Immobilization and Surfactant Enhanced Anthracene Biodegradation in Soil

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Abstract: The study was conducted to determine the extent of biodegradation of anthracene in salts solution (SS) as well as in environmental samples, focusing on methods used to enhance its biodegradation. A total of 32 fungal and bacterial isolates have been recovered from environmental samples by enrichment culture technique. The recovered isolates were assessed for their ability to degrade 10µg ml^{-1} of anthracene in pure cultures. Degradation of anthracene was assessed by determination of the residual substrate by HPLC method. Among the fungal isolates, *Fusarium oxysporum* was proved to be highly active, where 78% of anthracene had disappeared in environmental samples at a concentration of 10µg ml^{-1} within 7 days of incubation. While *Aspergillus terreus* was able to degrade 70% of anthracene under the same conditions. Degradation was enhanced significantly by immobilized fungal and bacterial cells; where immobilized *Fusarium oxysporum* degraded 83% of anthracene at a concentration of 10µg ml^{-1} as compared to 51% as free cells within 4 days of incubation. Addition of surfactant (Tween 80) to the culture of *Fusarium oxysporum,* 89% of the initial concentration of anthracene was degraded under the same conditions. The results showed that anthracene biodegradation might be rapidly decomposed by the addition of surfactants and/or immobilization of the degrading isolates.
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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemical compounds, which occur as a result of incomplete combustion of organic matter in automobile exhaust, petrochemical industry, or accidental spills during the transportation of petroleum (Sartoros *et al.,* 2005; Jacques *et al.,* 2008).

Since PAHs exhibit carcinogenic, teratogenic, mutagenic and other toxic properties (Crisafully *et al.,* 2008) they pose serious risks to the environment and human health (Yuan *et al.,* 2000). Anthracene is one among the 16 PAHs and was chosen for the study of PAH degradation because of its relative toxicity, and it is a probable inducer of tumors (Das *et al.,* 2008).

Although PAHs may undergo chemical oxidation, photolysis, bioaccumulation, volatilization and adsorption, microbial degradation is the major process affecting PAH persistence in nature (Cerniglia, 1993; Yuan *et al.,* 2000). Recently, bioremediation, which is expected to be an economic and efficient alternative method to other remediation processes such as chemical or physical ones, has been developed as a soil cleanup technique (Potin *et al.,* 2004). However, the bioavailability of PAHs to microorganisms in soil is a primary limiting factor, which may be resulted from PAH molecule stability and hydrophobicity, soil properties and soil–PAH contact time (i.e., aging) (Alexander *et al.,* 2000; Antizar-Ladislao *et al.,* 2006**).**

In soil habitats, filamentous fungi offer certain advantages over bacteria for bioremediation. The majority of studies have focused on white rot fungi, particularly *Phanerochaete chrysosporium* (Field *et al.,* 1995; Pointing, 2001). Non-lignolytic fungi, such as *Cunninghamella elegans* (Cerniglia, 1993) and *Penicillium janthinellum* (Launen *et al.,* 1995) can also metabolize a variety of PAHs to polar metabolites.

Many studies have been conducted to enhance the biodegradation of PAHs using surfactants to increase their solubility by decreasing the interfacial surface tension between PAHs and the soil/water interphase. When surfactant concentration is above the critical micelle concentration (CMC), micelle aggregates provide an additional hydrophobic area in the central region of micelles enhancing the aqueous solubility of PAHs (Li and Chen, 2009). Immobilized cells have been used and studied extensively for the production of useful chemicals (Ohta *et al.,* 1994; Chang and Chou, 2002) and the bioremediation of contamination from numerous toxic chemicals (Rahman *et al.,* 2006).

The study was designed to investigate methods used to enhance anthracene biodegradation in soil samples. These methods included addition of non ionic surfactant and immobilization of the degrading cells.

2. Materials and Methods

2.1. Isolation of degrading organisms

Enrichment culture technique was used for isolation of degrading microorganisms from soil samples (Ramadan *et al.,* 1990). Salts Solution (SS) composed of (g l^{-1} distilled water) 1 NH_4Cl, 0.2 KH_2PO_4, 0.38 K_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.05 $FeCl_3$ with the final pH adjusted to 6.8 and autoclaved at 121ºC for 20 min. Anthracene was added to the SS to the final concentration of 50 µg ml^{-1} which was previously dissolved in 19% acetone (Mohammadi and Nasernejad, 2009). After complete evaporation of acetone, 10 g of contaminated soil was added to the flask. Flasks were incubated on a rotary shaker at 30ºC at a speed of 150 rpm in the dark. This was replicated for four times. After 4 weeks of incubation, 1 ml of culture was spread over plates containing SS containing 50µg ml^{-1} anthracene and solidified with 2% Noble agar (Daane *et al.,* 2001). Plates were incubated at 28 ºC for 2 weeks. Colonies that grow on this media were purified and used in this study.

2.2. Anthracene degradation

To assess the capacity of the microbial isolates to degrade anthracene in soil, 10 g of soil was added to a flask containing 50 ml SS and 10µg ml^{-1} anthracene. Fresh culture of fungal isolates (*Fusarium oxyspoum, Fusarium moniliforme, Fusarium chlamydosporum* and *Aspergillus terreus)* were added to the SS flasks (10% v/v) according to (Bishinoi *et al.,* 2008), while 2.5 ml of 10^8 of fresh cultures of bacterial isolates (*Pseudomonas aureofaciens*, *Micrococcus varians, Lactobacillus coryniformis*) were added according to (Sepic *et al.,* 1997). All flasks were incubated at 30ºC on a rotary shaker working at 150 rpm in the dark. Uninoculated media with the same concentration of anthracene was used as a control. At different time intervals sample of both test and control were taken to determine the residual substrate by HPLC.

2.3. Immobilization techniques

The procedure of immobilization was carried out by the method described by Owabor and Aluyor (2008). Isolates were added to 10 g of activated carbon in 50 ml flaks containing SS. Flasks were incubated for 3 days on a rotary shaker at 30ºC at a speed of 150 rpm. Soil (10 g) was added to 50 ml SS containing 10µg ml^{-1} of anthracene. A control sample without activated carbon was set up alongside the experimental samples following the same procedure. The flasks were then incubated for another week under the same conditions.

2.4. Addition of surfactants

A non ionic surfactant (Tween 80) was used at a concentration above CMC, which was previously determined (Jayashree *et al.,* 2006). The experiment was carried out by the modified method of Bautista *et al.* (2009). Tween 80 at a concentration of 2% (above CMC) was added to a flask containing 10 g of soil and SS containing anthracene with the final concentration of 10µg ml^{-1}. The flasks were inoculated with the tested isolates and incubated at 30 ºC in a rotary shaker working at 150 rpm in the dark. Control experiment with SS and anthracene alone was carried out at the same time under the same conditions.

2.5. Quantitative analysis of anthracene

Determination of residual anthracene was carried out by HPLC (Yuan *et al.,* 2000). Residual PAHs were extracted with hexane (1:2), centrifuged for 10 min at 10000g, after phase separation 1 ml of hexane was filtered with millipore filters (0.45 µm) and transported into a sterile vial for evaporation of hexane. After complete evaporation of hexane, 1 ml of acetonitrile was added to the residue and the remaining anthracene was analyzed by HPLC using a UV-detector at 254nm.

2.6. Fungal identification

Identification of fungal isolates was done by the Micro Analytical Center in the Faculty of Science, Cairo University. The fungal isolates were identified as: *Fusarium oxyspoum, Fusarium moniliforme, Fusarium chlamydosporum* and *Aspergillus terreus.*

2.7. Statistical studies

Statistical studies were done by InStat program and applying paired t Test and One-way ANOVA.

3. Results

3.1. Isolation degrading organisms

A total of 32 fungal and bacterial isolates have been recovered from soil by enrichment culture technique. Isolates were isolated on Noble agar containing 50µg ml^{-1} of anthracene. Among the recovered isolates, 3 bacterial isolates: *Pseudomonas aureofaciens*, *Micrococcus varians, Lactobacillus coryniformis*; while 4 fungal isolates: *Fusarium oxyspoum, Fusarium moniliforme, Fusarium chlamydosporum* and *Aspergillus terreus*. The isolates were assessed on their ability to degrade anthracene as a sole carbon source, where the residual substrate was determined by HPLC.

3.2. Biodegradation studies

To determine the degradation of anthracene by fungal isolates in soil samples, 10 g of soil was added to SS with anthracene at a final concentration of 10µg ml^{-1} and inoculated with the different fungal isolates. After incubation in a rotary shaker at 30º C at speed of 150 rpm for 1 week, residual anthracene was evaluated by HPLC. Among the fungal isolates were *Fusarium oxysporum* and *Aspergillus terreus* which have showed a high degradation of anthracene within 7 days. Anthracene was degraded by *Fusarium oxysporum* with an initial concentration of 10µg ml^{-1} where 78 %

of the initial concentration had disappeared after 7 days of inoculation (Figure 1a), while *Aspergillus terreus* was able to degrade 70% of anthracene under the same conditions (Figure 1b).

Among the bacterial isolates were *Pseudomonas aureofaciens* and *Micrococcus varians* which have showed a high degradation of anthracene within 7 days. Anthracene was degraded by *Pseudomonas aureofaciens* with an initial concentration of 10µg ml^{-1} where 70 % of the initial concentration disappeared after 7 days of inoculation (Figure 2a), while *Micrococcus varians* was able to degrade 61% of anthracene under the same conditions (Figure 2b).

3.3. Immobilization:

Biodegradation of anthracene was carried out with free cells as well as immobilized cells on charcoal. The results obtained showed that there was a significant degradation of anthracene by all tested isolates on immobilized cells as compared to that of free cells within 4 days of incubation (Table 1) .

The degradation of anthracene was higher among fungal isolates than bacterial isolates, where cells of immobilized *Fusarium oxysporum* was able to degrade 83% of anthracene at a concentration of 10µg ml^{-1}, compared to free cell within 4 days of incubation 51% (Figure 3).

3.4. Degradation of anthracene in presence of surfactants

To investigate the effect of surfactants on enhancement of biodegradation of anthracene, non-ionic surfactant Tween 80 was used at a concentration above the CMC. Uppon addition of 2% of Tween 80 as a surfactant, degradation of anthracene was enhanced, where *Fusarium oxysporum* was able enhance significantly anthracene biodegradation compared to the control (without surfactant) (Figure 4). A significant difference of anthracene degradation between isolates in absence and presence of Tween 80 is shown in Table 2.

Table 1: Degradation of anthracene by immobilized and free cells of fungal and bacterial isolates

Name of degrading organism	% of anthracene degradation		
	free cells	immobilized cells	Significance (t-test)*
Fusarium oxysporum	51	83	S
Fusarium chlamydosporum	40	61	S
Fusarium moniliforme	35	68	S
Aspergillus terreus	35	78	S
Pseudomonas aureofaciens	48	72	S
Micrococcus varians	37	66	S
Lactobacillus coryniformis	41	67	S

*A significant difference between isolates with and without addition of surfactants at P< 0.0001.

Table 2: Degradation of anthracene by fungal and bacterial isolates in the presence and absence of Tween 80

Name of degrading organism	% of anthracene degradation		
	without Tween 80	with Tween 80	Significance (t-test)*
Fusarium oxysporum	78	91	S
Fusarium chlamydosporum	45	56	S
Fusarium moniliforme	60	75	S
Aspergillus terreus	68	90	S
Pseudomonas aureofaciens	70	89	S
Micrococcus varians	61	81	S
Lactobacillus coryniformis	40	60	S

*A significant difference between isolates with and without addition of surfactants at P< 0.0001.

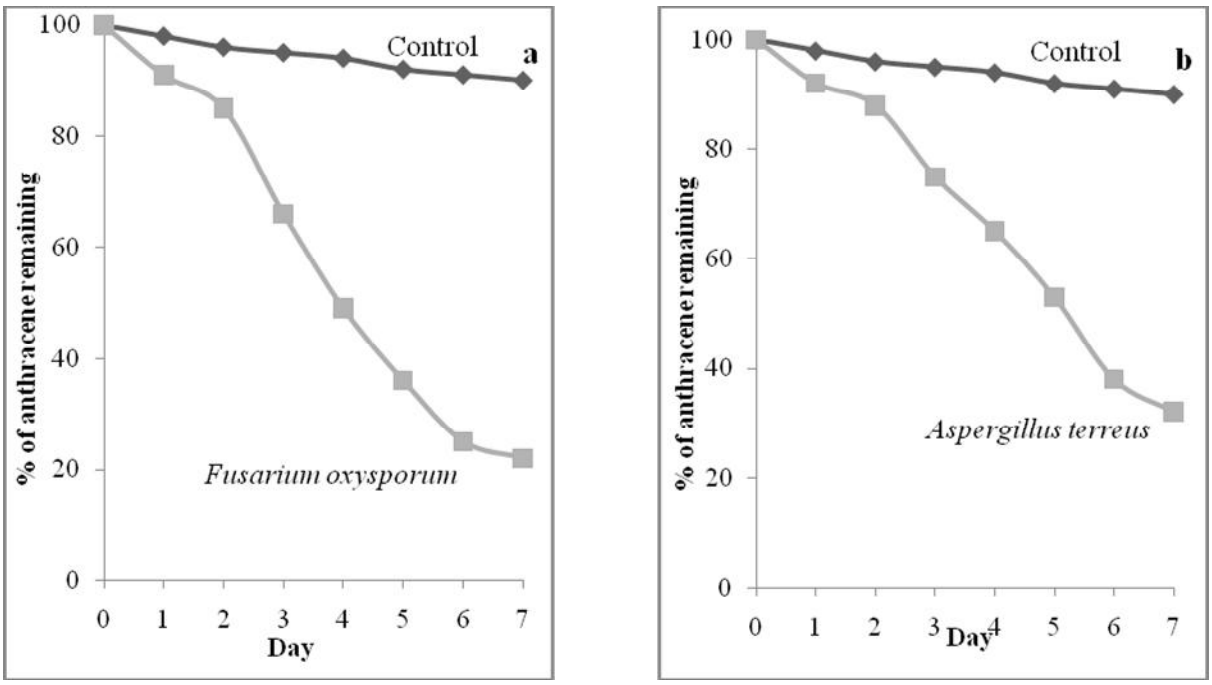

Figure 1: Degradation of 10µg/ml of anthracene by a) *Fusarium oxysporum* and by b) *Aspergillus terreus* in soil samples.

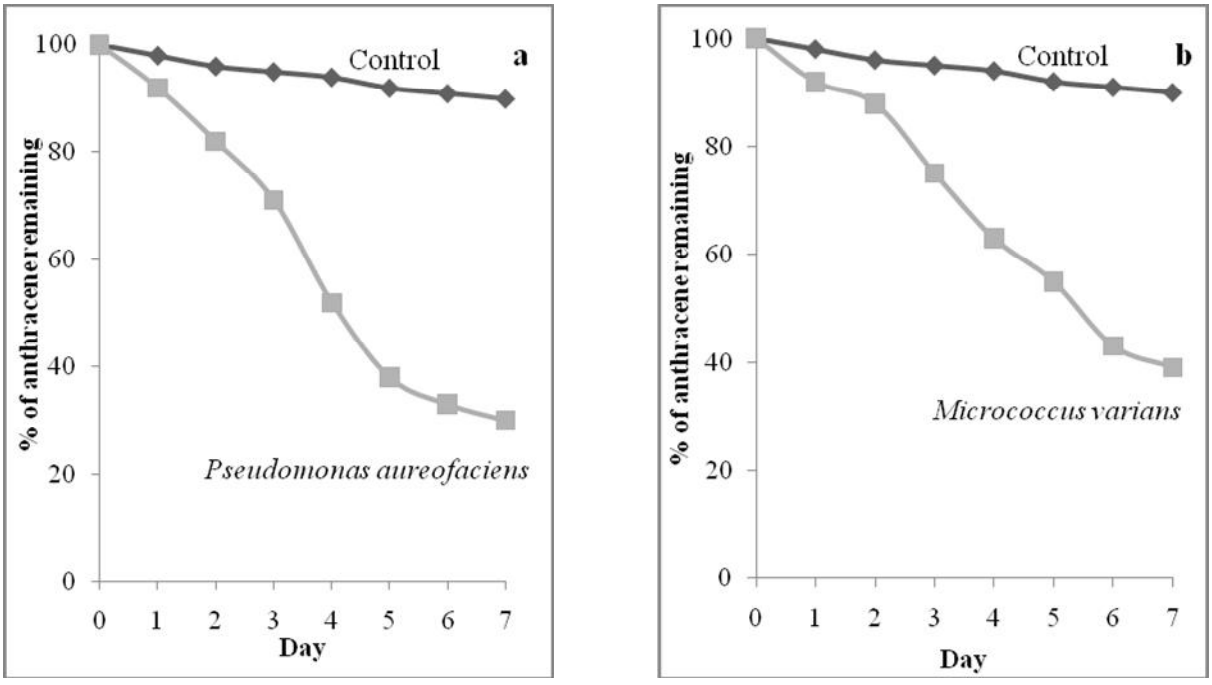

Figure 2: Degradation of 10µg/ml of anthracene by (a) *Pseudomonas aureofaciens* and by (b) *Micrococcus varians* in soil samples.

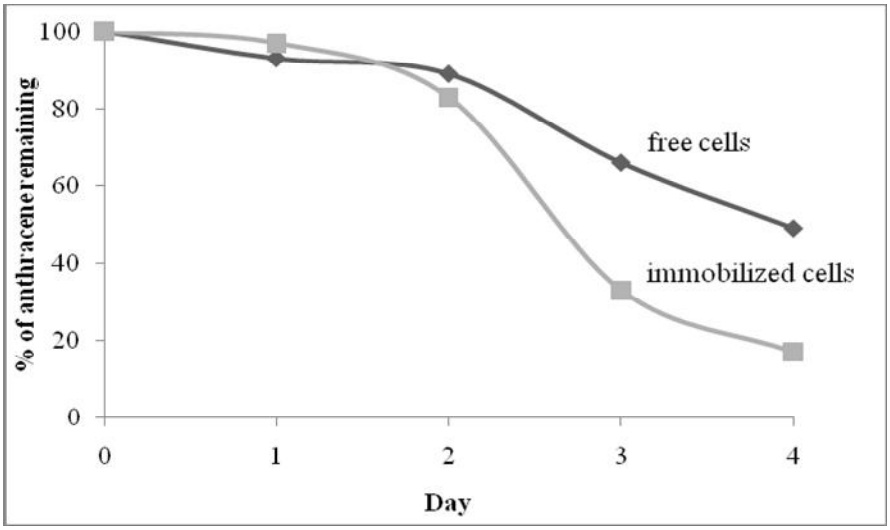

Figure 3: Degradation of 10µg/ml of anthracene by free and immobilized cells of *Fusarium oxysporum* in soil samples.

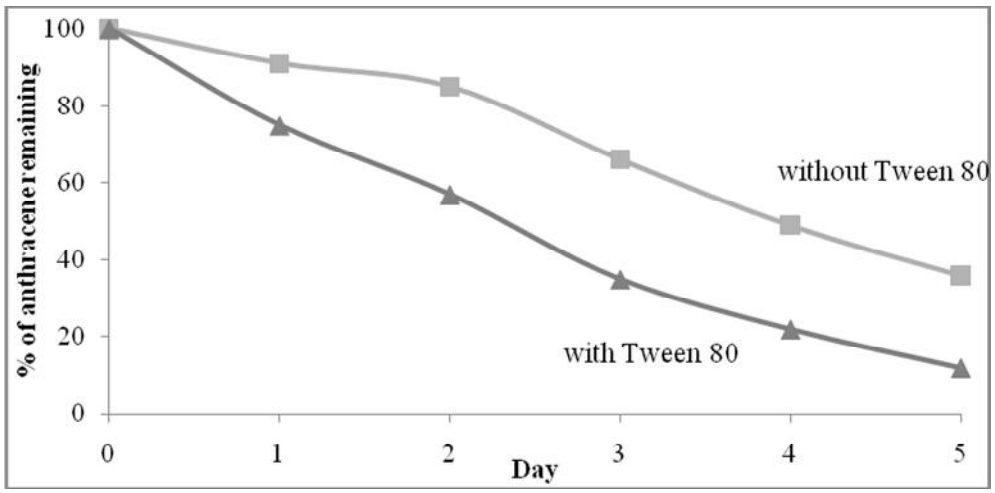

Figure 4: Degradation of 10µg/ml of anthracene by *Fusarium oxysporum* in presence and in absence of Tween 80.

4. Discussion

Polycyclic aromatic hydrocarbons (PAHs) are comprised of two or more fused aromatic rings. They are generally formed by incomplete combustion of fossil fuels and other organic material. They are known to be carcinogenic to humans and other organisms (Kim *et al.,* 2001) and posing serious risks to the environment and human health (Yuan *et al.,* 2000). Anthracene is one among the PAHs and was chosen for the study of PAH degradation because of its relative toxicity, and it is a probable inducer of tumors (Das *et al.,* 2008).

Anthracene as a polycyclic aromatic hydrocarbon has been shown to be hydrophobic due to the absence of polar groups (-OH groups) in their chemical structure (Mulder *et al.,* 2001; Bogan and Sullivan, 2003). As a result of this property, the slow rate of biodegradation was observed in anthracene. The bioremediation of a pollutant and its rate depends on the environmental conditions, number and type of degrading organisms, nature and chemical structure of the chemical compound being degraded. Some microorganisms can utilize PAHs as a source of carbon and energy (Ye *et al.,* 2011). The PAH-degrading microorganisms could be algae, bacteria, and fungi. It involves the breakdown of organic compounds through biotransformation into less complex metabolites (Hartiash and Kaushik, 2009).

The obtained results showed that the extent of anthracene degradation by fungi was more significant than that obtained by bacteria. Fungal isolate *Fusarium oxysporum* was able to degrade 78 % of the initial concentration of anthracene after 7 days of inoculation; while bacterial isolate *Pseudomonas aureofaciens* degraded 70 % under the same conditions. Degradation studies proved that fungal biodegradation is more

feasible than bacterial biodegradation. The enhancement of biodegradation by fungal species might be attributed to fungal mycelium which has a high surface area that can maximize both mechanical and enzymatic contact with insoluble substrates such as anthracene and invade a larger volume of soil (Potin *et al.,* 2004).

It is well known that immobilized cells have high capacity for metabolizing organic compounds and the bioremediation of contamination from numerous toxic chemicals. Therefore, the study was designed to use immobilized cells in anthracene degradation. The observed results showed that showed significant differences between the free cells and the immobilized cells in anthracene degradation. A significant reduction in anthracene concentration by immobilized cells of bacterial and fungal isolates was elucidated in Table 2. Such enhancement might be attributed to the presence of the adsorbents such as charcoal; the number of sites available for adsorption also increases. This results in the removal of anthracene from the liquid phase onto the solid which is the adsorbed phase. The overall effect is an increase in the decay rate of anthracene in the samples with adsorbent. This observation can be related to the result of the experimental study conducted by Owabor and Iyasele, (2006) and Owabor and Ogunsakin (2006). It has been suggested that immobilization provides a certain level of membrane stabilization and increased cell permeability, which is conceivably conductive to the bacteria's degradation capability on account of improved cell protection (Manohar *et al.,* 2001).

Low water solubility and adsorption to soil are two major factors of PAHs that limit their availability to microorganisms. Due to the hydrophobic of PAHs, some biostimulating strategies, including surfactants, were employed to improve the desorption of PAHs (Doong and Lei, 2003) and enhance the solubility of hydrophobic compounds (Boonchan *et al.,* 1998). The degradation of anthracene in presence of 2 % Tween-80 (above CMC) was enhanced, where *Fusarium oxysporum* was able to degrade up to 89% of anthracene, compared to 78% without surfactant. A significant difference of anthracene degradation, both bacterial and fungal isolates was observed in presence and absence e of Tween 80, as shown in Table 1.

Data showed that the addition of surfactants affects positively PAHs biodegradation, increasing the solubility of PAHs and allowing their progressive biodegradation up to concentration levels lower than the initial values. This effect was clearly observed considering the low solubility and biodegradation degree of PAHs when surfactant was not added. The explanation for this phenomenon is that surfactant molecules can accumulate along the air-liquid and liquid-liquid interfaces and thus reduce both surface tensions and interfacial tensions at the same time (Bautista *et al.,* 2009). At surfactant concentrations above the critical micelle concentration (CMC), the solubility of hydrocarbon increases linearly with surfactant concentration. Surfactants are able to improve the mass-transfer of hydrophobic pollutants from a solid or non-aqueous liquid phase into the aqueous phase by decreasing the interfacial tension and by accumulating the hydrophobic compounds in the micelles (Makkar and Rockne, 2003; Li and Chen, 2009).

Conclusion

We can conclude that using immobilized degrading cells in the presence of surfactant might enhance anthracene biodegradation in soil environment.

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