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ARTICLE

Optimization of bioethanol production from saccharified sweet potato root flour by co-fermentation of *Saccharomyces cerevisiae* and *Pichia* sp. using OVAT and response surface methodologies

Preeti Krishna Dash[1], Sonali Mohaptra[1], Manas Ranjan Swain[2], Hrudayanath Thatoi[3]*

[1]Department of Biotechnology, College of Engineering and Technology, Biju Patnaik University of Technology, Bhubaneswar-751003, India
[2]Department of Biotechnology, Indian Institute of Technology, Madras, Chennai-600036, India
[3]Department of Biotechnology, North Orissa University, Takatpur, Baripada-757003, India

ABSTRACT In recent years with the increase in price of fossil fuels, the demand of biofuel production from tuber crops such as sweet potato has become very important to meet the future energy crisis in developing countries. In the present study, fermentation of saccharified sweet potato root flour (SPRF) was carried out using co-culture of cells of *Saccharomyces cerevisiae* and *Pichia* sp. in immobilized condition for bioethanol production. Various growth parameters like incubation time, fermentation medium pH, incubation temperature and inoculum size were initially optimized using one variable at a time (OVAT) methodology. Then, 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). The maximum ethanol yield of 127.2 ± 2 g/kg of SPRF was obtained at pH 5 with an incubation period of 72 h at 30 °C by OVAT methodology. RSM further enhanced the bioethanol yield to 138.6 ± 3 g/kg of SPRF with an overall increase of 8.22% as compared to the OVAT method.

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Introduction

The bioconversion of biomass resources in large-scale, especially starchy materials to ethanol, was expected to find its application in the production of biofuel (Shigechi et al. 2004). In the recent years, increase in the price of the fossil fuel is one of the major reasons for search of renewable fuels to meet the future energy demand (Liimatainen et al. 2004). The major constraint for industrial bioethanol production includes the collection of the raw material and its processing costs (Vucurovic et al. 2009). Hence, studies were focused to search for raw materials with high carbohydrate content and efficiently developed transformation processes for enhanced bioethanol production to meet the fuel crisis (Dias et al. 2012; Soccol et al. 2010). Bioethanol produced from tuber crops like sweet potato, cassava, potato, etc., is a promising option since it contains sufficient amount of starch (15-37%) that could be hydrolyzed to sugars and then fermented to ethanol (Lin et al. 2010). Among the tuber crops, sweet potato (*Ipomoea batatas* L.) represents an important biomass resource for fuel ethanol production due to high density of starch compared to other forms of biomass (Roukas 1994). It contains starch (178 g/kg), total sugars (26 g/kg) and protein (3.2 g/kg) on fresh weight basis (Tian et al. 1991).

Generally, the starchy substrates require a reaction of starch with water and enzymes (hydrolysis) to break down the starch into fermentable sugars by a process known as saccharification. The hydrolysis process involves the mixing of starch with water to form slurry, which is subjected to heat treatment for the cell wall to rupture. Enzymes, like α-amylase and glucoamylase, which are responsible for the breakdown of the chemical bonds in the starch, are applied at various times during the heat treatment (Badger 2002). The α-amylase randomly hydrolyzes internal α-1,4-glycosidic bonds in starch, and liberates soluble dextrin and oligosaccharides that are more suitable for efficient conversion to glucose. This process is the dextrinization. It is followed by saccharification, in which glucoamylase hydrolyzes 1,4 and 1,6-alpha linkages in liquefied starch and thereby converting

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*Corresponding author. E-mail: hnthatoi123@gmail.com

the starch to sugar (Van der Maarel et al. 2002). The microorganisms utilized in fermentation for ethanol production should have attributes like high ethanol yield, high productivity and have the capacity to withstand high concentrations of ethanol (Von Sivers and Zacchi 1996). The *Saccharomyces cerevisiae* is the most preferred organism for utilizing part of hexose sugar present in the substrate during fermentation of ethanol; however, a substantial amount of the sugar remains unutilized. Thus, use of another strain as co-culture can be preferred over a single strain (Pornkamol and Friedrich 2010). Recently, *Pichia stipitis* has seen to be a promising microorganism for industrial application as it gives high ethanol yield (Du Preez and Prior 1985), and able to ferment a wider range of sugars (Du Preez et al. 1986).

In the fermentation, the microorganisms are immobilized with suitable matrix, in order to use the strain in perpetual basis for an economical bioethanol production. Use of the immobilized strains for fermentation of ethanol have several advantages over freely suspended cells as the immobilized cells can be easily separated and recycled further (Giordano et al. 2008; Zhou et al. 2008). Co-immobilization of different kinds of microorganisms within the same porous matrix is a simple technique, which reduces the energy input and increases the efficiency of substrate utilization and the rate of production (Lee et al. 2012).

The response surface methodology (RSM) is extensively used in bioethanol production as this model predicts experimental modifications like changes in operational conditions, various processing steps, which ultimately help in designing an experimental setup with minimum requirements and maximum yields (Uncu and Cekmecelioglu 2011). RSM comprises of a group of mathematical and statistical procedure that can be used to study the optimization of culture conditions and it has already been successfully applied for optimization of media and culture conditions in many fermentation processes for production of ethanol, enzymes and amino acids (Kar et al. 2009). The aim of this work was to develop a simultaneous single-step system for bioethanol fermentation from saccharified sweet potato root flour, using co-immobilized cells of yeast *S. cerevisiae* and *Pichia* sp. at submerged fermentation condition. Further optimization of the major fermentation parameters was carried out by applying OVAT and response surface methodology (RSM) for enhanced bioethanol production.

Materials and Methods

Microorganisms

Saccharomyces cerevisiae used for alcoholic fermentation in factories and *Pichia* sp. isolated from toddy samples in our laboratory (strain CET) were adopted as the experimental strains. Both strains were maintained on potato dextrose agar medium (PDA; g/l: potato, 200; dextrose, 20; agar, 15; pH 7) and stored at 4 °C 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 $CaCl_2$ for overnight at 4 °C. Similarly, preparation of immobilized *Pichia* sp. cells was carried out by using this method. For co-fermentation, the immobilized beads of *S. cerevisiae* and *Pichia* sp. that were prepared separately, were mixed together in equal proportions and used for further studies.

Multiplication of immobilized yeast cells

In order to obtain a high yeast cell density, the cells were allowed to grow on the beads for 24 h before used in the fermentation. The gel beads, containing the immobilized yeast cells, were immersed in yeast extract peptone dextrose (YEPD) agar medium, [(g/l): yeast extract, 5; peptone, 5; glucose, 20; agar, 15; pH 5.5]. The immobilized cells of *S. cerevisiae* (5.3 x 10^9 CFU/ml) and *Pichia* sp. (5.3 x 10^9 CFU/ml) were grown separately in 250 ml Erlenmeyer flasks, containing 100 ml sterilized YEPD broth at 30 ºC for 24 h, and were used for further ethanol production experiments.

Substrate

Sweet potato (*Ipomoea batatas* L.) was purchased from the local market of Bhubaneswar, Odisha, India during the month of February, 2013. The tubers were washed thoroughly, to remove the dust and other debris, peeled off and chopped into small pieces. These sieves were then placed in oven at 70 °C for 24 h, still the moisture content reduced to 11-12% and were grinded into flour using a mixture grinder (Bajaj Pvt. Ltd, India) at 250 rpm. The flour was sieved through a steel mesh to get 2-3 mm diameter size and stored in airtight container for further use.

Enzymatic saccharification of SPRF

The sweet potato root flour (SPRF) 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 (sweet potato root flour:water) for experiment. In the first step the slurry was dextrinized by addition of 32 µl Palkolase-HT (Maps Enzymes, Ahmadabad, India) at pH 5.5 and 90 °C for 2 h, and then it was cooled down to room temperature. In the second

step, 329.7 µl of Palkodex (Maps Enzymes, Ahmadabad, India) was added to the cooled dextrinized slurry, and it was incubated at pH 4.5 and 60 °C for 24 h for saccharification.

Ethanol fermentation from saccharified SPRF

Ethanol fermentation was conducted by co-culture of cells of *S. cerevisiae* and *Pichia* sp. under anaerobic conditions in an Erlenmeyer flask (250 ml) sealed with rubber stopper equipped with opening for CO_2 venting. The saccharified SPRF (100 ml) was inoculated with freshly harvested co-fermentation cells of *S. cerevisiae* and *Pichia* sp. (10% w/v, 5.3 x 10^9 CFU/ml) aseptically. Flasks (n = 3) were incubated in an incubator-cum shaker at 30 ± 2 °C for 120 h with a constant shaking of 100 rpm. The fermented broth was distilled to recover ethanol using alcohol distillation apparatus (Borosil Glass Works, Mumbai, India).

Study of fermentation parameter

The saccharified SPRF slurry was inoculated with co-immobilized cells of *S. cerevisiae* and *Pichia* sp. and incubated at different incubation times (24-120 h). The fermentation medium (saccharified 10% SPRF slurry) with pH 3-5.5 at 0.5 interval was inoculated with immobilized cells of *Saccharomyces cerevisiae* and *Pichia* sp. separately and incubated at 30 °C for 72 h.

The saccharified 10% SPRF slurry at pH 5 was inoculated with co-immobilized cells and incubated for 72 h at different temperatures (20-40 °C). The fermentation medium with pH 5.0 was incubated at 30 °C for 72 h by 10% inoculums of *S. cerevisiae* and *Pichia* sp. comprising with different proportions (2:1, 1:1, and 1:2).

Analytical techniques

The ethanol concentration of the fermentation medium was determined based on the density of the alcohol distillates obtained from the fermentation broth. The density of the distilled alcohol sample was calculated as following: ρ (g/kg) = (W_{sample}-W)/(W_{water}-W), where W_{sample} is the mass of pycnometer, containing distilled ethanol from the sample, *i.e.* filtrated mash (50 ml) and then filled up with distilled water up to the marker level. W is the mass of clean and dry pycnometer (50 ml); W_{water} is the mass of the pycnometer filled up with distilled water. Ethanol concentration of the fermentation liquid was determined by measuring the specific gravity of the distillate according to the procedure described by Amerine and Ough (1984).The pH was measured using a pH meter (Systronics, Ahmadabad, India) fitted with a glass electrode.

Calculations

The maximum theoretical ethanol yield from sugar was calculated according to the stoichiometric relation represented by Equation 1. Accordingly, 100 g of hexose produces 51.1 g of ethanol and 48.9 g of CO_2. The fermentation efficiency (Y_1) and average ethanol productivity rate (Y_2) were calculated according to Equation 2 and 3.:

Eq. 1.

$$C_2H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

Eq. 2.

Y_1 = Ethanol produced in fermentation (g/kg starch) x 100/
Ethanol produced in theoretical (g/kg starch)

Eq. 3.

Y_2 = Final ethanol concentration/Fermentation time

RSM experimental design and optimization

The effect of different factors responsible for the ethanol production from SPRF was optimized using response surface methodology. The statistical model was studied by using Central Composite Design (CCD) experiments. Incubation time (A), pH (B) and temperature (C) were taken as independent variables as shown in Table 4. These three parameters were chosen as they were seen to influence the ethanol productivity to a more extent. Ethanol concentration was chosen as the dependent variable. The twenty experiments based on CCD were carried out with different combinations of variables and the results were presented in Table 2. The response was measured in terms of ethanol production, which was taken as the dependent variable.

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 (4) as it represents the behavior of such a system more appropriately.

Eq. 4.

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

The 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,

Table 1. Effect of incubation period on bioethanol production.

Time interval (h)	Ethanol production (g/kg)	Total sugar (g/kg)
0	0	224.8 ± 0.4
24	27.8 ± 0.9	194.8 ±0.3
48	74.6 ± 1	173.2 ± 0.5
72	127.2 ± 2	120.7 ± 0.6
96	127.9 ± 1.1	114.4 ± 0.4
120	127.6 ± 0.8	110.4 ± 0.8

Table 2. Effect of fermentation medium pH on bioethanol production.

pH	Ethanol production (g/kg)	Total sugar (g/kg)
3	11.8 ± 0.9	218.8 ± 0.3
3.5	28.6 ± 0.1	199.2 ± 0.3
4	57.2 ± 0.6	170.7 ± 0.9
4.5	91.9 ± 1.1	151.4 ± 0.9
5	129.4 ± 5	110.9 ± 0.1
5.5	130.1 ± 0.1	100.2 ± 0.2

Table 3. Effect of temperature on bioethanol production.

Temperature (°C)	Ethanol production (g/kg)	Total sugar (g/kg)
20	47.6 ± 0.6	182.7 ± 1.2
25	81.9 ± 1.9	143.5 ± 1.5
30	131.6 ± 3	107.6 ± 3
35	131.8 ± 0.9	104.3 ± 0.2
40	131.9 ± 0.1	94.3 ± 0.6
45	132.1 ± 2	75.3 ± 1.2

B^2, C^2, AB, AC and BC are level of independent variables. Statistical significance of the model equation 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. The optimum ethanol production was identified in the three-dimensional plot.

Results and Discussion

Optimization of SSF parameters following OVAT methodology

In the present study, different process parameters influencing the ethanol production of single immobilized *S. cerevisiae* and *Pichia* sp. were studied in conical flasks using enzymatic saccharified SPRF as substrate. Then, use of immobilized cells of *S. cerevisiae* and *Pichia* sp. was studied for ethanol production in laboratory scale. Optimization of the various parameters of saccharified SPRF was carried out using OVAT and RSM technologies. Four process parameters (incubation time, temperature, pH, and inoculum size) were considered, as these parameters mostly influence the growth of both yeast strains. All the experiments for OVAT were carried out in triplicate and data in Table 1, 2 and 3 are presented as mean ± SE (standard error).

Table 4. 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 (°C)	C	25	30	35

Comparison of single immobilization and co-fermentation for bioethanol production

The ethanol production from enzymatic saccharified SPRF was studied using immobilized *S. cerevisiae* and *Pichia* sp., separately in conical flasks. Ethanol production of 75.1 ± 2 g/kg and 62.6 ± 2 g/kg was obtained from single immobilized *S. cerevisiae* and *Pichia* sp., respectively, whereas, the co-immobilized cells of *S. cerevisiae* and *Pichia* sp. produced 120.5 ± 2 g/kg of ethanol. Since the co-immobilized cells demonstrated higher ethanol yield than single cultures, further process optimization was carried out with co-immobilized cells of *S. cerevisiae* and *Pichia* sp.

Effect of incubation period on ethanol production

The effect of incubation period on ethanol production from SPRF by co-immobilized strains of *S. cerevisiae* and *Pichia* sp. was studied. Ethanol production increased sharply up to 72 h and then a steady state was obtained, which was decrease in the glucose uptake capacity of the immobilized yeast cells. The sharp increase in the ethanol concentration may be due to the rapid utilization of the glucose by the two separately immobilized yeast strains. The maximum ethanol of 127.2 ± 2 g/kg of SPRF was obtained at 72 h of incubation with total sugar consumption up to 53.7% (Table 1). This may be due to rapid increase in biomass and ethanol concentration (Swain et al. 2007). In a study by Mohanty et al. (2009), maximum ethanol concentration was obtained after 72 h of incubation

from mahula (*Madhuca latifolia* L.) flowers. McGhee et al. (1996) studied the bioethanol production from molasses by separately immobilized cells of *S. cerevisiae*, *S. uvarum* and *Zymomonas mobilis* in calcium alginate. The immobilized *S. cerevisiae* was found to be the best alcohol producer yielding 4.7 g ethanol/100 g 10% glucose solution within 72 h. Improved ethanol production from sweet sorghum was achieved to 29.7 g ethanol/100 g dry sorghum stalks by using *Fusarium oxysporum* mixed culture with *Z. mobilis* (Swain et al. 2013). These studies also suggest that immobilized yeast cells with 72 h incubation time generally present high ethanol yield, which is in good agreement with our current study.

Effect of pH on ethanol production

The pH value of the fermentation medium is a very important factor for bioethanol production (Pirselova et al. 1993). The effect of pH on ethanol production yield of co-fermenting yeasts from saccharified sweet potato root flour was studied by conducting batch experiments at different pH ranging from pH 3 to 5.5 (Table 2). Ethanol concentration increased drastically with the increase in pH up to 5 and decreased beyond this value. The maximum ethanol concentration of 129.4 ± 5 g/kg of SPRF was obtained at pH 5 with a decrease in the total and reducing sugar. In another study by Lee et al. (2012), ethanol production of 3.05% (v/v) was achieved at an initial pH 4 followed by 2.65% (v/v) at pH 5, from sweet potato by the co-immobilization of *A. oryzae* and *S. cerevisiae*. In a study by Neelakandan and Usharani (2009), increasing the pH from 4 to 6 resulted increased ethanol production with highest production yield at pH 6 from cashew apple juice by immobilized *S. cerevisiae.* According to Narendranath and Power (2005), yeasts having pH optimum between 4 and 6 can grow under pH range conditions of 2.5 to 8.5. It was also shown that no ethanol production exists lower than pH 4 (Graves et al. 2006). The effect of pH on ethanol production was studied by Pitt (1993) using mixed cultures of *Zymomonas mobilis* and *Paenibacillus* sp. on raw starchy material from sweet potato. In a similar study, ethanol production from wheat bran flour was investigated by co-culturing of *Aspergillus niger* and *Kluyveromyces marxianus* and the optimum pH was found to be 5.5 (Manikandan and Viruthagiri 2010). According to Clarence et al. (2010), the pH of the fermentation medium significantly affects the growth of the microorganisms and ethanol production.

Effect of temperature on ethanol production

The amount of bioethanol production depends on the optimal temperature, because it influences sugar utilization by yeast cells (Manikandan and Viruthagiri 2010). In the present study, increase in the fermentation temperature from 20 to 45 °C significantly affected the ethanol concentration, ethanol productivity and fermentation efficiency and sugar concentration (Table 3). Both low (20 °C) and high (45 °C) temperature had negative effect on ethanol production. The maximum ethanol concentration (131.6 ± 3 g/kg SPRF) was obtained at 30 °C (pH 5) with an incubation period of 72 h. The total and reducing sugars decreased with the increase in the ethanol concentration. This was probably due to the decrease in viable cell number above 30 °C, because at higher temperatures the yeast cells degrade (Periyasamy et al. 2009). According to Hashem and Darwish (2011), high temperature affects the growth of some yeast strains, which inhibit the ethanol fermentation. Temperature range of 25 to 30 °C is found to be optimum for ethanol production of mesophilic *S. cerevisiae* strains in SSF of sweet sorghum (Mamma et al. 1996). The effect of temperature on ethanol production from glucose was studied using calcium alginate-immobilized *Candida tropicalis* and *S. cerevisiae* (Jamai et al. 2007). It was observed that the fermentation capacity and the ethanol production reduced by 25% at 40 °C. In another study, Shuler and Kargi (2002) achieved high ethanol production at temperature range between 30-35 °C from glucose.

Effect of inoculum size on ethanol production

The size of inoculum in ethanol fermentation has a great importance for completion of the fermentation process. Maximum ethanol concentration of 131.4 ± 4 g/kg of SPRF was achieved at 10% (1:1 = *S. cerevisiae*:*Pichia* sp.) inoculum concentration by utilizing (73.4% by *S. cerevisiae* and 75.1% by *Pichia* sp) of total sugars. A study of Swain et al. (2013), concluded that the maximum ethanol was obtained with a concentration of 10% (1:4 = *Trichoderma* sp.: *S. cerevisiae)* in sweet potato flour by co-culture of *Trichoderma* sp.: *S. cerevisiae.* In another study by Guo et al. (2008) co-immobilized mixed cultures of *Kluyveromyces marxianus* and *S. cerevisiae* were used for bioethanol production from cheese whey powder and the results revealed that maximum ethanol was produced using equal volume cells of *K. marxianus* and *S. cerevisiae*. In general, 3-10% (v/v) inoculum of *S. cerevisiae* has been reported for maximum bioethanol concentration from various substrates such as mahula (Maiti et al. 2011) and cashew apple (Tahir et al. 2010).

Cycles of the co-fermentation for ethanol production

In this study, to investigate the fermentation efficiency of immobilized cell recycling, repeated-batch fermentations were carried out and the results revealed that the immobilized cells were successfully recycled for three more times without much affecting the final ethanol concentration. There was a 3% decrease in ethanol production in the fourth cycle, which indicated a decrease in the efficiency of the immobilized yeast

Table 5. Growth and fermentation kinetics of immobilized microbial (*S. cerevisiae* and *Pichia* sp.) cells.

Growth and fermentation kinetics	Immobilized cells
Final ethanol (P, g/l)	22.7
Final biomass concentration (X, g/l)	5.23
Specific growth rate (μ, h)	0.098[a]
Cell yield (Yx/s, g/g)	0.013
Ethanol yield (Yp/s, g/g)	0.579
Volumetric substrate uptake (Qs, g/l/h)	0.431
Volumetric product productivity (Qp, g/l/h)	0.643
Conversion rate (%) into ethanol	82.8

Yp/s = Mass of ethanol formed/Mass of glucose consumed; Yx/s = Mass of yeast cell formed/Mass of glucose consumed; Qs = Substrate (glucose) uptake (g) per litre of hydrolysate per hour; Qp = Product formed (g) per litre of hydrolysate per hour; [a]μ (h^{-1}) = Standardized value (0.098) for specific growth rate of micro organism (yeast) under specific substrate (glucose) consumption

Table 6. Ethanol production by Box-Behnken factorial design.

Run	Time (h)	pH	Temp. (°C)	Ethanol (g/kg)
2	48.00	4.00	25.00	84.3
14	120.00	4.00	25.00	43.75
1	48.00	6.00	25.00	56.72
5	120.00	6.00	25.00	36.
6	48.00	4.00	35.00	31.4
12	120.00	4.00	35.00	18.3
19	48.00	6.00	35.00	64.
4	120.00	6.00	35.00	29.7
7	23.46	5.00	30.00	124.3
11	144.54	5.00	30.00	21.2
16	84.00	3.32	30.00	93.1
20	84.00	6.68	30.00	91.5
10	84.00	5.00	21.59	102.3
17	84.00	5.00	38.41	106.2
15	84.00	5.00	30.00	138.7
18	84.00	5.00	30.00	138.7

cells for ethanol production and hence no further cycles were repeated. The cells not only survived, but also were active physiologically yielding almost equal volume of bioethanol up to three cycles (131.3 ± 2, 128.7 ± 2, and 128.2 ± 2 g/kg of SPRF) without hampering the ethanol productivity. In recent years, many workers have used immobilized cell systems to ferment a wide variety of carbohydrates to ethanol. A similar study using co-immobilized yeast cells by Ghorbani et al. (2011) was reported, in which the immobilized cells were active up to four cycles of fermentation. However, there are some more reports where the immobilized cells were active for still longer period as evidenced from more number of cycles.

In a study by Amutha and Paramasamy (2001), the co-immobilized gel beads of *Saccharomyces diastaticus* and *Z. mobilis* on liquefied cassava starch were stable up to seven successive batches for ethanol production. The advantage of using co-immobilized cells was that the used cells survived and were active on the support used for immobilization for four cycles of fermentation, which could save considerable time and energy (Amutha and Paramasamy 2001).

The growth and fermentation kinetics of immobilized (Ca alginate) cells of *S. cerevisiae* and *Pichia* sp. were studied (Table 5). The ethanol concentration (P) obtained by co-immobilized cells was 22.7 g/l, and the volumetric substrate uptake was found to be 0.431 g/l/h. The ethanol yield and volumetric product productivity (Q_p) and (Qs) were found to be 0.643 g/l and 0.431 g/l/h, respectively, for the co-immobilized cells. The final sugar to ethanol conversion rate was found to be 82.8%. However, the final biomass concentration (X) was 5.23 after a successful fermentation and maximum ethanol fermentation. The above parametric optimization study from co-immobilized *S. cerevisiae* and *Pichia* sp. indicates co-fermentation is an efficient technique for ethanol production from saccharified SPRF. Similar study carried out by Behera et al. (2010), showed that, the ethanol concentration (P) obtained with immobilized cells using agar-agar and calcium alginate were 25.2 and 25.75 g/l, respectively, whereas, the volumetric substrate uptake (Qs) were found to be 0.552 and 0.554 g/l/h, respectively.

Statistical optimization and model validation

For further enhancement of ethanol production, a Box-Behnken factorial design was performed as given in Table 6. Since, the most important physical factors affecting the final ethanol yield were the temperature, pH and incubation time, these parameters were considered for the RSM methodology. In the present experiment, the inoculum size seemed to be less important in ethanol production and therefore it was not included in the RSM plots. It was observed that the predicted values that were obtained for ethanol production was in good agreement with RSM plots. The effects of the fermentation variables and their possible interactions were studied by ANOVA. Coefficients of a full model were evaluated by regression analysis and tested for their significance. Suitable levels for these parameters were determined using a statistical 2^3 full factorial design. Twenty experiments were performed for evaluation of ethanol production parameters by using co-immobilized *S. cerevisiae* and *Pichia* sp. The highest ethanol concentration 138.6 ± 3 g/kg substrate was obtained at 30 °C for 84 h, corresponding to an ethanol volumetric production rate of 0.48 g ethanol/l/h. However, the Q_p value was lower at 84 h. ANOVA performed on this models demonstrates that the models are statistically valid with *p*-values lower than 0.0001. ANOVA for model terms and its significance (*p*-values lower than 0.05 indicated that model terms were significant) showed linear effect of temperature, pH and incubation time on ethanol production (Table 7). The absence of interactions

Table 7. 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	284100	9	31570.12	21.61	<0.0001
A (Incubation period)	97814.39	1	97814.39	66.94	<0.0001
B (pH)	9860.29	1	9860.29	6.75	0.0266
C (Temperature)	4273.00	1	4273.00	2.92	0.1180
AB	330.89	1	330.89	0.23	0.6444
AC	1531.53	1	1531.53	1.05	0.3301
BC	42.55	1	42.55	0.029	0.8679
A^2	73318.17	1	73318.17	50.18	<0.0001
B^2	86656.81	1	86656.81	59.31	<0.0001
C^2	42760.24	1	42760.24	29.26	0.0003
Residual	14611.57	10	1461.16		
Lack of Fit	14611.57	10	1461.16	14611.57	10
Pure Error	0.000	5	0.000		
Cor Total	298700	19			
R-Squared	0.9511				
Adj R-Squared	0.9071				

between factors (p>0.05) may lead to the assumption that factors have an additive effect on the response. The relationship between the response and variables was visualized by the response surface or contour plot to see the influence of the parameters. The quadratic polynomial equations to experimental data (from Eq. 4) can be described by the response surface plots for ethanol production. The proportion of total variation attributed to each fit can be evaluated by the value of *R*-squared (a value of *R*-square>0.75 indicate the aptness of the model) (Haaland 1989). The regression equation obtained after ANOVA indicated an *R*-squared value of 0.951 that was in good agreement with the adjusted *R*-squared of 0.907. This ensured a satisfactory adjustment of the theoretical values to the experimental data by this model. The lack of fit is significant; however, *R*-squared value is high indicating that the models are well adapted to the responses. Therefore, the model is suitable to predict optimum ethanol production from the sweet potato flour by using co-immobilized *S. cerevisiae* and *Pichia* sp. The optimum values of the selected variables for ethanol production were obtained by solving the regression equation.

The highest R^2 value that was obtained in response can be explained by the second order polynomial equation giving the ethanol (406.7 g/kg) as a function of time (A), pH (B) and temperature (C). Using the results of the experiments, the following second order polynomial equation was obtained:

$$R1 = 405.43 + 84.63 \times A + 26.87 \times B + 17.69 \times C - 6.43 \times A \times B + 13.84 \times A \times C - 2.31 \times B \times C - 71.33 \times A^2 - 77.54 \times B^2 - 54.47 \times C^2$$

$$R1 = -4270.94961 + 10.18413 \times \text{Incubation period} + 831.15691 \times \text{pH} + 130.11842 \times \text{Temperature} - 0.17865 \times \text{Incubation period} \times \text{pH} + 0.076868 \times \text{Incubation period} \times \text{Temperature} - 0.46125 \times \text{pH} \times \text{Temperature} - 0.055036 \times \text{Incubation period}^2 - 77.54430 \times \text{pH}^2 - 2.17886 \times \text{Temperature}^2$$

A satisfactory correlation can be seen between the observed values and the predicted values in the parity plot (Fig. 1).

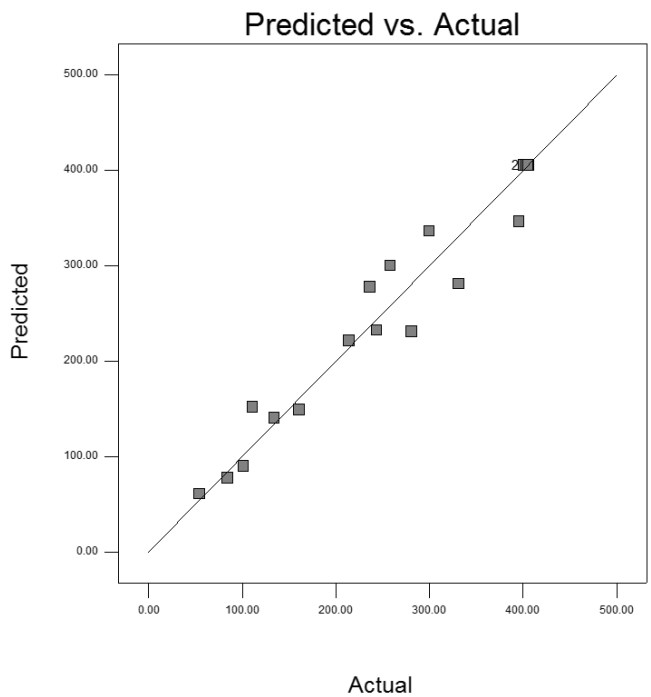

Figure 1. Parity graph showing the distribution of actual vs. predicted values of ethanol yield.

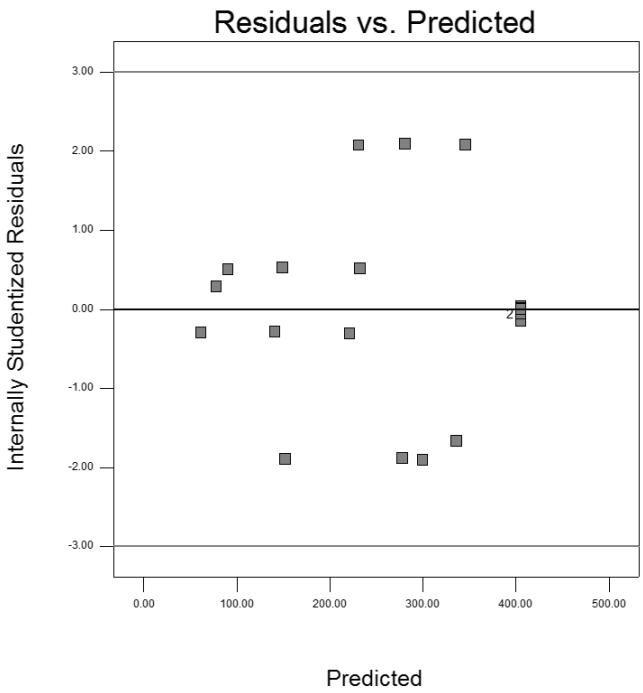

Figure 2. Parity graph showing the distribution of residual vs. predicted values of ethanol yield.

Additionally, the Parity graph showing the distribution of residual and predicted values of ethanol yield (Fig. 2). The clustered points around the diagonal line indicate good fit of the model since there is less deviation between the observed and predicted values. Figure 3 depicts the effects of the factors (incubation time, pH, and temperature) on ethanol production. The lines in the perturbation graph represent the influence and sensitivity of respective factors for ethanol production.

Interactions among the factors

Response surface was generated by plotting the response (bio-ethanol production) on the y-axis against any two independent variables on the x-axis, while keeping the other independent variables at zero level. Therefore, three response surfaces were obtained by considering the possible combinations. Figure 4 (a, b, c) represents the three-dimensional surface plots for the optimization conditions. The plot illustrates the main and the interactive effects of the independent variables on the dependent ones. The response surface plots were generated by plotting the response on the y-axis. Figure 4 (a) shows the effect of temperature and pH on ethanol production keeping the other variable (incubation period) at zero level. Ethanol production increased with the increase in temperature and pH, but it was observed that a further increase in these two variables reversed the trend. In the case of medium pH, ethanol production was increased up to pH 5 and then declined.

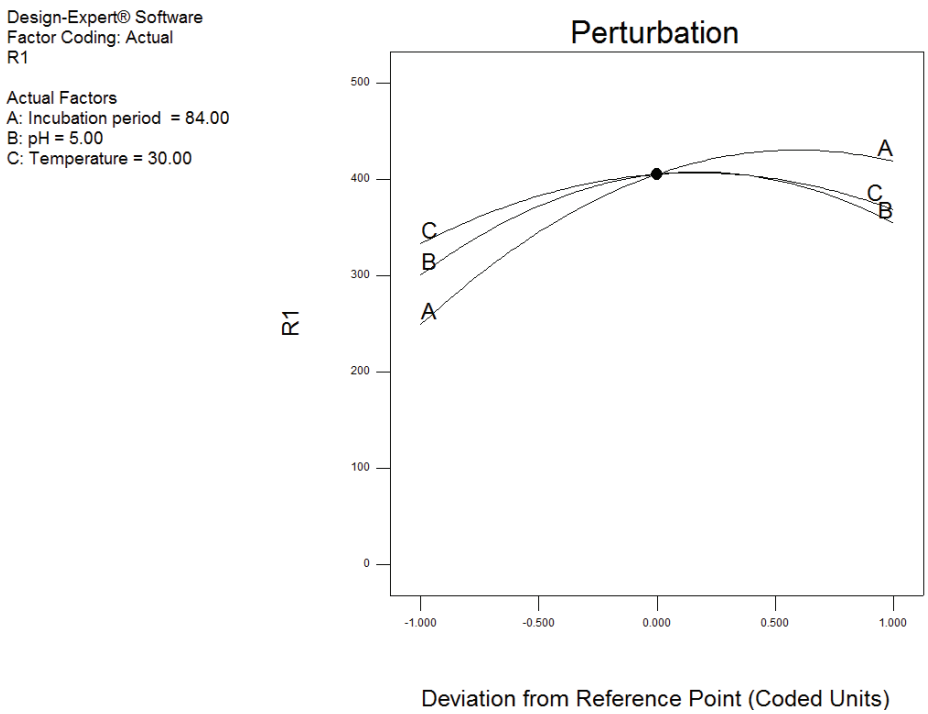

Figure 3. Perturbation graph representing the influence and sensitivity of respective factors for ethanol production.

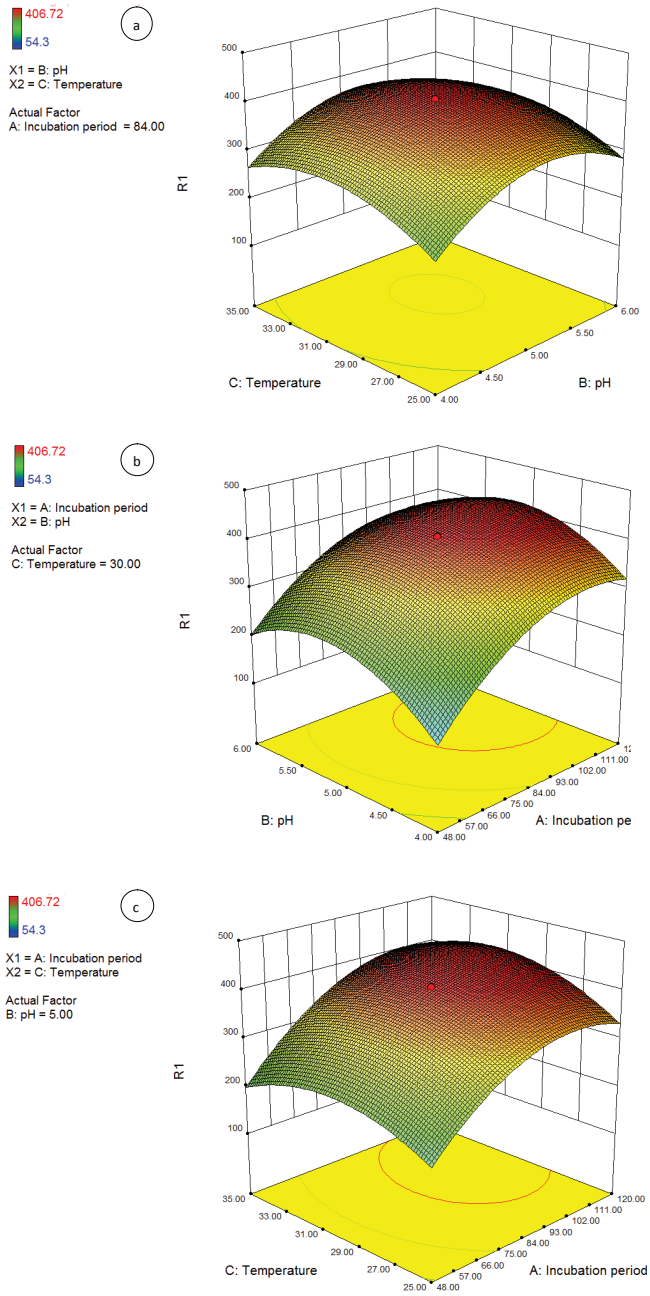

Figure 4. (a). Response surface plot of temperature vs. pH on ethanol production (time was kept constant at 72 h). (b). Response surface plot of pH vs. incubation period on ethanol production. (c). Response surface plot of temperature vs. incubation period on ethanol production.

When the level of incubation period was increased, a linear increase in ethanol production was recorded up to 84 h (Fig. 4b). The response between incubation period and temperature indicated that temperature at 30 °C was optimum with 84 h incubation period for bioethanol production (Fig. 4c).

The Model F-value of 21.61 implies the model is significant. Therefore, the model can be used to navigate the design space. The optimum values of temperature at 30 °C incubation time for 84 h, and at a medium pH of 5 were determined by the point prediction tool of the software with 98.93% validity. The results of the variables, temperature and pH agreed with the OVAT results, but a slight variation was observed for incubation time. For OVAT the incubation time was observed to be 72 h, whereas, for RSM it was predicted to be 84 h. Further, this variation did not hold any significant effect on ethanol production, with ethanol yields of 131.6 ± 2 and 138.7 ± 2 g/kg of substrate by OVAT and RSM, respectively. The present research shows that ethanol concentration can be increased in SPRF when fermented by co-immobilized cultures of *S. cerevisiae* and *Pichia* sp. under optimized medium and process parameters as developed by the response surface methodology. The result showed a similarity with the OVAT methodology results; hence, it could be inferred that the RSM model is useful to predict the optimization of the experimental conditions.

Conclusion

Sweet potato flour is plentily available in the Asia-Pacific regions, including Odisha (India). Being a cheap source of fermentable carbohydrate bio-resource, it could be employed to produce fuel ethanol. In the present investigation, maximum ethanol production from saccharified sweet potato flour in submerged fermentation was obtained at incubation period, 72 h; pH, 5; temperature, 30 °C; inoculums concentration [10% (1:1)] by using co-immobilized culture of *S. cerevisiae* and *Pichia* sp., which are active for three cycles. The present results revealed that co-immobilization is found to be a promising technique for bioethanol production from saccharified sweet potato flour as compared to pure culture and co-culture technique and it reduces the energy input and increases the efficiency of substrate utilization. Further, the ethanol concentration can be increased in SPRF, when fermented by co-immobilized cultures of *S. cerevisiae* and *Pichia.* sp. under optimized medium and process parameters as developed by the response surface methodology.

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