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Response Surface Optimization of Critical Medium Components for the Production of Lactic Acid by *Rhizopus arrhizus*

Dwivedi Naveen[1], Dwivedi Shubha[1], Majumder C.B.[2] and Mondal P.[2]
[1]Department of Biotechnology, S D College of Engineering and Technology, Muzaffarnagar-251001, UP, INDIA
[2]Department of Chemical Engineering, Indian Institute of Technology Roorkee, Roorkee-247667, UK, INDIA

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Abstract

Response surface methodology (RSM) was used to optimize fermentation medium for enhancing lactic acid production by Rhizopus arrhizus (RA). In the first step of optimization with Plackett- Burman design, in the second step, a 2^3 full factorial central composite design and RSM were applied to determine the optimal concentration of each significant variable. Three most effective medium constituents identified by initial screening method of Plackett- Burman were glucose, urea, and $MgSO_4$. Central composite design (CCD) and Response Surface Methodology (RSM) were used in the design of the experiment and in the analysis of results. This procedure limited the number of actual experiments performed while allowing for possible interactions between the three components. The optimum values for the tested variables for the maximum lactic acid production were glucose 10.97g/lit urea 0.135g/lit and $MgSO_4$ 7.22%. The maximum lactic acid production was 182.5g/lit. It was 65.7g/lit increased from basal medium.

Key words: Response surface methodology, central composite design, lactic acid, fermentation.

Introduction

Lactic acid or 2-hydroxypropionic acid was discovered by Swedish scientist Sheel in 1780 being first isolated from sour milk. It is an organic hydroxy acid whose occurrence in nature is wide spread. It was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881[1]. Lactic acid has a pleasant, sour taste, but no odor. It is completely miscible with water, alcohol and ether, although it is insoluble in chloroform, thus, it does not crystallize from solution as do other acids[2]. It is weak acid with good solvent properties and it polymerize readily for the production of polymers[2]. It, due to flavoring and preservation properties, is used as an acidulant particularly in dairy products, confectionary, beverages, pickles, bread and meat products. The cyclic dimmer of lactic acid is used as a raw material in the synthesis of biodegradable polymers for medical applications; and ethyl lactate is the active ingredient in many anti-acne preparations. Crude grades of lactic acid are used for the deliming of hides in the leather industry and it is utilized for fabric treatment in the textile and laundry industries. Lactic acid is generally produced by submerged fermentation. Normally optimization of medium composition is done, so as to obtain maximum yield from minimum possible inputs, they minimizing the amount of utilized components at the end of fermentation. No defined medium has yet been established for optimum production of lactic acid from *Rhizopus arrhizus* (RA). The conventional method of optimization involves varying one parameter at a time and keeping the others at a fixed level is extremely time consuming and expensive when a large number of variables are evaluated at different levels[3]. Response Surface Optimization of the three most effective constituents screened by Plackett-Burman design fairly reduces the total number of experiments required (only 20) and also manifests any possible interaction effect between the medium constituents. (N+1) No. of experiments in Plackett- Burman screening {where, N= No. of medium constituents plus number of dummy variables (D)} followed by a 2^3 factorial central composite design of experiment[4] and response surface optimization[5-7] was performed in the present investigation. All the experiments were carried out in triplicate and average values were reported. Hence the use of experimental factorial design and response surface methodology[8-9] already successfully applied in other fields is well suited to the study of the main and interaction effects of the factors on the production of lactic acid. The present investigation is aimed at optimization of medium components (Glucose, Urea, and $MgSo_4$) which have been predicted to play a very significant role in enhancing the production of lactic acid.

Material and Methods

Micro-organism and inoculation preparation: A new strain of Rhizopus arrhizus (RA) isolated in our laboratory was used as the producer of lactic acid. Inoculum was prepared by transferring 5 ml of suspension prepared from a fresh slant culture in to Erlenmeyer flask (250 ml) containing 50 ml of sterile inoculum seed medium. The inoculum seed medium was Sabouraud dextrose broth (SDB) having pH 6.5 consisting of dextrose and special peptone and sterilized at 15 psi for 15 minutes. The flask was kept on a rotatory shaker at 180 rpm at 30^0C.

Shake flask experiments: Five milliliters of 24 hrs aged inoculum of Rhizopus arrhizus (RA) was added to 50ml production medium in 250 ml Erlenmeyer flask and incubated on a rotatory shaker at 180 rpm at 30^0C for 72 hrs the composition of production medium comprising: glucose 10 gm/lit., Urea .2gm/lit., $MgSO_4$ .25gm/lit., KH_2PO_4 .65 gm/lit, $ZnSO_4$ 0.45 gm/lit, tween-80 1.0% and $CaCO_3$ 6.0 %. The initial pH of all media was adjusted to 6.5-7.0. $CaCO_3$ was sterilized by dry heat sterilization at 160°C for 30 min. before being added to the medium.

Experimental design and data analysis: Screening of Effective Medium Constituents: The classical method follows simultaneous optimization of each component by varying the concentration of only one of the component and keeping all other at a constant value. This results in a large number of experiments which is both costly and time consuming. Therefore, Plackett-Burman screening method was used for the purpose of screening the medium components that indeed effected the production. The design of the experiment and the effect of the medium constituents have been shown in table 1 and table 2 respectively. The high and low levels of medium constituents involved in Placket-Burman Design have also been shown in table 1. The effectiveness of the medium constituents was determined according to the test of significance (student's t test, P value and confidence level). Confidence level of 95% and more was considered to be significant. A significant effect on Lactic acid production was shown by glucose, urea and $MgSO_4$. The variations made on other constituents (KH_2PO_4, $ZnSO_4$, $CaCO_3$ and Tween 80) did not show any significant effect on the yield of Lactic acid. Effect of the medium constituents is calculated as per following equation:

$$E(x)_1 = 2\,(\Sigma H_i - \Sigma L_i)/N \qquad (1)$$

Where, N = no. of experiments (12, in this case), ΣH_i = sum of the yields of the experiments where the level of i^{th} constituent is high, ΣL_i = sum of the yields of the experiments where the level of the i^{th} constituent is low. Three constituents, glucose, urea and $MgSO_4$ were the ones screened out for optimization, keeping the rest of the constituents (KH_2PO_4, $ZnSO_4$, $CaCO_3$ and Tween 80) at a constant level in all the flasks in central composite design.

Central Composite Design (CCD) and Optimization: The central composite design as proposed by Box and Wilson[10], is shown in table 3. The experiment runs accordingly and the range and the levels of the variable constituents are as shown in table 4. The zero level (central value) chosen were 10gm/l for glucose, and 0.10gm/l for both urea and $MgSO_4$. The coded test factors for the development of regression equation were according to the equation:

$$X_i = (x_i - x_{ci}) / \Delta x_i \qquad (2)$$

Where, X_i is the coded value for the i^{th} independent variable, x_i is the natural value, x_{ci} is the natural value at the center point and Δx_i is the interval.

A total of 20 experiments were conducted that included eight cube points (1-8), six star points (9-14) and six replicas of the central points. This design was to fit the second order polynomial model. Design Expert, statistical software was used to perform the regression and the graphical analysis of the results obtained from the central composite design. A second order polynomial equation comprising linear, quadratic and interaction terms in the form as shown here was obtained.

$$Y = b_o + \sum b_i x_i + \sum b_{ii} x_i^{\,2} \sum b_{ij} x_i x_j \qquad (3)$$

where, Y is the lactic acid yield, b_0 is the intercept term, b_i is the coefficient for the linear effect due to x_i, b_{ii} is the coefficient for quadratic effect due to x_i^2 and b_{ij} is the coefficient for interaction effect due to $x_i x_j$.

Table-1
Plackett-Burman design for the experiment of 12 runs along with the yield observed

Trial No.	Variables											Yield (g/lit.)
	X1	X2	X3	X4	X5	X6	X7	D1	D2	D3	D4	
1	H	H	L	H	H	H	L	L	L	H	L	40.00
2	L	H	H	L	H	H	H	L	L	L	H	09.05
3	H	L	H	H	L	H	H	H	L	L	L	54.55
4	L	H	L	H	H	L	H	H	H	L	L	18.18
5	L	L	H	L	H	H	L	H	H	H	L	09.00
6	L	L	L	H	L	H	H	L	H	H	H	18.18
7	H	L	L	L	H	L	H	H	L	H	H	50.91
8	H	H	L	L	L	H	L	H	H	L	H	32.73
9	H	H	H	L	L	L	H	L	H	H	L	27.26
10	L	H	H	H	L	L	L	H	L	H	H	18.18
11	H	L	H	H	H	L	L	L	H	L	H	61.83
12	L	L	L	L	L	L	L	L	L	L	L	21.82
High	10%	0.4%	0.6%	1%	0.4%	6%	0.5%	-	-	-	-	
Low	1%	0.1%	0.025%	0.01%	0.025%	1%	0.1%	-	-	-	-	

D_1-D_4= Dummy variable, H= High, L= Low

Table-2
Effect estimates for Lactic Acid production from the result of Plackett-Burman design

Factors	Medium Components	Effect	S.E.	t-value	p-value	Confidence Level
X1	Glucose	28.811	3.04	9.47752	0.0000	100
X2	Urea	11.815	3.04	-3.8865	0.0025	99.75
X3	KH_2PO_4	00.325	3.04	-0.1069	0.9176	8.24
X4	$MgSO_4$	10.025	3.04	3.29769	0.0071	99.29
X5	$ZnSO_4$	02.708	3.04	0.89089	0.3921	60.79
X6	$CaCO_3$	-5.778	3.04	-1.9007	0.0838	91.62
X7	Tween-80	-0.905	3.04	0.1230	0.5421	74.65

Effect = Significant for confidence level >95

Table 3
Central Composite Design of 20 Experiments for Lactic acid production media

Sl. No	Glucose		Urea		$MgSO_4$		O. yield	P. yield	Residual
	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	g/l	g/l	
1	-1	5	-1	0.05	-1	0.05	49.55	63.624	-14.074
2	1	15	-1	0.05	-1	0.05	70.072	90.586	-20.514
3	-1	5	1	0.15	-1	0.05	72.73	76.381	-3.651
4	1	15	1	0.15	-1	0.05	105.023	88.977	16.046
5	-1	5	-1	0.05	1	0.15	95.78	65.284	30.496
6	1	15	-1	0.05	1	0.15	120.19	104.923	15.267
7	-1	5	1	0.15	1	0.15	98.76	99.33	-0.57
8	1	15	1	0.15	1	0.15	135.1	141.58	-6.48
9	-1.68179	0	0	0.10	0	0.10	48.51	27.226	21.284
10	1.68179	20	0	0.10	0	0.10	53.23	56.888	-3.658
11	0	10	-1.68179	0	0	0.10	44.1	22.398	21.702
12	0	10	1.68179	0.20	0	0.10	14	8.206	5.794
13	0	10	0	0.10	-1.68179	0	52.98	46.52	6.46
14	0	10	0	0.10	1.68179	0.20	182.5	165.206	17.294
15	0	10	0	0.10	0	0.10	158.23	154.317	3.913
16	0	10	0	0.10	0	0.10	156.96	154.317	2.643
17	0	10	0	0.10	0	0.10	159.36	154.317	5.043
18	0	10	0	0.10	0	0.10	158.372	154.317	4.055
19	0	10	0	0.10	0	0.10	162	154.317	7.683
20	0	10	0	0.10	0	0.10	162.05	154.317	7.733

Table-4
Boundaries of experimental domain and spacing of levels expressed in coded and natural units

Code Units	Glucose (gm)	Urea (gm)	$MgSO_4$ (gm)
-1.68179	0	0	0
-1.00000	05.00	0.05	0.05
0	10.00	0.10	0.10
1.00000	15.00	0.15	0.15
1.68179	20.00	0.20	0.20
Δx	5.0	0.05	0.05

Results and Discussion

Analysis of Response Surface Model (RSM): The results of the second order response surface model fitting in the form of analysis of variable (ANOVA) have been shown in table 5. The fisher F test with a very low probability value (P value ~ 0, > F = 5.07E-05) shows a very high significance of the regression model. The determination coefficient (R^2) in this case is 0.9135, which checks the goodness of fit of the model. The value of R^2 indicates that only 8.35 % of variations are not explained by the model. The value of adjusted determination coefficient (Adj. R^2= 0.8956) is very high and that indicates the high significance of the model and good correlation between the independent variable is signified by high value of correlation coefficient (R=0.9558). Lower value of coefficient of variation (CV = 21.70) indicates improved precision and reliability of the conducted experiment[11,12].

Table-5
Result of the second order response surface model (same for coded and uncoded test variables) fitting in the form of Analysis of variable (ANOVA)

	DF	SS	MS	F-test	P-value
Regression	9	48367.4	5374.2	20.33	0.000
Residual Error	10	2643.3	264.3	118.56	0.000
Total	19	51010.7			

Model coefficients and their significance: The significance of each coefficient was determined by p-values and T-values, which are listed in table 6. The greater the T-value and the smaller the p-value the more significant is the corresponding coefficient[13]. This implies that the quadratic main effects of glucose and urea are more significant than their first order effects, while first order effect of $MgSO_4$ is more significant than its quadratic effect.

Response equations and optimum values: The application of Response Surface Methodology yielded the following regression equation which is an empirical relationship between the logarithmic value of lactic acid yields and test variables in coded and uncoded units:

In coded units: $Y=159.18 + 7.69*A + 6.64*B + 27.77*C +2.96*AB + 0.99*AC - 5.03*BC - 24.97*A^2 - 36.16*B^2 - 9.89*C^2$

In uncoded units: $Y = -213.419 + 19.935*Glucose + 3108.564*Urea + 1507.952*MgSO_4 + 11.851*Glucose*Urea + 3.968*Glucose*MgSO_4 -2012.05*Urea*MgSO4 - 0.999*Glucose^2 -14465.036*Urea^2 -3955.09*MgSO_4^2$

Where Y is the response, that is, lactic acid concentration expressed in logarithmic values, and A, B and C are the coded values of the test variables (glucose, urea and $MgSO_4$ respectively).

Response surface plot as a function of two variables at a time maintaining all other variables at fixed levels are more helpful in understanding both the main and the interaction effects of the detected two factors. The yield values of different concentrations of the variables can also be predicted from the respective response surface plots (figure 1-3). The maximum predicted yield is indicated by the surface confined in the response surface diagram.

Figure-1 shows the response surface plot obtained as a function of glucose concentration vs. urea concentration, while all other variables are held at zero level. Increase in the yield of lactic acid was observed with an increase in glucose and urea concentration.

Figure-2 shows the response surface plot obtained as a function of glucose concentration vs. $MgSO_4$ concentration, while all other variables are maintained constant at zero level. There was an increase in yield of lactic acid with an increase in glucose concentration vs. $MgSO_4$ concentration.

Figure-3 shows the effect of urea concentration vs. $MgSO_4$ concentration, keeping all other variables at zero level. An increase in lactic acid production was observed with an increase in the concentration of urea and $MgSO_4$.

The production of lactic acid is predominantly influenced by glucose, urea and $MgSO_4$ concentrations. Glucose and urea are however the key nutrient materials which control the biosynthesis of lactic acid. At higher concentrations both glucose and urea tend to inhibit the production of lactic acid. Carbon catabolte repression may also be occurred due to high concentration of glucose. The optimal values of the test variables in coded units are as follows; A = 0.194, B = -0.047, C = 1.404 with the corresponding Y= 182.5. The natural values obtained by substituting the respective values of variables in regression equation are: glucose 10.97g, Urea 0.135g and $MgSO_4$ 7.22% with corresponding Y= 182.5g/L.

Conclusion

Response Surface Methodology was performed to optimize the medium components for lactic acid production of *Rhizopus arrhizus* (RA). A highly significant quadratic polynomial obtained by the central composite design was very useful for determining the optimal concentrations of constituents that have significant effects on lactic acid production. The production of lactic acid was 182.5 g/L under the optimal conditions while it was only 65.7 g/L from basal medium. After the optimization of medium components by above mentioned approaches the increment of lactic acid production was very appreciable, it was 116.8 g/L greater production of lactic acid than the production of basal medium. This methodology could therefore be successfully employed to any process, where an analysis of the effects and interactions of many experimental factors are required. The maximum information can be obtained by Central composite experimental design, while required very little amount of the individual experiments. The main effects and interaction of the factors are focused by the help of isoresponse curves. Thus, the optimization of many fermentation processes can be designed by same for smaller experimental design and less time consuming.

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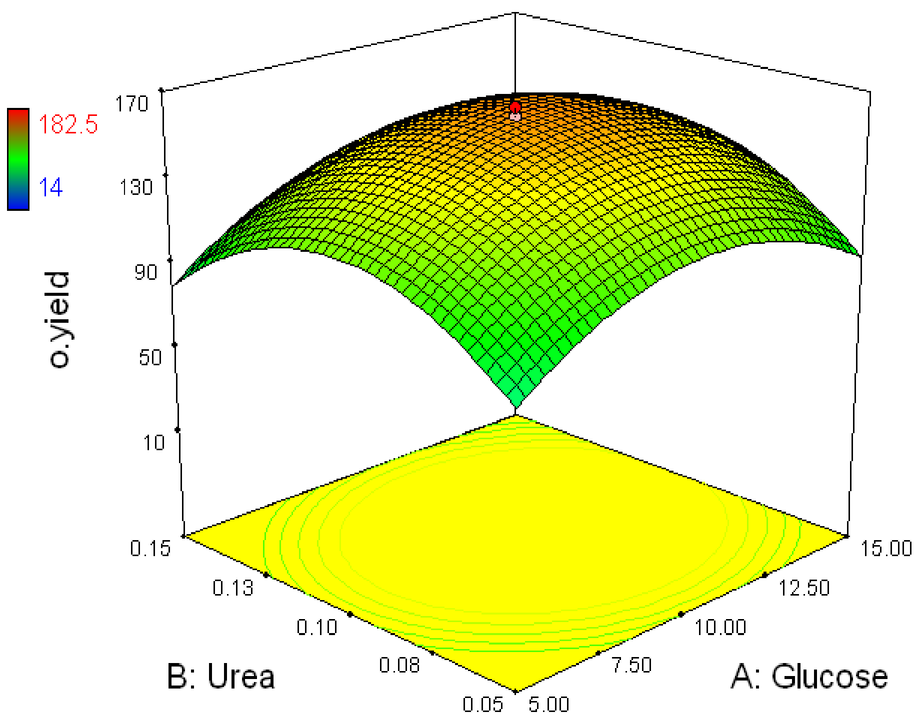

Figure-1

Response surface plot showing the effect on glucose concentration, urea concentration and their interaction effect on the production of lactic acid. Other variables are held at zero level

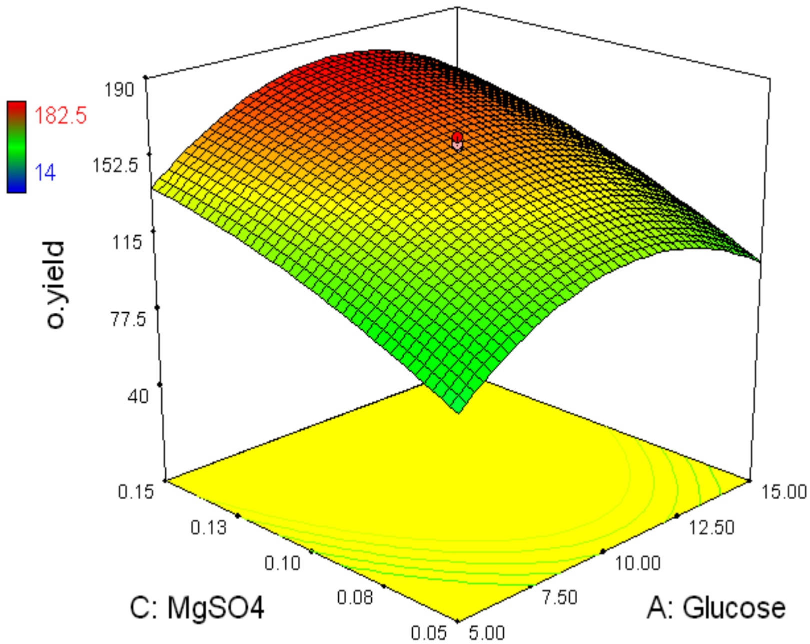

Figure-2
Response surface plot showing the glucose concentration, $MgSO_4$ concentration and their interaction effect on the production of lactic acid. Other variables are held at zero level

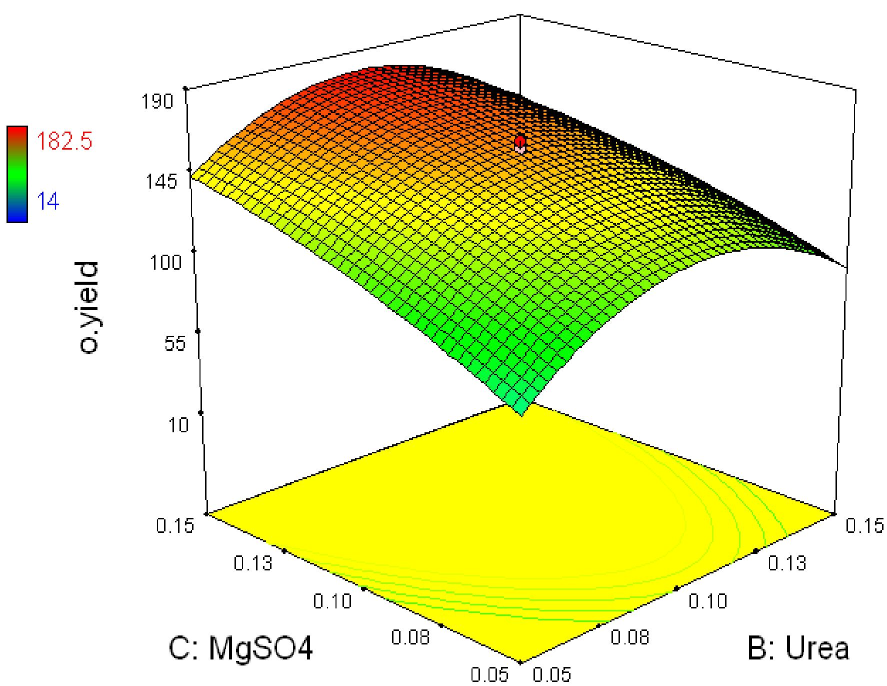

Figure-3
Response surface plot showing the effect on urea concentration, $MgSO_4$ concentration and their interaction effect on the production of lactic acid. Other variables are held at zero level