Accepted Manuscript

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PII: S0960-8524(19)30724-2
DOI: https://doi.org/10.1016/j.biortech.2019.121494
Article Number: 121494
Reference: BITE 121494

To appear in: *Bioresource Technology*

Received Date: 20 March 2019
Revised Date: 13 May 2019
Accepted Date: 14 May 2019

Please cite this article as: Joy, S., Rahman, P.K.S., Khare, S.K., Soni, S.R., Sharma, S., Statistical and sequential (fill-and-draw) approach to enhance rhamnolipid production using industrial lignocellulosic hydrolysate C_6 stream from *Achromobacter* sp. (PS1), *Bioresource Technology* (2019), doi: https://doi.org/10.1016/j.biortech.2019.121494

Statistical and sequential (fill-and-draw) approach to enhance rhamnolipid production using industrial lignocellulosic hydrolysate C_6 stream from *Achromobacter* sp. (PS1)

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Abstract

Statistical optimization using industrial rice-straw hydrolysate (C_6 stream) containing 5.0% total sugars was carried out for enhancing the rhamnolipid production from *Achromobacter* sp. (PS1) with subsequent adoption of a sequential fermentation approach with fill-and-draw operation for further increment. The interactive effects of six influential variables obtained from one-factor-at-a-time approach as sodium nitrate, yeast extract, ferrous sulphate, phosphate concentrations and agitation in presence of lignocellulosic hydrolyzed sugars as a basal medium using central composite design revealed the experimental rhamnolipid yield of 5.46 g/L at optimum conditions of total sugars 40 g/L (*w/v*), sodium nitrate 6.0 (g/L), yeast extract 2 (g/L), ferrous sulphate 0.2 (mg/L) and phosphate 1000mM at 100 rpm at 30°C in 8 days. The sequential approach further resulted in an overall yield of 19.35 g/L of rhamnolpid in five sequential-cycles with an increase of 258% over the batch process on account of nutrients replenishment and dilution of toxic by-products.

Key words: Rhamnolipid; Lignocellulosic hydrolysate; Response surface methodology; sequential (fill-and-draw) approach

1. Introduction

Consumer's general concern for the environmental impact has shown increased interests in green bio-based chemicals, resulting in the discovery of new and sustainable sources as global economy can no longer depend on fossil fuels (Ozdenkci et al., 2017; Amiri and Karimi., 2018). To bring about a transitional change from a fossil fuel-based economy to a more sustainable carbon-neutral bio-economy and to mitigate the climate changes, maximum efforts have to be carried out on the reduction of alarming levels of greenhouse gases (CHGs) - CO_2, CO, CH_4, N_2O and harmful gases such as non-methane hydrocarbons (NMHC), NOx, SO_2 etc generated due to fossil fuel or open field burning of agro-residues (Singh et al., 2016; Lohan et al., 2018). India being an agricultural country produces 611 million tons (mt) of crop residues annually, of which wheat and paddy residues constitutes 27–36 % and 51– 57 % respectively. Of this, 84% of crop residues from paddy-wheat system is alone burnt and the characterization analyses reveal that this burning contribute 20% organic carbon (OC) and elemental carbon (EC) into the atmosphere (Lohan et al., 2018; Singh and Basak., 2019) This practice of burning crop-residues can be prevented by their utilization as substrates for microbial growth after pretreatment as these prove to be an enormous source for monomeric sugars (C_5 and C_6) from its constituents cellulose (30-50%) and hemicellulose (20 - 40%) (De Bhowmick et al., 2018). Now-a-days, with several bio-refineries being established due to the mandate shift towards cleaner bio-energy systems, the utilization of lignocellulosic substrates has become the rationale design of each bio-refinery concept providing a sustainable solution for agricultural waste management and the production of several value-added bio-products (Chandel et al., 2018).

One such value-added bio-product is biosurfactant, the global market of which is projected to surpass $ 2.7 billion by 2024 growing at a CAGR of 5.5% from 2018- 2024 with consumption

expected to exceed over 540 kilo tons by 2024 (Global Market Insights, Inc). Biosurfactants have properties similar to chemical surfactants but are far more superior to them owing to their unique properties like higher biodegradability, low toxicity, ecological acceptability, increased surface activities, higher foaming, low critical micelle concentrations (CMC), high selectivity and specificity at extreme temperatures, pH and salinity ranges, creating their basic essential requirement in various industrial applications as in petroleum, textile, cosmetic, agriculture, pharmaceutical, and food processing industries.

To meet the requirement in all these industrial sectors, the large-scale production is desired which however is limited due to low product yields and higher production costs. Other important hindering factors include excessive foaming, comparatively expensive raw materials and downstream processing. Thus, the need of the hour is to adopt cultivation strategies using low-cost renewable feedstock like agricultural waste residues with media optimization, to improve industrial production of biosurfactant (Joy et al., 2019).

Statistical optimization under response surface methodology (RSM) using industrial lignocellulosic hydrolysate can be a suitable technique for medium optimization and to understand the various interactions among different parameters (Sathendra et al., 2019). Further, adoption of a sequential approach with a fill-and-draw strategy using statistically optimized medium can result in the enhancement of the yield. This sequential approach with a fill-and-draw strategy is a method in which half of the cultured broth is withdrawn and the remaining broth which serves as the seed is replenished with equal volume of fresh medium in order to maintain the cells in logarithmic phase (He et al., 2017).

Thus, the present study focusses on the use of central composite design (CCD) under response surface methodology (RSM) using lignocellulosic sugar hydrolysate generated from

rice-straw for the production of rhamnolipid (RL) biosurfactant from *Achromobacter* sp. (PS1). After understanding the interactions among different parameters and obtaining the optimum set of parametric conditions, the biosurfactant yield was further enhanced by adopting the sequential fill-and-draw strategy.

2. Material and Methods

2.1. Chemicals and lignocellulosic hydrolysate

All chemicals, solvents and reagents used were of analytical grade. Rhamnolipid standard JBR 215 (15 % solution in water) was purchased from Jeneil biosurfactant Company (Saukville, WI, USA). Lignocellulosic rice-straw hydrolysate (RSH) used in this study was obtained from Indian glycols Limited (IGL, Kashipur, Uttarakhand, India). The individual sugar composition of the C_5 and C_6 lignocellulosic hydrolysate streams was analyzed by high performance liquid chromatography (HPLC) (Agilent 1260, Palo Alto, CA) using a Biorad Aminex HPX-87H column equipped with a refractive index (RI) detector.

2.2. Microorganism

The microorganism used in the present study possesses a great potential for producing glycolipid biosurfactant with concomitant hydrocarbon degrading ability. It was identified as *Achromobacter* sp. (PS1) (NCBI accession no. KT735240), petroleum sludge isolate from, BPCL refinery, Mumbai, India (Joy et al., 2017). The culture was routinely sub-cultured and maintained on nutrient agar plates.

2.3. Biosurfactant production

The biosurfactant production was carried out in 250 mL Erlenmeyer flasks containing 50 mL of the lignocellulosic hydrolysate medium with essential nutrients dissolved in industrial C_5 and C_6 streams (pH 7.0) separately. The essential nutrients were: $NaNO_3$ (8.7g/L); KCl (1.1 g/L);

NaCl (1.1g/L); $FeSO_4.7H_2O$ (0.00028g/L); K_2HPO_4 (4.4g/L); KH_2PO_4 (3.4g/L); $MgSO_4.7H_2O$ (0.5g/L) and beef extract (0.16g/L) which were obtained from one-factor-at-a-time (OFAT) study during optimization in chemically defined minimal salt medium (MSM) (Joy et al., 2019). For cost-effective formulation of medium, beef extract was replaced by yeast extract which also resulted in almost similar yields. Inoculum was prepared from overnight grown cultures of *Achromobacter sp.* (PS1) in Luria broth (LB) (1 % *v/v*) at 30 °C and 150 rpm. An inoculum with optical density of 1.0 at 600 nm was used to inoculate the flasks followed by incubation at 30°C, 150 rpm for eight days.

2.4. Experimental design and statistical analysis

The individual C_5 and C_6 streams obtained from IGL industry after supplementing with nutrients were evaluated for rhamnolipid production potential. The hydrolysate favoring rhamnolipid production was further optimized statistically under response surface methodology (RSM). Central composite design (CCD) comprising of six parameters namely: (A) C_6 sugar hydrolysate, (B) sodium nitrate ($NaNO_3$), (C) yeast extract (D) ferrous sulphate ($FeSO_4$), (E) phosphate concentrations and (F) agitation rate, all varied at five different levels ($-\alpha$, –1, 0, +1, $+\alpha$) respectively was used (Table 1). A total of 40 experimental runs was obtained using the statistical software package, Design Expert (v.11.0, Stat-Ease, Inc., Minnaepolis MN, USA). The responses *i.e.* rhamnolipid production (R_1) and cell dry weight (R_2) were determined by fitting the second order polynomial equation which displays the effect of variables in terms of linear, quadratic and cross product terms as

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

where Y is the predicted response variable (rhamnolipid production / cell dry weight), β_0 constant terms, β_i linear terms coefficient, β_{ii} quadratic terms coefficient and β_{ij} interaction terms

coefficient, X_i and X_j symbolize the independent variable. To evaluate the statistical significance of the model, the regression and the graphical analysis of the experiments were generated and analysis of variance (ANOVA) was performed. The quality of fit of the model was expressed by the coefficient of determination (R^2) and its statistical significance was checked by F Test. The three-dimensional curves of the response surfaces were generated to visualize individual effects and the interactions between significant parameters. The statistical model was validated with respect to rhamnolipid production and cell dry weight under the conditions predicted by the model at shake flask level. The flasks were harvested at 8th day of incubation and rhamnolipid (RL) assay and cell dry weight analysis were performed as described below.

2.4.1. Analytical methods

The Rhamnolipid (RL) concentration was determined by the orcinol assay with slight modifications wherein approximately, 4 mL of the culture filtrate was acidified to pH 2.0 using 6N HCl and kept overnight for precipitation (Rahman et al., 2010) The precipitate obtained after centrifugation was extracted four times with 1 mL of diethyl ether which was evaporated to dryness and the residue was dissolved in 0.5 mL of deionized water for orcinol-sulphuric acid assay. From the correlation equation of pure rhamnolipids / rhamnose [y = (0.0139 X - 0.0058) x 0.68], the correction factor obtained ranges between 3.0 and 3.4. The value of correction factor is not exact, since the rhamnolipid biosurfactant is not composed of single molecule rather a family of congeners that have different molecular masses. Hence, in the current work an average correction factor of 3.2 was used for multiplication (George and Jayachandran., 2012; Joy et al., 2019). Cell dry weight was determined by centrifuging 1 mL culture broth at 10,000 rpm for 10 min. The cell pellet was then washed with 1 mL distilled water and dried at 90°C until a constant weight was recorded.

2.5. Extraction of biosurfactant

The bacterial cells were removed from culture broth by centrifugation (10,000 rpm at 4°C for 10 min). The collected supernatant was acidified with 6N HCl to pH 2.0 and kept overnight for precipitation. The precipitate was separated after centrifugation and extracted twice with a mixture of chloroform: methanol (2:1 *v/v*). The extracts were pooled and were concentrated under vacuum using a rotary evaporator (Joy et al., 2017).

2.6. Characterization of biosurfactant

The characterization of the biosurfactant was done by thin layer chromatography (TLC). A portion of the partially purified biosurfactant was solubilized in methanol and separated on a silica gel 60 F_{254} plates using a solvent system chloroform: methanol: acetic acid (65:15:2 *v/v/v*) and the separated spots were visualized with orcinol-sulphuric acid reagent (Joy et al., 2017).

2.7. Sequential rhamnolipid production based on a fill-and-draw strategy

Lower yields are generally a major hindrance for the production of rhamnolipid especially on a large scale. Hence, the sequential rhamnolipid production based on a fill-and-draw strategy was carried out for 9-18 days using the statistically optimized set of nutrients in industrial C_6 lignocellulosic sugar hydrolysate stream as basal medium with an aim to improve the rhamnolipid productivity and cost effectivity. In this method, the medium was initially cultured and the production was continued till the sugar in the medium was not completely utilized. Thereafter, half of the cultured broth was withdrawn and the remaining was used as the seed and mixed with equal volume of fresh sterilized culture medium. The withdrawn culture broth was analyzed for rhamnolipid yield. Thus, a similar feeding cycle was sequentially carried out at the onset of sugar depletion. The experiment involved three sets; Set A: initially containing C_6 sugar hydrolysate with 4% (*w/v*) sugar and fed sequentially with 2% (*w/v*) C_6 sugar hydrolysate; Set B:

containing initially 4% (*w/v*) sugar and fed sequentially with 4% (*w/v*) of sugars; Set C: containing initially 2% (*w/v*) sugar and sequentially fed with 2% (*w/v*) of sugars. Since set A and set B contained 4% (*w/v*) sugar, the flasks were initially cultured for 8 days followed by sequential feeding as per the sugar consumption while in set C since the initial concentration of sugar was 2% (*w/v*), the flasks were cultured for 3 days followed by sequential feeding at onset of sugar depletion. Conventional batch fermentation was also conducted in parallel for comparisons.

3. Results and Discussion

3.1. Optimization of rhamnolipid production using RSM

The composition (*w/v*) of the industrial lignocellulosic hydrolysate streams analyzed using HPLC was C_5:- glucose (0.83%), xylose (4.16 %) and C_6:- glucose (4.93%), xylose (0.32%) respectively. The individual C_5 and C_6 streams upon supplementation with essential nutrients, (obtained using OFAT approach) revealed production of 1.09 ± 0.26 g/L and 3.3 ± 0.15 g/L rhamnolipid with 2.1 ± 0.38 g/L and 4.6 ± 0.24 g/L of cell dry weight respectively. A parallel experiment using synthetic sugars having same composition as industrial C_5 & C_6 streams was also performed which resulted in almost similar RL production of 1.30 ± 0.24 g/L and 3.64 ± 0.25 g/L and cell dry weight of 2.4 ± 0.17 g/L and 4.8± 0.32 g/L respectively. Ramirez et al. (2016) reported a maximum rhamnolipid yield of 0.03 g/L from *Pseudomonas aeruginosa* using oil mill waste lignocellulosic hydrolysate containing 2.11 g/L of total sugars in comparison to 0.04 g/L yield in medium containing similar concentration of synthetic glucose. Hence, for statistical optimization using CCD, industrial lignocellulosic C_6 hydrolysate stream having total sugars of 50 g/L (*w/v*) was found a worth parameter to be used as basal medium in combination with other parameters as concentration of sodium nitrate, yeast extract, ferrous sulphate ($FeSO_4$),

phosphate along with agitation rate. The experimental responses showed highest RL yield of 4.68 g/L in run number 34 containing 40 g/L (*w/v*) total sugars, 8(g/L) sodium nitrate ($NaNO_3$), 1.6 (g/L) yeast extract, 0.2 (mg/L) ferrous sulphate ($FeSO_4$) and 1500 mM phosphate at 150 rpm agitation rate producing 4.95 g/L cell dry weight. The lowest yield of 1.37 g/L was observed in run number 16 with 10 g/L (*w/v*) total sugars, 8(g/L) sodium nitrate, 1.2 (g/L) yeast extract, 0.4 (mg/L) ferrous sulphate, and 2000 mM phosphate with agitation rate at 200 rpm resulting in 1.1 g/L cell dry weight (Table 2). In 2015, Prabu et al. reported statistical optimization of rhamnolipid production with maximum yield of 9.38 g/L from *P.aeruginosa* NCIM 2036 with utilization of 52 % of total initial reducing sugars (76 g/L) present in wheat straw hydrolysate supplemented with 8.7 g/L of sodium nitrate and 0.33 g/L of $MgSO_4$,. However, they observed a decreased in production of 1.4 times when the sodium nitrate concentration was increased to 10 g/L and $MgSO_4$, concentration was decreased to 0.238 g/L keeping same initial sugar concentration.

The regression analysis of the experimental data obtained after analysis of variance (ANOVA) resulted in second order polynomial equation in which the rhamnolipid yield (Y_1) and cell dry weight (Y_2) are represented by the equation (2) and (3)

$$Y_1 = 3.00 + 0.74A - 0.04B + 0.02C + 0.07D - 0.001E - 0.10F + 0.18AC - 0.13AF - 0.15BC + 0.17BF + 0.18DE + 0.20EF - 0.09F^2 \quad \textbf{(2)}$$

$$Y_2 = 3.74 + 1.04A + 0.14B + 0.14C - 0.03D - 0.03E - 0.17F - 0.04CF - 0.08A^2 - 0.03\,B^2 - 0.15F^2 \quad \textbf{(3)}$$

where A, B, C, D and E and F are concentrations (in terms of coded values) for total sugar, sodium nitrate, yeast extract, ferrous sulphate, phosphate and agitation rates respectively. Positive symbol mentioned in the above equations specify the synergistic effects and thus more

interaction towards response, whereas negative symbol indicates the antagonistic effects and thus less interaction towards the response (Behera et al. 2018; Anahas et al., 2019).

The coefficients of the regression models presented in Eq. (2) & (3) and the significance of each coefficient, determined by *p*-values are listed in Table 3 & 4 for rhamnolipid yield and cell dry weight respectively. From Table 3, it is apparent that, the variables C_6 sugar hydrolysate (A) and agitation (F) were found significant with *p*-values <0.05 for rhamnolipid production, while for cell dry weight, all variables were found significant. The coefficient estimates in Table 3, show positive effects of C_6 sugar hydrolysate (A), yeast extract (C) and $FeSO_4$ (D) on rhamnolipid yields while other variables showed negative effects. For cell dry weight, C_6 sugar hydrolysate (A), sodium nitrate ($NaNO_3$) (B) and yeast extract (C) showed positive effects.

The analysis of variance (ANOVA) of the reduced regression model for rhamnolipid yield and cell dry weight showed the *F*-values of 35.08 and 493.16 respectively (Table 3 & 4); implying that the models are significant. High degree of correlation between the experimental and predicted values was presented by determination coefficients (R^2) which revealed R-sq and (Adj) R-sq as high as 98.47 % and 96.94% respectively for rhamnolipid production. Similarly, R-sq and (Adj) R-sq value for cell dry weight had values of 99% each, explaining almost all the variability in the responses. The "Lack of fit F value" of 2.79 and 0.71 for rhamnolipid production and cell dry weight respectively implies that the lack of fit is not significant relative to the pure error.

The graphical representations of the regression equation (2) and (3) represented in the form of 3D response surface plots and 2D contour plots were constructed by plotting the relative effect of two experimental factors on the response while keeping the other factors constant. Circular contours indicate the negligible interactions while elliptical ones indicate interactions between

the variables (Hosseini et al., 2009). Figures 1a-d represents 3D response surface plots for the optimum conditions of rhamnolipid production and cell biomass respectively.

Fig. 1(a) shows that the simultaneous increase in concentration of C_6 sugar and yeast extract till 40 g/L and 2.0 g/L respectively enhanced the rhamnolipid production (experimental 4.68 g/L; predicted 4.81 g/L) indicating a vital role of carbon, as major portion of carbon is paved towards product synthesis (Mukherjee et al., 2008). Beyond, 40g/L, a slight lowering in RL production was observed (experimental value - 3.92 g/L; predicted 4.05 g/L). This might be due to catabolite repression imposed by sugar. Chen et al. (2007) also reported that the excessive feeding of glucose can result in the formation of acidic metabolites causing catabolic repression thereby limiting the rhamnolipid synthesis. With respect to the interactive effect of the organic and inorganic nitrogen source, the rhamnolipid yield was found to increase with increase in organic yeast extract than inorganic sodium nitrate (Fig. 1b). Qazi et al. (2013) also reported maximum biosurfactant production from *Pseudomonas putida* SOL-10 using yeast extract (1.5% *w/v*) along with urea and L-leucin (0.1% *w/v*) as compared to inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and potassium nitrate. This trend can be explained on the basis of the amino acids present in the yeast extract which might be supportive for various enzyme synthesis responsible for rhamnolipid production (Banihashemi et al., 2016). This observation can be supported by the results of Nurfarahin et al. (2018) who reported yeast extract to be a source of carbon supporting both cell growth and rhamnolipid production. Fig. 1c explains the interactive effect of agitation and C_6 sugar on rhamnolipid production while Fig.1d the interactive effect of agitation and yeast extract on cell dry weight. The interactive effect observed in both Fig. 1c & 1d, showed that low agitation rates supported both RL production and cell biomass. In rhamnolipid production increasing agitation results in the shear effect on cells and leads to

foaming due to the presence of surface active rhamnolipid molecules. The resulting foam inhibit cell growth and result in lower yields by reducing the bioavailability of substrates as well as the mass transfer efficiency of oxygen (Fontes et al. 2010; Long et al., 2016).

After understanding the interactions, the validation of the experimental model was performed by carrying out a batch run under optimal operating conditions of 40 g/L (*w/v*) total sugars, 6.0 (g/L) sodium nitrate, 2.0 (g/L) yeast extract, 0.2 (mg/L) ferrous sulphate, and 1000 mM phosphate at 100 rpm resulting in a experimental value of 5.46 g/L for rhamnolipid production which was close to the predicted value of 5.88g/L, thereby validating the model.

3.2. Sequential rhamnolipid production based on a fill-and-draw strategy

As is evident from Table 5, the total rhamnolipid yield, cell growth and $Y_{RL/S}$ (rhamnolipid yield per gram of sugar consumed) were higher in Set A in which the fermentation was carried out with initial 4% sugar followed by sequential feeding of 2% sugars after its depletion in 48hrs in comparison to Set B and Set C. In Set A, a maximum of 19.35 g/L of overall rhamnolipid was obtained in 5-cycles of sequential feed with a total utilization of 140 g/L of sugar after an incubation period of 18 days, resulting in $Y_{RL/S}$ of 0.138g/g. However, in set B, a maximum of only 11.86 g/L of rhamnolipid was achieved after 18 days of incubation period with a total utilization of 120 g/L of sugar with $Y_{RL/S}$ of 0.098 g/g. In Set C, the overall rhamnolipid yield was low (4.54 g/L) with $Y_{RL/S}$ of 0.056 g/g at end of 3rd feeding cycle. The higher yield observed in Set A may be due to the presence of well-maintained cells in the log phase. Also, sequential addition of substrate prevents catabolite repression or substrate inhibition due to replenishment of exhausted nutrients and the timely removal of the accumulated toxic by-products (Zhu et al., 2012; Bustos et al. 2018). Chong and Li (2017) reported that the sequential addition of nutrients is a key to control the substrate concentrations at minimal levels which

allows optimal microbial growth without catabolite repression or substrate inhibition, thereby diverting most of substrates for rhamnolipid formation.

Thus, using sequential feed strategy, an increase of 258 % in the overall rhamnolipid production was achieved in five cycles using 140 g/L total sugar in comparison to the conventional batch fermentation where a maximum of 5.4g/L rhamnolipid yield was obtained in 8 days using 40 g/L which remained constant thereafter. He at al. (2017) also reported 163 % increase in rhamnolipid production from *Pseudomonas aeruginosa* using sequential feeding method in comparison to conventional batch method. It has also been observed in sequential fed method, that an indefinite rhamnolipid productivity could not be achieved, rather, a drop in the rhamnolipid yield was observed beyond a particular generation cycle, here 5^{th} cycle which may be due to the strain degeneration (Fig.2). Thus, the overall rhamnolipid yield of 19.35 g/L obtained in our study is comparatively better and easy in approach compared to Bustos et al. (2018) wherein, a slight different strategy was followed using recycled biomass after various fermentation and extraction cycles resulting in only 2.7g/L of biosurfactant from *Lactobacillus pentosus* indicating the adoption of this strategy as remarkable for the enhancement of rhamnolipid yields as very limited reports have been focused on this approach.

From the perspective of economic feasibility of the production process for microbial surfactants, it has been reported that the selection of raw material and scale of production influences the production cost with a slight increase of 20-30% for high volume low-cost biosurfactants in comparison to chemical surfactants (de Gusmaoet al., 2010). This has been well documented by Lang and Wullbrandt (1999) wherein the cost of producing rhamnolipid from *Pseudomonas aeruginosa* using soybean oil as substrate showed inverse pattern with respect to scale-up production with USD 20/kg in 20 m^3 scale and USD 5/kg in 100m^3. As per the IGL

information, the cost of C_6 sugars from rice straw is rated at USD 0.26/Kg. In relation to this the rough estimate of 19.35g of rhamnolipid obtained from our *Achromobacter* sp. (PS1) isolate using lignocellulosic rice straw hydrolysate is valued to be USD 13 exclusive of all the consumables, utilities, labor and miscellaneous costs and calculated on basis of USD 0.7/g of Natsurfact rhamnolipid (Natsurfact., 2019). Thus, the production of biosurfactant using lignocellulosic sugars would serves to be a valuable bioproduct from an economical perspective of a biorefinery concept for the replacement of conventional synthetic surfactant.

3.3. TLC characterization of the biosurfactant

The TLC result of the extracted biosurfactant from *Achromobacter* sp. (PS1) produced in lignocellulosic hydrolysate showed a pattern similar to the standard rhamnolipid-Jeneil JBR 215 and to that produced in chemically defined medium, with two prominent spots at R_f of 0.36 and 0.74 on spraying with orcinol reagent (Fig. 3). The R_f values of 0.74 and 0.36 relate to monorhamnolipid and dirhamnolipid moieties respectively (Joy et al., 2017). Similar results were reported by Bhat et al. (2015) for Jeneil standard rhamnolipid. Monteiro et al. (2007) reported R_f 0.35 and 0.73 values for rhamnolipid produced from *Pseudomonas aeruginosa* when cultivated in a medium containing glycerol. Marcelino et al. (2017) also reported glycolipid type of biosurfactant with R_f value of 0.76 using lignocellulosic sugarcane bagasse hydrolysate produced by *Scheffersomyces stipites*.

4. Conclusion

The statistical optimization of lignocellulosic rice-straw hydrolysate medium under CCD resulted in 5.46 g/L of rhamnolipid with a 1.65 fold increase in comparison to the initial un-optimized conditions. Further, the rhamnolipid production using a sequential fill-and-draw approach enhanced the overall yield by 258%. Thus, this production strategy provides a cost

effective and ecofriendly approach towards enhanced biosurfactant production and its commercialization.

Acknowledgements

This work was supported by a grant provided by the Department of Science and Technology (DST), Science and Engineering Board (SERB), Govt. of India under a project file no. SB/YS/LS- 83/2013. Mr. Sam Joy also acknowledges the senior research fellowship (SRF) financial support, from the Council of scientific & industrial research (CSIR), India (file.no.09/915/0011/2018). We also acknowledge Mr. Umesh Joshi (Senior Manager) India Glycols Ltd. (IGL), Kashipur, Uttarakhand, India for providing us the lignocellulosic hydrolysates in good amounts.

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Legends of Figures

Fig. 1. Three-dimensional surface and contour plots of interactions between (**a**) C_6 sugar hydrolysate and $NaNO_3$, (**b**) $NaNO_3$ and yeast extract, (**c**) C_6 sugar hydrolysate and agitation on rhamnolipid yield (g/L) (**d**) effect of interaction of agitation and yeast extract on cell dry weight from *Achromobacter* sp. (PS1)

Fig. 2. Comparison of rhamnolipid yield in batch and sequential (Set A) fermentation approach

Fig. 3. TLC chromatograms: (**a**) Jeneil JBR-215 (rhamnolipid standard); (**b**) Rhamnolipid from *Achromobacter* sp. produced in chemically defined medium (PS1); (**c**) Rhamnolipid from *Achromobacter* sp. produced in lignocellulosic rice-straw hydrolysate

Fig. 1.

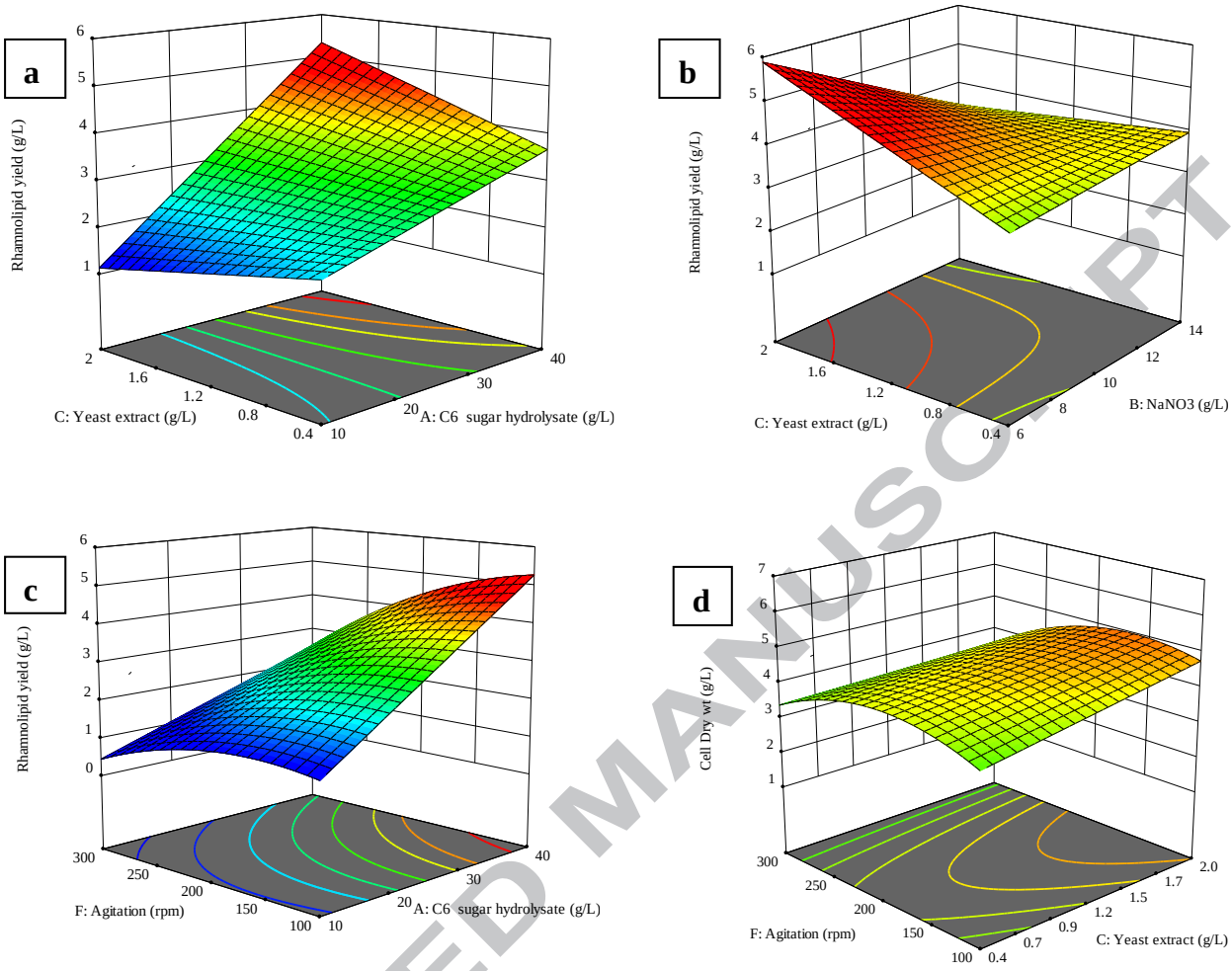


Fig. 2.

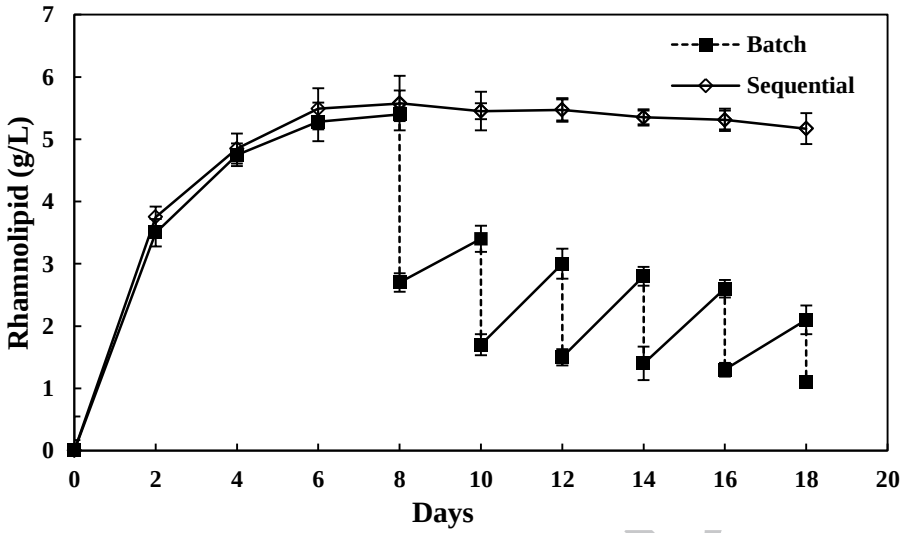
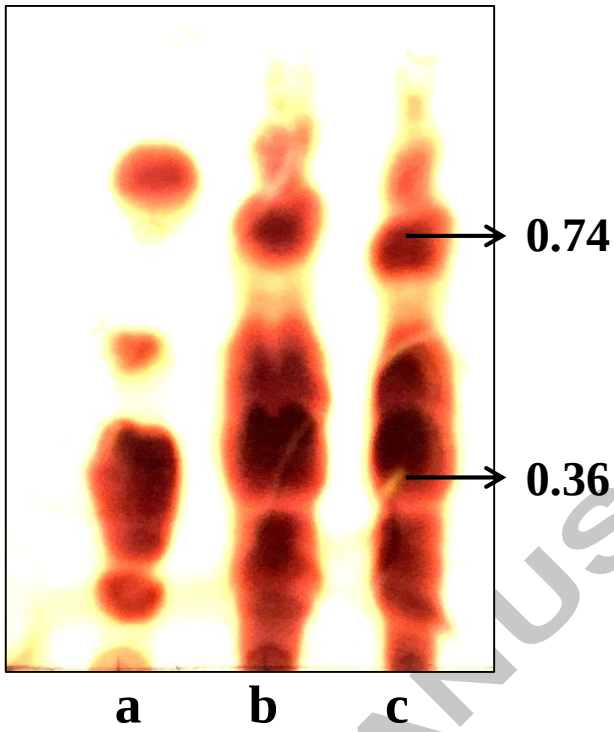


Fig. 3.



List of Tables

Table 1.

Variables	Units	Range of levels				
		-α	**-1**	**0**	**+1**	**+α**
C_6 sugar hydrolysate	**g/L**	10	20	30	40	50
$NaNO_3$	**g/L**	6	8	10	12	14
Yeast Extract	**g/L**	0.4	0.8	1.2	1.6	2.0
$FeSO_4$	**mg/L**	0.05	0.2	0.4	0.6	0.8
Phosphate	**mM**	1000	1500	2000	2500	3000
Agitation	**rpm**	100	150	200	250	300

Table 2.

		Factors						Response 1		Response 2	
Std	**Run**	**C_6 sugar hydrolysate (A) (g/L)**	**$NaNO_3$ (B) (g/L)**	**Yeast extract (C) (g/L)**	**FeSO4 (D) (mg/L)**	**Phosphate (E) (mM)**	**Agitation (F) (rpm)**	**Experimental Rhamnolipid yield (g/L)**	**Predicted Rhamnolipid yield (g/L)**	**Experimental Cell Dry wt. (g/L)**	**Predicted Cell Dry wt. (g/L)**
27	1	30	10	0.4	0.4	2000	200	2.82	2.95	3.50	3.44
34	2	30	10	1.2	0.4	2000	300	2.40	2.31	2.65	2.63
8	3	20	12	0.8	0.6	2500	150	2.17	2.26	2.36	2.48
12	4	40	12	0.8	0.6	2500	250	4.23	3.94	4.30	4.31
1	5	20	8	1.6	0.6	1500	250	1.94	1.74	2.17	2.21
29	6	30	10	1.2	0.05	2000	200	2.78	2.87	3.70	3.80
32	7	30	10	1.2	0.4	3000	200	3.12	3.00	3.60	3.66
39	8	30	10	1.2	0.4	2000	200	3.29	3.00	3.90	3.74
38	9	30	10	1.2	0.4	2000	200	3.01	3.00	3.70	3.74
24	10	50	10	1.2	0.4	2000	200	3.92	4.05	5.49	5.58
17	11	20	12	1.6	0.2	1500	150	2.20	1.89	3.00	3.00
20	12	20	12	1.6	0.6	2500	250	2.21	2.47	2.48	2.42
14	13	40	12	1.6	0.2	1500	150	4.44	4.05	5.10	5.08
15	14	20	8	1.6	0.6	2500	150	2.26	2.39	2.60	2.58
10	15	40	8	1.6	0.6	2500	250	4.13	4.12	4.26	4.21
23	16	10	10	1.2	0.4	2000	200	1.37	1.38	1.10	1.08
35	17	30	10	1.2	0.4	2000	200	2.98	3.00	3.60	3.74
6	18	40	12	1.6	0.2	1500	250	3.49	3.50	4.65	4.64
19	19	20	8	0.8	0.6	1500	150	2.54	2.46	2.26	2.27
13	20	20	12	0.8	0.2	2500	250	2.55	2.58	2.40	2.30
21	21	40	12	1.6	0.2	2500	150	3.38	3.26	4.89	5.01
11	22	40	8	0.8	0.6	2500	150	3.98	3.80	4.42	4.28
26	23	30	14	1.2	0.4	2000	200	2.55	2.90	3.90	3.90
7	24	40	8	0.8	0.6	1500	250	2.50	2.60	3.97	4.10
36	25	30	10	1.2	0.4	2000	200	3.05	3.00	3.70	3.74
4	26	40	12	1.6	0.6	1500	150	3.65	3.83	5.08	5.01
33	27	30	10	1.2	0.4	2000	100	2.55	2.76	3.30	3.38
30	28	30	10	1.2	0.8	2000	200	3.22	3.16	3.70	3.67
37	29	30	10	1.2	0.4	2000	200	3.09	3.00	3.80	3.74
25	30	30	6	1.2	0.4	2000	200	3.05	3.11	3.23	3.29
40	31	30	10	1.2	0.4	2000	200	2.89	3.00	3.79	3.74
18	32	20	8	0.8	0.2	1500	250	1.80	1.98	2.15	2.09
28	33	30	10	2	0.4	2000	200	2.80	3.06	4.10	4.05

9	34	40	8	1.6	0.2	1500	150	4.68	4.81	4.95	4.80
2	35	40	12	0.8	0.2	1500	150	3.84	3.92	4.79	4.70
16	36	40	8	0.8	0.2	2500	250	2.65	2.86	4.08	4.09
3	37	20	8	0.8	0.2	2500	150	2.04	1.89	2.30	2.27
22	38	20	8	1.6	0.2	2500	250	2.11	2.00	2.15	2.21
5	39	20	12	0.8	0.6	1500	250	2.48	2.32	2.3	2.30
31	40	30	10	1.2	0.4	1000	200	2.98	3.01	3.70	3.82

Table 3.

Source	Sum of Squares	df	Mean Square	F-value	p-value		Coefficient Estimate
Model	20.23	13	1.56	35.08	< 0.0001	significant	
A- C_6 sugar hydrolysate	13.03	1	13.03	293.68	< 0.0001		0.747
B-$NaNO_3$	0.0698	1	0.0698	1.57	0.2214		-0.049
C-Yeast extract	0.0184	1	0.0184	0.4153	0.5252		0.025
D-$FeSO_4$	0.1471	1	0.1471	3.32	0.0806		0.076
E-Phosphate	0.0001	1	0.0001	0.0021	0.9635		-0.001
F-Agitation	0.3083	1	0.3083	6.95	0.0142		-0.103
AC	0.6752	1	0.6752	15.22	0.0006		0.189
AF	0.3699	1	0.3699	8.34	0.0079		-0.138
BC	0.4442	1	0.4442	10.01	0.0041		-0.154
BF	0.5972	1	0.5972	13.46	0.0012		0.177
DE	0.6220	1	0.6220	14.02	0.0010		0.184
EF	0.8287	1	0.8287	18.68	0.0002		0.207
F^2	0.4017	1	0.4017	9.05	0.0059		-0.099
Residual	1.11	25	0.0444				
Lack of Fit	1.02	20	0.0509	2.79	0.1295	not significant	
Pure Error	0.0913	5	0.0183				
Cor Total	21.34	38					

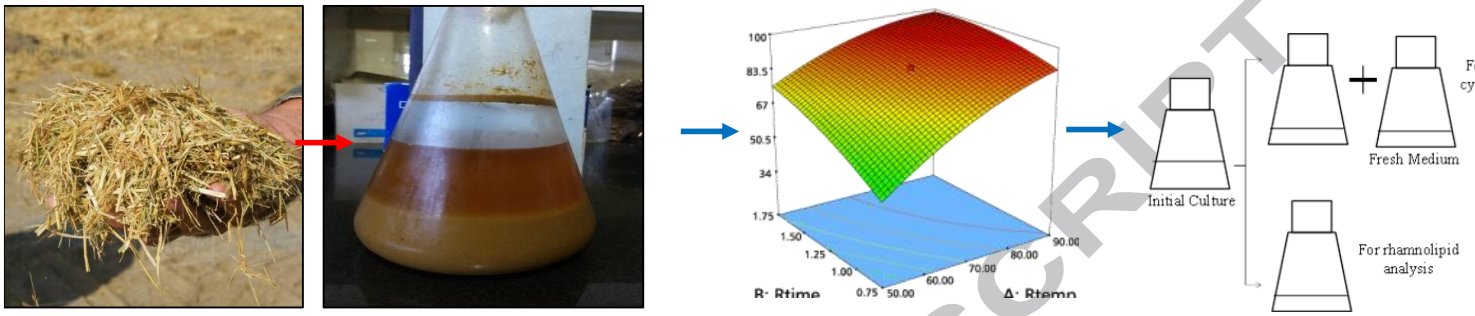
Table 4.

Source	Sum of Squares	df	Mean Square	F-value	p-value		Coefficient Estimate
Model	40.50	10	4.05	493.16	< 0.0001	significant	
A- C_6 sugar hydrolysate	32.67	1	32.67	3978.17	< 0.0001		1.04
B-$NaNO_3$	0.6097	1	0.6097	74.24	< 0.0001		0.14
C-Yeast extract	0.6195	1	0.6195	75.44	< 0.0001		0.14
D-$FeSO_4$	0.0342	1	0.0342	4.17	0.0503		-0.03
E-Phosphate	0.0404	1	0.0404	4.92	0.0346		-0.03
F-Agitation	0.9338	1	0.9338	113.70	< 0.0001		-0.17
CF	0.0450	1	0.0450	5.48	0.0263		-0.04
A^2	0.3209	1	0.3209	39.07	< 0.0001		-0.08
B^2	0.0391	1	0.0391	4.76	0.0373		-0.03
F^2	1.01	1	1.01	122.59	< 0.0001		-0.15
Residual	0.2382	29	0.0082				
Lack of Fit	0.1841	24	0.0077	0.7091	0.7438	not significant	
Pure Error	0.0541	5	0.0108				
Cor Total	40.74	39					

Table 5.

Set A			
Incubation time (Days)	**Rhamnolipid RL (g/L)**	**Cell dry weight CDW (g/L)**	**Total analysis**
8th Day (1st Feed)	5.43	4.94	✓ Total sugar utilized :140 g ✓ Total Rhamnolipid produced : 19.35g ✓ $Y_{RL/S:}$ 0.138 g/g
10th Day (2nd Feed)	3.41	2.64	
12th Day (3rd Feed)	3.07	2.21	
14th Day (4th Feed)	2.81	2.05	
16th Day (5th Feed)	2.52	1.94	
18th Day	2.11	1.89	
Set B			
Days	**Rhamnolipid RL (g/L)**	**Cell dry weight CDW (g/L)**	**Total analysis**
8th Day (1st Feed)	5.37	5.1	✓ Total sugar utilized :120 g ✓ Total Rhamnolipid produced : 11.86 g ✓ $Y_{RL/S:}$ 0.098 g/g
13th Day (2nd Feed)	3.53	3.05	
18th Day	2.96	1.82	
Set C			
Days	**Rhamnolipid RL (g/L)**	**Cell dry weight (CDW)**	**Total analysis**
3th Day (1st Feed)	1.02	1.48	✓ Total sugar utilized :80 g ✓ Total Rhamnolipid produced : 4.54 g ✓ $Y_{RL/S:}$ 0.056 g/g
5th Day (2nd Feed)	1.36	2.13	
7th Day (3rd Feed)	1.29	1.82	
9th Day	0.87	1.64	

Cost-effective Rhamnolipid Production Using Lignocellulosic Hydrolysate



Rice-straw | **Lignocellulosic rice-straw hydrolysate** | **Response surface methodology** | **Sequential fill-and-draw**

- Cost effective rhamnolipid production using lignocellulosic hydrolysate
- Statistical optimization by response surface methodology
- Approx.1.65 fold increase in rhamnolipid yield
- Adoption of sequential fill-and-draw approach to further improve rhamnolipid yield
- Achievement of overall 19.35 g/L rhamnolipid yield using sequential approach