

A Case Study to Evaluate the Role of Basiluses in Producing Biosurfactant and the Feasibility of MEOR

R. Mostafaei, M. Mohammadinia, F. Mirzaee Tabesh*

Graduated Msc. Petroleum Geology N.I.O.C, Exploration Directorate Tehran, Iran

* rashel.mostafaei82@gmail.com, faeze_tabesh@yahoo.com

Abstract

Bibi Hakimeh oilfield consists of more than 145 oil producing wells. Its Oligomiocene Asmari reservoir is dominantly made of limestone. The act of a reverse fault on the north flank of Bibi-Hakimeh Field caused a significant thickness reduction in Gachsaran formation in the way that in some drilled wells, members No. 2, 3, 4, 5 and 6 of Gachsaran cap rock have been totally eliminated. This causes locating Asmari reservoir in a shallower level and therefore lowers reservoir temperature in the North flank already have made the reservoir suitable for a microbiological enhanced recovery. The long term production of this reservoir caused a significant reservoir pressure drop. Therefore; the recovery has been performed using *Basilus* with Ex-Situ method. In this case study, the feasibility of surfactant production in several oil wells has been accomplished. A high temperature resistant *Basilus* has been selected to evaluate the production ability of biosurfactant. This bacterium has been chosen after performing all morphological, biochemical and genetic studies. This bacteria shows a good resistance against the temperature in such manner that its emulsification, surface tension and inter surface tension abilities do not change after 15 min in an autoclave process at 120° C. In the next step, the temperature, pH, Carbon, N₂ and other factors have been optimized for biosurfactant production. Considering the lithology of the reservoir using this type of bacterium. This can be a good way to produce lipopithidic biosurfactant by Ex-Situ method in Asmari to enhance oil recovery. The *basilus*, which has good resistance against temperature and acts well in pressurized environments, can be considered as a good candidate for tertiary enhanced oil recovery process. The best method to produce *basilus* in B5 is the Formislink method.

Key words: Bibi Hakimeh Oilfield, Asmari Reservoir and Oil Recovery

Introduction

Iran is in the 3rd and 4th position of gas and oil resources, respectively. Ten percent of all oil resources (132.5 bbl) and 16 percent of explored gas resources (971 tcf) around the world are located in Iran [1]. The first oil reservoir of the Middle East was explored in southern Iran, Masjed Soleyman in 1908. The explorations continued in 1937 (Kuwait) and 1938 (Saudi Arabia) [2]. Most of the Iranian reservoirs are located in Zagros as Persian Gulf basins. Also, in north eastern Iran (Kope Dagh) and North West (Dasht-e Moghan), some exploration operations are being done. About 43 percent of Iranian reserves are giant consisting of 64 oil and gas reservoirs.

90 percent and 10 percent of Iranian giant reservoirs are carbonates and sandstones, respectively, of which 53.12 percent are oil reservoirs and 46.87 percent are gas reservoirs. Actually, the correct portion of carbonate reservoirs to sandstones is 9 to 1 [3]. Use of microbes for oil enhanced recovery (MEOR) was first recorded in 1913 by Davis. In 1946, Zobell recorded a process for secondary enhanced recovery using non-aero microbes and mechanism of mineral substance solution. The first field test of MEOR was done in 1954 in it of the Arkansas fields. Although this was successful, was ignored because low cost oil resources were available. However, the instability of oil price and importance of

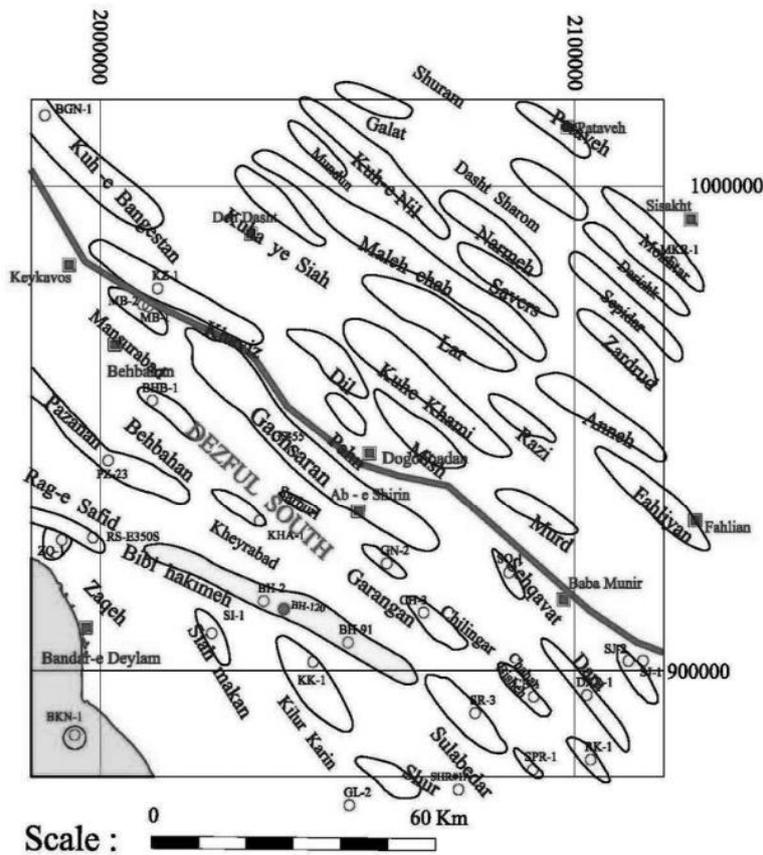
biotechnology in the 70s, resulted in paying attention to MEOR again. Since 1980, this method has become common in different countries because of increase in oil price.

In our country, numerous researches have been done during the last 10 years, but they haven't reached the industrial stage because of high amount of deepness and heat of the reservoirs.

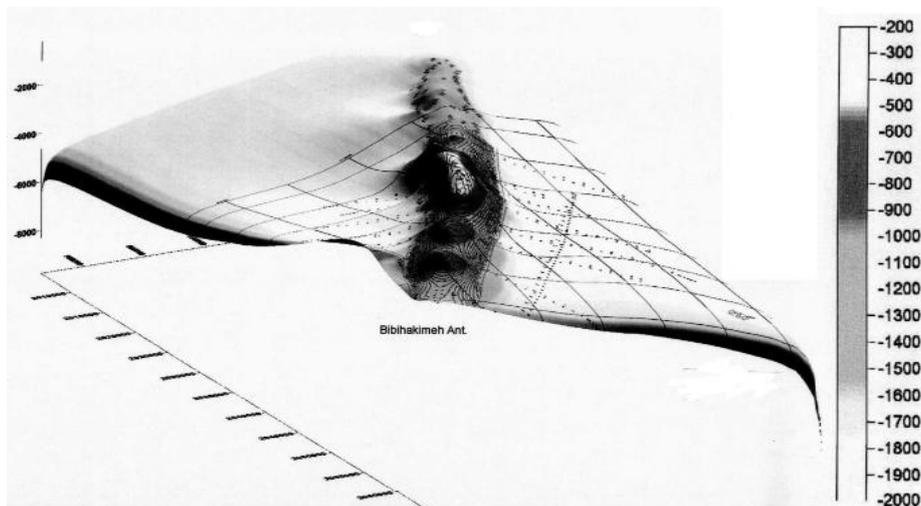
General characteristics of Bibi Hakimeh oilfield

Geophysical structure of Bibi Hakimeh is located in south Dezful, north of Kilur Karim and Siah Makan, and

south of Kheyr Abad, Garangan and Chilingar, with a northwest-south east trend and parallel with Sulabdar and rag-e safid fields (Map no.1) [2]. The youngest and the oldest outcrop of this anticline are Bakhtiari Formation and Mishan Formation respectively. In Bibi Hakimeh structure, Asmari Formation with dimension of 75*8 km and Sarvak Formation are the main and the second productive reservoirs. This field was explored in 1340 and has 2 reservoir Formations: Asmari and Bangestan. These 2 reservoirs have pressure relationship (Map no.2).



Map 1- Structural Geology Map of Bibi Hakimeh



Map 2- 3D Contour Map of Bibi Hakimeh Anticline

Asmari Formation is one of the important reservoir horizons from Iraq to Iran whose thickness has been decreased in northern rim of Persian Gulf. In Iraq it is called Jirob, but the general lithology is same as Asmari with index fossils such as: *Neoalveolina melo curdica* (Late Miocene), *Nummulites intermedius*, *Austrollina howchini* etc.

Based on the newest structural UGC of Fahliyan top, prepared by structural geology department of N.I.O.C, Bibi Hakimeh structure has dimensions of 4*61, with a vertical closure exceeding 1400 m. Drilling operations and the tests done in different wells of the filed show complex geological situation resulting from the movement of Arabian plate toward Iranian plate, which causes deep faults like Fore Thrust in most of the anticlines and folding. These faults have important effects on the quality of reservoir characteristics of Bibi Hakimeh structure & Back Thrust

Method of evaluating Asmari Formation from microbial growth point of view in Bibi Hakimeh oilfield:

In the current study, possibility of biosurfactant production in some of the wells were evaluated. For this purpose, a basillus with high temperature resistance was selected and the ability of biosurfactant production was studied. After morphological, biochemical and genetic studies, the bacterium was investigated.

Geological and reservoir properties of Asmari Formation in Bibi Hakimeh oilfield:

On the basis of tectonic, geological and reservoir studies in south Dezful, it looks as if the possibility of MEOR is weak, but among all the fields in south Dezful, Bibi Hakimeh has an appropriate situation.

Bibi Hakimeh oilfield is one of the main fields with numerous wells and an old history of production.

Geological and tectonic studies indicate thrust fault function in the northern part of the anticline causes decreasing thickness of Gachsaran Formation and deleting members, 2,3,4,5 and 6 in some parts. Therefore, decreasing in depth and heath toward north makes this part suitable for MEOR.

For production circumstances, we can point out some characteristic: API is 29.9, its specific gravity is about 0.87, and its average amount of sulfur is 1.6 %. From reservoir characteristics, 3 wells located in northern part of the anticline were studied. The results of pressure and temperature were investigated and then core samples were studied to obtain porosity and permeability. Finally, possibility of Basillus growth and biosurfactant production were investigated.

Sampling points

Sampling was only done from Asmari Formation of Bibi Hakimeh oilfield.

Sampling and samples transporting to the lab

Glasses with lid or cover were used for sampling. All the glassware were acidized before using and washed with tap water and distilled water and sterilized in an autoclave for 20 minutes (Table 1)

Table 1- Samples from Bibi Hakimeh Filed

Bibi Hakimeh Filed	1- Bibi well B ₁
	2- Bibi well B ₃
	3- Bibi well B ₅

Immediately after collection, the samples were transported to the lab and grown in an appropriate environment. Most of the samples were at temperature between 65-120 °C.

Pour plate microbial counting

In order to separate and purify bacteria, Pour Plate Technique was used on the plate count agar. After 48-72 hours of incubation at 30 °C, bacterium growth and number of colonies were counted. All the colonies were transferred to nutrient agar environment for more investigations. After growing, their morphological and biochemical characteristics were primarily identified. Our purpose was working on positive, hot, non-aerobic bacteria (Basillus). Thus, separation stages, identifying, and determining the products affecting enhanced recovery were different.

enrichment of producing biosurfactant Basiluses

To increase the number of Basiluses, which are producers of biosurfactant and their separation, all the samples were gathered from different resources and grown in a special environment of 1 % mineral salts (MSM) for degradation of petroleum. 30 ml fresh MSM was poured in a 50 ml erlenmeyer flask and was sterilized at 120°C for 20 minutes. Then, 0.3 ml (1%) sterilized petroleum was added using a filter (pore size = 0.45μ, Gelman Sciences). Also 0.5 gram soil with 0.5 ml water was added and was heated for two weeks at 30 °C.

Main components (gr/lit):

(NH₄)₂SO₄ 3.0, Na₂HPO₄ 2.2, NaCl 0.05, KH₂PO₄ 1.4, MgSO₄.7H₂O 0.6, CaCl₂ 0.02

Rare elements (gr/lit):

MnSO₄.4 H₂O 200, CuSO₄.5H₂O 705, ZnSO₄.7H₂O 525, Na₂MoO₄.2H₂O 15, CoCl₂.6H₂O 200, H₃BO₃ 15, NiSO₄.6H₂O 27

In the next stages, 24 hour planting was done fore bacterial separation.

Bacterial separation and purification

For bacterial purification, mineral agar was used to grow bacteria. This was prepared by adding 1.5 % agar to MSM. Spread Plate Technique on solid environment was used [4]. A sterilized filter covered by oil was located on the plate lid and the prolific plate was added.

Hydrocarbon vapors were gathered in the plate are always available for bacteria to be used as a carbon source [5]. Growth on solid environment was repeated consecutively until no separation of new bacteria occurred based on the appearance and morphological properties. It was made sure that the maximum separation of bacteria from the sample had been obtained [6].

Biosurfactant production is a suitable criterion for enhanced oil recovery. Among all the different innovated methods, hemolytic activity, which is attributed

to biosurfactant, has been selected as a primary criterion because of its high rate, being simple and having no need to have highly equipped lab facilities [7].

β Hemolysis with a transparent halo around colony, was considered as positive hemolytic activity and samples bearing positive hemolytic activity were used for the next studies [8].

Measurement of facial tension

Surface tension was measured by Du Nouy Ring Method and using tensiometer as other principle criterions. Also, OD45 was used with 0.8-0.9. For this purpose, each of selected were injected to 100 ml Erlenmeyer flasks bearing 50 ml growth environment of Yeast Extract 1gr/lit+ MSM. Then, 2 ml (1%) sterilized oil was added as a carbon source using a filter and the samples were put in a shaker with 200 rpm at 30°C for 48 hours. Surface tension measurement indicated that some of with hemolytic activity, produce biosurfactant in a reasonable amount, which are able to reduce surface tension less than 40 mN/m (or near 40) and are also able to produce reasonable amount of biosurfactant.

Facial tension measurement

Facial tension was measured as a principle criterion for producing Biosurfactant by Du Nouy Ring Method using a tensiometer. Planting species in nutrient brat environment with OD45= 0.8-0.9 was applied as productive liquid. To do this, each of the selected species were injected in to 100 ml Erlenmeyer flasks containing 50 ml sterilized implant environment of + MSM 1gr/lit Yeast Extract. Then, 2 ml (2%) sterilized oil were added using cortex filter as the carbon source to the mixture and it was put on the shaker at 30°C with 200 rpm. Facial tension measurement shows that some of the species having hemolytic activities produce proper amount of biosurfactant. The species, which are able to reduce facial tension to less than 40 mN/m, produce suitable amount of biosurfactant.

Measurement of emulsifier activity

In order to measure the activity of emulsifying, the method described by Cooper & Goldenberg was used [9].

Growth and producing measurement of biosurfactant in saline environment

To eleven 250 ml Erlenmeyer flasks in MSM plant environment, (sterilized in autoclave for 20 minutes at 121°C) 18, 16, 14, 12, 10, 8, 6, 24, 0, 20 g/L NaCl were added, respectively, .

Planting of species in nutrient brat with OD45= 0.8-0.9 was used as productivity liquid. 1 ml of productive liquid was injected in each of environments and was put on the shaker at 30°C with 200 rpm. Facial tension and emulsification activity for 48 planting in presence of different amount of salt was investigated.

Growth and production measurement of biosurfactant in different pHs

Six 250 ml flasks containing 50 ml MSM environment were prepared at pHs of 2,8,7,6 and 4. After sterilizing the environments, 5% productive liquid was injected

and was put on the shaker at 30°C with 180 rpm. Light absorption and facial tension were measured after 48 hours. Facial tension and emulsification activity of 72 bacteria planting in pHs of 10, 8,6,4,2 and 12 were measured. To adjust pH, 1 normal HCl and 1 normal NaOH were used.

Influence of different sources of carbon on biosurfactant growth and production

1% of carbon source (7% in 7 flask) containing crude oil, hexadecane, glycerol, molasses, mannose, sucrose, glucose and fructose was added to MSM planting environment and after sterilizing the environment, 5% of productive liquid was injected and was put on the shaker at 30°C with 100 rpm. 48 hour facial tension of each environment was measured as a criterion of biosurfactant production.

Different sources of nitrogen such as urea, peptone, yeast extract, sodium nitrate and sodium nitrite in amount of 0.3% were added instead of ammonium sulfate [10]. Then, after sterilizing the environment and injecting 5% productivity liquid, the bacteria were transported to the shaker at 30°C with 200 rpm. 48 hour facial tension of each environment was measured.

Environment factors and growth conditions such as temperature, aeration scale, access capability to O₂ and media type will affect cell growth and activation, which influence the production of biosurfactant and in most cases, is itself a biosurfactant stimulator.

Carbon source is a very important parameter Therefore, with changing in the first stage, the bio surfactant features change. Other features that change are iron, calcium, potassium and magnesium density.

Aeration in producing bio surfactant has a very important role. Producing biosurfactant is maximum when a few conditions are non-aerobic.

Temperature is an important factor in the effectiveness of biosurfactant production. However, biosurfactant shows resistance against temperature. Growth conditions are influenced by such factors as temperature, aeration scale, accessing capability to O₂ and media type with influencing cell growth and activation influence to produce biosurfactant.

In order to measure thermal resistance of biosurfactants they were exposed to high temperatures. It was observed that they resisted 15 minutes at temperatures of 100^o, 120^o and no changes on surface tension (ST), EC activation , cell suspension and a liquid for bacteria cultivation were reported.

To produce cell suspension and cultivation liquid, 500ml of cultivation was carried out for 24 hr in MSI environment having kerosene as carbon source and centrifuged for 10 minutes at 100 rpm. The surface liquid is gathered and remaining sediment is dissolved in 500ml physiological serum.

Discussion and results

cell count results related to Bacillus

After diluting oil samples and cultivating in agar implant

environment, separated sediments from the dilutions were counted. The results are shown in table 1. Note that a lot of bacteria and *Bacillus* grew in cultivated samples from oil wells (Table 2).

Table 2- Cell counts in oil samples

Oil well name	The result of pour plate	Number of bacteria in 1 ml of each sample
SB well B ₁	+	2.5×10^2
SB well B ₃	+	3.5×10^2
SB well B ₅	+	4.5×10^2

Primary segregation and sieving:

About 24 water and oil samples were prepared from various oil regions, based on surface characteristics and sediment features, 136 strains of bacteria were isolated, all of which are able to grow on MSI cultivation environment as carbon sources.

Because hemolytic activation is one of the bacteria features that produce biosurfactant, hemolytic activation with cultivation on agar environment is studied. In the same study, Hemolytic activation is used for primary segregation for separating reproductive bacteria for bio surfactant. Abu Ruwinda used this method in 1991 [11].

β hemolysis or transparent halo around settlement as positive hemolytic activation is accounted and the samples having positive activations are selected for the next study. Among separated strains, 36 strains can decompose erythrocytes and produce a β hemolysis halo on blood sugar environment and 100 remaining strains are lake hemolytic activation [12].

Based on results obtained on previous studies on reproductive biosurfactant bacteria, every bacteria strain that can reduce surface tension to less than 40 mN/m can be a beneficial strain for producing biosurfactant. Among 36 strains isolated, the surface tension of 8 strains can reduce to less that 40 mN/m and can be accounted as a proper candidate.

The strongest biosurfactant is one that is a peptide and lipid antibiotic with significant surface activation and is produced by *Bacillus subtilis* (Banat, Makkar and et al, 2000). Lichenysin, of licheniformis *Bacillus* by Yakimov in Germany is similar to biosurfactin and can reduce surface tension of environment from 72 mN/m to 28 mN/m (Yakimov, Kenneth and et al, 1995).

The isolated strains and bio chemical tests (table 2) are identified by morphological method (figure 1) by using the Bergey's classification of bacteria.

Selecting most appropriate strain

Surface tension cannot be an acceptable criterion for selecting the best isolated strain for final studies they have close surface tensions. To select the most appropriate strain(s), supplementary tests such as emulsion are carried out.

Emulsion activation with E_{24} and Cooper method is identified and is a way for indicating biosurfactant capability in the emulsion of various hydrocarbons.

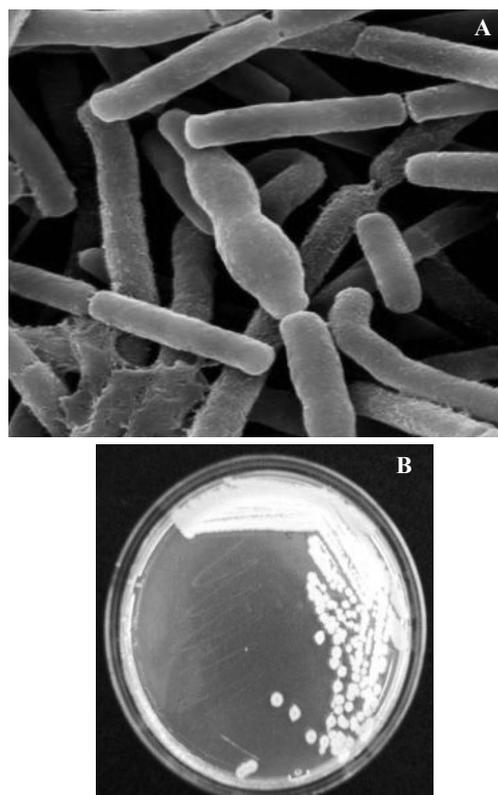


Figure 1- Licheniformis *Bacillus*
A: microscopic forms, B: colony

E_{24} for our best strain was 91% SBB5. Due to above results (morphological methods and biochemical tests) and having compared the results with the identification table for *Bacillus*, it can be said that our bacteria of interest is likinforis *Bacillus*. The minimum surface tension values obtained for the cultivation environments for likinforis *Bacillus* was 30 mN/m compared with values reported for other biosurfactant reproductive bacteria, these are in acceptable limits.

Surveying salt effectiveness on obtained bio-surfactant activation

To assess biosurfactants activities resulting from above strain, surface tension ST and emulsion activities, bacteria cultivation with densities such as 1%, 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20 sodium chloride were tested.

Often, strains isolated from oil sources in salt unit 2% to 4% have max ability for producing biosurfactant. With increasing percentage salt from 60% to 20, biosurfactant production is reduced. Max production of biosurfactant is related to strain SB B5, which can reduce surface tension to 30 mN/m in 4% salt in the observed sample. To assess effect of various concentrations of salt on likinforis *Bacillus* biosurfactant activities with increasing salt to 10%, a small change was observed in bio-surfactant activities.

Measuring growth and production of bio-surfactant in various pHs

To assess various pH effects on surface activity of strain biosurfactant production, *Bacillus* sp, surface tension,

intersurface tension and CMD, bacteria was cultivated for 48 hrs in pHs such as 2, 4, 6, 8, 10, 12 and then biosurfactant production was measured.

Chart 2 indicates the obtained results. pH effects on this strain indicated that in pH 6 to 8, it can produce biosurfactant the best production was in pHs between 6.5 to 7.5.

Effects of carbon source on producing biosurfactant by the Bacillus Licheniformis strain

Chart 3 indicates effects of 7 different sources of carbon such as glucose, petroleum, molasses, gasoline, mannose, fructose, sucrose and ketene on surface tension resulting from 48 hour-incubation of 8 biosurfactant productive strains of Bacillus Licheniformis.

This strain can grow and produce biosurfactant in various carbon sources. It can also grow and reduce surface tension in petroleum, gas oil, ketene, sucrose

fructose, glucose and molasses.

Simple carbon sources such as glucose, fructose, and sucrose are widely used by Bacillus separated from oil because of their simple structure and reduce surface tension and sucrose and glucose reduce this tension to 30 mN/m. Petroleum is the best carbon source.

Effectiveness of nitrogen sources on producing biosurfactant via strain Bacillus Licheniformis

Chart 4 indicates effectiveness of four nitrogen sources such as peptin, sodium nitrate, yeast distillate and ammonium nitrate on surface tension resulted cultivation of productive strain biosurfactant Bacillus Licheniformis in 48 hrs.

This strain can grow in all types of nitrogen sources and produce biosurfactant, which strain can reduce surface tension to 30mN/m (chart 4).

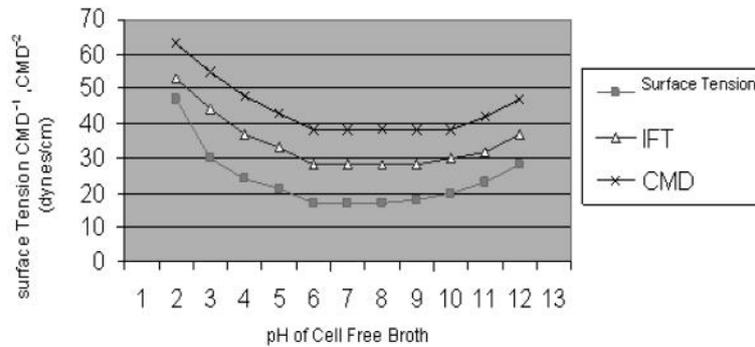


Chart 2- Curve of effects of various pH on biosurfactant activity produced by the Bacillus Licheniformis strain

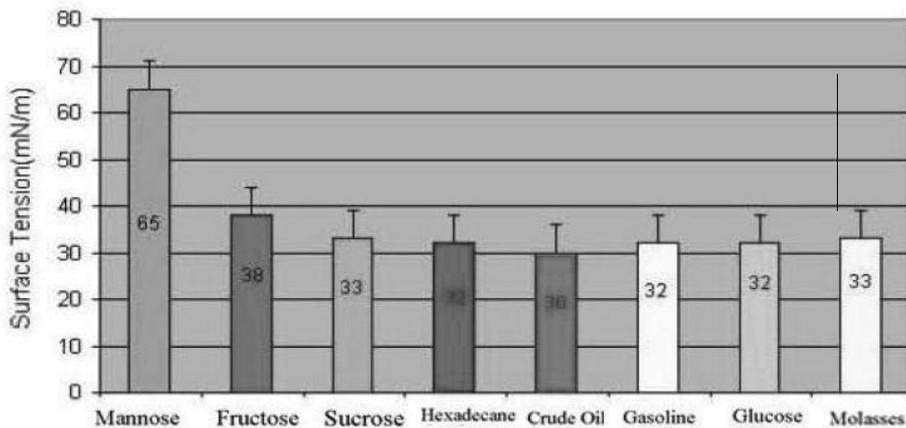


Chart 3- Effects of carbon sources on surface tension Bacillus sp incubated for 48 hrs

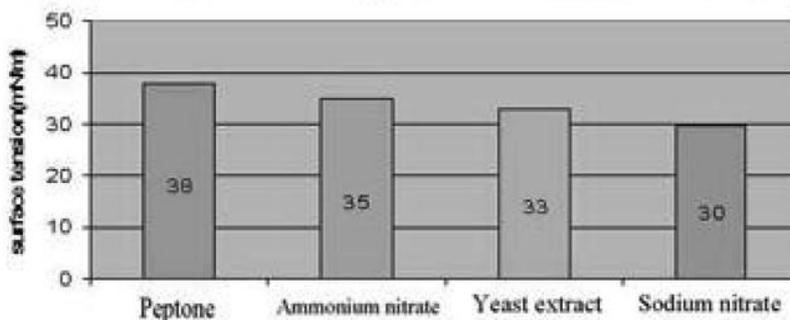


Chart 4- Effectiveness of nitrogen source on surface tension of cultivation 48 hrs of strain

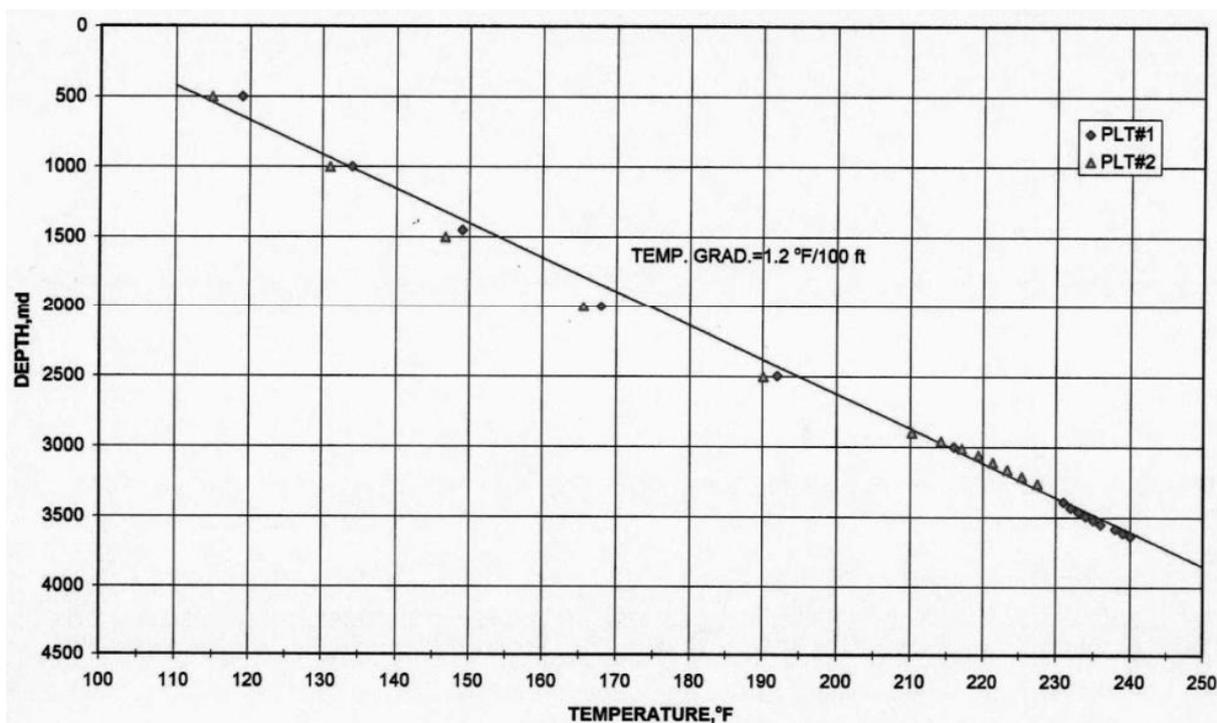


Chart 5- Temperature to depth in a well of the Bibi Hakimeh Oilfield

Measuring thermal resistance of biosurfactant obtained and assessing petrophysics situations and PVT of height source in Bibi Hakimeh Oilfield

Because of high thermal conditions in the oil source depth, biosurfactant used must be resistant to high temperature. An oil region such as Zagros is very deep and therefore temperature in the source is high and process of increasing cultivation must be as EX situ. The results indicate that microorganisms were isolated in high temperatures but reduced producing of its products.

In the field studied, average diameter of putting Asmari Formation in north mane of Bibi Hakimeh Oilfield is about 450 meters and average temperature in Asmari source compared to other squares with respect to building condition indicates significant reduction (chart 5).

To assess thermal resistance of biosurfactant obtained at high temperatures, the effects of 100°, 120° temperatures for 15 minutes on surface tension (ST), EC, cell suspension and cultivation liquid obtained from the Bacillus Licheniformis strain were studied and the results are indicated in table 4.

Table 4- Effectiveness of thermal conditions on activity of bio-surfactant produced by the Bacillus Licheniformis strain.

Licheniformis Bacillus			
Culture broth 100 °C/15min			
S.T	31.3	31.3	31.1
EC{%	55.0	55.0	55.0
Cell suspension 120 °C/15min			
S.T	30.4	30.3	30.6
EC{%	65.0	65.0	65.0

Aeration has an important role in production of biosurfactant. For example in the case studied, it was found that the highest quantity produced of biosurfactant was obtained under anaerobic conditions.

Temperature is the important factor. It is effective in production of biosurfactant, but biosurfactants show resistance to temperature. Therefore, emulsion capability, surface tension and intersurface tension in Bibi Hakimeh Oilfield do not change after 15 minutes in an autoclave at 120°.

The quantity of biosurfactant produced is related to bacteria growth and all the above factors are related to conditions available on Asmari source or environment for growing Thermo Phil bacteria.

Asmari formation aged oligomiocene and its lithology includes brown to gray fossil limestone and dolomite and bitumen and middle part of formation includes light brown limestone to cream soft to semi-severe limestone and its layer is gray toward green and light gray Marl.

In beneath part, there is cream and fossil limestone and semi-severe and white limestone. This formation as consistently laid on Pabdeh Formation.

One of the effective factors in producing biosurfactant is consumption of the material and in most cases, there is growth on carbohydrate, but this is not necessary for all microorganisms.

Carbon source is the important parameter changing product type. Therefore, with changing primary cases, biosurfactant changes. Other effective factors in producing biosurfactant are density concentration of iron, magnesium, calcium and potassium. Average rate of porosity is appropriate at the Asmari Formation for micro-organism growth such as Bacillus.

Results of storage characteristic and storage system in Asmari formation

Due to oil storage reserve in square and production reservoir from 1340, pressure reduction at the reservoirs, building condition and petrophysical conditions in oil field have been assessed.

PVT characteristic of storage reservoir with respect to conning system is dual.

A few years ago, pressure dropped in both systems, pressure drop is water conning and gas conning but gas conning is more rigorous. Therefore, to prevent the reoccurrence of these problems, horizontal and directional mining is used.

Increasing mining cost and mining time, supplying storage reservoir pressure and preventing the reduction of pressure can play significant roles in increasing the reservoirs lifetime.

Temperature in Asmari storage reservoir ranges from 65° to 120° C and it is possible to grow *Bacillus* with respect to storage conditions. Asmari formation has various breakings porosity quantity and water saturation in various wells in formation studied is 7 percent and less than 50 percent

Result

In north mean part of the field studied, thickness average of Asmari formation is about 450 meters and average temperature is significantly lower in Asmari source than in other fields with respect to the building conditions. Based on the results of morphological methods, biochemical and genetic tests we can conclude that our bacteria is *Bacillus licheniformis*. The minimum surface tension values obtained for strain cultivation environments for *Bacillus licheniformis* separated was 30 mN/m. Compared with values reported for other biosurfactant reproductive bacteria, these results are within acceptable range.

Conclusion

One of the best carbon sources is crude oil. pH effectiveness on producing biosurfactant from this type of bacteria indicated that it can produce biosurfactant at a pH of 6 to 8, but the best pH is between 6.5 to 7.5. We also found that emulsion activation for our best strain was 91% SBB5. Nitrogen is one of the best sources for growing and producing biosurfactant. Strain can reduce surface tension in 30 mN/m. Meanwhile, sucrose and glucose reduce this tension to 30 mN/m. The results of reservoir pressure reduction, structural and petrophysical conditions in oil fields have been assessed. According to PVT characteristics of the reservoir, it is a dual system with respect to the drive system. A few years ago pressure dropped in both systems, water and gas drive but gas drive is more rigorous. Then supplying reservoir with pressure and preventing its pressure reduction can play a significant role in increasing lifetime of the reservoir. Temperature in Asmari reservoir ranges between 65 to 120° C and it is possible to grow *Bacillus* with respect

to the reservoir conditions. Asmari Formation has various breaking formations, porosity and water saturation in various wells in formation studied is 7 percent and less than 50 percent.

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