Effect of Pseudomonas Bacteria Biosurfactants on Persian Gulf Crude Oil Water Contamination: Optimized Conditions of Effective Parameters

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Abstract
In this study, effective parameters on pseudomonas bacterial culturing are analyzed individually. Pseudomonas bacteria were applied for oil pollutants segregation from a polluted water sample of Persian Gulf. The bacteria were used for cleaning the sample so that the contents' chemical features could be determined. Salinity and pH parameters were put under observation in different conditions and the results are illustrated in diagrams. Concentration of biosurfactants and conditions of culture medium like oxygen amount and temperature were all determined and analyzed. The influencing parameters on laboratory scale bacterial culturing were varied individually in order to obtain the optimum conditions for oil pollutants cleaning. Taguchi experimental design method was then applied to optimize the bacterial culturing conditions. Salinity, pH amounts, and biosurfactants concentrations were chosen as the parameters that use different levels based on Taguchi method. Eventually, a pH of 8.5, salinity of %2.5 and biosurfactants concentration of %0.1 were determined as the optimum conditions in which bacteria were cultured and grown the most.

Key words: Culture media, Bacteria, Biosurfactants, Salinity, Persian Gulf, Crude oil

1. Introduction
Persian Gulf is one of the most polluted marine areas in the world due to activities like production from oil reservoirs, oil leakages, tankers transportations, industrial sewerages and garbage throw-offs, petrochemical production, associated countries’ rapid industrialization and absence of coherently academic management.
The area is so contaminated that it faces a vast immensity of diminishing destruction and deterioration dimensions.
In the past, people believed that sea space can inherently eliminate all pollutions left by people. And this has increased the amount of drains of pollutions to waters. In recent years, the attitude and concept of marine environment protection have been fostered for the reasons that all associated countries’ properties and profits deal with environmental health and hygiene especially of waters, and that no good future can be foreseen without partnership and scientific cooperation.
This notion is felt today more vital and crucial than any time else. Persian Gulf as a very main way for oil transports is the occupational path of about 40% of world oil tankers which has been always exposed to oil different pollutions. Its marine ecosystem is severely threatened by serious dangers. Thus any contamination –more importantly oil pollutions– has potentials to cause their own difficulties.
Annually, a vast amount of crude oil enters this gigantic water which exerts adverse effects on the Persian Gulf ecology because of floating on water and prohibition of oxygen entrance to sub layers. On the other hand, after light components vaporizations and getting denser the heavy crude oil moves down to remain in mid layers of the sea. So the fishes feed the polluted water and this can cause infections in both: humans or inhabitant plants and animals.

Oil tankers irritating accidents in seas since 1980, have alerted international environmental authorities around environmental adverse influence of these pollutions and so around the efforts of finding efficient solutions to solve their problems [1, 2].

Microbiological study has revealed that the nature itself has enough ability to eliminate these kinds of oil pollutions via a slow but effective procedure, this is while preparing optimum conditions can accelerate these natural processes in a perfect way [3, 4, 5]. The presence of oil hydrocarbons in the area, during complementary period has complemented some microorganisms to utilize these combinations as carbon and energy sources. On the basis of this suppose, for the purpose of finding a separating bacteria, sampling from Khurmusa oil located in Khoozestan province which was naturally exposed to this pollutant were carried out. As environmental factors are so effective on the growth and function of microorganisms to produce biosurfactants for separating and isolating oil, it is required to have a vast study on the role of environmental elements. Microorganisms are capable to synthesize biosurfactants from crude oil, pure hydrocarbons and also various kinds of non-hydrocarbon layers, simple carbohydrates and acids. Any biological method needs the environmental condition of area in different cases like salinity, pH and temperature [6, 7]. The well known strategy for biosurfactants application is actually biosurfactants production in continuous culture medias and their addition to polluted area [2, 7]. For motivation of native bacteria producing biosurfactants growth, the opted nutrition chemicals have to be injected to the basic culture media. Through all project stages, monitoring of pH variation and turbidity in wavelength of 680 nm indices is a criteria for bacteria growth and biosurfactants production [8, 9].

Application of biosurfactants in raising oil recovery is one of important methods of recovering a substantial amount of leaving oil. Increasing biosurfactant increases the potential of oil separated such that adding these biosurfactants had all important parts in oil environmental separation compared to chemical surfactants.

This study was conducted to assess a strategy for applications of biosurfactants included their production continuously in culture media and their addition to polluted regions of Persian Gulf crude oil contaminations.

This analysis of biosurfactants had more advantages than chemical surfactants such as lower toxicity, native chemical acceptance, separability and more suitable application in environmental separation of oil. This research was carried out with the purpose of optimizing oil absorption from environment with the method of Taguchi test design. The results illustrated that the best parameters in mentioned pollutant separation were pH and producing biosurfactant concentration of polluted area native microorganisms. Considering all these can find out that applying Taguchi test design method is able to be utilized with the aim of explaining oil separation process via biosurfactants.
2. Geography of the Region:

Mahshahr exportation port being more than 85 years old is located at extremity of Khurmusa natural water stream; at the end of Persian Gulf Iran southwest. This port due to its natural situation has been a suitable media for transferring crude oil produced by Manateq Naftkhiz Company and a new project product– a project which was carried out in Khark Island called Cham. The port tanks and jetties were applied for exportation of refined production of Abadan refinery.

2.1. Coastal Area:

This area has eight jetties among which five ones are presented to oil products exportations, one presented to ships harbor and finally one to loading and unloading of oil company goods. In this region there exist about 39 storage tanks with various capacities which are applied to transfer and store petroleum products such as SRG petrol, kerosene, oil, gas, light and heavy naphthas, Bandar Imam petrochemical feed and MTBE.

In these region five units of oil product pumping installations, ship-loading installations on jetties, control room, instrument installation and subsidiary instruments have been constructed.

2.2. Aqabeh Storage Tank Area:

The region has twenty four fuel oil storage tanks with different capacities for exportation of Abadan Refinery fuel oil which is delivered by a pipeline to the mentioned area. The most pollutions of sea water which are made by these installations are due to tanks pipelines balancing, pumps fails and balancing of ships in the sides of jetties in order to load products, as well.

3. Sampling:

At first, to observe area’s pollution with oil cuts, an initial visit was carried out in Mahshahr exportation port hence the jetties number one and six -which are in the entering channel to the ends of the path- were opted. Each jetty has 50 meters width. In addition jetty 1 and 6 respectively have 200 and 240 meters length. Water sampling was obtained from the depth of 3metersof gulf by 1.5 liter bottles. After numbering and specification recordings, bottles were sent to labs for related tests. The first stage was assessment of pH, TDS and salt amounts[10].

3.1. Enrichment

Water sampling was done in order to enrich and cultivate existing bacteria in water of the purposed area. The sample of water was placed in nutrition field for forty eight hours so that the bacteria could be able to get compatible with present situation and start to generate. The most common culture media that are used for bacteria cultivation is Blood Agar (B.A).

The second culture media, which is applied for this purpose, is Eosin Methylen Blou (EMB) media. This media is purchased commercially as well. The existing sample in nutrition field was injected to upper plates by injector and there, the bacteria were cultivated in the sample via a strilled loop with the presence of flame under hood. The plates were placed for twenty four hours in incubator at temperature of 37 °C.
3.2. Bacterial Culture Media Study

If bacteria grow only in B.A media, the bacteria are positive bacteria and in the case of growing in EMB media the bacteria are gram-negative bacteria. On the basis of initial studies it was distinguished that the most sea bacteria are gram-negative, and gram-positive bacteria population is usually 10% of total population of bacteria. It is recommended that gram-negative bacteria cell walls have more compatibility with sea area. A various category considering morphological matters in positive bacteria is Bacillus; while the category in negative one is Psedomonace [11, 10]

4. Morphology and Biochemical Characteristics

Rudimentary study was colony morphology on its color and its form. Some biochemical characteristics of bacteria such as reaction to negative coloration, catalyzation and oxidation were put under study and assessment during related tests for distinguishing bacteria species. These results are shown in table 1.

<table>
<thead>
<tr>
<th>Colony’s Features</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>Small and smooth</td>
</tr>
<tr>
<td>Color</td>
<td>Grey</td>
</tr>
<tr>
<td>Gram Staining</td>
<td>Negative</td>
</tr>
<tr>
<td>Gelatinize test</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxidize test</td>
<td>Positive</td>
</tr>
<tr>
<td>TSI test</td>
<td>Alk/Alk</td>
</tr>
<tr>
<td>Nitrate to nitrite</td>
<td>Positive</td>
</tr>
<tr>
<td>Activation</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxidative</td>
<td>Positive</td>
</tr>
<tr>
<td>Formative</td>
<td>OF test with glucose</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
</tr>
</tbody>
</table>

4.1. Biosurfactant Production in Bacteria Culture media

In order to produce biosurfactants, identified bacteria being separated from water are to be placed in another culture media. The new medium is a mixture of salt solution, hydrocarbon nutrients and carbohydrate nutrients. The composition of salt solution is given in table 2.

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>2.2 gr/lit</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.4 gr/lit</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.6 gr/lit</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>0.02 gr/lit</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0 gr/lit</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.08 gr/lit</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.02 gr/lit</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>1.0 gr/lit</td>
</tr>
<tr>
<td>Glucose</td>
<td>2% V/V</td>
</tr>
</tbody>
</table>

The salt solution pH was adjusted on the range between 7.0 to 8.5 close to that of Persian Gulf pH. Nutritious hydrocarbons contain 1.5% v/v light crude oil from Abadan refinery products and nutritious carbohydrates contain 2.0% v/v glucose [12, 13]
4.2. Preparing Substance and Instruments:
All applied chemicals are products of Merck company. The crude oil used, was received from Abadan refinery. Glucose and yeast extract were prepared in microbiologic labs. The applied instruments are as follows:
1. Incubator with shaker (model IKA KS4000 ic control)
2. pH meter
3. Spectrophotometer
4. Mixer
5. Oil content HORIBA
6. Balance
7. Autoclave
8. Laboratory vessels including Erlenmeyer, pipet, graduated cylinder, separating funnel and plates specified for bacteria cultivation
9. Steam bath.

4.3. Biosurfactant Production Stages:
Producing biosurfactants aimed; The culture media mixture including 100 cc of salt solution, carbohydrates nutrients and hydrocarbons nutrients were poured into an Erlenmeyer flask; then - being the flask head covered - the flask was put in Autoclave for 15 minutes at pressure of 15 bar and temperature of 120 °C. After cooling, after adding 10⁷ to 10⁸ bacteria per each mL to the Erlenmeyer flask. That is while bacteria adding occurred under the conditions of purely sterilized and below air chamber hood. The erlenmeyer flask containing the biosurfactant culture media and bacteria was held in an incubator with its shaker on for 7 days at a temperature of 30ºC and revolution of 120 rpm [5].

4.4. Biosurfactant Growth Study Procedure:
During a seven day surfactant production and growth period, the solution pH was checked by a pH meter instrument. In the case of any variation, the initial solution was brought by addition of NaOH and HCL 1N. To study and manipulate the biosurfactants growth, the amount of solution turbidity was measured by a spectrophotometer in a routine schedule duration [11].

4.5. Measurement of Oil Amount in Water:
The measurement of existing oil concentration in sea water sample and adding CCl₄ solvent by a volume equal to that of the obtained volume is performed. Twenty minutes were required for the shaker with a rotation of 2 ⁰cc rpm that all the oil particles which were in emulsion form in water could be absorbed to the solvent. Then this mixture was poured through a separator funnel, two phases were separated from each other and the solvent containing the whole oil is injected into the instrument.

4.6. Measurement of Oil Amount After Adding Biosurfactant:
A definite volume of sample was considered, after adding the produced biosurfactants, it was held in the shaker so as all oil emulsion particles in water to be combined with biosurfactants. After a residence, two phases were separated from each other. By using separating funnel the filtrated water was separated then injected into the instrument to calculate the remaining oil amount.
5. Results and Discussion: Studying effective factors on oil degradation process.

Among pollutant areas physical properties, pH, salinity and applied biosurfactant concentration can be brought in to comprehensive attention.

5.1. pH

Microorganisms usually inhabit in natural pH conditions. The optimum growth of most bactreia happens at a pH range of about 6 to 8.5. Generally too much activity or too much base situation prevents the growth of microorganisms. Sea water pH does not have so much fluctuations while oil often lays between 7.5 to 8.5 pH [14].

To assess the effect of pH on the amount of seperated oil via native bacteria producing biosurfactant, four amounts of pH 7.0, 7.5, 8.0 and 8.5 were defind on initial salt base culture media [12, 16]. The option of pH range of 7.0 to 7.5 was due to recommends of latest researches on this subject. The pH range of 8.0 to 8.5 was due to similarity of culture media conditions to pH of sampling location. Figure 1
In the next step, the pH of culture media was measured by means of a pH meter everyday. Then the amount was adjusted back to initial one. One of the signs of biosurfactant production is a raise in pH of the area.

5.2. Salinity

In order to produce a biosurfactant with the highest efficiency for elimination from the area and separation of crude oil from polluted ecosystem, it is needed that the amount of salt in culture media should be studied in different concentrations. Consequently, four salinity of 2.0%, 1.0%, 0.5% and 0.25% were used according to the observations so that the best salinity where the produced biosurfactants had more effects could be chosen.
In addition to daily manipulation of pH, another index called turbidity in wavelength of 680nm is utilized for addressing the effect of salinity on cell growth of biosurfactants [17]. According to figure 3-A to 3-D, it is derived that inpH=8.5 and salinity of 1.0%, the logarithmic phase of growth starts faster.

5.3. Applied Concentration of Biosurfactant

The applied concentration of produced biosurfactant is one of the other fundamental parameters affecting the separation of oil from polluted area. Dealing concentrations were 0.1%, 0.15%, 0.25% and 0.5% among which the most effect of oil absorption occurred at the concentration of 0.1% of applied biosurfactants[24] as well in their researches introduced 0.1% applied concentration of biosurfactants to be the best applied concentration.

5.4. Oxygen

Presence of oxygen is one of the important conditions for hydrocarbon microbiological separation however, availability of this factor in environmental separation of sea oil hydrocarbons, is often served as a limiting factor [14]. For this reason all the stages of procedure were played on shakers.

5.5. Temperature

Among the other important environmental factors in this research is the temperature of bacteria in incubator and that of the culture media for biosurfactant production where it is heated up[12]. Temperature of most sea waters are often between 20° to 35°C. Environmental separation of oil hydrocarbon in sea has been observed in all conditions of climate. In warm waters, vaporization of toxic hydrocarbons the rate of growth of microorganism increases increases but their toxicity degree decreases. While in waters of lower temperatures oil gets more dense and
this result in a decrease in contact surface area of micro organisms and also a decrease in the rate of environmental separation [14]. The temperature at which heating takes place must be close to the sampling area temperature. The temperature condition of this research for heating of samples in incubator was 32 °C.

5.6. Nutritious Elements (Nitrogen, Phosphorus)

One of the ways to encourage the environmental segregation of hydrocarbon is to adjust nutrition harrassment. Nitrogen and phosphor are the most important nutritious chemicals vital for the growth and reproduction of micro organisms whose amounts from various aspects affect this process. Lots of studies illustrate that the loss of the chemicals can comprehensively reduce the rate of environmental separation. Despite oil hydrocarbons prepare a vast source of carbon and energy for bacteria growing, they have losses in the amounts of nitrogen and phosphor needed for the processes [14]. The effect of produced biosurfactants from native bacteria on oil separation showed that the most amount of oil separation and the best clarity of polluted area happen when we apply these two sources in culture media [19].

To prepare nitrogen sources for production of biosurfactants being effective for oil absorption, a solution of NaNO₃ with a concentration of higher than 1 gr/lit was utilized [13]. For consumption of 1 gr/lit of crude oil the minimum of optimum amounts KH₂PO₄ were considered to be 0.1 gr/lit [1,9] but in upper amounts bacteria enters the logarithmic phase of growth.

6. Bahavior Specification of Biosurfactants in Oil Segregation:

Biosurfactants are amphiphilic compounds that can reduce the surface area and the stresses between surfaces by storages of immisible liquids and can so increase the solubility, environmental ability. They subsequently separate environmental insoluble combinations biologically. Chemical and biological surfactants play an important role in oil recovery and biological separation of pollutions. Biosurfactants have immediate effects on decreasing oil and water inner surface stresses. They are also able to break the viscosity of the oil and to separate water from oil hence. Oil emulsion, which are formed in both cases of oil in water and water in oil through oil exploration, production and recovery processes are representative of a basic form in oil companies [7].

An anti-emulsion process requires recovery of the oil from such emulsions. Traditional methods of anti-emulsion included centrifuge, thermal treating, additions of chemical solutions containing soap and oily acids [7].

Asintebacter and Psedomonas are common anti-emulsion species in bacteria culture mixture [20]. Subsequently producing these two species, biosurfactants have a good effect on separation of oil. Some of the destroyers of chemical emulsions may be polyglycoles, solphonic acid salts and polyhydric alcohols. The chemical materials reach an emulsion drop separation surface and destroy the stresses associated between two phases letting water drops to be coagulate and settle based on gravity [21]. Kind and amount of a suitable emulsion destroyer; the rate of combination of emulsion destroyers to emulsions and the resident time for separation of two phases and settling are important factors in breaking emulsions [7].
A big deficiency in chemical emulsion destroying methods is presence of these destroyers in liquid phase and their elimination from oil phase which may prevent a required emulsion formation in later steps [22]. Paying attention to figure 3D reveals that applied surfactantant the best concentration for oil segregation from polluted area segregation from sample was 0.1% which can bring as this result that in such concentration oil water emulsion had been destroyed and a suitable emulsion between biosurfactant and oil in so low concentration had been taken [18]. Biosurfactants are best known and suitable for emulsion activities even in very low concentration [23].
Figure 3. Study of different biosurfactant content variation influence on separation of oil from media.

7. Taguchi Optimization of the Results

In designing Taguchi test each system deals with independent parameters and different levels. This method simply analyzes the influence of independent parameters and their relation with each other. Among the advantages of this method one can serve the accuracy of tests, minority of variation in response amounts and applicability of result in practice [24]. After designing Taguchi test; using this method for three effective basic working parameters of pH, salinity and applied biosurfactant concentration, nineteen stages of test were carried out whose results are demonstrated in Table 3.
### Table 3. Recommended tests via Tagguchi method and their results

<table>
<thead>
<tr>
<th>pH</th>
<th>Salinity percentage</th>
<th>Biosurfactants concentration percentage</th>
<th>Results (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.25%</td>
<td>0.1%</td>
<td>51.1</td>
</tr>
<tr>
<td>7.0</td>
<td>0.5%</td>
<td>0.15%</td>
<td>46.47</td>
</tr>
<tr>
<td>7.0</td>
<td>1.0%</td>
<td>0.25%</td>
<td>48.8</td>
</tr>
<tr>
<td>7.0</td>
<td>2.0%</td>
<td>0.5%</td>
<td>31.0</td>
</tr>
<tr>
<td>7.5</td>
<td>0.25%</td>
<td>0.15%</td>
<td>50.8</td>
</tr>
<tr>
<td>7.5</td>
<td>0.5%</td>
<td>0.1%</td>
<td>48.0</td>
</tr>
<tr>
<td>7.5</td>
<td>1.0%</td>
<td>0.5%</td>
<td>26.1</td>
</tr>
<tr>
<td>7.5</td>
<td>2.0%</td>
<td>0.25%</td>
<td>50.0</td>
</tr>
<tr>
<td>8.0</td>
<td>0.25%</td>
<td>0.25%</td>
<td>36.1</td>
</tr>
<tr>
<td>8.0</td>
<td>0.5%</td>
<td>0.5%</td>
<td>16.3</td>
</tr>
<tr>
<td>8.0</td>
<td>1.0%</td>
<td>0.1%</td>
<td>22.9</td>
</tr>
<tr>
<td>8.0</td>
<td>2.0%</td>
<td>0.15%</td>
<td>25.9</td>
</tr>
<tr>
<td>8.5</td>
<td>0.25%</td>
<td>0.5%</td>
<td>25.5</td>
</tr>
<tr>
<td>8.5</td>
<td>0.5%</td>
<td>0.25%</td>
<td>21.6</td>
</tr>
<tr>
<td>8.5</td>
<td>1.0%</td>
<td>0.15%</td>
<td>15.2</td>
</tr>
<tr>
<td>8.5</td>
<td>2.0%</td>
<td>0.1%</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Each parameter effect was reported in terms of oil segregation amount from polluted sample in ppm scale. Oil concentration calculation was performed by oil content analysis instrument; gravity meter method were applied according to Betz standard for comparison. According to two parameters pH and consumed concentration of biosurfactant, it was found that it had the best result in separation and absorption of oil. On the basis of received results the best separation is achieved when pH=8.5, Salinity=2.5% and applied surfactant concentration was 0.1%. This is a confirmation for initial tests and also a validity for prevailing results and observations [25], which declare the best result of separation occurs at 0.1% applied concentration. In Figure 4 the effect of each parameter on oil separation was illustrated by applications of Tagguchi test design.

![Figure 4. Effect of parameters pH, salinity and biosurfactant concentration on separation of oil. Significant factor and interaction influences.](image)

### 8. Conclusion

Biosurfactants were applied to clean the polluted areas via the common strategy of being grown in bacterial culture plates and then added continuously to pollution sources. Application of biosurfactant in increasing oil recovery is one of the most important methods of recovering a
substantial amount of oil residue. Additions of biosurfactants increase degradation potential and oil segregation. The results illustrated that bacterial cell production via biosurfactants was much more effective than chemical surfactants. Biosurfactants are more advantageous due to their lower toxicity, native acceptance, biodegradability, and more useful applications in biological and separation of crude oil.

9. References


