# *Fluorene Removal by Biosurfactants Producing* Bacillus megaterium

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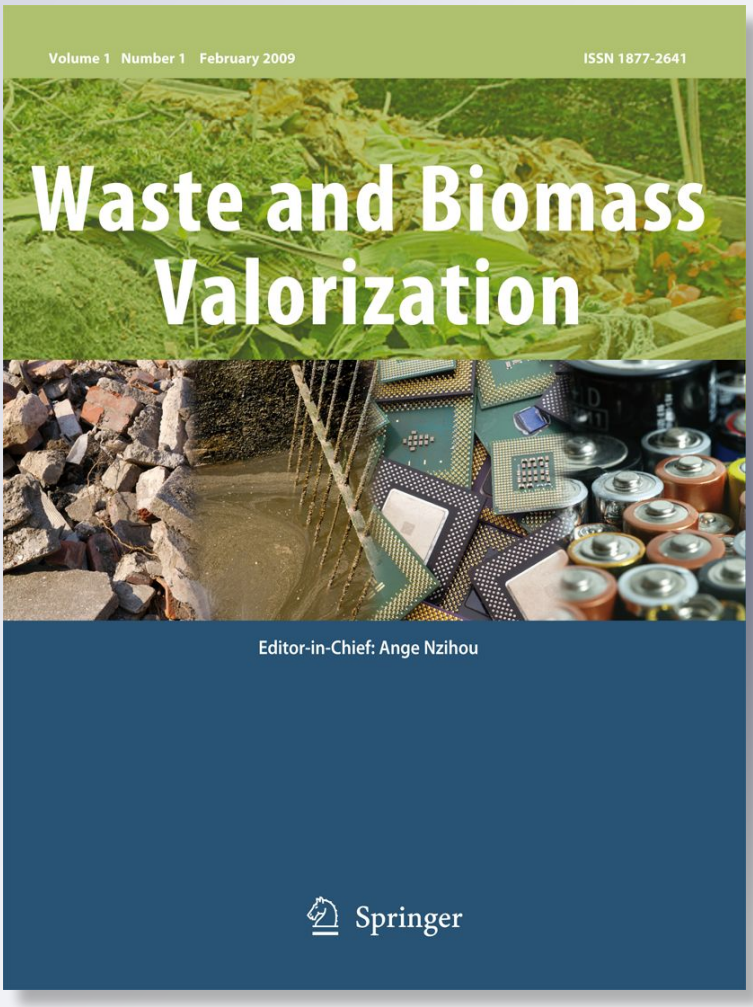


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ORIGINAL PAPER

# Fluorene Removal by Biosurfactants Producing *Bacillus megaterium*

**Nuning Vita Hidayati · Endang Hilmi · Abdul Haris · Hefni Effendi · Michel Guiliano · Pierre Doumenq · Agung Dhamar Syakti**

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**Abstract** This paper describes the study of a surfactant-producing bacterial strain of *Bacillus megaterium*. The study determined the conditions that favor the production of surfactant and how this bacterial strain functions in the biodegradation of fluorene. Crude biosurfactant was produced from *B. megaterium* on mineral salt media (MSM) supplemented with either acetate ammonium (MSM-AA) or crude oil (MSM-CO) as sole carbon source. The *B. megaterium* showed the highest crude biosurfactant yield ($2.99 \pm 0.11$ g $L^{-1}$) when grown on MSM-AA, while a yield of $2.63 \pm 0.04$ g $L^{-1}$ was found on MSM-CO. Biosurfactant activities were observed in both media with a $35.68 \pm 1.05$ and $28.48 \pm 0.39$ mN/m reduction in surface tension when using acetate ammonium and crude oil, respectively. FTIR spectroscopy showed that carbon substrates induce the same glycolipid classes for both MSM-AA and MSM-CO. The results clearly demonstrated that carbon substrates affect biosurfactant production in terms of yield, and that the increase of fluorene removal by approximately 1.5 and 2 compared to the control was due to the presence of the amended crude biosurfactant from MSM-AA and MSM-CO, respectively, after 28 days.

**Keywords** Bioremediation · Persistent · Contaminated sediment · Mangrove rehabilitation · Biotransformation · Indonesia

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## Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are known as potential contaminants of public concern, particularly with regards to health and environmental issues. Several authors have suggested that PAHs may cause harmful effects in various organisms from different species through PAH accumulation in the tissues, resulting in biological consequences such as the introduction of cytochrome P450 enzymes that disrupt DNA, early mortality, edema, disturbance of cardiac functions and deformities [1]. Aside from their potential harmfulness, PAHs are considered as the most persistent pollutants in soil and sediment [2]. Their persistence within ecosystems is explained by their physical and chemical properties, such as vapor pressure, water solubility, dissociation constant, partition coefficient, sorption to soil, and volatility from water and soil/sediment matrices as well as its susceptibility to oxidation, reduction, hydrolysis, photolysis and substitution that give them ubiquity and the ability to accumulate in living organisms and nature [3, 4]. In contrast, the fate of PAHs in water and soil varies from volatilization to adsorption on surfaces and degradation via biotic or abiotic processes [5]. For instance, microbial degradation of many PAH compounds has been demonstrated and is widely accepted as the remedial mechanism for most organic pollutants in the

environment [6]. One obstacle limiting the biodegradation rate of PAHs in the environment is their low solubility, resulting in low bioavailability to the organisms responsible for biodegradation [7, 8]. Fortunately, most hydrocarbon-degrading bacteria are capable of releasing enough biosurfactants to facilitate assimilation of these insoluble substrates [6].

Biosurfactants are microbial amphiphatic molecules that contain hydrophilic (water soluble) and hydrophobic (oil soluble) moieties that reduce the free energy of the system by replacing the higher energy bulk molecules at an interface [9]. Hydrophilic and hydrophobic moieties allow these molecules to increase the surface area of hydrophobic contaminants in soil or water, thereby increasing their aqueous solubility and consequently their microbial degradation [10].

*Bacillus megaterium* has been reported as being capable of breaking down persistent and recalcitrant PAH compounds such as pyrene [9]. The present investigation aims to study the surfactant-producing bacterial strain of *Bacillus megaterium.* The study determined the conditions that favor the production of surfactant by regulating the temperature, pH and salinity and showed how this strain can be applied to the biodegradation of low-solubility hydrocarbons such as fluorene in the presence of biosurfactant.

## Materials and Methods

### Bacteria

The bacterium used in this study, *Bacillus megaterium*, was originally isolated from polluted mangrove sediment collected near a petroleum refinery at Cilacap, Central Java, Indonesia [11].

### Chemicals

Fluorene (97%) was purchased from Sigma (Germany). Acetone, dichloromethane, heptane, methanol (chromasolve grade) and diethyl ether (puriss p.a.) were purchased from Fluka (Germany). GF/F filters (47 mm dia.) were purchased from Whatman (England). Bacto-yeast extract and bacto-peptone (Difco) were provided by BD Biosciences (San Jose, CA, USA).

### Media and Culture Growth Conditions

*Bacillus megaterium* was grown aerobically in a mineral salt medium (MSM) consisting of 14 g $L^{-1}$ of $NaNO_3$, 2 g $L^{-1}$ of $KH_2PO_4$, 4 g $L^{-1}$ of $K_2HPO_4$, 0.2 g $L^{-1}$ of KCl, g $L^{-1}$ of $MgSO_4{\cdot}7H_2O$, 0.02 g $L^{-1}$ of $CaCl_2{\cdot}2H_2O$, 0.024 g $L^{-1}$ of $FeSO_4{\cdot}7H_2O$, 5.0 g $L^{-1}$ of NaCl and 0.5 mL of trace element solution. The trace element solution was composed of 0.26 g $L^{-1}$ of $H_3BO_3$, 0.5 g $L^{-1}$ of $CuSO_4{\cdot}5H_2O$, 0.5 g $L^{-1}$ of $MnSO_4{\cdot}H_2O$, 0.06 g $L^{-1}$ of $MoNaO_4{\cdot}2H_2O$, 0.7 g $L^{-1}$ of $ZnSO_4{\cdot}7H_2O$ [12]. The medium was sterilized by autoclaving at 121 °C for 20 min. Different values for temperature (30 and 37°C), pH (6, 7, and 8), and the salinity of the growth medium (20 and 30 g $L^{-1}$) were tested to establish the optimal culture conditions for producing biosurfactants and conducting the biodegradation experiments. To ensure the experimental culture growth phase (expected in the exponential log phase), a pre-culture was prepared, then a cell suspension was taken to be inoculated. First, an aliquot of 20 mL of inoculums was transferred to an Erlenmeyer flask containing 500 mL of mineral salt medium (to produce the biosurfactant and also to be used later in the biodegradation experimentation), which was left to incubate on a rotary shaker incubator (150 rpm) for 7 days. Second, a control series conditioned at 30°C, pH 6, and salinity of 20 g $L^{-1}$ was prepared to enumerate viable cells using the Agar Plate Count Method and biomass dry weight determination. Concerning biomass, culture samples taken at different times were centrifuged at 8,500 rpm for 20 min at 4°C to remove the bacterial cells. The removed cells were collected and placed in an oven at 105°C for 18 h to obtain the microbial concentration expressed as gram of dry weight per litre.

### Preliminary Test of Biosurfactant-Producing *B. megaterium*

The blood agar test was used to screen a potential of biosurfactant-producing bacteria [13]. Fresh cultures from bacterial isolate were prepared by streaking on marine agar and were incubated at 37°C for 48 h. The fresh single colony of cultures was then re-streaked on blood agar and incubated at 37°C for 48–72 h. The bacterial colonies were then observed to determine the presence of a clear zone of hemolysis around the colonies on the blood agar.

### Production and Extraction of Crude Biosurfactants

*Bacillus megaterium* was grown aerobically in 300 mL of a mineral salts medium (MSM) in one-litre Erlenmeyer flasks at 150 rpm, temperature (37°C), pH (8), sand salinity (30 g $L^{-1}$). The first set in the reactor was inoculated by *B. megaterium* and was supplemented by 2% (v/v) ammonium acetate as a substrate, the resulting biosurfactant crude being labelled "BS-AA", while in the second set, 2% (v/v) crude oil was used as the source of energy and carbon for *B. megaterium* to produce "BS-CO". The size of the inoculums corresponded to 4% (v/v) of an aliquot of pre-culture in the culture medium. The amount of

biosurfactant produced in 7 days was determined by analysing the biosurfactant crude yield in the culture supernatant.

Filtered culture supernatants were obtained by centrifuging 50 mL samples at 11,000 rpm for 20 min at 4°C. Cultural supernatant was acidified using a 6 N HCl solution to obtain a pH of 2.0 and allowed to stand overnight at 4°C to achieve complete precipitation of the biosurfactant. In order to reduce diverse precipitation of bio-components including protein, the filtrate was then extracted using chloroform and methanol (2:1 v/v). The solvents were removed by rotary evaporation and the resulting residue was crude biosurfactant. The weight of the biosurfactant was expressed in terms of milligrams per milliliter (dry weight).

### Surface Tension Measurement

The reduction in surface tension of the culture medium was measured using a Krüss processor tensiometer. Surface tension was measured at room temperature after dipping the platinum ring in the culture supernatant solution long enough to reach equilibrium conditions. The measurement was repeated at least three times and an average value was used to express the surface activity of each sample.

### Fourier Transform Infrared (FTIR) Spectroscopy

Spectra were obtained using a Thermo Electron Nexus spectrometer equipped with a diamond crystal Smart Orbit$^{TM}$ accesssory. Spectra were recorded in attenuated total reflection (ATR) and were corrected by the ATR correction of the OMNIC$^{TM}$ software. All the spectra were acquired between 4,000 and 450 $cm^{-1}$ with 64 accumulations and a spectral resolution of 4 $cm^{-1}$.

### Fluorene Removal

The effect of the crude biosurfactants on fluorene degradation by *B. megaterium* (BM) was determined by growing the bacterium in MSM containing fluorene (50 mg $L^{-1}$) (BM + FLO) with and without the biosurfactants produced by *B. megaterium.* Solubility of fluorene in water was $1.2 \times 10^{-2}$ mmol $L^{-1}$, referring to the work of Pearlman et al. [14]. The next two experimental designs were "BM + FLO" + "BS-AA", referring to the use of biosurfacant produced by BM using ammonium acetate as the sole source of carbon, and "BM + FLO" + "BS-CO", designating the culture using crude oil as the carbon source, as described in the section *Production and extraction of biosurfactants*. Abiotic processes such as adsorption and oxidation phenomena were controlled using dead-cell controls prepared by adding mercury chloride (0.1 M). The entire experiment was conducted at 37°C, pH 8, and 30 g $L^{-1}$ of salinity in a reciprocal shaker (150 rpm) for 28 days of incubation time. Cell growth was determined by total plate count on marine agar. Fluorene stock (100 g $mL^{-1}$) was prepared in acetone to increase its miscibility with water. Fluorene residual concentration after microbial degradation was determined using liquid–liquid extraction with dichloromethane for 24 h and anthracene was added as an internal standard to quantify and correct the losses due to extraction [15]. Prior to chromatographic analysis, solvent extracts were dried over $Na_2SO_4$ and then concentrated using rotary evaporation followed by blow -down under a gentle stream of nitrogen.

Fluorene concentrations in the culture fluids were analysed using a gas chromatograph equipped with a flame ionisation detector. Samples were injected into an HP-5 MS column. The mobile phase was helium (90 kPa). The temperature gradient used in analysis was 50°C for 1 min, increased to 300°C at 8°C/min and lowered from 300 to 50°C at 40°C/min. The injection temperature was 270°C and the detector temperature was 300°C. The injected volume was 3 μL.

### Statistical Analysis

The means and standard deviations of fluorene removal percentages were obtained by analyzing independent triplicates for each time period of the experiment. ANOVA was used to sort out any difference in hydrocarbon biotransformation during the culture time course for significance levels of 5 and 10%. The statistical analysis was performed with Microsoft (Redmond, WA, USA) Excel software. For growth condition optimization analysis, the statistically significant effects of three variables were further analyzed by means of a factorial design for the main effects using Design Expert® Software version 8.0.4.

## Results and Discussion

### Growth Condition Optimization

*Bacillus megaterium* was cultured at different temperatures, pH values, and salt concentrations. During the 7 days of culturing, the bacterial counts increased from $2.36 \pm 0.6 \times 10^6$ to $5.62 \pm 1 \times 10^7$ CFU $mL^{-1}$ and $1.09 \pm 0.2 \times 10^6$ to $1.29 \pm 0.5 \times 10^8$ CFU $mL^{-1}$ at temperatures of 30 and 37°C, respectively. The different pH values resulted in an increase in the number of cells from $4.62 \pm 4.5 \times 10^5$ to $4.6 \pm 1 \times 10^7$ CFU $mL^{-1}$, $6.19 \pm 0.2 \times 10^6$ to $3.50 \pm 0.9 \times 10^8$ CFU $mL^{-1}$ and $5.6 \pm 5.4 \times 10^5$ to $1.29 \pm 1.8 \times 10^8$ CFU $mL^{-1}$ when pH values were 6, 7 and 8, respectively. A different

increase was noted when *B. megaterium* was cultured under two different conditions of salinity (20 and 30 g $L^{-1}$) ranging from $4.57 \pm 0.8 \times 10^5$ to $2.55 \pm 1.1 \times 10^7$ CFU $mL^{-1}$ and $1.09 \pm 0.2 \times 10^6$ to $1.29 \pm 0.5 \times 10^8$ CFU $mL^{-1}$. Relative control bacterial count indicated an arithmetic average of $15 \pm 0.5 \times 10^5$ CFU $mL^{-1}$. The study revealed that temperature and salinity had an equal impact on the growth of *B. megaterium* for 23.4% of total variation (11.7% each). pH accounted for only 7.6% of total variation. The different responses and interactions of all the variables were significant and responsible for 26.7% of the total variation in growth conditions ($p = 0.05$). The highest growth of *B. megaterium* was observed at 37°C, pH 8 and a salinity of 30%, which could constitute the optimum conditions for biosurfactant production.

## Preliminary Test of Biosurfactant-Producing Bacteria

In this study, a blood agar plate test was used to quickly screen the biosurfactant produced by *B. megaterium*. This method had already been used by several authors [12] to achieve the same purpose. Results were recorded based on the type of clear zone observed, i.e. $\alpha$-hemolysis when the colony was surrounded by a greenish zone, $\beta$-hemolysis when the colony was surrounded by a clear white zone and $\gamma$-hemolysis when there was no change in the medium surrounding the colony.

Due to the varying degree of solubility in the carbon sources and the molar proportion of carbon in each substrate, the experimental design was developed based on the concentration of carbon sources (2% v/v), since the amount of carbon empirically available was higher for CO than for AA. The results showed that bacteria on the blood agar plate formed a clear zone around the colony measuring 1.2 and 1.5 cm in diameter when the *B. megaterium* culture was amended by two different carbon sources, i.e. ammonium acetate and crude oil, respectively, while no clear zone was observed on the control. According to [13], the use of the blood agar test can be correlated with haemolytic activity and the clear zone on the blood agar plates may be affected by the concentration of biosurfactants, divalent ions and other hemolysins produced by the microbe.

## Microbial Growth and Biosurfactant Production

In this study, *B. megaterium* growth was investigated using two different carbon sources (ammonium acetate and crude oil). Microbial growth and profiles of the *B. megaterium* biosurfactant produced are shown in Figs. 1 and 2. As indicated, higher cell density was achieved in the medium containing ammonium acetate as compared to crude oil. These findings confirmed that acetate ammonium is a substrate more readily assimilated than crude oil in

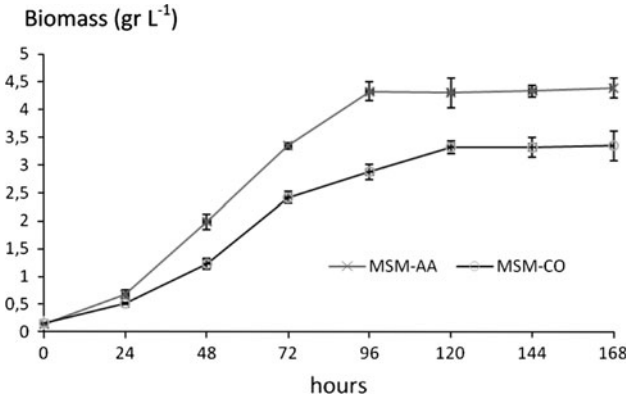


**Fig. 1** Microbial growth after 168 h of incubation with carbon sources consisting of ammonium acetate (MSM-AA) and crude oil (MSM-CO), where n = 3

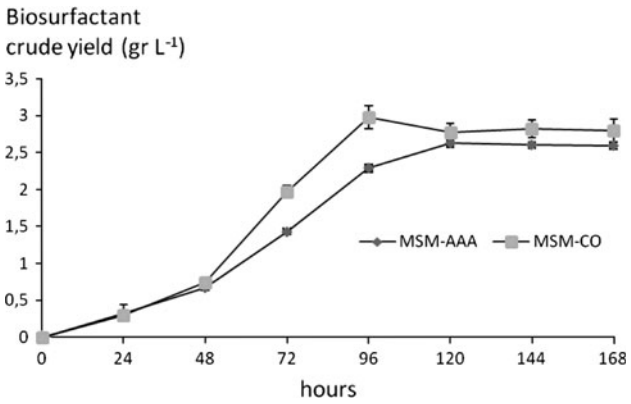


**Fig. 2** Biosurfactant crude production after 168 h of incubation of *B. megaterium* with carbon sources consisting of ammonium acetate (MSM-AA) and crude oil (MSM-CO), where n = 3

supporting cell growth. *B. megaterium* nonetheless displayed a significant ability to grow on the MSM-CO media, although the growth phase was inferior to that on MSM-AA. Up until now little information has been available to demonstrate the ability of *B. megaterium* to use crude oil as a source of carbon and energy.

Figures 1 and 2 can be used to determine both the growth phase and the crude biosurfactant production curve as a benchmark in order to estimate the harvest time of the crude biosurfactant yielded. In the crude oil substrate, *B. megaterium* began to produce biosurfactants after 24 h of growth and reached its maximum of $2.62 \pm 0.04$ g $L^{-1}$ at day 5 of the incubation period, while in the ammonium acetate substrate, *B. megaterium* reached a maximum production of $2.98 \pm 0.11$ g $L^{-1}$ after 96 h of incubation time (Fig. 2). ANOVA results show that crude biosurfactant production on ammonium acetate was significantly higher ($\alpha = 0.05$) than that obtained on crude oil.

This data is in agreement with several studies reporting that the difference in quantity and quality of the produced

biosurfacants results from the difference in the carbon substrates used. The different carbon sources induced cells to take different metabolic pathways, which in the end yielded biosurfactants displaying different structures [12]. Furthermore, Perfumo et al. [16] reported that microbes produce biosurfactants consisting of a mixture of various isoforms that vary in the carbohydrate and peptide part of the molecule, or in the chain length or branching of the lipid part. For instance, Thavasi et al. [12] described a difference in the amount of biosurfactant produced by *B. megaterium* in crude oil, waste motor lubricant oil and peanut oil cake. Among the three substrates used, biosurfactant production was highest when a peanut oil cake substrate was applied (7.8 g $L^{-1}$). Das et al. [17] investigated whether a marine strain, *B. circulans,* was capable of assimilating various carbon substrates to produce biosurfactants. To summarize their study, of the several carbon substrates tested, the production of crude biosurfactant was found to be greatest when using glycerol (2.9 ± 0.11 g $L^{-1}$), followed by starch (2.5 ± 0.11 g $L^{-1}$), glucose (1.16 ± 0.11 g $L^{-1}$) and sucrose (0.94 ± 0.07 g $L^{-1}$). The study described in this paper dealt with the same substrate as that investigated in the first study mentioned above, the results of both clearly indicating that carbon substrates affect the production of crude biosurfactant in both qualitative and quantitative analyses, the carbon substrate being an important factor that limits crude biosurfactant yield [12].

## Surface Tension

Surface tension of the culture broth decreased by 28.48 ± 0.39 and 35.68 ± 1.05 mN $m^{-1}$ in the crude oil and ammonium acetate substrates, respectively (Figs. 3, 4). This decreasing trend in surface tension might suggest the presence of biosurfactant produced by bacteria. Cameotra and Singh [8] reported that biosurfactants can reduce surface tension and such a reduction would indicate the effectiveness of the biosurfactants. Although the crude oil did not reach the maximum value of crude biosurfactant, the excreted biosurfactants displayed good surface activity in terms of surface tension reduction and the diameter of the clear zone. The effectiveness of a surfactant is determined by its ability to lower the surface tension, which is a measure of the surface free energy per unit area required to bring a molecule from the bulk phase to the surface [18].

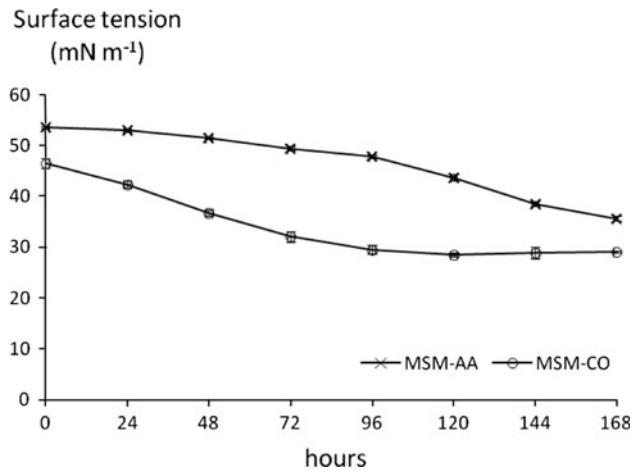


**Fig. 3** Surface tension reduction after 168 h of incubation with carbon sources consisting of ammonium acetate (MSM-AA) and crude oil (MSM-CO). (n = 7)

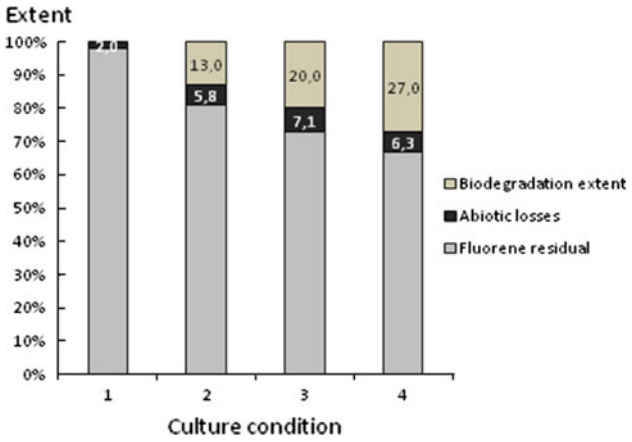


**Fig. 4** Percentage of fluorene biotransformation by *B. megaterium* in the different culture conditions: 1 (control), 2 (BM + FLO), 3 (BM + FLO + BS-AA), and 4 (BM + FLO + BS-CO) after 28-day of culture. (n = 2)

As regards the results, it appears that the crude biosurfactant produced by the crude oil substrate was more efficient than that of the ammonium acetate substrate, since the reduction in surface tension was much lower on the first than on the second. A possible explanation is that the different types of biosurfactant generated by the crude oil and ammonium acetate cultures might affect the ability of biosurfactant to reduce surface tension. Previous reports pointed out that the type of biosurfactant produced depends on the bacterial strain and the carbon source used [13–17]. Biosurfactants synthesized by bacteria may differ in quality and quantity when bacteria are grown on different substrates, thereby resulting in different surface tension values. Moreover, the reduction in surface tension may consequently increase the biological availability of hydrophobic compounds such as hydrocarbon compounds [8].

## Role of Crude Biosurfactants in Fluorene Removal

Degradation of fluorene (50 mg $L^{-1}$) by *B. megaterium* is shown in Fig. 4. The experimental data are presented in terms of arithmetic averages of two replicates. The biodegradation of fluorene was expressed as the percentage of

fluorene degraded in relation to the amount of the remaining compound in the appropriate abiotic control samples. Abiotic losses varied from 2 to 7.1% during the culture time course. After 28 days of incubation, 13, 20 and 27.3% of fluorene was biotransformed in the respective experimental designs, i.e. BM + FLO, BM + FLO + BS-AA and BM + FLO + BS-CO, respectively. These results indicated that the additional crude biosurfactants enhanced the degradation rate of fluorene, especially biosurfactants produced by bacteria amended with crude oil as the sole source of carbon and energy, as shown in the case of BM + FLO + BS-CO, enriched with biosurfactant produced on the crude oil substrate, which achieved the highest degradation rate.

Studies on the use of surfactants in bioremediation processes have demonstrated that biosurfactants can be employed successfully to facilitate PAH degradation and disperse hydrophobic compounds. Enhanced biodegradation is probably due to the increase in cell surface hydrophobicity after biosurfactants have been produced, which subsequently stimulates uptake via direct contact between cells and hydrocarbon droplets [19]. The authors suggest that crude biosurfactant from *B. megaterium* also increases cell hydrophobicity, which in turn affects the extent of fluorene degradation. Biosurfactant can actually influence the ability of bacteria to attach or detach to or from the target substrate [20, 21]. Accordingly, the study postulates that biosurfactants from two different sources of carbon may lead to two different types of biosurfactants, as stated by several authors [12].

Using spectral FTIR, the investigation revealed that the type of biosurfactant produced by *B. megaterium* when supplemented by acetate ammonium was similar to that obtained with crude oil. The infrared spectra (Fig. 5) of the crude biosurfactants showed characteristic bands of $CH_2$ and $CH_3$ groups, probably resulting from long-chain hydrocarbon lipids: C–H stretching bands between 3,000 and 2,800 $cm^{-1}$ ($\nu CH_2$; 2,924 and 2,856 $cm^{-1}$, $CH_3$; 2,965 $cm^{-1}$), C–H in-plane bendings at 1,463 and 1,379 $cm^{-1}$ ($\delta CH_2$, $\delta CH_3$) and $CH_2$ rocking at 720 $cm^{-1}$ (r $CH_2$) also confirmed the presence of alkyl groups. Interesting results can be seen in bands of the methyl groups (2,965 and 1,379 $cm^{-1}$), which were more intense for BS-CO, probably resulting from more ramified hydrocarbon chains. Carbonyl stretching bands ($\nu C{=}O$) appeared at 1,740 and 1,700 $cm^{-1}$ and were characteristic of ester (lipids) and carboxylic acid groups. Bands characteristic of carbohydrate were also observed: 3,421 $cm^{-1}$ (OH stretching, $\nu OH$) and 1,017 $cm^{-1}$ (C–O stretching, $\nu C–O$). In the spectrum region fingerprints were noted between 1,200 and 1,400 $cm^{-1}$, representing C–H and O–H deformation vibrations, characteristic of carbohydrates as one of the moiety glycolipid compounds. Consistent with this finding, neither changes in the intensity of single bands nor an overall change of intensity in the absorbance spectrum were noted for BS-AA and BS-CO. This may suggest that one of the possible forms of the surface-active compounds of *B. megaterium* (BS-AA and BS-CO) is a glycolipid, which could take the form of a rhamnolipid [22, 23]. The results of our findings contradict previous work [24], which

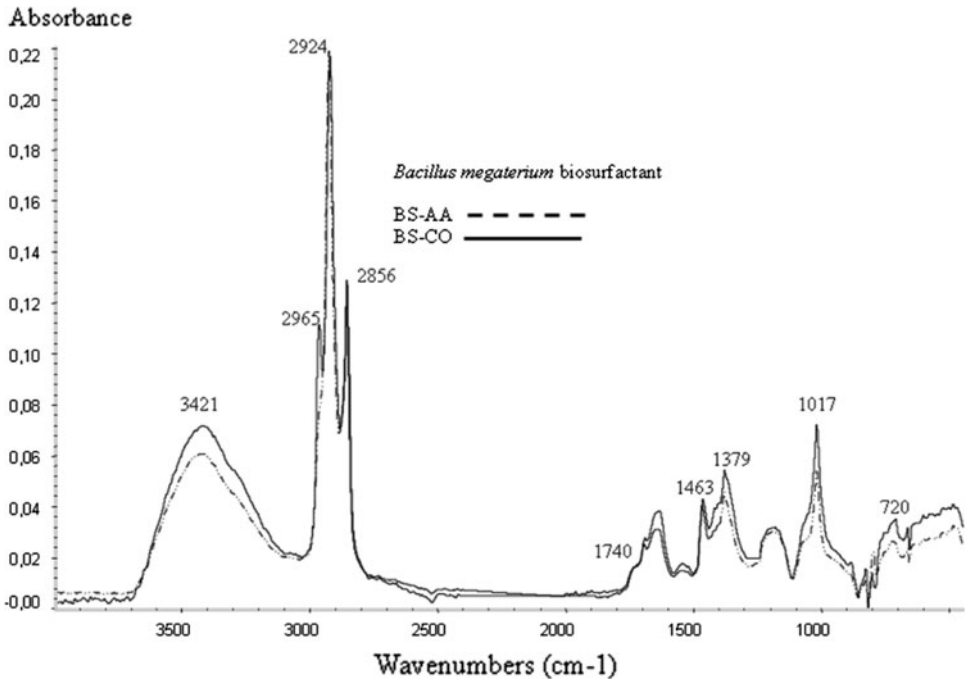


**Fig. 5** Comparison of FTIR for BS-AA and BS-CO extracts of *Bacillus megaterium*

reported that the type of biosurfactants produced depends on the bacterial strain and carbon source used, and may lead to differences in quality and quantity, explaining the differences in surface tension.

Biosurfactants play a critical role in driving this less polar compound to the aqueous phase, where they become readily available to the microorganism for degradation. Biosurfactants enhance solubility of PAHs due to the physical association between the active site of the molecules and the hydrophilic moiety of the aggregated biosurfactants or micelles [21]. The presence of biosurfactants also lowers surface energy, resulting in enhanced solubilization of the hydrocarbons [25]. Fluorene is a hydrophobic compound, as confirmed by its low solubility in the aqueous phase, where it attains 1.995 mg $L^{-1}$ at 20°C [26] or approximately 1.2 $\times$ $10^{-2}$ mmol $L^{-1}$ [14]. If we set this degree of solubility as a reference and compare it with our study, assuming that fluorine biodegradation occurs through a solubilisation mechanism, we can hypothesize that *B. megaterium* biosurfactant can effectively enhance fluorene solubility by approximately 3.2, 5, and 6.8 times in the case of BM + FLO, BM + FLO + BS-AA and BM + FLO + BS-CO, respectively. We therefore suggest that the presence of *B. megaterium* biosurfactant in a growth medium could enhance fluorene removal.

Beal and Betts [27] showed that rhamnolipid biosurfactant increased the solubility of hexadecane from 1.8 to 22.8 mg $L^{-1}$. In a study conducted by Cameotra and Singh [8], adding biosurfactants in a sludge oil bioremediation situation stimulated the bioremediation rate after 8 weeks of incubation. Cubitto et al. [28] also reported that biosurfactants yielded by *Bacillus subtilis* 09 significantly accelerated aliphatic hydrocarbon degradation. A similar effect was reported by Hickey et al. [29] regarding fluoranthene degradation by *Pseudomonas alcaligenes* PA-10. In a different case, Rahman et al. [30] examined bioremediation of *n*-alkanes in petroleum sludge where *n*-$C_8$–*n*-$C_{11}$, *n*-$C_{12}$–$C_{21}$, *n*-$C_{22}$–$C_{31}$ and *n*-$C_{32}$–*n*-$C_{40}$ were degraded 87.4, 80–85, 57–73, and 83–98%, respectively, after 56 days of bacterial incubation supplemented by nutrients and rhamnolipids.

Other research by Garcia-Junco et al. [2] indicated that adding rhamnolipids led *P. aeruginosa* to adhere to the phenanthrene molecule, thereby enhancing its bioavailability and biodegradation. Under aerobic conditions, bacteria can degrade most PAHs featuring less than five rings. It thus appears that interaction between the additional biosurfactant, even in the form of crude, and fluorene biodegradation displays very specific characteristics.

Makkar and Rockne [7] explained the effect of surfactant on the availability of organic compounds through three major mechanisms: dispersion of non-aqueous-phase liquid organics, leading to an increase in the contact area as a result of reduced interfacial tension between the aqueous phase and the non-aqueous phase; increased apparent solubility of the pollutant, due to the presence of micelles containing high concentrations of hydrophobic organic chemicals (HOCs); and enhanced transport of the pollutant from the solid phase, which may be caused by lowering of the surface tension of the soil particle pore water, interaction of the surfactant with solid interfaces, and interaction of the pollutant with single surfactant molecules. Lin and Li-Xi [9] reported that biosurfactant produced on a hydrocarbon substrate can also emulsify different hydrocarbons to a greater extent, confirming its applicability in controlling various types of hydrocarbon pollution. Emulsification enhances the biodegradation of hydrocarbons by increasing their bioavailability to the microbes involved.

## Conclusions

The present work demonstrated fluorene removal by *Bacillus megaterium* in the cultures using biosurfactant crude produced from two different carbon substrates (MSM-AA and MSM-CO). This type of carbon source could affect biosurfactant production in terms of yield (2.99 $\pm$ 0.11 g $L^{-1}$ when grown on MSM-AA and 2.63 $\pm$ 0.04 g $L^{-1}$ on MSM-CO), thus increasing the removal rate of fluorene by a factor of 1.5 and 2 for (BM + FLO + BS-AA) and (BM + FLO + BS-CO), respectively, after 28 days of culture, in comparison with the control (BM + FLO). The biosurfactant showed high physicochemical properties in terms of the surface tension reduction capacity up to 35.68 $\pm$ 1.05 and 28.48 $\pm$ 0.39 mN/m for (BM + FLO + BS-AA) and (BM + FLO + BS-CO), respectively. FTIR spectroscopy showed that carbon substrates induce the same glycolipid classes for both MSM-AA and MSM-CO. The study requires further investigation, however, to elucidate the group of biosurfactants and their structure to achieve a better understanding of how they can be used effectively. In the perspective of a full-scale bioremediation application, the findings in this study point to the massive culture of a single consortium or a combination of selected consortia of microorganisms (bioaugmentation) that evolve predictably to produce biosurfactants. In a zone chronically contaminated by petroleum hydrocarbons, such as the mangrove sediment where *B. megaterium* was isolated for this study, indigenous bacteria was readily adapted using a hydrocarbon substrate. Since certain authors have demonstrated that bioaugmentation may not efficiently remediate PAH-contaminated dredged sediments in slurry-phase bioreactors [31], we suggested using contaminated mangrove sediment as a source of the bioremediation agent "starter", which could potentially produce biosurfactants. These conditions could overcome the high cost of bioremediation

applications in the field based on the use of industrially produced biosurfactants.

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