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Plackett-Burman Design: A Statistical Method for the Optimization of Fermentation Process for the Yeast *Saccharomyces cerevisiae* Isolated from the Flowers of *Woodfordia fruticosa*

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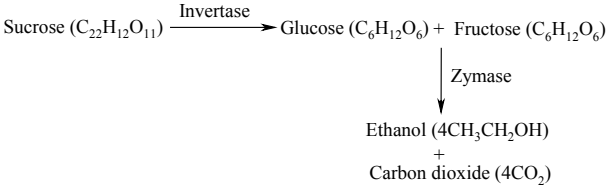
Abstract

Plackett-Burman design, a statistical method was successfully employed for the optimization of the fermentation process for the yeast *Saccharomyces cerevisiae*. The yeast used in the study was isolated from the flowers of *Woodfordia fruticosa* in our previous work. The effect of simultaneously varying the jaggery concentration, inoculums volume and incubation temperature on alcohol yield was studied with the help of the response surface methodology. The optimum conditions found were jaggery (40% w/v), inoculums volume (8% v/v) and temperature (30°C). After process optimization, alcohol yield was increased from 69.57 g/l to 95.84 g/l. The results demonstrated that the strain *S. cerevisiae* could be used efficiently as inoculums for the fermentation process at optimal conditions.

Keywords: Plackett-Burman design; *Saccharomyces cerevisiae*; Fermentation; Alcohol

Introduction

Alcohol is the important component produced during the development of fermentation based herbal formulations. These formulations are required to contain 40-80 g/l of alcohol. This alcohol causes the extraction of water insoluble active ingredients from the herbs [1,2]. The flowers of the plant *Woodfordia fruticosa* are used as inoculums during making of these formulations [3]. This process uses the natural yeasts *Saccharomyces cerevisiae* present on the flowers [4-5]. Yeast *S. cerevisiae* contains the enzyme invertase which causes the hydrolysis of sucrose to glucose and fructose. Enzyme *zymase* further converts glucose to alcohol and carbon dioxide [6]. 1 mole of sucrose converted to 4 mole of ethanol and 4 mole of carbon dioxide. The average conversion rate of total fermentable sugar to alcohol can be used to predict the level of alcohol in final product. The conversion rate used by European Union is alcohol produced (% v/v) is the sum of total reducing sugar (glucose and fructose) in g/l divided by 16.83 [7].



Yeast *S. cerevisiae* is capable of rapid rate ethanol production and glycolysis under optimum conditions [8]. The yield of alcohol in fermentation process can be increased by optimizing the experimental conditions. The use of factorial design for optimizing the fermentation process is well documented [9-11]. Batch type of fermentation process was employed because in continuous type, there is higher possibility of mutation and contamination. In this work, anaerobic fermentation was employed because during aerobic growth of *S. cerevisiae*, sugar and ethanol can be used as carbon and energy source and growth predominates over ethanol production. Under oxygen starvation conditions, the fermentative metabolic pathway always predominates [12]. Various factors affecting the fermentation process includes carbon source, sugar concentration, dissolved oxygen level, agitation rate, temperature, nitrogen source, pH, volume of inoculums, etc [13].

In this work, Plackett-Burman design 2^3 trial plus 3 central points was used for optimizing the fermentation process. We have selected three variables (i.e., jaggery concentration, incubation temperature and inoculums volumes added) as a possible causes for change in alcohol production. The range of variables was chosen from the data available in literature [14]. The correlation of these variables with response (alcohol generated) was statistically studied. These variables are expected to show the interactive results. The multivariate experiments using appropriate statistical designs will explain the effect of variables on fermentation process.

Materials and Methods

Sugar and chemicals

Commercial grade of jaggery was purchased from local market and was used as sugar for fermentation. Other chemicals and solvents used were of highest quality available.

Calibration graph of alcohol

To obtain calibration curve, a series of dilutions i.e. 0, 20, 40, 60, 80, 100 and 120 g/l was made using standard ethanol solution (99.99% v/v). 1 ml of each of the standard solution was added to different 50 ml volumetric flasks each containing 25 ml of potassium dichromate reagent. All the flasks were incubated at 60°C for 20 min and final volume was made up to the mark with distilled water. The absorbance of

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each solution was measured at 620 nm against distilled water as a blank. Calibration curve was plotted as concentration versus absorbance, and straight line equation was set up (Figure 1).

Yeast strain and maintenance

Yeast *S. cerevisiae* used in the study was the same which was isolated in our previous work. The strain was isolated according to the method suggested by Kurtzman and Fell [15] and Barnett [16]. The yeast culture was named as *S. cerevisiae* Jm.20 and maintained onto the MGYP agar media (3 g malt extract, 10 g glucose, 3 g yeast extract, 5 g peptone and 20 g agar per liter of water, pH 4.5), and preserved at 4°C for routine use.

Preparation of 12° brix jaggery media

About 6-7 g of jaggery was dissolved in 300 ml of distilled water. This media was then transfered to 250 ml measuring cylinder. Brix hydrometer (range: 10-20) was dipped into the media and brix value was observed. The brix value was adjusted to 12 by adding water or jaggery. pH of media was adjusted to 4.5 using 1 N HCl or 1 N NaOH solution. To this, 0.01% w/v of diaminophosphate (DAP) and 0.01% w/v of urea were added as a source of nitrogen, and media was autoclaved at a pressure 15 lbs and temperature 121°C for 15 min.

Evaluation of alcohol production capacity

Evaluation of alcohol production capacity of yeast was carried out using 50% w/v jaggery media. The inoculums for yeast were prepared by transferring one loopful of culture into 100 ml of sterile 12°brix jaggery media. The inoculums were incubated at 32.5°C in shaker incubator at 180 rpm. Cell growth (cells/ml) of yeasts in inoculums was measured.

After 48 h, 50 ml of inoculums (cell growth- 7.4×10^8 cells/ml) was added to 1 L conical flasks containing 450 ml of the sterile 50% w/v jaggery media. The media was incubated at 32.5°C till the alcohol production is stopped. The stopping of fermentation was indicated by no increase in alcohol production and no further consumption of substrate.

At the regular intervals of 24 h incubation, 15 ml fermentation broth was withdrawn and cell growth was measured using 1 ml of broth and remaining broth was centrifuged at 6000 rpm for 20 min. The supernatant liquid was used for analysis of alcohol and reducing sugar.

Kinetic of fermentation

Kinetic of fermentation was studied by investigating the relationship between alcohol production, substrate (reducing sugar) consumption and cell growth. Amount of alcohol produced was determined by dichromate oxidation method [17]. Amount of substrate consumed was determined by subtracting the amount of reducing sugar left from initial amount of reducing sugar. Amount of reducing sugar was determined by titration method [18]. Surface plot for alcohol produced was plotted against percent substrate consumed and cell growth. Regression equation for response was established using software STATISTICA (Version 8.0.360.0 English, StatSoft Inc., Tulsa, USA).

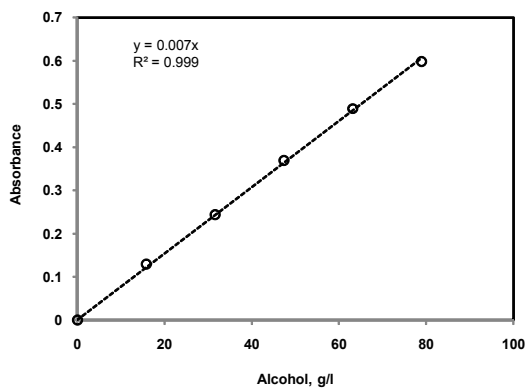

Figure 1: Standard calibration graph of alcohol by dichromate oxidation method.

Optimization of fermentation process

(i) Preparation of inoculums

The inoculum was prepared in the similar manner like that of method employed in evaluation of alcohol production capacity.

(ii) Experimental Design

Optimization of process for improving alcohol production was carried out using Plackett-Burman design 2^3 trial plus 3 central points. According to this design, total 11 trial batches were formed (8 main batches plus 3 central point's batches). All the batches were named as B-1 to B-11. For investigating the effect, each independent variable was studied at two levels, namely, "high" and "low". These levels define the upper limit and lower limits of the range covered by each variable. Three central points of all independent variable were also used in three separate batches. The values of coded levels of independent variables used in the experiment are listed in table 1.

(iii) Methodology

For each batch, in different 500 ml conical flasks, jaggery media were prepared by dissolving required amount of solid jaggery in distilled water. Each flask was autoclaved, cooled and inoculated with required volume of inoculums (cells content-7.1×10^8 cells/ml). These flasks were incubated at different testing temperatures for a period of 168 h. At a regular interval of 24 h, 15 ml of broth was withdrawn; centrifuged and supernatant liquid was used for the determination of amount of alcohol produced. Total volume of fermentation broth (i.e., jaggery media plus inoculums) used was 200 ml. The compositions of batches are given in table 2.

(iv) Study of response surface

The best method for the optimization of fermentation process is response surface methodology (RSM). This process will not only determine the optimum conditions, but also give the information necessary to design a process. It is a scientific approach for determining optimum conditions which combines special experimental designs with Taylor polynomial equation. The correlation of three independent variables (i.e., jaggery concentration $\{X_1\}$, inoculums size $\{X_2\}$ and temperature $\{X_3\}$) and dependent variable (alcohol $\{Y\}$) was studied. Response surface for each considered response was approximated by following polynomial equation using software STATISTICA (Version 8.0.360.0 English, StatSoft Inc., Tulsa, USA).

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3$$

Where Y is the response, β_0 is the constant (intercept) and β_1 to β_3

Variables	Code	Coded level		
		-1	0	+1
Sugar (%w/v)	X_1	40	50	60
Inoculums (%v/v)	X_2	8	10	12
Temperature (°C)	X_3	30	32.5	35

Table 1: Values of coded levels used in factorial design.

Batch	Coded variables			Natural variables			Alcohol produced (g/l)	
	X_1	X_2	X_3	X_1	X_2	X_3	Experimental	Predicted
B-1	+1	+1	+1	60	12	35	64.36	61.50
B-2	+1	-1	+1	60	8	35	61.34	57.06
B-3	-1	,-1	+1	40	8	35	78.28	79.54
B-4	-1	+1	+1	40	12	35	87.48	83.98
B-5	+1	-1	-1	60	8	30	66.86	66.93
B-6	+1	+1	-1	60	12	30	73.69	71.37
B-7	-1	+1	-1	40	12	30	94.56	93.85
B-8	-1	-1	-1	40	8	30	95.84	89.41
B-9	0	0	0	50	10	32.5	69.17	75.45
B-10	0	0	0	50	10	32.5	68.93	75.45
B-11	0	0	0	50	10	32.5	69.49	75.45
						Minimum	61.34	57.06
						Maximum	95.84	93.85
						Mean	75.45	75.45

Table 2: Study of variables on alcohol generation by first factorial design.

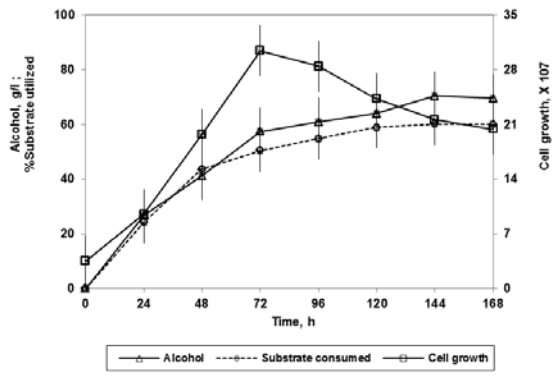

Figure 2: Calculated variation with time of alcohol produced, % substrate consumed and cell growth using 50%w/v jaggery and 10%inoculums added at 32.5°C

are the coefficient of the variables (slope times the X variables).

Ternary plot was prepared to examine the relations between four dimensions where three dimensions were of independent variables. In ternary plots, the triangular coordinate system in two dimensions was used to plot four variables (the components X, Y, and Z, and the response V).

Results and Discussion

Yeast *S. cerevisiae* used in the study was isolated in our previous work. The strain was evaluated for alcohol production capacity using jaggery media as a sugar. Alcohol production and substrate utilization was stopped after 168 h. This period was taken as a fermentation period in the process optimization. The alcohol (ethanol) production is always considered proportional to the consumption of reducing sugar. The rate of variation of alcohol production is given by equation:

$$\frac{dE}{dt}=R_{E/RS}\frac{dRS}{dt}$$

Where, E is ethanol concentration (g/l), $R_{E//RS}$ is rate constant of ethanol production and RS is the consumption of reducing sugar (% w/v).

The alcohol production curve for kinetic study was established by assuming a constant ethanol yield (Figure 2). The yeast produced 69.57 g/l of ethanol by free cells using 60% of initial substrate (reducing sugar) at 168 h under non shaking conditions (Table 3). Surface plot was plotted for alcohol against % substrate consumed and cell growth (Figure 3). The regression equation obtained for alcohol against percent substrate consumed{X_1} and cell growth {X_2} is given bellow.

$$\text{Alcohol}=-1.0685+4.1711X_1-0.1155X_2$$

Where, X_1 is the percent substrate consumed and X_2 is cell growth (cells/ml×10^7).

For optimizing the fermentation process, three independent variables (i.e., jaggery concentration, size of inoculums and temperature) were chosen as a possible cause for change in product yield. For optimization, total 11 trial batches were formed. Each batch consisted of different composition and was incubated at different temperature. After optimization of conditions, it could produce alcohol upto 95.84 g/l at the least set of conditions.

Experimental data showed that jaggery concentration (40% w/v), inoculums size added (8% v/v) and temperature (30°C) were optimum for maximum yield of alcohol. According to conditions, the amplitude of response value (alcohol) varied from minimum 61.34 g/l to maximum 95.44 g/l with mean value 75.45 g/l. Experimental amount and predicted amount of alcohol in various batches are shown in table 2. It is also observed that least and highest amount of alcohol was produced in batch B-2 (61.34 g/l) and B-8 (95.84 g/l), respectively. Comparative alcohol contents in various batches are shown in figure 4.

Alcohol production kinetic study of various trial batches indicated that there is slightly reduction in alcohol concentration after 148 h. this may be because of diauxic behavior of S. cerevisiae. This strain consumes ethanol as a source of carbon during fermentative metabolic pathway when glucose is no longer available in the media after lag-phase [12] (Figure 5). 3D surface plots and ternary graph of alcohol against different variables are shown in figure 6 and figure 7, respectively.

From the figures 8-10, it is clear that the effect of all variables on

Parameter/Batch		Before optimization	After optimization
Initial reducing sugar (g/l)		28.37	24.69
Rate of alcohol production (g/l/h)		0.414	0.570
Amount of reducing sugar after 168 h (%w/v)	Amount utilized	17.04	20.87
	Amount left	11.33	3.82
Amount of alcohol (g/l) after 168 h	Amount predicted*	80.59	98.70
	Actual amount produced	69.57	95.84
	Percent amount increased (% w/v)	-	2.62

*predicted by formula- Alcohol (%v/v)=Utilized total reducing sugar (g/l) ÷ 16.83

Table 3: Kinetic study of fermentation before and after optimization of fermentation process.

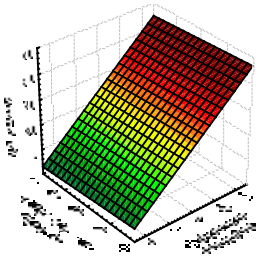

Figure 3: 3D Surface plots alcohol against %substrate consumed and cell growth.

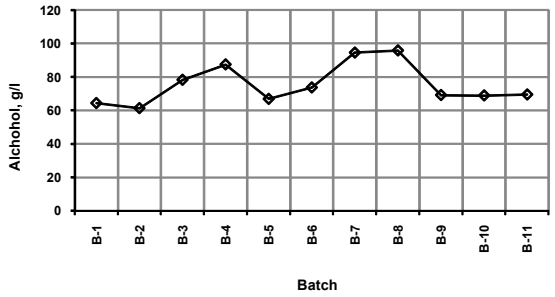

Figure 4: Final content of alcohol obtained in various batches after 168 h.

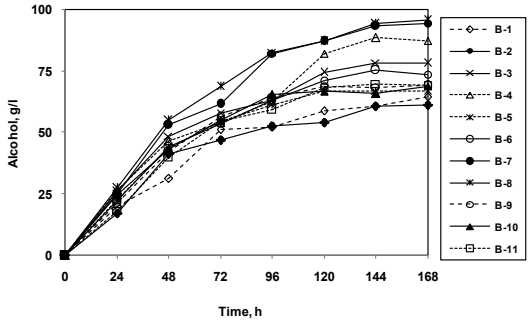

Figure 5: Alcohol generation kinetic in various batches during optimization process.

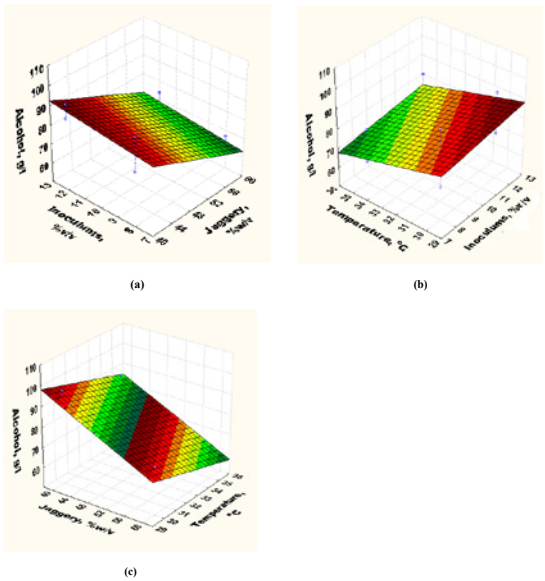

Figure 6: 3D Surface plots (a,b,c) of alcohol (g/l) against different variables.

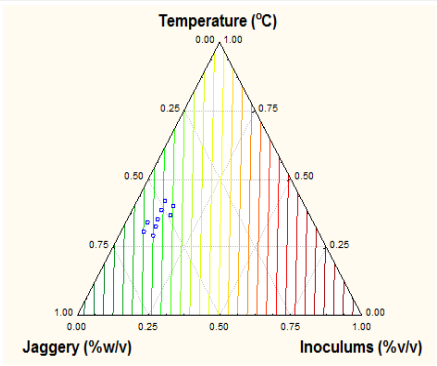

Figure 7: Ternary graph of alcohol (g/l) against different variables.

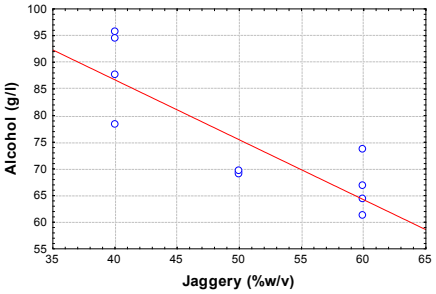

Figure 8: Relationship between alcohol generation and jaggery.

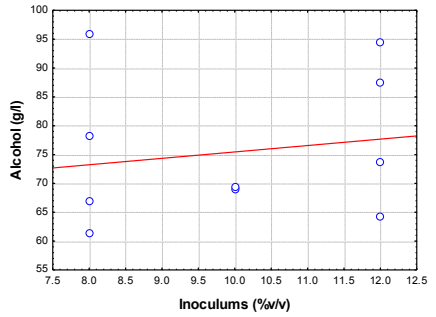

Figure 9: Relationship between alcohol generation and inoculums.

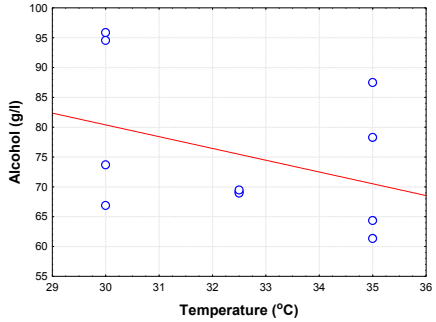

Figure 10: Relationship between alcohol generation and temperature.

alcohol production is significant. From the figure 11, it is observed that experimental values were closer to the predicted values. All the variables except interaction between jaggery and inoculums volume were statistically significant at 95% of confidence. While comparing all the variants, it was observed that all the variables cause the reduction of alcohols generation. However, variation in inoculums volume had very little effect on alcohol production. This is due to at higher sugar concentration, oxidation is suppressed and fermentation only takes place (Crabtree effect) [19]. Another reason may be, under anerobic conditions, high sucrose concentration in jaggery has detrimental effect on alcohol production. This may be due to decreased activity of enzyme *invertase* [20]. During fermentation, glucose is consumed first, followed by fructose and sucrose [21]. Catabolic repression of glucose

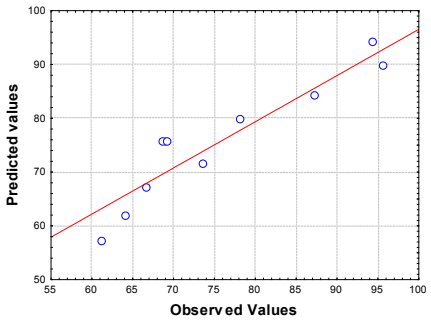

Figure 11: Relationship between predicted and observed values of alcohol generation.

Factor	Effect	Std.err.pure	t –value (2)	p-value
Mean	75.45	0.08	890.74	0.000001
Jaggery (1)*	-22.48	0.20	-113.14	0.000078
Inoculums (2)*	4.44	0.20	22.36	0.001994
Temperature (3)*	-9.87	0.20	-49.70	0.000405
1×2**	0.48	0.20	2.43	0.135826
1×3*	2.45	0.20	12.32	0.006524
2×3*	1.67	0.20	8.39	0.013899

Table 4: Effect estimates from the first factorial design.

N = 11	B Coefficient	Std.err.	t –value (7)	p-value
Intercept (B constant)	200.0650	18.6408	10.7326	0.000013
Sugar (%w/v)	-1.1239	0.1332	-8.4347	0.000063
Inoculums (% v/v)	-0.2031	0.0635	-3.1982	0.015103
Temperature (°C)	-1.9745	0.5329	-3.7046	0.007608

*P<0.05 (significant for a 95% confidence level)
*P<0.05 (significant for a 95% confidence level)
**P<0.15 (significant for a 85% confidence level)

Table 5: Model coefficient of alcohol generation determined by linear regression.

and fructose does not allow the formation of extracellular *invertases,* which converts sucrose to glucose and fructose which are subsequently consumed by yeast [22].

At increased temperature, alcohol production was greatly reduced. Fermentation efficiency of *S. cerevisiae* at high temperature (above 35°C) is low because of increased fluidity in membranes that changes the composition of fatty acid [23]. Our data indicated that 30°C incubation temperatures is the best temperature to achieve maximum yield. This data further confirm the temperature range cited in literature for yeasts growth [24].

The regression line expresses the best prediction of the dependent variable (Y), given the independent variables (X). However, nature was perfectly predictable, and was substantial variation of the observed points around the fitted regression line (Figure 11). From the results following regression equation was obtained to predict the amount of alcohol produced.

$$\text{Alcohol (Y)} = 200.0650 - 1.1239X_1 - 0.2031X_2 - 1.9745X_3$$

Where, X_1, X_2 and X_3 are the independent variables i.e. jaggery concentration (% w/v), inoculums volume (% v/v) and temperature (°C), respectively.

The p–level for all independent variables was in the range of 0.000013 to 0.015103. The value represents the probability of error that is involved in accepting our observed result. The lesser the p-level of results, the more we can believe that the observed relation between variables in the sample is a reliable indicator of the relation between the respective variables in the population. The statistical significance of results is an estimated measure of the degree to which it is "true" (in the sense of representative of the population). The p-value It indicate the probability of error involved in accepting our observed results as valid (i.e., as "representative of the population. The p-level of 0.05 (i.e., 1/20) indicates that there is a 5% probability that the relation between the variables found in our sample is a fluke (significant for a 95% confidence level). The t-test is the most commonly used method to evaluate the differences in means between two groups. It tells us the confidence interval of the true value. Statistical data and results of work are given in table 4 and table 5.

Conclusion

The optimization study to increase the product by controlling the variables was successfully performed using response surface methodology from the data obtained from Plackett-Burman design 2^3 trial plus 3 central points. From the response surface graphs the optimum conditions were selected to be jaggery (40% w/v), inoculums volume (8% v/v) and incubation temperature (30°C). The results states that the alcohol production is inhibited at higher jaggery concentration (60% w/v) and at higher incubation temperature (35°C). Varying the volume of inoculums between the range 8 to 12% v/v did not show any significant effect and hence inoculums volume added to media can be fixed at any value within this range. The highest amount of alcohol (i.e., 95.84 g/l) was obtained after process optimization. This value was close to the value obtained from model equation 98.70 g/l. Thus, finally we conclude that the strain *S. cerevisiae Jm.20* could be efficiently used as an inoculums for fermentation process at optimal conditions.

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