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# BIODEGRADATION OF OILY EFFLUENTS OF PETROLEUM INDUSTRY AND PRODUCTION OF BIOSURFACTANT: EFFECT OF INITIAL OIL CONCENTRATION

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## Abstract:

Biosurfactants have many applications in petroleum oil industry: they are used for oil storage tank cleaning, for reducing the viscosity of heavy oil, thereby facilitating recovery, transportation and pipelining. Also, they are used for microbial enhanced oil recovery either from residual oil in reservoirs or from oily wastewater.

The present study is an investigation of biodegradation of petroleum oil effluents using Pseudomonas aeruginosa ATCC 9027 for producing rhamnolipid biosurfactants. The processes were performed in a mechanically agitated, fully baffled air- sparged 10L glass fermenter with a 5L working volume. The effect of oil concentration (1, 1.5, 2, and 2.5%) on the efficiency of oil biodegradation, rhamnolipid production, surface tension and bacterial biomass were studied. The fermentation with 1.5% oil concentration gave the highest biodegradation for aliphatic hydrocarbons (98.85%), followed by 2% oil concentration (95% degradation), then with 2.5% oil concentration 80% degradation), and finally with 1% oil concentration (66.8% degradation). Also, the complete biodegradation of poly-aromatic hydrocarbons was achieved for all tested oil concentrations. On the other hand maximum rhamnolipid production of 2.7 g/L as rhamnose equivalent and lower surface tension (30.2mN/m) were achieved at 2% oil concentration.

**Keywords**: Petroleum oil, Biodegradation, Pseudomonas aeruginosa, Biosurfactant.

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# 1. Introduction

Oily wastewater generated by various industries creates a major ecological problem throughout the world. The traditional methods for the oily wastewater treatment are inefficient and costly. Surfactants can promote the biodegradation of petroleum hydrocarbons by dispersing the oil into the aqueous environment. The use of rhamnolipid-containing cell-free culture broth to enhance the biodegradation of crude oil and lubricating oil in a conventional aerobically-activated sludge system is studied. At 25ºC, the removal efficiency of crude oil was over 80% with the presence of rhamnolipids compared to 22.3% in the absence of rhamnolipds [1].

Anaerobic and aerobic laboratory biodegradation of crude oil has been studied separately. It is observed that the distribution of hydrocarbons; saturated hydrocarbons, aromatic hydrocarbons, resins, asphaltenes (SARA) fractions, interfacial tension between oil and water, and oil density are affected very differently in the two different systems [2].

Increasing supply of heavy crude oils, bitumen, vacuum distillation residues in most of oil producing countries has increased the interest in transportation and conversion of the high-molecular weight fractions of these materials into refined fuels and petrochemicals. It also increased the interest of conversion of the heavy fractions of crude oil, like vacuum distillation residues, to more valuable components.

It is indicated in the literature that biosurfactants can be used in upgrading petroleum vacuum distillation residues [3]. *Bacillus*-SB553, *Bacillus licheniformis*-Sa52, *Pseudomonas pseudomallei* SASI and *Bacillus polymyxa*-SR could biodegrade petroleum crude oil by 99.79, 98.95, 99.87 and 99.12 wt%, respectively after 15 days [4]. Forty-four strains with the ability to grow on waste lubricating oil as sole carbon source were isolated from soil-contaminated samples and identified. The surface-active compounds identified and the percentage loss of waste lubricating oil after 120h of incubation ranged from 7.87% to 45.8%; depending on the strain selected compared with uninoculated control [5].

A microbial surfactant (biosurfactant) especially rhamnolipid biosurfactant was investigated for its potential to enhance bioavailability and, hence the biodegradation of octadecane [6]; crude oil [1, 7, 8]; different carbon sources (n-hexadecan, paraffin oil, glycerol, molasses) [9]; xylene, benzene, n-hexane, Bombay high crude oil, kerosene, gasoline and diesel fuel [10]; Arabian light crude, Al-Shaheen crude, diesel and oil slops [11].

Also, many researchers studied the biodegradation of oily wastes as well as the production of biosurfactants [1, 4, 12-15].

## 2. Materials and Methods

### 2.1. Organisms:

All experiments were conducted with the *Pseudomonas aeruginosa* ATCC 9027, which was supplied by Egypt Microbial Culture Collection (EMCC), Faculty of Agriculture, Ain Shams University. *P. aeruginosa* was maintained on agar slants. The medium used for *P. aeruginosa* growth contains (g/l): peptone 5, glucose 5, ammonium chloride 1.07, Potasium hydrogen phosphate 1.5, and magnesium sulphate seven hydrate 0.37, with the addition of 0.5% (v/v) oil to adapt microorganism to the fermentation substrate and adjusted to pH 7 by addition of sodium hydroxide.

### 2.2. Raw materials:

The following materials are used in the present study:
Petroleum oil waste, obtained from Assuit refinery, Egypt; *L.rhamnose* (Fluka Chemie GmbH), anthrone (loba chemie PVT ltd.) and glycerol A. R (Riedel-de Haen GmbH). The characterization of the crude oil used is shown in Table 2.1.

**Table 2.1: General Characteristics of 29.88 API Gulf of Suez Mixed Crude oil**

| Test | Result |
|---|---|
| Specific gravity at 60 /60, $^{o}$F | 0.8768 |
| Gravity API | 29.88 |
| **Kinematic viscosity cat** | |
| At 37.8 $^{o}$C | 11.49 |
| At 30.0 $^{o}$C | 15.13 |
| At 25.0 $^{o}$C | 17.52 |
| At 20.0 $^{o}$C | 20.28 |
| Pour Point (max.), $^{o}$C | -1 |
| Vapour pressure reid at 100 $^{o}$F kg/ cm$^2$ | 0.46 |
| Sulfur content, % wt | 1.71 |
| Salt content, % wt | 0.0028 |
| Carbon residue conradson, % wt | 6.16 |
| Sediments, %wt | Nil |
| Wax content, %wt | 1.37 |
| Asphaltene, % wt | 2.89 |
| Ash content, %wt | 0.012 |
| Acidity (total), mg KOH | 0.12 |
| Hydrogen sulphied, ppm wt | Nil |
| Mercapton sulfur, ppm wt | 23 |
| **Distillation** | |
| Initial boiling point, $^{o}$C | 39 |
| Recovery to 100$^{o}$C, % vol | 7.0 |
| Recovery to 125 $^{o}$C, % vol | 11.0 |
| Recovery to 150 $^{o}$C, % vol | 15.5 |
| Recovery to 175 $^{o}$C, % vol | 19.5 |
| Recovery to 200 $^{o}$C, % vol | 24.0 |
| Recovery to 225 $^{o}$C , % vol | 27.5 |
| Recovery to 250 $^{o}$C, % vol | 32.0 |
| Recovery to 275 $^{o}$C, % vol | 36.0 |
| Recovery to 300 $^{o}$C, % vol | 43.0 |
| **Metal in ppm wt** | |
| Vanadium | 49 |
| Nickel | 32 |
| Iron | 5.3 |
| **Light Hydrocarbon, % wt** | |
| Ethane | 0.037 |
| Propane | 0.371 |
| Isobutane | 0.221 |
| n- butane | 0.850 |

**2.3. Media and Fermentation:**

The media used for petroleum oil biodegradation and rhamnolipid production consisted of an appropriate concentration of oil (1, 1.5, 2 and 2.5%) (v/v)), supplemented with (g/l): peptone 5, ammonium chloride 1.07, Potasium hydrogen phosphate 1.5, and magnesium sulphate seven hydrate 0.37. The fermentation broth was adjusted to pH 7 by the addition of sodium hydroxide.

The fermentation runs were performed in mechanically agitated, fully baffled air-sparged 10L glass fermenter with 5L working volume. The reactor was supplied with air to v.v.m. (volume of air per volume of liquid per minute). The reactor temperature was maintained at 37ºC using a water bath. Experiment were allowed to run for a time of approximately 360h.

**2.4. Analysis:**

The cell density was determined using a spectrophotometric method at 660 nm, using Shimadzu spectrophotometer model UV1601 UV-visible [16]. The pH values were determined using a digital pH meter, model H18314 membrane pH-meter (HANNA Instrument). Rhamnolipid concentrations were determined as rhamnose equivalent (RE) using a standard anthrone method [17]. The surface tension of the supernatant was determined by the Du Nouy method with a Digital Tensiometer; KT10ST (KRVSS, Hamburg, Germany). Identification and quantification of polycyclic aromatic hydrocarbons and n-alkanes were carried out using HP6890 plus GC equipped with FID detector, SE54 capillary column and splitless injector mode. The initial column temperature was 80ºC. After an initial hold time of 2 min the temperature was programmed to rise to 280ºC at a rate of $8^{O}$C $min^{-1}$ for 30 min. The injector and detector temperatures were 200ºC and 300ºC, respectively. Helium was used as a carrier gas at a flow rate of 2 ml $min^{-1}$.

**2.5. Characterization of the purified rhamnolipid product:**

Purified rhamnolipid was subjected to analysis with thin layer chromatography (TLC) and mass spectrometry. These two analysis techniques were performed using Fourier-Transform Infra Red (FTIR) mass spectrometry.

**2.5.1: Using Thin Layer Chromatograph (TLC):**

Checking the purity of the compounds was made by TLC (Kieselgel 60 F254 precoated plates, E. Merck, Dermastadt, Germany) and using chloroform as elution solvent. The spot was detected by exposure to UV lamp at $k_{max}$ 254 nm, and sample/solvent ratio (Rf) equals 0.6.

The results of TLC analysis confirmed the high purity of rhamnolipid biosurfactant and also indicated that *P. aeruginosa* produce one type only of biosurfactant.

**2.5.2: Using IR Spectrometer:**

The structure of the compound was confirmed by Nicolet iS5 FT-IR Spectrometer through Thermo Scientific OMNIC software, without solvent (Minia University, Egypt).

(FT-IR (No solvent) $\nu_{max}$ = 2921, 2852 (C-H), 1710 (C=O ester),
1461 (C=C), 1376$cm^{-1}$ (C-O))

Fig. (2.5.2.1) show the results of FTIR which indicated that the biosurfactant produced is a glycolipid type, i.e., rhamnolipid (RL).

## 3. Results and Discussions:

The rhamnolipid production from waste petroleum oil by aerobic fermentation using pseudomonas aeruginosa was performed at 37$^{o}$C and pH 7 for the object of studying the effect of initial substrate (oil) concentration.

The effects of the above variables have been reported as follows:

i. Rhamnolipid biosurfactant production as rhamnose sugar equivalent;
ii. Growth of cells as cell dry weight;
iii. Residual oil analysis; and
iv. Surface tension.

The conditions used in the experiments were selected to examine the effect of initial waste petroleum oil concentration 1, 1.5, 2, 2.5% oil ( v/v) or 8, 12, 16, 20 g/l, (w/v) on the rhamnolipid production, cell dry weight, surface tension and residual oil.

### 3.1. Effect of initial oil concentration on cell growth:

The results in Fig. 3-1 show the effect of initial waste petroleum oil concentration on cell growth. As shown from the figure the cell passes through three different phases.
The first phase or namely the lag phase in which the rate of cell growth is very low and may be constant over this period (24 -36h). This long time may be due to the fact that the microorganisms need a time at the beginning of fermentation to be adapted on its substrate (which is difficult to be utilized directly because of its water insolubility), and the new environmental conditions and also for preparing the cells for multiplication and growth. So this stage was found to be characterized by low secretion of cell metabolites (or products) and also with low substrate utilization rate.

The second phase of growth or namely exponential phase takes the time period of the next forty hours. In this phase the cell growth rate is very fast due to the rapid multiplication of cells and this phase is characterized by rapid substrate utilization and the secretion of primary metabolites.

The third phase of growth or namely stationary phase, begins after the exponential stage, from 240h, till the end of fermentation. In this phase the cell growth rate decades until it is equal to the decay or death rate and hence the cells concentration becomes constant. This phase is characterized by the secretion of secondary metabolites and low substrate utilization rate. The decrease in growth rate can be attributed to the exhaustion or limitation of one or some of the growth essential elements or due to product inhibition.

Fig. 3-1 also shows that as the initial substrate concentration increases the cell growth rate and biomass concentration increase rapidly at the stationary phase. However, the relative increase becomes insignificant at about 150 hours till a maximum cell dry weight was obtained after nearly 240h from 1.5% oil (3.5178 g/l) followed by 2% oil (3.1977 g/l). At the stationary phase, (264 h), 1, 1.5, 2 and 2.5 % oil gave about 2.8842, 3.4882, 3.1733 and 2.3999 g/l (Cell Dry Weight), CDW, respectively. Therefore initial substrate concentration of 1.5% oil is recommended for maximum cell growth and biomass production. These results are summarized in Table 3.1.

**Table 3.1: Effect of Initial Oil Concentration on Cell Growth**

| Time ,hr | 1% v/v oil | 1.5% v/v oil | 2% v/v oil | 2.5% v/v oil |
|---|---|---|---|---|
| 12 | 0.2970 | 0.4629 | 0.3756 | 0.1043 |
| 24 | 0.3655 | 0.5169 | 0.4105 | 0.1293 |
| 36 | 0.3815 | 0.5366 | 0.44 | 0.1317 |
| 48 | 0.4917 | 0.6325 | 0.7042 | 0.3118 |
| 72 | 1.1007 | 1.4437 | 1.2521 | 0.7917 |
| 96 | 1.566 | 1.8085 | 1.4988 | 0.9664 |
| 120 | 1.9887 | 2.3166 | 2.1438 | 1.3553 |
| 144 | 2.2766 | 2.7002 | 2.5714 | 1.7331 |
| 168 | 2.4169 | 3.1221 | 2.775 | 1.9112 |
| 192 | 2.5701 | 3.3609 | 2.9982 | 2.2008 |
| 216 | 2.6602 | 3.4472 | 3.1366 | 2.3885 |
| 240 | 2.8771 | 3.5178 | 3.1977 | 2.4115 |
| 264 | 2.8842 | 3.4882 | 3.1733 | 2.3999 |
| 288 | 2.8915 | 3.4801 | 3.1721 | 2.4031 |
| 312 | 2.8857 | 3.4756 | 3.1397 | 2.3952 |
| 336 | 2.8481 | 3.4694 | 3.13 | 2.3818 |
| 360 | 2.7862 | 3.4178 | 3.0977 | 2.372 |

### 3.2. Effect of initial waste petroleum oil concentration on rhamnolipid biosurfactant production:

Fig. 3-2 shows that the rhamnolipid production during the fermentation process passes through three different stages:

The first stage (first 72h) is characterized by very low cell concentration that may be due to the fact that at the first 48h of fermentation the cells are at their lag phase thus there are low concentration of cells. In the next 24 h all cells metabolism (substrate utilization) is only directed towards cell growth and no energy or substrate is directed towards the formation of any products other than the primary metabolites produced from the primary cell metabolic pathway and the only needed secondary metabolite (rhamnolipid) begins to release.

The second stage (72-240h) is characterized by high substrate utilization rate and high cell production rate and yield of rhamnolipid. This may be due to the fact that, the cells need to release rhamnolipid which emulsify and solubilize the water immiscible substrate and also to change the cell surface and make it more lipophilic to adhere to the emulsified oil droplets.

The third stage is characterized by constant rhamnolipid production at its maximum level. This may be due to the reduced ability of utilization of any more rhamnolipid by the cells as an easier substrate for them, and/ or because of the depletion of some essential nutrients for rhamnolipid production.

Also, the results in Fig. 3.2 indicate that the maximum rhamnolipid production as rahmnose equivalent is obtained from effluents with: 2% oil (2.68 g/l), followed by 1.5% oil (2.41 g/l),

then for 2.5 % oil (2 g/l). Fermentation of 1% oil gave lower rhamnose equivalent (1.7 g/l). All these results are taken at 140h of fermentation.
Therefore, the increase in initial substrate concentration results in a sharp increase in both rhamnolipid production rate and yield. This continues till a maximum concentration after which any increase will be a high load on the microorganisms thus, reducing the rhamnolipid production and yield.

### 3.3. Effect of initial waste petroleum oil concentration on the surface tension of the culture broth:

As shown in Fig. 3.3 the values of the surface tension of the culture broth pass through three stages. This is because they are affected mainly by the presence of both rhamnolipid bio surfactant and the residual oil in culture broth.

At the first stage (first 6 hours) the values of surface tension were high (about 71-78 mN/m, which is slightly higher than that of water). This is because the rhamnolipid concentration is very low and the oil concentration is high.

During the next 100 h of fermentation period (second stage) the values of the surface tension decrease sharply and this is due to more rhamnolipid production and more depletion of oil during the activity metabolite phase of the culture.

In the third stage the surface tension decreases to a value as low as (30-35 mN/m) after 240h and it keeps at this value during the rest of fermentation period. This phenomenon can be explained by the fact that the surface tension decreases as the biosurfactant concentration increases until the later reaches its critical micelle concentration CMC at which the surface tension will have its lowest value and no more decrease will take place even that rhamnolipid concentration continues to increase.

Also, the results show that, the increase in initial waste petroleum oil (substrate) concentration resulted in a decrease in surface tension. This observation can be explained by the fact that the increase in substrate concentration resulted in an increase in rhamnolipid production which leads to decrease in surface tension.

### 3-4. Effect of initial oil concentration on the efficiency of oil biodegradation:
### 3-4-1: Effect on poly aromatic hydrocarbon fraction:

Figures 3.4.1a – 3.4.1d show the effect of initial oil concentration (1%,1.5%, 2% and 2.5%) on the degradation of poly aromatic hydrocarbons for a fermentation period of 360h.

As shown from the figures, at 1% oil, benzene, toluene and xylene are biodegraded completely (100% removal) after 240h. Other poly aromatic hydrocarbons reach the range from 99.7 to 99.9% after 240 hours till the end of the fermentation process (360h). 1.5 and 2% oil concentrations gave a similar trend but 2.5% oil sample gave lower percentage removal (87.3%) at 288 h and it increased to 98.6% removal after 360 hours.

Therefore, the maximum initial oil concentration should not exceed 2% to achieve complete biodegradation of oil in the ensuing effluent.

### 3-4-2: Effect on Aliphatic hydrocarbon fraction:

Figures 3.4.2a- 3.4.2d represent the effect of initial oil concentration on the degree of the biodegradation of the aliphatic hydrocarbons fraction.
It is shown from the figures that by increasing the fermentation time from 240 to 360 h, the percentage removal of aliphatic hydrocarbons increased from 88.8 to 98 for 1% oil. Similarly, it increased from 99.7 to 100% for 1.5% oil concentration, from 94.4 to 99.9% for 2% oil and from 95.6 to 96.9% for 2.5% oil concentration at 288 and 360h, respectively.
Therefore, 1.5% initial oil concentration can be used to achieve complete oil biodegradation (100%) for both hydrocarbon fractions (for fermentation time of 360h), followed by 2% oil (99.9% removal).
An effluent with 2.5% oil concentration could not reach higher than 96.6% oil removal after 360h fermentation.

## 4. Conclusions:

Petroleum oil wastes from the effluents of petroleum industry can be biodegraded efficiently using *P. aeruginosa* with a higher percent removal of oil in the ensuing effluent (99.9 % for both poly aromatic and aliphatic hydrocarbon fractions). Also, it provides a high percentage yield of rhamnolipid (2.7 g/l). Thus, petroleum oil wastes could be a favorable substrate for the production of rhamnolipid while satisfying pollution control. An initial petroleum oil concentration of 2% is recommended since it satisfies both complete biodegradation of waste oil and higher production of rhamnolipid.

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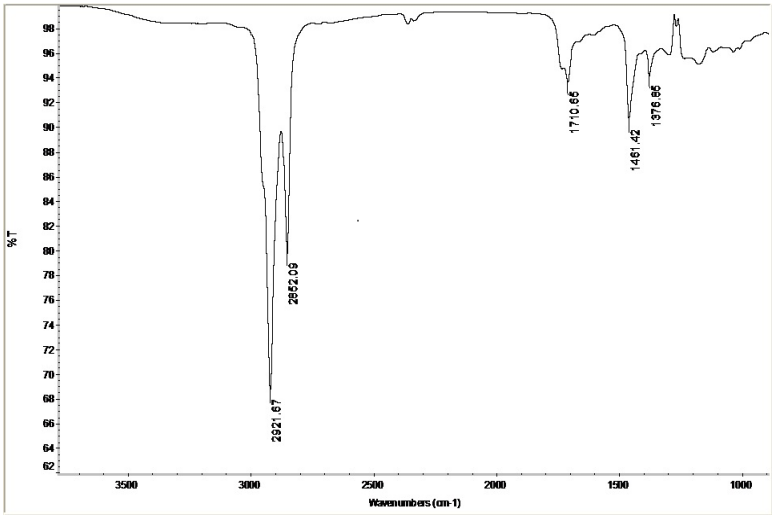


**Fig. 2.5.2.1: FTIR result of biosurfactant**

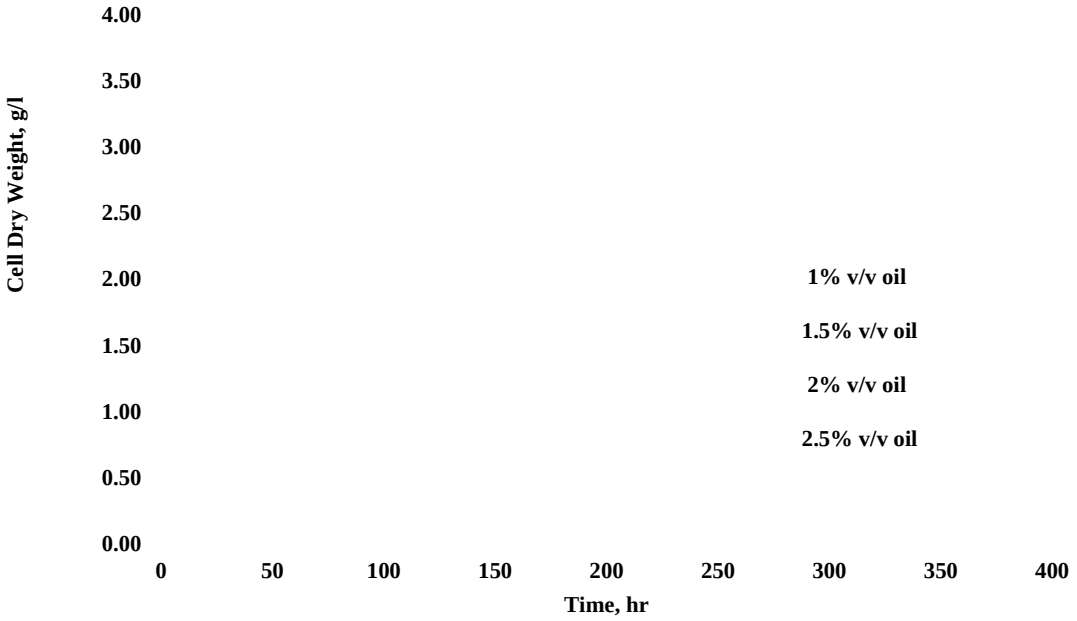


**Fig. 3-1: Effect of initial waste petroleum oil concentration on cell growth.**

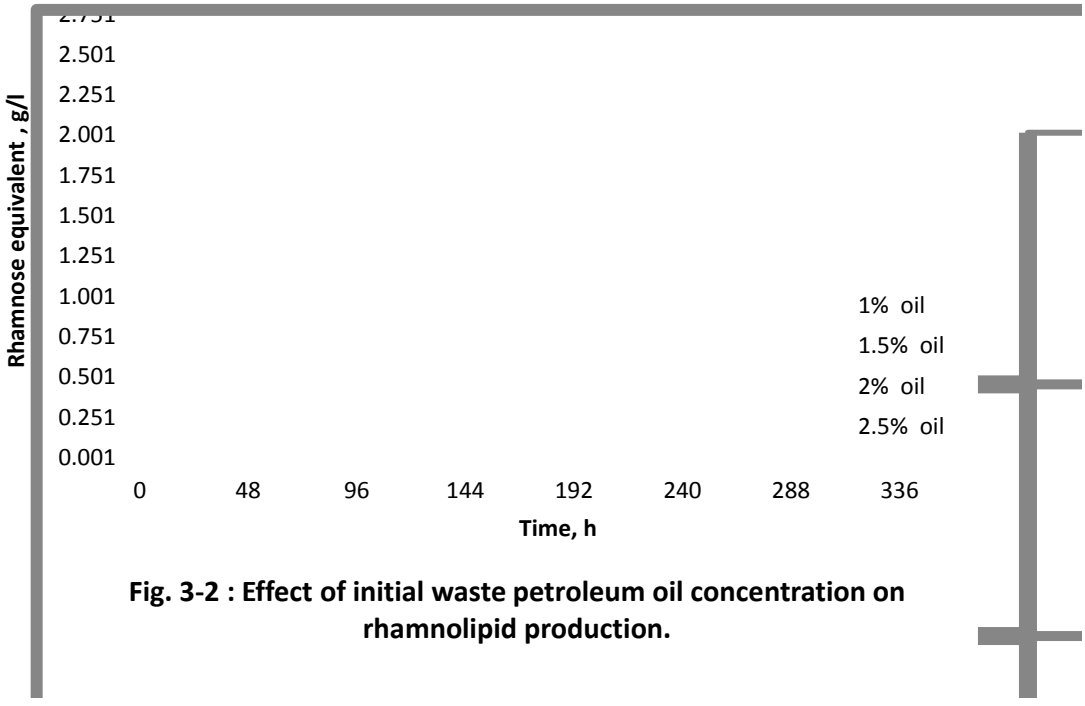


**Fig. 3-2 : Effect of initial waste petroleum oil concentration on rhamnolipid production.**

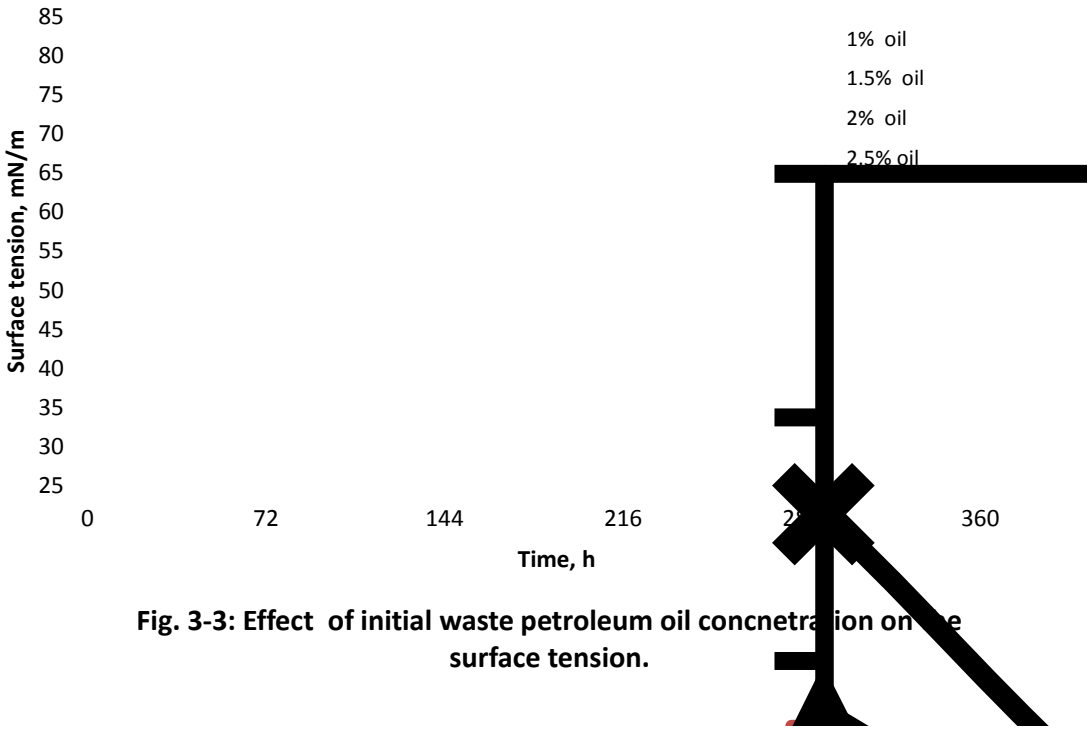


**Fig. 3-3: Effect of initial waste petroleum oil concnetration on the surface tension.**

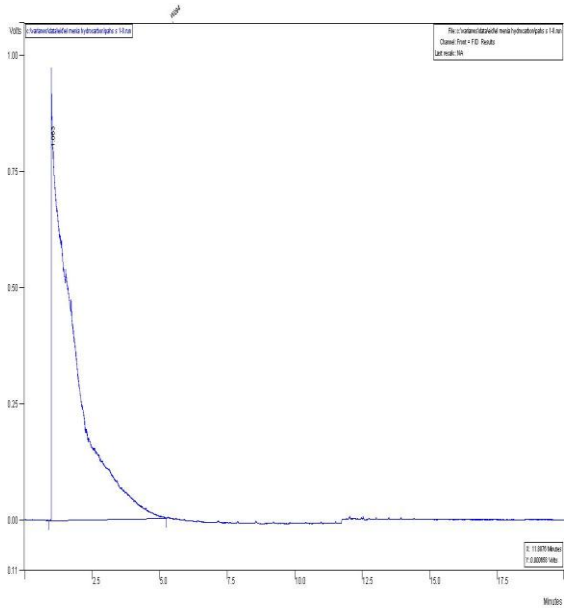

**Fig. (3.4.1a):**
Poly Aromatic hydrocarbon concentration for sample 1% Oil after 360 h

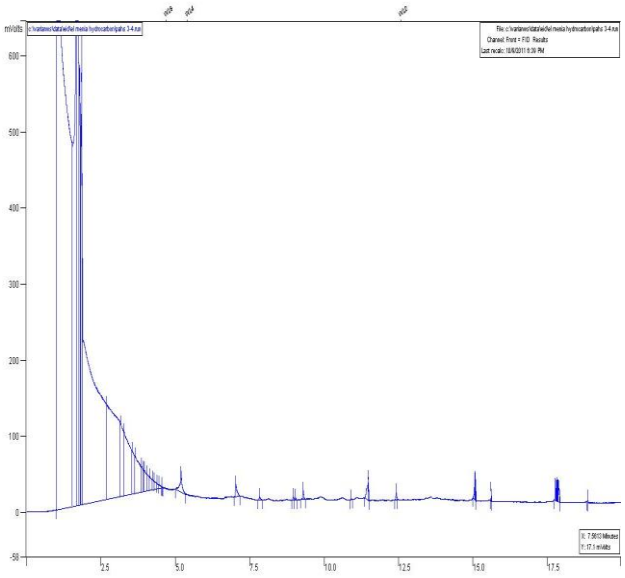

**Fig. (3.4.1b):**
Poly Aromatic hydrocarbon concentration for sample 1.5% Oil after 360h

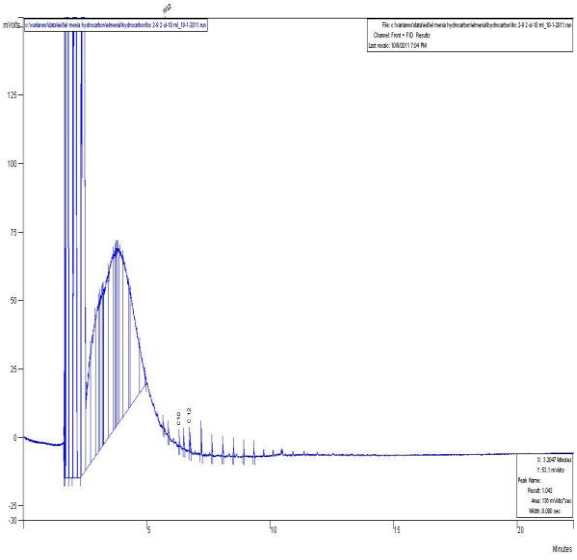

**Fig. (3.4.1c):**
Poly Aromatic hydrocarbon concentration for sample 2% oil after 360h

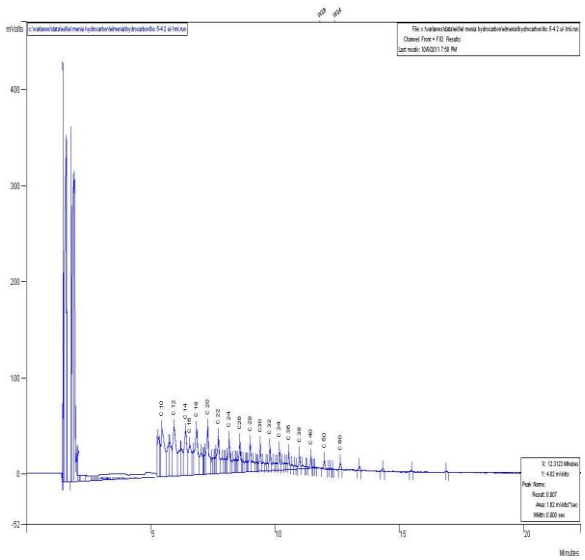

**Fig. (3.4.1d):**
Poly Aromatic hydrocarbon concentration for sample 2.5% oil after 360h

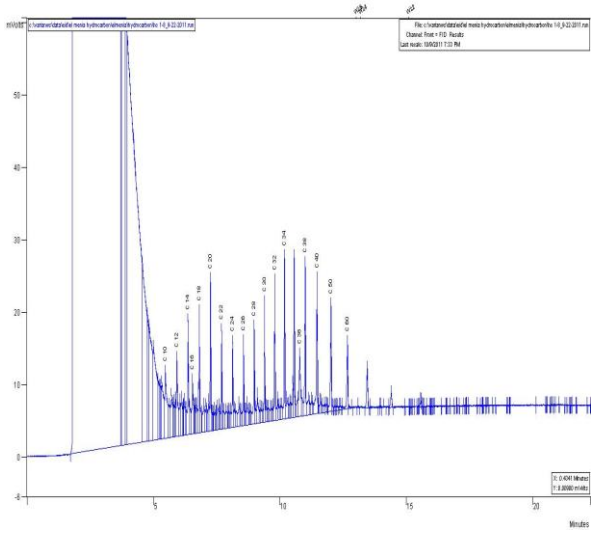

**Fig.(3.4.2a):**
Aliphatic hydrocarbon concentration for sample 1% oil after 360h

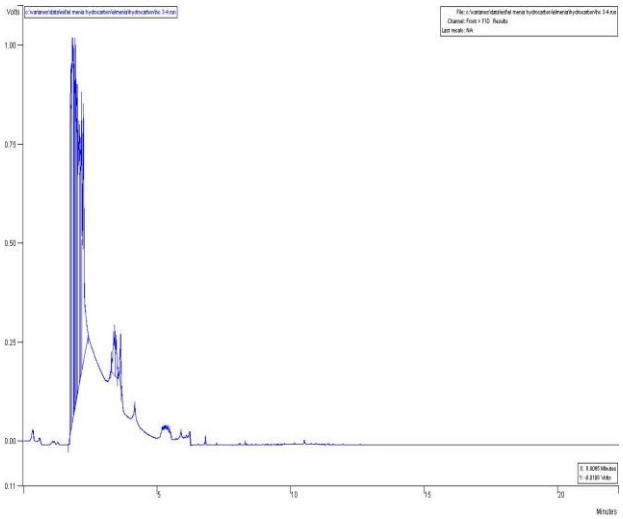

**Fig.(3.4.2b):**
Aliphatic hydrocarbon concentration for sample 1.5%oil after 360h

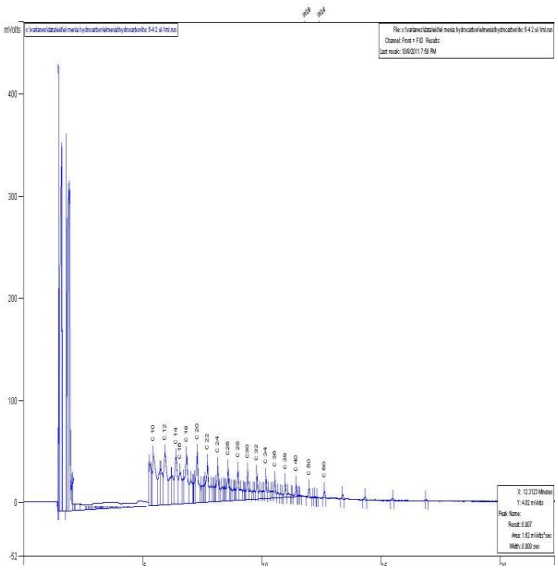

**Fig.(3.4.2c):**
Aliphatic hydrocarbon concentration for sample 2% oil after 360h

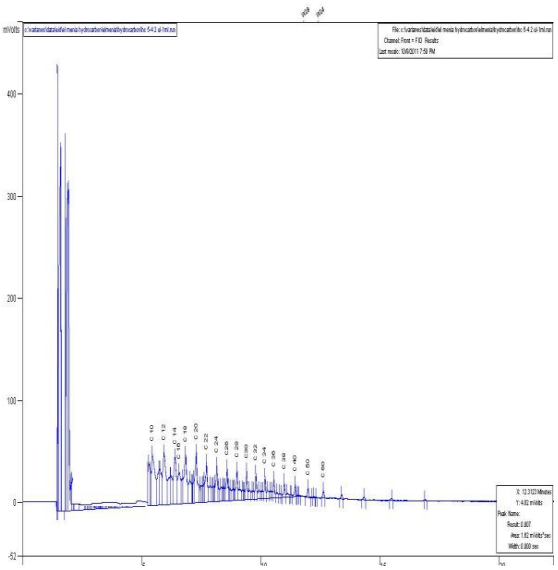

**Fig.(3.4.2d):**
Aliphatic hydrocarbon concentration for sample 2.5%oil after 360h