ORIGINAL ARTICLE

# Production, characterization, and optimization of rhamnolipids produced by *Pseudomonas aeruginosa* by solid-state fermentation

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## ABSTRACT

**Background:** *Pseudomonas aeruginosa* that produces biosurfactant (rhamnolipids) is used for emulsifying the oil-infested areas. The objective of the study was to isolate and identify a strain of environmentally important that can act on oil-contaminated soil. **Materials and Methods:** Isolation and identification of *P. aeruginosa* from oil-spilled soil isolated from Chennai. Rhamnolipid production was enhanced by fish oil. pH, temperature and incubation time were optimize using RSM, solid-state fermentation (sugarcane bagasse) was adopted for production of rhamnolipid. **Results:** The isolated strain of *P. aeruginosa* was subjected to optimization of cultural condition such as temperature, pH, and incubation time representing 35°C, 6, and 5 days, respectively. The solid-state fermentation was done to find the enzyme production using molasses and fish oil. The surfactant that was extracted was analyzed for surface tension ability to confirm the used as biodetergents. **Discussion and Conclusion:** From the above analysis, *P. aeruginosa* could be ideal candidate for the production of biosurfactant that can be used in the environmental oil slippage.

**Key words:** Fish oil, *Pseudomonas aeruginosa*, rhamnolipid, response surface methodology

## INTRODUCTION

A biosurfactant is a naturally produced surfactant that has both hydrophilic end (attracts water) and hydrophobic end (repels water and attracts non-polar chemicals). The hydrophobic part of the molecule is a long chain of fatty acids composed of hydroxyl fatty acids or α-alkyl beta hydroxyl fatty acids.[1-3] Most surfactants, including rhamnolipids, are also amphiphilic, meaning that they have a hydrophilic end and lipophilic end. The lipophilic end is attracted to oils.[4-6] Surfactants are chemicals that reduce the surface tension of water. Surfactants help as wetting agents, emulsifiers, and foaming agents and are used in soaps and wetting agents in sprays.[7-9] Biosurfactants are amphiphilic biological compounds produced as extracellular or as part of the cell membrane by *Pseudomonas aeruginosa* from various substances including sugars, oil, and wastes.[10] Rhamnolipid is one of the most commonly used biosurfactants with the ability of reduced surface tension of the water.[11]

Rhamnolipids are produced by the bacterium *P. aeruginosa* isolated from the oil-spilled soil.[12] They are constructed of rhamnose sugar combined with beta-hydroxy fatty acid. There are two major type of rhamnolipid; mono-rhamnolipid and di-rhamnolipid.[13-15] Mono-rhamnolipid has a single rhamnose sugar ring di-rhamnolipid have two rhamnose sugar ring. Biosurfactant use as bioremediation is the process of removing oils, metals, and other pollutants from soil, water, coastlines, and the seabed.[16,17] Rhamnolipids have been demonstrated to destroy the outer wall of various fungi in the spore state. Rhamnolipids kill some bacteria.[18-20] They also increase the susceptibility of certain Gram-positive bacteria to specific antibiotics.[21] Various studies have demonstrated that rhamnolipids help the treatment of numerous skin

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diseases.[22,23] Tests have shown that rhamnolipids suppress the spread of breast cancer cells.[24-26] Rhamnolipids in the right concentration are a hemolysin, meaning that they can break up red blood cells by lysis, destroying the blood cell wall.[27,28] Rhamnolipids have numerous applications in agriculture and farming, they are effective as a fungicide and bactericide for certain agricultural pests.

## MATERIALS AND METHODS

Soil sample was collected from different locations of Chennai. Serial dilution was performed to reduce the microbial load from the soil sample. By pour plate and streak plate technique, the individual colonies can be observed throughout the medium. Gram-staining, motility, and biochemical tests (indole test, methyl red test, Voges–Proskauer test, citrate utilization test [IMVIC], catalase, oxidase) were done for the identification of the organism.[29] *P. aeruginosa* grown on plate count agar was transferred to the seed culture medium.

Seed culture grown in 250 ml flask contains basal salt medium at 30°C at rotary shaker at 180 rpm for 14 h. Basal salt medium contains $MgSO_4 7H_2O$ - 0.5, $FeSO_4.7H_2O$ - 0.01, $KH_2PO_4$ - 1.0, $K_2HPO_4$ - 0.5, $NaNO_3$ - 2.0, $CaCl_2$ - 0.01, and KCl - 0.1.[30] The carbon source was sterilized separately by the steam sterilization process. About 1 ml of mid-exponential phase seed culture was inoculated into each 250 ml sterile Erlenmeyer flask containing 50 ml of sterile impregnating solution with 3% (v/v) glycerol. Flasks were incubated at 30°C at 200 rpm on the orbital shaker for 12 days. The impregnated solution contains per liter, 3.0 g - $KH_2PO_4$, 7 g - $K_2HPO_4$, 0.2 g - $MgSO_4.7H_2O$, and 1 g - $(NH_4)_2SO_4$ and glycerol.

### Response Surface Methodology

The physical paraments such as pH, temperature, and incubation time were optimized using the software JMP 10.[31,32] The design of experiments (DOE) was generated, with regard to the runs' experiment was performed, and the results were tabulated [Table 1]. The module used was central composite design with 16 runs.

### Solid-State Cultivation

In the standard cultivation, each 250 ml Erlenmeyer flask contained 10 g of dry sugarcane bagasse which had been dried at room temperature.[33] The flask was then autoclaved at 121°C for 15 min. About 50 ml of impregnated solution was inoculated with 2 ml of seed culture and mixed into bagasse [Figure 1]. Inoculated flask was incubated at 30°C. Further extraction of rhamnolipid from the sugarcane bagasse was performed. Surface tension of culture broth and surfactant solution was measured by capillary rise method.

### Extraction of Rhamnolipid

Each flask received 100 ml of distilled water, agitated for 1 h at 200 rpm at 30°C on orbital shaker, and then, filtered through cheese cloth; then, the excess liquid being squeezed out manually. This procedure done for 3 times; then, the sample was centrifuged for 10 min at 12,500 rpm. Then, the supernatant was extracted with at least 3 times with CHCL:methanol (3:1) with 15 ml of this solvent mix being used for each extraction [Figure 2]. The organic phase concentration at reduced pressured at 40°C, the crude extract containing the rhamnolipid. Using spectroscopic analysis, determining the production of rhamnolipid was performed.[34]

The sample pH is adjusted to 2.3 using 1 N HCl. The acidify sample was then extracted with 5-fold volume of chloroform. About 4 ml of chloroform extract was carefully removed.

### Rhamnolipid Analysis by Methylene Blue Analysis Procedure

Put in contact with a freshly prepared methylene blue solution containing 200 µl of the gram per liter methylene blue reagent

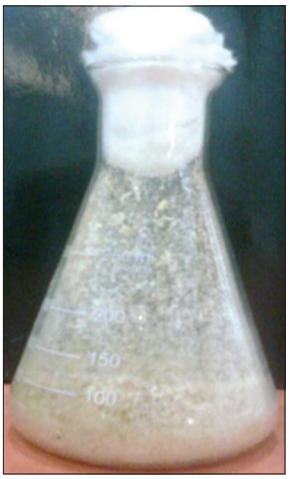

**Figure 1:** Solid-state fermentation

**Figure 2:** Extraction of rhampholipid

**Table 1:** DOE with results (response)

| Run | Pattern | pH | Temperature (°C) | Incubation time (days) | Response |
|---|---|---|---|---|---|
| 1 | −−− | 4 | 25 | 3 | 7.8 |
| 2 | +−− | 8 | 25 | 3 | 7.9 |
| 3 | 00a | 6 | 35 | 3 | 12.7 |
| 4 | −+− | 4 | 45 | 3 | 10.8 |
| 5 | ++− | 8 | 45 | 3 | 4.2 |
| 6 | 0a0 | 6 | 25 | 5 | 9.4 |
| 7 | a00 | 4 | 35 | 5 | 11.2 |
| 8 | 0 | 6 | 35 | 5 | 15.4 |
| 9 | 0 | 6 | 35 | 5 | 15.7 |
| 10 | A00 | 8 | 35 | 5 | 11.9 |
| 11 | 0A0 | 6 | 45 | 5 | 10.2 |
| 12 | −−+ | 4 | 25 | 7 | 9.1 |
| 13 | +−+ | 8 | 25 | 7 | 6.8 |
| 14 | 00A | 6 | 35 | 7 | 12.7 |
| 15 | −++ | 4 | 45 | 7 | 6.7 |
| 16 | +++ | 8 | 45 | 7 | 7.7 |

DOE: Design of experiments

and 4.9 ml of distilled water. pH of methylene blue had been preadjusted to 8.6 by adding the 50 mM borax buffer. It is then vigorously mixed for 4 min and the samples were left to stand for 15 min. The chloroform phase was transferred into a cuvette and the absorbance was measured at 638 nm with a ultraviolet-visible spectrophotometer.[35]

## RESULTS AND DISCUSSION

### Pour Plate Method in Plate Count Agar

After incubation of 48 h, in the plates containing bacterial culture of different dilution, green color colonies were observed.

### Streak Plate Method

In the first plate count agar, green, round colonies are observed [Figure 3]. In blood agar plate, β hemolytic colonies are observed. In the cetrimide agar plate, colonies show a characteristic green color.

After performing the Gram's staining, the slides were observed under light microscope at oil-immersion objective. The slide showed purple bacilli. This concluded that the slide contained Gram-negative bacteria. The bacterial suspension placed under the both cavity slides showed motility. The biochemical results show that indole positive, methyl red negative, Voges-Proskeur negative, citrate positive, catalase positive, and oxidase positive [Table 2].

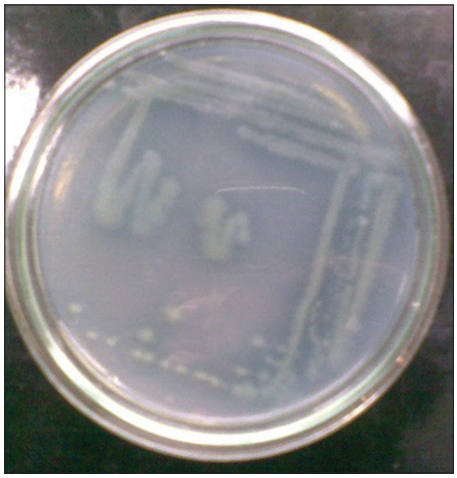

**Figure 3:** *Pseudomonas aeruginosa* on plate count agar

The effect of the carbon source on rhamnolipid produced by *P. aeruginosa* was investigated in the basal salt medium containing 1% (w/v) of variety of carbon sources such as glucose, glycerol, liquid paraffin, dextrose, lactose, and fish oil. The cell growth and emulsifying activity were measured after 48 h of cultivation. As shown in the Table 3, the fish oil was most effective carbon source for emulsifying activity among the carbon sources tested.

In the response surface methodology, the effective interaction of the components was studied and expressed in Figure 4 shown as contour plot, the interaction between various parameters was analyzed such as temperature versus pH (a), incubation time versus temperature (b), pH versus incubation time (c). Table 4 represents the relevant summary of fit as the square of regression is 95% that shows the significance of

the model and the adjusted $R^2$ is also similar to $R^2$ value. The analysis of variance shows the lack of fit is not significant and functionality of the model is good [Tables 5 and 6]. Figure 5 shows the curve fitting between the actual and predictable results as points present in the figure arranges to the central diagonal line that indicates that more the significant the results are.

Table 7 sorted parameter estimates report does not show the intercept. The effects are sorted by the absolute value of the *t* ratio, showing the most significant effects at the top. A bar chart shows the *t* ratio with vertical lines showing critical values for the 0.05 significance level. From the above estimate, its shows the interaction between the factors in combination with each other.

The basic use of constructing DOE in response surface methodology is to understand the interaction of components and also predictability of the component when they are combined in various concentrations [Figure 6]. The prediction

**Table 2:** Biochemical test

| Biochemical | Result |
|---|---|
| Gram-staining | Gram-negative bacilli |
| Motility | Motile |
| Indole | Positive |
| Methyl red | Negative |
| Voges–Proskauer | Negative |
| Citrate | Positive |
| Catalase test | Positive |
| Oxidase test | Positive |

**Table 3:** Comparative cell growth and emulsifying activity on various carbon source

| Carbon source | Cell growth at 650 nm | Emulsifying activity (unit) |
|---|---|---|
| Glucose | 0.6 | 13.5 |
| Dextrose | 0.4 | 12.4 |
| Lactose | 0.5 | 36.9 |
| Glycerol | 1.3 | 34.3 |
| Liquid paraffin | 0.9 | 26.0 |
| Fish oil | 1.8 | 168.4 |

**Table 4:** Relevant summary of fit

| | |
|---|---|
| $R^2$ | 0.954917 |
| $R^2$ adjustment | 0.937292 |
| Root mean square error | 1.919902 |
| Mean of response | 10.0125 |
| Observations (or sum Wgts) | 16 |

**Table 5:** Analysis of variance

| Source | DF | Sum of squares | Mean square | *F* ratio |
|---|---|---|---|---|
| Model | 9 | 130.32136 | 14.4802 | 3.9284 |
| Error | 6 | 22.11614 | 3.6860 | *P>F* |
| C. Total | 15 | 152.43750 | | 0.0550 |

DF: Degree of freedom

**Table 6:** Lack of fit

| Source | DF | Sum of squares | Mean square | *F* ratio |
|---|---|---|---|---|
| Lack of fit | 5 | 22.071138 | 4.41423 | 98.0939 |
| Pure error | 1 | 0.045000 | 0.04500 | *P>F* |
| Total error | 6 | 22.116138 | | 0.0765 |

DF: Degree of freedom

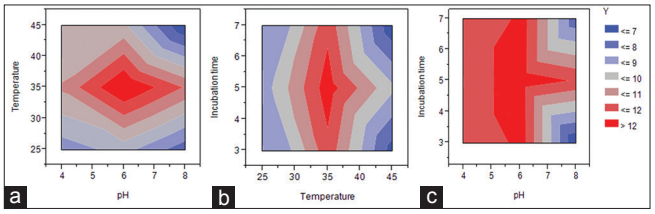


**Figure 4:** Counter plot against temperature versus pH (a), Incubation time versus temperature (b), pH versus incubation time (c)

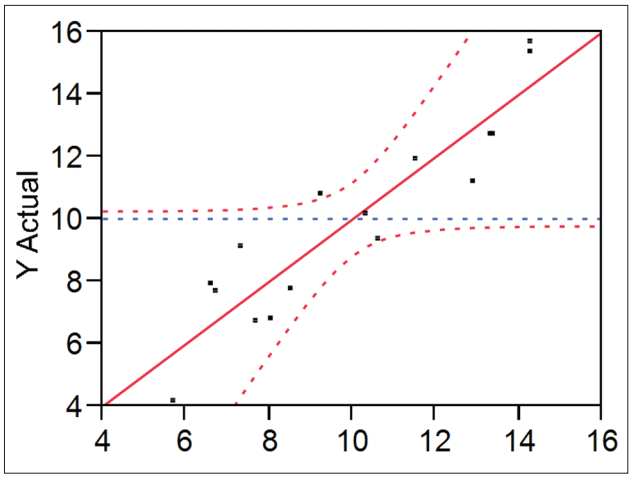


**Figure 5:** Actual by predicted plot with respect to the response

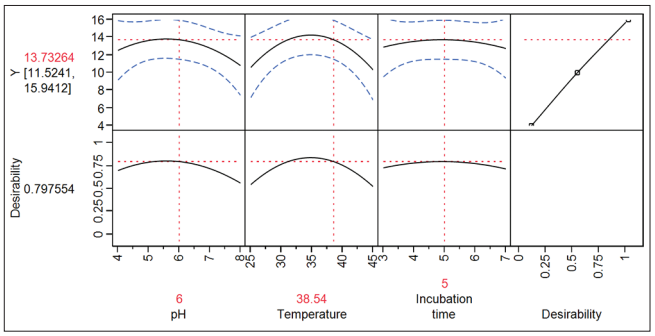


**Figure 6:** Prediction profiler

**Table 7:** Sorted parameter estimates

| Term | Estimate | Standard error | *t* ratio | *t* ratio | *P*>\|*t*\| |
|---|---|---|---|---|---|
| Temp*Temp | –3.815517 | 1.182433 | –3.23 | | 0.0180* |
| pH*pH | –2.065517 | 1.182433 | –1.75 | | 0.01313 |
| pH (4,8) | –0.71 | 0.607126 | –1.17 | | 0.02866 |
| pH*Incubation time | 0.65 | 0.678788 | 0.96 | | 0.03752 |
| Incubation time*Incubation time | –0.915517 | 1.182433 | –0.77 | | 0.04682 |
| pH*Temp | –0.425 | 0.678788 | –0.63 | | 0.05543 |
| Temp (25,45) | –0.14 | 0.607126 | –0.23 | | 0.08253 |
| Temp*Incubation time | –0.1 | 0.678788 | –0.15 | | 0.8877 |
| Incubation time (3,7) | –0.04 | 0.607126 | –0.07 | | 0.9496 |

**Table 8:** Effect of different fish oil concentration on cell growth and rhamnolipid

| Fish oil concentrated (ml) | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Cell growth | 3.7 | 6.0 | 7.7 | 9.8 | 13.0 | 14.3 |
| Rhamnolipid production | 3.9 | 5.7 | 9.8 | 10.0 | 14.0 | 10.2 |

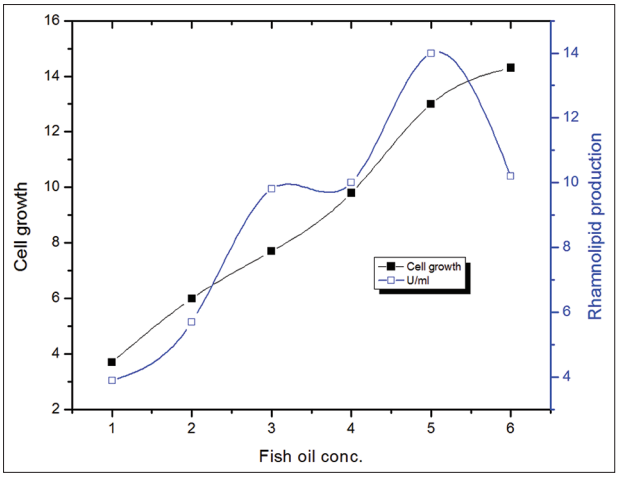


**Figure 7:** Effect of different concentration of fish oil on cell growth and rhamnolipid production

profiler explains about the interactive effects of component at different ranges.[32]

Effect of fish oil in the fermentation media [Table 8] shows the interaction of fish oil on the cell growth of *P. aeruginosa* and the amount of rhamnolipid produced by the organism into the fermentation media [Figure 7]. As the concentration increase in the initially, the growth and production were also increasing. At the concentration of 5 ml v/v in medium shows that the saturation and further a reduction in the production of rhamnolipid were occurring.

## SUMMARY AND CONCLUSION

In this present study, different strains of *P. aeruginosa* were isolated from oil-contaminated soil. The efficiency of this organism to produce rhamnolipid was investigated. Rhamnolipid was produced using different substrate by submerged and solid-state fermentation. Rhamnolipid production was optimized by altering the temperature, pH, and employing different carbon sources, different nitrogen sources. It was then extracted mechanically and chemically. These crude rhamnolipids, estimated by spectroscopic analysis, were profiled by methylene blue analysis process. The surface tension of rhamnolipid was determined by capillary rise method. This rhamnolipid can be commercially produced by fed-batch and continuous process, respectively.

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