International Journal of Molecular and Clinical Microbiology 7(2) (2017) 855-868

International Journal of
Molecular and Clinical Microbiology

Cloning and expression of rhl AB operon under the control of tac promoter in *E. coli*

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ARTICLE INFO

Article history:
Received 5 September 2017
Accepted 1 November 2017
Available online 1 December 2017

Keywords:
Biosurfactant;
Rhamnolipid;
E. coli BL12;
E. coli DH5α;
Transformation

ABSTRACT

Today, efforts go towards the replacement of chemical surfactants by natural biological biosurfactants (biosurfactant), as these materials are not carcinogenic and highly compatibile with the environment. One of the main classes of biosurfactants is rhamnose-containing glycolipid biosurfactant (rhamnolipids). This type of biosurfactants can be applied in many industries such as oil industry, pharmaceutical industry, food industry and esc. In the present study, with elimination of regulatory elements of monorhamnolipid operon, the suitable construct was made and transformed to *E. coli* BL12 and *E. coli* DH5α competent cells. Then the production of monorhamnolipid in recombinant *E. coli* BL12 harboring pET 12a vector and mono and di-rhamnolipid biosurfactant in recombinant *E. coli* DH5α harboring pTrc 99A evaluated by CTAB plate and thin layer chromatography (TLC). The relative production of mono and dirhamnolipid biosurfactant was determined by oil spreading method. Finally, the efficiency of the extracted biosurfactant was verified by E12 method in different salinity and pH conditions.

1. Introduction

Surfactants are known as surfactants, consisting of two hydrophilic and hydrophobic components. The presence of two components with opposite natures results in the penetration of biosurfactant in two hydrophilic and hydrophobic phases. Today, surfactants are the most widely used materials in various industries. Surfactants disrupt biological ecosystems. Some of these materials are carcinogenic and often toxic. Chemical surfactants are not biocompatible and this is one of the biggest disadvantages of these materials. On the contrary, biosurfactants have biological origin and lack many disadvantages mentioned for chemical surfactants. The chemical structure of these materials is such that they can be decomposed by other microorganisms. Biosurfacants are classified based on their chemical composition and microbial origin. Generally, the structure of a biosurfactant consists of a hydrophilic part including amino acids or anionic and cationic peptides or mono-, di-, or poly-saccharides, and a hydrophobic part including saturated or unsaturated fatty acids (Abolos et al., 2001; Abde-Mawgoud et al., 2011; Abdel-Mawgoud et al., 2010; Al-Arji et al., 2007). In biosurfactants, the hydrophilic part has the most diversity; it produces the wide

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spectrum of biosurfactants' performance in various aspects (Muligan, 2005; Henkel et al., 2017) and, accordingly, classifies biosurfactant. (Desai and Banat, 1917).

Rhamnolipids are one of the most famous types of glycolipid biosurfactants that were first discovered, isolated and described in 1947 by Jervis and Jhonson in pseudomonas pyocyanin bacteria. This biosurfactant consists of a sugar part and a lipophile part of the body, which is usually a dimer of 10-carbon-hydroxyl-fatty acids. Rhamnolipid biosurfactant is most commonly found in the forms of monorhamnolipid and dirhamnolipid. L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoat and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate are known as rhamnolipids 1 and 2, respectively (Adetunji et al., 2017). Rhmnolipid biosurfactant is a highly water-soluble compound that also has a high solubility in polar solvents such as alcohols. (Edward., 1965) Burger et al. Showed that the biosynthesis route of the Ramnolipid molecule results from the binding of Ramenose sugar to dimer fatty acids (Burger et al., 1963).

Two operons are related to the biosynthesis of two major rhamnolipids (dirhamnolipid and mono-rhamnolipid) (Rahim et al., 2001). The first and second operons are responsible for the biosynthesis of monorhamnolipid and dirhamnolipid, respectively. The operon which is responsible for monorhamnolipid biosynthesis consists of RhlI, RhlA, RhlB, and RhlR.

In previous reports, the beginning of rhamnolipid biosurfactant biosynthesis has been stated as a nutritional deficiency (Bauer et al., 2006; Bazire and Dufour., 2014; Burger et al., 1963). The reports indicate that the source of nitrogen is effective in the stimulation and the amount of rhamnolipid biosurfactant secretion in pseudomonas aeruginosa. As shown, the bacteria that use Nitrogen Nitrate source synthesize more amount of rhamnolipid biosurfactant than those that use ammonium as a source of nitrogen (Arino et al., 1996).

In addition, iron ion deficiency is very effective in the production of rhamnolipid biosurfactant so that low iron ion media can be effective in transcribing and expressing LasIR and RhLIR genes (Duan and Surette, 2007). Researchers have also shown that under nitrogen shortage, rhamnolipid biosurfactant biosynthesis is more rapid. In this case, the sigma factor involved in the transcription of genes of nitrogen deficiency conditions cannot be considered ineffective. Sigma 54 (RpoN) factor is involved in the transcription of genes whose products are needed in nitrogen deficiency. Reports have shown that this transcription factor is active in the biosynthesis of rhamnolipid biosurfactant (Carbera-Valladares et al., 2006; Totten et al., 1990). In the context of the regulation of the rhamnolipid biosynthesis operons expression, the nitrate level sensor elements affect the expression of the biosurfactant producing operons. Nitrate sensor elements include NarX/NarL. In experiments carried out by the researchers, a bacterial mutant was created from NarL and it was shown that the production of rhamnolipid biosurfactant in the mutant microorganism increased by 6 times (Van et al., 2007).

In *E. coli*, limitation of access to rhamnose sugar is one of the limiting factors for the production of this biosurfactant in manipulated bacteria. The maximum amount of biosurfactant production in a heterologous pseudomonas putida host in one of the reports was 60 mg/l; and according to some reports, the maximum amount of rhamnnolipid biosurfactant production in *E. coli* is 52.5 mg/L (Carbera-Valladares et al., 2006). In the present study, with the removal of regulatory elements of rhamnolipid biosurfectant producing operon, and the creation of a proper construct with RhlAB genes and transformation of bacteria prone to the production and identification of these beneficial compounds are reported.

2. Materials and Methods

The compounds used in this study are the chemical solutions used in this study which were purchased from Sigma Co.

2.1. Bacteria

The bacteria strain used in this study was *E.coli* DH5α, which was obtained from the National Institute of Genetics and Technology Engineering.

2.2. Plasmids

The plasmids used in this study were PET 23a and PTRC 99A.

2.3. Enzymes

RNase A, Proteinase K, Lysozyme enzymes from Roche Co., EcoRI, HindIII and Tango buffer enzymes from Fermentase Co., and Fast Start Taq DNA polymerase enzyme from CinnaGen Co. were used according to the manufacturer's instructions.

2.4. Laboratory kits

A high pure plasmid purification kit, Agarose Gel DNA Extraction Kit, and high pure PCR product kit from Roche Co. were used.

2.5. Primers

In this study, the primers presented in Table 1 were used for PCR with Genomic Pattern of Pseudomonas aeruginosa bacteria ATCC 9027.

Table 1. Sequence of the primers used.

	Primers
Forward	5'CCGGAATTCATGCGGCGCGAAAGTCTGTTG3'
Reverse	5'AAGCTTTCAGGACGCAGCCTTCAGCCAT3'

2.6. Plasmid DNA extraction using kit

Plasmid DNA extraction was performed according to the instructions of the kit manufacturer (Promega Co.).

2.7. Primer design

In the primer design, the site of shear with EcoRI was placed at the beginning of the Go primer and the site of shear with Hind III was located at the beginning of the Return primer. The primer was designed using the required information about the RhlAB genes found on the NCBI site and using the Oligo program.

2.8. Primer annealing temperature

The temperature of primer annealing to the template DNA (Tm) was obtained from the following equation.

$$Tm=[4(G+C)+2(A+T)]^{O}C$$

The following equation is used to calculate the Annealing Temperature, Ta:

$$Ta=Tm-4$$

The Ta temperatures obtained from the above relationship are used to perform PCR.

2.9. Cloning

Ramenosyl transferase 1 (2200 bp) gene was cloned after separation from the pseudomonas host in a pET 23a vector; then, it was used by dual-enzyme digestion method for cloning in pTRC 99A vector. These plasmids have a site resistant to ampicillin; this feature is used in the screening stage.

2.10. Sequencing

In order to obtain a high pure sample, a plasmid containing the rhamnosyl transferase 1 gene was again treated by a Large Scale method using a High Pure Plasmid Purification Kit. Then, the absorbance of the sample was read by a UV spectrophotometer at 260 and 280 nm wavelength. According to the length of the desired portion for the sequence (2200 bp), an equivalent volume of 30 micrograms of the sample was poured into a vial. Then, the plasmid was precipitated with 0.1% sodium acetate volume (3M, pH = 4.6) and 2.5 ethanol (100%) volume; it was dried after being washed with ethanol 100%.

Since the method used for the sequencing is only capable of reading 700 nucleotides, two samples were prepared from each plasmid, and each one was sent with a Go or Return primer for sequencing. For this, primers were diluted to 10 pmol/µl with sterile injectable distilled water. The sequencing was done automatically by the GenFanAvaran Co.

2.11. Extraction of all proteins in the cell

In order to extract the cellular proteins, at first, 1 ml of the cells was centrifuged at 6,000 rounds. The obtained precipitate was boiled in 100 microliters of sample solvent for 10 minutes and then centrifuged at 6000 rpm.

2.12. Detection of rhamnolipid biosurfactant by plate (CTAB)

The plate contains all the ingredients in an ordinary nutrient agar plate and with the same proportions, plus 20% Cetyl Ammonium Bromide (CTAB) and 0.005% methylene blue.

This plate has the ability to show colonies producing rhamnnolipid biosurfactant. The rhamnolipid produced by bacteria in the colony with CTAB surfactant, which, unlike rhamnolipid, is a cationic surfactant, creates a complex that turns to a blue-green color in the presence of a methylene blue colored substance. For this purpose, the bacteria were first cultured for 24 hours on a CTAB plate containing the IPTG material (for induction of manipulated bacteria). After this time, they are incubated for 48 hours at 4 °C. This incubation causes easier emergence of a blue-green halo around colonies producing rhamnolipid.

2.13. Extraction of rhamnolipid biosurfactant from the culture medium of manipulated bacteria

At first, the culture medium of manipulated bacteria were cleaned from the cell by centrifugation. Then the pH was adjusted to 2 with concentrated hydrochloric acid and incubated at 4 °C for 12-24 hours. At this pH, the biosurfactant molecules are almost unloaded and begin to precipitate. The cold application facilitates this precipitate. After this time, the precipitate was centrifuged at 10.200 rpm for 40 minutes. Then, the obtained precipitate was dissolved in ethyl acetate and a honey-color solution was obtained. The solution was condensed with an evaporator in vacuum.

2.14. Thin Layer Chromatography (TLC)

In this type of chromatography, a solution of chloroform/methanol/acetic acid 0.2 with ratio of 65/15/2 was used as a moving phase and silica-coated plates as a stationary phase. In this method, from the rhamnolipid extracted from manipulated bacteria and also the rhamnolipid extracted from the pseudomonas aeruginosa bacteria, two spots were placed in front of each other on the stationary phase at a distance of half a centimeter from the lower edge of the TLC paper. The stain was then dried at room temperature and placed in a tank containing a moving phase in such a way that the dried spots were placed a few millimeters above the moving phase. Then, the tank door is blocked with a piece of plastic and a stream of moving phase runs through the spots and carries the materials in them to a certain distance along based on the polarity. The TLC paper is then heated in an oven at 120 °C and the TLC paper can be removed from the oven after the appearance of yellowish green spots indicating the presence of rhamnolipid biosurfactant on the TLC paper.

2.15. E24 analysis

This method is used to measure the emulsion strength of surfactants. In this method, the volume of 1 ml of extracted rhamnolipid biosurfectant with 1 g/l concentration with an equal volume of hexadecane, which is a 16-carbon hydrocarbon, was vortexed for 2 minutes and the suspension was placed at room temperature for 24 hours. Multiplying the obtained number from the ratio of the height of the emulsified layer to the total height of the fluid inside the tube by 100, E24 surfactant is obtained. Also, this experiment was performed for supernatant of manipulated bacteria and non-construct bacteria (*Pseudomonas aeruginosa*, positive control and negative control *E. coli*).

3. Results

3.1. PCR result of RhlAB genes of pseudomonas aeruginosa ATCC 9027 with designed primers

Removing the regulatory elements using the Go primer with shear site for the restricting enzyme of EcoR1, and the return primer for the restricting enzyme of Hind3, the RhlAB operon was isolated from genomic DNA of *Pseudomonas aeruginosa* ATCC 9027. The isolated gene is an epron consisting of only two RhlA and RhLAB genes. (Fig. 1)

3.2. Cloning PCR product of RhlAB genes in pET 23a and pTC 99A vectors in DH5α bacteria

To carry out ligation process, the cut plasmid and the cut gene segment of Fig. 2 were mixed with a suitable concentration (1.5 µl of plasmid with dilution of 13.5 µm separated with a Large scale method, 2.3 µl of Insert, 1 µl of ligase enzyme and 1.2 µl of buffer), the tube was incubated at 14°C for 24 hours and the connection of segments was made.

20 µl of ligation product was combined with 150 µl of *E. coli* DH5α susceptible cells for transformation process, and then, the cells were cultured on a plate containing ampicillin and the formed colonies that were ampicillin-resistant

colonies were cultured in a mini prep method to extract the plasmid.

Performing the dual-enzyme digestion method on the pET 23a vector and the pure PCR product, and cloning process, the gene segment was cloned in this vector. At the same time, cloning in the pTRC 99A vector was also selected to perform a comparison and report the higher-performance vector for cloning. The isolation of the RhlAB gene from the pET 23a vector was accomplished by dual-enzyme digestion and this segment was cloned into the pTRC 99A vector. (Fig. 3)

The accuracy of the transformation and cloning was confirmed by the use of plasmid extraction with kit, carrying out dual-enzyme and multi-enzyme digestion reactions on the recombinant plasmid. (Fig. 4)

3.3. Electrophoresis of all manipulated cell proteins

After induction, one of the rhamnosyl transferase 1 enzyme subunits was not detected using a recombinant bacterial sample containing pET 23a, and none of the rhamnolipid biosynthesis complex subunits in the recombinant bacterial sample containing pTRC 99 A were observed on the gel. (Fig. 5 and 6)

3.4. Chromatography of thin layer rhamnolipid extracted from manipulated bacteria

Using thin layer chromatography method proved the presence of mono- and di-rhamnolipid in supernatant of manipulated bacteria. The monorhamnolipid used in this method is obtained using the acidification method described previously.

By the movement of the moving phase from the spots on the stationary phase, the materials in the spots will be separated from each other based on their hydrophobicity. The spots, which are actually biosurfectants extracted from manipulated bacteria, are placed and dried in front of each other on the stationary phase. Solvent movement causes separation of substances within the spots based on their tendency to moving phase. In this experiment, monorhamnolipid has a higher rate of progression than dirhamnolipid. (Fig. 7)

3.5. Emergence of bacteria producing rhamnolipid biosurfactant on CTAB plate

The bacteria producing the rhamnolipid biosurfectant will be identified using the CTAB plates. The rhamnolipid biosurfectant which is an anionic biosurfectant, interacts with CTAB present in the medium and turns into blue-green color in the presence of methylene blue. In this test, pET 23a vector-treated bacteria were considered as positive controls and the bacteria containing the pET 23a plasmid without RhlAB gene were considered as negative control (Figure 8).

3.6. Oil spill dispersion test for comparing the amount of biosurfactant in bacterial soup

Using the oil dispersion method, the relative level of bacteria supernatant biosurfectant can be evaluated. In this method, 10 µl of supernatant without bacteria cell is poured on a spot of crude oil spread on a water surface of a plate. The diameter of the halo created by the application of supernatant to the oily spot is directly related to the activity of the supernatant components (Fig. 9).

Harvesting manipulated bacteria supernatants, negative control bacteria and pseudomonas aeruginosa bacteria at 2, 6, and 13 hours after cultivation and using them in the method of oil spot dispersion, it was tried to compare the relative level of secretion of biosurfectant in the culture medium of these bacteria (Burger et al., 1963).

Regarding the graphs obtained from the oil dispersion test, it can be concluded that the production of rhamnolipid biosurfectant in manipulated bacteria was higher in all supernatant harvesting stages of bacteria in comparison with *Pseudomonas aeruginosa* bacteria and Bl21 and DH5α bacteria without RhlAB gene. This biosurfectant production starts from the logarithmic phase of the manipulated bacteria and is higher for the B21 bacteria in the rich LB medium rich compared to the minimal medium, and the production of rhamnolipid biosurfectant increases with time. Also, no rhamnolipid production was observed in LB medium and minimal medium for recombinant DH5α bacteria, and this bacterium started producing rhamnolipid only in LB

medium with glucose 2% and from logarithmic growth phase (Fig. 10).

3.7. E24 analysis

Using the E24 method, the effect of a surfactant on the emulsification of hydrocarbons can be detected. In the following experiment, we investigated the power of impure biosurfactant from manipulated bacteria with a concentration of 1 g/l in different pH and salinity levels. Higher emulsion layer (white layer) leads to higher biosurfactant power (Fig. 11 and Table 2).

Preparing different salt concentrations, the emulsification power of the biosurfactant was studied and no emulsification was observed at any of the concentrations (Fig. 12).

Table 2. Relation of pH and emulsification of rhamnolipid

10	**8**	**7**	**6**	**3**	
33%	67%	55%	44%	0	E24 (pET 23a)
38%	60%	56%	23%	0	E24 (pTRC 99A)

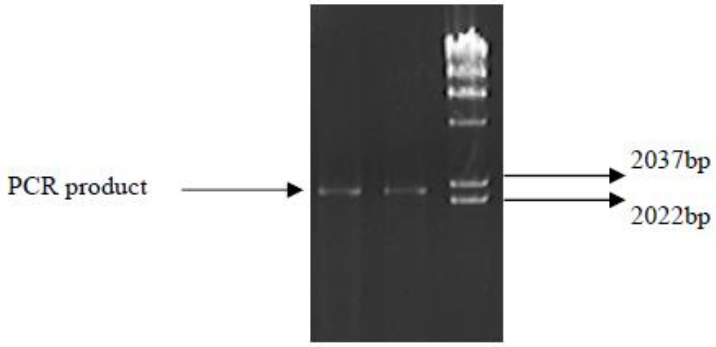


Figure 1. PCR produced by primers designed to proliferate the RhLAB genes of *Pseudomonas aeruginosa* ATCC 9027.

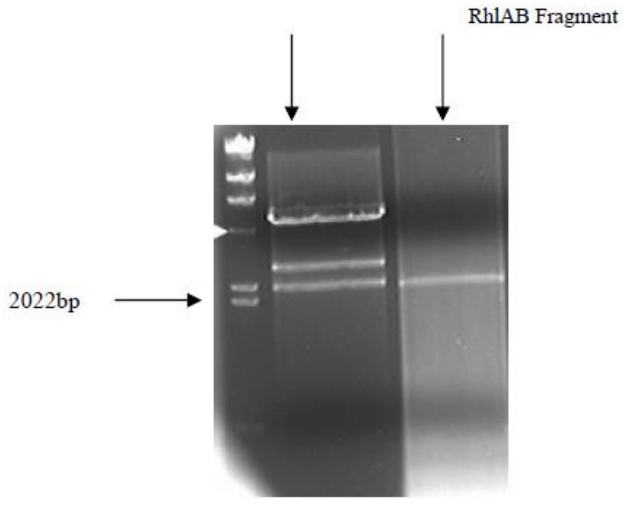


Figure 2. Dual-enzyme digestion of pET 23a plasmids extracted from transformed *E. coli*

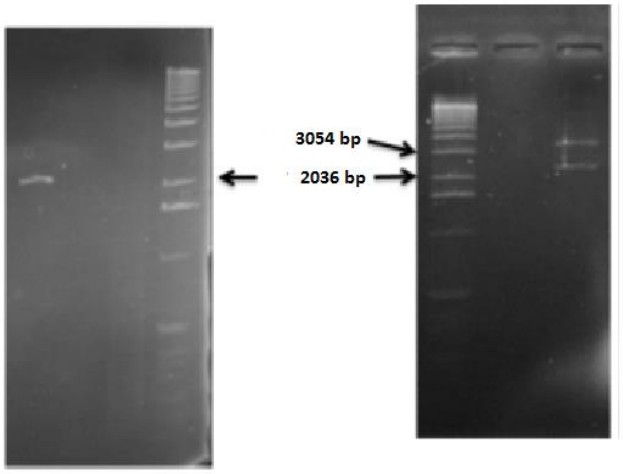


Figure 3. Dual-enzyme digestion pET 23a vector containing the RhlAB gene and the RhlAB gene recovery product on the agarose gel 1%.

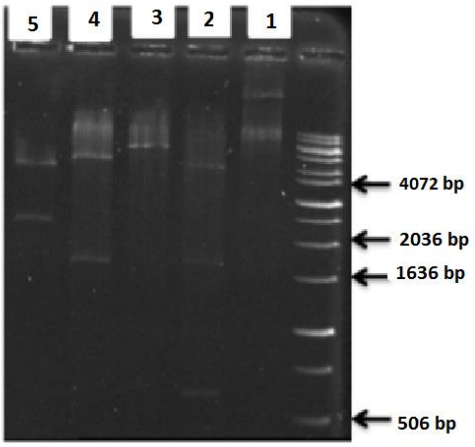


Figure 4. Cloning confirmation 1) pTRC 99A vector with RhlAB gene, 2) digestion with BamH1 and Hind3 enzymes and creation of segments with 4119, 1606, and 560 bp lengths, 3) digestion with Hind3 enzyme and creation of a segment with 6300 bp length, 4) digestion with Sma1 enzyme and creation of segments with 4283 and 1582 bp lengths, and 5) digestion with EcoR1 and Hind3 enzymes, and creation of segments with 2200 and 4100 bp lengths.

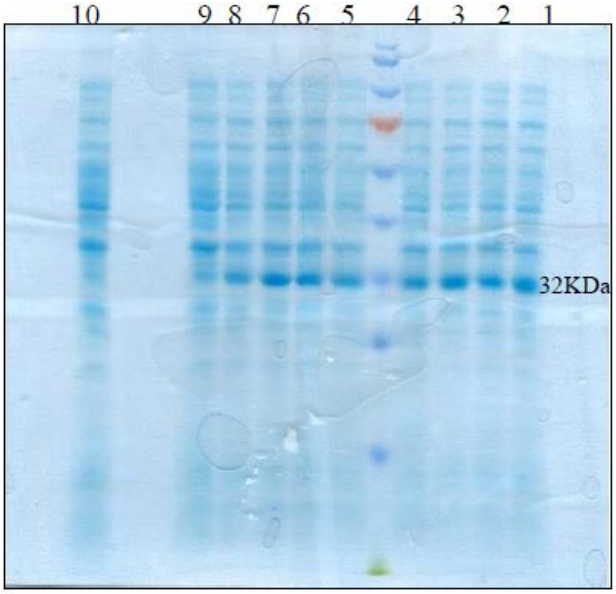


Figure 5. The recombinant bacteria carrying RhlAB genes in the pet 23a vector. Numbers 1 to 9 with concentrations of 0.1 to 0.9 mM IPTG. No. 10 with negative control cell proteins.

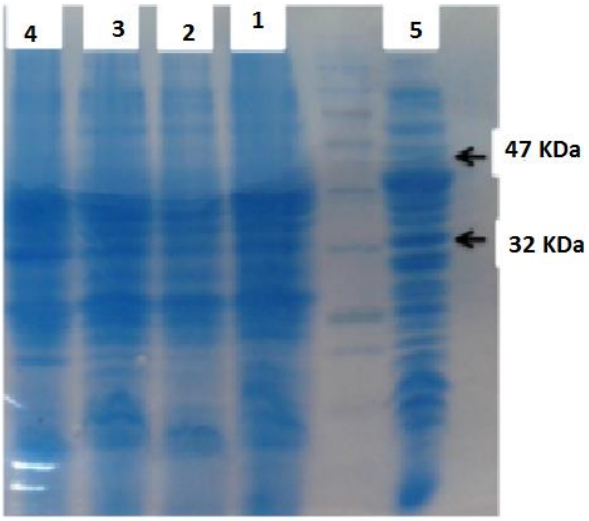


Figure 6. The recombinant bacteria carrying RhlAB genes in the pTRC 99Avector. Numbers 1 to 4 with concentrations of 0.1, 0.3, 0.7, and 1 mM IPTG. No. 5 with negative control cell proteins

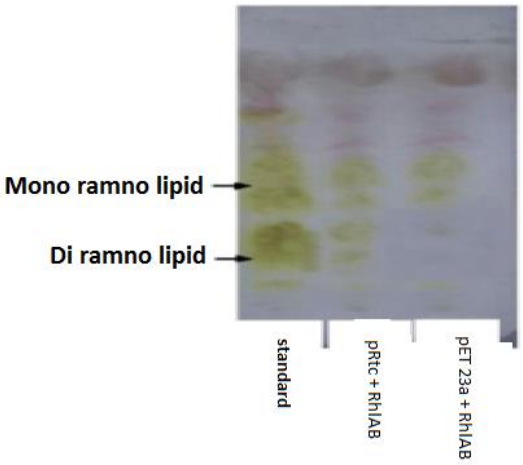


Figure 7. Chromatography of rhamanolipid thin layer extracted from manipulated bacteria.

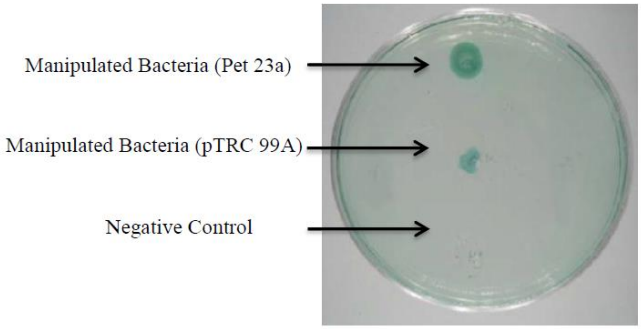


Figure 8: CTAB plate and the bacteria producing the rhamnolipid biosurfactant

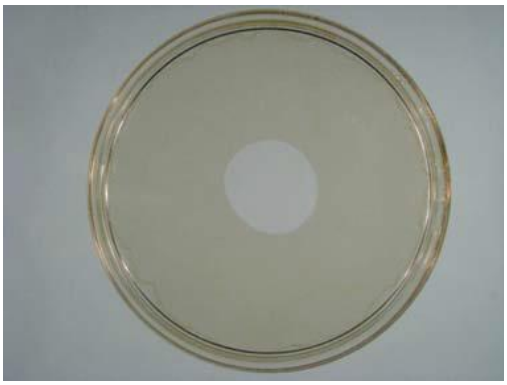
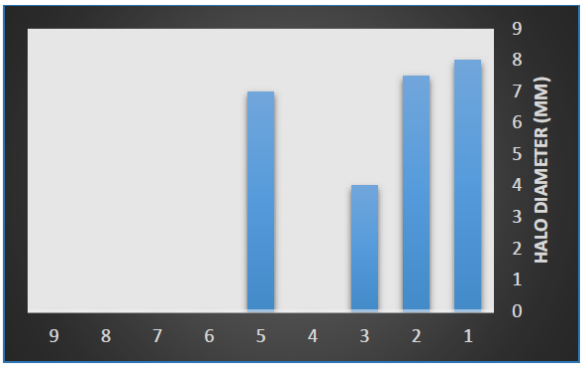
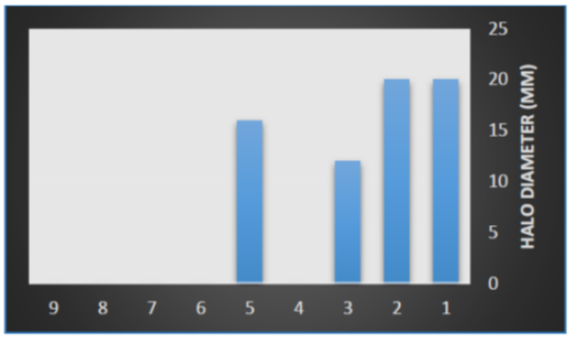


Figure 9: The halo created on the spot of oil from pouring supernatant containing biosurfactant

A



B



C

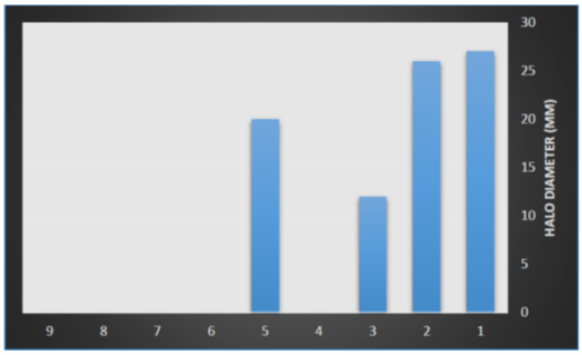


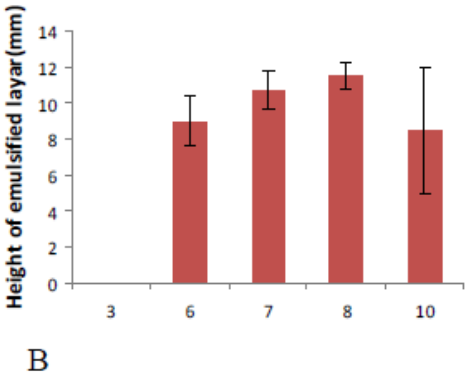
Figure 10.

A. Oil spot dispersion test using supernatant of manipulated bacteria and negative control at the second hour after culture. 1) The bacteria manipulated with the pET 23a vector along with the RhlAB gene in LB and glucose media, 2) the bacteria manipulated with the pET 23a vector along with the RhlAB gene in LB medium, 3) the bacteria manipulated with pET 23a vector along with the RhlAB gene in the minimal medium, 4) negative bacterial control, 5) the bacteria manipulated with pTRC 99A vector along with the RhlAB gene in LB and glucose media, 6) the bacteria manipulated with pTRC 99A vector along with RhlAB gene in LB medium, 7) the bacteria manipulated with pTRC 99A vector along with RhlAB gene in the minimal medium, 8) negative bacterial control, and 9) positive control of pseudomonas aeruginosa bacterium.

B. Oil spot dispersion test using supernatant of manipulated bacteria and negative control at the sixth hour after culture. 1) The bacteria manipulated with the pET 23a vector along with the RhlAB gene in LB and

glucose media, 2) the bacteria manipulated with the pET 23a vector along with the RhlAB gene in LB medium, 3) the bacteria manipulated with pET 23a vector along with the RhlAB gene in the minimal medium, 4) negative bacterial control, 5) the bacteria manipulated with pTRC 99A vector along with the RhlAB gene in LB and glucose media, 6) the bacteria manipulated with pTRC 99A vector along with RhlAB gene in LB medium, 7) the bacteria manipulated with pTRC 99A vector along with RhlAB gene in the minimal medium, 8) negative bacterial control, and 9) positive control of pseudomonas aeruginosa bacterium.

C. Oil spot dispersion test using supernatant of manipulated bacteria and negative control at the thiteenth hour after culture. 1) The bacteria manipulated with the pET 23a vector along with the RhlAB gene in LB and glucose media, 2) the bacteria manipulated with the pET 23a vector along with the RhlAB gene in LB medium, 3) the bacteria manipulated with pET 23a vector along with the RhlAB gene in the minimal medium, 4) negative bacterial control, 5) the bacteria manipulated with pTRC 99A vector along with the RhlAB gene in LB and glucose media, 6) the bacteria manipulated with pTRC 99A vector along with RhlAB gene in LB medium, 7) the bacteria manipulated with pTRC 99A vector along with RhlAB gene in the minimal medium, 8) negative bacterial control, and 9) positive control of pseudomonas aeruginosa bacterium.



A

Figure 11. Forms A and B are the level of rhamnolipid emulsification at different pHs.

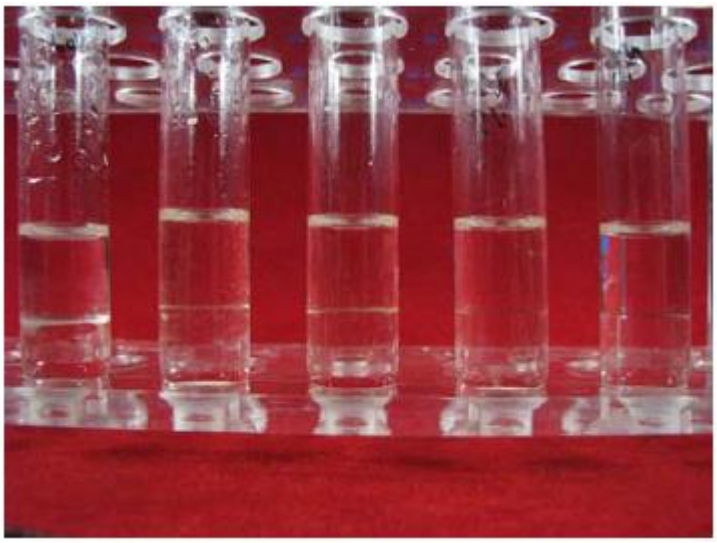


Figure 12. E24 rhamnolipid test at concentrations of 0.02, 0.04, 0.06, 0.08 and 0.1 g/l of sodium chloride.

4. Discussion

The biosurfactant manufacturer operon has certain regulatory elements that make the expression of the operon impossible at all times. These regulatory elements, as well as the factors that control them from higher level, are aware of the presence of bacteria producing biosurfectants and express the level of population density of fellows. Pseudomonads are bacteria with high power for growth and proliferation and, in general, for life under severe nutrient deficiencies, and so on. *Pseudomonas aeruginosa* bacterium which is an opportunistic pathogen, can live and reproduce in media where many bacteria are unable to grow. These bacteria can use a wide variety of materials as their carbon source. These bacteria can use soluble and insoluble hydrocarbons in water as substrates of their metabolic enzymes and use them as carbon sources. Pseudomonas aeruginosa bacterium, with this ability, can live in a wide variety of media in terms of carbon source and physicochemical properties. *Pseudomonas aeruginosa* is one of the few bacteria that has the ability to live close to hydrocarbons such as oil. This bacterium can use the simple and complex hydrocarbons in oil and use these materials as carbon and energy sources. Living conditions in the vicinity of these materials are not provided for all bacteria and the use of a substance such as crude oil as a carbon and energy source needs special physiological and metabolic conditions. Bacteria such as *pseudomonas aeruginosa* have these conditions and can use oil as an energy source. With the ability to produce rhamnolipid biosurfectants, these bacteria can use oil resources.

When the bacterium is forced to use hydrophobic sources or live within a dense population, it begins to secrete the rhamnolipid biosurfectant. In order to produce this biosurfectant on an industrial scale, there should be a way to produce this biomolecule in the logarithmic phase of bacterial growth. As mentioned, the expression of the operon producing the rhamnolipid biosurfectant occurs when the bacteria are depleted from the nutrients commonly used by bacteria and the bacterial dense population seek other resources to meet their needs. Due to the length of this process that occurs naturally within the *Pseudomonas aeruginosa* bacteria under the strict conditions mentioned above, the production of rhamnolipid biosurfectant, as occurring in the prokaryote itself, cannot be used for its industrial production in bioreactors. In the production of a biomolecule in a bioreactor, the production time, energy and consumables (including the nutrients used for microorganisms, the materials needed to maintain favorable conditions inside the bio-reactor, etc.) should be reduced to make the biomolecule production economical as much as possible. Shortening the expression time of the operon producing the rhamnolipid biosurfectant and producing this biomolecule in a shorter time than that which occurs naturally within the prokaryote itself, one can take a more effective step in the industrial production of this biosurfectant and reduce its price in the market.

Considering that it is possible to use inexpensive materials such as the consumed frying oils, low-priced vegetable oils, whey, molasses, etc. to feed the productive microorganism of the rhamnolipid biosurfectant (*Pseudomonas aeruginosa*), by reducing the time required to produce this biomolecule in bioreactors, we can save considerable energy in the reactor. Scientists have so far done a great deal for industrial production of this biomolecule, but there is still a long way to produce this biomolecule industrially. By genetic manipulation in other prokaryotes, scientists have tried to change the expression pattern of the operon producing rhamnolipid biosurfectant. The use of other prokaryotes as hosts of the rhamnolipid biosurfectant genes created the opportunity for researchers to examine the behavior of the genes of this operon in a different context. The expression of responsible genes in heterologous host hosts which have a metabolic, nutritional, physiological and genetic background differences with pseudomonas aeruginosa, may be able to provide a better and more productive solution, and ultimately industrial production of this biomolecule. (Arino et al., 1996; Edwards., 1965; Ochsner et al., 1995).

According to Urs A. Ochsner et al., recombinant *P. fluorescens*, which carries rhamnolipid operon-encoding plasmids, produces rhamnolipid at a rate of about 0.25 g/l under limited nitrogen conditions. According to the findings of these researchers, the highest yield was related to *Pseudomonas putida* strain

under tac promoter as 0.6 g/l. Also, in recombinant *E. coli*, the synthesis of active rhamnosyl transferase occurred, but rhamnolipid was not synthesized after RhlAB gene expression (Ochsner et al., 1995).

Daniel K et al., cloned coding sequence of rhamnosyl transferase from chain A (RhlA, 894 bp) and coding sequence of rhamnosyl transferase from chain B (RhlB, 1272 bp) as well as the sequence of N-acyl transcription regulation protein of homoserine lactone (726 bp) in *Pseudomonas chlororaphis*. These genes encode subunit A (297 amino acids), subunit B (423 amino acids) and R protein (241 amino acids). These proteins are only about 65% homologous in pseudomonas aeruginosa. Results of their findings showed rhamnosyl transferase 1 and mono-rhamnolipid expressions. The researchers then isolated the Rh1C gene from pseudomonas aeruginosa to express the rhamnosyl transferase 2 and produce diraminolipid; then, they expressed it in a vector controlled by the pseudomonas syringae promoter. Mono- and di-rhamnolipid were produced with ratios of 2:4, respectively (Soleiman et al., 2015).

According to the findings of Alexis Bazire et al., RmlA, RmlB, RmlC, and RmlD are responsible for the synthesis of dTDP-L-rhamnose from glucose-1-phosphate. While RhlA provides the acyl component by converting two molecules of the protein carrying hudroxylacyl-Acyl β (ACP) to an alkanoic acid β-D molecule (β-D-hidroxyalkanoyloxy). Eventually, RhlB is a rhamnosyl transferase that binds the L-rhamnose molecule to a HAA and synthesizes a mono-rhamnolipid. This can be the final product or the RhlC rhamnosyl transferase substrate for transferring the second rhamnose and synthesizing di-rhamnolipid (Bazire and Dufour., 2014).

Qinhong Wang et al., through the chromatogenetic integration by transposition, inserted the RHLAB gene into the pseudomonas aeruginosa chromosome of the mutant RhlA- and E. coli BL21, none of which was able to synthesize the rhamnolipid. Finally, the mono-rhamnolipid extracted from recombinant E.coli strain and the di-rhamnolipid extracted from the recombinant pseudomonas strain were analyzed and determined by TLC and HPLC-MS tests. The researchers also investigated the effect of salt and pH factors on the level of reduction of surface tension of extracted rhamnolipids (wang et al., 2007).

The results of this study showed that the expression of one of the rhamnosyl transferase 1 subunits in the recombinant bacterium Bl21 represents the plasmid pET 23a. Also, mono-rhamnolipid synthesis in this recombinant strain and the synthesis of di- rhamnolipid in DH5α strain containing RhlAB gene was confirmed by TLC and the activity of biosurfactants was examined using oil spot dispersion tests and E24 test and was compared with pseudomonas aeruginosa ATCC 9027.

Accordingly, the highest expression was observed in the recombinant E. coli of strain BL21, containing pet 23a vector and RhlAB gene, and this bacterium synthesizes the mono-rhamnolipid in LB + GLu, LB , and the minimal media. The E. coli bacterium of DH5α strain, containing the pTrc 99A vector, is performed only in the LB medium with 2% glucose, and rhamnolipid synthesis was not performed in any of the other two media. Expression of rhamnolipid operon in pseudomonas aeruginosa in the logarithmic phase was not observed in any of the media due to the presence of large regulatory elements.

Sampling supernatant bacteria at different growth hours, it was determined that the production of surfactants started from the early phases of the logarithmic phase and, over time, its amount increased in supernatant bacteria.

Due to the fact that in all published articles, RhlAB gene is introduced only responsible for expressing rhamnosyl transferase 1 and is responsible for the synthesis of only mono-rhamnolipid, in order to produce di-rhamnolapid in the absence of the Rh1C gene in our recombinant construct, it is likely that the rhamnosyl transferase 1 enzyme in the DH5α strain also plays the role of rhamnose transfer to the second mono-rhamnolipid.

Refereces

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