Production of Bio-ethanol by Enzymatic Saccharification through Response surface Method using Agro-waste

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Abstract: *A major constraint in the enzymatic saccharification of biomass for ethanol production is the cost of enzymes. Production cost of enzymes may be brought down by multifaceted approaches which include use of cheap lignocellulosic substrates for fermentation and production of enzymes, and use of cost efficient fermentation strategies like solid state fermentation (SSF). The focus of this study, firstly different agro-waste was washed with tap water for three times. Next wheat bran was screened from different agro-wastes. Then enzymatic saccharification of pre-treated wheat bran for high yield of reducing sugar using a purified enzymatic treatment from fungal isolate Aspergillus flavus PUF5. Central Composite Design was employed to plan experiments and optimize the enzymatic saccharification of wheat bran. Experimental results show that substrate amount, fermentation time and pH of the medium were main factors governing the enzymatic saccharification of wheat bran. The Model F value of 52.73 implied the Model was significant. Model P value in this study was < 0.01, which also indicated that the model was significant. The "Pred R–Squared" of 0.8982 was in reasonable agreement with the "Adj R-squared" of 0.9668, which indicated a good fitness of the model. On the basis of medium optimization, the quadratic model predicted that the maximum amount of reducing sugar was produced (36.4 mg/g) when the substrate amount, fermentation time and pH were 5.7 g, 16 h, and pH 6.5, respectively. The fermentation of the concentrated hydrolyzate (20 gL^{-1}) by Saccharomyces cerevesiae resulted in ethanol concentration of 7.9 gL^{-1}.*

Keywords: Response Surface Method, Bio-ethanol, Amylase, Saccharification, Agro-waste

INTRODUCTION:

Liquid bio-fuels, such as ethanol, produced from low-cost biomass are regarded as an attractive and alternative to fossil fuels to reduce dependence on oil and decrease carbon dioxide emissions, the prime cause of greenhouse effect (Demirbas, 2009; Hamelinck et al, 2005; Balat and Balat, 2009). Bio-ethanol production from lingo-cellulosic biomass is emerging as one of the most important technologies for sustainable production of renewable transportation fuels (Lin and Tanaka, 2006). Ethanol has a higher octane rating than gasoline and produces fewer emissions, therefore being widely recognized as a substitute and additive to gasoline. Due to these apparent advantages and also being a renewable alternative to existing transport fuels, there is now an increased interest in commercializing technologies for ethanol production from inexpensive biomass. In principle, ethanol could be obtained from any material containing simple or complex sugars. Currently, the main feedstock for bio-ethanol is starch-rich biomass (corn, wheat, and potato), hydrolysis of biomass is essential for generation of fermentable sugars which are then converted to ethanol by microbial action (Sukumaran et al, 2009; Serrano et al, 2009).

A promising approach to gradually introduce second generation bio-fuels may take advantage of the existing capacities and logistics of the present wheat-to-ethanol first generation processes (Palmarola-Adrados et al, 2005). Indeed, wheat bran contains a significant amount of sugars, such as hemi-cellulose, residual starch and cellulose, which could be converted to ethanol enhancing the overall alcohol efficiency of the plants (Favaro and Casella, 2013). In this perspective, wheat bran has the great potential to be a low-cost lingo-cellulosic feedstock for bio-ethanol and may be considered as a model of other cheap and abundant agricultural waste (Neves et al, 2006). Wheat bran starch can be hydrolysed by commercial amylases. Although the practice of converting starch to ethanol by an enzymatic process is a fairly mature technology, energy cost is high and the need to develop a more energy-efficient process is evident (Gray et al, 2006). A raw-starch hydrolysing and fermenting yeast could yield substantial cost reductions and improve the energy balance for the resulting one-step conversion of starch into ethanol. However, utilising also the hemi-cellulose/cellulose fraction of wheat bran could increase ethanol production but enzymatic hydrolysis is not enough to degrade these complex polysaccharides to simple sugars (Favaro et al, 2010). Reduction in production expense of ‘‘bio-ethanol’’ may also be obtained by good and proper technologies for saccharification which includes the use of better enzyme and optimized the conditions for hydrolysis.

Reduction in the cost of enzymes can be achieved only by conjunct efforts which address several points of enzyme production from the raw material used for production to microbial strain improvement. Use of low-priced raw materials and lucrative fermentation strategies like solid state fermentation can boost the economics of enzyme production (Sukumaran et al, 2009). In this present study, starch hydrolysis methods have been evaluated. Firstly, bran was subjected to liquefaction and saccharification in order to hydrolyze the starch fraction. The resulting material was separated into a glucose-rich liquid and a solid that was subsequently washed. This solid material, denoted starch-free bran (SFB), was then hydrolyzed by enzymatic hydrolysis (Jurado et al, 2009).

Materials and methods

Substrate collection

Ribbed gourd peel, banana peel, potato peel and wheat bran (≤5 mm mesh size) were used as a solid substrate for enzyme production. The substrate was collected from local market of Uluberia (Howrah, India). The substrate was stored at room temperature without any pre-treatment.

Substrate Pre-treatment

Banana peel, potato peel and wheat bran were washed with tap water for 4 to 5 times to remove the external sugars. Then finally used for the enzyme production.

Microorganism and inoculum preparation

The *Aspergillus flavus* PUF5 was grown on Czapek- Dox agar (composition in % w/v: glucose 5, sodium nitrate 0.2, magnesium sulfate 0.05, potassium chloride 0.05, iron (III) sulfate 0.001, di-potassium hydrogen phosphate 0.1, agar 1.75; pH 5.0) slants at 30°C for 5 days. Fully sporulated slants were used immediately or stored at 4°C for further use. A conidial suspension was prepared in sterile distilled water with a spore count of 10^6-10^7 spores/ml.

Enzyme production

Enzyme was produced from *Aspergillus flavus* PUF5 through solid state fermentation of ribbed gourd peel and wheat bran (3:2 ratio) under pre optimized condition. The extracted crude amylase enzyme (260U/ml) was used during the saccharification process.

Screening of suitable agro-waste for enzymatic saccharification

To screen suitable substrate for enzymatic saccharification process, potato peel, banana peel and wheat bran were taken. 4 g of substrate was moistened with 20 ml of buffer solution (50mM, pH-6) containing 0.5ml enzyme solution (substrate to moisture ratio 1:5 w/v) and incubated for 24h at 40°C. The most suitable substrate was chosen depending on the yield of reducing sugar.

Enzymatic saccharification following Central Composite Design (CCD)

The effects of substrate amount (g), incubation time (h) and pH on enzymatic saccharification process was evaluated through central composite response surface design. These factors were chosen as they showed influencing effects in OVAT (one variable at time) optimization (data not shown). Table 1 represents different selected factors where each variable was tested in three different coded levels: low (−1), middle (0) and high (+1). Table 2 represents a 17- trial of the experimental design.

Ethanol fermentation

Ethanol production was studied using the enzymatic hydrolysate of wheat bran extract. For ethanol production, submerged fermentation schharified wheat bran extract was concentrated at $20gL^{-1}$ of reducing sugars and sterilized at 121°C for 15 min. The microorganism was a lab based strain of *Saccharomyces cerevisiae*. To start the fermentation one loop of yeast culture was suspended in submerged in 250 ml Erlenmeyer flasks. The flasks were shaken at 80 rpm on a BOD incubator shaker at 40 ± 0.1°C for 72 h. The yeasts were centrifuged after the end of the fermentative cycle at 3000 rpm for 20 min. The concentration of total ethanol was determined using the dichromate method (AOAC 1990).

Results and discussions

Screening of suitable agro-waste for saccharification process

To screen suitable substrate for enzymatic saccharification process, potato peel, banana peel and wheat bran were used. The substrates were washed in distilled water, dried prior the experiment. From the result shown in fig 1 it was observed that among the substrates, wheat bran supported maximum yield of reducing sugar of 22.6 mg/g after 24h incubation under shaking condition. Wheat bran is one of the most abundant agricultural by-products, presents a low commercial value and most of it is being used as cattle feed and waste. In terms of total production, wheat is the second most important grain crop in the world. So the result clearly justifies the need to consider wheat bran as a complementary source of raw material for the production of bio-ethanol.

Optimization of enzymatic saccharification through CCD

The optimal level of the key factors and the effect of their interactions on enzymatic saccharification process were studied by central composite response surface design. Experimental design and results are

shown in Table 2. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was established to explain the α-amylase production:

$Y = 36.08+1.43A-77B+0.43C-0.79AB+0.44AC+1.66BC-3.36\ A^2-2.93B^2-2.95C^2$

Where A, B and C are the coded values of pH, substrate amount and incubation times, respectively. The analysis of variants (ANOVA) was conducted to test the significance of the fit of the second order polynomial equation for the experimental data as shown in Table. 3.

The Model F value of 52.73 implies the Model is significant. There is only a 0.01 % chance that a "Model F value" could occur due to noise. Model P value in this study is < 0.01, which also indicates that the model was significant. The "Pred R – Squared" of 0.8982 is in reasonable agreement with the "Adj R-squared" of 0.9668. A ratio greater than 4 is desirable. The ratio of 21.342 indicates an adequate signal. The response surface plots and the contour plots are shown in Figure 1. Shapes of response surfaces and contour plots indicate the nature and extent of the interaction between different factors (Prakash et al, 2008). Circular contour plots generally indicate a less prominent or negligible interactions, while elliptical contour plots indicates comparatively prominent interactions. Ferreira et al. (2009) showed that the maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram. Previous studies also reported that elliptical contours are obtained when there is a perfect interaction between the independent variables (Li et al, 2007; Xiao et al, 2007). On the basis of medium optimization, the quadratic model predicted that the maximum amount of reducing sugar was produced (36.4 mg/g) when the substrate amount, fermentation time and pH were 5.7 g, 16 h, and pH 6.5, respectively.

Bio-ethanol production

The extracted hydrolyzate obtained from CCD was used as a substrate for bio-ethanol production using *S. cerevisiae*. Fermentation was performed using 20 gL^{-1} of initial reducing sugar concentration and the ethanol production up-to 4 days with pre 24h of fermentation. Maximum ethanol concentration of 7.9 gL^{-1} was obtained. Currently, ethanol is widely considered to be one of the most important alternatives to petroleum. Agricultural wastes, due to their abundance and low cost, have become attractive raw materials for ethanol production (Tabka et al, 2006).

Conclusion

Lignocellulogic materials represent the most abundant and cost effective biomass in the world. Their use allows either the production of a valuable bio-fuel and the utilization of a variety of residues of domestic, agricultural, or industrial activities. In the current work, response surface methodology was applied for the optimization of bio-saccharification of wheat bran through purified amylase from *Aspergillus flavus* PUF5. The model developed for CCD had R^2 values of 0.8982 for saccharification process, which was in reasonable agreement with the "Adj R-squared" of 0.9668. The optimum values of the variables obtained from experimental results were: substrate amount- 5.7g, saccharificationtime-16h and pH– 6.5, when the maximum amount of reducing sugar produced was 36.4 mg/g. Fermentation of the concentrated hydrolyzate obtained after enzymatic hydrolysis by *S. cerevisiae* cells resulted in an ethanol concentration of 57.9 gL^{-1}.However, some other processes of pre-treatments have to be further investigated for increment of glucose concentration after enzymatic hydrolysis.

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Table 1. Factors coded with different levels for Box-Behnken response surface methodology

Independent variable	Code units	Coded variable level		
		-1	0	+1
pH	A	4	6	8
Substrate amount (g)	B	4	6	8
Incubation time (h)	C	8	16	24

Table 2. Box-Behnken experiments design with experimental and predicted values for alpha amylase production

Run	Factor 1 A : pH	Factor 2 B : substrate amount (g)	Factor 3 C : Incubation time (h)	Response 1 Reducing sugar (mg/g)	Predicted value	Actual value
1	4.00	4.00	8.00	26.8	27.05	26.80
2	8.00	4.00	24	29.1	29.03	29.10
3	8.00	8.00	8.00	24.2	24.18	24.20
4	8.00	4.00	8.00	30.7	30.61	30.70
5	6.00	6.00	29.45	29.1	28.46	29.10
6	6.00	6.00	16.00	35.5	36.08	35.50
7	6.00	2.64	16.00	29.2	29.07	29.20
8	4.00	8.00	8.00	23.1	23.77	23.10
9	2.64	6.00	16.00	25.2	24.17	25.20
10	6.00	9.36	16.00	27.2	26.49	27.20
11	6.00	6.00	16.00	36.4	36.08	36.40
12	8.00	8.00	24.00	28.9	29.24	28.90
13	6.00	6.00	2.55	27.2	27.00	27.20
14	4.00	4.00	24.00	23.1	23.71	23.10
15	8.00	8.00	24.00	26.4	27.08	26.40
16	6.00	6.00	16.00	28.8	28.99	28.80
17	6.00	6.00	16.00	36.2	36.08	36.20

Table 3. Analysis of variants (ANOVA) for alpha amylase in second order polynomial model

Source	Sum of squares	Degree of freedom	F value	Probe > F
Model	270.99	9	52.73	< 0.0001
A	28.00	1	49.04	0.0002
B	8.02	1	14.04	0.0072
C	2.54	1	4.46	0.0727
AB	4.96	1	8.69	0.0215
AC	1.53	1	2.68	0.1455
BC	22.11	1	38.73	0.0004
A^2	127.22	1	222.80	< 0.0001
B^2	97.11	1	170.08	< 0.0001
C^2	98.28	1	172.13	< 0.0001
Residual	4.00	7		
Lack of fit	3.55	5	3.18	0.2564
Cor total	274.98	16		

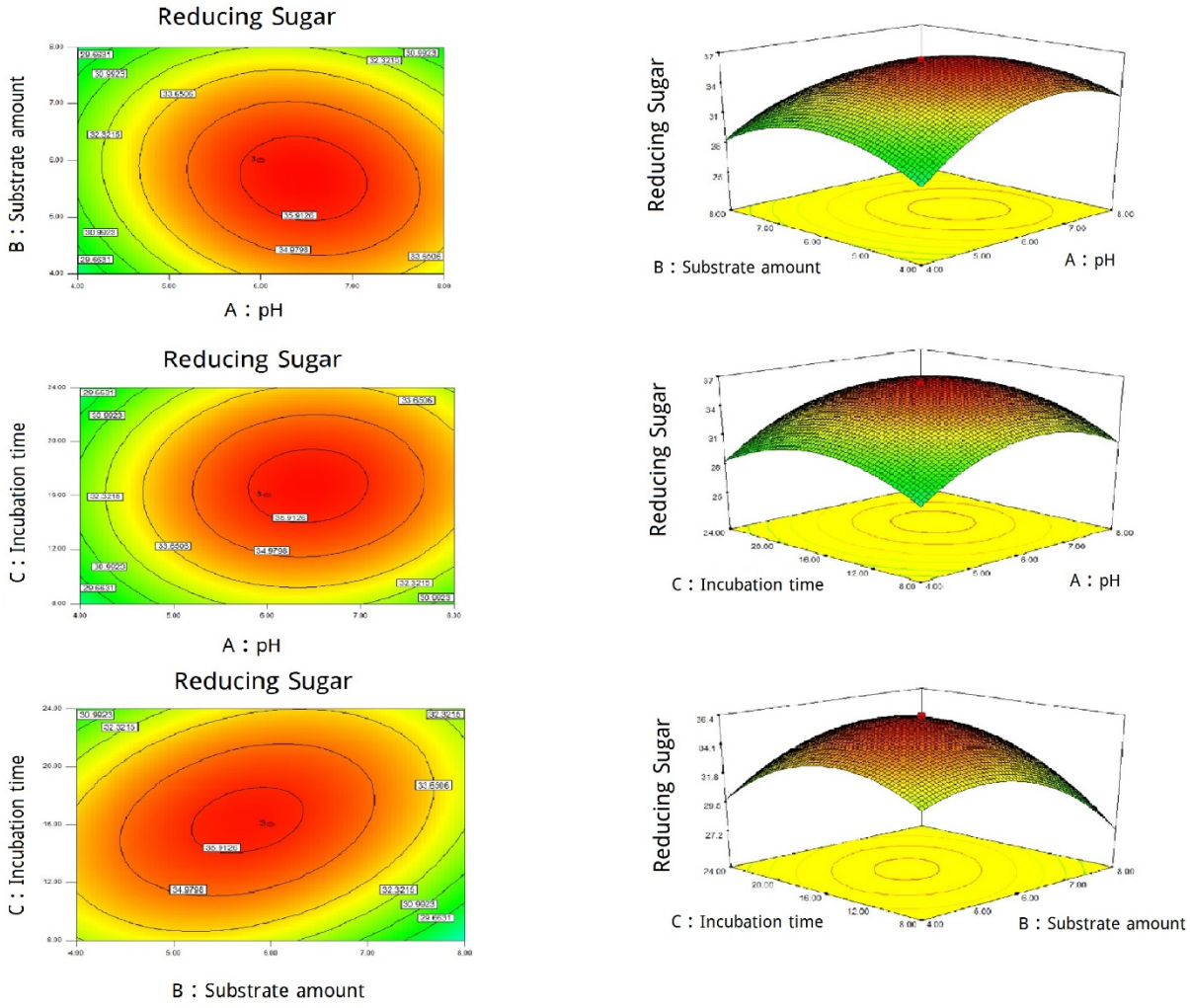

Figure 1. Response surface plots and the contour plots, showing the effects of different factors on alpha amylase production.