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Bioethanol Production from Saccharified Sweet Potato (*Ipomoea batatas* L.) Root Flour using Immobilized Cells of *Saccharomyces cerevisiae* and *Zymomonas mobilis* by RSM Method

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Abstract: ***With the increase in price of fossil fuels, the demand of bioethanol production from agricultural crops has become very crucial to meet the energy crisis in both developing and developed countries in future. Sweet potato is considered as an important agricultural crop due to its abundance and high amount of starch content. The present study focuses on bioethanol production from saccharified sweet potato root flour (SPRF) using co-immobilized cells of Saccharomyces cerevisiae and Zymomonas mobilis for bioethanol production by RSM method. The process parameters such as temperature, pH and incubation time were found to be the most favorable variables for the maximum ethanol production with box-behnken design of response surface methodology (RSM). Maximum ethanol of 90.6 g/kg of SPRF was obtained at pH 4.5 with an incubation period of 72 h at 32.5 °C by response surface methodology.***

Keywords: Sweet potato, Bioethanol production, Immobilization, Response surface methodology, Saccharomyces cerevisiae and Zymomonas mobilis

1. Introduction

With the increasing demand for ethanol, there is a considerable interest in developing biorenewable alternatives to substitute fossil fuels from non- conventional raw materials. Bioethanol contributes to diminish petroleum dependency, has positive effects on the environment generate and also new opportunities in the agricultural and agro-industrial sectors [1]. Now-a-days establishment of ethanol industry requires a cheap and sufficiently available feedstock to reduce the production costs which has been recognized as a critical point [2]. To meet the sake of the win-win prospect between the energy production and food security, today the fuel industry requires non-grain energy crops and agricultural biomass for ethanol production [3]. As the tubers contain sufficient amount of starch, they may be a suitable substrate for ethanol production [4]. The tuber crops like cassava, potato, sweet potato are most promising feed stock used in bioethanol production in worldwide due to their economic viability and availability [5, 6].

Sweet potato (*Ipomoea batatas* L.) is a cheap and readily available tuber crop in the tropical and temperate regions' in Indian sub-continent which mainly contains starch (178 g /kg), total sugars (26 g /kg) and protein (3.2 g/kg) on fresh weight basis [7]. As starch is a polysachharide, it can be hydrolysed to monomer units of carbohydrates for ethanol production by microorganisms in fermentation process [8]. The ethanol fermentation processes from starchy materials commonly involves two stages: (i) liquefaction of starch by a amylase (hydrolysis) enzyme and enzymatic saccharification of the low molecular weight liquefaction products such as dextrin to produce glucose by an glucoamylase (saccharification); (ii) fermentation of glucose to ethanol by ethanol producing microorganisms [9, 10].

The yeast, *Saccharomyces cerevisiae*, is the major ethanol producing microorganism has been used all over the world [11]. *Zymomonas mobilis*, is also an ethanol producing bacterium, as it shows several better fermenting characters like convertion of glucose to ethanol and CO_2, grows more rapidly and shows highest ethanol productivity at industrial-scale [12]. Now a days many researchers have been attempted to combine the two stage fermentation process in a single-step for bioethanol production but not on an industrial scale [13] because it is very difficult to optimize the conditions for one strain without affecting the other strains [14]. Therefore, co-immobilization different kinds of microorganisms within the same porous matrix and combination two stage fermentation process in a single-step used in bioethanol production which reduces the energy input and increases the efficiency of substrate utilization [15].

Response surface methodology (RSM) is an extensively used method in bioethanol production which comprises of a group of mathematical and statistical procedure that can be used to optimize different culture conditions in fermentation processes [16]. The model predicts experimental modifications like changes in operational conditions with minimum requirements and maximum yields [17]. The present work aims at developing a simultaneous single-step system for bioethanol fermentation from saccharified sweet potato, using co-immobilized cells of yeast *S. cerevisiae* and bacteria, *Zymomonas mobilis* by RSM for enhanced bioethanol production.

2. Materials and Methods

Strains and culture condition

Zymomonas mobilis MTTC 92 and *Saccharomyces cerevisiae* MTCC 120 strains were earlier used for bioethanol fermentation.. The *Z. mobilis* strain was maintained on Zymomonas specific medium ZSM [(g/L) yeast extract ,10; glucose, 20; $MgCl_2$, 10; NH_4SO_4 ,10 ; KH_2PO_4, 10; agar, 15 and pH 6-6.5] and the *S. cerevisiae* was maintained on malt-extract-yeast extract-glucose-peptone (MYGP) medium [(g/L): malt extract, 3; yeast extract, 5; peptone, 5; glucose, 20; agar, 15; pH 5.5]. Both the cultures were stored at 4 ^oC for further use.

Immobilization and co-fermentation

S. cerevisiae (3 x 10^9 CFU/ml) was mixed with 2.5% (w/v) Na-alginate solution and was added drop wise into 0.2 N $CaCl_2$ solution using a 50-ml syringe and beads of calcium alginate with entrapped cells, and were formed with a diameter of 3-4 mm. Then the beads were allowed to harden in 0.2 N $CaCl_2$ solution for overnight at 4 ºC. Similarly, preparation of immobilized *Z. mobilis* cells was carried out by using this method. For co-fermentation, the immobilized beads of *S. cerevisiae* and *Z. mobilis* that were prepared separately, were mixed together in equal proportions and used for further studies (*S. cerevisiae*:*Z. mobilis*=1:1).

Sample preparation and enzymatic saccharification

Fresh sweet potato was collected from the local market of Bhubaneswar, Capital of Odisha, India, during February-March, 2016. The collected sample was washed thoroughly to remove the dust and other debris, peeled off and chopped into small pieces. It was then placed in oven at 70 ^oC till the moisture content reduced to 11-12 % and grinded to powder form and sieved through a steel mesh to get 2-3mm diameter size.

SPRF (10%) slurry was prepared in 250 ml Erlenmeyer flasks with a working volume of 100 ml by adding tap water in a ratio of 1:10 for experiment. In first step for dextrinisation, the slurry was dextrinized by addition of 32 µl Palkolase- ®HT (a α-amylase) at pH 5.5 and 90°C for 1h and then slurry was cooled down to room temperature. In second step, for sachharification, a glucoamylase, Palkodex® (329.7 µl) was added to the dextrinized slurry at pH 4.5 and incubated for 24 h at 60 °C for saccharification.

Fermentation using RSM

RSM experimental design and optimization

Optimization of different growth factors responsible for the bioethanol production from sachharified SPRF was done by Response surface methodology. The statistical model was studied using Central composite design (CCD) experiments in which Incubation times (A), pH (B) and temperature (C) were taken as the independent variables (Table 1) where ethanol concentration was the dependent variable. In this experiment, these three parameters were chosen as they have showed great influence towards ethanol productivity. A total of fifteen experiments were carried out with different combinations of the independent variables and the response was measured in terms of ethanol production (Table 2).

Statistical analysis

The data obtained from RSM on total ethanol production were subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second order polynomial equation which represents the system more appropriately.

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \times \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where Y is response variable, β_0 is intercept, β_1, β_2, and β_3 are linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ are squared coefficient, $\beta_{1,2}$, $\beta_{1,3}$ and $\beta_{2,3}$, are interaction coefficient and A, B, C, A^2, B^2, C^2, AB, AC and BC are level of independent variables. Statistical significance of the model was determined by Fisher's test value, and the production of variance explained by the model was given by the multiple coefficient of determination, R squared (R^2) value. Design Expert ®software was used in this investigation. Three dimensional plots were obtained to study the interaction of one parameter with other.

3. Results and Discussion

Statistical optimization and model validation

During this experiment, incubation time, temperature and pH are the most important factors for bioethanol fermentation. So, these parameters were considered for bioethanol production by response surface methodology (RSM). The effects of the three variables and their possible interactions were statistically studied by ANOVA. Suitable levels for these parameters were determined using a statistical 2^3 full factorial design. Fifteen experiments were performed for evaluation of bioethanol production parameters by using co-immobilized cells of *S. cerevisiae* and *Z. mobilis*. The highest ethanol concentration of 90.6 g/kg of SPRF was obtained at pH 4.5 with an incubation period of 72 h at 32.5 °C. ANOVA (Table 3) was performed to check the validity of model (*p*-values< 0.001).

Determination of the influence of the process parameters between the response and variables is visualized by the response surface contour plot. The regression equation obtained after ANOVA indicates a *R*-squared value of 0.9835. This indicates a satisfactory adjustment of the experimental data with the theoretical values. Hence the *R*-squared value indicates that the model is suitable to predict optimum ethanol production from the sweet potato flour by using co-immobilized *S. cerevisiae* and *Z. mobilis* in which the optimum values of ethanol production were obtained by solving the regression equation.

The highest R^2 value was obtained in response which was explained by the second order polynomial equation producing maximum bioethanol of 90.6 g/kg where

incubation time (A), pH (B) and temperature (C). The second order polynomial equation was given below as:

R1 = -288.8 + 1.661X 72 + 88.7 X4.5 + 5.51 X32.5 - 0.01187X 72 X 72 - 10.06 X4.5X 4.5 - 0.0860 X32.5 X 32.5 + 0.0247X 72 X 4.5+ 0.00504 X72 X 32.5 + 0.121 X4.5X32.5

Interactions among the factors

The three-dimensional surface plots for the optimization conditions for maximum bioethanol prodution are given in **Figure 1 (a, b and c)** and represents the main and the interactive effects of the independent variables on the dependent ones. The **Figure 1(a)** shows the effect of temperature and pH on ethanol production keeping incubation period at '0' level. In case of medium pH, optimum ethanol production was increased up to pH 4.5 which gradually declined with increase in pH. When the level of incubation period was increased, a linear increase in bioethanol production was observed at 72 h **(Figure 1 b)**. Further the response between incubation period and temperature indicated that temperature at 32.5 °C was optimum with 72 h incubation period for maximum bioethanol production **(Figure1 c**). Fermentation conditions at temperature at 32.5 °C, incubation time of 72 h and medium pH of 4.5 were determined to be optimum conditions with 98.93 % validity.

4. Conclusion

The present results revealed that response surface method used for bioethanol production is found to be a promising technique for bioethanol production from saccharified sweet potato flour as substrate. The study demonstrated that the optimized parameters are pH 4.5 with an incubation period of 72 h at 32.5 °C with maximum ethanol yield of 90.6 g/kg of SPRF using co-immobilized cultures of *S. cerevisiae* and *Z. mobilis* by RSM methodology. Hence in conclusion, the co-immobilized cultures of *S. cerevisiae* and *Z. mobilis* can be a efficient microbial source for biorthanol production under optimized medium and process parameters as developed by the response surface methodology.

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Table 1: Coded levels of the independent variables for the design of the experiment

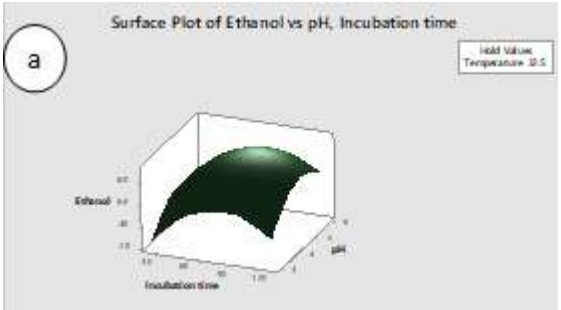
Independent variables	Symbols	Coded levels		
		-1	0	+1
Incubation period (h)	A	48	84	120
pH	B	4	5	6
Temperature (^oC)	C	25	30	35

Table 2: Ethanol production by Box-Behnken factorial design

Run	Incubation time(h)	pH	Temperature (^oC)	Ethanol (g/Kg)
1	120	4.5	20.0	18.8
2	72	4.5	32.5	90.6
3	72	3.0	45.0	53.8
4	24	6.0	32.5	35.5
5	72	4.5	32.5	90.6
6	72	6.0	45.0	72.8
7	120	3.0	32.5	22.2
8	72	3.0	20.0	40.8
9	120	6.0	32.5	34.6
10	24	4.5	45.0	44.8
11	24	3.0	32.5	30.2
12	72	6.0	20.0	50.7
13	24	4.5	20.0	26.8
14	72	4.5	32.5	90.6
15	120	4.5	45.0	28.9

Table 3: ANOVA for Response Surface Quadratic Model Analysis of Variance table (Partial sum of squares-Type III)

Source	Sum of squares	Df	Mean square	F-value	P-value
Model	6846.25	9	760.69	33.09	0.001
A (Incubation period)	950.48	1	950.48	41.35	0.001
B (pH)	271.44	1	271.44	11.81	0.019
C (Temperature)	865.28	1	865.28	37.64	0.002
AB	12.60	1	12.60	0.55	0.492
AC	36.60	1	36.60	1.59	0.263
BC	20.70	1	20.70	0.90	0.386
A^2	2759.41	1	2759.41	120.05	0.000
B^2	1892.15	1	1892.15	82.32	0.000
C^2	666.71	1	666.71	29.00	0.003
Lack of Fit	114.93	5	22.99		
Pure Error	0.000	2	0.000		
R-Squared	0.9835				
Adj R-Squared	0.9538				



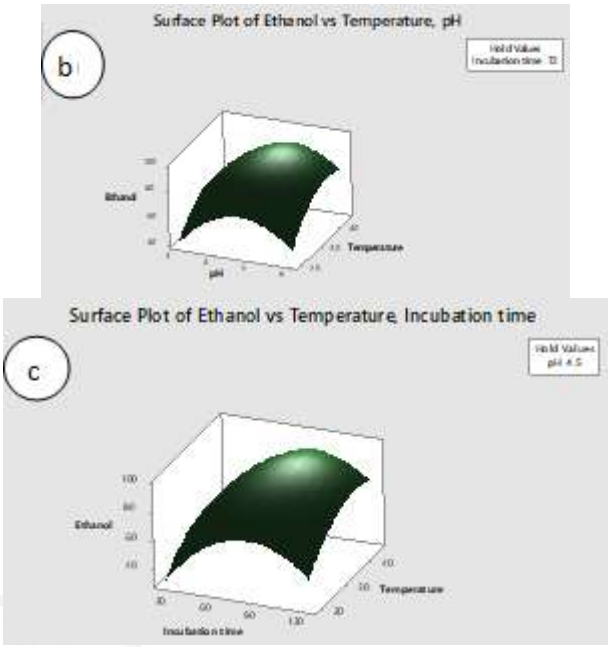


Figure 1(a): Response surface plot of temperature vs. pH on ethanol production, (b). Response surface plot of pH vs. incubation period on ethanol production, (c). Response surface plot of temperature vs. incubation period on ethanol production