Advanced Journal of Microbiology Research Volume 2012
Available online at http://advancedresearchjournals.org/AJMR

Full Length Research Paper

Screening and optimization of metal ions to enhance ethanol production using statistical experimental designs

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Accepted 14 March, 2011

Ethanol production using jaggery was enhanced in submerged fermentation when the effect of metal inducers was studied using the Plackett-Burman and Box-Behnken designs. *Saccharomyces cerevisiae* (NCIM 3288) was used as the fermenting organism. The Plackett-Burman design was used to initially screen seven of which the four elements were found to have significant effect on ethanol production. In the next stage, Box-Behnken design was used obtain concentrations of metal ion's that may be supplemented to get maximum ethanol in during production process. It was observed that ethanol yield has increased to 94.8 from 75.4g/l when supplemented with the critical concentrations of salts provided by the model. These were as follows (g/l): $FeSO_4$. 7H $_2O$ 0.0036, $MgSO_4.7H_2O$ 0.0033, $MnCl_2$. 4H $_2O$ 0.0017 and $ZnSO_4$.7H $_2O$ 0.0026, in the presence of 220 g/l of jaggery supplemented with $(NH_4)_2SO_4$ 2.612 g/l and KH_2PO_4 3.407 g/l, while the predicted concentration of ethanol as per the model is 95.35 g/l.

Key words: Jaggery, ethanol, Plackett-Burman design, Box-Behnken design, metal inducers.

INTRODUCTION

Ethanol has gained its importance not just as a chemical feed stock, an industrial solvent or a beverage, but in recent scenario; it is emerging as a fuel option for automobiles as gasohol. The quadrupling of the selling prices of crude petroleum by the Organization of Petroleum Exporting Countries (OPEC) since 1973 had a profound impact on fermentation processes for producing ethanol (Paul Dwight and Kavasmaneck, 1980). Since then, several renewable sources have been studied for producing ethanol, which included cane molasses (Sheoran et al., 1998; Nigam et al., 1998), agricultural wastes, grains (Wu et al., 2006; Zhan et al., 2006) and tubers (David and Zdravko, 1990). In the present study Jaggery, the natural sweetener made by the concentration of sugar cane juice and which account to 50% of the sugar eaten in India is used as the substrate. It is also produced in Sri Lanka, Thailand and Burma. Being the second largest producer of sugar cane (Rao and Kumar, 2005), India can look forward to the usage of jaggery as an alternative carbohydrate source to meet fuel demands. Along with the readily available fermentable sugars, jaggery also has metal ions, and vitamins like carotenes and nicotinic acid which may act as cofactor for better growth of the fermenting organism (Anand and Ashok, 2007). It does not require any pretreatment or hydrolysis and the metal ions concentration is non-toxic to yeast. *Saccharomyces cerevisiae* is the most common organism used for alcohol production, which, in addition to nitrogen and phosphorus sources, requires supplementation of metal inducers whose concentration must be optimized.

Jones et al. (1981), have listed out the various cations that may be used as supplements and their stimulatory effect on the physiology of fermenting organism. Iron, Zinc and Manganese are required as cofactors for several metabolic pathways (Morris, 1958), out of which Magnesium is known to influence the glucose uptake by microorganism (Sue and Horst, 1981) as well as its growth by regulating cell cycle (Graeme and John, 1980; Dombek and Ingram, 1986) . Zinc acts as a cofactor for many enzymes (Gottschalk, 1986; Auld et al., 1976) and also reduces higher alcohols formation (Gutierrez, 1993). Potassium, Cobalt and Magnesium are considered to be

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cofactors for glycolysis (Crane, 1975) while Copper, Zinc and Manganese are also reported to influence yeast biomass by activating phosphatases, increasing amino acid metabolism and fatty acid synthesis (Stehlik-Tomas, 2004) there by contributing to product yield. Sodium increases uptake of sugars (Jones and Greenfield, 1984) therefore contributes to increase in ethanol production.

The traditional method of optimization of parameters involves optimizing one parameter at a time. This is not only a time-consuming process, but often misses the alternative effects between components (Elibol, 2004). It also involves several experiments to determine the optimal levels, which may not give the exact values. These draw-backs may be avoided by using response surface methodologies of experimental designs like, Plackett-Burman (Srinivas et al., 1994; Ramesh, 2004) and Box-Behnken (Plackett and Burman, 1946; Flavia et al., 2006) designs. Plackett-Burman design employs a design that allows testing the largest number of factor effects with least number of observations. Full factorial designs try to work on all possible combinations of the factors, thereby increase the number of runs in the experimentation geometrically. Under these conditions, fractional factorial is used that sacrifice interaction effects so that main effects may still be completed correctly. According to Plackett and Burman (1946), their factorial design allows estimation of random error variability and test for the statistical significance of the parameter estimates. While Box-Behnken design is a 2-level factorial design, where contour plots are generated by linear or quadratic effects of key variables, and a model equation is derived fitting the experimental data to calculate the systems optimal response.

In the present work, the Plackett-Burman design was used to identify the metal ions that contributed significantly to ethanol production. Then using response surface methodology a model system was developed to optimize the concentration of metal ions in the production medium.

MATERIALS AND METHODS

Substrate

Jaggery was procured from the native makers of Anakapalii, A.P., India, and used as carbon source for the yeast. Its total sugars contents were estimated to be 80g/100g of jaggery.

Organism

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 composittion (g/l): Malt extract – 3, glucose – 10, yeast extract – 3, peptone – 5 and agar-agar 20. pH is maintained at 7.0, and the slants were incubated at $30^{o}C$ for 24 h. Subculturing was carried out once in a month and culture was stored at $4^{o}C$ (Mary Anupama, 2001).

To prepare the inoculum, a loopful of the organism was inoculated into 25 ml of medium taken in a 250 ml Erlenmeyer flask containing the same components as in the maintenance medium, except that agar was not added. The flask was incubated in an incubated orbital shaker at $30^{o}C$ and 200 rpm for 24 h. Five ml of the medium was then removed, centrifuged and inoculated into production medium.

Fermentation conditions

The 50 ml of basic production medium having composition as follows (g/l): jaggery- 200; $(NH_4)_2SO_4$- 2.6; and KH_2PO_4- 3.6 is taken in a 250 ml Erlenmeyer flask. It is an aerobic fermentation and the physical parameters like temperature was kept at $30 \pm 1^{o}C$, pH – 5 ± 0.5, agitation – 150 rpm and the inoculum added was 6 x 10^6 colony forming units(cfu)/ml.

Screening of trace elements

Although the substrate has some metal ions their concentrations according to reports in literature are low and hence, supplementations need to be done to enhance productivity (Anand and Ashok, 2007). The basic elements that can contribute to the growth of yeast, as well as act as inducers for enzymes of glycolytic and relevant pathways that contribute to ethanol production were identified from the literature (Jones et al., 1981). The elements chosen for this study, with their concentration ranges are as follows (g/l): $FeSO_4 . 7H_2O$ (0.002 to 0.006), $CaCl_2 . 2H_2O$ (0.001 to 0.003), NaCl (0.002 to 0.006), $CoCl_2$ (0.002 to 0.006), $MgSO_4 . 7H_2O$ (0.001 to 0.005), $MnCl_2 . 4H_2O$ (0.001 to 0.003) and $ZnSO_4 . 7H_2O$ (0.0005 to 0.0015). Stock solutions of the salts were prepared and added to the production medium before autoclaving as per the experimental design. All runs were carried out in duplicated and the average of the ethanol produced as on second day were presented in Table 1.

Analytical methods

Ethanol was estimated using gas liquid chromatography (GLC), equipped with a flame ionization detector and a stainless steel column packed with Poropack-Q (50 - 80) mesh (Nucon Engineers, India). The oven was maintained at 150^{o} C and the detector and injection ports were maintained at $170^{o}C$. The flow rate of carrier gas (nitrogen) flow rate was kept at 30 cm^3/min and the combustion gas was a mixture of hydrogen and air (Ratnam, 2003). Total sugar content was measured by the anthrone method (Jose et al., 1981).

Experimental designs

The Plackett-Burman experimental design is a factorial design used to demonstrate the relative importance of medium supplements. It considers the statistical interactions between variables to obtain maximum interferences for a minimum number of tests, thus reducing process variability, time of development and overall costs. In the present study, seven independent variables in eight combinations were organized according to the Plackett -Buramn design matrix (Table 2). For each variable, high (+1) and low (-1) levels were tested. All trials were performed in triplicate and the means of the response were considered. Using the data, Pereto charts were generated that revealed the most significant metal ions that can contribute to ethanol formation.

The Box- Behnken design allows estimating and interpreting the interactions between various variables at a time during an optimization process. It is suitable for exploration of such quadratic

Table 1. Plackett and Burman fractional factorial design.

Run	$FeSO_4.7H_2O$ (g/l)	$CaCl_2.2H_2O$ (g/l)	$MnCl_2.4H_2O$ (g/l)	$ZnSO_4.7H_2O$ (g/l)	$MgSO_4.7H_2O$ (g/l)	NaCl (g/l)	$CoCl_2$ (g/l)	Ethanol (g/l)
1	0.002	0.001	0.002	0.006	0.005	0.003	0.0005	69.2
2	0.006	0.001	0.002	0.002	0.001	0.003	0.0015	72.3
3	0.002	0.003	0.002	0.002	0.005	0.001	0.0015	75.8
4	0.006	0.003	0.002	0.006	0.001	0.001	0.0005	64.8
5	0.002	0.001	0.006	0.006	0.001	0.001	0.0015	77.8
6	0.006	0.001	0.006	0.002	0.005	0.001	0.0005	60.0
7	0.002	0.003	0.006	0.002	0.001	0.003	0.0005	71.8
8	0.006	0.003	0.006	0.006	0.005	0.003	0.0015	54.0
9	0.002	0.001	0.002	0.002	0.001	0.001	0.0005	83.0

Table 2. The Plackett-Burman design matrix representing the coded values for 7 independent variables.

Run	$FeSO_4.7H_2O$	$CaCl_2.2H_2O$	$MnCl_2.4H_2O$	$ZnSO_4.7H_2O$	$MgSO_4.7H_2O$	NaCl	$CoCl_2$
1	-1.00000	-1.00000	-1.00000	1.00000	1.00000	1.00000	-1.00000
2	1.00000	-1.00000	-1.00000	-1.00000	-1.00000	1.00000	1.00000
3	-1.00000	1.00000	-1.00000	-1.00000	1.00000	-1.00000	1.00000
4	1.00000	1.00000	-1.00000	1.00000	-1.00000	-1.00000	-1.00000
5	-1.00000	-1.00000	1.00000	1.00000	-1.00000	-1.00000	1.00000
6	1.00000	-1.00000	1.00000	-1.00000	1.00000	-1.00000	-1.00000
7	-1.00000	1.00000	1.00000	-1.00000	-1.00000	1.00000	-1.00000
8	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
9	-1.00000	-1.00000	-1.00000	-1.00000	-1.00000	-1.00000	-1.00000

Table 3. Process variables and levels.

Factors	Lower limit (-1)	Central point 0	Upper point (+1)
$FeSO_4.7H_2O$ (g/l)	0.002	0.004	0.006
$MgSO_4.7H_2O$ (g/l)	0.002	0.004	0.006
$MnCl_2.4H_2O$ (g/l)	0.002	0.004	0.006
$ZnSO_4.7H_2O$ (g/l)	0.001	0.003	0.005

responses and constructs a second-order polynomial model with very few runs. The number of experiments required according to this design is $N = k^2 + k + c_p$, where k is the factorial number and c_p is the replicate number of the centre point (Anderson et al., 2005). Table 3 lists the four variables (significant metal inducers as per Plackett-Burman design) studied and these were, X_1 is $FeSO_4.7H_2O$, X_2 is $MgSO_4.7H_2O$, X_3 is $MnCl_2.4H_2O$ and X_4 is $ZnSO_4.7H_2O$. All salts were added at concentrations of milligrams per liter. The manipulation responses of the input variables were evaluated as a function of the ethanol produced at the end of second day, which is indicated by Y. A three variable Box-Behnken four variables, as listed in Table 3. A total of 27 experimental runs design of response surface methodology (RSM) was used with the were carried out as per the design and second-degree polynomials (equation 1) were calculated with the statistical package (Stat-Ease Inc., Minneapolis, MN, USA) to estimate the response of the dependent variables.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4$$

In the equation Y is the predicted response, X_1, X_2, X_3 and X_4 are independent variables, b_0 is offset term, b_1, b_2, b_3 and b_4 are linear effects, b_{12}, b_{13}, b_{14}, b_{23}, b_{24} and b_{34} are interaction terms. Three-dimensional surface (3D) plots were drawn to illustrate the main and interactive effects of the independent variables on ethanol production. The optimum values of the selected variables were obtained from the software and also from the response surface plots.

RESULTS AND DISCUSSION

Based on literature reports the elements indicated in Table 1 were chosen for the present study. The results of the Plackett-Burman design (Table 2) identified the most significant elements amongst those selected and pereto chart effects are shown in Figure 1. It is evident from Figure 1 that elements $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, $MnCl_2.4H_2O$ and $ZnSO_4.7H_2O$, whose probability values

Table 4. Box-Behnken three variable experimental design.

S.No	X_1 (g/l)	X_2 (g/l)	X_3 (g/l)	X_4 (g/l)	Observed ethanol concentration (g/l)	Predicted ethanol concentration (g/l)
1	0.002	0.002	0.002	0.003	86.0	84.2381
2	0.006	0.002	0.002	0.003	78.0	75.6381
3	0.002	0.006	0.002	0.003	72.0	74.8547
4	0.006	0.006	0.002	0.003	70.0	72.2547
5	0.004	0.004	0.001	0.005	80.0	78.7464
6	0.004	0.004	0.003	0.005	76.0	74.7464
7	0.004	0.004	0.001	0.005	77.0	78.7464
8	0.004	0.004	0.003	0.005	73.0	74.7464
9	0.004	0.004	0.002	0.003	93.0	93.8083
10	0.002	0.004	0.002	0.001	81.0	80.8312
11	0.006	0.004	0.002	0.001	73.4	71.9312
12	0.002	0.004	0.002	0.005	73.0	74.9348
13	0.006	0.004	0.002	0.005	72.0	72.6348
14	0.004	0.002	0.001	0.003	85.0	87.8122
15	0.004	0.006	0.001	0.003	80.0	80.3789
16	0.004	0.002	0.003	0.003	77.9	77.9872
17	0.004	0.006	0.003	0.003	75.0	72.6539
18	0.004	0.004	0.002	0.003	94.0	93.8083
19	0.002	0.004	0.001	0.003	85.0	82.2080
20	0.006	0.004	0.001	0.003	74.0	73.1080
21	0.002	0.004	0.003	0.003	70.0	69.9330
22	0.006	0.004	0.003	0.003	66.0	67.8330
23	0.004	0.002	0.002	0.001	8.40	85.9104
24	0.004	0.006	0.002	0.001	80.0	79.7271
25	0.004	0.002	0.002	0.005	84.2	83.514
26	0.004	0.006	0.002	0.005	79.8	76.9306
27	0.004	0.004	0.002	0.003	94.0	93.8083

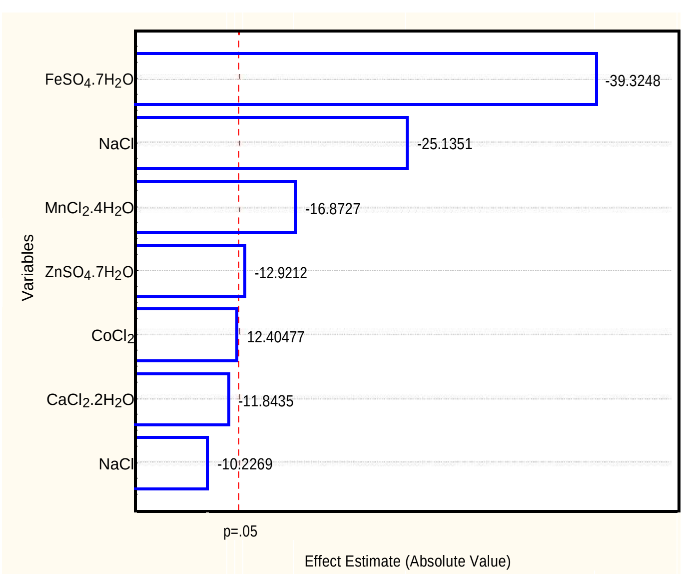

Figure 1. Pareto Chart of standardized effects for the Placket-Burman design.

are above 0.05, contributed significantly in enhancing the yield. The rest of the elements added to the substrate, which already had some micronutrients, may not contributed significantly to ethanol formation. Hence their probability values are below 0.05 and hence may be avoided.

Twenty- seven experimental runs were carried out according to Box-Behnken three variable designs with 3 replicates, for a period of three days. As per the design, various combinations of the four elements used, along with the results obtained, are summarized in Table 4. A quadratic equation was fitted to the data obtained as indicated in Table 4, using multiple linear regressions available in STATISTICA software (Equation 2). The significance of each co- efficient was determined by student's t-test and p-values which are listed in Table 5. The larger the magnitude of the t-value and the smaller the p-value, the more significant is the corresponding coefficient (Babu, 2007). This data implied that except for slight deviation in case of $ZnSO_4.7H_2O$, the rest all are highly significant. This is evident from their respective *p*- values,

Table 5. Model co-efficient estimated by multiple linear regression.

Term	Coefficient	Value	Std. error	t-value	p-value
Constant	b_0	71.92460	0.56874	126.4621	0.000000*
$FeSO_4$. $7H_2O$	X_1	-2.80000	1.10301	-5.0770	0.000050*
$MgSO_4$. $7H_2O$	X_2	-3.19167	1.10301	-5.7872	0.000010*
$MnCl_2$. $4H_2O$	X_3	-4.38750	1.35091	-6.4956	0.000002*
$ZnSO_4.7H_2O$	X_4	-1.29821	1.25070	-2.0760	0.050366
$FeSO_4$. $7H_2O$ x $FeSO_4$. $7H_2O$	X_1^2	5.87485	0.68345	17.1918	0.000000*
$MgSO_4$. $7H_2O$ x $MgSO_4.7H_2O$	X_2^2	2.65610	0.68345	7.7727	0.000000*
$MnCl_2$. $4H_2O$ x $MnCl_2$. $4H_2O$	X_3^2	4.39405	0.70689	12.4321	0.000000*
$ZnSO_4.7H_2O$ x $ZnSO_4.7H_2O$	X_4^2	3.48780	0.70689	9.8681	0.000000*
$FeSO_4$. $7H_2O$ x $MgSO_4$. $7H_2O$	$X_1 X_2$	1.50000	1.91047	1.5703	0.131293
$FeSO_4$. $7H_2O$ x $MnCl_2$. $4H_2O$	$X_1 X_3$	1.75000	1.91047	1.8320	0.081172
$FeSO_4$. $7H_2O$ x $ZnSO_4.7H_2O$	$X_1 X_4$	1.65000	1.91047	1.7273	0.098791
$MgSO_4$. $7H_2O$ x $MnCl_2$. $4H_2O$	$X_2 X_3$	0.52500	1.91047	0.5496	0.588390
$MgSO_4$. $7H_2O$ x $ZnSO_4.7H_2O$	$X_2 X_4$	-0.10000	0.191047	-0.1047	0.917618
$MnCl_2$. $4H_2O$ x $ZnSO_4.7H_2O$	$X_3 X_4$	2.38750	0.233984	2.0407	0.054047

* $p \leq 0.05$ indicating that the factors are significant.

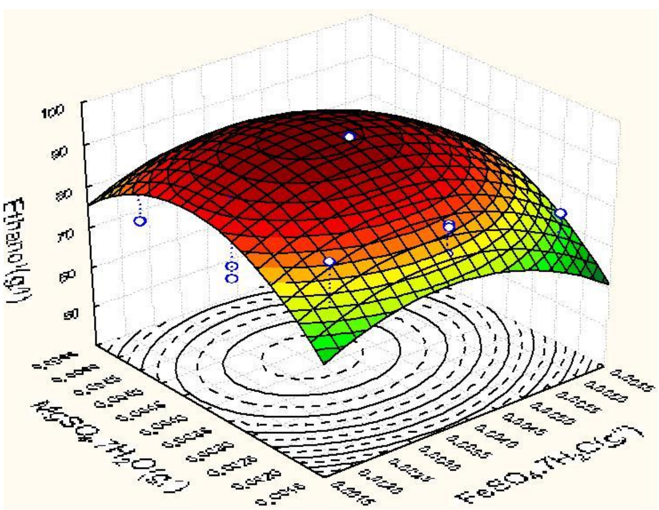

Figure 2. Effect of FeSO4.7H2O (X_1) and Mgso4.7H2O (X_2) on ethanol production (Y).

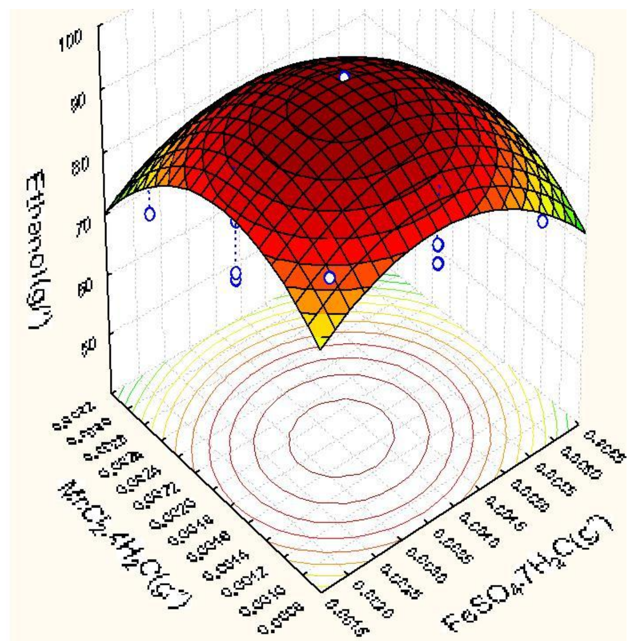

Figure 3. Effect of FeSO4.7H2O (X_1) and $MnCl_2$.4H2O(X_3) on ethanol production (Y).

which are lesser than or equal to 0.05 (Akhnazarova and Kafarrov, 1982; Khuri and Cornell, 1987). The best model for maximizing ethanol production by Response Surface analysis was the following quadratic polynomial model.

$$Y(g/l) = 71.924 - 2.8 X_1 - 3.191 X_2 - 4.387 X_3 - 1.298 X_4 + 5.87 X_1^2 + 2.65 X_2^2 + 4.394 X_3^2 + 3.487 X_4^2 + 1.5 X_1 X_2 + 1.75 X_1 X_3 + 1.65 X_1 X_4 + 0.525 X_2 X_3 - 0.1 X_2 X_4 + 2.38 X_3 X_4$$

The fit of the model was checked by the coefficient of determination R^2 which was calculated to be 0.9737) indicating that 97.37% of variability in the response could be explained by the model. By optimizing the above equation the following conditions were obtained. The maximum ethanol concentration predicted by the model was 95.35 g/l when supplemented with FeSO4. $7H_2O$(X_1) 0.0036 g/l, $MgSO_4$. $7H_2O$(X_2) 0.0033 g/l, $MnCl_2.4H_2O$(X_3 0.0017 and $ZnSO_4.7H_2O$(X_4) 0.0026 g/l. Experiments in triplicate were carried out at the above optimized condi-tions and an average response of 94.8 g/l ethanol was observed, which is very close to the predicted value. The excellent correlation between the predicted and mea-sured values of these experiments justifies the validity of

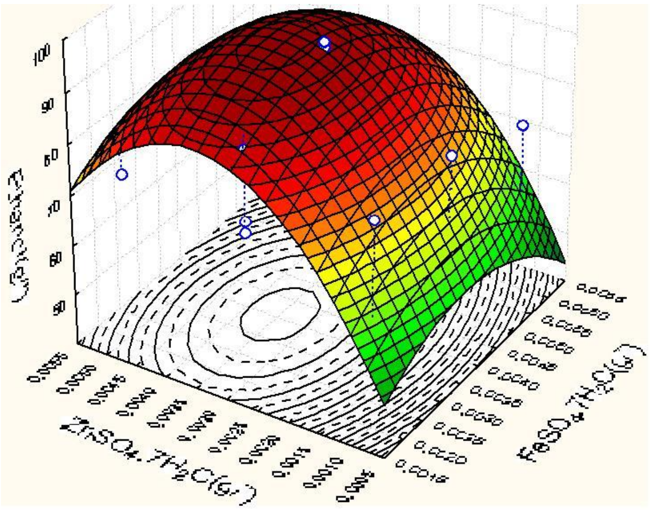

Figure 4. Effect of FeSO4.7H2O (X1) and ZnCl2.7H2O(X4) on ethanol production (Y).

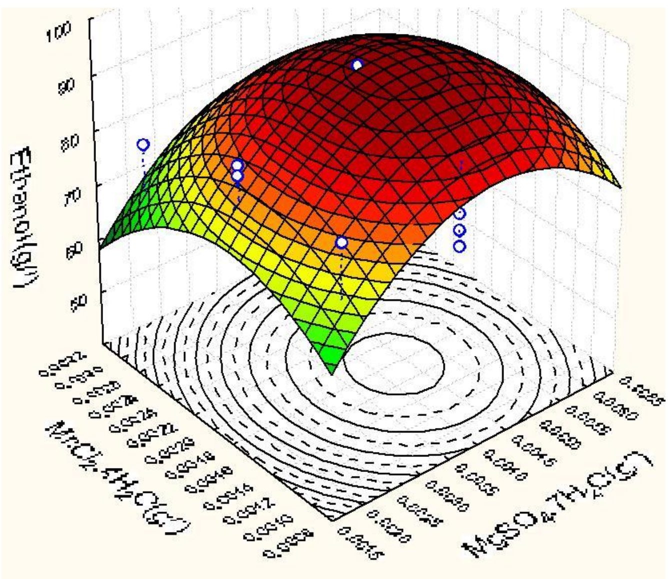

Figure 5. Effect of MgSO4.7H2O (X2) and Mnl2.4H2O(X3) on ethanol production (Y).

of response model and the existence of an optimum point.

The 3-D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables, and combined effects of each independent variable upon the response variable (Fi-gures 2 to 7). Figure 2, illustrates the 3D response surface based on the *Y* response against $FeSO_4.7H_2O$(X_1) and $MgSO_4.7H_2O$(X_2) with $MnCl_2.4H_2O$ and $ZnSO_4.7H_2O$ maintained at 0.002 g/l and 0.00322 g/l, respectively. An increase in $FeSO_4$ with a simultaneous increase in $MgSO_4$ led to an initial increase in ethanol

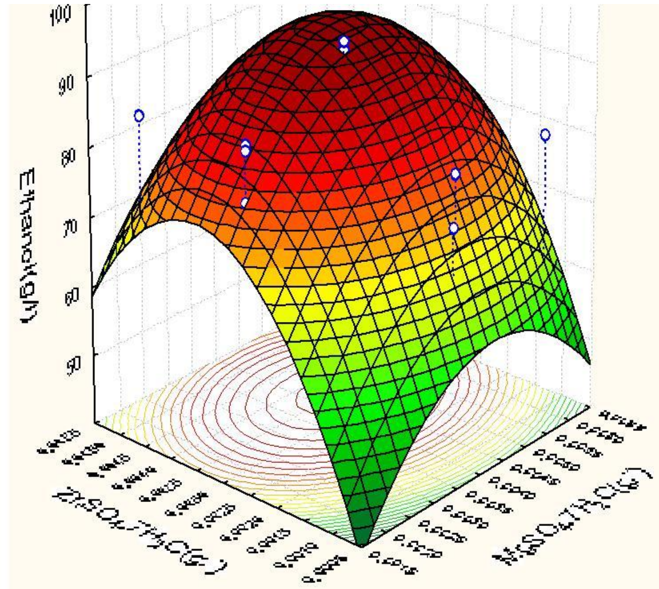

Figure 6. Effect of MgSO4.7H2O (X2) and ZnSO4.4H2O(X4) on ethanol production (Y).

formation until they reached their optimal values. The data obtained by varying concentrations of $FeSO_4.7H_2O$ (X_1) and $MnCl_2.4H_2O$ (X_3) keeping $MgSO_4.7H_2O$ and $ZnSO_4.7H_2O$ at 0.004 g/l and 0.00322 g/l respectively, is plotted in Figure 3. It shows that an initial increase in X_1 with a simultaneous increase in X_3 resulted in an increase in product formation. However an increase beyond this limit has affected the product formation. Figure 4 shows the response surface plot illustrating the effect of $FeSO_4.7H_2O$ (X_1) and $ZnSO_4.7H_2O$ (X_4) on ethanol formation keeping $MgSO_4.7H_2O$ and $MnCl_2.4H_2O$ at 0.004 and 0.002g/l respectively. The plot revealed that ethanol formation was low at lower as well as at higher concentrations of both the salts, and at a certain optimal value the yield will be high. Figure 5 shows the response generated with the data obtained by varying $MgSO_4.7H_2O$ (X_2) and $MnCl_2.4H_2O$ (X_3) keeping the variable $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$ at 0.004 and 0.00322 g/l. As evident from the graph maximum product formation was seen when 0.0034 g/l of $MgSO_4$ and 0.00167g/l of $MnCl_2$ were added to the production me-dium along with fixed concentrations of X_1 and X_4.

Figure 6 is plotted with the data obtained by varying $MgSO_4.7H_2O$ (X_2) and $ZnSO_4.7H_2O$ (X_4) on ethanol formation keeping $FeSO_4$ at 0.004 g/l and $MnCl_2$ at 0.002 g/l. From the response generated the optimal values of X_2 and X_3 at the fixed values of X_1 and X_4 were 0.0034 and 0.0028 g/l respectively. Figure 7 represents the response surface plots for ethanol formation by varying $MnCl_2.4H_2O$ (X_3) and $ZnSO_4.7H_2O$ (X_4) keeping both $FeSO_4.7H_2O$ and $MgSO_4$..$7H_2O$ at 0.004 g/l. An increase in the concentration of both the variables contributed to product formation until they reached an optimal value be-

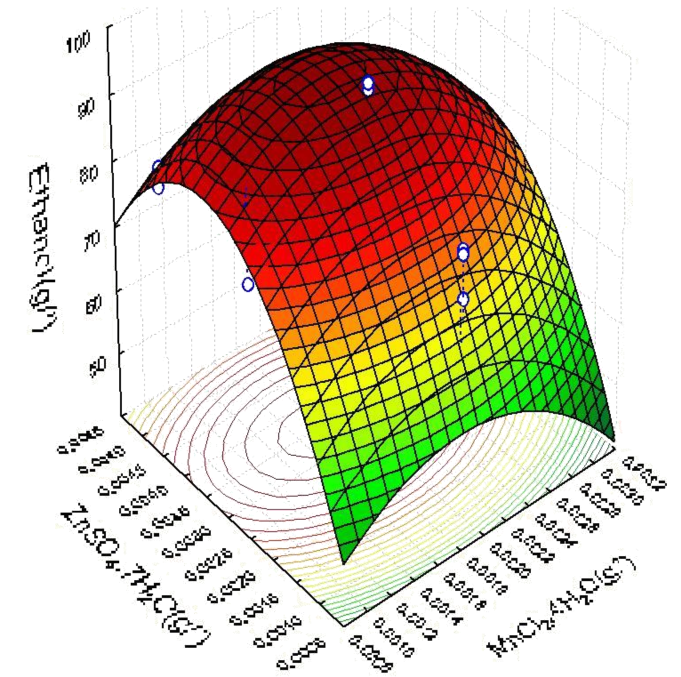

Figure 7. Effect of $MnCl_2.4H_2O$ (X_3) and $ZnSO4.7H_2O$ (X_4) on ethanol production (Y).

yond which the induction effect of the salts reverted and resulted in low ethanol formation. A final run was carried out by maintaining the critical values of elements and the ethanol concentration obtained was 94.8 g/l, which is very close to the predicted value, i.e., 95.35 g/l. concentrations of the four elements were determined using Box-Behnken design. The jaggery concentration was kept at 220 g/l with $(NH_4)_2SO_4$ and KH_2PO_4 substituted at 2.612 and 3.407 g/l concentrations. The critical values of the elements as revealed from the model were as follows (g/l): $FeSO_4$. $7H_2O$ 0.0036, $MgSO_4.7H_2O$ 0.0033, $MnCl_2$. $4H_2O$ 0.0017 and $ZnSO_4.7H_2O$ 0.0026 and the predicted product concentration was 95.35 g/l. A final run with the given optimal values was carried out which resulted in 94.8 g/l ethanol which is almost same as the predicted value. An R^2 value of 0.9737 was obtained which indicates that 97.37% variability could be explained by the model. The yield is higher when compared to studies carried out using pure sucrose (Belkis and Fazilet, 1998) and also avoids supplementation of insignificant elements.

ACKNOWLEDGEMENTS

We are grateful to the Head of the Department of Biotechnology, Dr. K. Balakrishnan and the management of ANITS for their constant support during the work.

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