American Journal of Life Sciences
2014; 2(6-3): 22-30
Published online January 26, 2015 (http://www.sciencepublishinggroup.com/j/ajls)
doi: 10.11648/j.ajls.s.2014020603.15
ISSN: 2328-5702 (Print); ISSN: 2328-5737 (Online)

Isolation, Cloning and Expression of Rhamnolipid Operon from *Pseudomonas aeroginosa* ATCC 9027 in Logarithmic Phase in *E. coli* BL21

Amin Jafari[1], Jamshid Raheb[1, *], Hassan Bardania[1], Behnam Rasekh[2]

[1]National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[2]Department of Petroleum Biotechnology, Biotechnology Research Center, Research Institute of Petroleum Industry, Tehran, Iran

Email address:
jam@nigeb.ac.ir (J. Raheb)
[*]Corresponding author

To cite this article:
Amin Jafari, Jamshid Raheb, Hassan Bardania, Behnam Rasekh. Isolation, Cloning and Expression of Rhamnolipid Operon from *Pseudomonas aeroginosa* ATCC 9027 in Logarithmic Phase in *E. coli* BL21. *American Journal of Life Sciences.* Special Issue: Microbiology Research. Vol. 2, No. 6-3, 2014, pp. 22-30. doi: 10.11648/j.ajls.s.2014020603.15

Abstract: The rhamnolipid operon from *Pesudomonas* strain with the native promoter was not expressed in logarithmic phase of *Ecoli*. The expression of rhamnolipid in logarithmic phase of growth whether the regulatory elements of the operon are eliminated or not was investigated. The rhamnolipid operon was identified in *Pseudomonas aeruginosa* ATCC 9027 and the rhlAB genes related to rhamnolipid were isolated and amplified by PCR. The PCR product was cloned in pET 23a expression vector and transferred into the *E. coli* BL21. The expression of rhlAB genes was analyzed and our results showed that the synthesis of monorhamnolipid occurred in logarithmic phase. In addition this data demonstrated a higher production of rhamnolipid in recombinant *Ecoli* Bl21compared to that indigenous *Pseudomonas aeruginosa* ATCC 9027.

Keywords: MEOR, Manipulated *E. coli* BL21, Monorhamnolipid, Logarithmic Phase

1. Introduction

Biosurfactants are known for their surface tension reduction activity with two different characteristics: hydrophobic and hydrophilic and therefore possess the ability to penetrate into two different phases to reduce the surface tension [15]. Some bacteria and fungi produce and utilize a group of biological surfactants which are called biosurfactant. *Pseudomonas aeroginosa* produce a kind of glycolipid biosurfactant, known as rhamnolipid. The bacteria apply surfactants to utilize the unusual carbon sources such as crude oil and hexadecane. Rhamnolipid molecule containing two moieties of rhamnose sugar and fatty group[1], a dimmer of two fatty acids that are usually medium long chain. *P. aeroginosa* produce two types of rhamnolipid which differ in the number of rhamnose sugar. The rhamnolipid molecule that has one or two molecule of rhamnose is called monorhamnolipid and dirhamnolipid respectively. This biomolecule is produced by some microorganisms such as *Burkholderia* and *P. aeroginosa* [2] and has a wide range of applications for the bacterial cells. Rhamnolipid biosurfactant can be applied as the wetting agent for friction reducing of bacterium and medium, that leads to easier surface movement for bacterial cells [7, 33]. Furthermore, rhamnolipid-producing bacteria can utilize this biosurfactant for competition in microbial population [1, 13] swarming, altering the cell surface hydrophobicity [4, 3], feeding [16] and etc. This green and biodegradable biosurfactant can functionally acts as synthetic surfactant that has not harmful effects on environment and health. This molecule can also be used in pharmaceutical industry, oil industry, food industry and etc [5]. The application of rhamnolipid in oil industry is due to its capability to remove the oil spills leaked from tankers, and this molecule can also remove heavy metals from the soils [8, 22]. Another application of this biosurfactant in oil industries is based on its ability to reduction of surface tension. In oil reservoirs, there is much crude oil which is viscose and hardly moveable. Running of heavy crude oil to the main reservoir is time consuming and, pumping of such viscose fossil

energy needs too much energy. On the other hand, thermal processes for enhancing oil recovery needs to high amounts of energy [31]. One of the solutions used for that problem is the application of synthetic chemical surfactants. These materials reduce interfacial tension between oil and aqueous phase. Using of the chemical surfactants causes reduction of oil viscosity and enhanced evoking of the heavy crude oil [30]. Application of chemical surfactants is not favorable method due to these methods are costly and have harmful environmental effects. Some of chemical surfactants are carcinogenic agents and interfere in the biological ecosystems [29]. Appling these harmful methods could replace with microbial enhanced oil recovery (MEOR) a moderate method, which applies the microorganisms produce and secret biosurfactants. MEOR is a method that can reduce viscosity of the crude oil for easier and faster extraction. In this method, microbial agents produce and secret some material such as biosurfactant which help evoking of the viscose oil [30].

Additionally as an auxiliary agent, rhamnolipid biosurfactant can be used in bio-desulfurization of oil. Desulfurization of oil is one of the major steps of refining for production of better and more desirable fossil fuel. There are several methods for desulfurization of the oil, such as hydro-desulfurization and oxidative desulfurization. These methods are costly and performed under hazardous conditions (high temperature and pressure) [27]. In addition, they need large amounts of some materials such as hydrogen peroxide (oxidative desulfurization) [14]. Application of rhamnolipid biosurfactant or engineered bacteria that produce rhamnolipid biosurfactant can provide more moderate economical situations for oil desulfurization. In nutshell engineered bacteria which produce and secret biosurfactant are more successful in bio-desulfurization because of accelerating two phase separation step in the process of bio-desulfurization through increasing of emulsification. Rhamnolipid as an effective and strong biosurfactant solely will be a suitable choice for enhancing oil bio-desulfurization [28]. According to the advantages and numerous applications of this molecule, large scale production of rhamnolipid is necessary for the industrial applications. The operon of rhamnolipid-producing enzymes has regulatory elements that can switch on the operon with increasing of bacteria density and increment of their concentration. Thus, in the existence of these regulatory elements the rhamnolipid production is cell number dependant [24, 25, 26]. The operons of the rhamnosyltransferases -enzymes that synthesis the rhamnolipids (mono and dirhamnolipid) - contain a regulatory gene called R gene. The product of this gene inhibits the transcription of operon when the bacterial cell is in logarithmic phase of growth [11]. When the number of cells increases Las quorum sensing system secrets a small signal molecule which is called 3-oxododekanoil homoserin lacton (3-O-C12-HSL) [18]. After the secretion of this signal molecule, another quorum sensing system which called Rhl, will be stimulated to increases the concentration of N-Butanoyl-HSL that can bind to the R protein and alter its conformation [6]. Complex of the R protein and the N-Butanoyl-HSL enhances the transcription of RhlAB and RhlC genes. The resultant mRNAs translated to the rhamnosyltransferase 1 and rhamnosyltransferase 2 which are responsible for synthesizing of monorhamnolipid and dirhamnolipid. According to the previous studies, the bacterial cells need a long period of time to start the secretion of rhamnolipids [24]. The purpose of this study is isolation of genes related to monorhamnolipid from identified bacterium *P. aeruginosa* PG201 [10], their insertion to pET 23a vector and finally cloning of new recombinant plasmid in *E. coli* BL21. Furthermore, production of monorhamnolipid by recombinant bacteria was evaluated and compared with indigenous bacterium *P. aeruginosa* in order to industrial applications.

2. Material and Methods

2.1. Materials

Restriction enzymes and an InsT/A clone PCR product cloning kit were purchased from Fermentas Company (Germany). Molecular weight markers and High pure plasmid purification kit, High pure PCR product purification kit, agarose gel DNA extraction kit were all obtained from Roche (Germany), all the chemical materials from Merck (Germany).

2.2. Bacterial Strains

All used bacterial strains in this research were supplied from NIGEB. *E. coli* BL21strian was applied as the bacterial strain for expression of rhamnosyl transferas 1 and production of monorhamnolipid. *E. coli* BL21 containing pET23a plasmid without RhlAB genes and *P. aeroginosa* PG201 were utilized as the negative and positive control respectively.

Bacterial strains were cultured in two media, Luria Broth (LB) containing 1% NaCl, 1% Peptone and 0.5% of Yeast Extract and a minimal medium including 0.1% Yeast Extract, 0.3% NaNO3, 0.25% K2HPO4 and 0.025% MgSO4.

2.3. SDS Page

10% resolving gel was applied for separation of protein A (32Kd) and protein B (41Kd). The separation of the protein components is performed in recombinant strain in a proper voltage and the gels were stained immediately. The destaining of the gel was done over night.

2.4. PCR Method

PCR was used for the identification and amplification of rhamnolipid genes. The proper primers were designed and restriction sites for EcoRI and HindIII introduced at the 5' ends of forward and reverse primers. Fast-start Taq DNA polymerase and a high fidelity kit were used for PCR. The gene amplification kit was provided by Corbett Research and used an annealing temperature of 65˚C. The PCR product in

the 2 kb region was purified and concentrated using a high pure PCR product purification kit.

2.5. Cloning

The PCR product containing monorhamnolipid genes was cloned into the T vector. This plasmid has carry out α-complementation. Cloning was carried out with an Ins T/A clone PCR product cloning kit that can clone fragments with sticky ends. The ligation stage was carried out by adding T4 DNA ligase to a solution containing the PCR product and the T vector for overnight at 16˚C. The ligation product was transformed into competent bacteria (*E. coli* BL21). After satisfied cloning of monorhamnolipid genes in T/A vector, monorhamnolipid genes were cloned in pet 23a vector for expression of cloned genes. Transformed bacteria (100 µl) were spread on an LB agar plate containing ampicillin, IPTG, and incubated at 37˚C. The presence of the monorhamnolipid genes was shown in the colonies by plasmid extraction and PCR.

2.6. Sequencing

The nucleotide sequence of the PCR bands was directly determined by automated sequencing 3700ABI (Gene fanavaran, Macrogene Seoul,Korea).

2.7. CTAB Plates

CTAB plates were used to detect the presence of rhamnolipid biosurfactant in the environment of the bacteria that produce rhamnolipid. CTAB (cethyle three ammonium bromide) as a cationic surfactant can react with the rhamnolipid as an anionic-surfactant rhamnolipid and leads to appearance of a blue-green color halo around of the rhamnolipid-producing colonies. The complex of rhamnolipid and CTAB is detected in the presence of methylen blue dye. This medium was composed of 0.5% yeast extract, 1% peptone, 1% NaCl 1.5% agar for solidifying of the medium IPTG and it also containing CTAB and methylen blue in ratio of 0.02% and 0.0005 %.

Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was applied for the separation of mono and di-rhamnolipid from each other. A mixture of chloroform, methanol and acetic acid 20% (65, 25, 2 ml) was applied as the mobile phase. The mobile phase runs and carries the material according to their tendency of them to the mobile phase. The silica sheets are utilized as the stationary phase of this chromatography. Using mobile phase, the materials that have more hydrophobicity can move more than the materials that have less hydrophobicity. The spots were located on the silica sheet, dried and the silica sheet was placed in the mobile phase. Moving of the mobile phase causes the separation of the fractions which are in the spots. When the mobile phase arrived in the top of sheet, the sheet was dried and treated with sulfuric acid/methanol (85/15 %), and then it dried again. This reagent alters the rhamnose part of monorhamnolipid biosurfactant to methyl furfural. The sheet was placed in oven with 120˚C to appearance the fractions. The rhamnolipid fraction is appeared with a light green color.

2.8. Oil Displacement

Oil displacement test is a rapid test can relatively determines the amount of a biosurfactant in the cell free bacterial supernatant. The supernatant of cultured biosurfactant-producing bacteria can make a colorless halo on the oil layer which spread on the water. The diameter of this colorless halo can relatively demonstrate the amount of biosurfactant in supernatant. The soups of bacteria (manipulated bacteria, *P. aeroginosa* PG201 and negative control bacteria) were separately centrifuged (in 6000 rpm) and the resultant supernatants used for oil displacement. Addition 10 µL of this supernatant on the surface of oil layer that is abroad on water dish (30 µL of the crude oil spread on water in a 10 cm plate dish), causes a halo on the oil surface. The diameter of the halo is dependent to the amount of biosurfactant in cell free soup.

2.9. Rhamnolipid Extraction

In an acidic environment the carboxyl group of the fatty part of the biosurfactant can absorb the hydrogen ions, and the net charge of the molecule will be zero in low pH. Therefore, biosurfactant molecules can sediment during an overnight, with acidifying (pH = 2) and cooling the cell free soup.

E24 Test

E24 test can determines the emulsification power of a surfactant. monorhamnolipid solution 1gr/L was applied for E24 test in different pH and salinity conditions (this monorhamnolipid was obtained from manipulated bacteria and not purified with silica gel column). In this assay an equal amount of rhamnolipid solution and hexadecane was gently vortexed (120 sec) and the resultant suspension incubated 24 hours in room temperature. The ratio of the height of emulsified layer to the total height must multiply 100 to result E24 index of the biosurfactant.

Tensiometry

The surface tension of cell free supernatant was measured by tensiometer K9 Kruss (Germany).

Column purification

The extracted rhamnolipid by acidification of the cell free supernatant of the manipulated bacteria will not be pure. Elimination of the impurities of this extracted biosurfactant provides a pure monorhamnolipid which is suitable for the machinery analysis. First 10 gr of silica powder was added to 100 mL of chloroform and this mixture was stirred 10 min to produce an equal suspension. The suspension poured into a glass column and the chloroform was allowed to exit. This action allows to the silica powder to compact and makes an equal gel layer for the purification of the extracted monorhamnolipid. 0.4 gr impure monorhamnolipid was dissolved in 2 mL of chloroform/methanol (1/1) and loaded on the top of the gel layer. The column was washed with 300 mL chloroform to eliminate the fatty impurities. After

chloroform washing, 450 mL of a solution containing chloroform and methanol in ratio of 50/5 was applied to elute pure monorhamnolipid.

FTIR Analysis

FTIR is a method that demonstrates the existence of chemical bonds due to their absorbance of infra red waves. Every chemical bond can absorb infra red waves by a definite energy and wave length. If the frequency of the infra red wave be equal with the frequency of the chemical bond, the chemical bond can absorb this infra red wave. The extracted monorhamnolipid was purified and a water-free tablet of sample analyzed by Bruker FTIR instrument.

Electrospray ionization (ESI) analysis

ESI analysis was performed to determining of definite mass of resultant monorhamnolipid. For that, the extracted and purified sample was analyzed by ESI neg Arginin 150-1300 method in MeOH/DCM 1:1 by instrument ICR Apex-Qe.

3. Results

Polymerase Chain Reaction (PCR)

The PCR was performed with designing primers and using genomic template from pseudomonas aeroginosa PG201. The primers were designed to amplify the rhlAB genes without regulatory elements. The result of partially sequencing and blasting of this amplified fragment (RhlAB gens) confirmed existence of RhlAB genes .

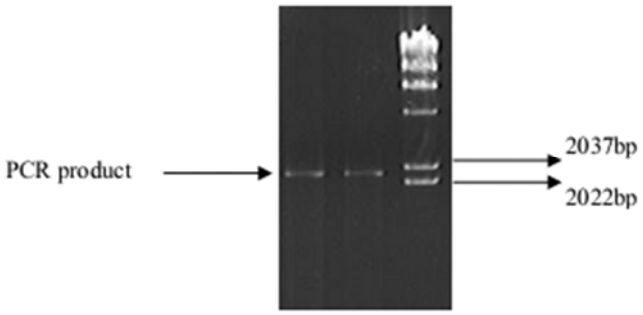

Figure 1. *PCR production for RhlAB genes of P. aeruginosa ATCC 9027*

Over expression of rhlAB operon

The over expression of rhlAB genes were studied after cloning and sequencing of the PCR fragment in T/A vector and sub cloning in pet 23a expression vector in E.coli BL21. The whole cell protein were extracted by boiling and centrifugation of recombinant bacteria. This extract were run on SDS gel for separating and detecting of two parts of monorhamnolipid complex. Monorhamnolipid complex consists of two protein A (32 Kd) & B(41 Kd) which are coded by RhlA & RhlB genes. The SDS page test demonstrated first part of RhlAB promoter (protein A) has expression sharply. The second protein , protein B was not detected obviously on the gel. Transcription of first gene which encodes 32 Kd protein is effected by T7 RNA Polymerase of pet 23a vector and also the native RBS of second gene that encodes 41 Kd protein is less known for *E.coli* BL21 ribosomes.

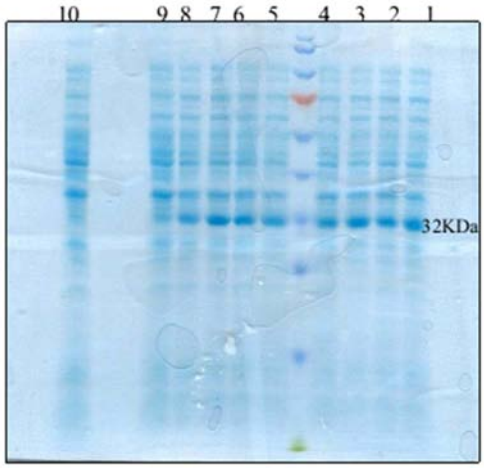

Figure 2. *Electrophoresis of Proteins of induced manipulating bacteria compared with negative control. Numbers of 1-9 are concentrations of 0.1 – 0.9 mM of IPTG. Number 10 is negative control cells.*

CTAB medium test

CTAB medium containing cethyl three ammonium bromides and methylen blue is a medium for detection of rhamnolipid producing bacteria. CTAB medium test demonstrated the manipulated bacteria produces rhamnolipid. In this test *P. aeroginosa* PG201 and *E. coli* BL21 (without RhlAB genes) were respectively applied as the positive and negative controls. After 48 hour incubation in 37ºC the plates were placed in cold room for 48 hour to appear the blue-green halo around of the rhamnolipid-producing colonies. This method showed that the manipulated bacteria produce rhamnolipid biosurfactant and secrets this biosurfactant around of colony. However, halo around the negative control bacteria was not observed.

Figure 3. *The CTAB plate method demonstrated the production and secretion of the rhamnolipid biosurfactant around of manipulated bacteria (Number 2) and pseudomonas aeroginosa (number 1). The negative control does not produce rhamnolipid biosurfactant (Number 3).*

Thin Layer Chromatography (TLC) analysis

The obtained result from TLC analysis can demonstrates the presence of monorhamnolipid in the extracted rhamnolipid from cell free supernatant of manipulated bacteria. A crude extract of rhamnolipid from *P. aeroginosa* was applied as the control for monorhamnolipid and di-

rhamnolipid. At first, the spots of crude rhamnolipid and extracted rhamnolipid were loaded on the silica sheet, then the mobile phase (chloroform/methanol/acetic acid 20% in amounts of 65,15,2 mL respectively) was run and fractioned the extracted rhamnolipid. After the separation of fractions, the TLC sheet was placed into H2SO4 and methanol (with ratio 85 to 15 %) for seconds. Finally heating of sheet for several minutes in 120 ºC causes appearance of the monorhamnolipid and di-rhamnolipid in different levels on the silica sheet. Two green spots -with RF 0.29 and 0.49 related to di-rhamnolipid and monorhamnolipid respectively- were detected in the control side of silica sheet, and a green spot with RF 0.49 was observed in the side of our sample. Therefore, the presence of monorhamnolipid was confirmed by TLC method.

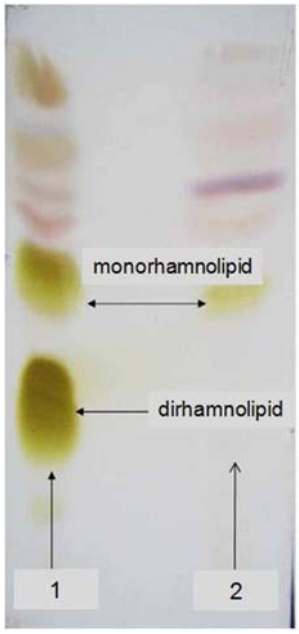

Figure 4. *The result of TLC method demonstrated presence of monorhamnolipid biosurfactant in the extract of the manipulated bacteria supernatant. The left column is the extracted crude rhamnolipid of Pseudomonas aeroginosa as our positive control.*

E24 Test

Obtained results from this analysis demonstrated emulsification power of impure monorhamnolipid is obviously related to pH condition, like the other ionic surfactant. The impure monorhamnolipid had better emulsification power around pH 7 and 8 in this test. The best E24 index for impure monorhamnolipid was gained around pH 8.

FT-IR analysis

FT-IR analysis was used to evaluation of the pure extracted monorhamnolipid from the manipulated bacteria around and acquired results showed that the 3380 peak (A) is for stretching free OH in the compound. The region between 2825-2925 peaks (B) is related to aliphatic bond stretch. Moreover, 1737 peak (C) is for the C=O stretch due to the ester functional group, and 1455.52–1380.65 peaks (D) and 1033-1300 peaks are showed bending of O–H bands in the carboxylic acid group and C–O–C stretching in the rhamnose respectively.

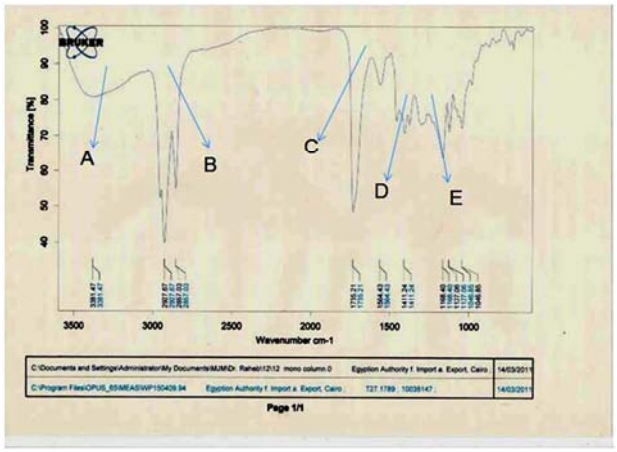

Figure 5. *FTIR analysis of the pure extracted monorhamnolipid from the manipulated bacteria around .the 3380 (A) is for stretching free OH in the compound. The region between 2825_2925 (B) related to aliphatic bond stretch.1737(C) is for the C=O stretch due to the ester functional group. 1455.52–1380.65(D) bending of O–H bands in the carboxylic acid group and 1033_1300 is related for C–O–C stretching in the rhamnose.*

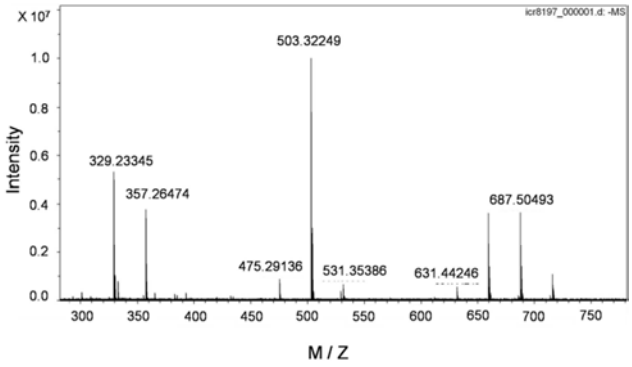

Figure 6. *ESI analysis of purified monorhamnolipid. 475.29136 peak is related for monorhamnolipid with a fatty part which contains 8 and 10 carbon fatty acids. 503.32249 peak is related for monorhamnolipid with 10 and 10 carbon fatty acids with chemical formula of C26H47O9. 531.35386 peak is related for monorhamnolipid with 10 and 12 carbon fatty acids.*

Electrospray ionization (ESI) mass spectrometry analysis

Electrospray ionization (ESI) mass spectrometry was performed to determine molecular mass of extracted and purified molecules. Obtained result showed that peak at m/z 503 is related to a monorhamnolipid with two 10 carbon saturated fatty acids. This monorhamnolipid contains most volume of monorhamnolipid biosurfactant which were extracted from manipulated bacteria.

Oil displacement assay

By this method the amount of secreted biosurfactant was relatively detected. Obtained result of oil displacement demonstrated that manipulated bacteria secreted monorhamnolipid biosurfactant in logarithmic phase of growth. Monorhamnolipid production in manipulated bacteria is higher than the positive and negative controls. The manipulated bacterial soup was collected at hours 2, 6 and 13 after the induction of the recombinant cells (also the soup of

negative and positive controls were collected at these times). After collecting of the bacterial soups, they were centrifuged and the resultant supernatants were applied for the oil displacement method. Obtained results from oil displacement method showed that the production of monorhamnolipid biosurfactant in manipulated bacteria is higher than the positive and negative control. The production of monorhamnolipid biosurfactant was occurred in logarithmic phase of manipulated bacterial growth and in LB medium is higher than the minimal medium. On the other hand, monorhamnolipid production by manipulated bacteria increased from 2 to 13 hour after culturing the bacterial cells.

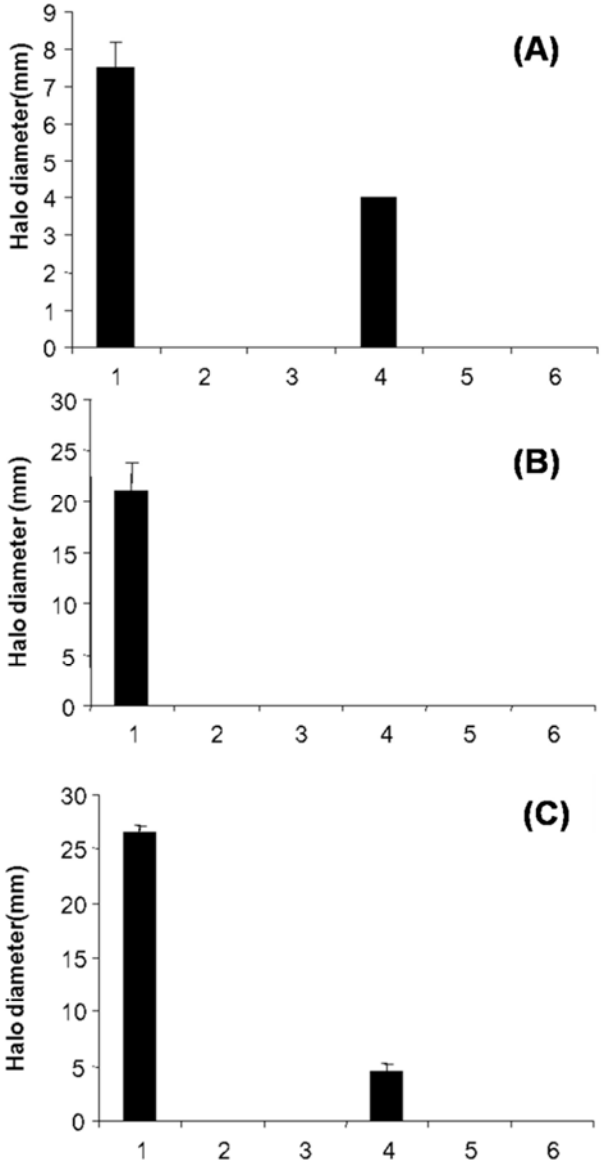

Figure 7. *Result of oil displacement after 2 (a), 6 (b) 13 (c) hour after induction: 1-manipulated bacteria in LB, 2-positive control in LB, 3-negative control in LB, 4-manipulated bacteria in minimal medium, 5-positive control in minimal medium, 6-negative control in minimal medium.*

Tensiometry

Tensiometry data showed that the surface tension of the cell free supernatant of manipulated bacteria is lower than the negative and positive controls in both media. The supernatants were gained when the cells were in the logarithmic phase.

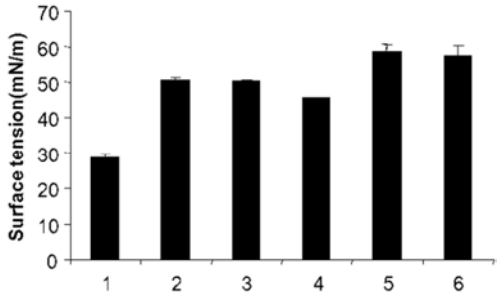

Figure 8. *Surface tension obtained by tensiometry analysis of the cell free supernatant of 1-manipulated bacteria in LB, 2-positive control in LB, 3-negative control in LB, 4-manipulated bacteria in minimal medium, 5-positive control in minimal medium, 6-negative control in minimal medium.*

4. Discussion

Nowadays fossil energies such as oil are very expensive and hard to gain. In an oil reservoir after the customary extraction of the oil, much heavy crude oil will be remained in the rock pores of around the producing well. By the customary extraction of the oil, 20% of total capacity of the reservoir will be evoked. by using of water steam as an auxiliary agent, 5% of total remained oil after first extraction will be extracted. Over 70% of total oil is still in the reservoir [21]. Remaining of so much heavy crude oil as an expensive and dwindling fossil energy will be a drastic problem for oil industries. There are many oil reservoir in the world which are needed a better method for much oil production. Chemical surfactants as auxiliary agents are used for more production of a reservoir. Usually more than one chemical surfactant is used to evoke more oil. A range of Different synthetic surfactants are utilized for mobility of different hydrocarbons which are existed in crude oil [20]. This method is an expensive method and it is unfavorable for our environment too. Chemical surfactants can interfere in biological ecosystems and some of them are carcinogen [22]. Additionally using of the chemical surfactants enhances the extracted oil cost. Enhancement of this fossil energy cost will raise the cost of other oil products. Microbial enhancement oil recovery (MEOR) as a low cost and environmental friendly method is attracted scientist's attention. In this method low cost materials are used and the product of microbial population has not harmful effect on our environment. *P. aeroginosa* is a micro organism that produces Rhamnolipid as a biosurfactant can be useful in MEOR process. As a biosurfactant, rhamnolipid like many of biosurfactants has high activity with low concentration. Rhamnolipid has a low critical micelle concentration (CMC) 10-30 mg/L, it can be effective in low amounts. *P. aeroginosa* is an opportunistic pathogen that causes many diseases. A more secure organism such as *E. coli* is better for industrial purposes. Environmental pollution with *E. coli* is

more secure than *P. aeruginosa* pollution. We used *E. coli* Bl21 for expression of monorhamnolipid operon and production of monorhamnolipid biosurfactant. With elimination of regulatory elements of monorhamnolipid operon, we were expected higher production of monorhamnolipid biosuffactant in a more suitable micro organism for industrial purposes like oil industries.

The production and secretion of monorhamnolipid biosurfactant was analyzed by different methods such as TLC, FTIR and ESI and obtained results showed that manipulated bacteria in this research produce monorhamnolipid biosurfactant. In *P. aeroginosa* the active complex of rhamnolipid biosurfactant synthesizer is located in preplasmic and inner membrane of the bacterium [23]. Production and secretion of monorhamnolipid by manipulated bacterium *E. coli* BL21 demonstrated that the active monorhamnolipid synthesizer complex exists in manipulated bacteria in a suitable situation that can correctly produce monorhamnolipid biosurfactant and secretion of monorhamnolipid biosurfactant occurs. The secretion of monorhamnolipid biosurfactant can also demonstrate that the cellular membrane structure and properties of manipulated bacteria is appropriate for exiting of the biosurfactant from the manipulated *E. coli* BL21bacterium. ESI mass espectometry method demonstrated that the produced monorhamnolipid by the manipulated *E. coli* BL21 is mainly a monorhamnolipid which has two 10 carbon saturated fatty acids. *P. aeroginosa* as a natural host of rhamnolipid genes prefer to use 10 carbon saturated fatty acids in its rhamnolipids (mono and dirhamnolipid) [17]. These results showed manipulated bacteria are similar with *P. aeroginosa* bacterium in selecting fatty acids for production of rhamnolipid biosurfactant. The acquired results from culturing of manipulated bacteria in two media (LB medium and minimal medium) exhibited the production of monorhamnolipid biosurfactant in manipulated bacteria is higher than the other bacteria (negative and positive control). The supernatants of manipulated, negative and positive control bacteria cells are collected in their logarithmic phase and oil displacement method demonstrated that monorhamnolipid production of manipulated bacteria cells in LB medium is higher than the minimal medium. Also these results showed that monorhamnolipid production by recombinant bacteria is higher than the positive control *P. aeroginosa* PG201. Higher production of monorhamnolipid in LB medium by manipulated bacteria verified that monorhamnolipid production is not dependant to the food conditions like *P. aeroginosa* [9]. The production of rhamnolipid biosurfactant by *P. aeroginosa* in some minimal media is higher than the rich media like LB. In this experiment, minimal medium was applied to trigger the positive control bacteria for better and higher production of rhamnolipid biosurfactant. With applying the minimal medium, we supposed that the production of rhamnolipid biosurfactant by *P. aeroginosa* should be higher than manipulated bacteria but the results were against of our prediction. Manipulated bacteria produced higher amount of biosurfactant than positive and negative controls in minimal and LB media. This production was increased by passing time and is not dependant to the food conditions such as iron and nitrogen concentration [19, 12]. Food starvation is not effective on the production of monorhamnolipid biosurfactant in manipulated cells that have truncated operon. Addition to the oil displacement method, obtained results from Tensiometry emphasized above results. Therefore, tensiometry and oil displacement methods exhibited that the production of monorhamnolipid in manipulated bacteria is occurred in logarithmic phase, however *P. aeroginosa* produces and secrets rhamnolipid biosurfactant in stationary-phase of growth [24]. Higher production of monorhamnolipid biosurfactant in manipulated bacteria demonstrated that production is not dependant to the time and food conditions. For industrial production of a biological product in fermentor, it is important that the production time be short as possible. In a shorter time of production, the cost of our product will be more economical. Also, with production of monorhamnolipid biosurfactant by manipulated bacterial cells in logarithmic phase of growth, the rate of rhamnolipid-synthesizing is in a high speed. In logarithmic phase of bacterial growth, bacterial cells are in a situation that allows them to be in a highest biosynthesizing rate. Therefore, manipulated bacterium in this research is cost-effective and has high efficiency for industrial applications.

2881	cgttctaccg	ccgcggcctg	caggcctacc	tggcctggca	gaagcagaca	cgccagtgga
2941	tcgatgaaag	ccacttgaac	gacgatgatc	gagcccgcgc	ccacttcctg	ttcaacctga
3001	tcaacgatgc	gctggcgccc	agcaactcgc	tgctcaatcc	gctggcggtc	aaggagctgt
3061	tcaacaccgg	cggccagagc	ctggtacgcg	gcgtggccca	cctgctcgac	gacctgcgtc
3121	acaacgatgg	gctgccgcgt	caggtggacg	agcgcgcctt	cgaagtgggc	gttaacctgg
3181	ccgcaacccc	tggcgcagtg	gtatttcgca	acgagctgct	agagctgatc	cagtactcgc
3241	cgatgagcga	aaagcagcac	gcacgcccac	tgctggtcgt	gccgcctcag	atcaaccggt
3301	tctacatctt	cgacctcagc	gcgaccaaca	gcttcgtcca	gtacatgctc	aaaagcggct
3361	tgcaggtgtt	catggtcagt	tggagtaacc	ccgacccacg	ccaccgtgaa	tggggccttt
3421	ccagctatgt	gcaagccctg	gaggaagcgc	taaatgcctg	ccgcagtatc	agcggcaacc

Acknowledgments

This study was financially supported by National Institute of Genetic engineering and Biotechnology (NIGEB), Tehran, Iran. The authors thank Dr. Habib Abbasi for consulting in some parts of this work.

3481	gcgaccccaa	cctgatgggt	gcctgtgccg	gcggcctgac	catggccgca	ctgcaaggcc
3541	atctgcaagc	caagaagcaa	ttgcgccggg	tacgcagtgc	cacctatctg	gtcagctggc
3601	tggacagaaa	gttcgaaagc	ccggccagcc	tgttcgccaa	tgagcagacc	atcgaagcgg
3661	ccaagcgccg	cttctatcag	cgcggcgtgc	tggacggtgg	cgaagtggcg	cggatcttcg
3721	cctggatgcg	gcccaacgac	ctgatctgga	actactgggt	caacaactac	ctgctcggca
3781	agacgccgcc	ggcgttcgac	atcctgtact	ggaatgccga	cagcacgcgc	ctgcccgccg
3841	cgctgcatgg	cgacctgctg	gagtttttca	agctcaaccc	cttgacctac	gcgtccgggc
3901	tggaggtgtg	cggtaccccg	attgacctgc	agcaggtcaa	tatcgacagc	tctaccgtgg
3961	ccggcagcaa	cgaccacatc	acgccatggg	acgcggtgta	ccgctcggcc	ttgctgctgg
4021	gtggcgagcg	gcgcttcgtg	ctggccaaca	gcgggcatat	ccagagcatc	atcaacccgc
4081	caggcaaccc	caaggcctac	tacctggcca	accccaagca	gagcagcgac	ccacgcgcct
4141	ggttccacga	cgccaagcgc	agtgaaggca	gctggtggcc	gttgtggctg	gggtggatca
4201	ccgcacgctc	cggcctgctc	aaggcgccgc	gcactgaact	gggcaacgcc	acttacccac
4261	cgctaggccc	cgcgccaggc	acctacgtgc	tgacccgatg	a	

References

[1] Abalos, A., Pinazo, A., Infante, M. R. M., Casals, García, F. and Manresa, A., 2001. Physicochemical and antimicrobial properties of new rhamnolipids produced by Pseudomonas aeruginosa AT10 from soybean oil refinery wast. Langmuir 17 (5): 1367-1371.

[2] Abdel-Mawgoud, A. M., Lépine, F. and Déziel, E., 2010. Rhamnolipids: diversity of structures, microbial origins, and roles." Appllied Microbiology and Biotechnology 86(5): 1323-1336.

[3] Al-Tahhan, R. A., Sandrin, T. R., Bodour, A. A. and Maier, R. M., 2000. Rhamnolipid-induced removal of lipopolysaccharide from Pseudomonas aeruginosa: effect on cell surface properties and interaction with hydrophobic substrates. Appllied Environmental Microbiology 66(8): 3262-3268.

[4] Arino, S., Marchal, R. and Vandecasteele, J. P., 1998. Involvement of a rhamnolipid-producing strain of Pseudomonas aeruginosa in the degradation of polycyclic aromatic hydrocarbons by a bacterial community. Journal of Applied Microbiology 84(5): 769-776.

[5] Banat, I. M., Makkar, R. S. and Cameotra, S. S., 2000. Potential commerical applications of microbial surfactants. Appllied Microbiology and Biotechnology 53(5): 495-508.

[6] Bjarnsholt, T. a. G., M., 2007. The role of quorum sensing in the pathogenicity of the cunning aggressor Pseudomonas aeruginosa. Anal Bioanal Chem. 387(2): 409-414.

[7] Caiazza, N. C., Shanks, R. M. and O'Toole, G. A., 2005. Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. Journal of Bacteriology 187(21): 7351-7361.

[8] Catherine, N. M., Raymond, N. Y. and Bernard, F. G., 2001. Heavy metal removal from sediments by biosurfactants. Journal of Hazardous Materials 85(1-2): 111-125.

[9] Chayabutra, C., Wu, J. and Ju, L. K., 2001. Rhamnolipid production by Pseudomonas aeruginosa under denitrification: effects of limiting nutrients and carbon substrates. Biotechnology Bioengineering 72(1): 25-33.

[10] Gallardo, M., Ferrandez, E. A., De Lorenzo, V., Garcia, J. L. and Diaz, E., 1997. Designing recombinant Pseudomonas strains to enhance biodesulfurization. Journal of Bacteriology 179(22): 7150-7160.

[11] Gerardo, M., Katy, J., Brenda, V. and Gloria, S.C., 2003. Mechanism of Pseudomonas aeruginosa RhlR transcriptional regulation of the rhlAB promoter. Journal of Bacteriology 185(20): 5976-5983.

[12] Glick, R., Gilmour, C., Tremblay, J., Satanower, S., Avidan, O., Déziel, E., Greenberg, E. P., Poole, K. and Banin, E., 2010. Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in Pseudomonas aeruginos. Journal of Bacteriology 192(12): 2973-2980.

[13] Haba, E., Pinazo, A., Jauregui, O., Espuny, M. J., Infante, M. R. and Manresa, A., 2003. Physicochemical characterization and antimicrobial properties of rhamnolipids produced by Pseudomonas aeruginosa 47T2 NCBIM 40044. Biotechnology and Bioengineering 81(3): 316-322.

[14] Jiang, Z., L. H., Zhangy, H., Li, C., 2011. Oxidative Desulfurization of Fuel Oils Chinese Journal of Catalysis 32: 707–715.

[15] 15 Karanth, N. G. K., Deo, P. G. and Veenanadig, N. K., 1999. Microbial production of biosurfactants and their importance. Current Science 77(1): 116–123.

[16] Koch, A. K., Käppeli, O., Fiechter, A. and Reiser, J., 1991. Hydrocarbon assimilation and biosurfactant production in Pseudomonas aeruginosa mutants. Journal of Bacteriology 173(13): 4212-4219.

[17] Kun, Z. and Charles, O. R., 2008. RhlA converts b-hydroxyacyl-acyl carrier protein intermediates in fatty acid synthesis to the b-hydroxydecanoyl-b-hydroxydecanoate component of rhamnolipids in Pseudomonas aeruginosa. Journal of Bacteriology 190(9): 3147-3154.

[18] Latifi, A., Foglino, M., Tanaka, K., Williams, P. and Lazdunski, A., 1996. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Molecular Microbiology 21(6): 1137-1146.

[19] Luis, H., Guerra S., Othmar K. and Armin F., 1996. Dependence of Pseudomonas aeruginosa continous culture biosurfactant production on nutritional and environmental factors. Applied Microbiology and Biotechnology 24(6): 443-448.

[20] Mazaheri-Assadi, M., Tabatabaee, M. S., (2010). Biosurfactants and their Use in Upgrading Petroleum Vacuum Distillation Residue: A Review. Int. J. Environ. Res. 4: 549-572.

[21] Meyer, R. F. and Attanasi, E. D., 2003. Heavy Oil and Natural Bitumen – Strategic Petroleum Resources. U.S. Geological Survey, Fact Sheet 70-03.

[22] Mulligan C. N. 2005. Environmental applications for biosurfactants. Environmental Pollution 133(2): 183-198.

[23] Ochsner, U. A., Fiechter, A. and J. Reiser, 1994. Isolation, characterization, and expression in Escherichia coli of the Pseudomonas aeroginosa rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. Journal of Biological Chemistry 269: 19787_19795.

[24] Ochsner, U. A., R., J., 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in Pseudomonas aeruginosa. Proceedings of the National Academy of Sciences (PNAS) 92(14): 6424-6428.

[25] Pearson, J. P., Pesci, E. C. and Iglewski, B. H. 1997. Roles of Pseudomonas aeruginosa las and rhl quorumsensing systems in control of elastase and rhamnolipid biosynthesis genes. Journal of Bacteriology 179(18): 5756-5767.

[26] Pesci, E. C., Pearson, J. P., Seed, P. C. and Iglewski, B. H. 1997. Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. Journal of Bacteriology 179(10): 127-3132.

[27] Philp, J, C, T. Y., Guerinik, K., Al-Saleh, M, A., Sanders P, F., 2008. From Petroleum Microbiology to Biotechnology: Prospects for Saudi Aramco. Saudi Aramco Journal of Technology: 87-97.

[28] Raheb, J, H. M., Saadati, M, Rasekh, B, Memari, B., 2009. The enhancement of biodesulfurization activity in a novel indigenous engineered Pseudomonas putida. Iran Biomed J. 13: 207-213.

[29] Rosenberg, E., R. E. 1999. High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol 52: 154-162.

[30] Singh, A, V. H. J. and Ward, O. P., 2007. Surfactants in microbiology and biotechnology: Part 2. Application aspects. Biotechnol Adv. 25: 99-121.

[31] Teodoriu, C., F. G. and Espinel, A., 2007. Letting Off Steam and Getting Into Hot Water – Harnessing the Geothermal Energy Potential of Heavy Oil Reservoirs. 20th World Energy Congress–Rome.

[32] Tremblay, J., Richardson, A. P., Lépine, F. and Déziel, E., 2007. Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour. Environmental Microbiology 9(10): 2622-2630.