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# Characterization of emulsification activity of partially purified Rhamnolipids from *Pseudomonas fluorescens*

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**ABSTRACT:** In recent years natural biosurfactants have attracted attention because of their low toxicity, biodegradability, and ecological acceptability. Rhamnolipids is composed of rhamnose sugar molecule and ß-hydroxyalkanoic acid. Soil isolates of *Pseudomonas fluorescens* was used to characterize substrate (Mustard oil, Soybean oil, Olive oil, Palm oil, Sunflower oil and Coconut oil) for highest rhamnolipid production. Highest yield of biosurfactants was obtained from Soybean oil as 7.16 g/L by *P. fluorescens*. The estimated value of rhamnolipids was 0.437 g/L using Soybean oil from *P. fluorescens* by Orcinol method. Bacterium was capable of emulsifying a wide range of vegetable oils. The emulsification activity was found stable up to 72 hours. Upon characterizing C:N ratio of higher rhamnolipid was obtained at C:N ratio of 40 (93.75%) for *P. fluorescens* using Soybean oil as carbon source and Ammonium chloride as nitrogen source. It was revealed that partially purified rhamnolipid of *P. fluorescens* showed highest emulsification at optimum temperature 4°C for Palm oil, Sunflower oil and Coconut oil (90% all), at pH 6 and pH 7 in Palm oil, Sunflower oil and Coconut oil (90% all) and for 5% NaCl concentration in palm oil (94.44%).

**KEYWORDS:** Rhamnolipid biosurfactant, *Pseudomonas fluorescens*, emulsification activity.

## 1 INTRODUCTION

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surface or excreted extracellularly 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 (Priya and Usharani, 2009). Biosurfactants have several advantages, including low toxicity, high biodegradability low irritancy and compatibility with human skin. Biosurfactant have gained importance in the field of enhanced oil recovery, environment bioremediation, food processing and pharmaceuticals. Microbial biosurfactant are classified by its chemical composition and microbial origin (Desai *et al*., 1997). Low molecular weight (glycolipids or glycopeptides) can diminish surface tension, but does not form stable emulsion. On the other hand, biopolymers are less affection, and have a considerable specificity for substrate. Petroleum-derived hydrocarbon degrading microorganisms can produce biosurfactant to increase bioavailability and degradation. Biosurfactant are produced by organism, in order to metabolize water immiscible substrates, allowing its absorption, emulsification or dispersion. For the microorganism, production of biosurfactant is an advantage in soil, giving them advantages in specific condition (Ron *et al*., 2002).

Microbial biosurfactant are mainly produced by aerobic microorganism, using carbon as sources carbohydrates, hydrocarbons, animals or vegetable oil or a mixture of them. Biosurfactant can be intracellular (remain attached to the cell well) and can be excreted to the media. When the biosurfactant are intracellular, their structure includes membrane lipids and promote the transport of insoluble substrates through the membrane; when they are extracellular, the biosurfactant help on the surface of lipids, proteins and carbohydrates (Prabhu *et al*., 2003). The main difference in the chemical nature of the different biosurfactant molecules is in hydrophilic head, allowing for wide range of variation in their physical and biological properties (Lu *et al.*, 2007).

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Rhamnose is an unusual sugar that is found primarily in plants and some bacteria. Unlike most natural sugars, it is found in L configuration instead of the usual D configuration. It forms a major structural component of plant cell walls and is also bound to other compounds, such as phenolics (Thaniyavarn et al., 2006). In some gram negative bacteria, the sugar is bound to lipids. Both the pure compound and the lipid component have a number of uses in the pharmaceutical, agricultural, and cosmetic industries. One highly important and widespread use of this sugar in plants is as a component of polysaccharides known as rhamnogalacturonans (such polymers are important for plant cell wall structural integrity and comprise part of pectin, one of the substances that holds plant cell walls together. These are long chains of L-rhamnose mixed with galacturonic acid). There are different types of rhamnogalacturonans that vary in their degree of branching and components, which may include other sugars.Other bacteria utilize rhamnose combined with lipids in their polysaccharides, resulting in compounds called rhamnolipids. Those produced by the gram negative bacterium *Pseudomonas aeruginosa* are used commercially (Hamid *et al.,* 2006**)**. At least one other type of gram negative bacterium has been genetically engineered to produce a larger percentage of rhamnolipid in the exopolysaccharide surrounding the cells to facilitate improved extraction of the compound for industrial uses (Linhardt *et al*., 1989).

Rhamnolipids have the properties of a surfactant, meaning they can mix with oil and water. Most such compounds have been made from petroleum products in the past. These naturally based compounds are considered a green alternative to older products, and are often used in cosmetics, pharmaceuticals, and agriculture (Rahman *et al.*, 2002). In addition to the direct use of rhamnolipids, they are also used as commercial sources of rhamnose sugar. It is not ideal to have plants as a source of an industrial compound, since their availability may be limited. Rhamnolipids are mainly produced by bacteria of the genus *Pseudomonas*. Rhamnolipids are composed of one or two hydrophobic ß-hydroxy fatty acids, which are linked through a ß-glycosidic bond to one or two rhamnose molecules forming the hydrophilic moiety (Wang *et al*., 2007). According to the number of rhamnose moieties, mono and di-rhamnolipidsare differentiated. The fatty acids alkyl chain length in *P.aeruginosa* can vary from C8 to C14. A rhamnose test is available for intestinal permeability in humans (Wittgens *et al*., 2011).

Application of biosurfactant and biosurfatant-producing bacteria in bioremediation and phytoremediation has been studied. Due to their biodegradability and low toxicity, they are very promising for use in environmental technologies. Optimized growth condition using cheap renewable substrates (agro-industrial wastes) and novel, efficient method for isolation and purification of biosurfactants could make their production more economically feasible. Another important aspect regarding biological bioremediation technologies is the use of biosurfactant in the process on large scale. To facilitate this process,a new techniques should be developed such as foams or micro-foams in conjunction with biosurfactants (Płaza *et al*. (2011). Rhamnolipids with one sugar molecule are referred to as mono-rhamnolipids, while those with two sugar molecules are di-rhamnolipids **(**Gunther *et al.,* 2005). *P. fluorescens* is capable of growth and rhamnolipid production using a range of different carbon sources; however, the highest levels of rhamnolipid production result from using vegetable-based oils (Santos *et al*., 2010). The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils (Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil, Sunflower oil) to produce rhamnolipid-type biosurfactant, as substrates respectively (Santaanna *et al.,* 2002).

The objectives of study were to partially purify rhamnolipids from *P. fluorescens* and screen and characterize emulsification activity of rhamnolipids at different physiochemical parameters.

## 2 MATERIALS AND METHODS

For the present investigation *Pseudomonas fluorescens* was used and procured from the Microbial Culture Collection Bank (MCCB), Department of Microbiology and Fermentation Technology, SHIATS. Allahabad and maintained on nutrient agar slant.

Nutrient broth was used for preparation of the inoculum. The composition of the Nutrient broth used was as follows: beef extract 3.0 g, peptone 5.0 g, sodium chloride 5.0 g in a litre of distilled water. The seed culture was prepared in a test tube containing 10 ml of Nutrient broth medium by inoculating 1 loopful spore suspension and cultivated with agitation (150 rpm) at 30°C for one day.

### 2.2 Biosurfactant production from *Psuedomonas fluorescens*

2% inoculum in Nutrient broth was transferred into a Bushnell haas medium with the following composition (g/900ml): $KH_2PO_4$ 0.5 g, $Na_2HPO_4$ 0.5 g, NaCl 5.0 g, $NH_4Cl$ 1.0 g, $MgSO_4.7H_2O$ 0.1 g with 100 ml distilled water containing 2% substrate whose pH was adjusted to 7 ± 0.2. Then Bushnell haas broth containing 2% of inoculum and 2% of substrate [Palm oil (K S Oils Ltd.), Olive oil (Figaro Ind.), Coconut oil (Parachute Oils), Mustard oil (Sunrise Spices Ltd.), Soybean oil, Sunflower oil (both

from Fortune cooking Oil)] was incubated at 30°C under aerobic condition in a shaking incubator (Remi Instruments Ltd., India) at 200 rpm for 72 hours to 96 hours to obtained the highest biosurfactant concentration.

**2.3 Partial Purification of Rhamnolipids from *Psuedomonas fluorescens***

The culture was refrigerated at 4°C and then centrifuged (Remi Instruments Ltd., India) at 8500 rpm for 30 minutes to remove the cells and filtered with sterile whatman No.1 filter paper. The clear sterile supernatant was served as crude biosurfactant. The biosurfactent was recovered from the cell free culture supernatant by cold acetone precipitation; 2 V of chilled acetone was added and allowed to stand for 10 hours at 4°C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone after which it was re-dissolved in sterile double distilled water.

**2.4 Screening of rhamnolipids for biosurfactant activity**

Biosurfactant activity of *P. fluorescens* was detected by using Oil displacement assay, Emulsificaiton activity and Drop Collapse assay in six different oils namely Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil and Sunflower oil.

**2.4.1 Oil displacement assay**

The 15 ml of distilled water was added to a Petri dish (15 cm in diameter) followed by the addition of 20 µl of oil to the surface of water and 10 µl of supernatant of culture broth of *Pseudomonas fluorescens* was inoculated to oil drop. The oil showed displacement and a clearing zone was formed. The diameter of this clearing zone on the oil surface correlates to biosurfactent activity.

**2.4.2 Emulsificaiton activity ($E_{24}$)**

Sterilized biosurfactant solution (2ml) was added into each test-tube (in a set of three) containing the substrate (Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil, Sunflower oil) 2ml. The content of the tubes was vortexed uniformly for 2 minutes and left undisturbed for 24 hours. The volume of oil that separated after 24h, 48h and 72h of standing was measured that showed the ability of a molecule to form a stable emulsion. The emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed in percentage.

EI = (Height of the emulsion layer/ Total height) ×100

**2.4.3 Drop Collapse assay**

Oil was added to a calibrated microscopic slide, which for 1 hour at room temperature. The culture supernatant (5µl) was added to the surface of the oil dropped on slide, the shape of the drop on the oil surface was noted within 1 minute. The culture supernatant that collapsed the oil drop indicated positive drop collapse test otherwise the test was considered negative. Here distilled water with uninoculated oil drop was used as negative control.

**2.5 Characterization for emulsification activity of rhamnolipids at different physical parameters**

The applicability of biosurfactants in several fields depends on their stability at different parameters.

**2.5.1 Quantification of rhamnolipids**

For analysis, rhamnolipids were extracted using 100µl of orcinol-assay of cell free culture broth and 500µl of ethyl acetate. Sample was mixed at vortex mixer (Icon, India) with a subsequent phase separation by centrifugation at 10000 rpm for 1 min. The upper rhamnolipids containing phase was transferred to a new reaction tube. This procedure was repeated three times. Finally the organic solvent was removed by evaporation under agitation.

**Orcinol assay**

The evaporated rhamnolipids were dissolved in 100µl distilled water, subsequently 100µl of 1.6% orcinol solution and 800µl of 60% sulphuric acid in distilled water was added. The samples were incubated at 80°C for 30 min. and 1000 rpm orbital shaking in an incubator. After cooling to room temperature the samples were measured at 420 nm using a

colorimeter (Aimil Photochem Ltd., India) in comparison to different concentration of the commercial rhamnlipid. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose by comparing the data with those of rhamnose standards between 0 and 0.5 g/L and expressed as rhamnose equivalents, RE.

**2.5.2 Effect of Carbon-Nitrogen Ratio on rhamnolipid production and emulsification**

Carbon source used were Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil, Sunflower oil (2%,3%,4%,5%,6%V/V each) with $NH_4Cl$ as nitrogen source. For evaluation of Nitrogen sources on bio emulsification $NH_4Cl$ was employed at a concentration of 1, 2, 3, 4, 5 (g/l each) with the optimum carbon source for 24 hours. The effect of different Carbon, Nitrogen sources and emulsifying activity was carried out by the C/N ratio. Treatment control was set. Then emulsification activity was measured by $E_{24}$ assay.

**2.5.3 Effect of temperature on emulsification stability**

To observe the effect of temperature on bio emulsification for supernatant (2ml of partially purified biosurfactant was mixed with 2ml of Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil, Sunflower oil) followed by incubation at different temperatures (4°C, 10°C, 30°C, 70°C and 100°C) for 24 hours. Treatment control was set. Then emulsification activity was measured by $E_{24}$ assay.

**2.5.4 Effect of pH on emulsification stability**

To observe the effect of pH on bio emulsification for supernatant (2ml of partially purified biosurfactant was mixed with 2ml of Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil, Sunflower oil) followed by incubation at different pH ranges (5, 6, 7, 8 and 9 ) for 24 hours. Treatment control was set. Then emulsification activity was measured by $E_{24}$ assay.

**2.5.5 Effect of salinity on emulsification stability**

To observe the effect of salinity on bio emulsification for supernatant (2ml of partially purified biosurfactant was mixed with 2ml of Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil, Sunflower oil) followed by incubation at varied concentration of NaCl (1%, 2%, 3%, 4% and 5%) for 24 hours. Treatment control was set. Then emulsification activity was measured by $E_{24}$ assay

## 3 RESULTS

**3.1 Production and partial purification of Biosurfactant from *P. fluorescens***

A typical time course profile of the bacterial growth of the biosurfactant mixture produced by *P. fluorescens* in Bushnell haas medium, which was performed at 30°C under aerobic condition in a shaking incubator at 200 rpm for 72 hours at pH 7.0. The highest biosurfactant yield of 7.16 g/l was obtained after 72 hours of incubation using soybean oil and ammonium chloride as carbon and nitrogen sources, respectively.

**3.2 Screening of rhamnolipids for biosurfactant activity**

**3.2.1 Oil displacement assay of partially purified biosurfactant from *Pseudomonas fluorescens***

*P. fluorescens* showed highest oil displacement for soybean oil (2.5mm) followed by 2mm for mustard oil, 1.5mm for olive oil and followed by 0.7mm for sunflower oil and coconut oil.

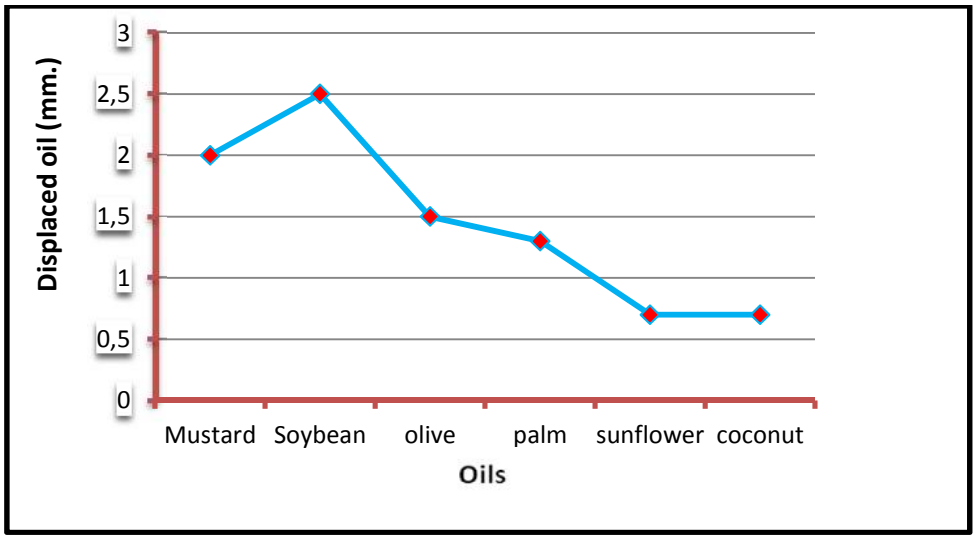


***Fig.3.1 Oil displacement assay***

### 3.2.2 Drop collapse assay of partially purified biosurfactant from *Pseudomonas fluorescens*

The partially purified biosurfactant from *P. fluorescens* showed a positive drop collapse assay that evidenced the biosurfactant activity. A positive drop collapse test by the isolates showed a preliminary indication of the biosurfactant activity of the bacterial cell that clearly indicated production of biosurfactant by the bacterial cell. The positive drop collapse assay also revealed about the extracellular production of the biosurfactant and its surface active nature.

### 3.2.3 Emulsification activity of partially purified biosurfactant from *P. fluorescens* on selected vegetable oils

*P. fluorescens* showed maximum emulsion formation on Mustard oil and Coconut oil (80% both) in 24 hours and minimum on olive oil (15%) in 72 hours. At 24 hours all six different oils *viz.,* Palm oil, Olive oil, Coconut oil, Mustard oil, Soybean oil, Sunflower oil showed the maximum emulsification index, and it gradually decreases up to 72 hours.

On analysing the data using two way ANOVA, the differences in data was found significant due emulsification and non significant due to oil. Critical difference (CD) was 21.77 for emulsification time.

***Table 3.1 Emulsification activity of partially purified biosurfactant from Pseudomonas fluorescens***

| Emulsification at different time | Emulsification Index (%) of Partially purified rhamnolipid by *P. fluorescens* | | | | | |
|---|---|---|---|---|---|---|
| | Mustard oil | Soybean oil | Olive oil | Palm oil | Sunflower oil | Coconut oil |
| $E_{24}$ | 80 | 70 | 75 | 50 | 73.33 | 80 |
| $E_{48}$ | 60 | 60 | 50 | 30 | 57.14 | 50 |
| $E_{72}$ | 33.33 | 55 | 15 | 25 | 33.33 | 50 |

***Table 3.2 Comparison table against CD value of emulsification at different time for Pseudomonas fluorescens***

| **CD = 21.77** | **$M_3$** 35.28 | **$M_2$** 51.19 |
|---|---|---|
| **$M_1$ 71.39** | S **36.11** | NS 20.2 |
| **$M_2$ 51.19** | NS 15.91 | - |

Where, $M_1$, $M_2$ and $M_3$ are the means of $E_{24}$, $E_{48}$ and $E_{72}$ respectively. From the above Comparison table, it is evident that there is significant difference between the mean pair ($M_1$, $M_3$) and non significant was observed for ($M_2$, $M_3$); ($M_1$, $M_2$).

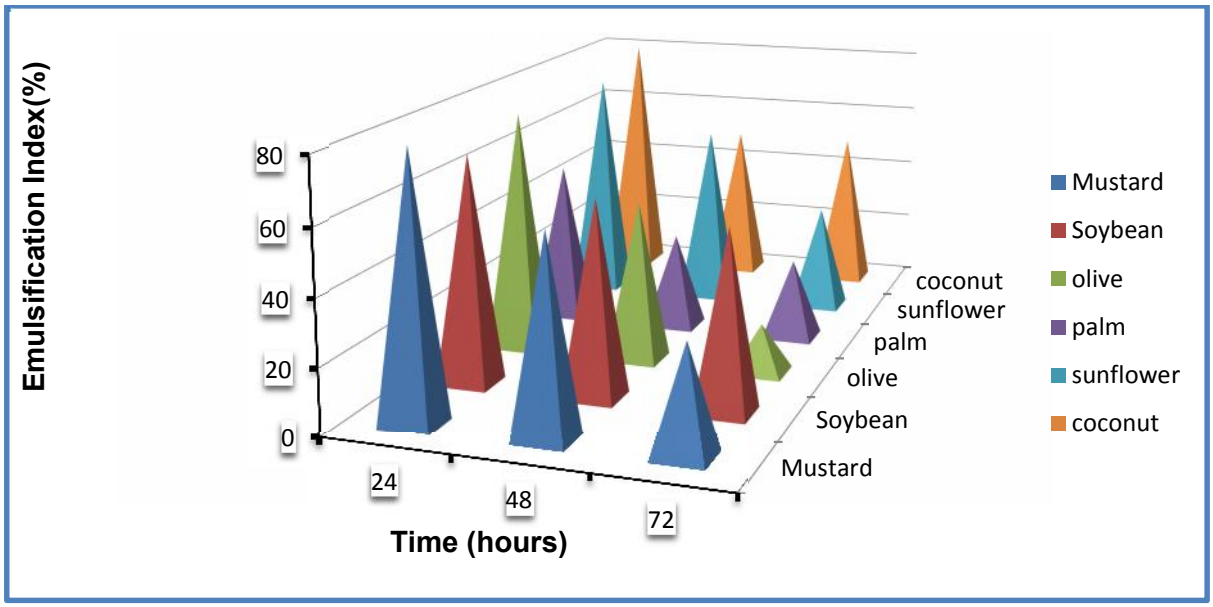


***Fig.3.2 Emulsification activity of partially purified biosurfactant from Pseudomonas fluorescens***

### 3.3 Characterization for emulsification activity of rhamnolipids at different physical parameters

The Biosurfactant from *P. fluorescens* was charecterized for emulsification activity of partially purified biosurfactant at different physical parameters *viz.,* quantification, C/N ratio, temperature, pH and NaCl concentration.

#### 3.3.1 Quantification of rhamnolipids

*P. fluorescens* showed highest yield for soybean oil (0.437 g/L) followed by 0.299 g/L for coconut oil, 0.289 g/L for palm oil, 0.233 g/L for mustard oil, 0.187 g/L for sunflower oil and 0.108 g/L for olive oil. In *Pseudomonas* sp. maximum yield obtained by *P. fluorescens.*

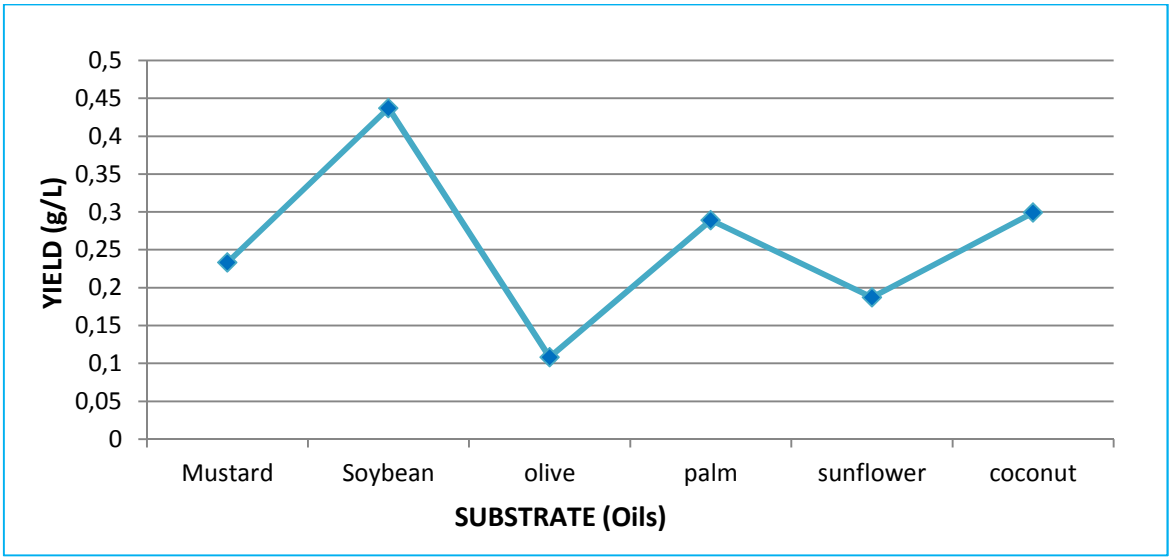


***Fig. 3.3 Quantification of rhamnolipids***

#### 3.3.2 Characterization for stability of emulsification activity at varying C/N ratio by partially purified rhamnolipid

*P. fluorescens* was charecterized for emulsification at different C/N ratio *viz.,* 10, 13, 20 and 40. *P. fluorescens* showed highest emulsification index on C/N ratio 10 (92%) followed by C/N ratio 13 (80%), C/N ratio 20 (70%) and C/N ratio 40 (65%).

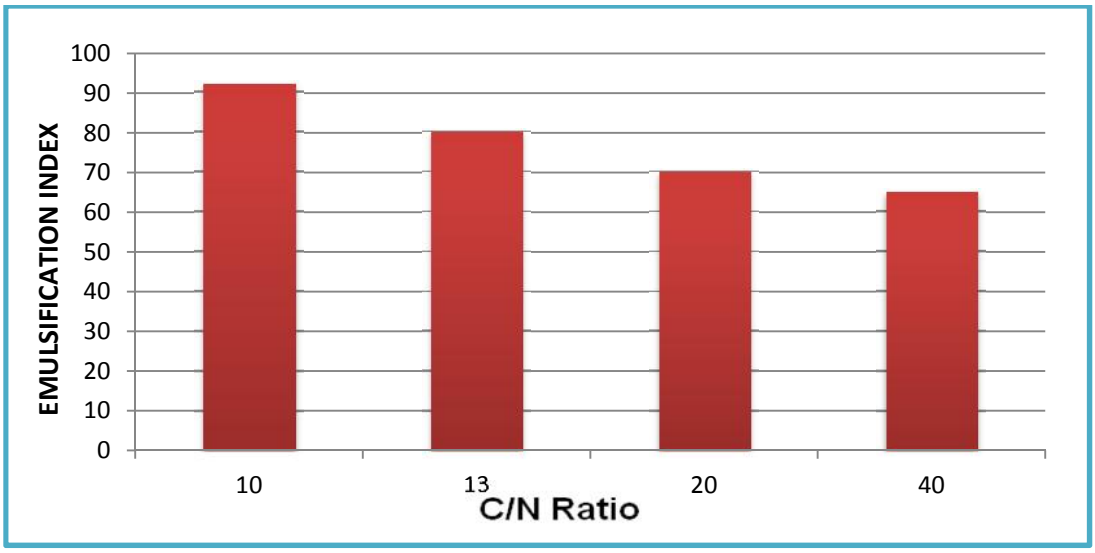


***Fig.3.4 Characterization for stability of emulsification activity at varying C/N ratio by partially purified rhamnolipid***

### 3.3.3 Effect of temperature on emulsification stability

The applicability of biosurfactants in several fields depends on their stability at different temperatures. The stability of biosurfactant was tested over a wide range of temperature. Cooling of biosurfactant to 4°C caused no significant effect on the biosurfactant performance. The emulsification activity was quite stable at the temperatures used ($E_{24}$=90%). *P. fluorescens* showed emulsification index on different hydrocarbons namely Mustard oil, Soybean oil, Olive oil, Palm oil, Sunflower oil and Coconut oil at different temperatures *i.e*. 4°C, 30°C, 40°C, 70°C and 100°C was observed. The highest emulsion formation was observed at 4°C on Palm oil, Sunflower oil and Coconut oil (90% all) followed by Soybean oil (66.67%), Mustard oil (73.33%) respectively. Optimum temperature 40°C for Mustard oil and Soybean oil, 30°C for olive oil and 4°C for Palm oil, Sunflower oil and Coconut oil.

On analysing the data using two way ANOVA, the differences in data was found significant due to temperature and non significant due to oil. Critical difference (CD) was 25.48 for temperatures.

***Table 3.3 Characterization for stability of emulsification activity of partially purified rhamnolipid by Pseudomonas fluorescens at varying temperature***

| Emulsification at different temperature | Emulsification Index (%) of partially purified rhamnolipid by *P. fluorescens* | | | | | |
|---|---|---|---|---|---|---|
| | **Mustard oil** | **Soybean oil** | **Olive oil** | **Palm oil** | **Sunflower oil** | **Coconut oil** |
| **4°C** | 40 | 46.67 | 66.67 | 90 | 90 | 90 |
| **30°C** | 44 | 53.33 | 73.33 | 83.33 | 60 | 80 |
| **40°C** | 52 | 66.67 | 33.33 | 41.67 | 80 | 70 |
| **70°C** | 48 | 33.33 | 26.67 | 66.67 | 50 | 20 |
| **100°C** | 40 | 26.67 | 13.33 | 66.67 | 0 | 10 |

***Table 3.4 Comparison table against CD value of temperatures for Pseudomonas fluorescens***

| **CD=25.48** | **$M_5$ 26.11** | **$M_4$ 40.78** | **$M_3$ 57.28** | **$M_2$ 65.67** |
|---|---|---|---|---|
| **$M_1$ 70.56** | S **44.45** | S **29.78** | NS 13.28 | NS 4.89 |
| **$M_2$ 65.67** | S **39.56** | NS 24.89 | NS 8.39 | |
| **$M_3$ 57.28** | S **31.17** | NS 16.5 | | |
| **$M_4$ 40.78** | NS 14.67 | | | |

Where, $M_1$, $M_2$, $M_3$, $M_4$ and $M_5$ are the means of the temperatures 4°C, 30°C, 40°C, 70°C and 100°C respectively. From the above Comparison table, it is evident that there is significant difference between the temperature mean pairs ($M_1$, $M_4$); ($M_1$, $M_5$); ($M_2$, $M_5$); ($M_3$, $M_5$) and non significant was observed between the temperature mean pairs ($M_1$, $M_3$); ($M_1$, $M_2$); ($M_2$, $M_4$); ($M_2$, $M_3$); ($M_3$, $M_4$) and ($M_4$, $M_5$).

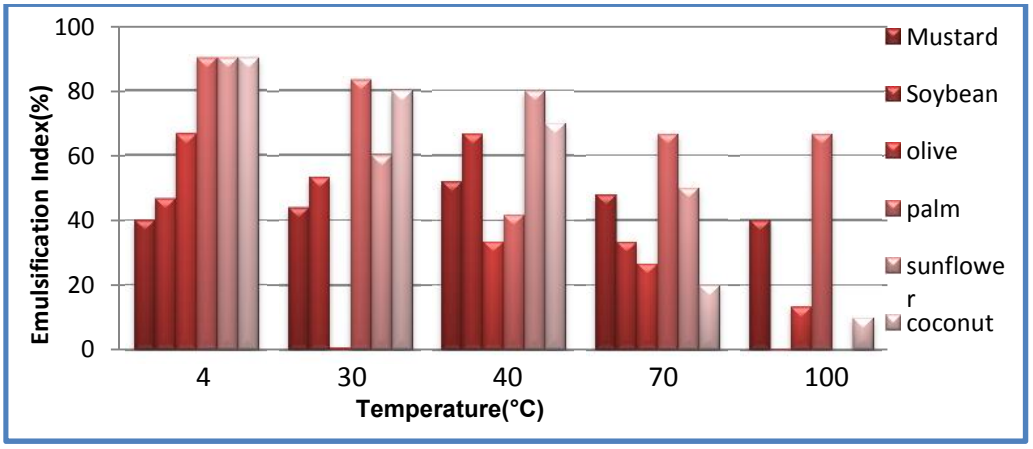


***Fig. 3.5 Characterization for stability of emulsification activity of partially purified rhamnolipid by Pseudomonas fluorescens at varying temperature***

### 3.3.4 Effect of pH on emulsification stability

The surface activity of the crude biosurfactant remained relatively stable to pH changes between pH 5 and 9, showing higher stability at pH 6 and neutral pH 7 At pH 9, the value in emulsification activity ($E_{24}$) showed almost more than 50% emulsification activity. *P. fluorescens* showed emulsification index on different hydrocarbons namely Mustard oil, Soybean oil, Olive oil, Palm oil, Sunflower oil and Coconut oil at different pH *i.e*. 5, 6, 7, 8 and 9 was observed. The highest emulsion formation was observed at pH 6 and pH 7 on Palm oil, Sunflower oil and Coconut oil (90% all) respectively. Optimum pH for Mustard oil was 5 and pH 8 for Soybean oil, pH 5 for olive Oil, pH 6 for Sunflower oil, pH 7 for Palm oil, pH 6 for Sunflower oil and Coconut oil.

On analysing the data using two way ANOVA, the differences in data was found non significant due to pH and oil.

***Table 3.5 Characterization for stability of emulsification activity of partially purified rhamnolipid by Pseudomonas fluorescens at varying pH***

| Emulsification at different pH | Emulsification Index (%) of Partially purified rhamnolipid by *P. fluorescens* | | | | | |
|---|---|---|---|---|---|---|
| | Mustard oil | Soybean oil | Olive oil | Palm oil | Sunflower oil | Coconut oil |
| **5** | 66.67 | 53.33 | 88.24 | 40 | 80 | 50 |
| **6** | 53.33 | 40 | 58.82 | 75 | 90 | 90 |
| **7** | 40 | 46.67 | 35.29 | 90 | 40 | 60 |
| **8** | 53.33 | 66.67 | 58.82 | 70 | 60 | 60 |
| **9** | 46.67 | 40 | 70.59 | 65 | 80 | 50 |

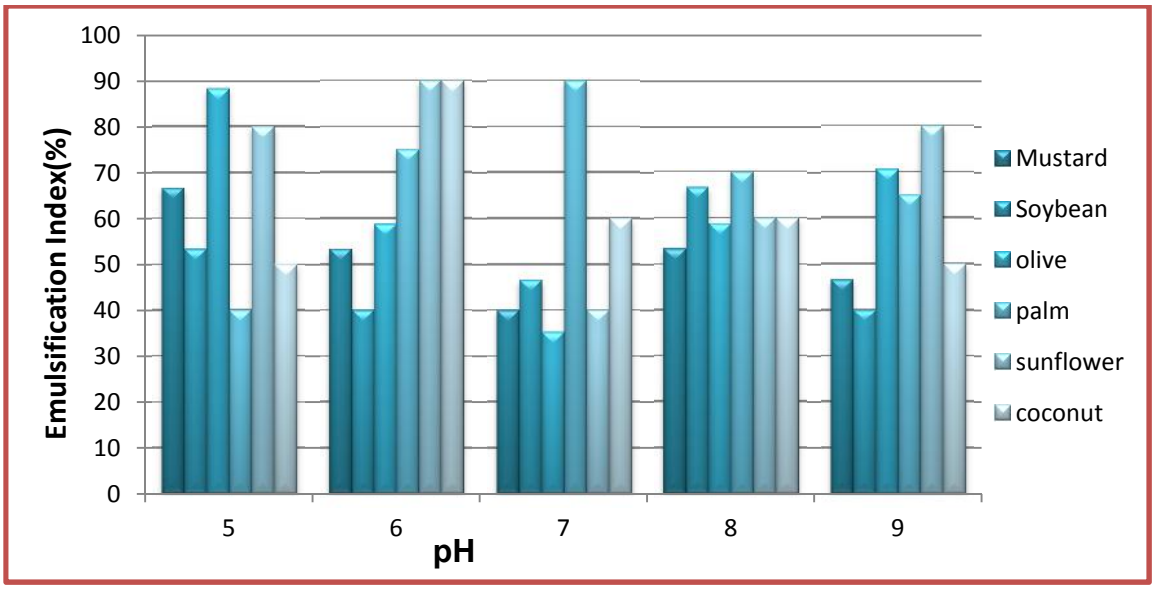


***Fig. 3.6 Characterization for stability of emulsification activity of partially purified rhamnolipid by Pseudomonas fluorescens at varying pH***

### 3.3.5 Effect of salinity on emulsification stability

The effect of sodium chloride addition on rhamnolipid was studied. Optimum stability of biosurfactant was observed at 5% NaCl concentration. Little changes were observed in increased concentration of NaCl of 1% and 5% (w/v). *P. fluorescens* showed emulsification index on different hydrocarbons namely Mustard oil, Soybean oil, Olive oil, Palm oil, Sunflower oil and Coconut oil at different NaCl concentration *i.e*. 1%, 2%, 3%, 4% and 5% was observed. The highest emulsion formation was observed at 5% NaCl concentration on Palm oil (94.44%). Optimum NaCl concentration for Mustard oil was 1% and 4%, for Soybean oil was 2%, 3%and 4%, for olive oil was 4%, for Palm oil was 5%, for Sunflower oil was 2% and Coconut oil was 1%.

On analysing the data using two way ANOVA, the differences in data was found non significant due to salinity and oil use.

***Table 3.6 Characterization for stability of emulsification activity of partially purified rhamnolipid by Pseudomonas fluorescens at varying salinity***

| Emulsification at different Salinity concentration | Emulsification Index (%) of Partially purified rhamnolipid by *P. fluorescens* | | | | | |
|---|---|---|---|---|---|---|
| | Mustard oil | Soybean oil | Olive oil | Palm oil | Sunflower oil | Coconut oil |
| **1%** | 75 | 70 | 70 | 40 | 70 | 90 |
| **2%** | 65 | 75 | 60 | 66.67 | 80 | 80 |
| **3%** | 60 | 75 | 70 | 83.33 | 60 | 80 |
| **4%** | 75 | 75 | 80 | 88.89 | 60 | 75 |
| **5%** | 60 | 25 | 50 | 94.44 | 80 | 70 |

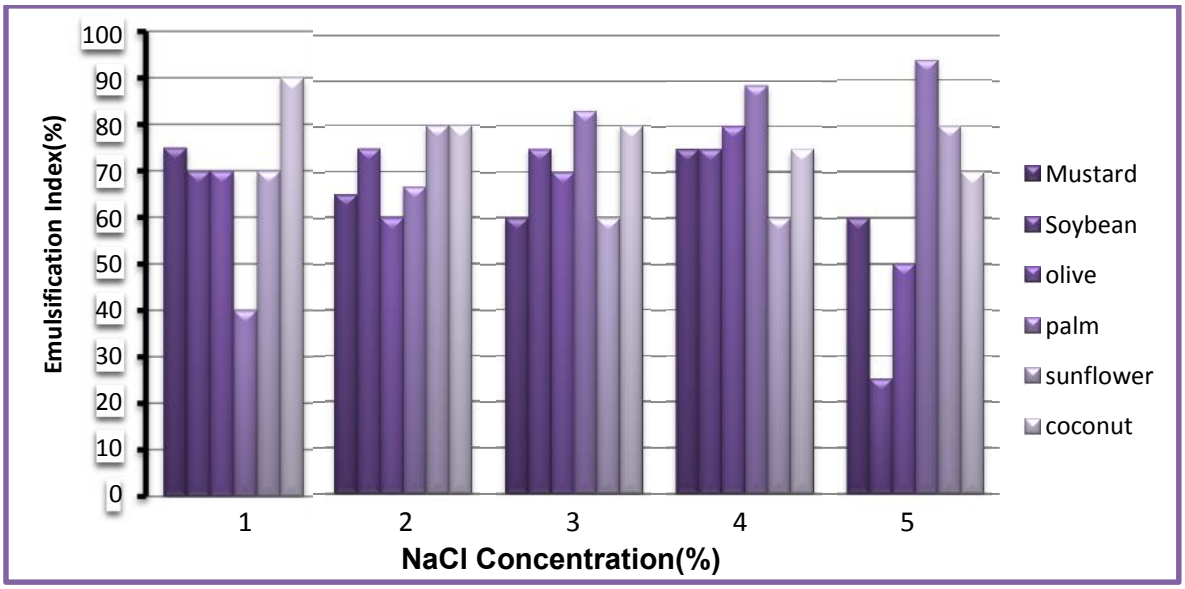


***Fig. 3.7 Characterization for stability of emulsification activity of partially purified rhamnolipid by Pseudomonas fluorescens at varying salinity***

## 4 DISCUSSION

In a similar study conducted by Pornsunthorntawee *et al*. (2008); Priya and Usharani (2009) the oil displacement assay was used to screen *P. fluorescens* for bio surfactant activity. The oil spread assay was used to screen bio surfactant activitiy against vegetable oil, kerosene, petrol and diesel. The study revealed similar results as in the present investigation. In other study conducted by Nishanthi *et al.* (2010) the diameter of clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. Pure biosurfactant has a linear correlation between quantity of surfactant and clearing zone diameter. The approach was used for screen the biosurfactant activity of partially purified rhamnolipids.

In the present investigation the highest displacement activity was recorded for soybean oil while least was for coconut oil by *P. fluorescens.* This might be due to the different type of viscosity possessed by the oils.

The drop collapse assay relies on the destabilization of liquid droplets by surfactants. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. The hydrocarbon degraders normally produce surfactants which adhere effectively to hydrophobic substrate. Many of the surfactants are reported to be basically lipids. Hence lipid content were analyzed which indicated the potential degradation of oils.

Recently, biosurfactants have gained numerous industrial and environmental applications which frequently involve exposure to extreme conditions. As a result, researchers have focused on isolating and screening strains that are able to produce biosurfactant under extreme environments, especially for MEOR and bioremediation purposes. In this study shown that *P. fluorescens* was produced rhamnolipid at pH values from 5 to 9 and at temperatures 4°C to 100°C, NaCl concentrations 1% to 4% (w/v). Kumar *et al.* (2012); Kumar *et al.* (2009); Priya and Usharani (2009) reported optimal growth for biosurfactant production by *P. fluorescens* was 37°C.

In the present investigation the highest emulsion formation was observed at 5% concentration of sodium chloride in Coconut oil. It might be due to the increases in intracellular concentration of Rhamnolipid parallel to the external concentration of sodium chloride. So, an increase in the intracellular concentration of Rhamnolipid can be resulted of increases in biosurfactant activity, also increases in retention by cytoplasmic membranes, or decreased dissimilation of fat globulli. Hence, increases the NaCl concentration decreases cellular efficacy, increases in viscosity of the aqueous phase and the increasing viscosity of the oil phase resulted in favourable emulsification activity. A similar study reported by Kokare *et al.* (2012) and Kumar *et al.* (2009) the effect of sodium chloride addition on biosurfactant activity. Optimum stability was observed at 3% NaCl concentration. In other study conducted by Christova *et al.* (2004) and Rahman *et al.* (2002) produced 0.97-2.7 g/l rhamnolipid by different species of *Pseudomonas* using glucose and waste fry oils as carbon source. The orcinol assay was used for direct asses of the amount of glycolipid in the biosurfactant. In the present investigation the highest yield were obtained from Soybean oil while least was obtained from mustard oil by *P. aeruginosa* and olive oil for *P. fluorescens.*

This might be due to Soybean oil suppliments increased the biomass and rhamnolipid production to several folds then other oils.

A more work by Rahman *et al.* (2002) showed *P. fluorescens* have higher emulsification activity in soybean oil. Emulsion formation probably due to the production of secondary metabolites which could interfere with emulsion formation and the adsorption of surfactant molecules at the oil-water interface. The reason behind the degradation of oil and hydrocarbon by *Pseudomonas* sp. is the nature of biosurfactant produced by them. As it know that surfactants are amphiphilic molecules consisting of a hydrophilic and hydrophobic domain. The surface tension and viscosity plays an important role, the more viscous in the liquid there is more chance of binding of partially purified biosurfactant as it increases the surface tension. The partition between two phases in a heterogeneous system increases the apparent solubility of a hydrophobic compound in water.

*P. fluorescens* strain is able to produce bioemulsifier at a wide range of temperatures, NaCl concentrations, pH values. Since biosurfactants are valuable products in industrial applications, determining the optimum conditions for improvement of the biosurfactant yield is very important from an economical point of view. The optimum pH for biosurfactant production was 7. The produced rhamnolipid has excessive oil spreading, emulsification and surface activity properties. With soybean oil and other vegetable oils it created an oil displacement zone of 3.0 mm, and reduced the surface tension and interfacial tension of the medium. According to the obtained results, it was found that temperature, pH, salinity and type of surfactant were the major factors affecting the biodegradation rate and surface activity of *P. fluorescens.* Further, the *P. fluorescens* had the ability to produce biosurfactant in the presence of long chain n-alkanes, light and heavy vegetable oil as the sole carbon source. This enables *P. fluorescens* to have potential to degrade these toxic compounds, even at relatively high concentrations. In contact with vegetable oil, it showed more growth and a higher percent of degradation than the individual strains of *P. fluorescens*. Some previous works reported that combinations of produced biosurfactants by microbial production are more effective than individual biosurfactants in lowering surface tension, improving the bioavailability of hydrocarbon and increasing the extent of degradation. *P. fluorescens* emulsifies the hydrocarbon and creates rhamnolipid which increases the solubility of crude oil in the aqueous phase. The combination of these major factors increases the bioavailability of crude oil. The ability of *P. fluorescens* to produce biosurfactant in contact with crude oil promotes its role in oil recovery, and decreasing the residual oil saturation. The highest biosurfactant production was achieved when $NH_4Cl$ was used as the sole source of nitrogen. This result was compatible with previous works where the maximum biosurfactant yield was obtained with sodium nitrate as the sole nitrogen source. The obtained data also showed that maximum biosurfactant yield was attained using a C/N ratio of 10:1. Nitrogen limitation improves biosurfactant production and the ratio of carbon to nitrogen must be balanced in the culture medium. It is also reported that a C/N ratio lower than 11 would maximize rhamnolipid production by *Pseudomonas* sp. The biosurfactants produced by the *P. fluorescens* increased the viscosity of the aqueous phase and reduced the viscosity of the oil phase. This leads to a favourable mobility control and emulsifying index, which have potential applications in the oil industry. The results obtained from the oil recovery tests suggest that the produced biosurfactants by the *P. fluorescens* can efficiently mobilize the trapped oil under extreme conditions. The strain of *P. fluorescens* produced a high molecular weight bioemulsifier which increased the viscosity of the aqueous phase. It has also been reported that the *P. fluorescens* strain produces a high amount of gas per mole of substrate that utilized. Gas production has been mentioned as an important mechanism for oil recovery which increases the pressure of the core sample, swells the crude oil and reduces its viscosity. The simultaneous effects of the increasing viscosity of the aqueous phase and the decreasing viscosity of the oil phase resulted in favourable mobility ratios. On the other hand, the Pseudomonas sp. strain produced rhamnolipid biosurfactcnt which had a substantial effect on the reduction of oil/water interfacial tension and recovering the trapped oil. IFT reduction is responsible for the major part of oil recovery from very small pores under the MEOR process. When the salinity and temperature was increased, the oil recovery decreased.

## 5 CONCLUSION

*P. fluorescens* uses Soybean oil as the best carbon source for biosurfactant production. For isolate physical parameters *viz.,* optimum C/N ratio (10), temperature (4°C), pH (6, 7) and NaCl concentration (5%) for biosurfactant activity were analyzed. Biosurfactant obtained by *Pseudomonas* species could be used in bioremediation and phytoremediation. The study highlighted the potential of bacteria that could be used specially for hydrocarbon polluted area and oil spills. The biosurfactants in mixtures were able to produce microemulsions for a wide range of oils that are applicable for vegetable oil extraction for biofuel application, hard surface cleansers, drug delivery and detergents.

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