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Optimization of nutritional composition and fermentation conditions for cellulase and pectinase production by *Aspergillus oryzae* using response surface methodology

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Abstract

Cellulase and pectinase are enzymes having a broad industrial and commercial application. These enzymes can be produced from solid state fermentation using agro–industrial residues as substrates. The objective of this study is to optimize nutritional composition and fermentation conditions for the production of both cellulase and pectinase by *Aspergillus oryzae* under solid state fermentation using response surface methodology. Soybean residue, sucrose, urea and $MnSO_4.H_2O$ were good nutrients for *A. oryzae* producing both cellulase and pectinase. Using Box-behnken design with four factors (moisture content, pH, fermentation temperature and fermentation duration) at three level, the optimum condition for *A. oryzae* producing the highest cellulase and pectinase activities (6.01 FPU/gds and 139.56 U/gds, respectively) was 67% of moisture content, pH 5.9, 33°C and 71.8 h of fermentation. As a result, the activities of both cellulase and pectinase produced by *A. oryzae* under optimized conditions in this study significantly increased as compared to the traditional one-at-a-time optimization.

Introduction

Solid state fermentation (SSF) is identified as a fermentation process occurring in the absence or near-absence of free water by employing a natural solid substrate. Many microorganisms are capable of growing on solid substrates such as filamentous fungi, which can grow to significant extent in the absence of free water. Agro-industrial residues are generally considered as suitable substrates for the production of enzymes, especially cellulase and pectinase, in solid state fermentation (Pandey *et al*., 2003).

Cellulases and pectinases are two enzymes which hydrolyze two main substances in plant cells, cellulose and pectin, respectively, into glucose. These enzymes are widely produced by fungi, bacteria, and protozoans. *Aspergillus niger* in the most commonly used fungal species for the industrial production of pectinase (Kotzekidov, 1991), whereas cellulases can be produced by various kinds of fungi (Enari *et al*., 1983). Cellulases and pectinases are known to have a broad industrial and commercial application. Cellulases can be used in food, animal feed, textile, fuel, chemical industries, paper and pulp industry, protoplast production, genetic engineering, and pollution treatment, whereas pectinases are applied in juice processing (extraction and clarification), vegetable oil extraction, alcoholic beverage processing and other food industries (Bhat *et al.*, 2000; Kumar *et al*., 2011).

The production of cellulase or pectinase by microorganisms has been reported to be highly affected by the composition of the growth medium and fermentation conditions. The nutritional composition sources such as carbon and nitrogen sources and mineral compositions as well as physical parameters such as temperature, pH, moisture content and incubation time were found to be the main factors affected the production of cellulase and pectinase (Urmila *et al*., 2005; Vinod *et al*., 2006; Kathiresan *et al*., 2006; Polyanna *et al.*, 2011). Although cellulase or pectinase production has been widely studied and reported using different cheap raw materials such as sugar cane bagasse (Silva *et al*., 2002), rice husk (Bo-Hwa *et al*., 2010), rice bran (Bo-Hwa *et al*., 2010), grapefruit peel (Sarvamangala *et al.*, 2006), soybean (Des *et al.*, 2003) and other food processing waste (Zhen *et al*., 2000) under solid state fermentation, these studies have been done for the production of cellulase or pectinase separately and independently. Recently, cellulose and pectinase were used for the liquefaction and saccharification of biomass or food processing such as coffee bean fermentation or peppercorn fermentation, both cellulase and pectinase were necessarily used to hydrolyze cellulose and pectin in biomass or remove the husks of coffee or

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peppercorn grains. Commonly, commercial cellulase and pectinase were mixed together to use for food processing, which increases the cost of products (Wilkins *et al*., 2007). Therefore, it is necessary to study on the production of both cellulase and pectinase by the same microorganism under same fermentation conditions, which can degrade both cellulose and pectin of the husk of grain for producing high quality of product.

Response surface methodology (RSM) has been used as one of the most practical optimization methods. This method is used to identify effects of individual variables and to efficiently seek the optimum conditions for multivariable system (Kumar *et al*., 2011). The objective of this study is to optimize nutritional compositions (substrate, carbon, nitrogen and metal sources) and fermentation conditions (temperature, pH, moisture content and incubation time) for *Aspergillus oryzae* producing both cellulase and pectinase under solid state fermentation using a combination of one factor at a time approach followed by response surface methodology using Box–Behnken design.

Materials and Methods

Materials

Aspergillus oryzae, isolated from pepper plant and identified using universal 28S rRNA primer for fungi, was used in this study. The strain was propagated extracted malt agar, incubated at 30°C for 5 days. After this period, the sporulated cultures were stored at 4°C or used for the inoculation of culture medium.

Rice husk and soybean residue were collected from local markets. Materials were dried at 70°C for 24 h and then ground to small particles size. Grapefruit peel and sugar cane bagasse were collected from local markets. They were washed in tap water and then followed the same procedure described above. Rice bran was obtained as a commercial product, then dried and used.

Standard condition for solid state fermentation

Solid state fermentation was carried out according to the method previously described by Leda *et al.* (2000). A 250-ml Erlenmeyer flask containing 10 g of substrate, 0.1 g glucose as carbon source, 0.1 g yeast extract as nitrogen source and 0.1 g $CaCl_2$ as metal source was used for fermentation. Citrate phosphate buffer (pH 6) was used to adjust moisture content of medium to 65%. The medium was then sterilized at 121°C for 30 min. The Erlenmeyer flask was inoculated with 10^8 spores from spore suspension prepared in Tween-80 (0.1%, v/v) and then incubated at 30°C for 48 h.

Optimization of medium composition by one factor at a time method

Different solid substrates (rice bran, rice husk, soybean residue, grapefruit peel or sugarcane bagasse), carbon sources (glucose, sucrose, maltose, sodium acetate or pectin), nitrogen sources (yeast extract, peptone, urea, tryptone or ammonium sulfate), and metal sources ($CaCl_2$, $ZnSO_4$, $MgSO_4$, $MnSO_4$. H_2O or KCl) were used to optimize the medium for producing high activities of both cellulase and pectinase by *A. oryzae*. For each condition, the one factor was changed while other factors were used as same as the above standard condition. Firstly, 10 g of each substrate (rice bran, rice husk, soybean residue, grapefruit peel or sugarcane bagasse), 0.1 g glucose as carbon source, 0.1 g yeast extract as nitrogen source and 0.1 g $CaCl_2$ as metal source were used for fermentation in order to determine effect of substrates on production of cellulase and pectinase by *A. oryzae*. After the best substrate was determined, it was used to determine effect of carbon sources on the production of cellulase and pectinase by *A. oryzae* with changing the carbon sources (glucose, sucrose, maltose, sodium acetate or pectin) in the standard condition for fermentation. The best nitrogen and metal sources were also determined in the same way.

Box-Behnken design

Box-Behnken design was used to optimize the fermentation conditions for all variable factors. Four important parameters, initial moisture content (X_1), initial pH (X_2), incubation temperature (X_3) and incubation time (X_4) were chosen as the independent variables and cellulase activity (Y_1), pectinase activity (Y_2) were the dependent response variables. Three different levels were studied for each independent variable including initial moisture contents at 60, 65 and 70%; initial pHs at 5, 5.5 and 6; incubation temperatures at 25, 30 and 35°C; and incubation times for 48, 60 and 72 h as shown in Table 1. Total 27 experiments were required for four independent variables.

The quadratic response function for n variables with interaction terms was considered for the mathematical relationship between independent and dependent variables.

$$Y=\beta_0+\sum_{i=1}^{n}\beta_i X_i+\sum_{i=1}^{n}\beta_{ii} X_i^2+\sum\sum_{i<j=1}^{n}\beta_{ij} X_i X_j$$

Where: Y is the measure response; β_0, β_i, β_j and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j are the coded value of the i^{th} and j^{th} independent variables. The variables X_iX_j represents the first order interaction between X_i and X_j for (i<j).

Pectinase and cellulase activities

Pectinase activity was determined by measuring the release of reducing groups using 3, 5-dinitrosalicylic acid (DNS) method with pectin as a substrate (Jonaina *et al*., 2010). A volume (1000 µl) of substrate (0.5% solution of citrus pectin) in acetate buffer (pH 4.4) was incubated at 40°C for 15 min. Then, 500 µl of enzyme extract was added and the mixture was incubated at 40°C for 6 min. The absorbance was measured in spectrophotometer at 540 nm (Miller, 1956). One pectinase unit was defined as an amount of enzyme that liberates 1 µmol of D-galacturonic acid per minute of reaction (U = mol/min). The enzymatic activity was expressed as units per gram of dry substrate (U/gds).

Cellulase activity was measured by determination of filter paper cellulase activity (FPA) according to US National Renewable Energy Laboratory (NREL) (Adney *et al*., 1996). The reaction solution was incubated at 50°C for 60 min, and the content of released reducing sugar was assayed using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1956). One unit (U) of enzyme activity was defined as an amount of enzyme required to release 1 µmol of reducing sugar per minute under assay conditions. The enzymatic activity was expressed as units per gram of dry substrate (FPU/gds).

Statistical analysis

Analysis of variance (one-way ANOVA) was performed using Duncan's multiple-rang test to compare treatment means at $P < 0.05$ using SPSS software version 16 (SPSS Inc., USA).

Design Expert software (version 7.0.0, STAT-EASE Inc., Minneapolis, MN, USA) was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots. The validity and adequacy of the predictive models were done by experimental analysis at suggested optimum conditions by the design expert.

Results and Discussions

Optimization of components medium

Cellulase and pectinase activities produced by *A. oryzae* under solid state fermentation (SSF) with

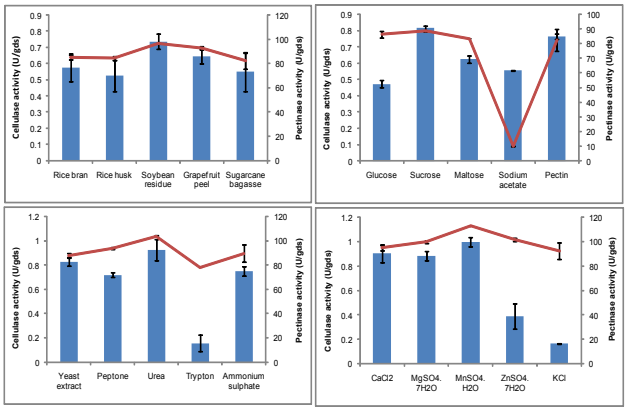

Figure 1. One factor at a time optimization of nutritional compositions for producing high activities of both cellulase and pectinase by *A. oryzae*. A, Substrates; B, Carbon sources; C, Nitrogen sources; D, Metal sources. The bars show cellulase activities (U/gds) and the lines show pectinase activities (U/gds).

different solid substrates including rice bran, rice husk, soybean residue, grapefruit peel or sugarcane bagasse are shown in Figure 1A. The results show that soybean residue was the best substrate for *A. oryzae* producing both cellulase and pectinase activities (0.74 ± 0.05 FPU/gds and 96.34 ± 0.82 U/gds, respectively), which were significantly higher than those obtained using other substrates. Kumar *et al.* (2011) reported that both cellulase and pectinase produced by *A. niger* NCIM having maximum activities with combination of substrates of wheat bran, corn bran and kinnow peel at ratio of 1:2:1. Another study also indicated that wheat bran was the best substrate for *A. oryzae* CCT3940 producing pectinase (Eloane *et al.*, 2004).

Carbon sources play a vital role in the cell metabolism and synthesis of cellulase and pectinase (Gautam *et al*., 2010). The effect of carbon sources on the enzyme production by *A. oryzae* was investigated (Figure 1B). Sucrose was found to be the best carbon source for both cellulase and pectinase production by *A. oryzae*. The highest cellulase and pectinase activities produced by *A. oryzae* were 0.82 ± 0.02 FPU/gds and 88.61 ± 0.44 U/gds, respectively. Another study reported that cellulose was as a carbon source for *A. niger* produced maximal cellulase production (Gautam *et al*., 2010).

Different nitrogen sources such as yeast extract, peptone, urea, tryptone and ammonium sulfate for the production of cellulase and pectinase by *A. oryzae* were investigated (Figure 1C). Cellulase and pectinase activities produced by *A. oryzae* were the highest when the urea was used as a nitrogen source (0.92 ± 0.09 FPU/gds and 104.07 ± 0.16 U/gds, respectively). Other studies showed that peptone was the best nitrogen source for pectinase production by *A. niger* (Neeta *et al*., 2011), whereas $NaNO_3$ and NH_4NO_3, peptone, NH_4SO_3 and $NH_4H_2PO_3$ were the

Table 1. The coded level of variables chosen for the experiments

Variable	Coded	Range and level		
		-1	0	+1
Initial moisture content, (%)	X_1	60	65	70
Initial pH	X_2	5	5.5	6
Incubation Temperature (°C)	X_3	25	30	35
Incubation time (h)	X_4	48	60	72

Table 2. Coefficients of the response function to predict cellulase and pectinase activities by regression analysis

Factor	Coefficient Estimate	
	Cellulase activity	Pectinase activity
Intercept	3.39	147.48
X_1	-0.21	8.57
X_2	0.59	2.35
X_3	0.79	-7.63
X_4	0.76	10.75
X_1X_2	0.21	-0.06
X_1X_3	0.42	0.12
X_1X_4	0.26	1.70
X_2X_3	0.13	-0.95
X_2X_4	0.12	2.94
X_3X_4	1.08	-3.34
X_1^2	0.73	8.27
X_2^2	1.45	1.38
X_3^2	-0.98	-16.52
X_4^2	-0.20	-12.77

X_1: Initial moisture content, X_2: Initial pH, X_3: Incubation temperature, X_4: incubation time

best nitrogen source for production of cellulase by *Trichoderma reesei* 1433 (Khare *et al*., 2011), *A. niger* (Acharya *et al*., 2008), *A. niger* YL 128 (Mohammed *et al*., 2010), respectively.

Effect of metal sources in producing both cellulase and pectinase by *A. niger* is given in Figure 1C. $MnSO_4.H_2O$ was found to be the best metal source for A. oryzae producing the highest cellulase and pectinase activities (1.00 ± 0.04 U/gds and 113.2 ± 0.00 U/gds, respectively). However, NaCl and KCl were good metal sources for *A. niger* producing cellulase (Mohammed *et al*., 2010), and NaCl was a suitable metal for *A. niger* producing maximal pectinase activity (Vinod *et al*., 2006).

Box-Behnken design

Fermentation conditions including initial moisture content, initial pH, incubation temperature and incubation time were also important for the growth of *A. oryzae*, which were optimized by using Box-Behnkent design. Table 1 shows the four independent variables (actual values) and dependent variables (cellulase and pectinase activities) in the design matrix.

The polynomials proposed models for cellulase and pectinase activities under SSF conditions regressed by considering the significant terms. The data was analyzed by multiple regression analysis and the regression coefficients for equation were determined (Table 2).

$$Y_1 = 3.39 - 0.21X_1 + 0.59X_2 + 0.79X_3 + 0.76X_4 + 0.21X_1X_2 + 0.42X_1X_2 + 0.26X_1X_3 + 0.13X_2X_3 + 012X_2X_4 + 1.08X_3X_4 + 0.73X_1^2 + 1.45X_2^2 - 0.98X_3^2 - 0.20X_4^2$$

Table 3. Analysis of variance for the response surface quadratic model

Source	Cellulase			Pectinase	
	df	F-Value	Prob > F	F-Value	Prob > F
Model	14	4.57	0.0061*	8.75	0.0003*
X_1	1	0.80	0.39	21.63	0.0006*
X_2	1	6.38	0.0266*	1.62	0.23
X_3	1	11.35	0.0056*	17.17	0.0014*
X_4	1	10.34	0.0074*	34.03	0.0001*
X_1X_2	1	0.71	0.42	0.00	0.97
X_1X_3	1	2.80	0.12	0.00	0.95
X_1X_4	1	1.10	0.31	0.75	0.40
X_2X_3	1	0.25	0.62	0.23	0.64
X_2X_4	1	0.22	0.64	2.26	0.16
X_3X_4	1	18.63	0.0010*	2.93	0.11
X_1^2	1	1.37	0.26	2.88	0.12
X_2^2	1	5.46	0.0376*	0.08	0.78
X_3^2	1	2.50	0.14	11.49	0.0054*
X_4^2	1	0.10	0.76	6.86	0.0224*
Residual	12				
Lack of Fit	10	0.96	0.61	3.04	0.27
R^2		0.84		0.91	

Table 4. Optimization of process variables with respect to cellulase and pectinase activities under SSF

		Optimum values (in a range)	Optimum values (targeted)
Variables	Initial moisture content (%)	67.21	67
	Initial pH	5.91	5.9
	Incubation temperature (°C)	32.95	33
	Incubation time (h)	71.86	71.8
		Predicted values	Experimental values
Responses	Cellulase activity (FPU/gds)	6.69	6.01
	Pectinase activity (U/gds)	144.38	139.56

$$Y_2 = 147.48 - 8.57X_1 + 2.35X_2 - 7.63X_3 + 10.75X_4 - 0.064X_1X_2 + 0.12X_1X_2 + 1.70X_1X_3 - 0.95X_2X_3 + 2.94X_2X_4 - 3.34X_3X_4 + 8.27X_1^2 + 1.38X_2^2 - 16.52X_3^2 - 12.77X_4^2$$

where Y_1 and Y_2 are the predicted response of cellulase and pectinase activities, respectively. X_1, X_2, X_3 and X4 are codes independent variables of initial moisture content, initial pH, incubation temperature and incubation time, respectively.

Table 3 shows the results which were tested by the Fisher's statistical test for the analysis of variance (ANOVA) using Design Expert software. The fitness and adequacy of the model were judged by the coefficient of determination (R^2). The R^2 which can be defined as the ratio of the explained variation to the total variation was measured by degree of fit. The closer of the R^2 value to unity, the better the empirical model fits the actual values. The coefficients of determination, R^2, were 0.84 and 0.91 for the regressed models predicting the cellulase and pectinase activities, respectively, suggesting a good fit for the model under SSF. The significance of each coefficient was determined using the F-test and p-value (Table 3). The F value of 4.56 and 8.74 for cellulase and pectinase activities, respectively, implying that the model was significant ($p < 0.05$). In this case, X_2, X_3, X_4, X_3X_4 and X_2^2 for cellulase activity and X_1, X_3, X_4, X_3^2 and X_4^4 for pectinase activity were found to be significant model terms. The lack of fit measured the failure of the model to represent data in the experimental domain at points

which were not included in the regression. The non-significant value of lack of fit (> 0.05), 0.61 and 0.27 for cellulase and pectinase respectively, revealed that the quadratic model is statistically significant for the response.

The software used second order model to optimize the responses. Predicted values of different responses on optimal conditions (in the range constraint) for models are given in Table 4. When constraint in the range was selected then the optimal conditions were found as initial moisture content of 67.21%, pH of 5.91, incubation temperature of 32.95°C and incubation time of 71.86 h under SSF, but in practice, they are difficult to maintain the recommended conditions during processing and some deviations were expected. Therefore, optimal conditions were targeted as initial moisture content of 67%, pH of 5.9, incubation temperature of 33°C and incubation time of 71.8 h under SSF. Different values of responses on targeted optimal conditions for models are given in Table 4. As a result, cellulase and pectinase activities produced by *A. oryzae* under targeted optimal conditions were 6.01 (FPU/gds) and 139.56 (U/gds), respectively. The obtained data were not significantly different with predicted cellulase and pectinase activities (6.69 (FPU/gds) and 144.38 (U/gds), respectively) meaning that the practical fermentation condition above can be acceptable to apply to obtain the highest cellulase and pectinase activities under the same condition. Thus, the production of both cellulase and pectinase with the highest activities was successfully produced by *A. oryzae* under SSF in this study.

Conclusion

A solid medium containing soybean residue, sucrose, urea and $MnSO_4$ was found to be suitable for the production of both cellulase and pectinase by *A. oryzae.* The highest cellulase and pectinase activities (6.01 FPU/gds and 139.56 U/gds, respectively) were achieved with initial moisture content of 67%, pH of 5.9, incubation temperature of 33°C and incubation time for 71.8 h. The findings of this study might be applied in a large scale for production of both cellulase and pectinase with cost-effective using cheap substrate (soybean residue).

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