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Utilization of spent wash for optimum production of biosurfactant using response surface methodology

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Abstract

The production of a biosurfactant by *Bacillus subtilis* MTCC 1427 was investigated using Design of Experiments software. The Central Composite Design (CCD) - Response surface methodology (RSM) was applied to understand the interactive effects of 16 experiments carried out for 100 hours in submerged fermentation for the production of biosurfactant. Further, the effect of 3 variables, i.e. pH, substrate concentration (spent wash) and inoculums volume on the biosurfactant activity was investigated. The optimum conditions of the variables were determined and found to be pH 6.0034, spent wash concentration 20.0341 % and inoculum volume of 7.0869 ml. The $p<0.05$ indicated that model was significant. Regression coefficient R2 of 0.9821 indicated excellent evaluation of experimental data by second order polynomial regression model. Applying RSM, the surface tension could be reduced from 72 mN/m to 29.436 mN/m, suggesting that the RSM to be an ideal tool for optimization of fermentation parameters in the production of biosurfactant. This study revealed that the exclusive utilization of spent wash without supplement with a refi8ned nutrient source is feasible and could ensure the economic sustainability of biosurfactant production.

1. Introduction

Microbial surfactants are the biochemical compounds in the extracellular secretions of bacteria, yeast and fungi. They consist of hydrophobic and hydrophilic groups and are capable of reducing the surface and interfacial tension [1]. Biosurfactant find their major applications variety of industrial applications. They also have lot of scope in environmental applications viz., bioremediation [2-3] and wastewater treatment [4]. Biosurfactants are called Green chemicals because of their biological origin and eco-friendliness. Biosurfactant are classified based on their microbial origin and chemical function. They are classified as glycolipid, lipoproteins, polysaccharides, fatty acids and neutral lipids [5]. Most of the biosurfactants are glycolipids such as rhamonolipd produced from Pseudomonas *aeruginosa* [6]**,** sophorolipids from *Candida species* [7]. Surfactin, a lipopeptide is one of most efficient biosurfactant and is produced by various strains of *Bacillus subtilis* [8,9,10]. Efficiency of these surface active compounds is largely due to their ability to withstand and be active at extremely temperature, pH and salinity. Biosurfactant facilitate efficient biological degradation of aliphatic and aromatic hydrocarbon [11], which either wise, are non biodegradable because of poor water solubility. By reducing the surface tension (ST) of water they make the molecules wettable and increase the surface area of the molecule in which microorganisms grow [12]. During past decade biosurfactant have gained considerable attention because of their low toxicity, biodegradability, ecological acceptance. The cost of production is the bottle neck issue. The choice of a suitable low cost raw material can account for 10- 30 % of the overall cost [13]. Recent studies on biosurfactant production from renewable wastes such spent wash, curd whey, wheat bran, Olive oil, Molasses [14-16] has enhanced the economic viability of the large scale production.

Distillery spent wash which has low pH, very high BOD, COD and toxic chemical substances are an environmental hazardous pollutant [17]. Treatment of spent wash by physical and chemical treatment methods have proved to be unsuitable because of the less cost effectiveness and are time consuming. There is a need to evolve an alternative treatment method which is environmental friendly and cost effective. In this context, microbial biodegradation would be the best substitute to other methods because of its efficiency and availability of diverse micro flora. Adequate research efforts have been put into this field [18]. In this current study our endeavor has been to use this hazardous spent wash as a medium to grow *Bacillus subtilis* MTCC1427 and produce biosurfactant which in turn during the process itself will reduce the break down the spent wash. So it is strictly beneficial in the sense, 1. The hazardous pollutant becomes the medium, 2. Surfactant is produced 3. The pollutant is turned less hazardous. In this study we have also tried applying Response Surface Methodology RSM, which is a statistics based approach, for designing the optimization of experiments. The current trend is to use RSM for various applications such as building models, evaluating the interactive effects of various factors in experiments and obtaining optimum conditions for required responses [19]. Though, RSM has been widely used in fermentation media optimization [20-23] it is hardly been applied either in biosurfactant production or spent wash biodegradation studies. The objective of present study is to 1. Optimize growth condition using distillery spent wash as substrate; 2. Select optimize process parameters for maximum production of biosurfactant using Design expert software; 3. Seek the significance of the model and validate it.

2. Material and Methods

2.1.Microorganism

The microbial strain namely *Bacillus subtilis* MTCC 1427 used in the present study was collected from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandīgarh, India. The organism *Bacillus subtilis* MTCC 1427 was provided with only the spent wash as substrate to grow on. The stock was maintained at 4 °C on spent wash fortified agar media with proper sub culturing at an interval of 30 days.

2.2. Inoculum preparation and Growth conditions

An inoculum was prepared using a loop of bacterial cells transferred to 250 ml flask containing spent wash medium as substrate which had been autoclaved at 121 °C for 20 minutes and incubated in a rotary shaker set at 30° C and 150 rpm for 24 hours. Then, 250 ml Erlenmeyer flasks containing 100 ml of the culture media was incubated in a rotary shaker incubator at 150 rpm at various inoculums volumes, spent wash concentration and pH of the medium as specified in Tab 3 for 100 hours. Cultures were harvested by spinning at 15000 rpm at 4° C in Remi cooling centrifuge. The cell free supernatant was collected and used as biosurfactant.

2.3. Growth media: Spent wash

Distillery spent wash was collected from Samson's Distillery, Duggavati, Davanagere, Karnataka. Spent wash were stored in freezer at 4 °C till further use. Spent wash at different concentration was used as the substrate.

2.4. Measurement of Surface Tension

The ST of biosurfactant was determined [24] at room temperature using a Clean and dry Traubel Stalagnometer mounted in vertical plane by using burette stand. The number of drops falling down between upper graduated end and the lower graduated end of the instrument was counted serially for both the liquid i.e., distilled water and biosurfactant. The process was repeated three times and means were calculated. The ST of the biosurfactant in mN/m was estimated from the following formula:

Surface tension of biosurfactant = ys(n2/n1).)(p1/p2)dyne/cm

ys = Density of sample

n1 = No. of drops of biosurfactant

p1 = Density of biosurfactant

y2 = Surface tension of water

n2 = No. of drops of water

p2 = Density of water

2.5. Optimization of independent variables using Response surface methodology (RSM)
RSM offers a statistical design of experiment to assess influential parameter that ultimately leads to peak process performance and the discovery of optimum conditions at minimum cost. To optimize pH, spent wash concentration, inoculum volume, a Central Composite Design (CCD) was used. The coded factors and their levels are given in Tab 1. CCD for three factors and five levels give 16 sets of experiments which were conducted to determine the optimum condition for biosurfactant production. The statistical software package Design Expert (version 9.0.4. State Ease, Minneapolis, MN trial version) was used to design and analyze the experiments. RSM uses a second order polynomial equation (Eqn1).

$$Y=\beta_0+\beta 1A+\beta 2B+\beta 3C+\beta 1\beta 3A+\beta 1\beta 2AB+\beta 1\beta 3AC+\beta 2\beta 3BC+\beta 1\beta 1A^2+\beta 2\beta 2B^2+\beta 3\beta 3C^2 \quad \text{..... Eqn1}$$

Where Y is response variable (dependent), β0 is an intercept (constant), β1, β2 and β3 are linear coefficients. β1 β2, β1 β3, β2 β3 are interaction coefficients of a model. Squared coefficients are β1β1, β2β2 and β3β3 respectively. Terms A, B, C, AB, AC, BC, A2, B2 and C2 are level of independent variables

2.6. Statistical analysis and Modeling
Suitable statistical model were chosen to model the interactions among the different experimental variables and their effect on ST reduction. Statistical significance of the model equation was determined by F-test value. The response, measured after 100 hours incubation period was modeled with an overall mean and quadratic regression model, respectively. The results obtained were subjected to further analysis by Analysis of Variance (ANOVA) to assess the significance of each variable on the ST of the biosurfactant produced. The extent of variance that could be explained by the model was determined by the multiple coefficient of determination, R2 value. The "p" value is the probability of getting a test statistic at least as extreme as the one that was actually observed, assuming the null hypothesis is true. At a "p" value less than 0.05, the null hypothesis is rejected for the central composite and it is concluded that the two-factor interaction models are more significant than linear main effects models. Later an experiment was run using the optimum values for variables given by response optimization in order to validate the predicted optimum values of variables for minimum response.

3. Results and discussion
Using DOE, the interactive effect of the parameters can be obtained. In this study to determine interactive effect of three process parameters on biosurfactant production was studied using central composite design (Table 1). The optimization of biosurfactant was done by considering cubic factor of 2^3=8, 2 center points and 6 star points leading to a total of 16 runs in single set of experiment. These second order polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design expert software. The experimental and predicted values of ST are given as Table 2. In order to check statistical significance of the second order model equation, F-test (ANOVA) was done and data is shown in Table 3

ST =29.9299 + 1.91168 * A + 1.44329 * B + -0.971418 * C + 3.56588 * AB + -2.11162 * AC + -0.670875 * BC + 0.77471 * A^2 + 3.96023 * B^2 + 6.28944 * C^2

The ANOVA results of the quadratic regression model indicate the model was significant. The F value 36.67 indicates that model was significant. There seems to be only 0.01% chance that F value could occur due to noise. The "p" values indicates the significance of the model as well, smaller the "p" values more significant is the model. Probability greater than F values and smaller than 0.05 shows that model term is significant. The "p" values as represented in Tab 3 show that among test variables used in the study A, B, C, AB, AC, B^2, C^2 are significant model terms. The predicted R^2 of 0.8596 was in reasonable agreement with the adjusted R^2 of 0.9554. Adequate precision measures the signal to noise ratio. This model can be used to navigate the design space. The fit of the model was also expressed by the coefficient of regression R^2 which was found to be 0.9821 indicating that 98.21 % the variability in the response could be explained by the model. The closer the value of R (correlation coefficient) to 1, the better is the correlation between the experimental and predicted values. Here the value of R^2 (0.9821) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. This implies that the prediction of experimental data is quite satisfactory. The Coefficient of Variation (CV) indicates the degree of precision with which the experiments are compared. Higher the value of CV lower is the reliability of experiment. Here a lower value of CV (3.65) indicates greater reliability of the experiments performed. The figure 1 shows close agreement of predicted and observed values of ST which are along diagonal.

Graphical representation of RSM and Interactions among the factors

Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels are helpful in knowing interaction effects of them. Such response surface plots allow for easy interpretation of experimental results and the predication of optimum conditions. The 3-D and contour plots can be used to determine the level of interaction between the variables. An elliptical contour shape shows a perfect interaction between the two variables, while a circular contour shows a non-interactive effect on the system response. In this study the response surface plots for ST are shown as in Figures 1, 2 and 3 respectively. The response contour plots showed ellipses for all the variable pairs plotted in Fig 1, 2 and 3 showing complete interaction. The response surface plot as Figure 1 represents two independent variables that is pH and spent wash concentration, keeping inoculum volume at center level. There was clear peak showing that optimum point was within design boundary. There was increase in value of pH with increase in concentration of spent wash to get minimum ST value. From this surface plot it was observed that decrease of ST value as increasing spent wash concentration 13.5-24.5 % and pH in the range of and 5-6.5 respectively. Three dimensional interactions between pH and inoculum volume for response ST at fixed concentration of spent wash is shown as Figure 2. The depression at the center indicates considerable decrease in ST in the pH range of 5 to 8 and inoculum volume of 5.6 to 7.4 ml. The plot depicting the interaction between spent wash concentration and inoculum volume shows initial increase in ST value with increasing spent wash concentration (8 to 30). However, the ST value dropped once the inoculum volume of 6.084 ml reached. Bento et al., obtained 49.5 mN/m ST in a culture medium containing minerals salts and yeast extract by *B. pumilus* [25]. Santos et al used a culture medium based on soybean oil and found that, *P. fluorescens* reduced the ST up to 49.5 mN/m [26] .A study by Dubey KD [27] reported biosurfactant yields when the bacterial strains were grown in curd whey followed by distillery waste, fruit processing waste and sugar industry effluent. The ST of the fermented wastes reduced from an initial range of 56-60 mN/m to 27-39 mN/m.

Process Optimization

The optimization of the combination of three independent factors (i.e. pH, surface tension and inoculum volume) for the best response in this study was carried out by using the numerical optimization of the Design-Expert software. Numerical optimization is a process, where desired goal for each process variable and response is worked out. The weight can give more or less importance on an individual goal relative to the others. The input variables can be set to maximize, minimize, target, within range or none while the response is often set to minimum or maximum. In this analysis substrate concentration (concentration of spent wash %), pH and inoculum volume were set within range. The response was set at minimum, since the desirable optimum is the combination of the independent variables that will give the maximum reduction in ST. Design-Expert software searches for number of solutions to match the set criteria from the most to the least desirable, desirability ranges from zero to one. Hence, the optimum condition for the maximum ST reduction of 29.436 mN/m was found to be at a pH of 6.0034, a substrate concentration of 20.031 % and an inoculum volume of 7.08698 ml.

Validation of Experiments

Validation of experimental model was carried out by taking optimum conditions obtained by the regression model. The optimum conditions were pH of 6.0034, a substrate concentration of 20.031 % and an inoculum volume of 7.08698 ml. The experiments were performed in triplicates and the results were compared. The surface tension value obtained was 29.348 mN/m and results obtained from experiments were close to the actual response (29.436) predicted by the regression model, which demonstrate the validity of the model.

Table 1: Independent factors and level for response surface analysis

Independent variables	Levels				
	-α	-1	0	1	+α
pH	3.999	5.0	6.5	8	9.02
Spent wash Conc. (%)	0.5	8	30	19	37.5
Inoculum (ml)	1.956	4	10	7	12.04

Table 2: Design matrix with observed and predicted values of response.

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**p-value**	
					Prob> F	
Model	681.25	9	75.69	36.67	0.0001	**Significant**
A-pH	49.91	1	49.91	24.18	0.0027	
B-Spent wash conc.	28.45	1	28.45	13.78	0.0099	
C-Inoculum	12.89	1	12.89	6.24	0.0466	
AB	101.72	1	101.72	49.28	0.0004	
AC	35.67	1	35.67	17.28	0.0060	
BC	3.60	1	3.60	1.74	0.2347	
A^2	5.56	1	5.56	2.69	0.1519	
B^2	145.29	1	145.29	70.39	0.0002	
C^2	366.46	1	366.46	177.54	< 0.0001	
Residual	12.38	6	2.06			
Lack of Fit	12.38	5	2.48			
Pure Error	0.000	1	0.000			
+Cor Total	693.63	15				

Table 3: Analysis of variance (ANOVA) results of quadratic model for surface tension (Y)
R^2=0.9821, Adjusted $R^{2=}$0.9554, C.V. % =3.65, PRESS=97.36

Run	Factor 1	Factor 2	Factor 3	Surface Tension	
	A: pH	B:Spent wash conc.	C:Inoculum	Observed Value	Predicted Value
		%	ml	m/Nm	mN/m
1	5	8	4	39.001	39.35
2	8	8	4	41.583	40.27
3	5	30	4	35.878	36.45
4	8	30	4	50.712	51.63
5	5	8	10	43.474	42.98
6	8	8	10	35.598	35.44
7	5	30	10	35.656	37.39
8	8	30	10	44.055	44.12
9	3.9773	19	7	29.989	28.91
10	9.0226	19	7	34.846	35.34
11	6.5	0.50027	7	37.543	38.70
12	6.5	37.4997	7	45.312	43.56
13	6.5	19	1.95462	49.465	49.35
14	6.5	19	12.0453	46.566	46.09
15	6.5	19	7	29.879	29.93
16	6.5	19	7	29.879	29.93

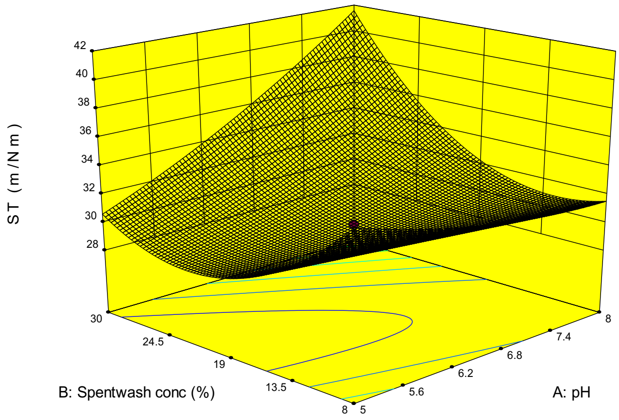

Figure 1 Response surface plot of pH vs. spent wash concentration on surface tension

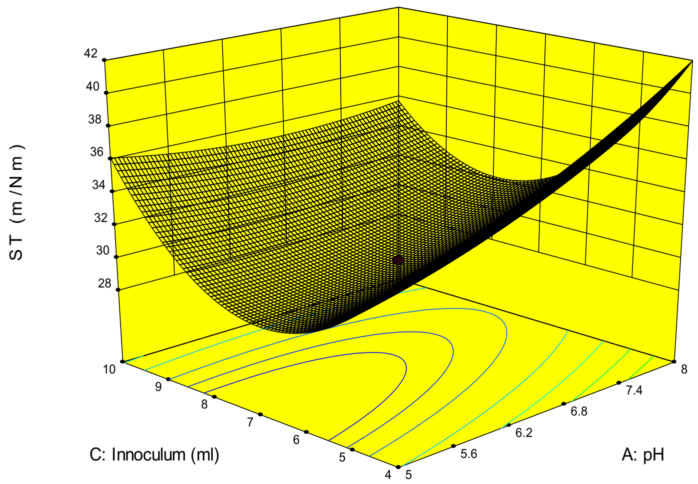

Figure 2 Response surface plot of pH Vs. Inoculum on surface tension

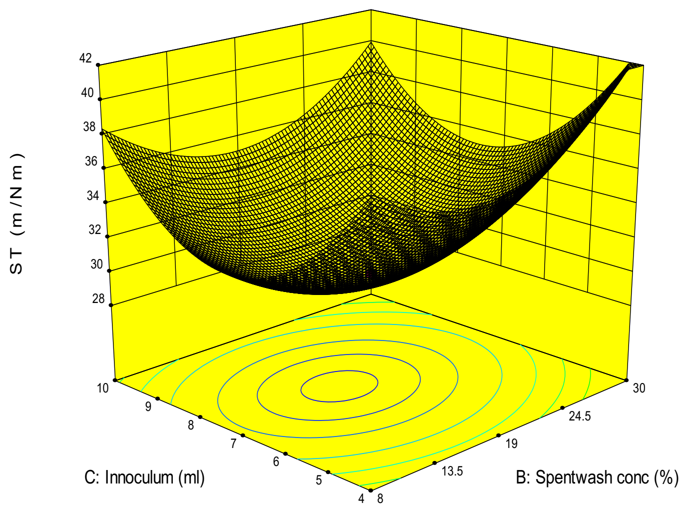

Figure 3 Response surface plot of spent wash concentration vs. inoculum on surface tension

Conclusion

In the current investigation a *Bacillus subtilis* strain, was used for the production of biosurfactant using spent wash as substrate and three parameters were selected for process optimization. Interaction of variables such as pH (5-8), spent wash concentration (8-30 %) and inoculum (4-10 ml) was studied to arrive at an optimal combination which would bring about maximum reduction in surface tension. Quadratic model developed for predicting the surface tension values and optimum conditions of three variables was obtained by Design expert software. The R^2 (0.9821) value shows the model to be significant. The optimum conditions for the production of biosurfactant were found to be pH of 6.0034, spent wash concentration 20.0341 % and an inoculum volume of 7.08698 ml. The predicted results and experimental results were complimentary. It can therefore be concluded that Response Surface Methodology as a statistical tool, is ideal for optimizing the fermentation parameters like pH, substrate concentration and inoculum volume.

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References

1. N G K Karanth, Deo PG and Veenanadig NK, *Curr Sci.* 77 (1999) 116-123.
2. NA Jayalatha and Veena-Kumara-Adi, *International Journal of Current Trends in Science and Technology* 7 (10) (2017). 20329-20335.
3. F Ahimou, P.Jacques, M.Deleu, *Enzyme Microb Technol.* 7 (2000) 749-754.
4. Veena-Kumara-Adi and NGK Karanth, *Asian Journal of Microbiology Biotechnology and Environmental Science*. 10(3) (2008) 505-508.
5. I M Banat, RS Makkar, SS Cameotra, *Appl. Microbiol. Biotechnol*. 53 (2000) 495-508.
6. Daverey and K Pakshirajan, *Appl. Biochem. Biotechnology.* 158 (2008) 663-673.
7. J R Dutta, P KR Banerjee, *Process biochem.* 39 (2004) 2193-2198.
8. G Gong, Z Zheng, H Chen, C Yuan, P Wang, L Yao and Z Yu, *Food Biotechnol*. 47 (1) (2009) 27–31
9. N Kosaric, *Pure Appl .Chem*. 11 (1992) 1731-1737.
10. A C Moran, Olivera, Commendatore, L Esteves and Sineriz, *Biodeg.*1 (2000) 65-71.
11. J D Hamme Vam, Singh OP Ward, *Biotech Adv.24* (2006) 604-620.
12. M K Nagaraj, K.S Jagadeesh, AR Alagawadi, *Indian J. Microb*.48 (2008) 41-48.
13. M Nistschke, SG Costa. *Biotechnol Prog*. 21 (2005) 1593-1600.
14. R S Makkar and SS Cameotra, *Appl. Microbiol. Biotech*. 58(4) (2002) 428-434.
15. K Dubey and Asha. *J World Journal of Microbiology and Biotechnology*.17(1) (2001) 61-69.
16. R Marchant and Ibrahim M Banat, *Trends in Biotechnology* 30 (11) (2012) 558-565.
17. N K Veenanadig, MK Gowthaman and NGK Karanth, *Bioprocess Engineering.* 22(2) (2000) 95-99.
18. Priya T. and G Usharani, *Bot. Res. Internat*. 2(4) (2009) 284-287.
19. R M Usha, NK Rastogi, KA Appaiah, *Micro Bio.* 21 (2011) 739-74.
20. Y H Xoing, JZ Liu, LN Song, Ji *Biochem Eng*. J. 21 (2004)27-32.
21. A Robertiellow *Agricultural Wastes*.4 (1982) 387-385.
22. E Z Ron and E Rosenberg. *Curr. Opin. Biotech*. 13 (2002) 249-252.
23. Veena-Kumara-Adi, B Keerti and A Prabhu, *Int J Recent Sci Res*. 9(5), (2018). 26688-26692.
24. W Z Wei, Zhenh Y, Liu, Z Zhu. *J fermentation Bioeng.* 26 (4) (1998) 395-399
25. F M Bento, FAO Camargo, B Okeke, WT Frankenberger-Júnior, *Braz J Microbiol.* 34 (1)(2003). 65-68
26. S C Santos, L G Fernandez, JC Rossi-Alva, MRA Roque, *African J Biotechnol* .9(35) (2010) 5704-5711.
27. K V Dubey *Indian J of Applied research*. 3(9):20 (2013) 281-284.