Studies on the Effects of Bioprocess Parameters and Kinetics of Rhamnolipid Production by *P. aeruginosa* NITT 6L

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Biosurfactants are gaining popularity in recent times due to lower toxicity, biodegradability, environmental compatibility and activity in extreme conditions. An air isolate was isolated previously for biosurfactant production in our laboratory, and characterized and named as *P. aeruginosa* NITT 6L. The biosurfactant thus produced was characterized to be surface-active rhamnolipid. This paper presents the study of the influence of various bioprocess parameters such as agitation, aeration and inoculum volume on rhamnolipid production by the isolate. Kinetics of rhamnolipid production in optimized media and process conditions were investigated. The rhamnolipid production was found to be increased after nitrogen depletion during stationary phase. The maximum rhamnolipid concentration of about 7.65 g L^{-1} was achieved after 96 h. Logistic model was found to be satisfactory in fitting the microbial growth. Emulsification activity of the crude rhamnolipid extract with different hydrocarbons was studied. The crude extract of rhamnolipid reduced the surface tension of water from 71.4 to 27.5 mN m^{-1}, and CMC was about 11 mg L^{-1}. Also, the usefulness of the extracted rhamnolipid produced under optimal conditions was investigated for remediation of crude oil contaminated soil. Soil washing with 0.3 % rhamnolipid removed about 71 % of crude oil present in sand samples within 24 h.

Key words:
bioprocess, *P. aeruginosa*, rhamnolipid, kinetics, oil removal

Introduction

Rhamnolipids are surface-active metabolites produced mainly by *Pseudomonas* sp. They are widely studied glycolipid biosurfactants that possess the ability to reduce surface tension of water from 72 mN m^{-1} to values below 30 mN m^{-1}, and interfacial tension of water/oil systems from 43 mN m^{-1} to values of about 1 mN m^{-1}.[1] They are produced using a number of substrates such as glucose, glycerol, mannitol, succinate, citrate, pyruvate, molasses, vegetable oils like olive oil, sunflower oil, palm oil, soya bean oil, rapeseed oil, starch-rich waste from potato processing, cassava flour waste, lactic whey, distillery waste, hydrocarbons, soap stock, oil refinery waste, fruit processing waste, crop residue and agroindustrial waste etc.[2–4]

Rhamnolipids are used as a source for rhamnose, for production of high-quality flavor compounds[5] and in a number of applications in the cosmetic and healthcare industries,[6,9] biodegradation and bioremediation of xenobiotics,[6–8] biocontrol[6,7,9] etc. Inspite of their potential applications, rhamnolipids could not compete with chemical surfactants due to their production cost and limited productivity by microorganisms. This issue could be overcome with the use of a higher yielding strain, a process with low capital and operating costs, optimizing process parameters, and media with use of cheap substrates for production, controlled systems, and culturing strategies with minimal or manageable by-products.

The yield, type, composition, surface, and emulsification activity of a biosurfactant depends not only on the producer strain, but also on the nature of the carbon source, the nitrogen source, as well as the C:N ratio, nutritional limitations, and culture conditions of the microbe such as temperature, aeration, agitation, divalent cations and pH.[10–13]

An air isolate was screened previously for biosurfactant production in our laboratory. The isolate was found to be *P. aeruginosa* and the biosurfactant was characterized as a rhamnolipid in the previous study. The effect of various carbon and nitrogen sources on rhamnolipid production with this isolate was studied using Plackett-Burman design and an optimized production media was formulated using Central Composite Design. Also, the interaction between various nutritional factors was analysed using Response Surface Methodology.[14] The rham-

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nose yield obtained with this optimized media using CCD was 1.36 mg mL^{-1}, nearly 15 times higher compared to unoptimized media (0.088 mg mL^{-1} of rhamnose yield).[14] The product yield can still be enhanced if optimized fermentation conditions of agitation, aeration, inoculum volume and production time are employed. Hence, in this work, an investigation was carried out to study the effect of cultivation conditions like agitation, aeration and inoculum volume on rhamnolipid production by *P. aeruginosa* NITT 6L. The time course profile for biomass growth, substrate consumption and rhamnolipid production was monitored in a shake flask culture. Also studied was the rhamnolipids efficacy in removing crude oil from contaminated soil by soil washing. Thus, this study will help in overcoming the limitations in commercial utilization of microbial surfactants.

Materials and methods

Microorganism used

A previously isolated and characterized culture *Pseudomonas aeruginosa* NITT 6L was used for this study. The strain was maintained in nutrient agar plate at 4 °C and sub-cultured regularly.

Production media used

Seed culture was prepared by inoculation of a loop of bacterial colony in nutrient broth. Media for rhamnolipid production was optimized using statistical design of experiments,[14] and consisted of 40 g L^{-1} glucose, 3.5 g L^{-1} sodium nitrate, 0.2 g L^{-1} magnesium sulphate, and 3 mg L^{-1} $FeSO_4$, 2 g L^{-1} KH_2PO_4, 5 g L^{-1} K_2HPO_4, 0.1 g L^{-1} NaCl. Glucose was autoclaved and added to medium separately. Ferrous sulphate was sterilized through filter sterilization. Initial pH of the media was adjusted to 7.00.

Extraction and estimation of rhamnolipid

0.5 mL of supernatant sample was extracted with 1 mL chloroform: methanol (2:1 v/v). The organic phase was evaporated and 0.5 mL of deionized water was added. Rhamnolipid yield was expressed in terms of rhamnose concentration in mg mL^{-1}. The rhamnose concentration was calculated from standard curves prepared with L-rhamnose (0–100 mg L^{-1}) using phenol-sulphuric acid method.[15]

Effect of agitation on rhamnolipid production

Effect of agitation was studied at three different agitation speeds 100, 150 and 200 rpm in 250 mL flasks containing 100 mL production media. These flasks were inoculated with 2 % overnight seed culture at an initial pH 7 and incubated at 37 °C for 72 h in rotary shaker. Rhamnose concentration in the supernatant was estimated as before.

Effect of aeration

To study the effect of aeration, the volume of production media was varied in the range from 25, 50, 75, 100, 125, 150 and 175 mL in 250 mL conical flasks corresponding to volumetric oxygen percentage of 90, 80, 70, 60, 50, 40 and 30 %, respectively. These flasks were inoculated with 2 % overnight seed culture at an initial pH 7 and incubated at 37 °C, 200 rpm for 72 h. Rhamnose concentration in the supernatant was estimated as before.

Effect of inoculum volume

Effect of inoculum volume on rhamnolipid production was studied at different inoculum percentages, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 %. Overnight seed culture was used as inoculum and inoculated in 250 mL flasks containing 75 mL production media, thereby maintaining 70 % aeration level at an initial pH 7. The cultures were incubated at 37 °C, 200 rpm for 72 h. Rhamnose concentration in the supernatant was estimated as before.

Time course profile for rhamnolipid production from *P. aeruginosa*

Time course profile of substrate consumption, growth and rhamnolipid production by *P. aeruginosa* NITT 6L was monitored. For this study, 1000 mL flasks containing 300 mL sterile production media were used with an initial pH 7 and 3.5 % inoculum. These flasks were incubated at 37 °C, 200 rpm with 70 % aeration. The kinetics was monitored during a period of 144 h by evaluation of growth, pH, rhamnose concentration and glucose consumption. Samples of culture medium were withdrawn from the flasks at appropriate time intervals and used for the above mentioned analysis. Bacterial growth was monitored by measuring the absorbance of the broth at 600 nm. Dry cell weight was also calculated and expressed as biomass concentration in g L^{-1}. Residual glucose concentration was measured using DNS method. Rhamnolipid was expressed as rhamnose concentration in g L^{-1} and quantified using phenol-sulphuric acid method.

Modelling of bacterial growth using logistic model

A significant amount of the rhamnolipid was produced after the biomass attained stationary phase,[2] thereby exhibiting secondary metabolite character. Therefore, logistic model[16] was used to fit the biomass data.

$$\frac{dx}{dt} = \mu_{max} X\left(1 - \frac{X}{X_{max}}\right) \quad (1)$$

On integrating and rearranging the above equation to obtain an explicit function for biomass

$$X = \frac{X_0 \exp(\mu_{max} t)}{1 - \frac{X_0}{X_{max}}(1 - \exp(\mu_{max} t))} \quad (2)$$

If the model Eq. (1) fits the data, a straight line is obtained when ln $[X/(X_{max}–X)]$ versus t is plotted. Then μ_{max} can be obtained from the slope of straight line equation.

Crystal structure of crude extract of rhamnolipid

The crystalline appearance of the extracted rhamnolipid was examined under a light microscope at magnification of 40X.

Emulsifying activity of produced rhamnolipid

To check the emulsifying activity on various oils and hydrocarbons, 3 mL of each was added to 2 mL of rhamnolipid solution (0.001 g mL^{-1}) and vortexed at high speed for 2 minutes. After 24 h, the emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100.[17]

$$E_{24} = \frac{\text{Height of emulsion layer}}{\text{Height of total solution}} \cdot 100 \quad (3)$$

Critical micelle concentration of crude rhamnolipid

An efficient surfactant is characterized as having the ability to reduce surface tension of water from 72 mN m^{-1} to 35 mN m^{-1}, as well as a low Critical Micelle Concentration (CMC) value. CMC of a surfactant is defined as minimum concentration of surfactant essential to initiate the micelle formation and produce maximum surface tension reduction,[2] and above this value, there is no further decrease in surface tension. 0.1 % crude rhamnolipid solution was used as stock solution and appropriately serially diluted within the range of 100–0 mg L^{-1}. The surface tension values of each dilution were then measured using Tensiometer (Dataphysics) by Wilhelmy plate method. The CMC of the crude rhamnolipid was estimated from the intercept of two straight lines extrapolated from the concentration-dependent and concentration-independent sections of a curve plotted between rhamnolipid concentration and surface tension values.[18]

Crude oil removal

In the first set of experiments, 0.001 g mL^{-1} rhamnolipid solution was utilized to wash crude oil contaminated sand samples. The sand (75–85 mesh) was added with 10 % (w/w) of crude oil collected from a local automobile industry and maintained at room temperature for 3 days. Then, 5 g of sand samples were washed with rhamnolipid solution for 18 h, 20 h, and 24 h at 200 rpm, 30 °C. In the second set of experiments, 0.002 g mL^{-1} and 0.003 g mL^{-1} rhamnolipid solution was utilized to wash the sand samples contaminated with crude oil for 24 h.

After washing, the aqueous solution was removed, and the sand was dried at 50 °C for 24 h. The sand sample was washed twice with dichloromethane, and the solvent was evaporated at 50 °C. The residual oil was determined gravimetrically, and the percentage of oil removal was calculated using the following equation:

$$\text{Crude oil removal (\%)} = (O_i - O_r)/O_i \cdot 100 \quad (4)$$

where O_i is the initial crude oil in the soil (grams) before washing with rhamnolipid, and O_r is the crude oil remaining in the soil (grams) after washing with rhamnolipid.[17]

Results and discussion

Agitation and aeration rates are highly correlated with oxygen transfer efficiency in shake flasks. Oxygen transfer is one of the key factors in aerobic fermentation for the oxidation of substrate. Hence, by means of agitation, an adequate oxygen transfer rate can be achieved which in turn improves the yield of rhamnolipid. In this study (Fig. 1), with the increase in agitation speed the rhamnolipid production increased and it was found to be higher at 200 rpm. Results are concurrent with work of Silva *et al.*[19] and highlighted the influence of agitation speed in rhamnolipid biosynthesis.

It is evident from Fig. 2 that, as the level of aeration increases, the rhamnolipid production increases up to 70 %, after which the production starts to decline. But the biosurfactant production by *Pseudomonas aeruginosa* UCP0992 using glycerol

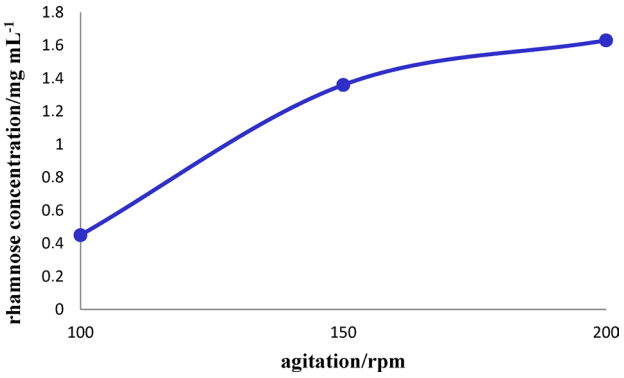

Fig. 1 – *Effect of agitation in rhamnolipid production*

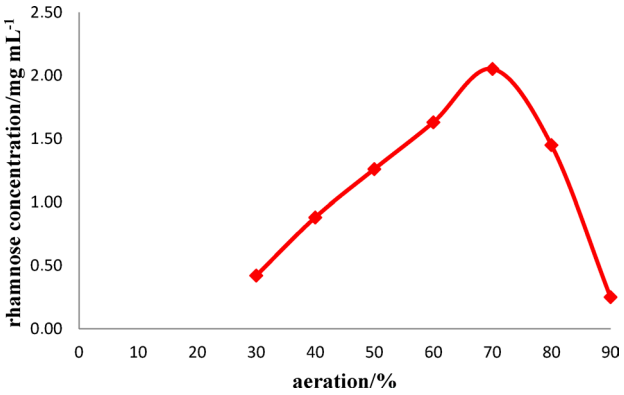

Fig. 2 – *Effect of aeration in rhamnolipid production*

as substrate[19] remained unaffected by aeration rate. On the other hand, Abdel-Mawgoud *et al.*[20] reported that maximum surfactin production by *Bacillus subtilis* BS5 occurred at an aeration percentage of 90 % and a sharp decline in the production upon decrease of aeration.

From Fig. 3, it was observed that the inoculum volume did not have a significant effect on rhamnolipid production by the isolate except at 1 % inoculum. Since a slight increase in rhamnose concentration was observed with 3.5 % inoculum volume compared to others, this inoculum volume was used for further studies.

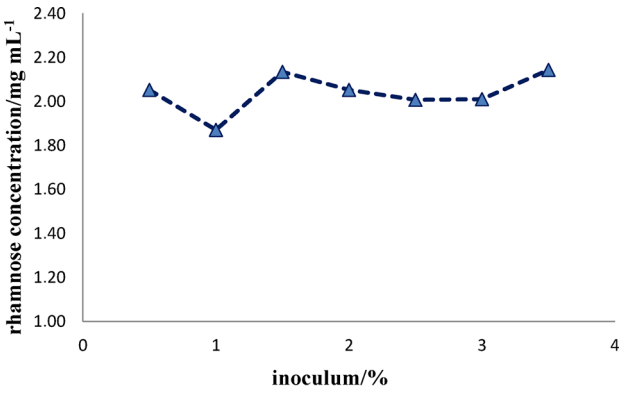

Fig. 3 – *Effect of inoculum volume in rhamnolipid production*

Fig. 4 shows the time course profile of glucose consumption, bacterial growth and rhamnolipid production in production media containing glucose as carbon source and sodium nitrate as nitrogen source. During the exponential phase, which extended up to 48 h, rhamnolipid production with cell growth was found to be low. But after the attainment of stationary phase of cell growth, the production started to increase at a higher rate[21,22] and maximum product accumulation occurred after 96 h of cultivation (2.25 g L^{-1} corresponding to rhamnose concentration).[23] Rhamnolipid yield was then calculated by a coefficient of 3.4 obtained from the correlation of pure rhamonlipids/rhamnose (1.0 mg of rhamnose corresponds approximately to 3.4 mg of rhamnolipids).[24] The limitation of nitrogen in media favours the product accumulation in the culture broth.[22] With reduced levels of nitrogen, bacterial growth is limited which favours the production of metabolites, whereas with excess nitrogen source, the substrate will be directed towards cellular growth, limiting the accumulation of the product. Decrease in rhamnolipid production was observed after 96 h of fermentation, due to depletion of glucose from the broth as shown in Fig. 4.

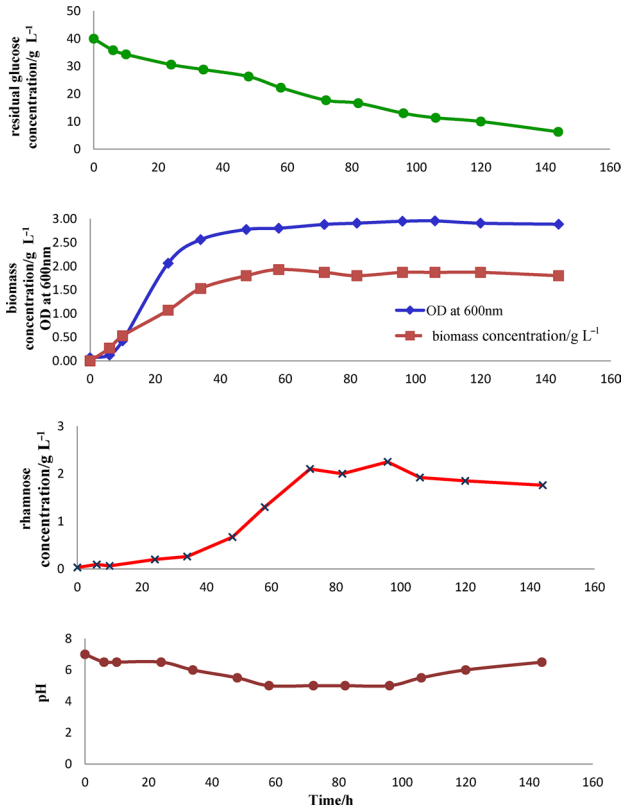

Fig. 4 – *Time course profile for glucose consumption, bacterial growth, rhamnolipid production and pH changes in production media containing glucose and sodium nitrate as carbon and nitrogen sources, respectively*

With a hydrophilic carbon source like glucose, it was hypothesized that the biosynthesis of rhamnolipids proceeds as follows:[25] The sugar moiety is directly derived from the carbon source, but the lipid component is synthesized de novo. Biosynthesis of the lipid components of RLs proceeds through the classical pathway of fatty acid synthesis from 2-carbon units using fatty acid synthases of type-II (FAS II).

The following pathway[25] explains how L-rhamnose is probably synthesized when the bacteria are grown with glucose as the carbon source. Robertson *et al.*[26] and Olvera *et al.*[27] found that phosphoglucomutase (AlgC) converts D-glucose-6-phosphate into D-glucose-1-phosphate, which is then used by

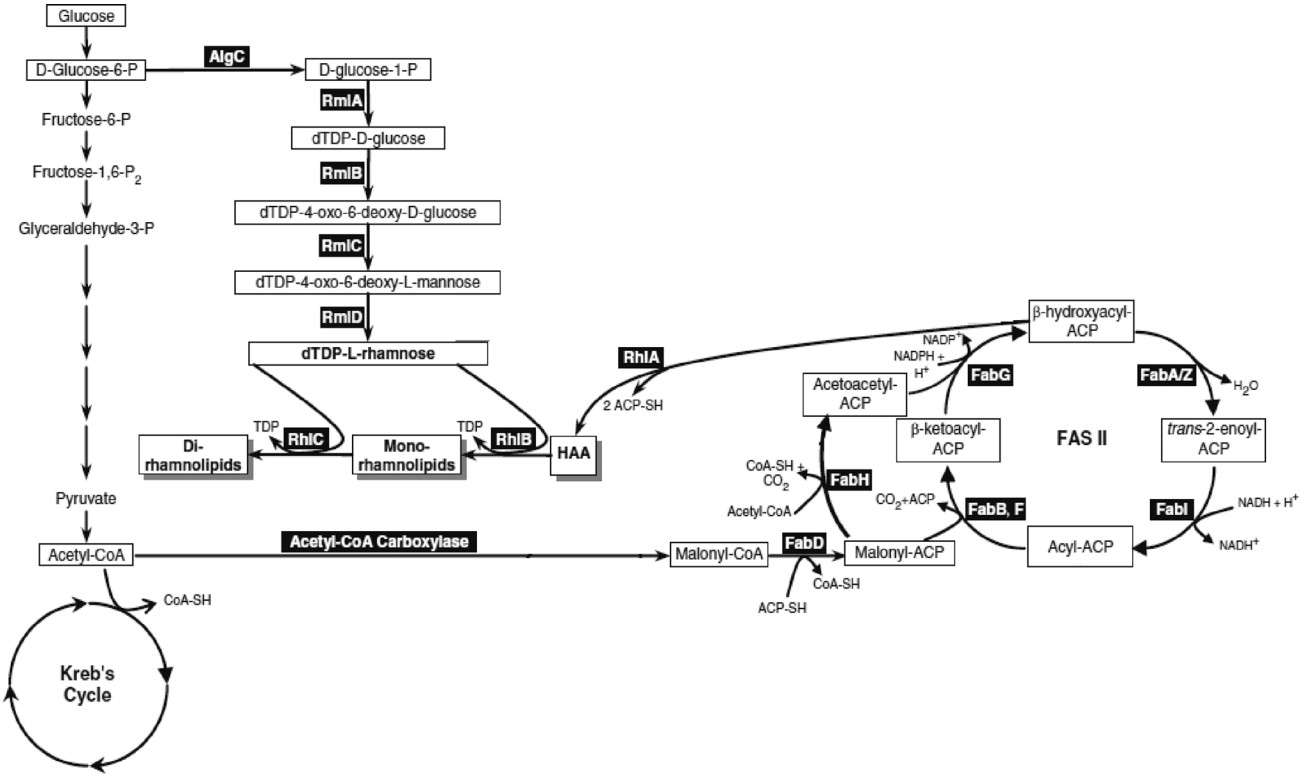

Fig. 5 – *Probable metabolic pathway of rhamnolipid biosynthesis in P. aeruginosa using glucose as carbon source. AlgC: phosphomannomutase, RmlA: glucose-1-phosphate thymidylyltransferase, RmlB: dTDP-D-glucose 4,6-dehydratase, RmlC: dTDP-4-dehydrorhamnose 3,5-epimerase, RmlD: dTDP-4-dehydrorhamnose reductase, FabD: malonyl-CoA:ACP transacylase, FabH, FabB and FabF: β-ketoacyl-ACP synthetases, FabG: NADPH-dependent β-ketoacyl-ACP reductase, FabA, FabZ: β-hydroxyacyl-ACP dehydratases, FabI: NADH-dependent enoyl-ACP reductase, HAA: 3-(3-hydroxyalkanoyloxy) alkanoic acid, RhlA: 3-(3-hydroxyalkanoyloxy)alkanoate synthetase, RhlB: rhamnosyltransferase 1, RhlC: rhamnosyltransferase 2.*[25]

RmlA, RmlB, RmlC, and RmlD to produce dTDP-L-rhamnose. dTDP-L-rhamnose is the precursor for the L-rhamnose.

Then, Rhamnosylation of fatty acid chains proceeds as follows in order to form rhamnolipids:[28]

2β –Hydroxydecanoyl – CoA
→ β – hydroxydecanoyl – β – hydroxydecanoate + + 2 CoA-SH

TDP –1 – rhamnose + β – hydroxydecanoyl – – β – hydroxydecanoate
→ TDP +1– rhamnosyl – β – hydroxydecanoyl – – β – hydroxydecanoate

TDP –1 – rhamnose + 1 – rhamnosyl – – β – hydroxydecanoyl – β – hydroxydecanoate
→ TDP +1– rhamnosyl –1 – rhamnosyl – – β – hydroxydecanoyl – β – hydroxydecanoate

The first reaction involves dimerization of two β-hydroxydecanoic acid chains. Then, the dimer undergoes two sequential rhamnosylation reactions with two different rhamnosyltransferases: rhamnosyltransferase 1 (RhlB) in reaction (2) and rhamnosyltransferase 2 (RhlC) in reaction (3). Fig. 5 shows the probable metabolic pathway of producing rhamnolipid using glucose as carbon source.[25] Table 1 presents the comparative analysis of the results of batch fermentation in this study with some published reports in the literature. The variations in the rhamnolipid production can be attributed to the variations in the strains, substrates and fermentation conditions. Fig. 4 shows the pH profile of the rhamnolipid production. Initial pH 7 drops to 5 after 58 h of fermentation, and then increases again after 106 h. Biomass growth data was fitted to logistic model (Fig. 6). The maximum specific growth rate was found to be 0.1023 h^{-1}.

The crystal structure of the extracted rhamnolipid was visualized using light microscope. 40X magnification of rhamnolipid crystals are shown in Fig. 7. Feather-like crystals of rhamnolipid were observed.

The emulsifying activity of 0.1 % crude rhamnolipid was checked against different hydrophobic substrates as shown in Fig. 8. The biosurfactant was able to form emulsion with all hydrophobic substrates tested, of which diesel and paraffin emulsions were stable up to several weeks. As the rhamnolipid product was able to emulsify aromatic hydrocarbons like hexane, paraffin, and diesel efficiently, it is a potential candidate for bioremediation

Table 1 – *Comparative analysis of rhamnolipid yield obtained in various studies*

Substrate	Microorganism used	Initial substrate conc.	Fermentation duration (h)	Maximum rhamnolipid conc.	Reference
Mannitol	*P. aeruginosa* 19SJ	2 %	150	1.7 g L^{-1} RE	Deziel *et al.*, 1996[29]
Molasses and corn steep liquor	*P. aeruginosa* GS3	Molasses – 7 %, corn steep liquor – 0.5 %	96	0.24 g L^{-1} RE	Patel *et al.*, 1997[30]
Ethanol	*P. aeruginosa* IFO 3924	30 g L^{-1}	168	3.7 g L^{-1}	Matsufuji *et al.*, 1997[31]
Fish oil	*P. aeruginosa* BYK-2 KCTC 18012P	25 g L^{-1}	216	17 g L^{-1}	Lee *et al.*, 2004[32]
Glucose	*P. aeruginosa* J4	4 %	120	1.73 g L^{-1}	Wei *et al.*, 2005[33]
Diesel	*P. aeruginosa* J4	6 %	120	1.3 g L^{-1}	Wei *et al.*, 2005[33]
Kerosene	*P. aeruginosa* J4	5 %	120	0.71 g L^{-1}	Wei *et al.*, 2005[33]
Glucose	*P. chlororaphis* NRRL B-30761	2 %	120	1 g L^{-1}	Gunther *et al.*, 2005[34]
Unused soybean frying oil	*P. aeruginosa* mutant EBN-8	2 %	168	3.1 g L^{-1}	Raza *et al.*, 2006[35]
Casamino acid	*P. aeruginosa* 181	5.3 g L^{-1}	65	3.6 g L^{-1} RE	Al-Araji *et al.*, 2007[13]
Glucose + Glycerol	*P. aeruginosa* EM1	Glucose – 30.5 g L^{-1}, Glycerol 18.1 g L^{-1}	Not mentioned	12.6 g L^{-1}	Wu *et al.*, 2008[36]
Sunflower oil	*Thermus thermophilus* HB8	15 g L^{-1}	40	0.3 g L^{-1}	Pantazaki *et al.*, 2010[37]
Oleic acid	*Thermus thermophilus* HB8	9.4 g L^{-1}	60	0.25 g L^{-1}	Pantazaki *et al.*, 2010[37]
Glucose	*P. aeruginosa* ATCC 9027	3 %	103	1.3 g L^{-1} RE	Clarke *et al.*, 2010[38]
Sunflower oil	*P. aeruginosa* PAO1	250 g L^{-1}	120	37 g L^{-1}	Müller *et al.*, 2011[39]
Molasses distillery wastewater	*P. aeruginosa* GIM32	--------	64	2.6 g L^{-1}	Li *et al.*, 2011[40]
Glycerol	*P. aeruginosa* ATCC 53752	30 g L^{-1}	94	1.07 g L^{-1} RE	Avili *et al.*, 2012[41]
Glucose	*P. aeruginosa* NITT 6L	40 g L^{-1}	96	2.25 g L^{-1} RE	This study

of pollutants and in clean-up of oil spillage. Emulsification of vegetable oils suggests its usage in the food, pharmaceutical and cosmetic industries.

The crude rhamnolipid was effective in reducing the surface tension of water from 71 mN m^{-1} to 27.5 mN m^{-1}. From the plot 'surface tension versus

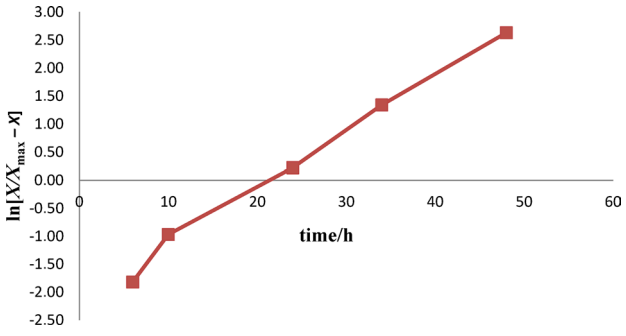

Fig. 6 – *Plot of ln [X/(X_{max} – X)] versus time*

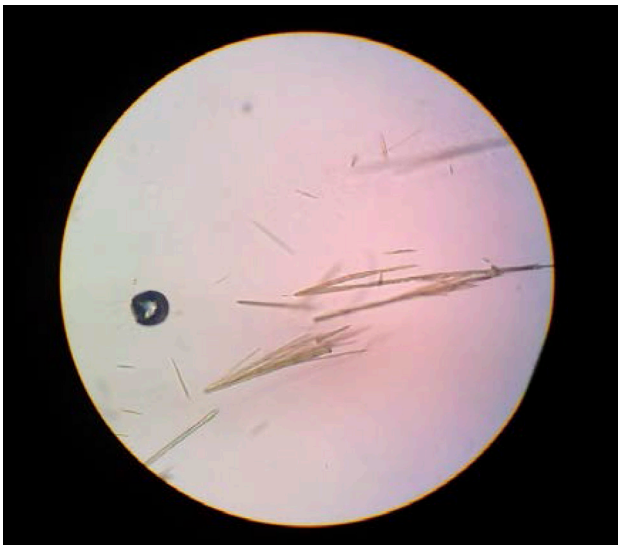

Fig. 7 – *Microscopic observation of crystals of rhamnolipid from Pseudomonas aeruginosa NITT 6L*

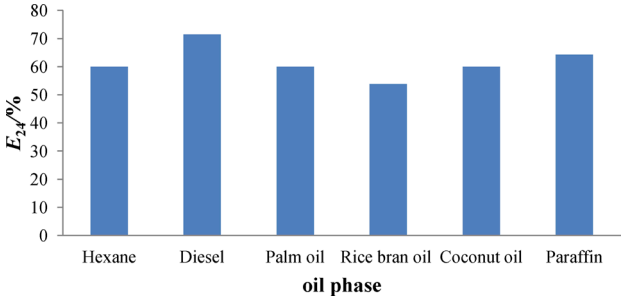

Fig. 8 – *Emulsification activity of rhamnolipid produced by P. aeruginosa NITT 6L*

rhamnolipid concentration' (Fig. 9), CMC was found to be 11 mg L^{-1} which is in agreement with the available literature on rhamnolipids reported to exhibit a wide range of CMC values ranging from 10 to 234 mg L^{-1} and surface tension from 25 to 31 mN m^{-1}.[17–19,21] Low CMC indicated that a lower amount of biosurfactant was required to reduce the surface tension to minimum value.

The effectiveness of this rhamnolipid in remediation of soil from crude oil contamination was investigated. Property of emulsification using crude extract of rhamnolipid was exploited for application of soil washing. Washing of crude oil contaminated soil with 0.1 % rhamnolipid solutions resulted in removal of up to about 43 % of the crude oil within 24 h. With increase in soil washing time, the percentage of oil removal from the soil also increases (Table 2). Also, as evident from Table 3, the percentage of oil removal from the contaminated soil increases with concentration of rhamnolipid. Using 0.3 % rhamnolipid solution, a maximum of 71 % crude oil removal was achieved for soil washing time of 24 h. Hydrocarbon removal from soil is credited to the ability of the biosurfactant to stabilise oil/water emulsions and increase hydrocarbon solubility.[7]

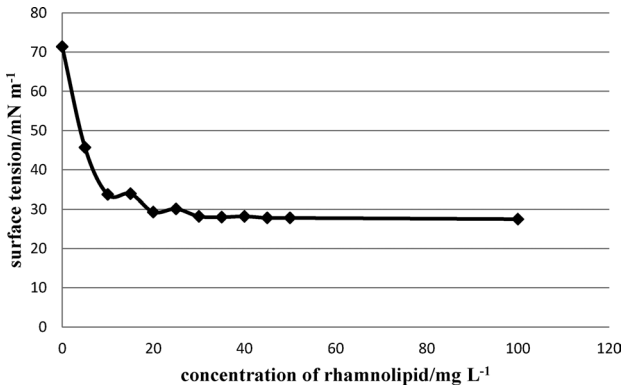

Fig. 9 – *Plot of surface tension versus crude rhamnolipid concentration*

Table 2 – *Crude oil removal from soil using 0.1 % rhamnolipid solution*

Soil washing time (h)	Crude oil removal (%)
18 h	34
20 h	39.7
24 h	42.8

Table 3 – *Crude oil removal from soil using different concentrations of rhamnolipid*

Rhamnolipid concentration (%)	Crude oil removal (%)
0.1	42.8
0.2	61.1
0.3	70.7

Conclusion

It could be concluded that for the maximum production of rhamnolipids from *P. aeruginosa* NITT 6L in the optimized fermentation media, bioprocess parameters like agitation rate, aeration and inoculum volume are to be at 200 rpm, 70 % and 3.5 % (v/v) respectively. From the time course profile of rhamnolipid production, it can be seen that maximum production of rhamnolipid of about 7.65 g L^{-1} occurred after 96 h of fermentation. This implies that the yield enhancement of about 25 times is achieved as a result of process optimization. Logistic model was found to be a good fit for biomass growth data. The CMC of the crude biosurfactant was estimated to be 11 mg L^{-1} and the surface tension of water was reduced to a minimum of 27.5 mN m^{-1}. Further, the application of this rhamnolipid in the removal of crude oil from contaminated soil was investigated. The study showed 0.3 % rhamnolipid was efficient in removing about 71 % of the crude oil from the soil after 24 h of soil washing.

Nomenclature

C:N – Carbon to nitrogen ratio
$FeSO_4$ – Ferrous sulphate
KH_2PO_4 – Potassium dihydrogen phosphate
K_2HPO_4 – Dipotassium hydrogen phosphate
NaCl – Sodium chloride
v/v – Volume/volume
w/v – Weight/volume
DNS – Dinitro Salicylic acid
X – Biomass concentration, g L^{-1}
t – Time, h
X_o – Initial biomass concentration, g L^{-1}
X_{max} – Maximum biomass concentration, g L^{-1}

μ_{max} – Maximum specific growth rate, h^{-1}
E_{24} – Emulsification index
CMC – Critical Micelle Concentration, mg L^{-1}
RL – Rhamnolipid

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