OPTIMIZATION OF FERMENTATION MEDIUM FOR THE PRODUCTION OF ETHANOL FROM JAGGERY USING BOX-BEHNKEN DESIGN

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ABSTRACT: The production of ethyl alcohol using *Saccharomyces cerevisiae* NCIM 3288 was found significant when jaggery is used as the carbon source. From the various physical parameters studied, the effect substrate concentration was found to play a key role, and the other two important chemical supplements tested, i.e., Nitrogen and phosphorus sources were also found to be significantly contributing to product concentration. A response surface analysis predicted a substrate concentration of 23.56 % (w/v), $(NH_4)_2SO_4$- 2.612 g/l and KH_2PO_4- 3.407g/l as the optimal conditions keeping the rest of the rest of the physical parameters at their optimal conditions as obtained from the preliminary studies. A maximum ethanol concentration of 6.15%(w/v) was obtained at the end of third day of fermentation.

Key words: Jaggery, Ethanol, Box-Behnken design, optimization of medium.

INTRODUCTION

Ethyl alcohol production is known for over three centuries (Smith and Ferdinand, 1940) and it has gained its importance as an important chemical feed stock for various industrial solvents (Rose, 1967). In the recent scenario, with the emerging fuel crisis, it is used as a fuel option, as Gasohol (Sybik Parker ,1999, Wheals, et al., 1990). Alcohol is produced from various saccharine, starchy as well as lignocellulosic materials (Krishna and Chowdary, 2003). Some substrates used include glucose (Chen, 1981), lactose (Mawson and Karen Coster, 1993), sucrose (Caylak Belkis et al.,1998) cane molasses (Suresh Sharma et al., 1980), fruit pulps, starch from cereal grains (Thomas Mullins and Christopher, 1987), cellulose (Krishna and Chowdary, 2003), hemicellulose as well as agricultural wastes (Wheals, et al., 1990). *Saccharomyces cerevisiae* is the common distiller used (Chen,S.L, 1981), while the other microorganisms used include include *S.diastaticus*, *Zymomonas mobilis* (Mohagheghi et al., 2004; Kademi and Baratti, 1982) and the thermo tolerant *Kluveromyces sp* (Krishna and Chowdary, 2003).

Usage of multivariant techniques has increased since the past few years during the optimization of processes (Joa, et al., 2007; Ratnam,et al., 2003; Ratnam, et al., 2005; Akhnazarova and Kafarrov, 1982; Khuri and Cornell, 1987; Sarat Babu Imandi, et al., 2007; Cochran and Cox, et al., 1968). Using design of experiments based on response surface methodology, the input levels of each parameter, as well as the product yield may be estimated. Central composite, Box-Behnken and Doehlert designs are among the principal response surface methodologies used in experimental design. In the present study, the effect of substrate concentration, $(NH_4)_2SO_4$ and KH_2PO_4 levels were optimized. Using Box-Behnken design of experiments, a mathematical correlation between jaggery concentration, $(NH_4)_2SO_4$ and KH_2PO_4 were determined to get optimal ethanol yield.

MATERIALS AND METHODS

Substrate: Jaggery procured from the native makers of Anakapalii, A.P., India, was obtained and used as carbon source for the yeast.

Microorganism: *Saccharomyces cerevisiae* NCIM 3288 obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India was used through out the study.

Growth conditions: Yeast strains were maintained in MGYP slants having a composition (%): Malt extract – 0.3, glucose – 1.0, yeast extract – 0.3, peptone – 0.5 and agar-agar 2.0. pH is maintained at 7.0, and the slants were incubated at 30^{o}C for 24h. Subculturing was carried out once in a month and culture was stored at 4^{o}C.

Inoculum preparation: The organism is inoculated into medium containing same components as in the maintenance medium except that agar-agar is not added to it. The 25ml medium is inoculated with a loop full of culture and kept in an incubated orbital shaker at 30°C and 200rpm for 24hours. 5ml of the medium is then taken, centrifuged and inoculated into production medium.

Optimization of physical parameters: The physical parameters that were optimized and their range chosen during the preliminary study are: effect of substrate concentration (Jaggery)- 10 to 50 % (w/v); pH- 2 to 8; inoculum level- 4 to 14 x 10^6 cells/ml, agitation - 50 to 250 rpm, temperature- 20 to 40°C.

Effect of chemical parameters: The two important chemical parameters chosen during preliminary studies are Nitrogen source ($(NH_4)_2SO_4$) and Phosphorus source (KH_2PO_4).

Analytical techniques: Ethanol concentration is estimated by dichromate oxidation method (Neish, 1952).

Experimental design: Box-Behnken design is suitable for exploration of quadratic response surfaces and constructs a surface order polynomial model, thus helping in optimizing a process using a small number of experimental runs. This design requires an experimental number according to $N= k^2 + k + cp$, where (k) is the factor number and (cp) is the replicate number of the central point. This is a spherical, revolving design, which is viewed as a cube (figure 1), which consists of a central point and the middle points of edges. It can also be viewed as consisting of three interlocking 2^2 factorial designs and a central point (figure 2). Second degree polynomials (equation 1) were calculated with the statistical package to estimate the response of the dependent variables.

$$Y= b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$
$$+ b_{12}x_1x_2 + b_{23}x_2x_3 + b_{13}x_2x_3 \quad - \text{Eqn-1.}$$

Where Y is predicted response, x_1, x_2 and x_3 are independent variables, b_0 is offset term, b_1, b_2, b_3 are linear effects, b_{11}, b_{22}, b_{13} are interaction terms. A three variable Box-Behnken design of response methodology is used in this investigation. From the results of preliminary experiments, the following factor levels were selected. Substrate concentration (10 to 30% (w/v)), $(NH_4)_2SO_4$ (1 to 5 g/l) and KH_2PO_4 (2-6g/l) as listed in Table 1 were considered during the study.

Table 1: Process variables and levels

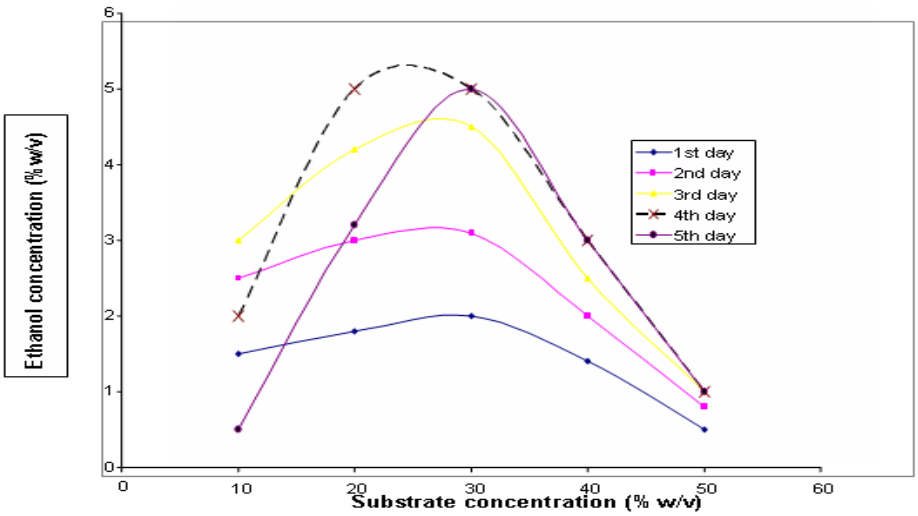
Factors	Lower limit (-)	Central point 0	Upper point (+)
Substrate concentration % (w/v)	10	20	30
$(NH_4)_2SO_4$ (g/l)	1	3	5
KH_2PO_4 (g/l)	2	4	6

RESULTS AND DISCUSSION:

Preliminary studies:

Effect of physical parameters: Effect of substrate concentration on ethanol production was studied by varying the percentage of jaggery between 10 to 50%, keeping pH at 5, temperature 30°C, inoculum level at 6 x 10^6 cells/ml and agitation 100rpm. An initial increase in product concentration is observed for 10 and 20% concentrations (Figure 1).

Figure 1. Effect of substrate concentration on ethanol production



A maximum of 3% on 3rd day for 10% concentration and 5% on 4th day for 20% were observed. Increase in fermentation days did not lead to any improvement in product concentration instead a decreasing trend was observed.

Usage of 30% jaggery also did not improve product concentration and further increase had negative effects, which may be due to high osmotic pressure caused by the high sugar content (Kademi and Baratti, 1982).

Since the total sugar percentage in jaggery is 8% (g/v), a 20% substrate concentration may be used for further studies.

Fermentation process is highly sensitive to changes in pH and the effect of this parameter is studied by varying the pH of the fermentation broth between 2 to 8 by adjusting it using 0.5N HCl and 0.5N NaOH. The percentage of jaggery is kept at 20%, temperature at 30°C, agitation 100 rpm and inoculum level at 6 x 10^6 cells/ml. Yeast are known to exhibit activity over a range of pH, i.e., between 5 and 7 (Jones, et al., 1981), a similar trend is observed in this study. pH values above and below the mentioned range were found to effect yeast growth and led to decrease in product concentration (figure 2).

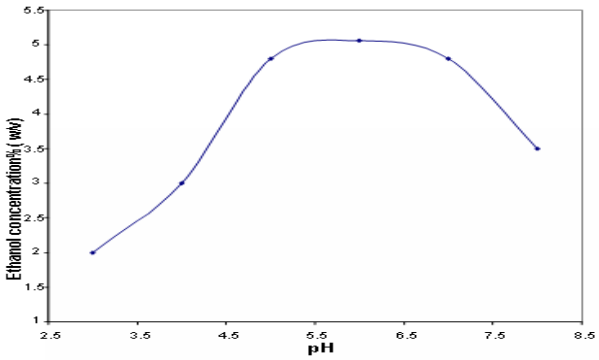


Figure 2. Effect of pH on Ethanol prodcution

Effect of inoculum level on fermentation was observed by varying it between 4 x 10^6 to 14 x 10^6 cells/ml keeping substrate concentration at 20%, temperature at 30°C, pH 5 and agitation at 100 rpm. It was observed that 6 x 10^6 cells/ml gave better results when compared to others, but the difference is not much considerable. Though addition of high inoculum levels is known to increase product concentration, it is also known to contribute to fusel alcohol (Watson and Hough, 1966; Huseyin Erten, et al., 2006), hence distillation was carefully carried out at 90°C and then product content is estimated.

The optimum temperature for fermentation is found varying temperature between the range 20 to 40°C. Results indicated that exposure to 30°C for a period of 3 days keeping rest of the parameters at their optimal level and rpm at 100 resulted in 5 %(w/v) ethanol as compared too others. Exposure to lower temperatures is known to give a high final ethanol concentration (Ayrappa, 1970) when compared to others, as it is known to reduce the loss due to evaporation. But the rate of fermentation is very slow.

And when higher temperatures are used they increase the rate of substrate conversion, but the loss due to evaporation if high.Effect of agitation on ethanol is monitored by varying rpm between 50 to 250, keeping rest of the parameters at their optimal levels. Agitation of 50 and100 rpm was found to give better results by the end of 3rd day as compared to others and the product concentration was found to be 5.2 %.

Agitation is required to ensure uniform exposure of organism to the available substrate and to increase dissolved oxygen content. It has been reported earlier that ethanol production rate is maximized at an O_2 uptake rate of 9 – 12 million moles /L/hr and there by increase product content ((Thomas, 1985; Mary Welch Baillargeon, et al., 2005). Therefore an rpm of 100 may be suggested as the optimal value.

Effect of Chemical parameters: Effect of Ammonium Sulphate as the Nitrogen source was studied by varying its concentration between 1 to 7g/l keeping rest of the parameters at their optimal conditions. Ammonium Sulphate acts as a nitrogen source and its substitution is important as a balanced carbon to nitrogen ratio is known to regulate glycolytic pathway that directly contributes to product formation (Daniel, et al., 2005). Optimal ethanol yield is obtained when $(NH_4)_2SO_4$ is added at a concentration of 3 g/l, and the product yield is 5.6 %. Addition of lower concentration resulted in low product formation, while higher values might have resulted in unfavorable conditions to the organism.

Potassium Dihydrogen Phosphate is used as the Phosphorus source and its concentration is varied between 1 to 7 g/l. maximum product concentration of 5.3 is obtained when 4g/l of KH_2PO_4 is added to the optimized medium. No nitrogen source is substituted at this stage. Higher and lower values of the salt were found have reverse effect. Phosphorus concentration controls synthesis of lipids and carbohydrates and maintains the integrity of the cell wall (Jones, et al., 1981).

Out of the above studied parameters the three most critical ones that regulate ethanol production, i.e., substrate concentration, Nitrogen and Phosphorus levels are considered for designing.

Response Surface Methodology:

15 experimental runs were carried out according to Box-Behnken three variable designs for a period of three days as per the design and various combination of the three parameters, i.e., substrate concentration, nitrogen and phosphorus levels the results of which were summarized in table 2. A quadratic equation was fitted to the above data, using multiple linear regressions available in STATISTICA software (Eq. (2)).

The significance of each co-efficient was determined by student's t-test and p-values which are listed in table 3. The larger the magnitude of the t-value and the smaller the p-value, the more significant is the corresponding coefficient (Akhnazarova and Kafarrov, 1982; Khuri and Cornell, 1987).

This data imply that first order main effect of substrate concentration, second order main effect of substrate concentration and nitrogen source are highly significant as is evident from their respective p-values, which are lesser than or equal to 0.05. The best model for maximizing ethanol production by Response Surface analysis was the following quadratic polynomial model.

Table 2: Box-Behnken three variable experimental design

Serial No.	Coded variable			Natural variable			Alcohol (%w/v)	
	x_1	x_2	x_3	X_1	X_2	X_3	Experimental value	Predicted value
1	1	1	0	10	1	4	3.5	3.417
2	1	-1	0	30	1	4	5.5	5.45
3	-1	1	0	10	5	4	3.25	3.297
4	1	1	0	30	5	4	4.58	4.662
5	-1	0	-1	10	3	2	3.89	4.045
6	1	0	-1	30	3	2	5.41	5.53
7	-1	0	1	10	3	6	3.5	3.38
8	1	0	1	30	3	6	5.45	5.295
9	0	-1	-1	20	1	2	5.5	5.427
10	0	1	-1	20	5	2	5.5	5.297
11	0	-1	1	20	1	6	5.1	5.302
12	0	1	1	20	5	6	4.45	4.522
13	0	0	0	20	3	4	6.02	6.016
14	0	0	0	20	3	4	6.05	6.016
15	0	0	0	20	3	4	5.98	6.016

Table 3: Model co-efficient estimated by Multiple linear regression.

S.No	Coefficient	Std. error	t-value	p-value
Intercept	4.63	0.056	82.388	0.0000
X_1	0.85	0.1378	12.334	0.000062
X_2	0.596	0.1014	11.75	0.000078
X_3	-0.227	0.1378	-3.301	0.021447
X_1^2	0.3085	0.1014	6.083	0.001736
X_2^2	-0.225	0.1378	-3.2649	0.022323
X_3^2	0.131	0.1014	2.5836	0.049214
X_1X_2	-0.1675	0.1949	1.7186	0.146314
X_2X_3	0.1075	0.1949	1.1030	0.320255
X_1X_3	-0.1625	0.1949	1.6673	0.156318

$$\text{Ethanol \%(w/v)} = 4.63 + 0.85x_1 - 0.227x_2 - 0.225x_3 + 0.59x_1^2 + 0.308x_2^2 + 0.13x_3^2 - 0.167x_1x_2 + 0.07x_1x_3 - 0.162x_2x_3. \quad \text{-EQ-2.}$$

The fit of the model was checked by the coefficient of determination R^2 which was calculated to be 0.98581, indicating that 98.58% of variability in the response could be explained by the model. By optimizing the above equation the following conditions were obtained. The maximum ethanol production is equal to 6.22%(w/v), optimum substrate concentration (x_1) is 23.56%(w/v), optimum concentration of nitrogen source (x_2) is 2.612 g/l and optimum concentration of phosphorus is 3.407 g/l. Experiments in triplicate were carried out at the above optimized conditions of jaggery, $(NH_4)_2SO_4$ and hKH_2PO_4 and an average response of 6.1%(w/v) ethanol yield was observed, which is very close to the predicted value. The excellent correlation between the predicted and measured values of these experiments justifies the validity of response model and the existence of an optimum point.

Figures 3 to 5 represent the response surface and contour plots for the optimization of medium constituents of ethanol production. The effect of substrate concentration and nitrogen levels on ethanol production were shown in figure 3. An increase in substrate concentration with $(NH_4)_2SO_4$ resulted in an increase in ethanol concentration until an optimum value i.e., 20% (w/v) jaggerys and 3g/l Nitrogen source.. And a further increase in the nitrogen source was found to be unfavorable for the production in ethanol and there by a decreasing trend was observed. The interactions between $(NH_4)_2SO_4$ and KH_2PO_4 on ethanol production were plotted in figure 4.

An increase in KH_2PO_4 along with a steady increase in $(NH_4)_2SO_4$ resulted in an increase in product concentration until they are added at their optimal values 4g/l and 3g/l respectively. Further increase had a reverse effect on the product formation as it may be unfavorable for the organism. The centre point of figure 5 reveals the optimal values of substrate and phosphate sources that may be added to get optimal concentration of the desired product, which is 20% (w/v) jaggery and 4g/l KH_2PO_4. Any further increase in both the Carbon and Phosphorus sources proved to revert the production.

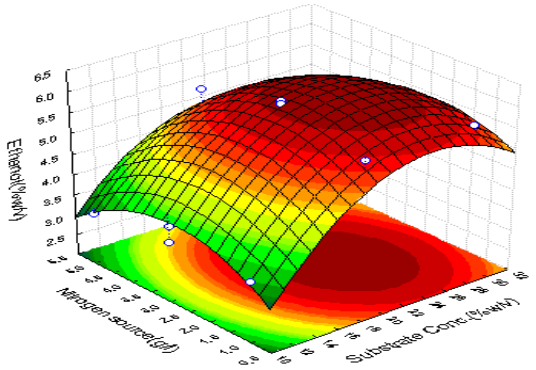


Figure-3: Response surface and contour plot of substrate Conc Vs Nitrogen level on ethanol production

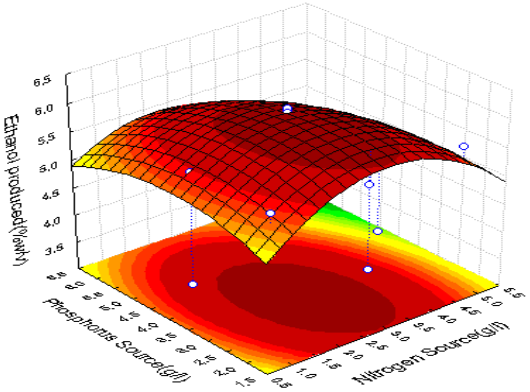


Figure-4: Response surface and contour plot of Nitrogen Vs Phosphorus levels on ethanol production

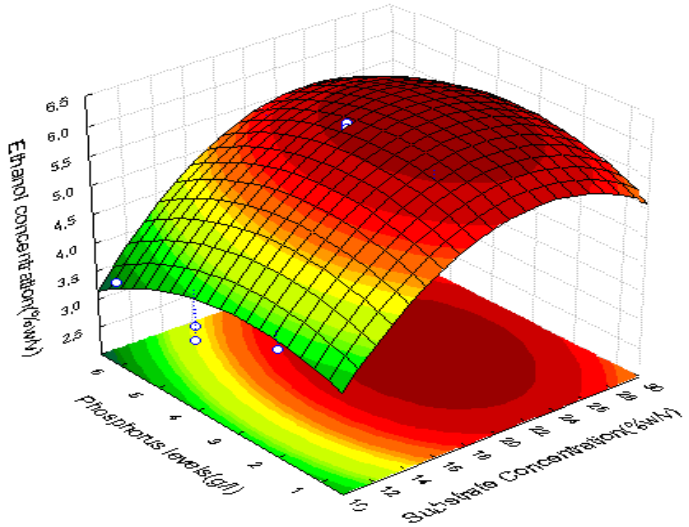


Figure-5: Effect of substrate concentration Vs Phosphorus on ethanol production

CONCLUSION

In the present investigation the parameters that could contribute to enhance ethanol production are carried out in the preliminary studies of which three critical ones were identified. They include, effect of jaggery concentration, Ammonium sulphate and Potassium dihydrogen phosphate levels. A Box-Behnken design was used to exactly identify the optimal concentrations of three parameters that can result in optimal yields of product. 15 experimental runs were carried out according to the chosen experimental design and a quadratic equation was fitted. The optimum ethanol yield obtained by solving the equation is 6.23% and the yield obtained is 6.15%, at 20% substrate concentration which account to a 1.6% increase in product concentration. It maybe concluded that the preliminary experimentation was carried out with exact precision. Substitution with metal inducers, amino acids and chelating agents may further contribute To increase in product concentration.

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