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Metabolism and removal of anthracene and lead by a *B. subtilis*-produced biosurfactant

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ABSTRACT

Most of effluents discharged to the environment contain toxic contaminants such as aromatic compounds and heavy metals which are considered hazardous to the nature and living organisms. In this study, *Bacillus subtilis* resistant to anthracene and lead was isolated from Persian Gulf sediments. Biosurfactant production was demonstrated using three methods, drop collapse, blood agar and oil spreading. Evaluation of optical density by spectrophotometer showed the bacterial growth in presence of 30 mg/l of anthracene and 50 mg/l of lead. Considerable proportion of anthracene (69.95%) was reduced after 120 h and the maximum percentage of lead absorption (82%) was observed after 150 min. The results indicated that the isolated bacterium was capable of removing anthracene and lead.

1. Introduction

The entrance of huge amounts of contaminants such as oil compounds and heavy metals via the wastewaters of industries to the coastal and aquatic ecosystems have caused serious problems [1]. Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic compounds with two or more benzene rings. The Environmental Protection Agency has introduced these compounds as dangerous pollutants because of their high stability and toxicity [2]. Anthrancene is an aromatic hydrocarbon, with low solubility in the water and high accumulation affinity in organism tissues [3,4]. Allowable limit of anthrancene identified by Canadian Council of Ministers of the Environment [5] for sediment is 46.9 μg/kg.

Heavy metals are also considered as a large group of environmental pollutants. Increases in heavy metal concentration, to levels higher than standard amounts identified by CCME, may lead to adverse effects on aquatics and humans [6,7]. Lead is an unnecessary toxic metal that may cause erythrocyte abnormality and reproductive disorders in aquatics. Damages to the nervous system and gills are considered the chronic and acute effects of lead, respectively [8]. Furthermore, human osteoporosis may resulted from calcium replacement by lead in bones [9,10]. Allowable limit of lead identified by W.H.O. (World Health Organization) [11] for seafood and C.C.M.E. (Canadian Council of Ministers of the Environment) [5] for sediment is 0.5 and 30.2 mg/kg dry weight (dw), respectively.

Using biological techniques for eliminating of heavy metals and aromatic compounds from marine ecosystems could be reasonable. Many studies have been conducted on the biosorbents ability such as fungi, yeasts, algae and bacteria [12–14]. However, bacteria represented more absorption ability due to their small size, high rate of growth and reproduction and active biosorption sites like peptidoglycan [1].

Persian Gulf is one of the main ways of energy and goods transition. Decrease in the distribution and dispersion of contaminants due to the low depth and limited contact of the Persian Gulf with other aqueous ecosystems cause to pollutants to remain in the Persian Gulf for a long time. This research is structured on isolation and identification of an indigenous bacterium and evaluation of its ability in removal of lead and anthracene.

2. Materials and methods

Samples were collected from the surface layer of sediments using grab in the Persian Gulf. Sediment samples were transported to laboratory in sterile glass containers on ice. Mineral salt medium (MSM) (containing 30 mg/l concentration of anthracene) and nutrient agar (containing 100 mg/l of the metal) were used for isolation of anthracene and lead resistant bacteria, respectively [15–17].

2.1. Identification procedures

Morphological characteristics of bacterial colonies were examined

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macro-microscopically. Initial identification of isolates was done using Gram-staining. Moreover, biochemical tests were used such as the potassium hydroxide test (KOH), D-carboxylase test, catalase test, oxidase test, lactose test, Simon citrate test, Voges Proskauer test (VP), Methyl Red test (MR), Triple Sugar Iron Agar test (TSI), Sulfur Indole Motility Media test (SIM), phenylalanine test, urease test and mac-Conkey test were used [18].

2.2. Biosurfactant production

To determine surfactant-producing bacteria, three methods including drop collapse, blood agar and oil spread were applied. Plating cells on blood agar showed the blood hemolysis, production of biosurfactant and a bright halo around the colonies [19–22]. For drop collapse test, 2 µl of mineral oil was placed in each well of micro plates. After an hour, 5 µl of culture medium was added to each well. The potential of surfactant production was determined based on distribution of oil drops on mineral oil surface [23]. Distilled water (50 ml) was poured in the plate and 20 µl of crude oil was added to the plate. Then 10 µl of the culture medium was added on the surface of crude oil. The surfactant production was identified based on the diameter of produced halo [19].

2.3. The measurement of bacterial growth in the presence of anthracene

MSM with 30 mg/l of anthracene and 3 ml of bacterial suspension was incubated at 30 °C [24]. The optical density was measured in 24 h intervals for 5 days using spectrophotometer (UV/Vis 2100, UNICO company, USA) [16]. The optical density of the bacterial suspension increase with the increase in anthracene consumption by the bacteria and bacterial growth.

2.4. The measurement of bacterial growth in the presence of lead

Bacterial suspension (1 ml) was added to 20 ml of lysogeny broth (LB) containing 50 mg/l of lead concentration. Control samples (free of metal) were also considered. All samples were incubated at 30 °C with shaking [25].

Blank solution (LB broth) (3 ml) was applied for calibration of spectrophotometer. Then 0.6 ml of the solutions containing different concentrations of lead was diluted with 2.4 ml of LB broth. Bacterial growth was determined at 600 nm wave length for 5 days in triplicates [25].

2.5. Anthracene degradation

The bacterial suspension (500 µl) was inoculated with 100 ml of MSM medium (containing 30 mg/l of anthracene. The flasks then were agitated on the shaker at 150 rpm for 5 days [26]. The remained amount of anthracene in the culture medium was monitored with High-Performance Liquid Chromatography (HPLC) (K2000 model, Knauer Co) according to MOOPAM (1999) method. Briefly, the samples were performed by adding 250 ml hexan/dichloromethane for 8 h and 20 ml potassium hydroxide (2 M) for two additional hours. The normal hexane then was added to the mixture. The organic phase (extract) was filtrated through glass wool. Next, it was dried with anhydrous sodium sulfate and was concentrated to about 1 ml using a rotary evaporator (Heidolph 2, Germany). For anthracene measuring, the extract was loaded onto a silica/alumina column. Finally two fractions (F1: aquatic phase and F2: organic phase) were separated. F2 separately was concentrated to about 1–1.5 ml under a moderate nitrogen flow. Then, samples were dried under a pure nitrogen flow and 20 ml acetonitrile was added to concentrate F2. Finally, F2 was injected to the HPLC.

2.6. Lead biosorption experiment

Metal solutions amended with 50 mg/l of lead were prepared in 250 ml erlenmeyer flasks and pH was adjusted on 6 using HNO_3 and NaOH. Then one ml of bacterial suspension was inoculated and the flasks were put on shaker incubator at 160 rpm. 5 ml of metal solutions was taken in 30 min time intervals and centrifuged at 4000 rpm for 10 min [27,28]. Finally the evaluation of metal absorbance was performed by atomic absorption spectrophotometer (Savanta AAΣ model) for 150 min. The potential of isolated bacterium in removal of lead was determined by reduction of remained lead concentration in flasks from the initial metal concentration.

2.7. Statistical analysis

Parametric (One Way ANOVA, Tukey post-hoc) and Non-parametric (Kruskal–Wallis and Mann–Whitney U) tests were performed to determine the significant differences between groups. Statistical analysis was performed using SPSS16.00. Significance level was set at 0.05.

3. Results

The isolated bacterium which resisted to both lead and anthracene was Gram-positive and belong to *Bacillus* sp. Table 1 shows the results of biochemical tests used for bacterium identification.

The ability of *Bacillus subtilis* to produce biosurfactant was confirmed using three techniques. The results were presented in Table 2.

B. subtilis started its growth on the first day of incubation with 30 mg/l of anthracene. The optical density reached 0.51 after 8 days. However, the bacterial growth in the control sample was 0.2.

The bacterium in presence of 50 mg/l lead grew more quickly and the maximum optical density was observed 72 h after the inoculation. There was no significant difference in optical density between the control sample and lead solution.

According to Fig. 1, reduction of the anthracene concentration in a solution inoculated with *B. subtilis* was similar to that in the control sample at the first day. After 24 h, the biological removal of anthracene was quickly continued up to 48 h later. Anthracene degradation reached to 9.015 ± 1.393 mg/l after 120 h of incubation.

The isolated bacterium started to absorb lead at the first moments of measurement and most of the metal was removed from solution in the first 30 min. Bacterial absorption of lead at a concentration of 50 mg/l was observed until the last minutes and the metal decreased to 9 ± 0.3 mg/l. The maximum percentage of lead removal was

Table 1
Biochemical features of the bacterium.

Bacillus subtilis (Rods-Gram-positive)	
LD	–
SIM	+
TSI	+
MR	–
VP	–
KoH	–
catalase	+
oxidase	+
PD	–
Urease	–
Citrate	+
Lactose	–
MC	–

Lysine Decarboxylase test (LD), Sulphide Indole Motility medium (SIM), Triple Sugar Iron test (TSI), Methyl Red test (MR), Voges-Proskauer Test (VP), Potassium hydroxide test (KOH), Phenylalanine Deaminase test (PD), MacConkey test (MC).

Table 2
The ability of *Bacillus subtilis* to produce biosurfactant.

Blood agar	+ +
Drop collapse	+
Oil spreading	1.8 cm

+ Incomplete hemolysis.
+ + complete hemolysis with diameter < 1cm.

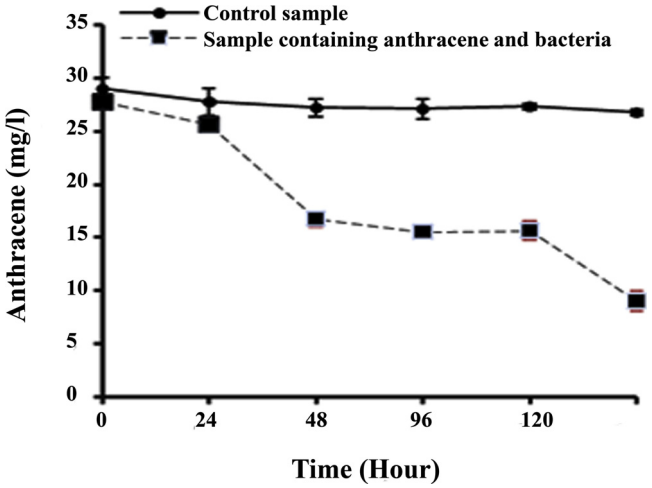

Fig. 1. Biodegradation of anthracenen by *B. subtilis* at a concentration of 30 mg/l.

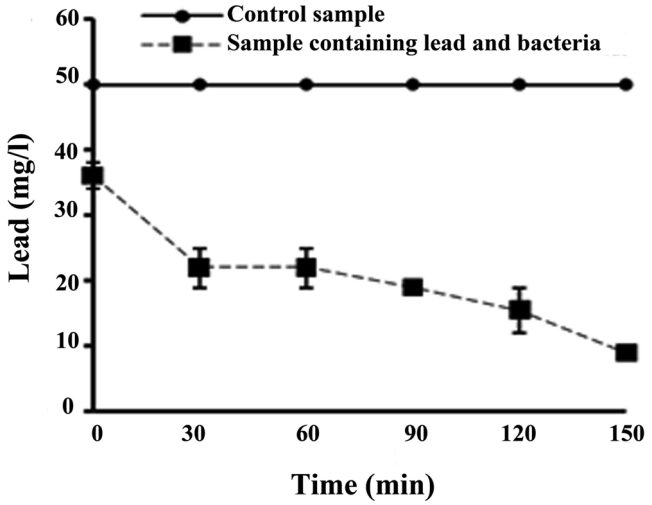

Fig. 2. Biosorption of lead by *B. subtilis* at a concentration of 50 mg/l.

calculated as 82% (Fig. 2).

4. Discussion

In the last decades, the possibility of using microorganisms such as fungi, alga, yeast and bacteria to eliminate pollutant from aquatic environments has been subjected in many researches [29–31]. In the present study, *B. subtilis* was identified as a bacterium resistant to anthracene and lead. The *Bacillus* genus has been isolated and purified from the soil, water and sediment contaminated with different pollutants such as oil compounds and heavy metals by several researchers [1,32]. Obuekwe et al. [33], presented *Bacillus* as one of the major degraders in the desert of Kuwait due to its high ability to clean the oil compounds using its different degrading enzymes. Four bacterial genus including *Bacillus*, *Pseudomonas*, *Alcaligenes* and *Vibrio,* with ability of crude oil degrading, were isolated by Zahed et al. [34] from the water samples collected from coastal areas of the north west of Malaysia. *Bacillus* genus was introduced by several researchers as the bacteria resistant to different heavy metals such as lead [35,36].

The results also showed that *B. subtilis* could grow in a medium containing 30 mg/l of anthracene after adaptation to culture conditions. This was in agreement with other studies which have also represented that some native bacteria such as *B. subtilis* from the tidal areas were able to grow on anthracene [37,38]. In the present study the growth of *B. subtilis* in lead solutions was entered to log phase without any delay and reached to maximum optical density after 72 h. Kim et al. [27] studied the growth of *Bacillus* spp CPB4 in solutions with 50, 100, 200 and 400 mg/l of lead. These researchers reported that this bacterium grow rapidly in a solution with 50 mg/l of lead and the maximum OD achieved after 62 h of the inoculation. This result was in agreement with those obtained in the present study.

In this study, the biosurfactant production ability of different bacteria was assessed using three techniques and *B. subtilis* was introduced as a biosurfactant producer. Das et al. [39] found that *Bacillus* sp. could produce biosurfactant in solution with anthracene as a sole source of carbon. Actually, in the aqueous medium coated by the oil (organic phase), the bacteria caught inner the oil droplets and therefore, it could not obtain the oxygen required for metabolism. But the surfactant producer bacteria could release the biosurfactant that in turn emulsify the oil compounds [40]. Additionally, various carbon resources have a significant influence on the biosurfactant production. They also can effect on the bacterial activity and may inhabit the bacterial growth and surfactant production. Then, the increase in the bacterial ability to produce surfactant strongly depends on the medium nutrients and the growth rate of the microorganism [41].

The present study demonstrated the *B. subtilis* ability to use anthracene as a sole source of carbon and degrade 69.95% of that in 120 h. The ability of *B. subtilis* to decompose different hydrocarbons such as pyrene, crude oil and phenanthrene has been also presented in previous studies [42–44]. This high capacity of the bacterium may be due to the species diversity and its spores in the environment which guarded the bacteria from severe environmental conditions. Anthracene and other organic matters are considered as a nutritional source for bacteria. Specific sensors activated in the bacteria in medium containing hydrocarbons, enabled them to bind to these organic compounds and emulsify and transport them into their body [45]. Generally, biodegradation of hydrocarbon compounds closely relate to production of biosurfactant by the organism. Several researchers have indicated the increase in degradation ability of different organisms in presence of biosurfactant [46–50].

According to previous studies, the Bacillus genus has a considerable capability in the heavy metals removal [27,36,51]. This genus include Gram-positive, aerobic, spore producers bacteria with the ability of heavy metal absorption because of the special binding sites in their cell wall (such as Teichoic and Teichronic acids) [52]. In the present study, the isolated bacterium from the sediment samples was able to eliminate 82% of lead concentration in 2.5 h. The maximum absorption of lead was occurred after 30 min of the inoculation. Tunali et al. [35] also reported the fast adsorption of copper and lead ions by Bacillus occurred in the first 15 and 30 min of measurement.

5. Conclusion

In general, various species of Bacillus have been isolated and reported by several investigators possibly due to its high resistance to wide range of oil hydrocarbons and heavy metals. Based on the mentioned characteristics of *B. subtilis* in this paper, the authors offer the use of this species in oil and heavy metal pollution removal in aquatic ecosystems such as the Persian Gulf.

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