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Optimization of Growth of *Rhodobacter Sphaeroides* Using Mixed Volatile Fatty Acids by Response Surface Methodology

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Abstract—A combination of photosynthetic bacteria along with anaerobic acidogenic bacteria is an ideal option for efficient hydrogen production. In the present study, the optimum concentration of substrates for the growth of *Rhodobacter sphaeroides* was found by response surface methodology. The optimum combination of three individual fatty acids was determined by Box Behnken design. Increase of volatile fatty acid concentration decreased the growth. Combination of sodium acetate and sodium propionate was most significant for the growth of the organism. The results showed that a maximum biomass concentration of 0.916 g/l was obtained when the concentrations of acetate, propionate and butyrate were 0.73g/l,0.99g/l and 0.799g/l, respectively. The growth was studied under an optimum concentration of volatile fatty acids and at a light intensity of 3000 lux, initial pH of 7 and a temperature of 35˚C.The maximum biomass concentration of 0.92g/l was obtained which verified the practicability of this optimization.

Keywords—Biohydrogen, Response Surface Methodology, *Rhodobacter sphaeroides*, Volatile fatty acid

I. Introduction

FOSSIL fuel, the major global energy resource, causes global atmospheric pollution problems during combustion.

Hydrogen is an ideal, clean and sustainable energy source for the future because of its high conversion and nonpolluting nature. At present, hydrogen is produced mainly from fossil fuels, biomass and water using chemical or biological processes. Biological hydrogen production processes are found to be more environment friendly and less energy intensive as compared to thermo chemical and electrochemical processes. These processes are mostly controlled by either photosynthetic or fermentative bacteria [1]. The combination of photosynthetic bacteria with fermentative bacteria can provide a system for hydrogen photo production from residual carbohydrates such as organic wastes. In such a coupled system, anaerobic fermentation of organic wastes produces low-molecular-weight organic acids in a first step, which are then converted to hydrogen by photosynthetic bacteria at the expense of light energy, in a second step [2]. The overall yield of hydrogen in such a two-stage process was found to be higher compared to a single stage process [3].

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The main aqueous products from dark acidogenesis are acetate, propionate and butyrate, while formate, lactate, valerate, and caproate are also produced as minor acidogenic products [4]. Among species of *Rhodobacter*, *Rhodobacter sphaeroides* (formerly known as *Rhodopseudomonas sphaeroides*) has been studied most widely for hydrogen production [5, 6, 7]. In studies involving an approach of the latter kind with *R. sphaeroides* several parameters such as substrate concentration, light intensity and initial pH have been optimized with respect to hydrogen production [8,9, 10].Hydrogen production by *Rhodopseudomonas capsulata* from three individual VFAs, i.e. acetate, propionate and butyrate, and a mixture of the three, was also investigated [11].

The one-factor-at-a-time design has two main drawbacks, one is it does not take into consideration the interactions among different factors; it involves a relatively large number of experiments, which makes it laborious and time-consuming to carry out the experiments, especially when the number of factors is large [12]. On the contrary, factorial design is able to study the effects of more than one factor at two or more levels. The experimental design generally includes various combinations of different factor levels, which enables it to depict the interactions among different factors and to be more efficient to deal with a large number of factors. Factorial design can be classified into two categories: full factorial design and fractional factorial design. The number of runs for a full factorial design increases geometrically as the number of factors increases ,when the effects of a large number of factors are to be studied simultaneously, a great many runs of experiment are required. Generally, this will constitute a larger experiment that is not economically and practically feasible [13]. Fractional factorial design provides an alternative when the number of runs for a full factorial design is too large to be practicable. Taguchi design, Plackett–Burman design, central composite design and Box–Behnken design are fractional factorial designs that were used a lot for fermentative hydrogen production processes. Box– Behnken design provides an economical alternative to the central composite design [14].

In the present study, Box –Benhken design is used for growth optimization of *Rhodobacter sphaeroides* using mixed volatile fatty acid as substrate. The individual maximum substrate concentration for Sodium acetate, Sodium propionate and sodium butyrate were already found by single parameter optimization. The optimum concentration of three for maximum growth of the organism is found by applying response surface methodology.

II. Materials and Methods

A. Bacteria

R.Sphaeroides (NJ 345) was kindly provided by Dr. Ch. Sasikala, Environmental Microbial Laboratory, JNTU-Hyderabad, India. The organism was grown photoheterotrophically with malate (30mM) and sodium glutamate (10mM) as carbon and nitrogen sources, respectively (using Biebl and Pfenning's basal media) at 34˚C and about 2000–2500 lx light intensity under anaerobic environment and maintained as stock culture.

B. Growth of Culture

The carbon source malate was replaced by Sodium acetate, Sodium Butyrate and Sodium propionate and the concentration of each is according to the design values given by RSM. Initial pH of the growth medium was maintained at 7.0.The culture was grown at a temperature of 35˚C,and at a light intensity of 3000lux in anaerobic environment.

C. Analysis of Growth

Bacterial growth was measured by monitoring the increase in absorbance at 660 nm (Elico, Bio-spectrophotometer). All experiments were repeated three times. The bacterial cell concentration was determined spectrophotometrically, it was found that an absorbance at 660 nm of 1.0 is equivalent to a cell density of 0.66 g dry weight per liter culture under experimental conditions.

D. Experimental Design

Box–Behnken design is a three-level fractional factorial design developed by Box and Behnken [15]. The design can be thought of as a combination of a two-level factorial design with an incomplete block design. In each block, a certain number of factors are put through all combinations for the factorial design, while other factors are kept at the central levels. Box–Behnken design provides an economical alternative to the central composite design, because it has less factor levels than the central composite design and does not contain extreme high or extreme low levels. Experiments with three initial Sodium acetate concentration namely 0.4,0.8,1.2 g/l, Sodium propionate concentration namely 0.5,1.0,1.5g/l and Sodium butyrate concentration namely,0.44,0.88,1.32g/l were employed simultaneously covering the spectrum of variables for the growth of the organism in the design.

For statistical calculations, the relation between the coded values and real values are described as follows,

$$x_i = \frac{X_i - X_0}{\Delta X} \qquad (1)$$

where x_i is the coded value of the i^{th} variable, X_i is the uncoded value of the i^{th} test variable and X_0 is the uncoded value of the i^{th} test variable at the centre point.

For response surface methodology, a second-order polynomial model (Eq.2) is usually proposed to describe the effects of various factors on the response based on experimental results from the Box–Behnken design.

TABLE I
Range and Levels of the Variables

Variables	Code	Range and Level		
		-1	0	1
Initial Sodium Acetate Concentration(g/l)	X_1	0.4	0.8	1.2
Initial Sodium Propionate Concentration(g/l)	X_2	0.5	1	1.5
Initial Sodium Butyrate Concentration (g/l)	X_3	0.44	0.88	1.32

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad (2)$$

where Y is the predicted response, β_0 is the offset term, β_i ,β_{ii} ,β_{ij} are coefficients of linear, quadratic, and cross products of X_1,X_2 and X_3 on response. A statistical design package, Minitab 15 is used for regression analysis of the data obtained and to estimate the coefficients of the second-degree polynomial equation.

The estimated second-order polynomial model can be displayed as a surface plot and a contour plot, by varying only two factor levels, while keeping other factor levels constant. The surface plot and contour plot will visually show the response over a region of interesting factor levels. In addition, they will indicate how sensitive the response is to the change of each factor levels and to what degree the factors interplay as they affect the response. Based on the analysis of variance (ANOVA) of the estimated model, terms which have significant effects on the response can be determined. The levels of the variables and the experimental design are shown in Table 1and Table 2 respectively. In this study, the experiment design contains 15 trials and the value of the responses was the mean of triplications.

III. Results and Discussion

The coded values of the test variables and the experimental results of biomass concentration yield are given in Table1. Multiple regression analysis of the experimental data yielded the following regression equation for the biomass concentration.

$$Y=0.9023-0.0603X_1-0.0304X_2-0.0889X_3-0.1624X_1^2-0.1357X_2^2-0.2377X_3^2-0.1502X_1X_2-0.0077X_1X_3+0.0025X_2X_3 \qquad (3)$$

where Y is the biomass concentration, X_1 is the initial sodium acetate concentration, X_2 is the initial sodium propionate concentration , X_3 is the initial sodium butyrate concentration . The value of regression coefficient (R^2= 99.5%) which is closer to one indicates that the correlation is best suited in predicting the values of the production system and the predicted values are found to be closer to the results. The results obtained from CCD namely the T distribution, the P values, and the parameter estimates are given in the Table 3.

The P values are used as a tool to check the significance of each of the coefficients, which in turn, may indicate the patterns of the interaction among the variables. Larger the magnitude of T and smaller the value of P indicate, that the corresponding coefficient is more significant. From the coefficient of individual variables it was found that the increase in initial concentration of three substrates (X_1 ,X_2, X_3 the negative sign of -0.0603,-0.0304,-0.0889) decreased the biomass production. The linear effect of initial acetate concentration(X_1) and initial propionate concentration (X_2) was found to be highly significant (P=0.000, 0.001) on biomass concentration. The coefficient of quadratic terms of all initial substrate concentrations (X_1*X_1, X_2*X_2, X_3*X_3) (P=0.000) was found to be significant. The interactive effect of initial acetate concentration and initial propionate concentration(X_1*X_2) was found (P=0.000) to be more significant than the other two interactions. The interactive effect of initial acetate and initial butyrate concentration, and initial propionate and butyrate was found to be less significant.

Table 4 shows the analysis of variance (ANOVA) summary of model for the biomass concentration. ANOVA is required to test the significance and adequacy of the model .The mean squares are obtained by dividing the sum of squares of each of the two sources of variations, the model and the error variance, by the respective degrees of freedom. The Fishers variance ratio F value = (Sr^2 / se^2) is the ratio of the mean square owing to regression to the mean square owing to error .It is the measure of variation in the data about the mean. Here the ANOVA of the regression model demonstrates that the model is highly significant as evident from the calculated F value (107.69) and a very low probability value (P model<F,=0.001). It was observed that the coefficient for linear and squared effect and interactive are highly significant (P=0.000).

The graphical representations of the regression equation called the surface were obtained using the Minitab software package. The response surfaces can be used to predict the optimum range for different values of the test variable from the circular or elliptical nature of the contours. The circular nature of the contour signifies that the interactive effects between the variables were not significant and elliptical nature confirms the significance. Figure 1-3 shows the response surface contour and wire frame plot for the three interactive effects with the yield. Figure 1 shows the interactive effect of initial Sodium acetate and Sodium propionate concentration on biomass concentration. The elliptical nature of the contour indicates that this interaction is significant on the response. Figure 2 shows the interactive effect of initial Sodium acetate and sodium butyrate concentration on biomass concentration. The elliptical nature of the contour indicates that this interaction is significant on the response. Figure 3 shows the interactive effect of initial Sodium propionate and butyrate on biomass concentration. It was evident from the circular nature of the contour that the interaction between the individual variables is not significant. The response surfaces also find the optimum range of process variables.

TABLE II
THE BOX-BEHNKEN EXPERIMENTAL DESIGN WITH EXPERIMENTAL AND PREDICTED VALUES

Run	X_1	X_2	X_3	Biomass Concentration (g/l)	
				Experimental	Predict
1	-1	0	-1	0.653	0.6507
2	0	-1	-1	0.643	0.6437
3	-1	-1	0	0.543	0.5383
4	-1	0	1	0.461	0.4679
5	1	-1	0	0.712	0.7245
6	0	-1	1	0.49	0.4813
7	1	1	0	0.365	0.3633
8	1	0	1	0.336	0.3453
9	1	0	-1	0.559	0.5447
10	0	1	-1	0.563	0.5844
11	-1	1	0	0.797	0.7843
12	0	1	1	0.42	0.4121
13	0	0	0	0.905	0.9023
14	0	0	0	0.912	0.9023
15	0	0	0	0.890	0.9023

TABLE III
SIGNIFICANCE OF REGRESSION COEFFICIENTS FOR BIOMASS CONCENTRATION

Model term	**Parameter estimate(coefficients)**	**T**	**P**
constant	0.9023	67.621	0.000
X_1	-0.0603	-7.373	0.001
X_2	-0.0304	-3.717	0.014
X_3	-0.0889	-10.876	0.000
X_1*X_1	-0.1624	-13.503	0.000
X_2*X_2	-0.1357	-11.279	0.000
X_3*X_3	-0.2377	-19.759	0.000
X_1*X_2	-0.1502	-13.002	0.000
X_1*X_3	-0.0077	-0.671	0.532
X_2*X_3	0.0025	0.216	0.837

TABLE IV
ANALYSIS OF VARIANCE (ANOVA) FOR THE SELECTED QUADRATIC MODEL.

Source of variation	**Degrees of freedom**	**Sum of squares**	**F**	**P**
Regression	9	-0.518717	107.89	0.000
Linear	3	0.099612	62.16	0.000
Square	3	0.328539	205.01	0.000
Interaction	3	0.090565	56.51	0.000
Residual error	5	0.002671	6.38	0.138
Total	14			

The sequential quadratic programming in MATLAB 7 was used to solve the second-degree polynomial regression equation 2. The optimum values of test variables corresponding to the maximum biomass concentration (0.916 g/l) in coded units as X_1=-0.1731, X_2= -0.018, X_3= -0.1841 and they were converted into uncoded units for the actual values. Maximum hydrogen yield of 0.92 (g/l) was obtained under optimum conditions. This value agrees closely with the values from the response surface analysis (0.916 g/l) confirming that the RSM using statistical design of experiments can be effectively used to optimize the process parameters and to study the importance of individual, cumulative and interactive effects of the test variables in the biomass concentration.

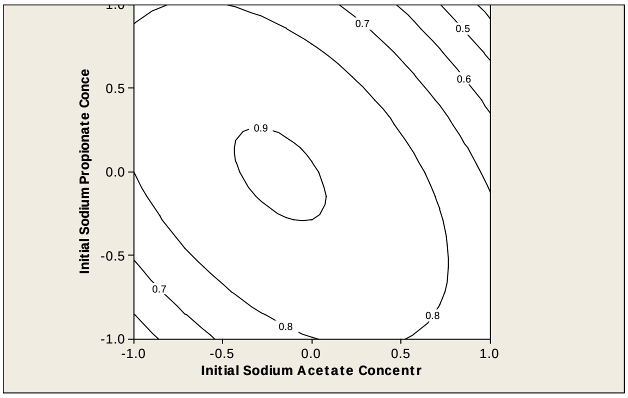

Fig. 1. Contour plot showing the interactive effect of sodium acetate concentration and sodium propionate concentration

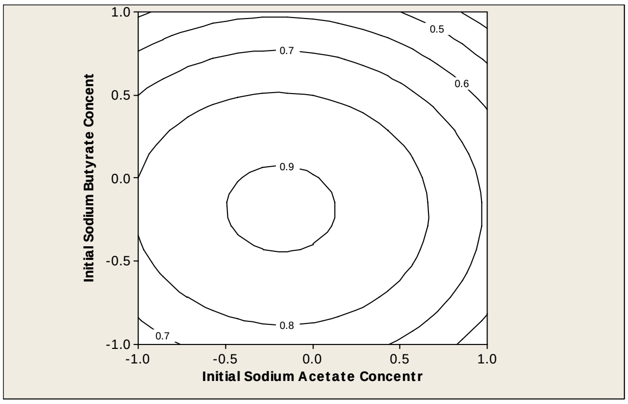

Fig. 2. Contour plot showing the interactive effect of sodium acetate concentration and sodium butyrate concentration

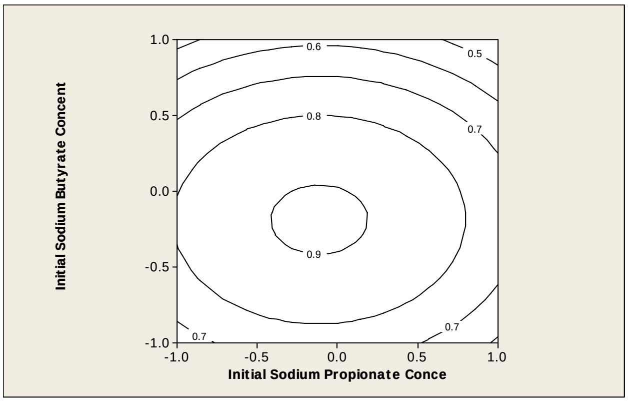

Fig. 3. Contour plot showing the interactive effect of sodium propionate concentration and sodium butyrate concentration

IV. CONCLUSIONS

Effect of initial sodium acetate, sodium propionate and sodium butyrate concentrations on biomass concentration by *Rhodobacter Sphaeroides* was investigated in batch tests and the optimization was done by response surface methodology with a Box-Benhken design.. The results suggested that statistical design methodology offers an efficient and feasible approach for identifying the optimal conditions for maximum biomass production and to analyse the individual and interactive effect of process parameters. The proposed model equation illustrated the quantitative effect of variables and also the interactions among the variables on the response. The optimal conditions are: initial sodium acetate concentration 0.73g/l, propionate 0.99g/l and butyrate 0.799 g/l. The maximum biomass concentration of 0.92g/l was obtained while conducting the experiments at optimum conditions which verified the practicability of this optimization.

REFERENCES

[1] D. Das and T. N. Veziroglu, "Hydrogen production by biological processes: a survey of literature", International Journal of Hydrogen Energy, vol. 26, 2001, pp. 13–28.

[2] M. J. Barbosa, J. M. S. Rocha, J. Tramper and R.H. Wijffels, "Acetate as a carbon source for hydrogen production by photosynthetic bacteria" Journal of Biotechnology, vol.85, 2001, pp.25–33.

[3] K. Nath, M. Muthukumar, A. Kumar and D. Das, "Kinetics of twostage fermentation process for the production of hydrogen", International Journal of Hydrogen Energy, vol.33, 2008, pp.1195–1203

[4] H.H.P. Fang and H. Q. Yu, "Mesophilic acidification of gelatinaceous wastewater", Journal of Biotechnology, vol. 93, 2002, pp. 99–108.

[5] H. G. Zhu, T. Wakayama, Y. Asada and J. Miyake, "Hydrogen production by four cultures with participation by anoxygenic phototrophic bacterium and anaerobic bacterium in the presence of NH^{+4}", International Journal of Hydrogen Energy, vol. 26, 2001, pp.1149–1154.

[6] E. Fascetti, E. D'Addario, O. Todini and A. Robertiello, "Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes", International Journal of Hydrogen Energy, vol.23, 1998, pp. 753–760.

[7] X. Y. Mao, J. Miyake and S.Kawamura, "Screening photosynthetic bacteria for hydrogen production from organic acids", Journal of Fermentation Technology, vol.64, 1986, 245–249.

[8] T. Arik, U. Gunduz, M. Yucel, L. Turker, V. Sediroglu and I. Eroglu, "Photoproduction of hydrogen by Rhodobacter sphaeroides O.U.001", In *Hydrogen energy progress XI, Proceedings of the 11th WHEC, vol. 3. Stuttgart,* Germany: International Association for Hydrogen Energy, 1996, pp. 2417–24.

[9] I. Eroglu, K. Aslan, U. Gunduz, M. Yucel and L. Turker, "Substrate consumption rates for hydrogen production by Rhodobacter sphaeroides in a column photobioreactor" Journal of Biotechnology, vol. 70, 1999, pp. 103–13.

[10] K. Sasikala, C.V. Ramana and P.R. Rao, "Environmental regulation for optimal biomass yield and photoproduction of hydrogen by Rhodobacter sphaeroides O.U. 001", International Journal of Hydrogen Energy, vol. 16, 1991, pp. 597–601.

[11] X.Y. Shi and H.Q. Yu, "Conversion of individual and mixed volatile fatty acids to hydrogen by Rhodopseudomonas capsulata", International Biodeterioration & Biodegradation, vol. 58, 2006, pp. 82–88.

[12] M. Kennedy and D. Krouse, "Strategies for improving fermentation medium erformance: a review", Journal of Industrial Microbiogy Biotechnolgy, vol. 23, 1999, pp. 456–475.

[13] J. T. Luftig and V.S. Jordan, "Design of experiments in quality engineering", NewYork, McGraw-Hill; 1998.

[14] Jianlong Wang, Wei Wan, "Experimental design methods for fermentative hydrogen production: A review", International journal of hydrogen energy, vol. 34, 2008, pp. 235-244.

[15] G. E. P. Box AND E. W. Behnken, "Some new three level designs for the study of quantitative variables", Technometrics, vol. 2, 1960, p.455–475.