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Utilization of Agro Residue Corncob for Production of Acetone-Butanol Using *Clostridium acetobutylicum* and Process Optimization through RSM

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Abstract

Acetone-Butanol-Ethanol (ABE) fermentation is an important industrial process for acetone and butanol production. In early 1950 due to the rise of cheaper petrochemical synthesis and increased cost of fermentation raw materials, these are predominately produced through chemical synthesis which relies on crude oil supply. With the growing concerns of environmental issues, depleting fossil resources and increasing crude oil price, interest has returned to fermentative production, not only as a chemical but also as an alternative biofuel. To overcome the limitations of conventional ABE fermentation such as low titer and high substrate cost emphases areas under research are utilization of renewable and low-cost feedstocks, development of novel fermentation processes, alternative product recovery technologies and metabolic engineering of solvent-producing microorganisms. In the present study the rotatable central composite design and response surface technique that was employed for optimization work in many studies is successfully used. The optimum conditions for acetone production of corncob were found as temperature 36.71°C, pH 4.16 and sugar (g/l) 99.94, butanol production of corncob were found as temperature 35.44°C, pH 4.79 and sugar (g/l) 91.96.

Keywords: *Clostridium acetobutylicum*; CCD; RSM

Introduction

ABE stands for Acetone-Butanol-Ethanol Fermentation generally used in industry to produce solvents using microorganisms [1]. Mostly the species of clostridium are used in ABE fermentation *viz*: *C. acetobutylicum* and *C. aurantibutyricum*. The typical acetone/butanol/ethanol ratio is 3:6:1. In 15-18 g/L ABE production in a conventional ABE fermentation the amount of butanol is 10-13 g/L [2]. Cellulosic biomass is heterogeneous and consists of complex network of different components like cellulose, hemicellulose and lignin [3]. Agricultural crop residues are high in cellulose and hemicellulose content and low lignin content than wood that makes it very much suitable substrate for ABE fermentation [4]. Lignocellulosic wastes like corn cob can be exploited for the production of ABE for blend fuels and solvents as well [5]. The biological conversion of lignocellulosic biomass to fuel, offers potential economic and environmental advantages over traditional fossil based fuels. Conversion of lignocellulosic materials into ABE involves hydrolysis of cellulose into fermentable sugars and then, subsequent fermentation of sugars into ABE. Corn cob is a potential feedstock for ABE production, as its cheap, wide and large availability throughout the year in India [6].

To produce ABE from biomass feedstocks, pretreatment is required to fractionate different carbohydrate polymers [7]. During pretreatment, hemicelluloses may be hydrolyzed to their monomeric constituents and lignin-hemicellulose-cellulose interactions partially disrupted. Therefore, the purpose of pretreatment is to remove and separate hemicellulose from cellulose, to disrupt and remove the lignin component, to decrease the crystallinity of cellulose, to increase the accessible surface area of cellulose and to increase the pore size of cellulose to facilitate the penetration of hydrolysis agents [8]. A method use in the pretreatment of biomass basically affects the rate of hydrolysis and the level of enzymatic action for maximum theoretical yield of acetone-butanol [9]. Enzymatic hydrolysis of cellulose is usually carried out by cellulase enzymes [10]. During hydrolysis, cellulose is degraded into the reducing sugars that can be fermented by yeasts or bacteria to ethanol [11].

The Response Surface Methodology (RSM) is a statistical tool used for modeling and optimization of multiple variables and determines optimum process conditions by combining experimental results [12]. To start with, statistically designed experiments were performed and regression coefficient was estimated to check the efficacy of the model [13]. In the present study the main objective is to optimize the response surface that is influenced by various process parameters for production of acetone and butanol. RSM also quantifies the relationship between the controllable input parameters and the obtained response surfaces. The application of experimental design and response surface methodology in bioprocesses can result in improved product yields, reduced process variability and development time and overall costs. The Central Composite Design (CCD) is the most popular of the many classes of RSM designs and chosen for the present work due to some of its properties like; a CCD can be run sequentially, it can be naturally partitioned into two subsets of points; the first subset estimates linear and two-factor interaction effects while the second subset estimates curvature effects, it is very efficient, providing much information on experiment variable effects and overall experimental error in a minimum number of required runs and it is very flexible.

Material and Method

Microorganism and maintenance

The strain *Clostridium acetobutylicum* (NCIM No. 2877) used

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in this study were obtained from National Collection of Industrial Microorganism (NCIM) National Chemical Laboratory, Pune, Maharastra, India. Growth medium (Cooked meat) was prepared in 100 ml Erlenmeyer flask. The medium composition was (g/100 ml): Beef extract 4.5 g, dextrose 0.2 g, proteose peptone 2 g, NaCl 0.5 g. The pH of the medium was adjusted to 7.2 with NaOH and HCl before autoclaving. The medium was incubated at 37°C for two weeks under anaerobic condition. The agar slant was also prepared and maintained at 4°C. The microorganism was sub cultured at regular intervals of 30 days.

Hydrolysis of corncob

Corncob was collected from local market (Kanpur) and dried in the sunlight. After grinding it was given a particle size approximately 1 mm thickness and is used as a raw material in this study. The average composition was determined according to standard methodology [14] and found as 32% cellulose, 35% hemicelluloses, 20% lignin, 4% ash. 50 g corncob was suspended in 1 L dilute sulfuric acid (20 ml sulfuric acid in 980 ml distilled water and final volume adjusted to 1 L) in a Erlenmeyer flask followed by autoclaving at 121°C for 1 h . The lost water was replaced by adding distilled water to the mixture. After autoclaving the mixture was cooled to room temperature followed by adjusting pH to 5 with 1 N NaOH. Finally the mixture was incubated at 45°C for 72 h with agitation at 80 rpm. Tubes are centrifuged to separate residual biomass and sugar content

After incubation the hydrolyzed mixture was filtered twice (Whatman no.41) to remove sediments. The filtered solution was stored in a pre-sterilized screw capped bottle at 4°C and used for fermentation studies to be conducted later. The hydrolyzate was used for reducing sugar analysis by 2,5-dinitrosalicylic acid method [15]. The hydrolysate contained approximately 30-32 g/L total reducing sugar.

Experimental design and RSM

In the experimental plan, Response Surface Methodology (RSM) was utilized to optimize the hydrolysis process and a 23 rotatable Central Composite Design (CCD) was adopted in order to fit a second order model and the design consisted of 20 set of experiments. It included eight experiments for factorial portion (2k=8, where k is the number of independent variables, 3 in this case), six experiments for axial points (2k=6) and six replications of the center point used to check the reliability of the data for lack of fit test. The second order model was selected for predicting the optima point and expressed as

$$Y=\beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}A\,B + \beta_{13}A\,C + \beta_{23}BC$$

where, Y represents response variable i.e. Acetone or Butanol (gL). β_0 is offset value, β_1, β_2 and β_3 are coefficients of linear terms, β_{11}, β_{22} and β_{33} are coefficients of quadratic terms and β_{12}, β_{13} and β_{23} are coefficients of interactive terms. The effect of variables, Temperature (A), pH (B) and sugar concentration (C) were studied on acetone-butanol generation. Regression analysis and graphical analysis were performed using Design Expert v.8.0.7.1 (Stat-Ease Inc. Minneapolis) software.

Fermentation

Fermentation studies were conducted in 250 ml Erlenmeyer flask. Studies with sugar substrate at various levels 40-100 g/l, adjusted pH and temperature were conducted in 250 ml Erlenmeyer flask. 50 ml filtered sterilized fermentation media was transfer to 250 ml pre-sterilized Erlenmeyer flask. After 48 h the Erlenmeyer flask was inoculated with 5 ml actively growing 15 days old culture. During fermentation sample was taken and prepared for Acetone Butanol analysis [16].

Analytical procedure

During the fermentation samples were withdrawn after 48 h for analysis. The concentrations of solvent were determined by using gas chromatography (Netel, MICRO 9100) equipped with the Flame Ionization Detector (FID). Separation was achieved by using a 2 m capillary column and nitrogen as the carrier gas. The column temperature was held at 65°C to 140°C with 10 min final hold. The temperature of the injector and detector was set at 220°C and 270°C respectively. Samples (1 ml) were acidified with a drop of HCl (5% v/v). The acidification was necessary to ensure that Butyrate and acetate were in the acid forms, the injection volume was 1 µl.

Statistical modeling

According to the experimental plan, range and levels of independent variables, temperature (A), pH (B) and sugar concentration (C) studied for the acetone butanol production of corncob are shown in Table 1. The coded values of all independent variables and the experimental value of the two response variables Ya, for acetone and Yb, for butanol (g/L) are presented in Table 2. The coefficients were calculated by using Design Expert v.8.07.1.

The quadratic model in terms of coded variables was found as

$$Ya=+7.40+0.25*A-0.61*B+1.26*C+0.28*A*B+0.075*A*C-0.100*B*C-1.58*A^2-0.58*B^2-0.75*C^2$$

$$Yb=+17.48+0.60*A-2.10*B+2.27*C-0.12*A*B-0.050*A*C-2.47*B*C-3.47*A^2-2.52*B^2-2.83*C^2$$

S. No.	Factor	Coded values	Actual values	
			Min	Max.
1	Temprature (°C), (A)	-α,-1,0,+1,+α	26.59	43.41
2	pH (B)	-α,-1,0,+1,+α	3.32	6.68
3	Sugar (g/l) (°C)	-α,-1,0,+1,+α	19.55	120.45

Table 1: Independent variables.

Run no.	Coded values of the variables			Experimental value acetone	Experimental value butanol
	A	B	C		
1	-1	-1	-1	4.1	6.3
2	1	-1	-1	3.7	6.6
3	-1	1	-1	2.5	6.1
4	1	1	-1	3.5	8.3
5	-1	-1	1	6.4	14.4
6	1	-1	1	6.6	16.9
7	-1	1	1	7.5	6.7
8	1	1	1	5.7	6.3
9	-1.682	0	0	3.2	6.2
10	1.682	0	0	2.2	8.3
11	0	-1.682	0	6.7	13.5
12	0	1.682	0	4.4	6.4
13	0	0	-1.682	2.8	4.9
14	0	0	1.682	7.3	13.2
15	0	0	0	7.2	17.7
16	0	0	0	7.7	17.2
17	0	0	0	7.2	17.7
18	0	0	0	7.4	16.7
19	0	0	0	3.2	17.8
20	0	0	0	7.5	17.9

Table 2: Central composite design consisting of 20 experiments with the experimental and predicted response.

To fit the response function and experimental data, regression analysis was performed and second order model for the response (Ya) was evaluated by ANOVA which is presented in Tables 3 and 4. The regression for the response was statistically significant at 90.45 of confidence level. The "Pred R-Squared" of 0.9232 is in reasonable agreement with the "Adj R-Squared" of 0.9769. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The very high signal to noise ratio of 24.485 indicates that the chance of the values could be due to noise is very less. The Model F-value of 90.45 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob >F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 3.66 implies there is a 9.03% chance that a "Lack of Fit F-value" this large could occur due to noise. Lack of fit is bad -- we want the model to fit. This relatively low probability (<10%) is troubling.

Std. Dev.	0.3	R-Squared	0.9879
Mean	5.42	Adj R-Squared	0.9769
C.V. %	5.47	Pred R-Squared	0.9232
PRESS	5.56	Adeq Precision	24.485

Source	Sum of square	df	Mean square	F value	P value Prov>F	
Model	71.47	9	7.94	90.45	<0.0001	Significant
A-Temperature	0.89	1	0.89	10.11	0.0098	
B-pH	5.01	1	5.01	57.02	<0.0001	
C-Sugar	21.58	1	21.58	245.83	<0.0001	
AB	0.61	1	0.61	6.89	0.054	
AC	0.045	1	0.045	0.51	0.4904	
BC	0.08	1	0.08	0.91	0.3623	
A^2	36.14	1	36.14	411.7	<0.0001	
B^2	4.78	1	4.78	54.47	<0.0001	
C^2	8.17	1	8.17	93.03	<0.0001	
Residual	0.88	10	0.088			
Lack of Fit	0.69	5	0.14	3.66	0.0903	Not significant
Pure Error	0.19	5	0.038			
Cor Total	72.35	19				

Table 3: Analysis of variance (ANOVA) for Acetone.

Source	Sum of square	df	Mean Square	F value	P value Prov>F	
Model	503.8	9	55.99	92.65	<0.0001	Significant
A-Temperature	4.84	1	4.84	8.01	0.0178	
B-pH	60.48	1	60.48	100.09	<0.0001	
C-Sugar	70.18	1	70.18	116.14	<0.0001	
AB	0.13	1	0.13	0.21	0.659	
AC	0.02	1	0.02	0.033	0.8593	
BC	49	1	49	81.09	<0.0001	
A^2	173.5	1	173.5	287.1	<0.0001	
B^2	91.16	1	91.16	150.86	<0.0001	
C^2	115.69	1	115.69	191.44	<0.0001	
Residual	6.04	10	0.6			
Lack of Fit	4.98	5	1	4.7	0.0573	Not significant
Pure Error	1.06	5	0.21			
Cor Total	509.91	19				

Table 4: Analysis of variance for response surface quadratic model of Butanol.

The "Pred R-Squared" of 0.9232 is in reasonable agreement with the "Adj R-Squared" of 0.9769. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 24.485 indicates an adequate signal. This model can be used to navigate the design space.

The Model F-value of 92.65 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob >F" less than 0.0500 indicate model terms are significant.

In this case A, B, C, BC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 4.70 implies there is a 5.73% chance that a "Lack of Fit F-value" this large could occur due to noise. Lack of fit is bad -- we want the model to fit. This relatively low probability (<10%) is troubling.

Std. Dev.	0.78	R-Squared	0.9881
Mean	11.46	Adj R-Squared	0.9775
C.V. %	6.79	Pred R-Squared	0.9141
PRESS	43.82	Adeq Precision	22.242

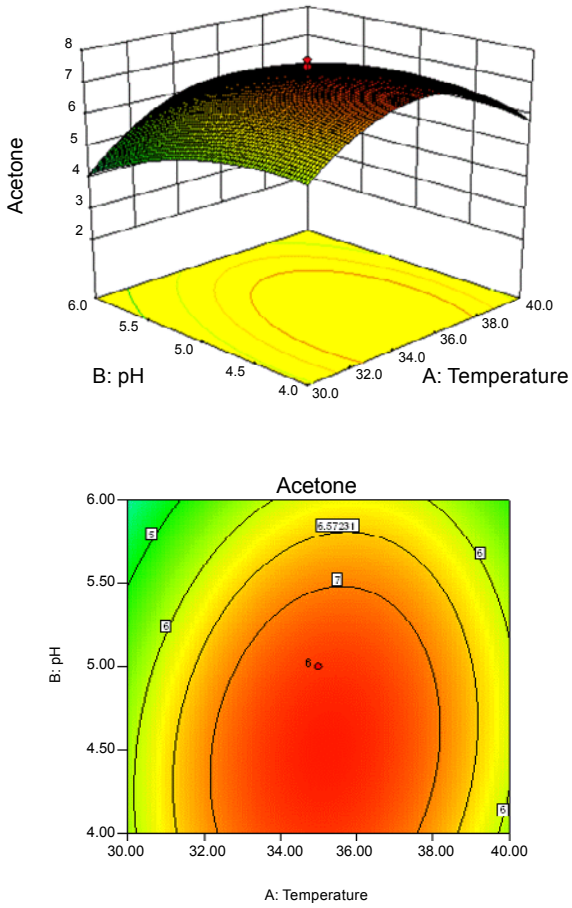

Figure 1: Effect of Temperature and pH on acetone production (A) Surface graph (B) Contour graph.

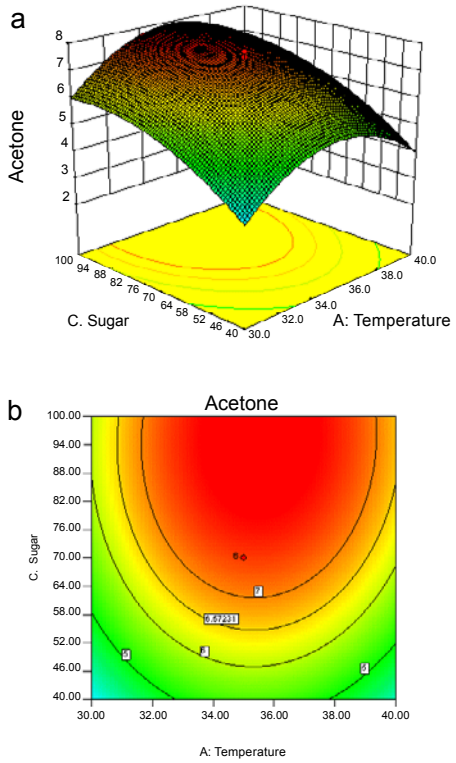

Figure 2: Effect of Temperature and Sugar on acetone production (A) Surface graph (B) Contour graph.

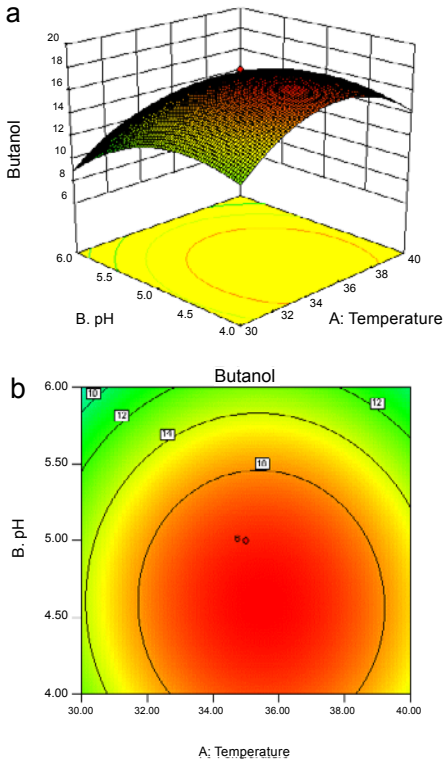

Figure 4: Effect of Temperature and pH on butanol production (A) Surface graph (B) Contour graph.

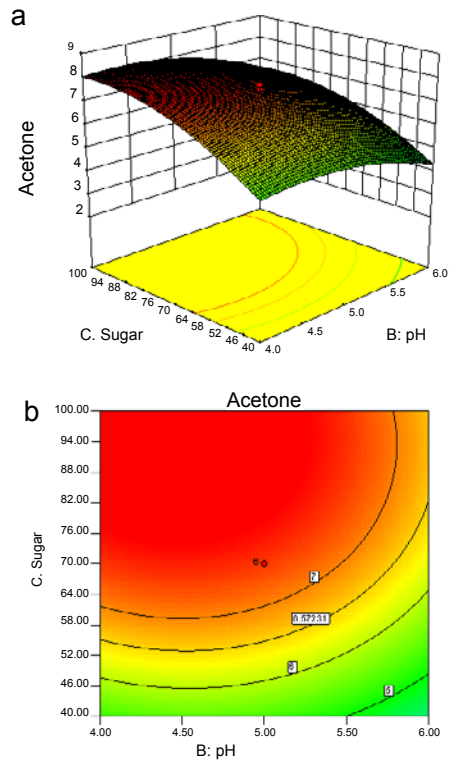

Figure 3: Effect of pH and Sugar on acetone production (A) Surface graph (B) Contour graph.

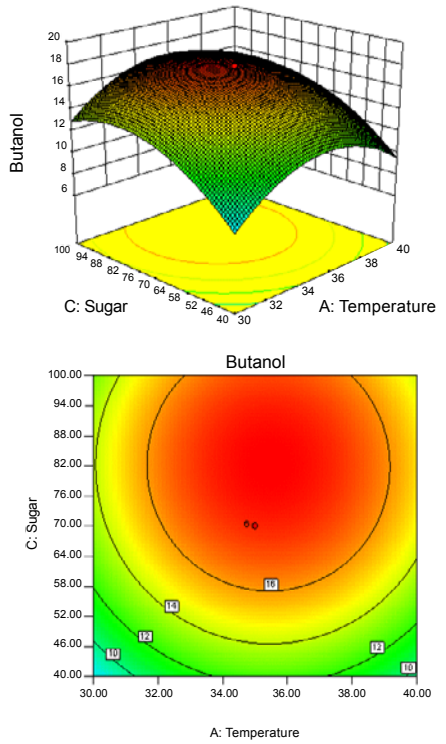

Figure 5: Effect of Temperature and Sugar on butanol production (A) Surface graph (B) Contour graph.

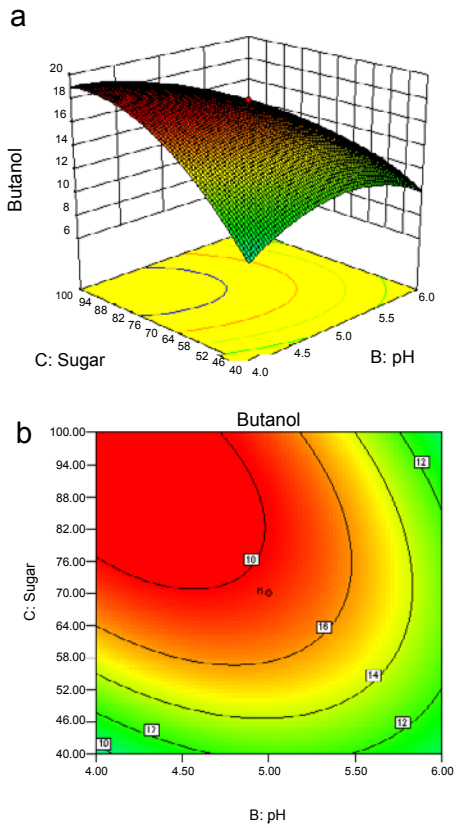

Figure 6: Effect of pH and Sugar on butanol production (A) Surface graph (B) Contour graph.

The "Pred R-Squared" of 0.9141 is in reasonable agreement with the "Adj R-Squared" of 0.9775. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 22.242 indicates an adequate signal. This model can be used to navigate the design space (Figures 1-6).

Conclusion

The rotatable central composite design and response surface technique that was employed for optimization work in many studies was successfully used in the present investigation. The optimum conditions for acetone production of corncob were found as temperature 36.71°C, pH 4.16 and sugar (g/l) 99.94, butanol production of corncob were found as temperature 35.44°C, pH 4.79 and sugar (g/l) 91.96.

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