P in aged soil is thought to be slow due to the strong sorption of the substrate on soil particulates (Hughes et al., 1997). The potential of Phanerochaete sordida to degrade PAHs in a creosote-contaminated soil was investigated under field conditions and none of those PAHs with five rings or more, was removed (Davis
et al., 1993), while a combination of bioaugmentation and biostimulation in landfarming increased the removal of B[a]P to 87% in 16 months. In the present study, 83.8% of B[a]P was rapidly removed from the contaminated soil in 42 days under thermophilic condition, owing to the application of biosurfactants and biosurfactants producing bacteria, indicating a potential for field application.

3.2.5. Emulsifying activity in composting material

To elucidate the mechanism responsible for the enhanced biodegradation of PAHs by biosurfactants or biosurfactants producing cells, emulsifying activity of composting mass was analyzed and presented in Figure 9. The emulsifying activity of soil was about 30 EU. The addition of pig manure initially increased the emulsifying activity to about 120 EU since the pig manure contained large amount of organic matters which may act as emulsifying agents. However, the emulsifying activity decreased sharply to the same level as the Control at the end of the composting period, due to the degradation of the emulsifying agents. Both the addition of the biosurfactants or inoculation of BU03 significantly increased the emulsifying activity. The emulsifying activity of the treatment with inoculation of BU03 was higher and lasted longer than those with the addition of the biosurfactants. Therefore the possible mechanism responsible for the effects of BU03 on biodegradation of PAHs might be due to the production of biosurfactants following the inoculation of BU03, as supported by the increase in emulsifying activity. Biosurfactants might increase the solubility and desorption of PAHs as indicated in the batch experiments. As a result, the bioavailability of PAHs was increased and the biodegradation of PAHs was consequently promoted.

The direct application of biosurfactants may not be a practical approach for large scale application, since the production and recovery of biosurfactants is expensive. On the other hand, the inoculation of biosurfactants producing microorganisms into the composting mass resulted in a higher removal of B[a]P, which is likely a more practical and cheaper alternative for remediation of PAHs contaminated soils.

4. CONCLUSIONS

Biosurfactants produced by an isolated strain, A. calcoaceticus BU03 were more effective in enhancing the solubility of PHE and B[a]P than synthetic surfactants Tween 80 and Triton X100, as well as biosurfactants produced by P. aeruginosa ATCC 9027. In a bench scale thermophilic composting system for remediation of
PAHs contaminated soil, addition of the biosurfactants and the inoculation of BU03 significantly enhanced the degradation rate of B[a]P to 3.85 and 4.95 mg kg\(^{-1}\) day\(^{-1}\), respectively, which are higher than most of the studies to date. Results from the present study gave sufficient evidence to affirm that addition of the biosurfactants or inoculation of biosurfactants producing microorganism i.e., \textit{A. calcoaceticus} is an effective method to enhance the bioremediation of soil contaminated by PAHs.

5. ACKNOWLEDGMENT

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6. REFERENCES


