Pathaka and Nakhate. J Bioprocess Biotech 2015, 5:5
DOI: 10.4172/2155-9821.1000229

Journal of Bioprocessing & Biotechniques

Research Article Open Access

Optimisation of Rhamnolipid: A New Age Biosurfactant from *Pseudomonas aeruginosa* MTCC 1688 and its Application in Oil Recovery, Heavy and Toxic Metals Recovery

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Abstract

Rhamnolipid is a new age biosurfactant, commonly produced biotechnologically with *Pseudomonas aeruginosa* in batch cultivations whereas novel substrates like karanja oil and soybean oil cake were employed, giving yield of 3.609 gm/lit of rhamnolipid, which shows effective and enhanced production of rhamnolipid, compared to other vegetable oil as a carbon sources, mentioned in the literature, at optimised pH of 7.0 and optimised substrate concentration at 3.0%. The optimum yield in terms of substrate was observed as 3.609 gm/lit of rhamnolipid produced per 5.255 ml of oil consumed, while yield in terms of biomass was observed as 3.609 gm/lit of rhamnolipid produced per 2.5 gm of dry biomass. The chloroform:methanol (2:1) extraction system was found to be the best solvent extraction system, where 83% of the rhamnolipid was recovered. The Rhamnolipid was successfully applied for the heavy and toxic metals recovery, where rhamnolipid reduces heavy metal concentration to 73%, 65% and 71% for $FeCl_3$, $ZnSO_4$ and $Pb(NO_3)_2$ respectively, while 43% in the case of toxic metal i.e. NaF. The produced rhamnolipid was found efficient in recovering 31% no-edible oil from oil sludge.

Keywords: Rhamnolipid; Karanja oil; Soybean oil cake; Heavy metal; Toxic metal; Oil recovery

Introduction

Biosurfactants

"Biosurfactants" are microbially produced, structurally diverse group of surface active biochemical molecules. The huge diversity of biosurfactants makes them an interesting moiety for application in many areas including agriculture, health care, public health, food, waste utilization, and environmental pollution control, like degradation of hydrocarbons present [1].

Microbially produced biosurfactants are adequate to fulfil many of the roles for which petrochemical and oleochemical surfactants are currently used [2]. The global market for surfactants is approximately 15 million tonnes per annum with a global average annual growth of approximately 3 per cent [3]. As demand on "crude oil" supplies increases the use of "sustainable biosurfactants" instead of petrochemically derived surfactants becomes more fascinating.

Biosurfactants often have interesting characteristics not possessed by petrochemical or oleochemical surfactants, like their application in "pharmaceuticals", "bioremediation", "food processing", nevertheless, for this to happen actually in an industrial scale, there needs to be the improved development of fermentation processes and downstream separation techniques for effective biosurfactant production [1,4,5]. Downstream separation techniques require deep knowledge, as they contribute approximately 60% to the total cost of biosurfactant production [6]. Current research and industrial interest rests in many biosurfactants, including "surfactin" obtained from *Bacillus subtilis*, hydrophobin proteins from various filamentous fungi such as *Schizophyllum commune* and *Trichoderma reesei*, and rhamnolipids from *Pseudomonas aeruginosa* [7,8].

Rhamnolipid

Rhamnolipids are "anionic glycolipids" consisting of "L-rhamnose" and "β-hydroxy fatty acids" produced by *P. aeruginosa* strain. The hydrophilic rhamnose moiety is attached by a glycosidic linkage to the lipid fatty acid tail. Rhamnolipids are generated as a mixture of different rhamnolipids [2,6,9].

It has been explained that, the rhamnolipid can be produced from various microorganism, but the *Pseudomonas aeruginosa* is found to be the most effective and prominent strain responsible for the production of rhamnolipid [9,10]. Rhamnolipid is one of the most important biosurfactant, as it can used for the various purposes. The problems, that hinder the bulk production of rhamnolipid are, the use of cheap raw materials, and effective downstreaming. Various journals reported the use of vegetable oils, waste oil etc. as a carbon source. Some other journals reported use of cheap whey, or molasses as a carbon source [11].

For the production of rhamnolipid, the search of low cost source ended at the plant derived oil, i.e. karanja. Nowhere in the literature, is it reported so far, that uses karanja oil as a source for the production of rhamnolipid. Karanja oil contains more amounts of fatty acids, which can be made available to the microorganism easily, and high production of rhamnolipid can be expected. Waste soybean oil cake is the complete waste, and generally used as an animal feed. Hence, use of such oil cake will definitely decrease the bulk production cost [12,13]

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Received April 20, 2015; **Accepted** May 22, 2015; **Published** May 27, 2015

Citation: Pathaka AN, Nakhate PH (2015) Optimisation of Rhamnolipid: A New Age Biosurfactant from *Pseudomonas aeruginosa* MTCC 1688 and its Application in Oil Recovery, Heavy and Toxic Metals Recovery. J Bioprocess Biotech 5: 229 doi:10.4172/2155-9821.1000229

(Figure 1).

Many researchers reported that, the rhamnolipid is late-growth phase product, and its production increases in the stationary phase. The results obtained from our research supports the findings of such researchers like SD Wadekar et al. [14]. From the graphs, explained later in the report, it was crystal clear that, rhamnolipid production starts at the late growth phase, indicating that, microorganisms utilises most of the carbon sources available first, and then produces product. The sudden increase in production was observed during the stationary phase. The media used contains less amount of phosphorus source in order to stringent the growth condition, hence ultimately microorganism growth ceases, and the culture enters into the stationary phase and high production can be achieved [15].

The production costs of biosurfactants, compared to synthetic compounds, are at least 50 times higher, on the surfactant market. It is estimated that the production costs of rhamnolipids produced in 25-200 m^3 scale is at about 6-25 US$/kg. Compared with petrochemical bulk surfactants like ethoxylates or alkyl polyglycosides, ranging at 1-3 US$/kg, rhamnolipids are not competitive in this field [3].

Material and Methodology

Inoculum preparation procedure

First step in the inoculum preparation is the culture revival. The *Pseudomonas aeruginosa* MTCC 1688 mother culture was revived according to the standard protocol given in the various microbiological journals. The mother culture was carefully inoculated into the freshly prepared nutrient broth, strictly under aseptic conditions in vertical laminar air flow.

After the successful microbial transfer, the 100 ml shake flask, containing nutrient broth was kept in Incubator for 24 hrs to grow the microorganisms. After 24 hrs, a loop full of inoculation from nutrient broth was taken and spread over the nutrient agar plate using quadrant technique. Four nutrient agar plates were kept at 4°C for further use.

Gram staining procedure

In order to check the Gram's nature of the microorganism, Gram staining was performed. The drop wise addition of crystal violet stains the bacterial in blue colour, while Gram's iodine is used to fix the crystal violet stain. Decolourisation occurs with addition of 70% ethanol, followed by addition of counter stain Safranin, which stains bacteria in pink colour.

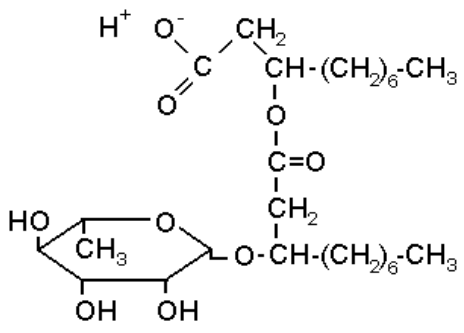


Figure 1: Rhamnolipid structure.

Shake flask cultivation

Cultivation experiment was carried out in 500 ml shake flask. As discussed in literature review, cheap carbon source had to use, hence, plant oil karanja was used, as it is a cheap hydrocarbon source. Soybean oil cake is also a waste, which is used as another hydrocarbon source. Glucose is used as a standard carbon source. The phosphate regulation was carried out by adapting minimal salt media (MSM) composition from Cameotra et al. [16]. Carbon to the nitrogen ratio i.e. C:N was maintained 20, by using $NaNO_3$ as a nitrogen source.

The optimization strategy was carried out by varying the percentage of carbon source used. 1%, 3% and 5% of carbon sources mentioned above were examined for the highest production of rhamnolipid. At the same time, pH of the media was varied using 0.1N HCl and 0.1N NaOH. Shake flask was operated at pH 5, pH 7 and pH 9 respectively after the Carbon source and its percentage optimisation.

The optimum carbon source, its concentration and optimum pH was calculated for highest rhamnolipid production. The other operating conditions, like temperature, agitation speed were properly maintained. The operating temperature was set 30°C and agitation speed was maintained 150 rpm in remi orbital shaker-incubator. During the cultivation period, biomass concentration, substrate concentration and rhamnolipid concentration were checked every after 24 hrs. After obtaining higher productivity, the cultivation days were calculated and the scale up was done (Figure 2).

Determination of biomass concentration

There are various methods which can be used for the determination of biomass. The cell dry mass was measured and calculated. The technique was carried out through following steps:

10 ml culture broth was taken

↓

Centrifugation carried out at 8000 rpm for 10 min.

↓

Pellet was collected and suspended in 0.9% saline tube

↓

Proper vortexed in order to re-suspend solid particles

↓

Allowed to settle the solid particles

↓

Filtered and dried the sample in order to measure dry mass

Determination of glucose concentration

Glucose is the simplest carbon source, utilised by almost all the microbes for their growth. The glucose decreases with the course of time, hence its concentration over the course of time is required for the calculations of yield of the product i.e. rhamnolipid product formed per unit substrate consumed. Product $Y_{p/s.}$ The glucose concentration was measured by using standard DNSA curve.

DNSA method was invented by Miller in 1959. DNSA method is basically used to detect free aldehyde groups present in the sugar, i.e. to detect reducing sugar. Oxidation of aldehyde group results into the formation of carboxyl group, which then reduce to 3-amino, 5-nitro

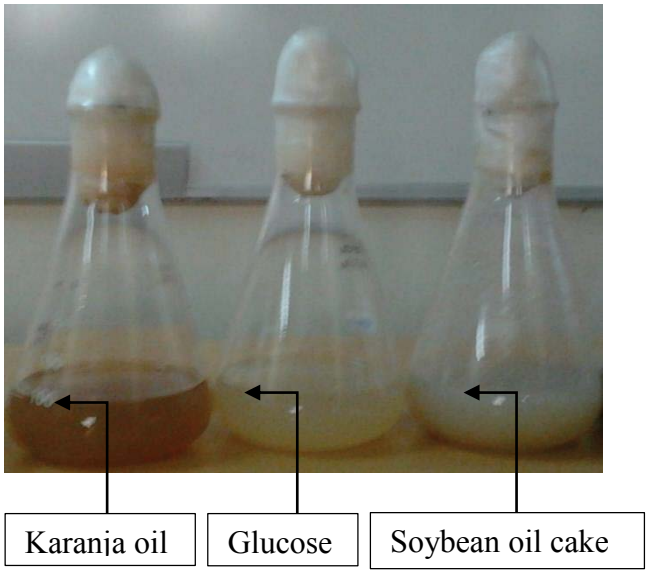


Figure 2: Shake flask study.

Tube No.	Amount of stock added	Amount of D/W added	Final volume	Dilution	Concentration
1	0 ml	10 ml	10 ml	0	0 mg/ml
2	0.25 ml	9.75 ml	10 ml	1:40	2.5 mg/ml
3	0.5 ml	9.5 ml	10 ml	1:20	5 mg/ml
4	1 ml	9 ml	10 ml	1:10	10 mg/ml
5	2 ml	8 ml	10 ml	1:5	20 mg/ml
6	5 ml	5 ml	10 ml	1:2	50 mg/ml
7	10 ml	0 ml	10 ml	1:1	100 mg/ml

Table 1: Preparation of dilutions for glucose standard curve using DNSA method.

salicylic acid in presence of DNSA, which is calculated.

The stock solution of 100 mg/ml was prepared and further dilutions were made as mentioned below. 3 ml of DNSA solution was added to 3 ml of test sample and mixture was heated at 90°C for 15 min. Then, 1 ml of potassium-sodium tartarate i.e. Rochelle salt was added to solubilise the colour. The mixture was then allowed to cool down and OD was measure on colorimeter at 540 nm.

Determination of free fatty acid content

Another important substrates used for the rhamnolipid production, were karanja oil and soybean oil cake. Both the substrates are plant originated and contains high amount of unsaturated fatty acid, i.e. oleic acid. Hence free fatty acid content in terms of oleic acid was calculated. The hexane was added in order to extract oil residues from broth culture (Table 1).

The procedure was followed as given below:

10 ml culture broth was taken

↓

Centrifugation carried out at 8000 rpm for 10 min

↓

Pellet was collected and suspended in 0.9% saline tube

↓

Proper vortexed in order to re-suspend solid particles

↓

Allowed to settle the solid particles

↓

Filtered and dried the sample in order to measure dry mass

The titration readings were noted down and acid value was calculated from the equation,

$$\text{Acid Value} = \frac{(56.1 \times \text{Burette reading} \times \text{Normality of KOH})}{\text{Weight in grams}}$$

Rhamnolipid detection [14]

Rhamnolipid detection was carried out using orcinol reagent. The culture broth was centrifuged at 8000 rpm for 15 min and the supernatant as collected. The supernatant was then treated with orcinol reagent, containing concentrated H_2SO_4 which causes L-rhamnose moiety to separate it out from lipid moiety and the L-rhamnose concentration can be calculated. This is the indirect way of detection of rhamnolipid, as pure rhamnolipid was not available.

Orcinol Reagent was produced by adding 53% H_2SO_4 solution to 0.19% orcinol solution. For each 1 ml sample, 9 ml orcinol reagent was summed. The formed mixture was then heated at 70°C for 30 min. and then allowed to cool down for 30 min. The optical density was

Tube No	Amount of stock added	Amount of D/W added	Final volume	Dilution	Concentration
1	0 ml	10 ml	10 ml	0	0 mg/ml
2	0.25 ml	9.75 ml	10 ml	1:40	0.25 mg/ml
3	0.5 ml	9.5 ml	10 ml	1:20	0.5 mg/ml
4	1 ml	9 ml	10 ml	1:10	1 mg/ml
5	2 ml	8 ml	10 ml	1:5	2 mg/ml
6	5 ml	5 ml	10 ml	1:2	50 mg/ml
7	10 ml	0 ml	10 ml	1:1	10 mg/ml

Table 2: Preparation of dilutions for L-rhamnose standard curve using orcinol reagent.

System used	Phase	O.D. at 540 nm
Ethyl acetate	Organic phase	0.41
	Aqueous phase	0.15
Chloroform: Methanol	Organic phase	0.48
	Aqueous phase	0.12

Table 3: Solvent extraction readings.

Process used	O.D. at 540 nm
Column chromatography	0.51
Rotary vacuum chromatography	0.48

Table 4: Different process readings.

S.No.	Stretching present	Wavelength (cm^{-1})
1	C-H	2930.18
2	C-H	2856.55
3	C=O	1734.35
5	CH3	1401.17
6	C-H/O-H deform	1384.98
7	OH deform	1315.45
8	C=O stretching	1041.63
9	C-H deform	874.07

Table 5: Structural analysis of rhamnolipid using FTIR.

then measured at 520 nm. The standard L-rhamnose 10 mg/ml stock solution was prepared (Table 2).

CTAB agar method

CTAB is the cetyl trimethyl ammonium bromide method. This is also called methyl assay for rhamnolipid detection. Concentration of "anionic surfactant" from the mixture of solution can be dictated. CTAB, being "cationic surfactant", bind with the rhamnolipid, which is "anionic surfactant", forming insoluble ion precipitation. This "insoluble ion precipitation" was detected by appearance of 'dark blue' colour on 'light' background (Table 3).

The CTAB agar was prepared by using components mentioned in tables 4 and 5. After autoclaving, petri-plates were poured and allowed to get solidified. After solidification, agar gel was punctured at proper positions using borer, and culture broth supernatant was added into the well using micropipette. The plate was then kept for the 24 hrs incubation and the precipitation zone around the hole were observed.

Production scale up

Production scale up was done by moving from shake flask to fermenter. For the scale of the production, 2 litre fermenter was used. The important operating conditions which were chosen include, Agitation speed, which was maintained 150 rpm. The temperature was maintained 35°C throughout. Aeration rate was maintained 0.8 vvm and pH was kept 7 throughout the process.

Before sterilisation, all the probes and Heating jacket was removed properly. All the filters and a sampling port were taken out and washed properly with alcohol. All the reactor assembly was washed neatly through water and then wiped with alcohol. The reactor assembly was then filled with 1.4 litres of fermentation media, mentioned in table 4. Then, the reactor assembly along with the fermentation media was autoclaved for 45 min at 20 lbs pressure. After the successful sterilisation, the assembly was allowed to cool down (Figure 3).

After cooling down, the fermenter was then inoculated with seed media, prepared already. The 100 ml Seed media was firstly prepared to grow the 10% microbes, to give them desired environment. In the middle of the exponential phase, this seed media was transferred aseptically to the fermenter. For the preparation of Seed media, minimal salt media was used.

After the successful transfer of seed media, all the probes and filters were assembled to the reactor. For regulation of pH during the fermentation course, 0.1M H_2SO_4 and 0.1N NaOH were prepared, and attached to the reactor. The reactor was then started and operated for continuous 7 day. From starting the fermenter, after every 24 hrs, sampling was done.

Down streaming of product

Down streaming is very important aspect in the fermentation process. The down streaming process was followed as me mentioned below.

Solvent extraction

First important step in the down streaming of rhamnolipid fermentation was solvent extraction. For the solvent extraction method, two different solvent systems were used, including ethyl acetate and chloroform:methanol system.

The solvent extraction scheme is shown below:

Culture broth

↓

Treatment with hexane in case of oil as a substrate

↓ ← RL detection through orcinol method

Centrifugation

↓

Collect supernatant

↓

Acid precipitation with 0.6 M HCl, pH 2

↓

Solvent extraction method

With ethyl acetate With chloroform:methanol

↓

Collect organic phase as a crude extraction and calculate RL concentration

Collect organic phase as a crude extraction and calculate RL concentration

For ethyl acetate solvent system, ethyl acetate:extract ratio was used 1:1, while using chloroform:methanol:extract, 2:1:1 ratio was used. The extraction efficiency and partition coefficient was also calculated for both the systems by calculating the rhamnolipid concentration present in the organic phase.

Chromatography

Chromatography is one of the major down streaming technique, which purifies rhamnolipid. Paper chromatography was used as an analytical technique, to calculate the retention factor, i.e. R_f value, while another chromatography, i.e. column chromatography was performed to purify the rhamnolipid.

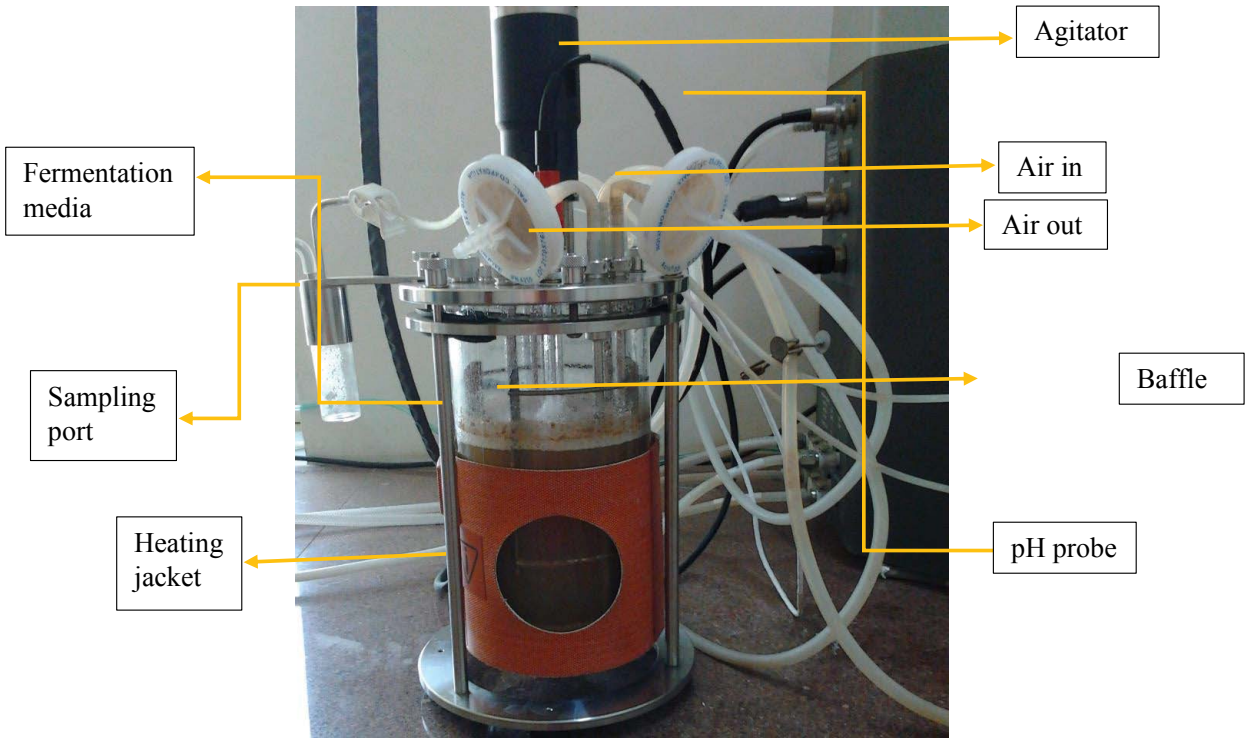


Figure 3: New Brunswick fermenter of 2 lit. capacity.

Paper chromatography: For paper chromatography, the mobile phase used was chloroform:methanol in ratio 2:1. Chromatography paper was marked initially for the sample loading. The sample was loaded at the marking position. The paper was then put into the chromatography jar. After some time, the sample runs on the chromatograph paper in up-ward position, leaving a mark. Hence, the distance travelled by sample to the mobile solvent was calculated.

Column chromatography: Column chromatography was performed with using silica-60 as a packing material or stationary phase. The silica gel-60 was packed tightly under the column. Primary cleaning was done by eluting the methanol from the column. As soon as the methanol was completely eluted, the crude sample was run in a column along with mobile phase. The mobile phase used was chloroform:methanol in 3:2 ratios. The methanol in the mobile phase was used to remove the hydrophobic impurities. Hence, the light brown coloured sample, which was eluted after the dark brown coloured sample, was collected and rhamnolipid concentration was measured.

Rotary vacuum evaporator

The concentrated product was collected by using the rotary vacuum evaporator (RVE). The collected crude sample was loaded in the round bottom flask of the rotary vacuum evaporator, integrated with vacuum system. The flask was rotated at the speed of 5 rpm, and the temperature was set 70°C, in order to evaporate the water content from the sample, using integrated vacuum. The concentrated sample was then analysed, using FTIR technique.

Emulsification index [9,10]

The emulsion index was calculated in order to check the capacity of formation of emulsion by rhamnolipid. The emulsion index was calculated after 24 hours; hence it is also called E_{24}. Emulsion index was measured in percentage by diving the height of the emulsion to the total height of the mixture. The followed procedure is described below.

6 ml D/W+2 ml immersion oil

↓

The sample was vortexed for 10 min

↓

The mixture was allowed to settle down

↓

2 ml crude rhamnolipid sample was added

↓

Again the sample was vortexed for 10 min and settled for 24 hrs

Emulsion was observed

Emulsion index stability [10,15]

The same procedure as described for the emulsion index was carried out for emulsion index stability test. The only difference with the previous technique is that, the crude rhamnolipid was kept at various temperatures and various pH. Rhamnolipid was kept at 4°C, 30°C and 80°C for around 30 min and the above procedure was carried out. Similarly, rhamnolipid was kept at pH 3, pH 7 and pH 9 for 30

minutes, and its emulsification index was calculated.

Oil spray assay [16,17]

Another important, but simple technique for the detection of emulsion capacity of rhamnolipid is oil spray assay. The following procedure was carried for the same.

20 ml D/W was poured in petri plate

↓

Immersion oil drop was added on the same D/W

↓

Rhamnolipid of 200 µl was added on the immersion drop

↓

Emulsion formation was observed

Heavy and toxic metal recovery [18]

The three major heavy metals along with the one toxic metal were recovered from the solution prepared. Three important heavy metals used were, $Pb(NO_3)_2$, ZnCl, $FeCl_3$. The toxic metal recovered was NaF. The standard concentration of heavy metal produced was 100 mg/ ml. The recovery procedure is given below for the 10 ml of the sample used each (Figure 4).

Standard metal conc. of 10mg/ml was taken 9 ml in test tube

↓

1 ml crude rhamnolipid was added in the sample

↓

The mixture was vortexed and allowed to settle for 2 hours

↓

The mixture was filtered through Whatman filter paper

↓

The weight of Whatman filter paper before and after the filtration was measured

The percentage of efficiency of removal of metals i.e. η were calculated as,

$$\eta = \frac{(\text{Initial heavy metal} - \text{Final heavy metal})}{\text{Initial metal concentration}} \times 100$$

Oil recovery [17,19]

Figure 4: Standard concentration of metal.

Rhamnolipid is very efficient in the oil recovery from oil sludge. The efficiency rhamnolipid for oil recovery was observed from the following protocol.

Components were mixed properly

↓

10 ml of immersion oil mixed with hot dry sand

↓

10 ml crude rhamnolipid was added

↓

The whole mixture was vortexed for 10 min and allowed to settle for 5 min

↓

Whole mixture was centrifuged at 6000 rpm for 20 min

↓

Different layers were observed, including oil emulsion

Results

Pseudomonas aeruginosa MTCC 1688 strain was successfully revived under the aseptic conditions. The green coloured pigment formation was observed, after 48 hrs of microbial inoculation, indicating presence of *Pseudomonas aeruginosa*. The strain was then identified using Gram staining procedure (Figures 5 and 6).

Gram staining

The pink coloured and rod shaped microorganisms were observed under the oil-immersion 100× lens of the microscope. Hence, gram staining procedure shows Gram-negative nature of *P. aeruginosa* (Graphs 1 and 2).

Shake flask cultivation

Shake flask optimisation study was carried out using 3 different substrates, i.e. glucose, karanja oil and soybean oil cake. The rhamnolipid production during the course of fermentation was determined by L-rhamnose standard curve. The equation of the straight line was obtained as, y=0.1712x+0.0042 (Graphs 3-5).

After analysing above experimentation, It has been observed that, 3% concentration of a substrate is efficient in rhamnolipid production. Hence, taking 3% substrate concentration, the experimentation for pH optimisation was carried out (Graphs 6-8).

After the shake flask studies, the data was analysed for higher rhamnolipid production, at a different substrates, their concentration and pH. After the successful data analysis of shake flask, the production study was carried out in 2 litre New-Brunswick fermenter, applying optimum conditions from shake flask (Figures 7-10) (Graph 9).

Fermentation studies

Confirmation of rhamnolipid production: Retention factor, i.e. R_f value=5.3/10=0.53

Solvent extraction

Ethyl acetate:extract ratio was used 2:1 for the extraction, and chloroform:methanol:extract ratio used was 2:1:1 (Figures 11-13) (Tables 3-5).

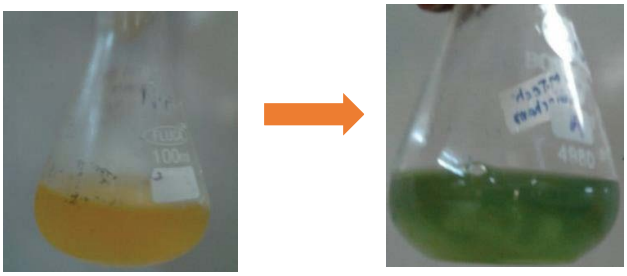


Figure 5: Change of color of *P. aeruginosa* inoculated nutrient broth after 48 hrs.

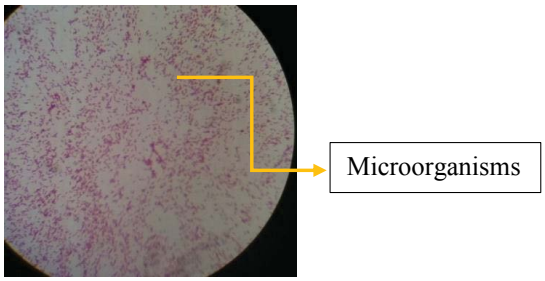
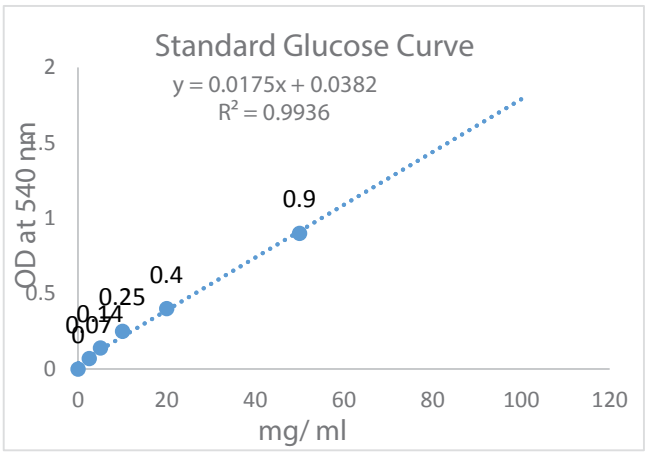


Figure 6: Gram staining.



Graph 1: Standard glucose curve.

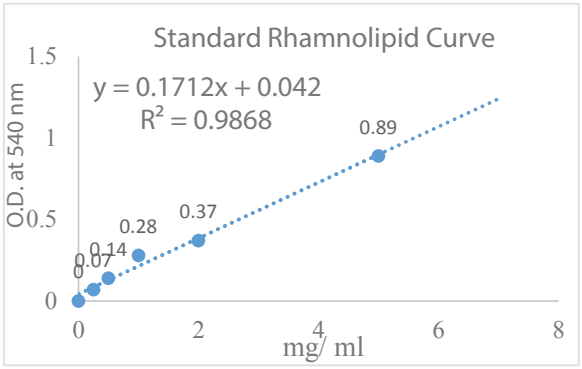
Structural analysis using FTIR

The FTIR spectrum analysis of the rhamnolipid, produced from *Pseudomonas aeruginosa* MTCC 1688 on 3% soybean oil cake as a substrate, is given below [9] (Table 6).

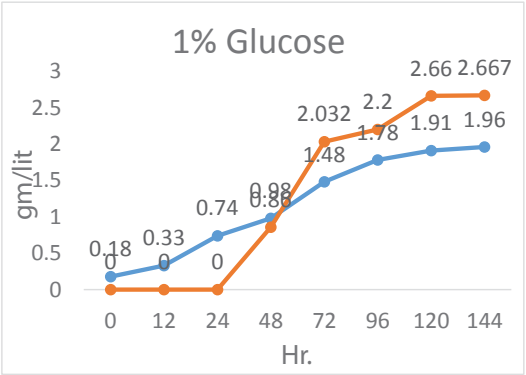
The data obtained was compared with the literature present, as well as standard spectrum data available, and found that, almost same stretching and deforms were observed.

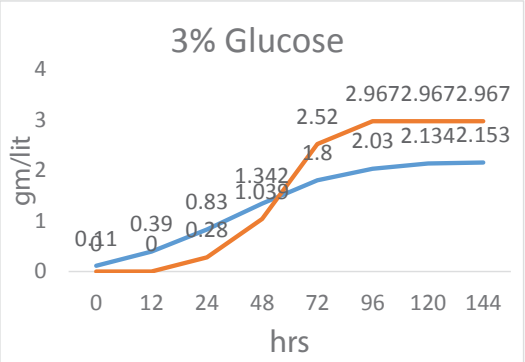
Emulsification index

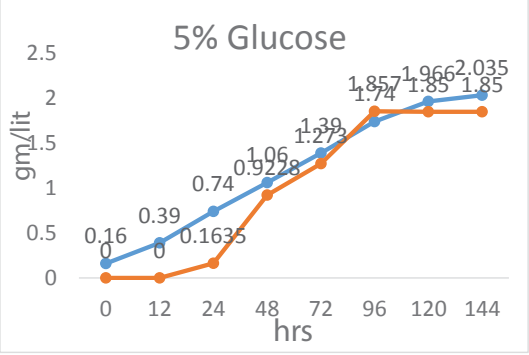
The emulsification index is determined (Figures 14-16), E_{24}=59.64.



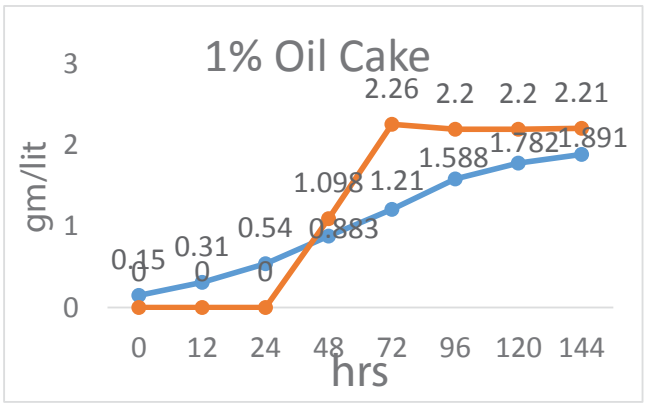
Graph 2: Standard rhamnolipid curve.

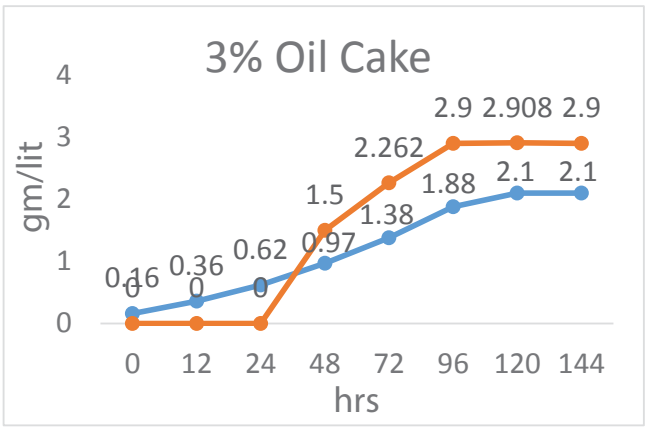


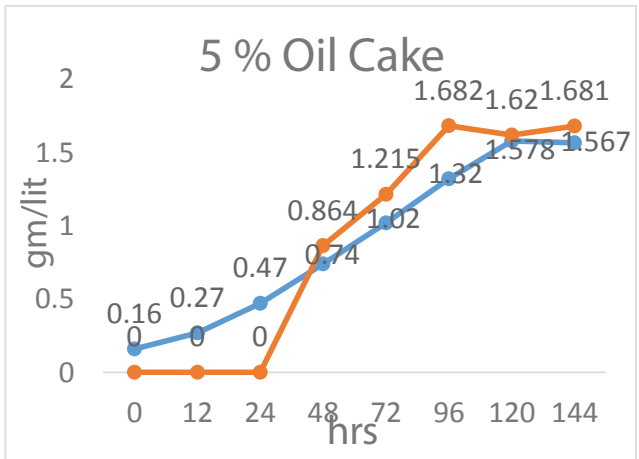




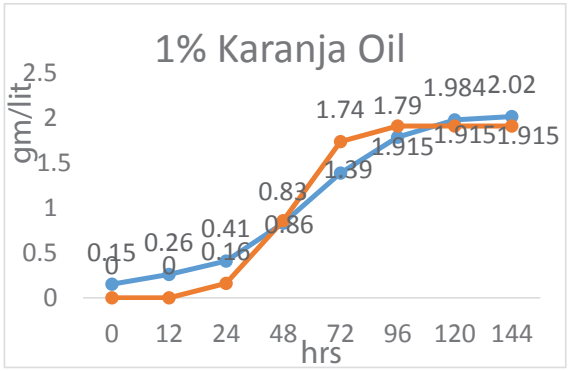
Graph 3.1- 3.3: Shows dry biomass concentration and rhamnolipid production at a glucose concentration of 1%, 3% and 5%, where line shows dry biomass concentration and line shows rhamnolipid concentration.

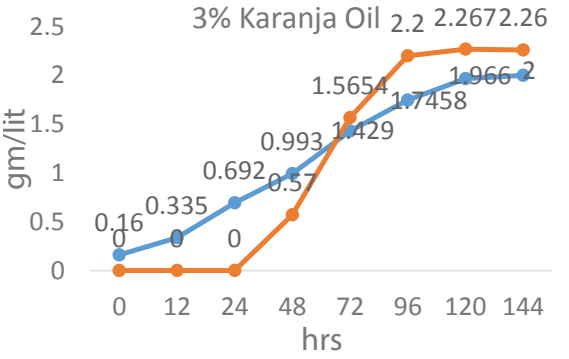


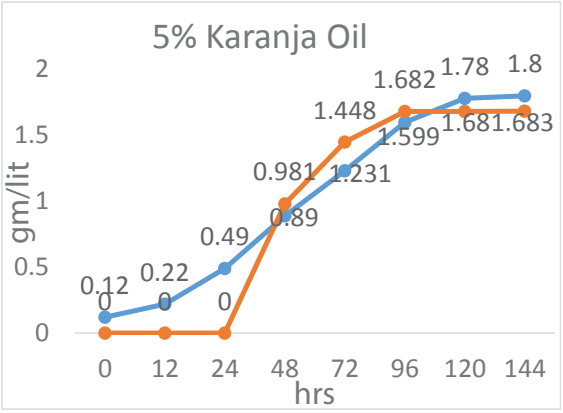




Graph 4.1- 4.3: Shows dry biomass concentration and rhamnolipid production at a soybean oil cake concentration of 1%, 3% and 5%, where line shows dry biomass concentration and line shows rhamnolipid concentration.







Graph 5.1- 5.3: Shows dry biomass concentration and rhamnolipid production at a karanja oil concentration of 1%, 3% and 5%, where line shows dry biomass concentration and line shows rhamnolipid concentration.

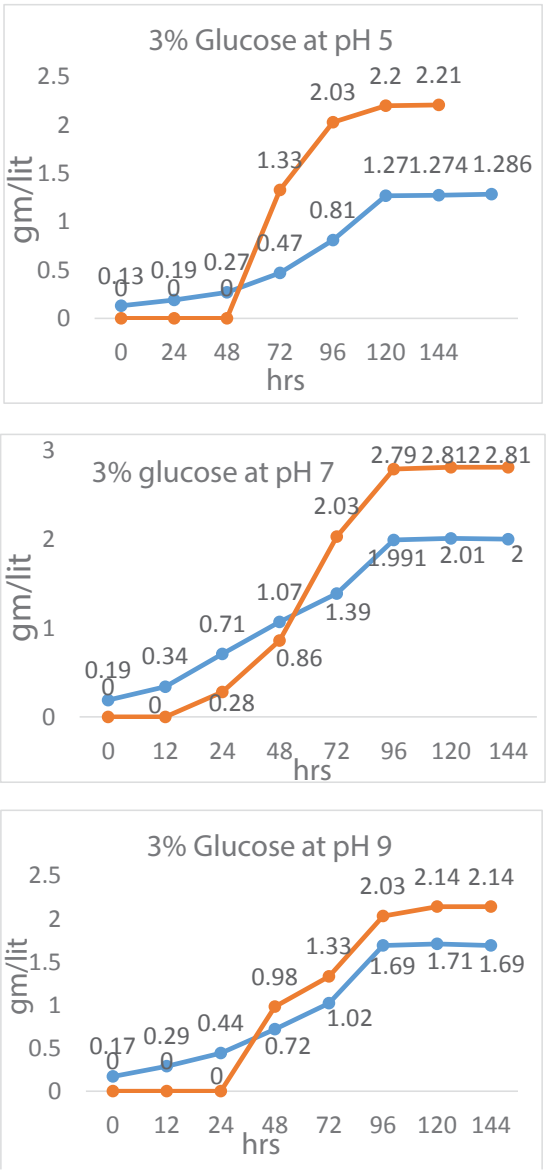
Emulsification index stability: Emulsification index stability at various pH and temperatures is plotted in Graphs 10 and 11.

Heavy and toxic metal recovery

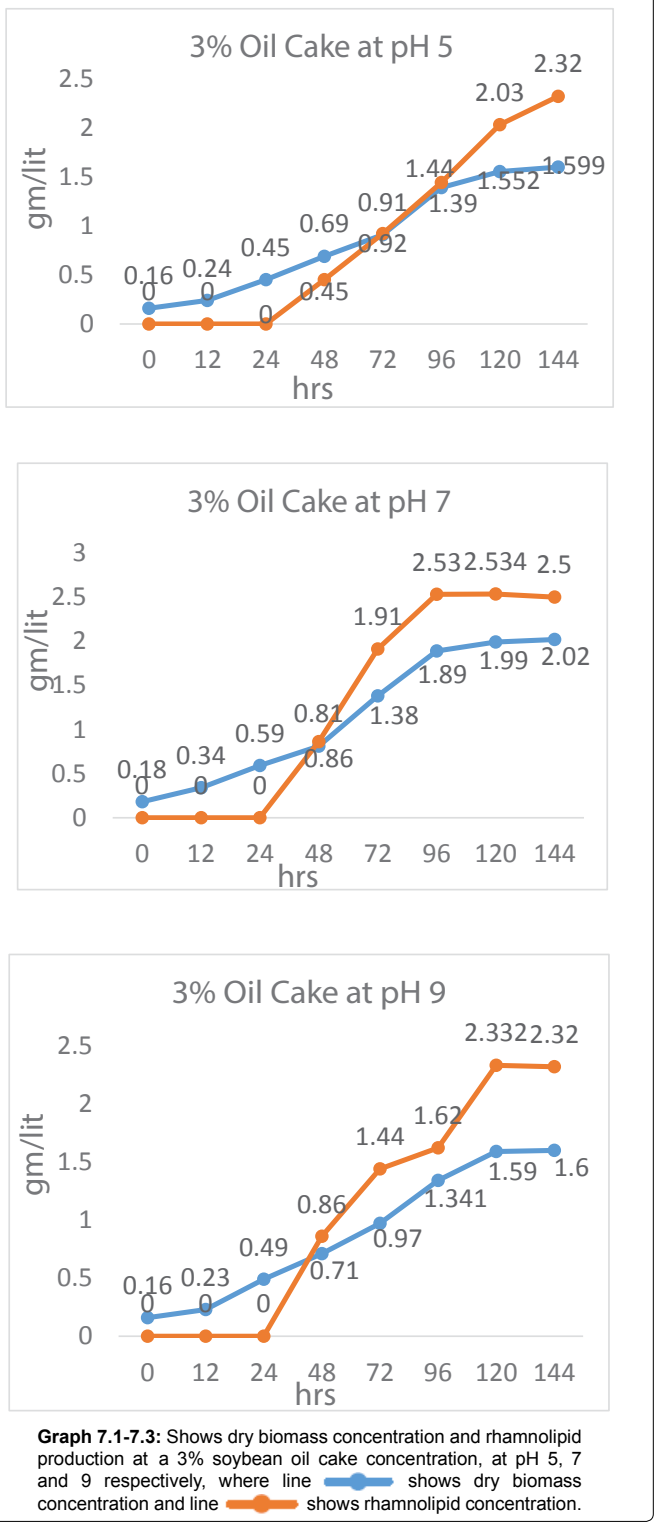
The recovery of heavy $FeCl_3$, $ZnSO_4$, $Pb(NO_3)_2$ was done at concentration 73%, 65%, 71% respectively and NF at concentration 43% (Tables 6 and 7, Figures 17 and 18).

Conclusion

The results show that, the rhamnolipid was successfully produced from *Pseudomonas aeruginosa* MTCC 1688 strain. The strategic search for cheap and effective substrate ends in karanja oil and soybean oil cake, which shows effective and enhanced production of rhamnolipid,

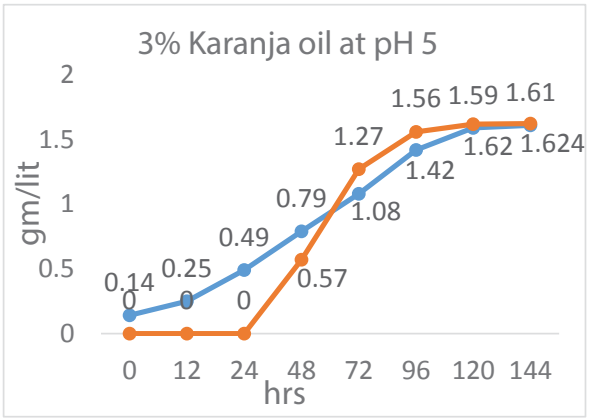


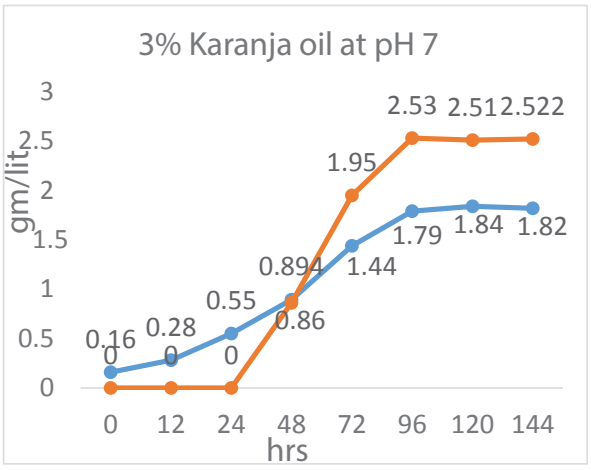
Graph 6.1- 6.3: Shows dry biomass concentration and rhamnolipid production at a 3% glucose concentration, at pH 5, 7 and 9 respectively, where line shows dry biomass concentration and line shows rhamnolipid concentration.

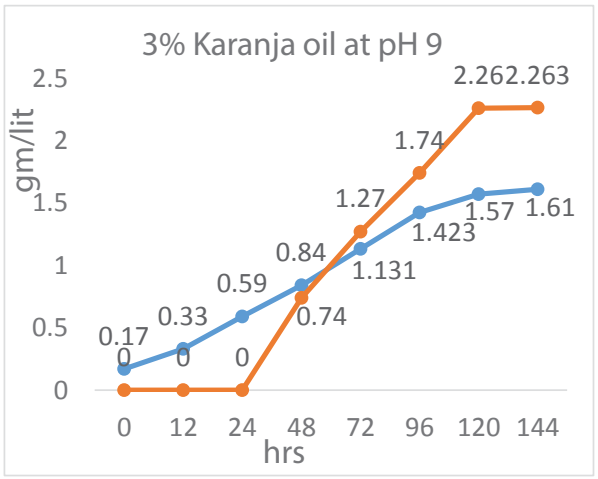


Graph 7.1-7.3: Shows dry biomass concentration and rhamnolipid production at a 3% soybean oil cake concentration, at pH 5, 7 and 9 respectively, where line shows dry biomass concentration and line shows rhamnolipid concentration.

at optimised pH of 7 and optimised concentration of 3%, compared to the various past researches. The optimum yield in terms of substrate was observed as 3.609 gm/lit of rhamnolipid produced per 5.255 ml of oil consumed, while yield in terms of biomass observed as 3.609 gm/lit of rhamnolipid produced per 2.5 gm of dry biomass. The chloroform:methanol extraction system was found to be the best solvent extraction system, where 83% of the rhamnolipid as recovered. The rhamnolipid was successfully applied for the heavy metals and toxic metals recovery, where rhamnolipid reduces heavy metal concentration to 73%, 65%, 71% for $FeCl_3$, $ZnSO_4$, $Pb(NO_3)_2$ respectively, while 43% in the case of toxic metal i.e. NaF. The produced rhamnolipid was found efficient in recovering 31% oil from oil sludge. Although rhamnolipid production has been intensively studied since the 1990´s, rhamnolipids have not widely succeeded in substituting synthetic surfactants; rather their use is restricted to specific applications where biocompatibility is required. The main reason for this situation can be found in the high costs for synthesis and downstream processing of rhamnolipids. The development of new production processes is the key issue in overcoming these economic obstacles.







Graph 8.1- 8.3: Shows dry biomass concentration and rhamnolipid production at a 3% Karanja Oil concentration, at pH 5, 7 and 9 respectively where line shows dry biomass concentration and line shows rhamnolipid concentration.

Figure 7: Foam formation during the fermentation.

Figure 8: Foam formation using magnetic stirrer after the fermentation in order to enhance bio surfactant production.

Figure 9: CTAB- methylene blue agar plate.

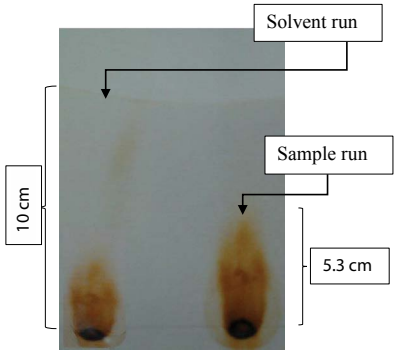
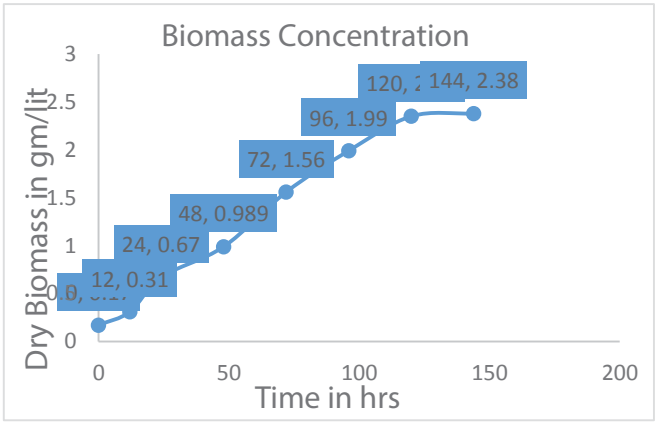
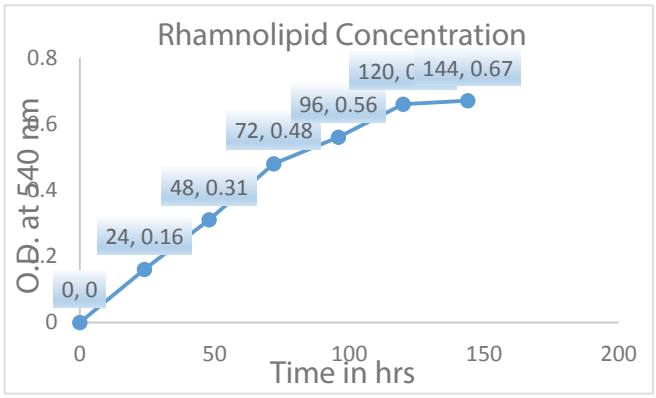


Figure 10: Paper chromatography result.





Graph 9.1- 9.3: Shows dry biomass concentration and rhamnolipid production at a 3% soybean oil cake concentration, at pH 5 in fermenter.

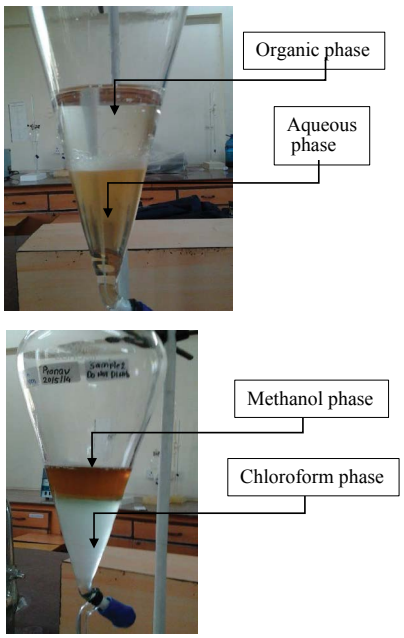


Figure 11: Solvent extraction using separating funnel with different extraction schemes

Figure 12: Column chromatography setup.

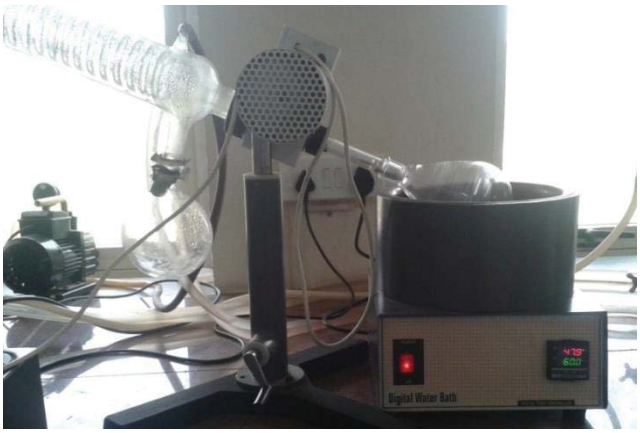


Figure 13: Rotary vacuum evaporator setup.

S. No	Metal	Wt. of Filter paper before (gm)	Wt. of Filter paper after filtration and drying (gm)	Difference (gm)	Efficiency of metal recovery
1	$FeCl_3$	1.244	1.271	0.027	73%
2	ZnSO4	1.273	1.308	0.035	65%
3	$Pb(NO_3)_2$	1.302	1.331	0.029	71%
4	NaF	1.242	1.285	0.043	43%

Table 6: Calculation table for heavy and toxic metal recovery.

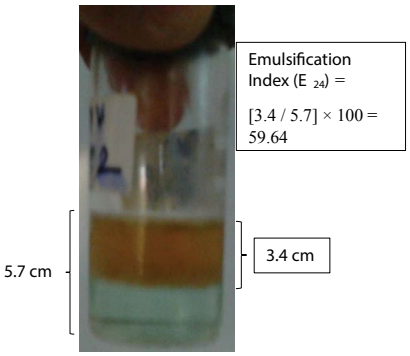


Figure14: Formation of emulsion.

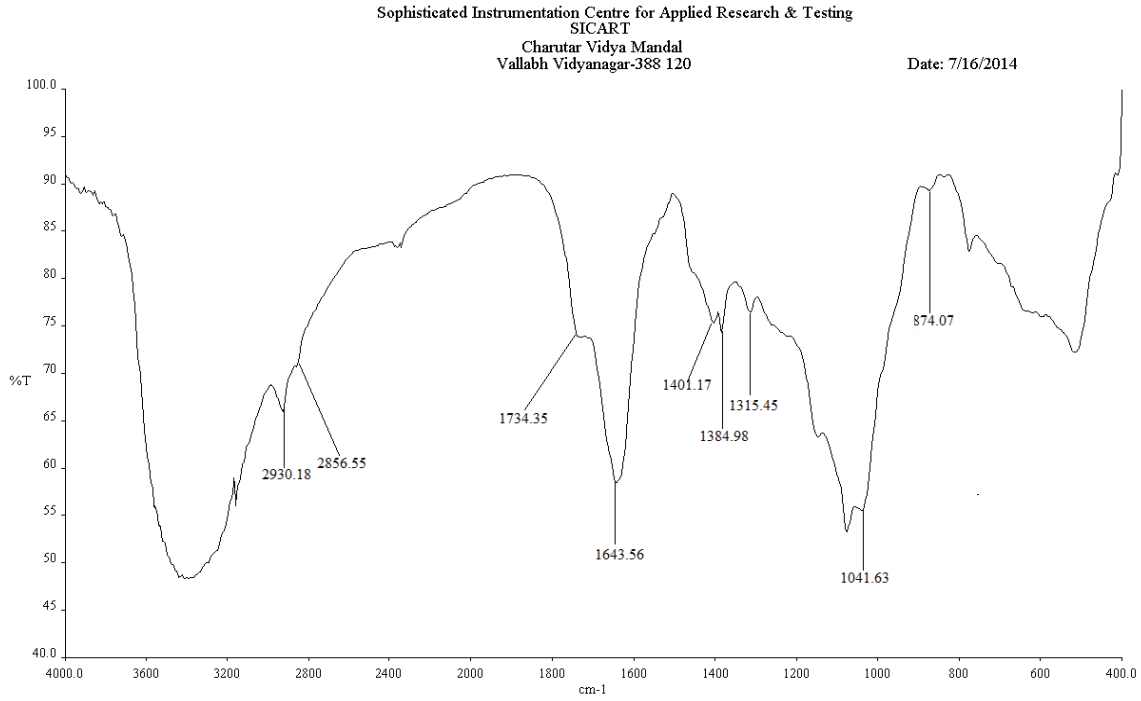


Figure15: FTIR graph.

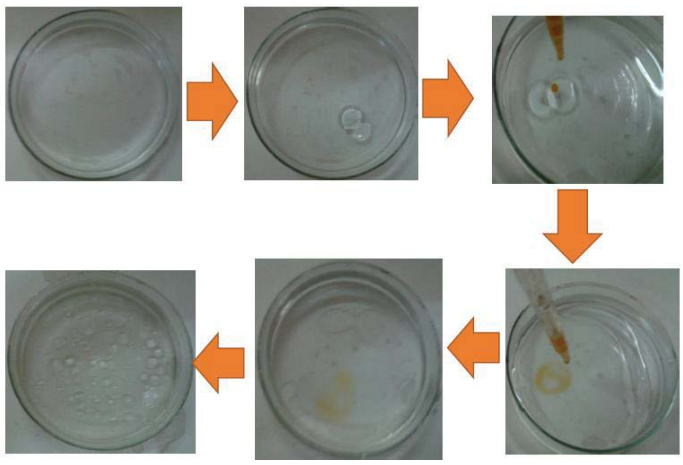
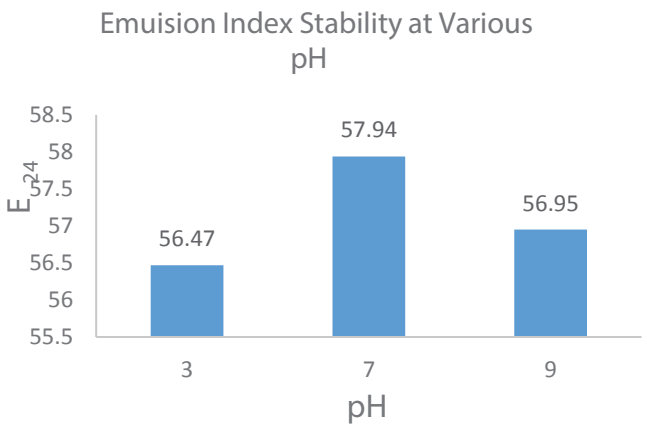
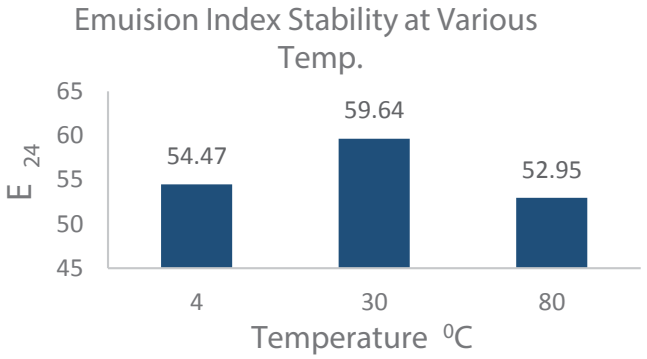


Figure 16: Formation of emulsion via oil spray technique.



Graph 10: Emulsion index at various pH.



Graph 11: Emulsion index at various temperatures.

S. No.	Experimental parameter	Value
1	Amount of sand taken	10 gm
2	Volume of oil added	10ml
3	Volume of RL added	10 ml
4	Vortexing time	5 min
5	Centrifugation speed	6000 rpm
6	Hexane used	10 ml
7	Oil recovered	3.1 ml
8	Percentage of oil recovered	31%

Table 7: Percentage recovery of oil from sludge.

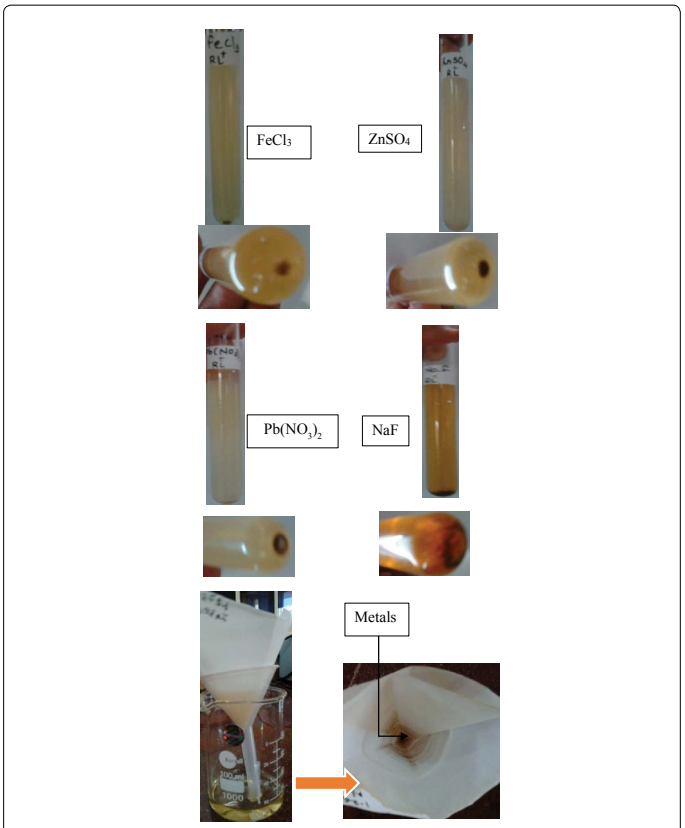


Figure 17: Heavy and toxic metal recovery process, showing recovery of $FeCl_3$, $ZnSO_4$, $Pb(NO_3)_2$and NF respectively

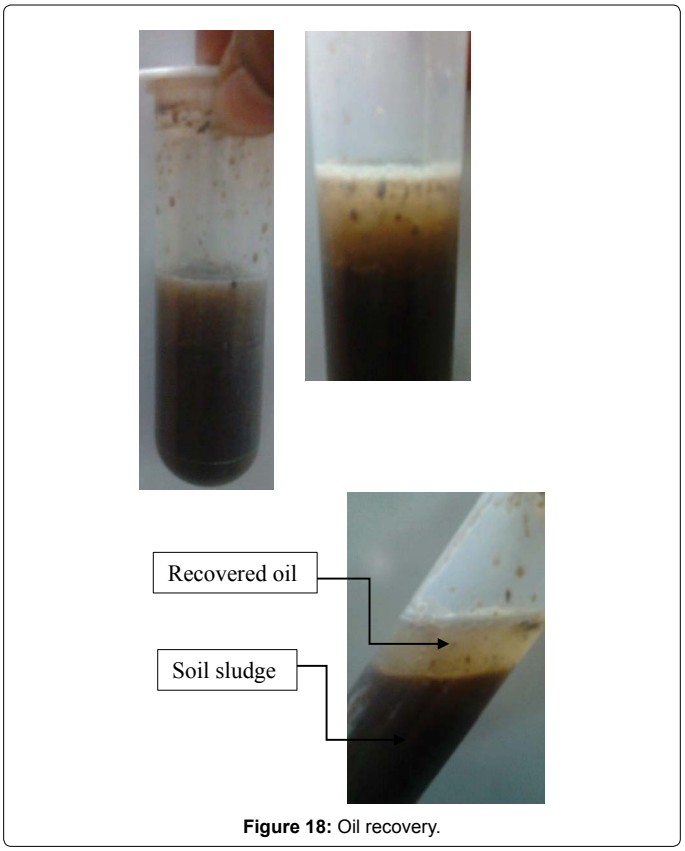


Figure 18: Oil recovery.

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