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Response Surface Methodological Analysis on growth of *Clostridium pasteurianum*

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Abstract: The individual and interactive effects of initial pH, temperature and initial substrate concentration on the growth of *Clostridium pasteurianum* was investigated in this study. Statistics based experimental designs are applied to optimize the process parameters for biomass concentration. The biomass concentration (g/l) was evaluated at various levels of initial substrate concentration, temperature and initial pH by applying Response Surface Methodology (RSM). RSM was adopted to investigate the mutual interaction between the variables and to identify the optimal values that bring out maximum biomass concentration. The optimal conditions for maximal biomass concentration (g/l) are as follows: initial substrate concentration 12.5 g/l, temperature 34.9°C and initial pH 4.9. Experiments carried out under the statistically designed experimental conditions confirmed the optimality since maximum biomass concentration of 0.28 (g/l) was obtained at the optimum conditions.

Keywords: Hydrogen, Glucose, Dark fermentation, Response Surface Methodology.

Introduction

A large proportion of the world energy needs are being covered by fossil fuels, which have led to an accelerated consumption of these non renewable resources. This has resulted in both, the increase in CO_2 concentration in the atmosphere and the rapid depletion of fossil resources. The former is considered as the main cause of global warming and associated climate change, whereas the later will lead to an energy crisis in the near future (1). Recently diverse alternative fuels have been proposed to substitute fossil fuels. Hydrogen is one of these alternative fuels, that is recognized as a promising future energy carrier. It is considered as a clean fuel since it does not have carbon, sulphur or nitrogen that causes pollution during combustion (2).

Hydrogen may be produced by a number of processes including electrolysis of water, therrmocatalytic reformation of naphtha and biological process. Biological production of hydrogen is an exciting new area of technology that offers the potential production of usable hydrogen from a variety of renewable resources. Biological systems provide a wide range of approaches to generate hydrogen, which include direct bio-photolysis, include direct bio-photolysis, photo fermentations and dark fermentations (3). The use of dark fermentative consortia presents several advantages such as high hydrogen generation rates, (~100 times more than with photo synthetic cultures) continuous hydrogen generation at a sustained rate (since it does not depend of light energy) generation of metabolites of commercial interest (such as organic acids and solvents), no oxygen limitation (because it is an anaerobic process) and it can use complex organic waste as a substrate in non sterile conditions (4,5).

Bacteria known to produce hydrogen include species of *Enterobacter, Bacillus* and *Clostridium*. Carbohydrates are the preferred substrates for hydrogen producing fermentations. *C. Pasteurianum, C. Butyricum* and *C. beijerinkii* are high hydrogen producers, while *C. propionicum* is a poor hydrogen producer (Hawkes *et al.,* 2002). *C. pasteurianum* is a classic hydrogen and VFA producer, but its

metabolism can be directed away from biomass concentration and towards solvent production at high glucose concentrations (12.5%W/V) by CO which inhibits Fe-hydrogenase, and by limiting Fe concentrations (6).

Temperature, one of the most important ecological factors, influences all kinds of physiological activities of microorganism and conversion rate of fermentation product. The pH of the environment has great effects on vital activities of the microorganism, one is that it leads to a change of cell membrane charge to further influence nutritious substance ingestion of micro organism, another one is that it influences enzyme activity during metabolic process, and the other is it changes nutritious substance supply and toxicity of harmful substances in habitat. During molasses fermentation when pH is below a given value ,butyric acid fermentation is converted to ethanol type fermentation(7).

A conventional "change one factor at a time" method was always used for multifactor design. This single dimensional search is laborious, time consuming and incapable of reaching the optimum since it ignores the interaction among the variables. RSM has been proposed to take into account the individual factors and their interactive effects. The RSM is a statistical technique for designing experiment, building models and evaluating the effects of several factors. It also finds optimum conditions for desirable responses and reduces the number of runs (8,9,10). /The present study focuses on the effect of initial substrate concentration, initial pH and temperature on biomass concentration of the bacterium *Clostridium pasteurianum,* using response surface methodology with a Central Composite Design (CCD)

Materials and Methods

Clostridium pasteurianum **(MTCC-146)** was obtained from the Institute of Microbial Technology, Chandigarh. This strain was grown in cooked meat medium with vitamin K in screw-capped tubes .The organism was kept under the designed experimental conditions obtained from Central Composite Design.(CCD).

Experimental Design

An orthogonal 2^3 factorial central composite experimental design with six star points (α=1.68) and six replicates at the centre point, all in duplicates, resulting in a total of 20 experiments were used to optimize the chosen key variables for the growth of the organism. Experiments with five initial substrate concentration namely 2, 4, 6, 8 and 10 g/l, initial pH namely 4.0,4.5,5,5.5 and 6.0 and five different temperature namely, 25°C,30°C, 35°C, 40°C and45°C were employed simultaneously covering the spectrum of variables for the biomass concentration in the Central Composite Design. In order to describe the effects of initial substrate concentration (X_1), temperature (X_2) and initial pH (X_3) on biomass concentration batch experiments were conducted. The coded values of the process parameters are determined by the following equation.

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

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. The range and levels of the individual variables are given in Table1.

The experimental design was given in Table 2 along with experimental data and predicted responses. Biomass concentration (g/l) is the response. The behaviour of the system is explained by the following second order polynomial

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

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 14 is used for regression analysis of the data obtained and to estimate the coefficients of the second-degree polynomial equation. The equations are validated by the statistical tests called the analysis of variance (ANOVA), to determine the significance of each term in the equation fitted and to estimate the goodness of fit in each case. Response surfaces were drawn to determine the individual and interactive effects of test variables on biomass concentration. The optimal values of the test variables are first obtained in coded units and then converted to uncoded units.

Table 1: The range and levels of the independent variables selected for the biomass concentration.

Independent variable	Range and levels				
	$-\alpha$	-1	0	1	α
Initial substrate concentration (X_1, g/l)	2	7	12	17	22
Temperature (X_2,°C)	25	30	35	40	45
Initial pH (X_3)	4	4.5	5	5.5	6

Results and Discussion

The coded values of the test variables and the experimental results of biomass concentration are given in Table2. Multiple regression analysis of the experimental data yielded the following regression equation for the biomass concentration.

$$Y=0.26663-0.01032X_1-0.00246X_2-0.00586X_3-0.04101X_1^2-0.0198X_2^2-0.01626X_3^2+0.005X_1X_2+0.005X_1X_3-0.01X_2X_3 \quad \ldots\ldots (3)$$

where Y is the biomass concentration, X_1 is the initial substrate concentration, X_2 is the temperature, X_3 is the initial pH. The value of regression coefficient (R^2= 95.0%) 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 increased initial substrate concentration (X_1 with the positive sign of 0.01032) increases the biomass concentration within the given range. As initial pH(X_3) and temperature(X_2) increased the yield decreased. The linear effect of initial substrate concentration (X_1) was found to be highly significant (P=0.016) on biomass concentration. The coefficient of quadratic terms of initial substrate concentration, initial pH and temperature (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 pH and temperature(X_2*X_3) was found (P=0.057) to be more significant than the other two interactions.

Table 2: The orthogonal, real values of process parameters along with experimental and predicted values of the biomass concentration

Run No.	Orthogonal values			Real values			Biomass concentration (g/l)	
	X_1	X_2	X_3	Initial Substrate Concentration (X_1)	Temperature (X_2)	Initial pH(X_3)	Experimental	Predicted
1	1.6817	0.0000	0.0000	10	35	5	0.18	0.168
2	-1.0000	1.0000	1.0000	4	40	5.5	0.16	0.151
3	0.0000	0.0000	0.0000	6	35	5	0.26	0.267
4	0.0000	0.0000	0.0000	6	35	5	0.28	0.267
5	0.0000	0.0000	1.6817	6	35	6	0.22	0.211
6	1.0000	-1.0000	1.0000	8	30	5.5	0.2	0.206
7	0.0000	0.0000	0.0000	6	35	5	0.26	0.267
8	1.0000	1.0000	-1.0000	8	40	4.5	0.22	0.213
9	-1.0000	1.0000	-1.0000	4	40	4.5	0.2	0.193
10	0.0000	0.0000	0.0000	6	35	5	0.26	0.267
11	-1.0000	-1.0000	1.00000	4	30	5.5	0.18	0.186
12	-1.0000	-1.0000	-1.0000	4	30	4.5	0.2	0.186
13	0.0000	0.0000	0.0000	6	35	5	0.26	0.267
14	0.0000	0.0000	-1.6817	6	35	4	0.22	0.230
15	1.0000	-1.0000	-1.0000	8	30	4.5	0.18	0.188
16	1.0000	1.0000	1.0000	8	40	4	0.18	0.192
17	0.0000	-1.6817	0.0000	6	25	5	0.22	0.215
18	0.0000	0.0000	0.0000	6	35	5	0.28	0.267
19	0.0000	1.6817	0.0000	6	45	5	0.2	0.206
20	-1.6817	0.0000	0.0000	4	35	5	0.12	0.133

Table 3.Significance of regression coefficients for biomass concentration

Model term	Parameter estimate(coefficients)	T	P
Constant	0.26663	49.675	0.000
X_1	0.01032	2.897	0.016
X_2	-0.00246	-0.692	0.505
X_3	-0.00586	-1.645	0.131
X_1*X_1	-0.04101	-11.830	0.000
X_2*X_2	-0.0198	-5.711	0.000
X_3*X_3	-0.01626	-4.691	0.000
X_1*X_2	0.005	1.075	0.308
X_1*X_3	0.005	1.075	0.308
X_2*X_3	-0.01	-2.149	0.057

Table 4: Analysis of variance (ANOVA) for the selected quadratic model.

Source of variation	Degrees offreedom	Sum of squares	Mean square	F	P
Regression	9	0.032748	0.003639	21.01	0.000
Linear	3	0.002005	0.000668	3.86	0.045
Square	3	0.029543	0.009848	56.86	0.000
Interaction	3	0.001200	0.000400	2.31	0.138
Residual error	10	0.001732	0.000173	2.25	0.197
Total	19	0.03448			

Table 4 shows the analysis of variance (ANOVA) summary of model for the production of hydrogen. 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 (21.01) and a very low probability value (P model<F,=0.000). It was observed that the coefficient for linear and squared effect was highly significant (P=0.045,P=0.000) when compared with the interactive effect(P=0.138). Parameter estimates of individual effects of substrate concentration, pH and temperature reveals that the biomass concentration increases with increase of pH and temperature but decreases with increase of substrate concentration.

The graphical representations of the regression equation called the response surfaces were obtained using the Minitab 15 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 biomass concentration. Figure 1 shows the interactive effect of initial substrate concentration and temperature on biomass concentration. The circular nature of the contour indicates that this interaction is not significant on the response. Figure 2 shows the interactive effect of initial substrate concentration and initial pH 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 temperature and initial pH 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.

The sequential quadratic programming in MATLAB 7 was used to solve the second-degree polynomial regression equation 3. The optimum values of test variables corresponding to the maximum biomass concentration in coded units as X_1=0.1156, X_2= -0.0071, X_3= -0.1603 and they were converted into uncoded units for the actual values. Maximum biomass concentration of 0.26(g/l) was obtained under optimum conditions. This value agrees closely with the values from the response surface analysis (0.2677 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.

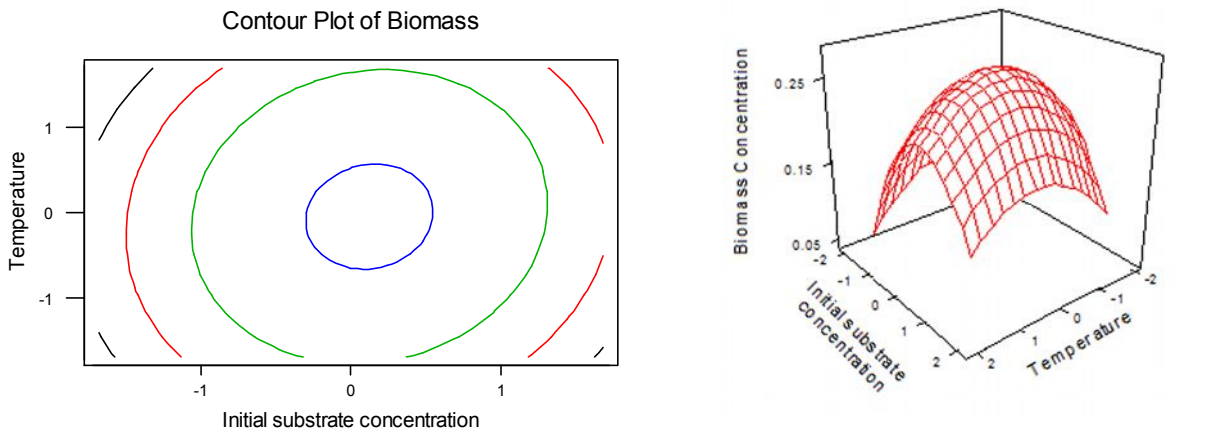

Fig.1. Interactive effect of initial substrate concentration and temperature on biomass concentration

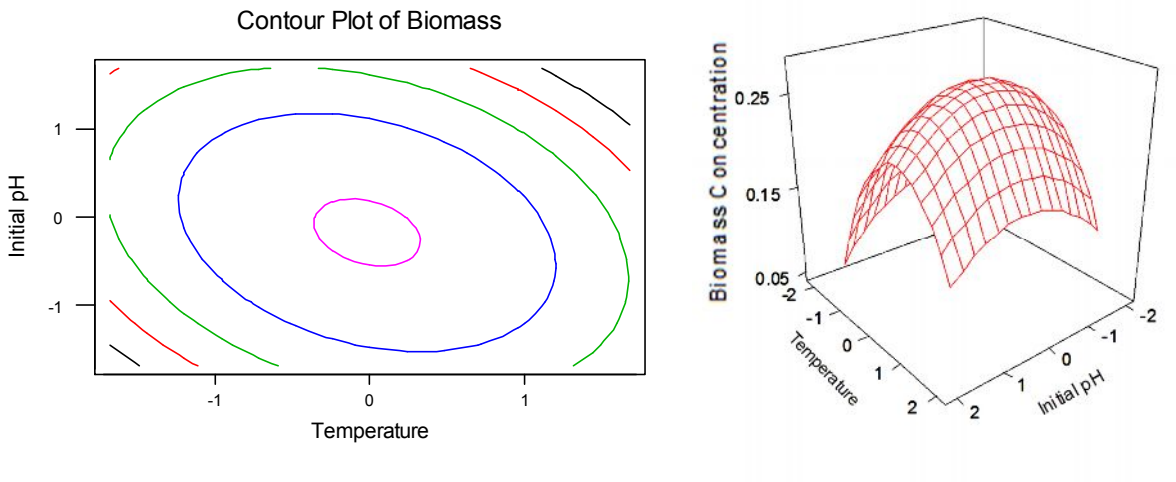

Fig.2. Interactive effect of temperature and initial pH on biomass concentration

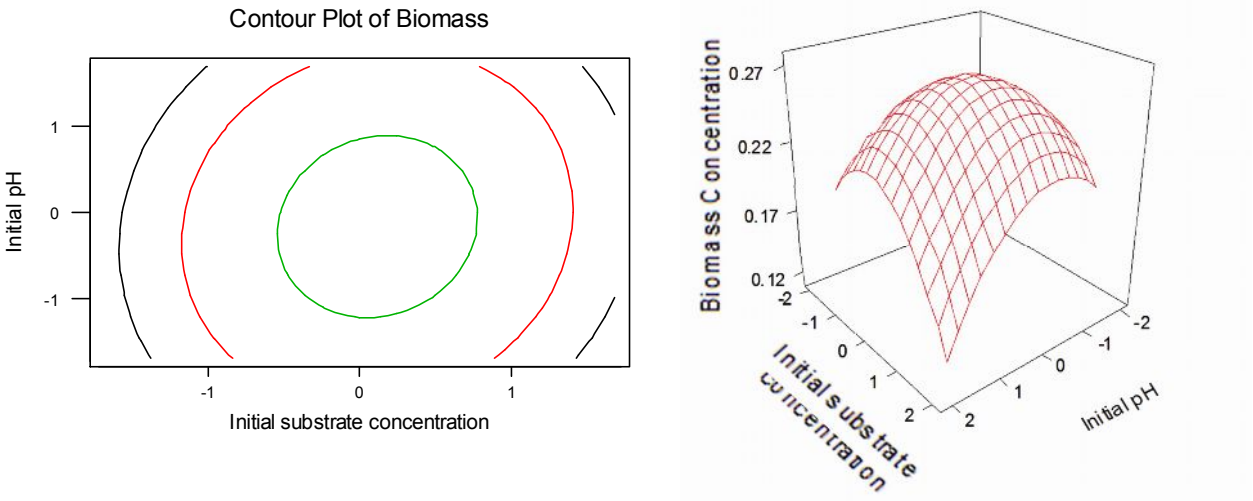

Fig.3. Interactive effect of initial pH and initial substrate concentration on biomass concentration

Conclusion

Effect of temperature, initial pH and initial substrate concentrations on biomass concentration by *Clostridium pasteurianum* was investigated and the optimization was done by response surface methodology with a central composite design. Temperature, initial pH and initial substrate concentration had impact on biomass concentration individually and interactively. The results suggested that statistical design methodology offers an efficient and feasible approach for identifying the optimal conditions for maximum biomass concentration 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 biomass concentration. Initial substrate concentration and initial pH are found to influence the biomass concentration than the temperature. The optimal conditions are: initial substrate concentration 12.4g/l, temperature 35^0C and initial pH of 4.9. Under the optimal conditions, the experimental biomass concentration of 0.26 (g/l) matched well with the predicted value of 0.267 (g/l) which verified the practicability of this optimum strategy.

References

1. Kadpan, I.K. and Kargi, F., Bio-hydrogen production from waste materials. Enzyme and Microbial Technology, 2006, 5, 569 – 582.
2. Veziroglu, T.N. and Barbir, F., Hydrogen- The wonder fuel. International Journal of Hydrogen Energy, 1992, 17, 391-404.
3. David Levin, B., Lawrence Pitt. and Murray Love**.** Bioydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy 2004,29,1000 -1013.
4. Das, D. and Veziroglu, TN. Biohydrogen production by biological process: a survey a literature, International Journal of Hydrogen Energy 2001,26:13-28
5. Liang, T, M., Cheng, S,S. and Wu KL**.** Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. International Journal of Hydrogen Energy 2002,27,1157-65
6. Dabrock Hawkes F.R, Dinsdale R, Hawkes DL, Hussy I, Sustainable fermentative production Challenges for process optimization. International journal of Hydrogen Energy, 2002, 27,1339 -47.
7. Yongfeng Li., Nan qi, ren., Ying Chen. and Guo Xiang Zheng. Ecological mechanism of fermentative biomass concentration by Bacteria. International Journal of Hydrogen Energy, 2007, 32,755-760
8. Yang Mu., Gang Wang. and Han-Qing Yu. Response surface methodological analysis on biohydrogen production by enriched anaerobic cultures. Enzyme and Microbial Technology,2006, 38, 905 -913.
9. Jianlong Wang, Lalei Wan. Optimization of fermentative hydrogen production process by response surface methodology International Journal of hydrogen energy 2008, 33, 6976-6984.
10. Wan-Qian Guo, Man-Qi Ren, Xian-Jing Wang. Optimization of culture conditions for hydrogen production by *Ethanoligenens harbinense* B49 using response surface methodology ,Bio resource Technology, 2009,100, 1192-1196.

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