Original Article

APPLIED FOOD BIOTECHNOLOGY, 2016, 3 (2): 83-89
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Isolation and Identification of Crude Oil Degrading and Biosurfactant Producing Bacteria from the Oil-Contaminated Soils of Gachsaran

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Abstract

Background and Objectives: Petroleum hydrocarbons are harmful to the environment, human health, and all other living creatures. Oil and its byproducts in contact with water block sunshine to phytoplanktons and thus break the food chain and damage the marine food source. This study aims to isolate the crude oil degrading and biosurfactant producing bacteria from the oil contaminated soils of Gachsaran, Iran.

Materials and Methods: Isolation was performed in peptone-water medium with yeast extract. Oil displacement area, emulsification index and bacterial phylogeny using 16S rRNA analysis were studied.

Results and Conclusion: Three isolates were able to degrade the crude oil. In the first day, there were two phases in the medium; after a few days, these three bacteria degraded the crude oil until there was only one phase left in the medium. One strain was selected as a superior strain by homogenizing until the medium became clear and transparent. This method confirmed that the strain produces biosurfactant. According to the morphological and biochemical tests, the strain isolated from the oil contaminated soils is a member of *Bacillus subtilis*, so to study the bacterial phylogeny and taxonomy of the strain, an analysis of 16S rRNA was carried out, and the phylogenic tree confirmed them. The results verified that oil contaminated soils are good source for isolation of the biosurfactant producing bacteria.

Article Information

Article history
Received 17 Oct 2015
Revised 10 Feb 2016
Accepted 24 Feb 2016

Keywords
Bacillus,
Bioremediation,
Biosurfactant,
Crude oil,
Gachsaran

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1. Introduction

Release of petroleum hydrocarbons to natural environments causes contamination and toxicity problems [1]. The embryo toxicity of hydrocarbons has been reported so that women exposed to high benzene levels experience menstrual abnormalities, decrease in ovary size and potential fertility reduction [2]. After breaking the food chain because of forming a thin layer of petroleum hydrocarbons on the surface of water, this theory was confirmed by observing the impact of hydrocarbons' waste on phytoplankton communities [2]. Toxic hydrocarbons may indirectly contaminate food supplies. In areas far from urban or industrial activities, high level of poly aromatic hydrocarbons found in unprocessed foods reveals the background contamination. In the neighborhood of industrial areas or along the highways, the contamination of vegetation can be ten-fold higher than in rural areas. The waxy surface of vegetable and fruits can accumulate low molecular mass of poly aromatic hydrocarbons mainly through surface adsorption. The adsorbed poly aromatic hydrocarbons finally finish in fresh water or marine sediments. The method of smoking and drying and cooking of food is commonly thought to be the main source of contamination by poly aromatic hydrocarbons [3]. Soil pollution by petroleum hydrocarbons brings up critical issues regarding the world-wide environmental and health concerns. This leads to further attention with

respect to investigation of innovative and environmental compatible technologies for its remediation [4]. Washing with acid or synthetic surfactant adversely affects the chemical and physical structure of soils, thus limiting its reuse. The ultimate goal of any remediation process must not only be to remove the contaminant from the polluted soil but also to refurbish the soil health so that it restores its potential to support the natural flora and fauna [5]. The primary mechanism for elimination of hydrocarbons from contaminated sites is biodegradation by the natural populations of microorganisms [6].

The enhancement of natural biological degradation processes in what is termed "bioremediation" can be a preferred cost-effective method of removing contaminant hydrocarbons from oil-contaminated environments [1]. Bioremediation, which is the use of microorganisms consortia or microbial processes to degrade and detoxify environmental contaminants, is also amongst these new technologies, which derives its scientific justification from the emerging concept of Green Chemistry and Green Engineering [7]. The success of bioremediation of oil spill not only depends on the ability of the strains but also on the physical, chemical and biological conditions of the contaminated environment [8].

To maximize the process in bioremediation technologies, two main approaches have been explored: biostimulation, in which nutrients are added to stimulate the intrinsic hydrocarbon degraders, and bioaugmentation, in which microbial strains with specific degrading abilities are added to work cooperatively with normal indigenous soil microorganism. Petroleum and petroleum products are complex mixtures consisting of thousands of compounds. The capability of hydrocarbons to microbial attack is ranked in the following order: n-alkanes>isoalkanes>low molecular weight aromatics>naphthenes. It is generally accepted that a single microorganism is not capable of degrading all compounds from such mixtures [6]. Factors influencing bioremediation are: pH, temperature, metals, toxic compounds, water content, geological characters, nutrient availability, external electron availability, bioavailability of pollutants, co-metabolism, gene expression, and bioaugmentation [7].

Many species of aerobic bacteria, microscopic fungi and yeasts can utilize hydrocarbons of crude oil and derived products. However, within the last decades, it has been established that anaerobic microbes such as sulfide reducing bacteria and denitrifying bacteria are also capable of growing on alkanes as a unique organic substrate [9]. A large number of bacteria that use poly aromatic hydrocarbons have been isolated; some of them belonged to genera Bacillus [10]. In addition, bacillus species are spore forming microorganisms that are characterized for probiotic properties [11]. For example, *Bacillus subtilis* is isolated from the soil of Iran and has been used in probiotic formulations [12,13] So, due to producing biosurfactant, it could be added as a starter culture in fermented products for detoxification of food sources contaminated with poly aromatic hydrocarbons**.**

Bioremediation is effective and environmental friendly but it often takes time and is not cost-effective on treating large volumes of polluted materials. The major difficulty in bioremediation of oil-contaminated soil is the bioavailability or mass transfer limitation of the oil pollutants in the soil, causing poor food-microorganism contact and thus poor biodegradation efficiency. Petroleum hydrocarbons are highly hydrophobic with low water solubility that attach to soil particles, reducing the bioavailability of oil compounds to microorganisms, and thereby limiting the rate of mass transfer for biodegradation.

The key process to enhance the bioavailability of oil contaminants is to transport the pollutant to the aqueous bulk phase. One of the effective ways to increase the bioavailability (or solubility) of petroleum hydrocarbon pollutants in soil is using surfactants to enhance desorption and solubilization of petroleum hydrocarbons, thus facilitating their assimilation by microorganisms. When compared with synthetic surfactants, biosurfactants, in general, exhibit greater environmental compatibility, better surface activity, lower toxicity, and higher biodegradability. Therefore, biosurfactants seem to be better candidates for the use in bioremediation of contaminated soil and subsurface environments. In addition, biosurfactants could easily be produced from renewable resources via microbial fermentation, making it an additional advantage over chemically synthetic surfactant [4]. Biosurfactants are produced by a number of microbes growing in petroleum hydrocarbons [14]. They have also been useful in oil washing for secondary oil production and to clean oil pipes and oil reservoirs. The use of synthetic surfactants in soil bioremediation can create problems because of their low biodegradability and toxicity; in addition, they could accumulate in ground waters [1].

Biosurfactants are surface-active amphipathic molecules, and have a wide structural diversity, ranging from glycolipids, lipopeptides and lipoproteins to fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants. These molecules retain their activity at extremes of temperatures, pH and salinity conditions [15]. To decrease the utilization of synthetic surfactants in the food industry, the utilization of biosurfactants has become of a great interest taking into account their low toxicity, biodegradability, and potential to be produced from economic and natural sources [16-17]. Some biosurfactant producing microorganisms such as yeasts can produce biosurfactants in the presence of different carbon source; this changes the structure and properties of the produced biosurfactant, which can be appropriate in particular applications. The great advantage of using yeasts is the "Generally Regarded as Safe" status, which allows the using of byproducts from these microorganisms in the food and pharmaceutical industies [18]. Most of the biosur-

factants represent physiochemical properties and characteristics such detergency, emulsification, de-emulsification, foaming and wetting. They are able to reduce superficial and interficial tension between solids, liquids and gases [19]. Different emulsifiers could be used to improve bread volume such as a biosurfactant of *B. subtilis*. The addition of this emulsifier increases significantly specific volume, and consequently, decreases density.

This specified class of biological products enables the emulsifiers to concentrate at the oil-water interphase and thus contribute to the increased stability of a thermodynamically unstable system [17]. The aim of this study is isolation of biosurfactant producing and crude oil degrading bacteria from the oil contaminated soils of Gachsaran as well as the study of biosurfactant activity by qualitative screening methods.

2. Materials and Methods

2.1. Sampling

Oil contaminated soils were collected from the surrounding areas of Gachsaran's oil fields (well no. 5, Binak, belonging to the "Oil and Gas Production Company of Gachsaran", Iran). The samples were poured into separate sterile plastic containers, and transported aseptically to our laboratory, and stored at 4˚C. There were two oil-polluted soil samples and each of them was coupled with the crude oil from the same area (sample A, pH=8.43, and sample B, pH=7.87).

2.1.1. Preparation of culture medium

Culture medium 1 was made up of 0.5% peptone water (Merck), 0.3% yeast extract (Merck) and 1.5% agar-agar (Merck) in distilled water.
Culture medium 2 was made up of 0.5% peptone water and 0.3% yeast extract in distilled water.
Culture medium 3 was made up of 0.5% peptone water, 0.3% yeast extract and 0.5% urea (pharmaceutical) in distilled water. The pH of the media was adjusted to 6.8 at 25˚C using 1N NaOH.

2.2. Isolation and identification of crude oil-degrading and biosurfactant-producing bacteria

2.2.1. Isolation

One gram of each sample was added to 99 ml of sterile distilled water in a 250 ml flask. Then tween 80 (the final concentration was 0.01%) was added to the flask and the pH was adjusted using 1N NaOH. The flasks were incubated at 30˚C on a shaker incubator (TAIYEC) at 150 rpm for 12-24 hours. The suspensions were centrifuged. The supernatants were serially diluted from 10^{-1} to 10^{-5} in distilled water, and 100 µl of each dilution was spread onto the sterile culture medium 1. All the plates were incubated at 37˚C for 24 hours. Two types of colonies of sample A and two types of colonies of sample B were observed on the plates, and pure cultures of each morphologically distinct colony were obtained by repetitive streaking onto the culture medium 1.

2.2.2. Study of decomposition of crude oil by isolated strains

Each isolate was inoculated to the culture medium 2. After incubation at 30˚C with the shaking rate of 150 rpm for 24 hours, all the isolates were centrifuged at 10000 ×g at 4˚C. The supernatants were eliminated and the sediments were washed by saline (0.9% NaCl) three times. Then1.5×10^{8} $CFUml^{-1}$ of the isolates were inoculated to 100 ml of the culture medium 3 in a 250 ml flask (pH A=8.43 and pH B=7.87) with 1 $\%vv^{-1}$ crude oil (A crude oil for the isolates of samples A, and B crude oil for the isolates of samples B) as the sole source of carbon and energy. These flasks were incubated at 30˚C on the shaker incubator at 150 rpm for a week. The flasks were monitored on a daily basis (through observation homogenizing and becoming clear of the culture medium and crude oil layer).

2.2.3. Biosurfactant producing test

a. Oil displacement activity

In this technique, 50 ml of distilled water was put in a Petri dish, and 20 µl of crude oil was added to it, forming a thin layer on the surface. By adding 10 µl of bacterial suspension containing biosurfactant, there formed be a clear area in the oil layer, started to expand by increasing the biosurfactant concentration. The oil displacement area was measured according to the area of clear zone in the shape of a circle [20].

b. Emulsification index (E_{24})

In this study, crude oil was added to the cell free supernatant in a ratio of 1:1 and vortexed vigorously for two minutes. After 24 hours of incubation at room temperature, the height of the emulsified layer was measured. The emulsification index (E_{24}) was estimated as:

$$E_{24}\ (\%) = (H_{EL}/H_S) \times 100$$

Where, E_{24} is the emulsification activity after 24 hours, H_{EL} is the height of the emulsified layer, and H_S is the height of the total liquid column [21].
On the basis of the 2 and 2.3, the best crude oil-degrading and biosurfactant-producing bacteria were selected, and the result of sections 2 was named ZH1, and maintained on the nutrient agar slants at 4˚C.

2.2.4 Biochemical tests

Gram staining, cell shape, spore forming ability, motility, growth in 1-7% NaCl, colony color and growth in aerobic and anaerobic conditions were studied, and some biochemical tests such as catalase, citrate, nitrate, starch hydrolysis and CHO fermentation were done.

2.2.5. DNA isolation for 16S rRNA analysis and sequencing

DNA of the ZH1 was extracted by High Pure PCR Template Preparation Kit- 11 796 828 001- Roche. PCR was also carried out by the kit CinnaGen PCR

MAster Mix. 2X Cat No. PR8252C. The amounts of PCR is as follows: Master Mix 25 µl, Forward Primer (27F) 1 µl, Reverse Primer (1510R) 1 µl, Template DNA 1-2 µl, Sterile Deionized Water 50 µl. PCR program are as follows: 1; 110ºC, (55s), 72ºC (1 min), 4; 72ºC (10 min), 5; store 8ºC. BLAST-n analysis was carried out at the National Center for Biotechnology Information (NCBI) server. Phylogenetic relationships were studied based on the similarity of the 16s rRNA sequence of ZH1 to the reference strains using Neighbor-joining method and Geneious 8 software.

2.2.6. Study of the beet molasses for producing biosurfactant

Finally, the identified isolate was inoculated to the culture medium 2 with 1% crude oil (A), and after incubation at 30˚C and 150 rpm for 24 hours, the isolate was centrifuged at 10000 ×g at 4˚C. The supernatants were eliminated and the sediments were washed by saline (0.9% NaCl) three times. Then 1.5×10^{8} CFUml^{-1} of the isolate were inoculated to 100 ml of the culture medium 3 in two 250 ml-flasks (pH =8.43) (each flask containing only one carbon source) with 1% vv^{-1} crude oil (A) and 1% vv^{-1} beet molasses (Urmia factory). The flasks were then incubated at 30˚C and 150 rpm. After two days of incubation, oil displacement area and E_{24} were studied.

3. Results and Discussion

In this study, we isolated four distinct microbial colonies. One of the isolates of sample B was not able to degrade the crude oil and the other one was able to do it; however, in sample A, all isolates were able to degrade the crude oil. So that in the first day, there were two phases in the medium, after a few days, these three bacteria degraded the crude oil until there was only one phase left in the medium. Among them, one strain was selected as a superior strain (ZH1) by homogenizing until the medium became clear and transparent. There are several methods for recognition of biosurfactant production. Most of the screening methods for biosurfactant producing microbes are based on the interfacial or surface activity. Many of the screening methods created so far rely on the interfacial activity of the biosurfactant, and some of the screening methods are based on the hydrophobicity of the cell surface [22]. In this study, we managed to determine the oil displacement area and the emulsification index for ZH1. The oil displacement area was in the range of 23.74-70.84 cm^2 for the crude oil.

The diameter of the oil layer forming on the surface of distilled water was 10cm, and the diameter of the clear area for 10-40 µl of the bacterial suspension containing biosurfactant was 5.5-9.5 cm. Figure 1 shows the oil displacement area in a petri dish.

There is a linear relationship between the oil displacement area and the amount of biosurfactant of ZH1. Figure 2 shows this relationship (R^2=0.9726).

Morikawa et al. [20] reported that the area of clearly formed oil displaced circle was measured as the activity of surfactants. The oil displaced area formed by the activity of surfactants showed linear relations to the amount of surfactants tested [20]. In addition, Varjani et al. [23] reported score ranging "+" to "++++" corresponding to the partial to complete displacement of crude oil. The oil displaced area formed by the activity of surfactants shows linear relationship to the amount of surfactants tested.

Figure 1. The oil displacement area by ZH1 (right and left are different in the amount of suspension containing ZH1 (µl). The right circle contains more suspension.

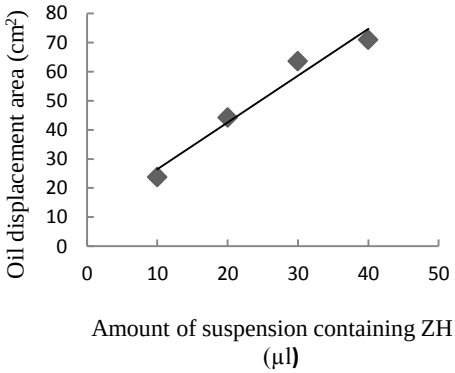

Figure 2. Linear curve of the oil displacement activity of ZH1

Table 1 shows some properties of ZH1. It was preliminarily identified as *B. subtilis*, and 16S rRNA ribotyping confirmed that the isolated bacterium belongs to *B. subtilis*. The phylogenic tree of ZH1 can be seen in Figure 3. Besides their role in hydrocarbon bioremediation and microbial enhanced oil recovery, these biological products have potential uses in agriculture and in the cosmetic, pharmaceutical, detergent, food, textile, paper and paint industries [24].

Table1. Classical characterization of ZH1

Confirmative test	ZH1
Gram staining	+
Cell shape	Rod
Spore forming	+
Motility	+
Catalase	+
Citrate	-
Nitrate	+
Starch hydrolysis	+
Growth in 1-7 % NaCl	+
CHO fermentation	Glucose, Fructose, Sucrose
Colony color	White
Growth in aerobic and anaerobic conditions	+

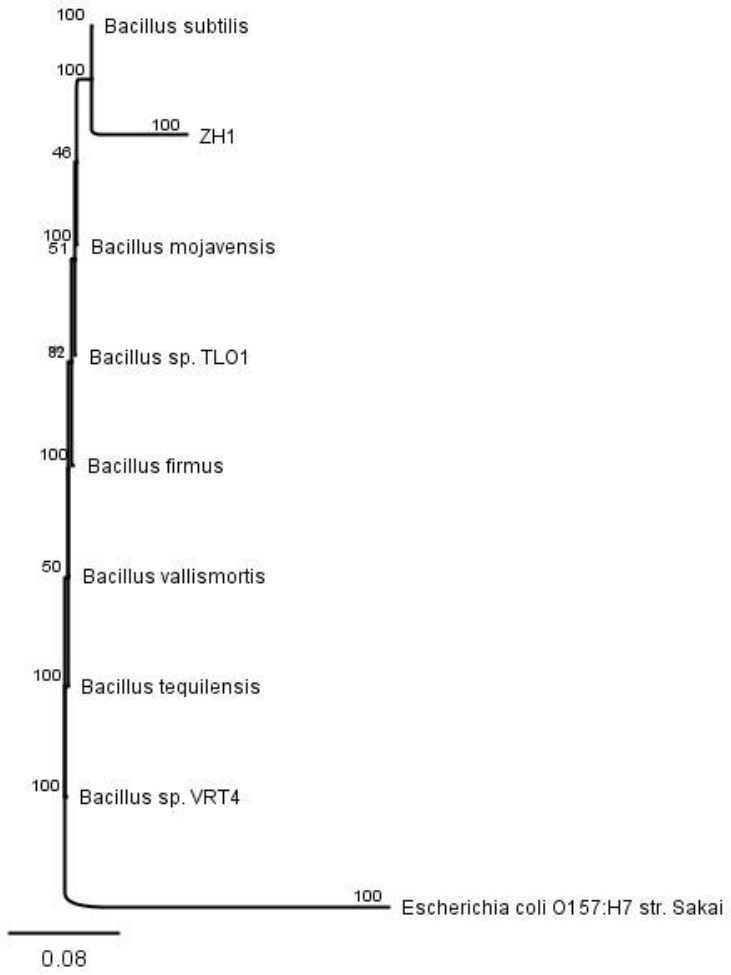

Figure 3. Phylogenetic analysis of the 16S rRNA gene of *Bacillus spp*. using the neighbor-joining method

Lipopeptides are among the most favorite and efficient biosuaractants. They are mostly synthesized by the strain belonging to the Bacillus genera, especially *B. subtilis* strains and are classified into three main classes: surfactin, iturin and fengycin. Many properties of these biosurfactants such as surface and emulsification activity, the foaming, the wetting, the anti-adhesive and the antimicrobial activities are caused using of them in food processing and formulation [17]. *B. subtilis* is usually being utilized for oral bacteriotherapy and bacterio-prophylaxis of gastrointestinal disorders. *B. subtilis* is normally found in the soil, and the soundness of spores is thought to enable passing across the gastric barrier. In addition, the clinical effects of *B. subtilis* as an immunostimulatory agent in a variety of disease have been recorded [25].

Bacillus species' surfactants facilitate the process of emulsification of hydrocarbons in aqueous phase by forming micelles, thereby enhancing their availability for microbial uptake and degradation [14]. In the present study, E_{24} for ZH1 was calculated as 34%. Similarly, Pathak et al. [26] reported E_{24}=33.3 ±0.5 for *B. subtilis* K1.

Biosurfactant produced by *B. subtilis* ZH1 should be produced by cheap substrate, and here we studied beet molasses for producing the biosurfactant. Of the two different carbon sources (crude oil and beet molasses), beet molasses was better carbon source for biosurfactant producing induction by ZH1 due to its high oil displacement activity and emulsification index. The oil displacement area of ZH1 with molasses was 28.26-94.98 cm^2 compared to 23.74-70.84 cm^2 for crude oil as carbon source. The emulsification activity of ZH1 with molasses as carbon source was approximately 50% comparing to 34% for crude oil as carbon source. Oil displacement activity and emulsification activity are two screening methods that both rely on the interfacial activity of the biosurfactants; however, they do not measure it directly [22]. Plaza et al [27] reported that the surface active properties of the cell-free supernatant of *B. subtilis* were the best with molasses. So beet molasses as a cheap substrate is comparable with crude oil, and could be used as a feasible source of biosurfactant production in practice.

4. Conclusion

The soil of south of Iran is a promising area for the isolation of crude oil degrading microorganisms and their application in bioremediation. The isolate is a candidate for the study of optimization of biosurfactant producing, especially when using the appropriate carbon source such as molasses.

5. Acknowledgement

The authors would like to thank the Oil and Gas Producing Company of Gachsaran, Iran for encouragement.

6. Conflict of interest

The authors declare that there is no conflict of interest.

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