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Research Article

Bioremediation of oil contaminated soil using rhamnolipid produced by *Pseudomonas* sp. NBP08

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Background: In the present investigation rhamnolipid production was attempted with *Pseudomonas* sp. NBP08 using groundnut oil cake as a substrate under solid state fermentation conditions.

Methods: Optimized conditions for rhamnolipid production using Box-Behnken 43 experimental design were 8.24, 8.49, 7.30, and 31.96 for groundnut cake, inoculation load, pH and temperature, respectively.

Results: Kinetic parameters observed in optimized media are specific growth rate k (h-1) 0.091, time to reach RL max (h-1) 84, biomass X (mg g-1) 8.2, RL max concentration mg g-1 2.2, RL content (%) 11.3 and productivity (mg g-1h-1) 022. Enhanced oil recovery (EOR) experiments showed the highest percentage of hydrocarbon removal of 85.6% was observed at 2% rhamnolipid concentration.

Conclusion: The optimized biosurfactant used in the present study has shown promising results in terms of 85 % hydrocarbon removal, and this can be used for the large-scale bioremediation of hydrocarbon contaminated soils.

Keywords: *Pseudomonas sp. NBP08, rhamnolipid, groundnut oil cake, solid state fermentation, Box-Behnken design, enhanced oil recovery*

Introduction

Bacteria of genus *Pseudomonas* are gram negative, aerobic gammaproteobacteria having versatile electron accepting capability and can grew under aerobic environments (Palleroni, 2010). *Pseudomonas* sp. is known to possess a wide range of metabolic diversity and are able to colonize a wide range of niches (Madigan and Martinko, 2005). Matthijs et al. (2007) reported rhamnolipid production in *Pseudomonas aeruginosa* capable of exopolysaccharide production and biofilm formation. The biosurfactant production in *P. fluorescens* from olive oil is reported to have antimicrobial activity along with good foaming and emulsifying activity and as documented by Abouseoud et al. (2007). Biosurfactants are amphiphilic compounds capable of reducing surface and interfacial tensions and can increase the surface area of insoluble compounds including hydrocarbons by increased mobility and bioavailability (Rahman et al., 2003). Among these biosurfactants, rhamnolipid type biosurfactant comprise of a mixture of one or two rhamnoses attached to β-hydroxyalkanoic has excellent emulsifying power with a variety of hydrocarbons and vegetable oils and can reduce the interfacial tension of water/oil systems from a value of 43 to below 1mN/m. (Abalos et al., 2001; Lang and Wullbrandt, 1999; Soberón-Chávez et al., 2005). Rhamnolipid are the one among the promising class of biosurfactants owing to their low toxicity, high emulsifying activity and biodegradability (Shatila et al. 2020). The major factor that limits the commercialization of any biosurfactant including that of rhamnolipid is their production cost. In this context, there has been an increased interest in the usage of low-cost raw materials such as plant derived oils, oil wastes, starchy substrates, lactic whey and distillery wastes as raw materials since they account for about 10-30% of production cost (Mukherjee et al., 2006). Recently, Asmadi et al. (2024) used Palm oil mill effluent sludge oil

as a low cost and environmentally friendly raw material for the rhamnolipid production. Taking this into account the present study was undertaken with the objectives such as: To optimize the rhamnolipid production using the cheaply available groundnut cake waste using *Pseudomonas* sp. NBP08. Determination of bacterial growth and kinetics of rhamnolipid production was studied in RSM optimized media. Use of partially purified rhamnolipid was attempted for enhanced oil recovery (EOR) using artificially contaminated sand with 10% of crude oil.

Materials and methods

Biosurfactant producing bacteria

Biosurfactant producing bacteria *Pseudomonas* sp. using mineral medium salt (MSM) supplemented with 2% of spent engine oil as carbon source and incubated at 30°C.

Solid state fermentation

Groundnut oil cake used as a substrate in the present study was obtained from a local market in the Tirunelveli, Tamilnadu, India and dried at 60 °C for 36 h. The cake was blended to fine powder using a blender, sieved and stored at room temperature in a moisture free container. Solid state fermentation was carried out in a 250 mL Erlenmeyer flask containing the substrate at different concentrations and the moisture content was maintained at 60% with the addition of M9 minimal salt medium. The substrate was sterilized and added with different inoculation loads and maintained with different temperatures according to the outputs generated by Box-Behnken 43 experimental design.

Experimental design for optimization of biosurfactant production

Box-Behnken 43 experimental design was developed with four variables (Groundnut cake, inoculation load, pH and temperature) as provided in Table 1. The response variable was emulsification index (EI24%) = % height of the emulsified layer (mm) / total height of the liquid column (mm) (Bodour & Miller-Maier, 1998; Morikawa et al., 2000; Youssef et al., 2004). From the experimental data according to this design, a second-order polynomial regression model equation was derived as given below.

Table 1. Box-Behnken factorial 4^3 experimental design

Factor	Name	Level	Low Level	High Level
A	Groundnut Cake	5.50	2.75	8.25
B	Inoculation load	6.50	4.00	9.00
C	pH	6.50	4.00	9.00
D	Temperature	30.00	20.00	40.00

$$Y=\beta2+\beta1A+\beta2B+\beta3C+\beta4D+\beta11A2+\beta22B2+\beta33C2+\beta44D2+\beta12AB+\beta13AC+\beta14AD+\beta23BC+\beta24BD+\beta34CD$$

(where Y: predicted response (Emulsification Index, %EI24), β0: intercept, A: Groundnut cake, B: inoculation level, C: pH, D: Temperature, β1, β2, β3 and β4 are the linear coefficients; β11, β22, β33, and β44 are the squared coefficients; β13, β14, β23, β24, β34 are the interaction coefficients; A2, B2, C2, D2, AB, AC, AD, BC, BD, CD are the interaction between the variables as significant terms.

Growth and Rhamnolipid production kinetics in RSM optimized medium. Bacterial growth was monitored by plate count method in TSA maintained at 28±2 °C for 48 h. Biosurfactant production in the optimized media was monitored using Du Noüy Ring Tensiometer. Emulsification activity was measured as described earlier. Rhamnolipid production was quantified as rhamnose equivalents (RE) using phenol-sulphuric acid method with a standard curve prepared using rhamnose. Biomass extraction, recovery and quantification were done according to the methods of (Oliveira et al., 2004). Briefly, after growth, about 5 g of the fermented solids was vigorously agitated with 15 ml of distilled water for 20 min, and solid residues washed with 10 ml water and filtered. The combined filtrates were centrifuged for 30 min at 5000 rpm and washed with water twice. The final bacterial pellet was used for dry weight determination after filtering and drying at 60 C.

The specific growth rate of *Pseudomonas* sp. NBP08 under solid stat fermentation was calculated using the formulae $\mu = (\log10\ N - \log10\ N0)\ 2.303) / (t - t0)$. The samplings were taken at an initial time period of 20, followed by another sampling done at 60 h.

Kinetics parameters such as Time to reach RL max (h), Biomass X (mg/g), RL max concentration mg/g, RL content (%) and Productivity (mg/g/h) were determined as described by Oliveira et al. (2004).

Experiment for enhanced oil recovery:

Biosurfactant suitability for the remediation was tested with contaminated sand prepared with 10% of spent engine oil according to method described by Silva et al. (2010). Samples of 20 g of 0.1–0.2 mm fractions of the contaminated sand were transferred to 250 ml Erlenmeyer flasks, which were submitted to the following treatments: addition of 40 ml distilled water (control) or 40 ml of the cell-free broth or 40 ml of a solution of the isolated rhamnolipid at different concentrations such as 1, 2 and 3%concentrations. The samples were incubated on a rotary shaker at 150 rpm, 24 h, and at 27 °C, centrifuged at 9000 rpm for 20 min for separation of the laundering solution and the sand. Triton X-100 and SDS at concentrations of 2% were maintained as possible controls. The amount of oil residing in the sand after the impact of biosurfactant was determined gravimetrically.

Results

Optimization of biosurfactant production using Response Surface Methodology

In the present study, four variables namely concentration of substrate (Groundnut cake), inoculation load (Log Cfu), pH and temperature were selected to study the influence of these factors on rhamnolipid production and Box-Benhen design was used with three equally spaced values coded as -1, 0, + 1. The model was designed with the result of 29 runs and the experimental and $\%EI_{24}$ is provided in Table 2.

Table 2. Box-Behnken factorial 4^3 experimental design of independent variables and their corresponding experimental and predicted yields of emulsification activity (EI_{24}%)

Run	Factor 1 A: Groundnut Cake	Factor 2 B: Inoculation load Log CFU	Factor 3 C: pH	Factor 4 D: Temperature	Response 2 Observed %EI24
1	8.25	6.5	9	30	55.6
2	5.5	6.5	6.5	30	48.6
3	5.5	6.5	6.5	30	49.5
4	2.75	6.5	9	30	29.8
5	5.5	6.5	6.5	30	41.4
6	2.75	6.5	6.5	40	38.6
7	5.5	6.5	4	20	30.1
8	2.75	6.5	4	30	25.2
9	8.25	9	6.5	30	65.9
10	5.5	9	6.5	40	56.6
11	5.5	6.5	4	40	33.6
12	5.5	4	6.5	40	36.8
13	5.5	6.5	9	40	44.6
14	5.5	4	9	30	34.9
15	5.5	4	4	30	34.6
16	5.5	4	6.5	20	32.6
17	8.25	4	6.5	30	53.2
18	5.5	6.5	6.5	30	34.6
19	2.75	6.5	6.5	20	24.8
20	8.25	6.5	6.5	40	54.2
21	8.25	6.5	6.5	20	49.4
22	2.75	4	6.5	30	27.4
23	5.5	6.5	9	20	34.6
24	8.25	6.5	4	30	50.6
25	5.5	9	4	30	32.8
26	2.75	9	6.5	30	29.4
27	5.5	6.5	6.5	30	47.4
28	5.5	9	9	30	55.8
29	5.5	9	6.5	20	31.4

The second-order polynomial equation was constructed to evaluate the influence of each individual input parameter on biosurfactant production through regression analysis.

Second order polynomial equation for $\%EI_{24}$ = 44.3 +12.8 * A+4.366* B +4.033* C +5.125 * D+ 2.675 * AB +0.1 * AC-2.25 * AD +5.675 * BC + 5.25 * BD +1.625 * CD+ 0.758 * A^2 -0.829* B^2-4.479 * C^2-3.841*D^2

ANOVA was used to compute the significance of each parameter/variable for biosurfactant production (Table 3). The Model F-value of 11.46 shows the model is significant, indicating a 0.01% chance that an F-value of this much higher could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, BC, BD, C^2, D^2 are significant model terms. The "Lack of Fit F-value" of 0.31 implies the Lack of Fit is not significant relative to the pure error. The "Pred R-Squared" of 0.7263 is in reasonable agreement with the "Adj R-squared" of 0.8395 since the difference is less than 0.2. The optimal concentration based on this design is Groundnut cake 8.24, inoculation load 8.49, pH 7.30, and temperature 31.96.

Table 3. ANOVA for Response Surface Quadratic model for analysis of variance table

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	3227.08	14	230.51	11.46	< 0.0001	significant
A-Groundnut Cake	1968.64	1	1968.64	97.88	< 0.0001	
B-Inoculation load	228.81	1	228.81	11.38	0.0046	
C-pH	195.21	1	195.21	9.71	0.0076	
D-Temperature	315.19	1	315.19	15.67	0.0014	
AB	28.62	1	28.62	1.42	0.2527	
AC	0.040	1	0.040	1.989E-003	0.9651	
AD	20.25	1	20.25	1.01	0.3327	
BC	128.82	1	128.82	6.41	0.0240	
BD	110.25	1	110.25	5.48	0.0345	
CD	10.56	1	10.56	0.53	0.4806	
A^2	3.73	1	3.73	0.19	0.6733	
B^2	4.46	1	4.46	0.22	0.6450	
C^2	130.14	1	130.14	6.47	0.0234	
D^2	95.73	1	95.73	4.76	0.0467	
Residual	281.58	14	20.11			
Lack of Fit	123.94	10	12.39	0.31	0.9372	not significant
Pure Error	157.64	4	39.41			
Cor Total	3508.66	28				

The 3D surface plot between different variables showing significant interaction for rhamnolipid production is given in Figure 1a, b. The experimental and predicted value for $EI_{24\%}$ was near to a straight line (Figure 1c).

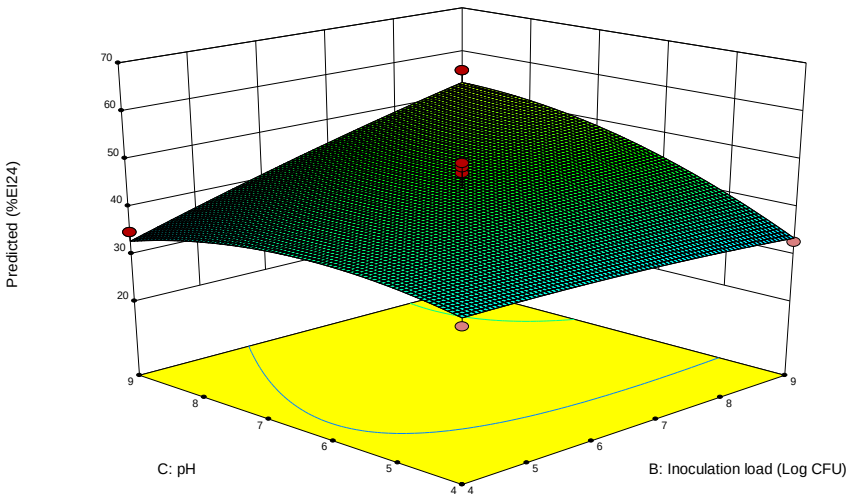

Figure 1a

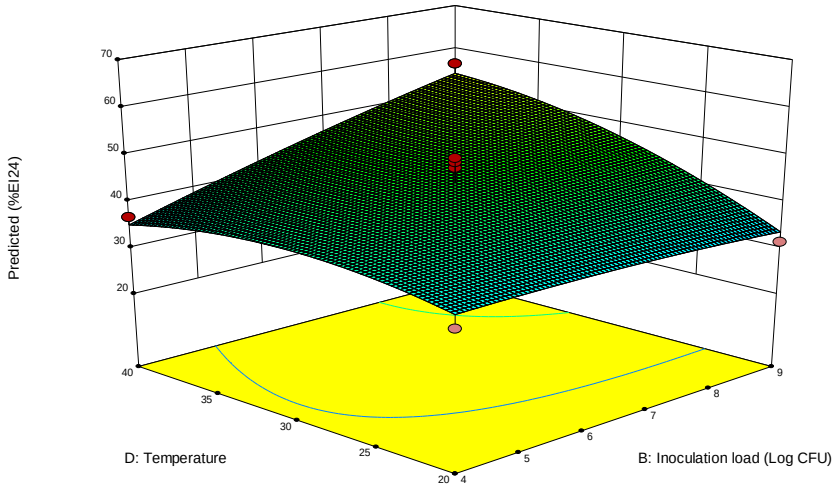

Figure 1b

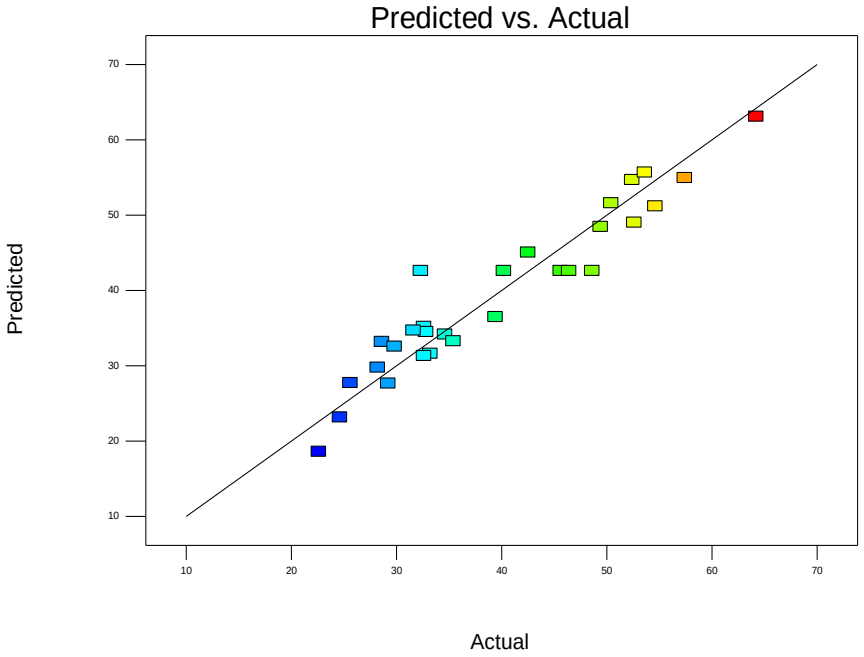

Figure 1c

Figure 1. Three-dimensional contour plot for rhamnolipid production expressed as emulsification index EI24% as a function of (a) pH and inoculation load (b) temperature and inoculation load Predicted vs. actual values for rhamnolipid production based on RSM

Bacterial growth and rhamnolipid production kinetics

Growth and rhamnolipid production in optimized medium using Box-Behnken design was studied and the results are presented in Fig S1. When grown in RSM optimized media *Pseudomonas* sp. NBP08 showed an increase bacterial growth up to a period of 54 h (7.4 log Cfu g-1), followed by a gradual decrease. No increase in biomass yield (8.2 gL-1) after a period of 96 h and no increase in rhamnolipid production (2.2 mg g^{-1} CDW) after a period of 84 h was observed. Surface tension reduction of 28.6 mN/m was observed at 24h and emulsification index of 65.6 was observed at 90h. Bacterial specific growth and kinetics of biosurfactant production were worked out based on the data provided in Fig S1 and the results on specific growth rate k (h^{-1}), time to reach RL max (h^{-}), biomass X (mg g^{-1}), RL max concentration mg g^{-1}, RL content (%), and productivity (mg $g^{-1}h^{-1}$) are provided in Table 4.

Table 4. Growth and kinetics of rhamnolipid production for NBP08 grown in RSM optimized media under submerged fermentation conditions

Parameters	Values
Specific growth rate k (h^{-1})	0.091
Time to reach RL max (h)	84
Biomass X (mg/g)	8.2
RL max concentration mg/g	2.2
RL content (%)	11.3%
Productivity (mg/g/h)	0.022

Application of biosurfactants for enhanced oil recovery (EOR)

In the present study the isolated rhamnolipid biosurfactant was found to be successful in removing the hydrocarbon from the adsorbed sand. The highest percentage of hydrocarbon removal of 85.6% was observed at 2% rhamnolipid concentration and further increase in the rhamnolipid concentration did not have any significant influence on the removal of the hydrocarbon. Interestingly, the cell free broth was also able to remove engine oil in successful quantities similar to SDS and triton X-100 (Figure 2).

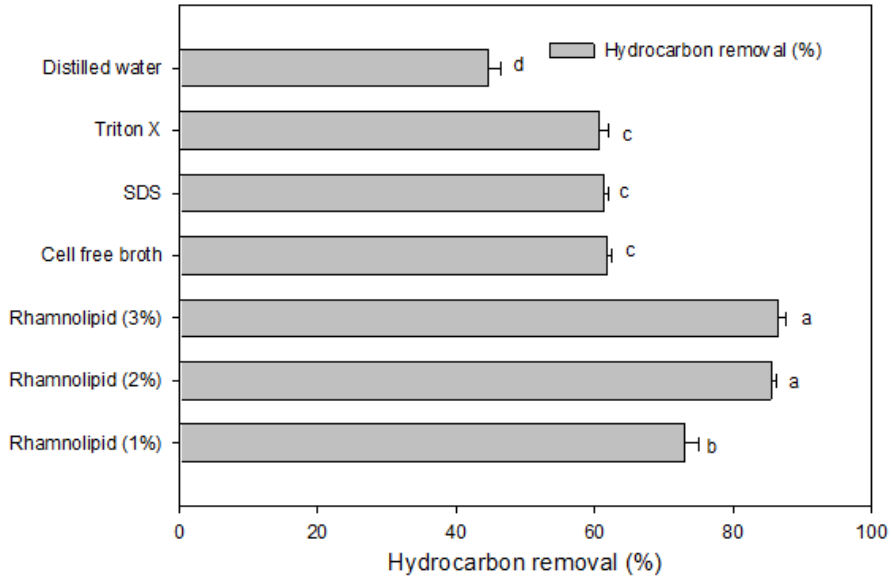

Figure 2. Effect of rhamnolipid treatment on the crude oil removal from contaminated sand compared with different surfactants, cell free broth and distilled water. Different lower-case letters after values indicate that there is a significant difference at a *P* value of 0.05 as determined by DMRT

Discussion

These results show that the combination of inoculation load with pH and temperature can enhance the rhamnolipid production using groundnut cake as a raw material. Different oil cakes such as coconut oil cake, gingerly oil cake, mahua oil cake, etc., serve as sources of carbohydrates, proteins a lipids has been preferred over various sugars and hydrocarbons due to their cost effectiveness (Banat et al., 2014; Nalini and Parthasarathi, 2014; Thavasi et al., 2007). Ebadipour et al. (2015) reported RSM approach through box-behnken design to optimize the biosurfactant production using the low-cost agricultural waste corn steep liquor (CSL). Recently Zhao et al. (2023) reported the usage of RSM for the optimization of rhamnolipid production using Peanut meal, $CaCl_2$, and $MgSO_4$ using solid-state fermentation and these authors reported a 31 % increase post-optimization. In this study, it was observed that RSM optimized groundnut cake media inoculated with *Pseudomonas* sp. NBP08 showed an increase bacterial growth up to a period of 54 h, the highest biomass yield, biosurfactant production and surface tension reduction was also observed in this period. Safari et al. (2022) reported that the biosurfactant produced by *P. aeruginosa* PTCC 1340 using rice bran oil as a carbon source reduced the water surface tension from 70.46 to 25.86 mN/m. Kiran et al. (2010) reported the possibility of using oil seed cake as a substrate for the production of a glycolipid type biosurfactant using *Nocardiopsis lucentensis* MSA04.

The isolated rhamnolipid biosurfactant is found successful in removing the hydrocarbon from the adsorbed sand. Numerous authors (Amani, 2015; Eskandari et al., 2009; Wang et al., 2007) have demonstrated the usefulness of rhamnolipid type biosurfactants in the EOR. Derguine-Mecheri et al. (2021) reported that biosurfactant produced by *Rhodotorula* sp. YBR has proven great potential in the remediation of hydrocarbons polluted soil with a removal rate >95%.

Conclusion

In the present study, the production of rhamnolipid type biosurfactant was optimized using ground oil cake with Box-Behnken method. Growth and kinetics of biosurfactant production were studied in the optimized medium. Rhamnolipid has also shown promise in the removal of crude oil to the adsorbed soil particles. From the present study, the cost of rhamnolipid production could be drastically reduced by the use of cheaply available agro based materials.

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Author contributions

Manoharan Melvin Joe: designed the experiment, and proof read. Abitha Benson: Carried out this experiment, gathered the data and drafted this manuscript.

Conflict of interests

The authors declare no conflict of interest.

Ethics approval

Not applicable.

AI tool usage declaration

The authors did not use any AI and related tools to write this manuscript.

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