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Research Article

STUDY OF RHAMNOLIPIDS AND ITS PRODUCTION FROM PETROCHEMICAL WASTE

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Abstract

The present study was aimed to screen and characterize the potential Biosurfactant producing microbes from petrochemical waste samples. The Biosurfactant was isolated from the organism *Pseudomonas aeruginosa* and it was identified as Glycolipid by different spectral studies *viz.*, Thin Layer Chromatography (TLC), Gas Chromatography - Mass Spectrometry (GC-MS) and DNA Sequencing. Among all the 5 different strains, K_4 strain was identified as well emulsifying organism, which was produced Glycolipid as a Biosurfactant. *Pseudomonas aeruginosa* may play an important role in the natural degradation of hydrocarbon contaminants in the environment and have potential use in accelerated bioremediation processes.

Article History

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1. Introduction

Biosurfactants are amphiphilic compounds, mostly microbial cell surfaces or excreted extra cellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively. Biosurfactants are produced by different microorganisms such as bacteria, fungi and yeast. Biosurfactants have gained importance in the fields of enhanced oil recovery, environment bioremediation, food processing and pharmaceuticals. Surfactants are molecules that concentrate at interfaces and decrease surface and interfacial tensions (Rosenberg *et al.,* 1999). The amphiphiles that form micelles can be potentially used for surface chemical works are termed as SURFace ACTive AgeNTS or Surfactants. The surfactants find applications in an extremely wide variety of industrial processes involving adhesives, emulsification, foaming, flocculating, wetting and forming agents, detergency, dispersing or solubilisation, lubricants and penetrants (Mulligan and Gibbs, 1993).

The total surfactant production has exceeded 2.5 million tons in 2002 (Deleu and Paquot, 2004) for many purposes such as polymers, lubricants and solvents. The hydrophobic part of the molecule is a long chain of fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids. The water soluble end (hydrophilic end) can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces. As a result,

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surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily (Bodour and Miller-Maier, 2002). Due to the presence of surfactant, less work is required to bring a molecule to the surface and the surface tension is reduced. The ability to reduce surface tension is a major characteristic of surfactant. From the total surfactants output, about 54 % of them was consumed as household or laundry detergents, with only 32 % destined for industrial use (Cameotra and Makkar, 1998). Physicochemical behavior, charge-type, solubility and adsorption behavior are some of the most important selection criteria for surfactants (Mulligan, 2004). Biosurfactant is a structurally diverse group of surface-active molecule synthesized by microorganisms. Their capability of reducing surface and interfacial tension with low toxicity and high specificity and biodegradability, lead to an increasing interest on these microbial products as alternatives to chemical surfactants (Banat *et al*., 2000).

At present, biosurfactants plays an important application in petroleum-related industries which is use in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludges (Bertrand *et al*., 1994; Daziel *et al*., 1996). It has been focused here that improving the method of biosurfactant production and characterizing the major properties of the biosurfactant are highly important in the commercial application of biosurfactant.

2. Materials and Methods

Collection of sample

Soil samples were collected from the petroleum storage tank at reliance petrol bulk in Ashok nagar, Chennai. Samples were stored in mesophilic temperature (25 ºC – 40 ºC). Five grams of soil samples were weighed and inoculated in 50 ml of R_2B medium and were incubated at 25 ºC on a shaker at 200 rpm for 72 hours. After incubation, 1 ml of broth was transferred into sterile petriplate, over that 20 ml of R_2A medium poured. This plate was incubated at 25 ºC for 48 hours. Plate was examined and preliminary identification of isolates made on the basis of colony morphologies.

Crude oil

The crude oil samples used in this study the sample was autoclaved (121 ºC, 15 lbs for 15 minutes) separately in bottles before being added aseptically to the growth medium.

Growth and Maintenance of Bacterial Isolates

Inoculum Preparation

Pure colony of each bacterial isolates was transferred aseptically from agar plate into R_2B liquid medium using a sterile wire loop. The inoculated medium was then incubated at either 37 ºC or 45 ºC at 200 rpm in orbital shaker until the culture reached an Optical density (O.D - 600) of between 0.5 - 0.8, prior to use as inoculums.

Screening of Biosurfactant Producing Bacteria

Blood Hemolysis Test

Fresh cultures from five bacterial isolates were prepared by streaking on Nutrient Agar and incubate at 37 ºC for 24 hours. The fresh single colony of cultures was then restreaked on Blood agar and incubated at 37 ºC for 48 - 72 hours. The bacterial colonies were then observed for the presence of clear zone of hemolysis around the colonies on Blood agar. Hemolytic organisms were sub cultured and used for further studies. Results were recorded based on the type of clear zone observed i.e. α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear white zone and γ-hemolysis when there was no change in the medium surrounding the colony (Saravanan *et al.*, 2012).

Experiment for biosurfactant production

In this experiment of biosurfactant production, screened bacterial broths are inoculated into the mineral salt medium. One ml of broth cultures were inoculated into the 50 ml mineral salt medium containing 1 ml of crude oil as carbon and energy source in 250 ml Erlenmeyer

flasks. These flasks were incubated for 7 - 9 days at 25 -30 ºC in shaker at 200 rpm. In this experiment, a control flask used for identify the level of crude oil degradation. This control flask was having miner salt medium, crude oil and without inoculum. The effective isolate was identified by DNA sequencing (Chandankere *et al.,* 2013).

Extraction of biosurfactant

After 7 days incubation, bacterial cells were removed from surfactant containing medium by centrifugation at 10,000 rpm for 15 minutes. Bacterial cells were settled down, supernatant collected from the centrifuge tube. Supernatant was adjusted to pH of 2.0 by adding 6N HCl for the precipitation of the biosurfactant and kept at 4 ºC overnight. The biosurfactant was extracted with mixture of chloroform: methanol (2:1 ratio) solvent systems added to the supernatant containing tube. The tubes are fitted in rotary evaporator, sediment crude biosurfactant occur in the tube (Tabatabaee *et al.,* 2005).

Analytical Methods

Thin Layer Chromatography (TLC)

Preliminary characterization of the biosurfactant was done by Thin Layer Chromatography method. A portion of the crude biosurfactant was separated on a silica gel plate using $CHCl_3$:CH_3OH:H_2O (70:10:0.5, v/v/v/) as developing solvent system with different colour developing reagent. Such colour developing reagents are

1) Ninhydrin reagent (0.5 g of ninhydrin in 100 ml of anhydrous acetone) was used to detect lipopeptide biosurfactant as red spots.
2) Anthrone reagent (1 g of anthrone in 5 ml of sulfuric acid mixed with 95 ml of ethanol) to detect glycolipid biosurfactant as yellow spot.

Gas Chromatography-Mass Spectrometry (GC-MS)

Biosurfactant extracted from well emulsified flask was rediluted with chloroform and the sample was analyzed by GC-MS with the help of Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Madras. The GC-MS reports were recorded.

DNA Extraction

Bacterial cultures were grown in Luria Bertani Medium at 28 ^{o}C for 12 – 13 hrs. Then, 0.5 ml aliquots of cultures were centrifuged at 1000 rpm for 20 min, followed by washing the pellets with sterile double-distilled water. The pellets were each suspended in 0.8 ml of sterile double-distilled water and boiling water for 10 min to obtain the denatured bacterial DNA. After centrifugation at 3500 rpm for 10 min, 15 µl of the supernatant was used as template.

PCR Amplification

The 16S rDNA fragments were amplified with primers which are specific for universally conserved bacterial 16S rDNA (Iwamoto *et al.,* 2000). Each PCR mixture (total volume, 25 µl) consisted of 2.5 µl of 10 × PCR buffer, 0.7 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM for each), 0.085 µl of each primer (74 AM), 0.2 µl of Taq polymerase (5 U/µl), 15 µl of the above template and the adequate amount of sterile double-distilled water added to reach the desired volume. A hot start PCR was performed at 95 ^{o}C for 10 min, and touchdown PCR was performed as follows: the denaturing temperature of each cycle was carried out at 94 ^{o}C for 1 min. The annealing temperature was initially set at 68 ^{o}C and was then decreased by 0.5 ^{o}C every cycle until it reached 58 ^{o}C. Twenty additional cycles were carried out at 58 ^{o}C. Primer annealing was performed using the scheme described above for 1 min and primer extension was carried out at 72 ^{o}C for 3 min. The final extension step was at 72 ^{o}C for 10 min.

Sequencing

PCR products were subjected to electrophoresis on 2 % agarose gel in TBE buffer. Amplicons were purified using QIA quick PCR purification kit (QIAGEN) and subjected to cycle sequencing using ABIPRISM Big Dye Terminator V3.1 cycle sequencing kit. Post cycle sequencing purification was done using dye ex 2.0 spin kit (Qiagen) and eluted DNA was dried in a vacuum

centrifuge, resuspended in 20 µl of hi-diformamide (ABI) and denatured at 94 °C for 2 mins and quenched on ice. Sequencing was performed on ABI PRISM 310 sequence alignment and the sequences were compared for their homology by CLUSTAL W.

3. Results and Discussion

The study was initiated with basic identification based on cellular and colony morphologies, followed biochemical characteristics of these bacteria. This study was also sought to the preliminary characterization of the crude biosurfactant produced by means of their physicochemical properties. Characterization studies included Emulsification activity, Thin layer chromatography (TLC) and Gas Chromatography - Mass Spectrometry (GC-MS) analysis.

Analytical Method

Thin Layer Chromatography

Biosurfactant was isolated by acid precipitation method at pH 2 was identified by TLC method. From the three different strains (K_1, K_3 and K_4,), a drop of samples were placed on the TLC plates. The glycolipid compound was detected as yellow spot on the TLC plate after spraying with Anthrone reagent (Figure - 1). The K_4 strain was produced yellow colour spot in the TLC plate. This indicates the production of biosurfactants. Remaining two strains were not produced any colour spots. The results were presented in the Table - 1. Blood agar lysis was used in this study since it is widely used to screen for biosurfactant production and in some cases, it was used as primary method for screening purpose (Yonebayashi *et al*., 2000; Youssef *et al*., 2004). Mulligan *et al*. (1984) had recommended this method as a preliminary screening method. In addition, the hemolytic assay was a simple, fast and low - cost method for the screening of biosurfactant producers on solid medium. The results of the blood hemolysis for the bacterial isolates were presented in Table - 2.

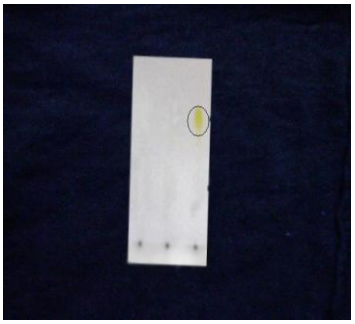


Figure - 1: Thin Layer Chromatography - K4 strain Produced yellow colour spot

Table - 1: Thin Layer Chromatography

Strains	Reaction
K_1	No spot
K_3	No spot
K_4	Yellow colour spot was appeared (Glycolipid)

Table 2: Results for Blood Hemolysis Test

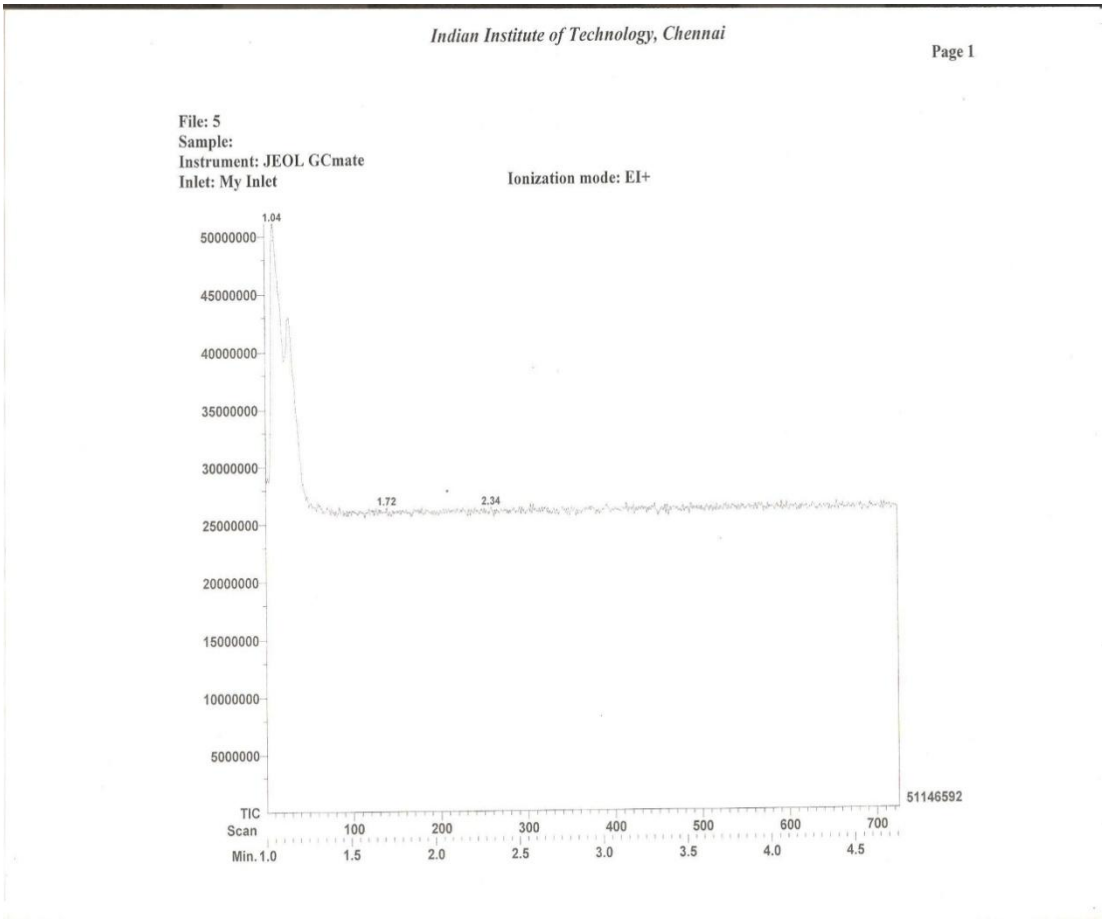
Strains	Response
K_1	α –hemolysis
K_3	β –hemolysis
K_4	β –hemolysis

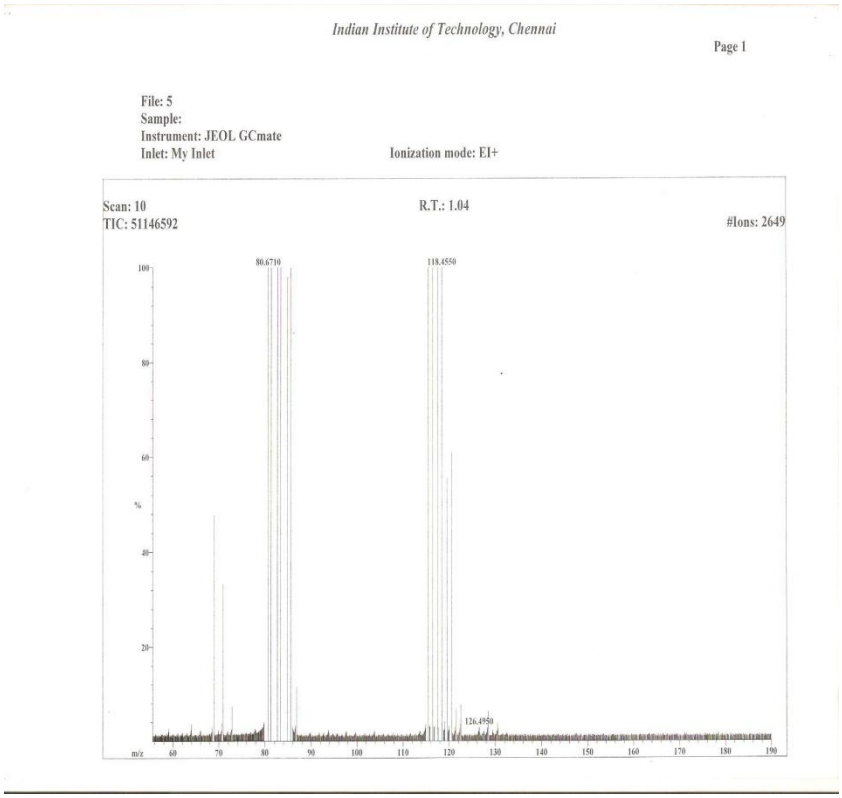
Gas Chromatography-Mass Spectrometry (GC-MS)

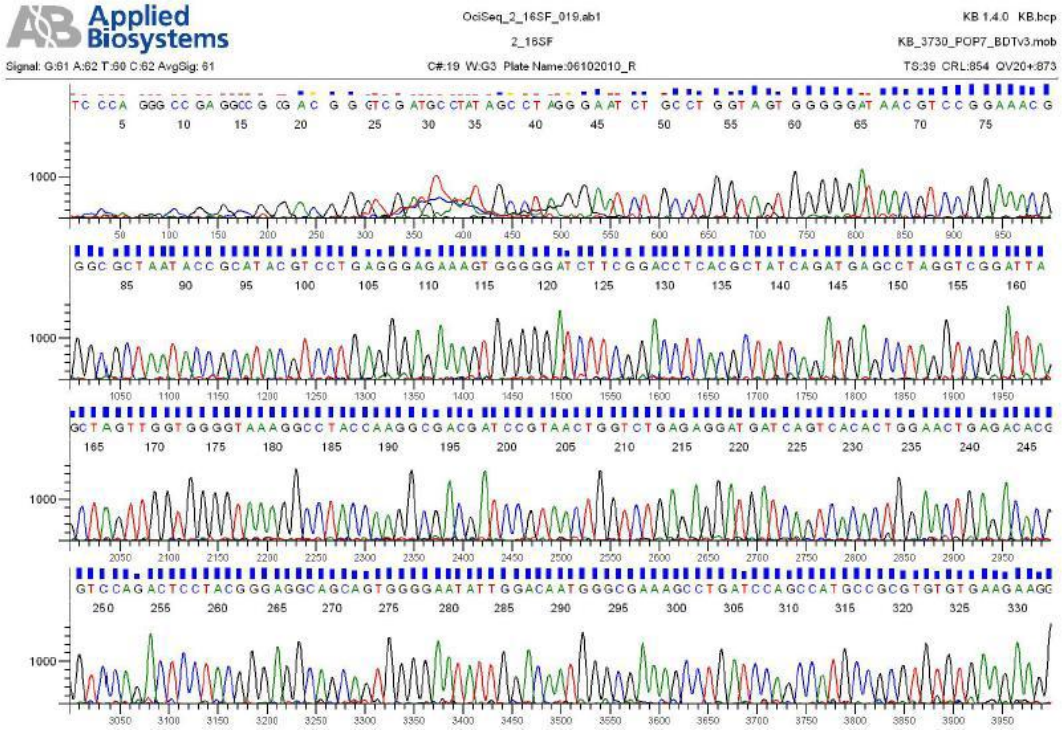
According to the GC-MS report, 5 components were present in our crude biosurfactant sample. It was confirmed by 5 peaks were found in the GC-MS report. More study requires for the identification of specific molecular weight of 5 particular components present in the crude biosurfactant.

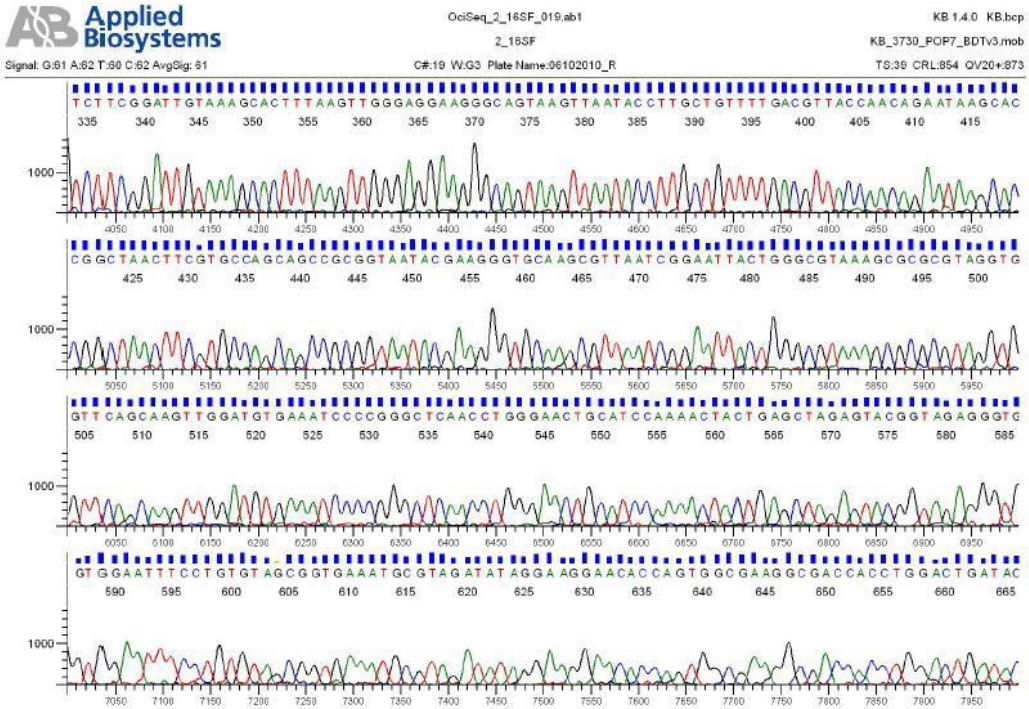
DNA Sequencing

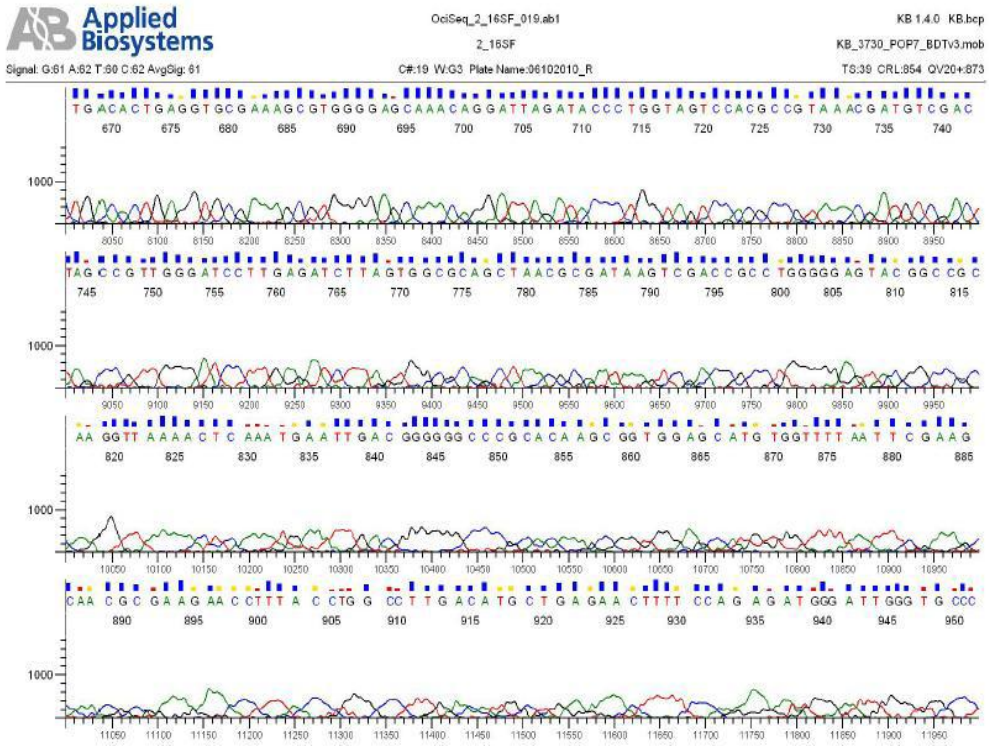
The K_4 strain was identified as well emulsifying organism, which was produced glycolipid as a biosurfactant. This organism was subjected to DNA Sequencing for organism identification 16S rRNA sequencing isolates showed comparative sequences of analysis suggest that isolates belong to the *Pseudomonas* group, so K4 isolate was identified as *Pseudomonas aeruginosa* according to the sequencing report.

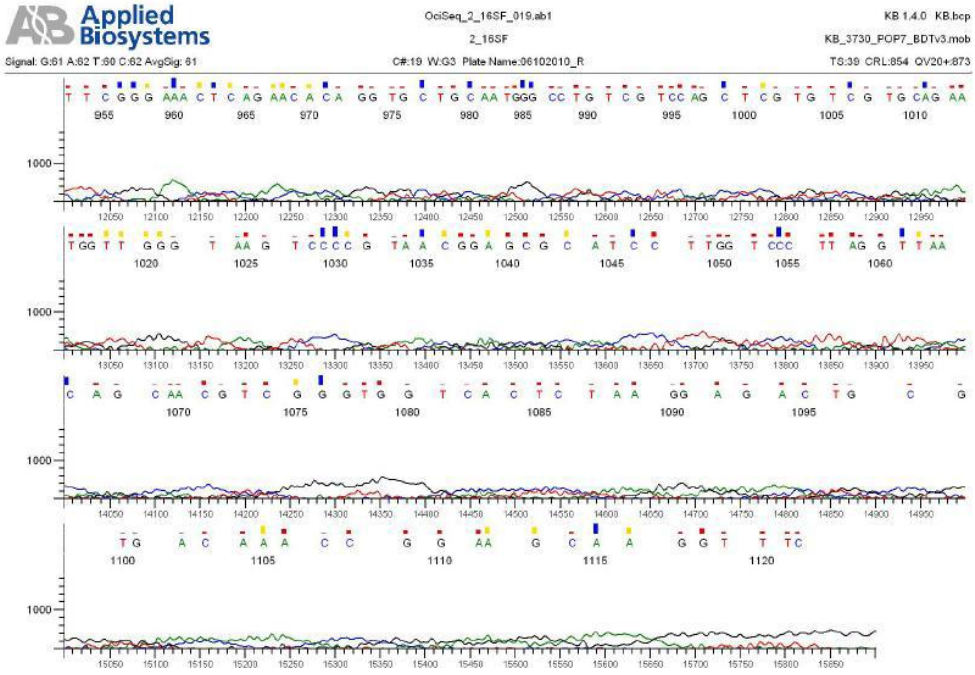












Based on the results obtained from the Blood hemolysis test screening method used in this study, to select for the most potential bacteria capable of producing biosurfactant. A qualitative assay to determine biosurfactant producer was also developed based on their ability to cause hemolysis of red blood cells. Screening of biosurfactant producers via this method was previously outlined (section 3) that only those isolates which showed β-hemolysis were considered to be the potential biosurfactant producing microbes (Bernheimer and Avigad, 1970; Carrillo *et al*., 1996). The estimation of this test was based on the fact that surfactants interact strongly with cellular membranes and proteins. Exotoxins called hemolysins cause lysis of the red blood cells.

4. Conclusion

The isolated strain K4 was produced glycolipid. It was conformed through Thin Layer Chromatography method. The *Pseudomonas aeruginosa* only produce rhamnolipid such a kind of glycolipid. So that glycolipid was conformed as rhamnolipid. The rhamnolipid have higher biosurfactant activity than surfactin produced by *Bacillus subtilis.* Some properties of rhamnolipid commonly used several applications. It has high solubility and bioavailability. As rhamnolipid, an effective biosurfactant, has been reported to stimulate the biodegradation of hydrocarbon. Rhamnolipid also act as heavy metal removers. *Pseudomonas aeruginosa* may play an important role in the natural degradation of hydrocarbon contaminants in the environment and have potential use in accelerated bioremediation processes.

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