RSC Advances

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Journal:	*RSC Advances*
Manuscript ID:	RA-ART-04-2015-005800.R1
Article Type:	Paper
Date Submitted by the Author:	01-Jun-2015
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Microbial rhamnolipid production in wheat straw hydrolysate supplemented with basic salts

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Abstract

Rhamnolipids are important glycolipids that find applications in the areas of crude oil bioremediation, enhanced oil recovery, food and pharmaceutical applications. The economic feasibility of rhamnolipid production mainly depends upon the cost of the substrate. Lignocellulosic biomass is a potential substrate for the production of several microbial metabolites and can also be used for rhamnolipid production. For the utilization of sugars from lignocellulosic biomass, the polymeric carbohydrates need to be hydrolysed for releasing the fermentable sugars for rhamnolipid production. In this study, pretreatment of wheat straw was carried out using sulphuric acid, phosphoric acid and ammonia. All the pretreated substrates were subjected to enzymatic hydrolysis using cellulases, produced by *Trichoderma reesei* NCIM 1186. Maximum reducing sugar yield (509.33 mg/g dry pretreated substrate) was obtained in case of biomass treated with 0.2% sulphuric acid at 150 ^{o}C for 15 min which was further used for rhamnolipid production by *Pseudomonas aeruginosa* NCIM 2036. The highest rhamnolipid production of 9.38 g/L was obtained in sugar hydrolysate (mainly containing cellobiose) supplemented with $MgSO_4$, Na_2HPO_4, $FeSO_4$ and $NaNO_3$. The production of rhamnolipid by *P. aeruginosa* NCIM 2036 using pure cellobiose as the sole carbon source was demonstrated. The current study showed that lignocellulosic biomass can be used as an alternative cost-effective substrate for rhamnolipid production.

Keywords: Rhamnolipid, *Pseudomonas aeruginosa*, wheat straw, cellobiose, nutrients

1. Introduction

The use of microbial biosurfactants as alternatives to chemical surfactants has gained considerable attention owing to their ability to solubilize hydrocarbons.[1] Biosurfactants consist of the hydrophobic and hydrophilic groups that confer them the property to accumulate between fluid phases, thereby reducing surface and interfacial tension.[2] In addition, biosurfactants are biodegradable and less toxic than chemical surfactants and retain activity under wide ranges of temperatures, pH and salinity.[3] Due to several advantageous features, biosurfactants can be used for diverse applications such as enhanced oil recovery, oil spill clean-up, emulsification, wetting, foaming, and cleansing.[4,5] The use of microbial biosurfactants during the enzymatic hydrolysis of biomass was found to be beneficial in increasing the reducing sugar yields.[6] Moreover, the addition of rhamnolipids to cultures of *Penicillium expansum increased* the production of cellulolytic enzymes.[7]

Biosurfactant properties (structural and chemical) and production is greatly influenced by the choice of microorganism, substrate and process conditions.[8] Among different biosurfactants, rhamnolipids produced by different strains of *Pseudomonas* sp. have been studied extensively.[9] Chavez and Maier[9] mentioned that the production of rhamnolipids by *Pseudomonas* usually occurred at the onset of stationary phase and high rhamnolipid yields were obtained during fed batch cultivations. Several substrates of synthetic or natural origin and even industrial wastes have been used to produce rhamnolipids.[10] Recently, rhamnolipid produced from a synthetic substrate (glycerol), albeit at lower concentrations of 1.62 g/L, by a genetically engineered strain of *Pseudomonas aeruginosa,* was used for enhanced crude oil recovery in simulated oil reservoirs.[11] Another report indicated that crude oil itself could be used as a naturally occurring carbon source for rhamnolipid production, although the reported concentrations (20 mg/L) were lower.[12] Higher rhamnolipid concentrations of 13.93 g/L were produced by *Pseudomonas* SWP-4 with waste cooking oil as the substrate.[13]

Although the waste streams are inexpensive, they are less preferred for rhamnolipid production due to their non-uniform compositions and dilute nature leading to inconsistent product formation.[10]

Due to their cheaper cost and abundant availability, lignocellulosic biomass can be used as an inexpensive feedstock for rhamnolipid production. The use of biomass would however include the unlocking of fermentable carbohydrates by thermochemical pre-treatment followed by enzymatic hydrolysis.[3,14] The selection of suitable thermochemical methods for biomass pretreatment is critical to improve the fermentability of sugar hydrolysates. The dilute acid process has been widely used for the pretreatment of various biomass varieties. The sugar hydrolysate generated via dilute acid hydrolysis has been found to be fermentable for producing bioethanol, lipids, triacylglycerols etc.[15,16] In order to overcome nutrient deficiency of sugar hydrolysate, macro and micro nutrient supplementation has been tried for improving microbial product formation.[17] Earlier studies have indicated that magnesium, phosphorus, iron and nitrogen sources are important factors for microbial rhamnolipid production.[18,19] For cellobiose rich sugar hydrolysates, selection of microbial strains with beta-glucosidase (cellobiases) activities is essential to produce value added fermented products. Although, strains of *Pseudomonas* can utilize diverse carbohydrates, reports related to their cellobiose utilization are few, possibly due to lower cellobiase activities.[20,21]

In the present study, sugar hydrolysate generated after enzymatic hydrolysis of pretreated wheat straw was used for rhamnolipid production. The concentrations of nutrients ($MgSO_4$, Na_2HPO_4, $FeSO_4$ and $NaNO_3$) to be added to wheat straw hydrolysate were optimized using central composite design (CCD) based response surface methodology (RSM). The production of mono and di rhamnolipids was confirmed by Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) spectroscopy.

2. Material Methods

2.1 Microbial growth and inoculum preparation

Pseudomonas aeruginosa NCIM-2036 (ATCC 19429) and *Trichoderma reesei* NCIM 1186 (ATCC 26921) were procured from National Culture of Industrial Microorganisms (NCIM), Pune, India and both stored until use at 4 ^{o}C. *Pseudomonas* inoculum preparation was done by inoculating a loopful of culture from the slants into 10 ml sterile nutrient broth (Himedia) to obtain cell growth (O.D: 0.6 @ 600 nm) after incubation at 30 ^{o}C for 24 h. The cell suspension was used as innocula for fermentative rhamnolipid production. *Trichoderma* inoculum preparation was done by inoculating a loopful of culture from the slants onto a Potato Dextrose Agar (Himedia) slant and incubated at 30 ^{o}C for 72 h hours till visible growth was observed. Inoculum for cellulase production was prepared by scrapping spores from the slants into sterile water.

2.2 Enzyme production

Cellulase enzyme production was carried out as described by Das *et al.*[22] by culturing *Trichoderma reesei* NCIM 1186 under solid state fermentation (SSF). Medium preparation for SSF was carried out by mixing the autoclaved wheat bran with Czapek dox minimal media[23] at 1:1 (w/v) ratio and incubated for 96 h at 30 ^{o}C. On completion, the grown cultures were extracted with sterile distilled water in 1:2 (w/v) ratio. The liquid extract was centrifuged at 5,000 rpm, 4 °C for 10 min for removing insoluble material. The cellulase activity of the supernatant was determined as per the standard protocols.[24,25] The endoglucanase, exoglucanase and xylanase activities of the supernatant were found to be 25 IU/gds, 7.29 IU/gds and 121.25 IU/gds, respectively. The supernatant was further used for enzymatic hydrolysis of pretreated wheat straw.

2.3 Wheat straw

Wheat straw (approx. avg. particle size of 0.5 mm) was procured from local sources around Bangalore, India and dried in an oven at 60 ^{o}C for 48-72 h, till a constant biomass weight was obtained.

2.4 Thermochemical pretreatment

Wheat straw pretreatment was carried out using 0.5% (v/v) sulphuric acid, 0.2% (v/v) phosphoric acid or 20% (v/v) ammonia at 150 °C for 15 min in a 500 mL PARR reactor. After every pretreatment, the biomass was washed with distilled water and dried overnight at 60°C until constant biomass weight was obtained. The dried biomass was further used for enzymatic hydrolysis.

2.5 Enzymatic hydrolysis of pretreated wheat straw

To pretreated wheat straw, the required quantity of enzyme (18.67 FPU/g of dry pretreated biomass) was added to obtain solid loadings of 15% (w/v) and incubated at 50 ^{o}C for 24 h. After hydrolysis, samples were withdrawn and tested for reducing sugar concentrations by using an Ultra-High Pressure Liquid Chromatography (Agilent 1290 UHPLC) by the method described earlier.[26] The sugar-hydrolysate was used for rhamnolipid production.

2.6 Cellobiose Utilization

To determine rhamnolipid production from pure cellobiose, *Pseudomonas aeruginosa* NCIM 2036 was cultured in LB medium[27] with cellobiose (7 g/L) (Himedia, India) as the sole carbon source and incubated at 32°C upto 72 hr. Samples were collected periodically and cellobiose utilization was determined by using an Ultra-High Pressure Liquid

Chromatography (Agilent 1290 UHPLC) by the method described earlier.[26] The production of rhamnolipid was determined by the orcinol method.

2.7 Rhamnolipid fermentation

200 mL sugar hydrolysate was inoculated with 1% (v/v) of 24 h old *Pseudomonas* sp. culture and incubated at 30 ˚C, 150 rpm for 72 h. Samples was periodically withdrawn and tested for microbial growth (dry weight basis method) and rhamnolipid production (Orcinol method).

2.8 Optimization of rhamnolipid production

Optimization of rhamnolipid production from *Pseudomonas* sp was carried out using CCD based RSM. Optimization of rhamnolipid production was carried out by varying following parameters $MgSO_4$ (143-333 ppm), Na_2HPO_4 (5000-9000 ppm), $FeSO_4$ (30-90 ppm) and $NaNO_3$ (8000-10000 ppm). All experiments were carried out in triplicates. Table 1 shows the experimental design and response for rhamnolipid production. The experimental data were analyzed by the Response Surface Regression (RSREG) method to fit the second-order polynomial equation (SAS, 1990):

$$Y = \beta k0 + \sum i=1\ 5\beta kixi + \sum i=1\ 5\beta kiixi2 + \sum i=1\ 4\sum j=i+1\ 5\beta kijxixj \quad (1)$$

Where, Y is the response (rhamnolipid yield); βk0, βki, βkii and βkij are constant coefficients and xi, xj are the coded independent variables, which influence the response variables Y. This response is preferred because a relatively few experimental combinations of the variables are sufficient to estimate potentially complex response function.

2.9 Recovery of Rhamnolipid

Rhamnolipid extraction from wheat straw hydrolysate was carried out as per the method described by Zhi-Feng *et al*.[28] At regular intervals, samples were taken from the fermentation

media and the pH of the broth was adjusted to pH 8 by addition of 1.0 M NaOH and centrifuged at 13,756 rcf for 10 min. On centrifugation, the pH of the supernatant was readjusted to pH 2 by addition of 1.0 M HCl followed by extraction in cold ethyl acetate at a ratio of 1:5. After extraction, the solvent was evaporated using a Buchi Rotavap at 70 ^{o}C, 120 mbar and the residue was used for further purification.

2.10 Analysis

2.10.1 Orcinol method

Rhamnolipid concentration was determined by the orcinol method as described by Rahman *et al.*[29] A standard curve was plotted using Rhamnose (Sigma) as the standard.

2.10.2 Cell growth determination

Growth of *Pseudomonas aeruginosa* during rhamnolipid production was determined by measuring culture densities, at 600 nm.

2.10.3 Emulsification index test

Emulsification index and interfacial tension (IFT) of rhamnolipid samples were determined by the method described by Noh *et al.*[30] and Christova *et al.*[31], respectively.

2.10.4 Preparative TLC

For the separation of rhamnolipid from lignocellulosic hydrolysate, preparative TLC method was used.[32]

2.10.5 FTIR measurements

The Infrared spectra of the partially purified product were recorded using the JASCO FTIR instrument and the IR spectra are presented in % transmittance with respect to wave numbers (cm^{-1}).

2.10.6 NMR measurements

The partially purified product was characterized by ^{1}H NMR using Jeol 400 MHz NMR spectrometer. ^{1}H NMR spectra were recorded using tetramethylsilane (TMS) as an internal standard.

3. Results and Discussion

3.1 Enzymatic hydrolysis of pretreated wheat straw

The dilute acid pretreatment of lignocellulosic biomass causes effective removal of hemicellulose, making the residual biomass amenable to enzymatic hydrolysis.[33,34] In our previous study[35], the optimum conditions for wheat straw pre-treatment were determined by subjecting the biomass to different (0.1-1%) acid (sulphuric and phosphoric) and ammonia (1-20%) concentrations for varying periods (5-120 min) and temperatures (120 ^{o}C-180 ^{o}C) (Data not shown). The pretreated biomass was subjected to enzymatic hydrolysis with *Trichoderma* cellulases at 50 ^{o}C for 24 h and the reducing sugar concentrations were measured. Dilute sulphuric acid as a catalyst for biomass treatment has been previously found to be effective for hydrolysis and suitable due to the requirement for milder operating conditions. Likewise, in the current study, the use of 0.2% sulphuric acid at 150 ^{o}C for 15 min for wheat straw pretreatment resulted in maximum sugar yield of 509.33 mg/g of dry pretreated biomass (Data not shown) after 24 h of enzymatic hydrolysis and the sugar hydrolysate was used as the media for microbial rhamnolipid production.

3.2 Rhamnolipid production from pure cellobiose

To confirm the utilization of cellobiose by the selected strain of *Peudomonas aeruginosa*, the microorganism was cultured in minimal media containing cellobiose as the sole carbon source. After inoculation of the *Pseudomonas* sp. culture, a lag phase was observed till up to 12 h, after which the cells started growing exponentially up till 48 h and later entered the

stationary phase. Rhamnolipid production was detected from the beginning of the exponential phase, reached maximum concentration of 3.12 ± 0.35 g/L after 60 h of incubation and thereafter remained constant (Fig. 1). At the end of fermentation (72 h), approximately 30% (w/v) cellobiose remained unutilized in the medium.

3.3 Rhamnolipid production in sugar hydrolysate

The fermentation of sugar hydrolysate containing 98% (w/v) cellobiose, 1% (w/v) glucose and 1% (w/v) xylose by *Pseudomonas* sp. (without nutrient supplementation) results in lower (1.79 ± 0.2 g/L) rhamnolipid production. The lower rhamnolipid production in sugar hydrolysate can be attributed to the nutrient deficient conditions, which are essential for optimum microbial growth and metabolite formation.[36,37] For enhancing the fermentability of sugar hydrolysate, the optimum concentrations of selected micronutrients (magnesium and iron) and macronutrients (nitrogen and phosphorus) were determined through CCD based RSM.

3.4 CCD based RSM for optimization of rhamnolipid production by *Pseudomonas aeruginosa*

The design matrix of the variables in uncoded units along with response has been given in Table 1. Using the designed experimental data (Table 1), the second-order polynomial model for the rhamnolipid production is shown as follows:

$$\text{Rhamnolipid (g/L)} = -89.03 - 7.72 \times MgSO_4 - 2.38 \times Na_2HPO_4 + 0.07 \times FeSO_4 + 25.15 \times NaNO_3 + 1.39 \times MgSO_4 \times MgSO_4 + 0.20 \times Na_2HPO_4 \times Na_2HPO_4 - 1.39 \times NaNO_3 \times NaNO_3 + 0.07 \times MgSO_4 \times MgSO_4 + 0.03 \times MgSO_4 \times NaNO_3 - 0.06 \times Na_2HPO_4 \times NaNO_3 \quad (2)$$

Based on the experimental response, runs 17 and 25 had the maximum and minimum rhamnolipid production, respectively. The ANOVA results of second-order response surface

models for rhamnolipid production have been given in Table 2. From ANOVA analysis of regression model, at 20 degree of freedom, F-value was 93.54 and p-value was <0.001. From the F and P values it indicated that the quadratic regression model for rhamnolipid production was significant. The 'goodness of fit' for the model was checked by the determination of coefficient (R^2). The R^2 value provided a measure of the variability in the actual response values that could be explained by the experimental factors and their interactions. A value of one represents the ideal case at which 100% of the variation in the observed value can be explained by the model. In this case, the mathematical model was found to be reliable with R^2 value of 98.72%, whereas, the adjusted R^2 value of 97.66% indicated that only 2.34% of the total variations were not explained by the model.

The 3D response surface plots represent the regression equation. Figures 2, 3 and 4 represent the 3D response surface plots for the optimum conditions of rhamnolipid production. Each figure represents the effect of two variables on rhamnolipid production. Fig. 2 indicates that maximum rhamnolipid production of 9.45 g/L was obtained on addition of 90 ppm $FeSO_4$ and 8760 ppm $NaNO_3$. Fig. 3 depicts that the maximum rhamnolipid levels were attained at 143 ppm of $MgSO_4$ and 8760 ppm of $NaNO_3$. Fig. 4 similarly shows the effect of varying $MgSO_4$ and $FeSO_4$ concentrations on rhamnolipid production. Overall, the supplementation of hydrolysate with 143 ppm of $MgSO_4$, and 90 ppm of $FeSO_4$ (micronutrients) and 9000 ppm of Na_2HPO_4 and 8760 ppm of $NaNO_3$ resulted in maximum rhamnolipid production. Under optimized conditions (with nutrient supplementation) the maximum predicted product concentration of 9.45 g/L was similar to the experimental response (9.38 g/L). At the end of fermentation, around 48% (w/v) of the initial sugars remained unutilized. The Liquid hydrolysate containing about 76 g/L of reducing sugars (initial) produced 9.43 g/L of rhamnolipid by utilizing 39.52 g/L of reducing sugars. The unutilized sugars remained in the media was about 36.48 g/L.

3.5 *Pseudomonas* cell growth in sugar hydrolysate

The growth of *Pseudomonas aeruginosa* in sugar hydrolysate supplemented with macro and micro nutrients was determined (Fig. 5). After inoculation, the cells continued to grow steadily till the 24th hour of incubation, beyond which the increase in cell mass remained relatively constant. The rhamnolipid production was initiated after the 12th hour of inoculation and attained maximum levels by the 68th hour of incubation. The maximum biomass (0.912 OD_{600}) and rhamnolipid (9.35 g/L) concentrations were attained after 72 h of incubation. It was observed that rhamnolipid production was initiated during the mid exponential phase of *Pseudomonas* cell growth and continued to rise even after the cells had attained the stationary phase.

In the present study, rhamnolipid concentrations obtained in sugar hydrolysate were marginally higher than the previously reported levels of 6 g/L, 4 g/L and 6.6 g/L in synthetic media containing glucose, mannitol or sunflower oil respectively.[38,39,40]

3.6 Rhamnolipid characterization

3.6.1 Emulsification index and interfacial tension

The rhamnolipids produced by *P. aeruginosa* in sugar hydrolysate reduced the culture medium surface tension to values around 3 mN/m, with emulsifying indexes of 56 % against 64% for CTAB (chemical surfactant).

3.6.2 Fourier Transform Infrared Spectroscopy (FTIR) studies of the partially purified product

The FTIR spectrum of the partially purified product is present in Table 3. The IR bands could be assigned to frequencies due to C-C stretching of SP^3 and SP^2 carbons, C-H, Carbonyl groups from esters, pyrnal (oxygen in six-membered ring) groups, C-O groups

present in the rhamnolipid product. The assignments of the major IR bands observed over the product have been tabulated (Table 3). The presence of C-C, C-H, C-O, C=O, RCOO-, C-O-(ring) were confirmed. Similar observations were previously reported by Rahman *et al.*[41] Therefore, FTIR analysis indicated the presence of mono and di rhamnolipids in the extracted sample.

3.6.3 Characterization of the partially purified product by ^{1}H NMR

The ^{1}H NMR spectrum of the partially purified product is presented in Fig. 6. The following assignments are made based on the ^{1}H chemical shifts (Table 4). From the ^{1}H NMR data, the chemical shifts could be assigned to different protons of the functional groups present in the rhamnolipids. The ^{1}H NMR spectrum indicated that the sample predominantly contained mono-rhamnolipids along with di-rhamnolipids. These NMR observations are in agreement with the earlier report.[42]

The peaks reported in the literature for a mixture of mono and di-rhamnolipids ranging from 7 – 9 ppm were attributed to the –CH2-CH- linkage (region III).[39] The NMR spectrum in the range 7-8 ppm observed in the present study could be attributed to the presence of mono-rhamnolipids predominantly. Nevertheless, the di-rhamnolipid also has –CH2-CH- linkage which could overlap in the same chemical shift region. In the present study, there are very negligible peaks between the ranges 8 – 9 ppm. The signals between 7-8 ppm were assigned specifically to mono-rhamnolipid and the rest of the signals (8-9 ppm) were attributed to di-rhamnolipid.[42] However in the present study, it could be observed in the Table 4 that the absence of peaks or very weak NMR signals in the range 8 to 9 ppm indicated the presence of di-rhamnolipid in lesser quantities (region III).

Therefore, from the ^{1}H NMR spectra, it could be concluded that the partially purified product contained mono-rhamnolipids in predominance and lesser quantities of di-

rhamnolipids.

4. Conclusion

The present study showed for the first time, the successful production of microbial rhamnolipids in lignocellulosic sugar hydrolysate. The fermentability of hydrolysate was improved by the addition of selected nutritional supplements at specific concentrations. The rhamnolipid concentrations obtained in our study were comparable to the reported levels obtained using relatively expensive substrates.

Acknowledgement

Authors thank Mrs. Kusum Kumari Vsiwakarma for her support in biosurfactant analysis. The authors acknowledge Hindustan Petroleum Green Research and Development Centre (HPGRDC), Bangalore, India for the financial support to conduct this research.

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Figure captions

Fig. 1 *Pseudomonas aeruginosa* cell growth (-●-) and rhamnolipid production (-○-) from pure cellobiose (-■-).

Fig. 2 RSM plot showing the effect of $FeSO_4$ and $NaNO_3$ on rhamnolipid production.

Fig. 3 RSM plot showing the effect of $MgSO_4$ and $NaNO_3$ on rhamnolipid production.

Fig. 4 RSM plot showing the effect of $MgSO_4$ and $FeSO_4$ on rhamnolipid production.

Fig. 5 *Pseudomonas aeruginosa* cell growth (--●-) and rhamnolipid production (- ○-) from lignocellulosic hydrolysate at 30 ^{o}C for 72 h.

Fig. 6 NMR spectrum of partially purified rhamnolipid sample.

Table 1 Experimental design (conditions and responses) for rhamnolipid production from lignocellulosic hydrolysate

Run order	$MgSO_4$ (ppm)	Na_2PO_4 (ppm)	$FeSO_4$ (ppm)	$NaNO_3$ (ppm)	Rhamnolipid production (g/L)	
					Experimental	Predicted
1	143	5000	30	10000	7.9	7.926
2	333	5000	30	9000	9.15	9.136
3	143	9000	30	8000	8.30	8.358
4	333	9000	30	10000	7.95	7.998
5	143	5000	90	8000	8.55	8.447
6	333	5000	90	10000	8.2	8.087
7	143	9000	90	10000	8.0	7.952
8	333	9000	90	8000	8.6	8.519
9	333	5000	30	10000	7.70	7.695
10	143	9000	30	10000	7.75	7.631
11	333	9000	30	8000	8.60	8.598
12	143	5000	90	10000	8.40	8.47
13	333	5000	90	8000	7.75	7.937
14	143	9000	90	8000	8.35	8.43
15	333	9000	90	10000	8.10	8.168
16	143	7000	60	9000	9.30	9.334
17	333	7000	60	9000	9.35	9.263
18	238	5000	60	9000	8.70	8.652

19	238	9000	60	9000	8.8	8.795
20	238	7000	30	9000	7.20	7.207
21	238	7000	90	9000	7.50	7.44
22	238	7000	60	9000	7.84	7.909
23	238	7000	60	9000	7.90	7.909
24	238	7000	60	8000	6.80	6.661
25	238	7000	60	10000	6.30	6.373
26	238	7000	60	9000	7.90	7.909
27	238	7000	60	9000	7.85	7.909
28	238	7000	60	9000	7.95	7.909
29	238	7000	60	9000	7.85	7.909
30	238	7000	60	9000	7.84	7.909
31	238	7000	60	9000	7.96	7.909
32	238	7000	60	9000	7.92	7.909

Table 2 ANOVA analysis of RSM model for rhamnolipid production from lignocellulosic hydrolysate

Source	DF[a]	Seq SS[b]	Adj SS[b]	Adj MS[c]	F	P
Regression	14	12.6602	12.6602	0.9043	93.54	<0.001
Linear	4	0.545	8.2662	2.06655	213.75	<0.001
Square	4	11.4148	11.4777	2.86942	296.8	<0.001
Interaction	6	0.7004	0.7004	0.11673	12.07	<0.001
Residual Error	17	0.1644	0.1644	0.00967		
Lack-of-Fit	9	0.1466	0.1466	0.01628	7.32	0.005
Pure Error	8	0.0178	0.0178	0.00223		
Total	32	12.8246				
R^2	98.72%	97.66%				

[a]: Degree of freedom

[b]: Sum of square

[c]: Mean square

Table 3 Assignment of FTIR bands to different groups present in the rhamnolipid sample

(Wavenumber, cm^{-1})	**Finger Print**	**Product Mixture containing Mono- & Di-Rhamnolipids**
3300 - 3500	-OH due to water/moisture	Broad band at ~ 3500 cm^{-1}
2850-3000	Aliphatic C-C and C-H bond stretching.	2926, 2854 cm^{-1}: Symmetricand Asymmetric Stretching of C-C and C-H bonds.
1710-1760	Carboxylic Acid/Ester	1757 cm^{-1}: -RCOO-
1500-1350	Bending vibrations of OH in Carboxylic acid	1456-1374 cm^{-1}
~ 1200	C-C stretching	1244 cm^{-1}
~1000	C-O Stretching	1097; 1050 cm^{-1}
~900-950; 830-850	–O–C– (ring)	~910 cm^{-1}; 847 cm^{-1}

Table 4 ^{1}H NMR peak assignments to the partially purified product obtained from rhamnolipid sample. The assignments are based on previous literature[31]

Functional group	**Chemical shift (ppm) Assignment based on previous literature[31]**	
	Mono Rhamnolipid	**Di Rhamnolipid**
$–CH_3$ (terminal)	0.879-0.939	0.862 and lower
$-CH_3$ (ring)	1.255 – 1.37	1.21 (small)
$–(CH_2)n–$	1.255 – 1.37	1.21 (small)
$–CH_2–COO–$	2.41, 2.671 - 2.772	2 - 2.079, 2.31-2.334
4'-H	3.457	3.312
2', 3', 5'- H	3.605 – 3.65	3.727 - 3.78
1' - H	4.204 - 4.261	4.148 – 4.176
–O–CH–	4.714	4.907-5.013
–COO–CH–	5.345-5.37	Traces
$–CH_2$ CH–	7.21-7.718	7.21-7.29

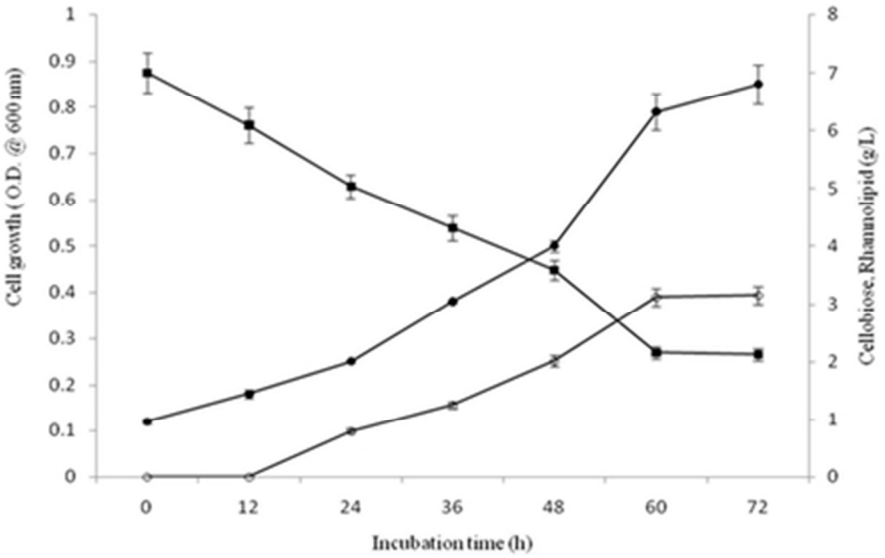

Fig. 1

38x27mm (300 x 300 DPI)

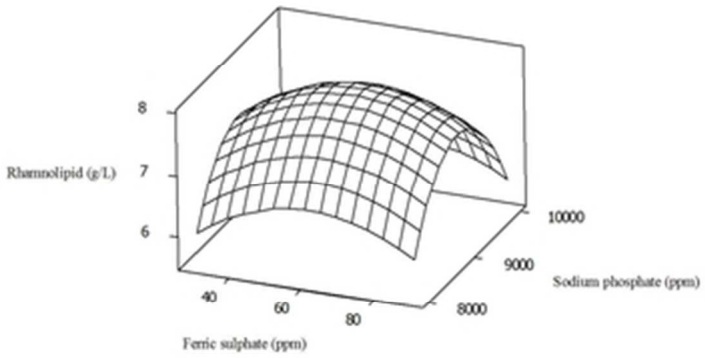

Fig. 2

34x20mm (300 x 300 DPI)

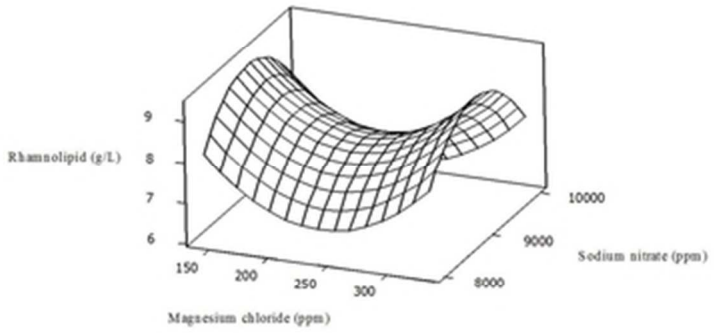

Fig. 3

33x20mm (300 x 300 DPI)

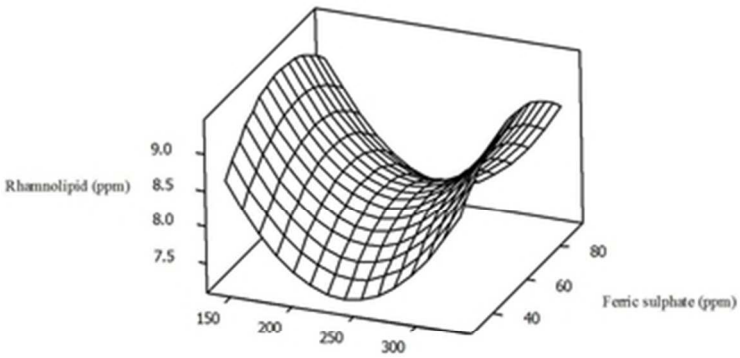

Fig. 4

34x20mm (300 x 300 DPI)

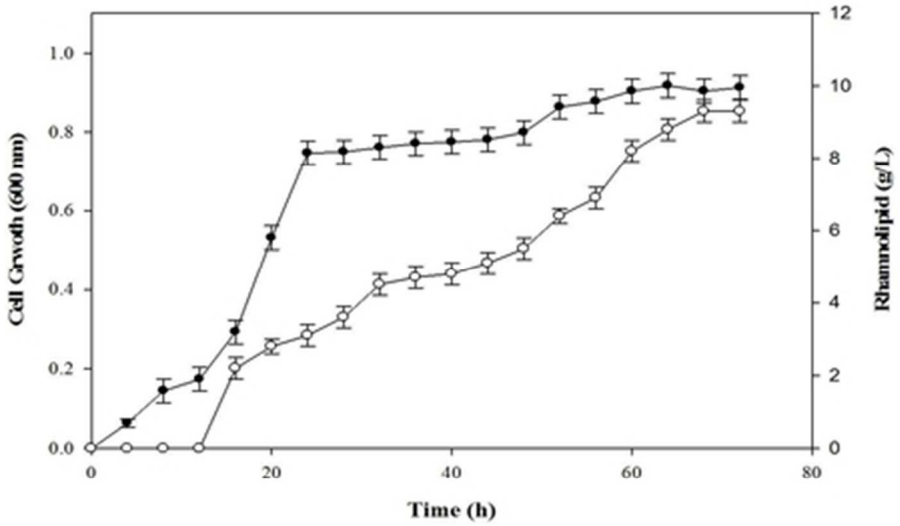

Fig. 5

38x26mm (300 x 300 DPI)

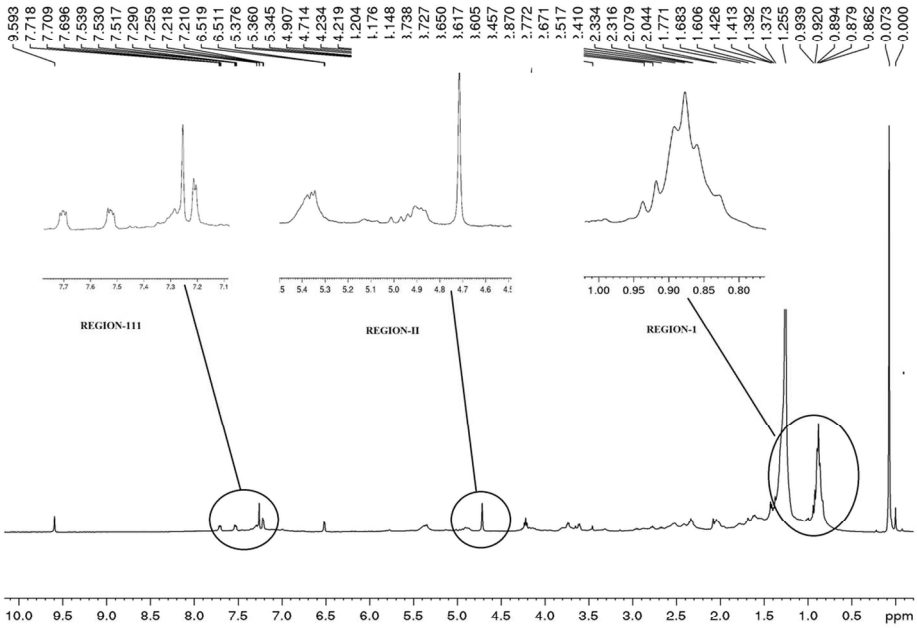

Fig. 6

110x85mm (300 x 300 DPI)