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Statistical optimization of endo-polygalacturonase production by overproducing mutants of *Aspergillus niger* in solid-state fermentation

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Abstract

Statistically based experimental designs were applied for endo-polygalacturonase production by mutated strains of *Aspergillus niger* using apple pomace waste in solid-state fermentation (SSF). The Plackett-Burman design was used to search for the main factors. Urea was found to be the most significant factor ($p<0.05$) among these variables. To study the mutual interactions between variables and find the optimum medium, Doehlert design was performed to investigate the effect of the medium components, apple pomace, glucose and urea. Using the optimized medium, the maximal activity of endo-polygalacturonase production was found to be 4.266 U. The Plackett-Burman design coupled with Doehlert design were proved to be the potent tools in optimizing medium composition for producing endo-polygalacturonase.

Keywords: Doehlert design, Plackett-Burman design, solid-state fermentation

Introduction

Apple pomace is a byproduct of juice extraction industries and being highly biodegradable; its disposal is a serious problem. Apple pomace is a heterogeneous mixture consisting of peel, core, seed, calyx, stem and soft tissues. Since it is a rich source of carbohydrates, acids, fibers, vitamin C and minerals, its disposal as a waste in the environment is a huge loss of precious natural resources (Smock *et al*. 1950, Joshi *et al*. 1990). However, its nitrogen deficient nature makes it inadequate as an animal feed. Therefore, its utilization in one or other forms is the immediate necessity from the economic and environmental protection point of view.

Endo-polygalacturonase is a heterogeneous group of enzyme that degrade pectin). Numerous studies have been done to utilize apple pomace waste for producing endo-polygalacturonase enzyme by *Aspergillus niger* in solid-state fermentation (Berovic and Ostroversnic, 1997). Due to complexity and non-homogeneity of solid state fermentation medium, there is a necessity of applying statistical designs to maintain process parameters like temperature, moisture content and pH (Taragano *et al*. 1999).

Statistically based experimental designs like response surface methodology are more efficient in experimental biology, as variables are tested simultaneously. Moreover, the interactions between different variables can be estimated. Doehlert design matrix presents the advantage of being easily expanded in both the variables space and the experimental space. Doehlert designs are easily applied to optimized variables and offer advantages in relation to Central Composite and Box–Behnken designs used in response surface analysis. They need fewer experiments, which are more efficient and can move through the experimental domain.

The objective of this work is to optimize solid-state fermentation medium to produce endo-polygalacturonase with apple pomace as substrate using a statistically based experimental designs as Plackett-Burman design and Doehlert design.

Materials and Methods

Maintenance and growth of microorganism:

Aspergillus niger GHRM5 was taken from the Center for Biotechnology, Andhra University, India. Originally the strain was isolated by mutating the *Aspergillus niger* NCIM 548 which was procured from National Chemical Laboratory, Pune, India. The *Aspergillus niger* GHRM5 was maintained on potato dextrose agar (PDA) slants. The slants were incubated at 30 °C for 6 days and stored at 4 °C. The stains were subcultured every 6-7 weeks.

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Solid-state fermentation medium (SSF):

The enzyme was produced by inoculating approximately 5 x 10^6 spores/ml of 3 days old culture of mutated strain *Aspergillus niger* GHRM5. The solid-state fermentation medium consists g/100g: urea, 0.3; $(NH_4)_2SO_4$ 1.26, K_2HPO_4 0.65, $MgSO_4$ 0.02, $FeSO_4$ 0.029, apple pomace 2, sugar cane bagasse (SCB) 23.1 and 70 ml distilled water. The final concentration of the fermentation medium was maintained with glucose 10 g/l. The fermentation medium and pretreated sugar cane bagasse (bagasse was washed with hot water to eliminate sucrose and other soluble materials) were sterilized at 121 °C for 15 min separately. After cooling down, both fractions were mixed. Approximately 5 * 10^6 spores were inoculated with 0.1ml fermentation medium. The flasks were incubated at 30 ± 1°C. Samples (10 g) of fermented material were taken after 3 days, mixed with 10 ml distilled water and pressed in a manual press. The liquid extract was kept at 4°C for enzymatic assays (Maldonado *et al*. 1998).

Analytical Method

0.2 ml of 1 % pectin solution, 2.0 ml of sodium citrate buffer of 0.1 M concentration, pH 5.0 and 1.0 ml of liquid extract was added. The reaction mixture was incubated at 35 ± 1°C for 25 min. After 25 min, 1.0 ml of this reaction mixture was withdrawn to test tubes containing 0.5 ml of 1 M sodium carbonate solution, the concentration of reducing sugars were determined by the dinitrosalicylic acid (DNS) method (Miller. 1959) using galacturonic acid as a reference. One enzyme unit of endo-polygalacutronase (EC 3.2.1.15) is the number of micromoles of reducing sugars measured in terms of galacturonic acid, produced as a result of the action of 1.0 ml of enzyme extract in 1 minute at 35 ± 1 °C. All the activity measurements were made in triplicates (Dhillon *et al*. 2004). All chemicals were procured from Sigma-Aldrich, USA.

Experimental design and data analysis

Plackett-Burman design

The purpose of the first optimization step is to identify which ingredients of the medium have significant effect on endo-polygalacturonase production. The Plackett-Burman statistical experimental design is very useful in screening the most important factors. This design does not consider the interaction effects between the variables and is used to screen the important variables affecting endo-polygalacturonase production. The design matrix was developed according to Montgomery (1997). The total number of experiments to be carried out according to Plackett-Burman is K+1, where K is the number of variables (medium components and environmental factors). Each variable is represented at two levels, namely a high level denoted by '+' and a low level denoted by '–'. The high level of each variable is far enough form the low level so that a significant effect, if exists, is likely to be detected.

The experimental design for screening of medium components is shown in Table 1. The rows in the Table 1 represent the eight different experiments and each column represents a different variable. For each experimental variable, high (+) and low (-) levels are tested. All experiments are performed in duplicate and the average of the maximal endo-polygalacturonase enzymatic activity is taken as the response. Apple pomace and glucose are not included in Plackett-Burman design as they have already shown significant effect on endo-polygalacturonase production in preliminary studies (Unpublished preliminary results).

Table 1: The Plackett-Burman experimental design matrix for screening of medium components for endo-polygalacturonase production by *Aspergillus niger* GHRM5

X_1, urea at high concentration (+) of 0.3 % and low concentration (-) of 0.05 %; X_2, K_2HPO_4 (high, 0.65%; low, 0.1%); X_3, $(NH_4)_2SO_4$ (high, 1.26%; low, 0.5%); X_4, $FeSO_4$ (high, 0.001%;low, 0.029%); X_5, $MgSO_4$ (high, 0.001%; low, 0.02%); X_6, X_7 are dummy variables

Run No.	Variable Level							Response
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	*Enzyme Activity* (U)
1	-	-	-	+	+	+	-	1.156
2	+	-	-	-	-	+	+	3.09
3	-	+	-	-	+	-	+	2.327
4	+	+	-	+	-	-	-	3.433
5	-	-	+	+	-	-	+	2.766
6	+	-	+	-	+	-	-	3.029
7	-	+	+	-	-	+	-	2.916
8	+	+	+	+	+	+	+	3.884
9	-	-	-	-	-	-	-	1.598

The Plackett-Burman design is analyzed using Statistica Version 6.0 software (StatSoft, USA) to estimate the significant factors. The Pareto chart of standardized effects is drawn to detect the most significant variables inside the experiment. The Pareto chart analysis is a simple but powerful way of identifying the significant variables. It amounts to construct a histogram of variables highlighting the significant variables by crossing the p-value line (0.05 level of significance). The p-values are calculated by performing analysis of variance (ANOVA).

Doehlert experimental design

Once the variables having the greatest influence on the response are identified, Doehlert experimental design is used to optimize the levels of these variables. The Doehlert design allows the description of a region around an optimal response and contains k^2+k+1 points for k variables (Sautour et al. 2001). For three variables, a set of 13 experiments is required and in that case, one of the properties of the Doehlert design is the uniform distribution of the experiments in a three-dimensional space. Thus, 12 experiments are equidistant from a central experiment having the coded values (0, 0, 0) and are distributed on a sphere with a radius of 1.

In this study, enzyme activity is estimated, taking into account the influence of three factors (i.e. variables) urea (X_1), apple pomace (X_2), and glucose (X_3). The natural values and the corresponding coded values are used for setting up the experiments and the model respectively. The experimental design and coded levels are shown in Table 2.

Table 2: Experimental design obtained by applying the Doehlert design methodology for three factors and experimental values for endo-polygalacturonase production

Runs	Experimental Value (Coded Value)			Enzyme Activity (U)	
	Urea (%) X_1	Apple Pomace (%) X_2	Glucose (g/l) X_3	Observed	Predicted
1	2.1 (+1.000)	10 (0.000)	10 (0.000)	0.3556	0.297773
2	0.1 (-1.000)	10 (0.000)	10 (0.000)	3.6978	3.755627
3	1.6 (+0.500)	19 (+ 0.866)	10 (0.000)	1.2800	1.137858
4	0.6 (-0.500)	1 (- 0.866)	10 (0.000)	1.9913	2.133518
5	1.6 (+0.500)	1 (- 0.866)	10 (0.000)	0.6044	0.600028
6	0.6 (- 0.500)	19 (+ 0.866)	10 (0.000)	3.0578	3.062222
7	1.6 (+0.500)	13 (+ 0.289)	15 (+ 0.816)	1.7422	1.942184
8	0.6 (-0.500)	7 (- 0.289)	5 (-0.816)	2.2755	2.075516
9	1.6 (+0.500)	7 (- 0.289)	5 (-0.816)	0.8177	0.879949
10	1.1 (0.000)	16 (+ 0.577)	5 (-0.816)	1.5644	1.702136
11	0.6 (-0.500)	13 (+ 0.289)	15 (+ 0.816)	4.2667	4.204471
12	1.1 (0.000)	4 (- 0.577)	15 (+ 0.816)	2.7022	2.564464
13	1.1 (0.000)	10 (0.000)	10 (0.000)	3.4489	3.454800
14	1.1 (0.000)	10 (0.000)	10 (0.000)	3.3950	3.454800
15	1.1 (0.000)	10 (0.000)	10 (0.000)	3.5200	3.454800

Analysis and interpretation of the results

Multiple regression analysis based on the least square method was performed using Statistica Version 6.0 software (StatSoft USA). The analysis concerned the linear and quadratic effects of the three factors and their interactions. Thus, the equation giving enzyme activity is a second-order polynomial model with 10 coefficients (b_0, b_1, b_{12}…..… b_{23}):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$$

Where X_1, X_2 and X_3 are coded factors studied.

The significance of the coefficients is evaluated by multiple regression analysis based upon the *F*-test with unequal variance ($P<0.05$) (Nair et al., 1997).

Results and Discussion

Screening of medium constituents for endo-polygalacturonase production

The experimental design (Table 1) for the various treatments was based on preliminary work. The plan included eight experiments and two concentration levels for each factor. The factors designated X_1-X_5 represent medium constituents, with X_6, X_7 being dummy variable. The Pareto chart of standardization histogram graph (Figure 1) showed that only urea (X_1), with significance level ($p<0.05$), crosses the p-line and was considered to significantly influence endo-polygalacturonase production by *Aspergillus niger* GHRM5. X_4 variable was far from the p-line confirming the insignificance of the variable inside the experiment. X_2 and X_3 were considered significant next to urea (X_1) with significance level ($P<0.0649$) and $P<0.0631$) respectively (Table 3).

Optimization of screened medium constituents for endo-polygalacturonase production

Based on the results of the Plackett-Burman design (P-B design) and preliminary studies, the selected variables are urea from P-B design

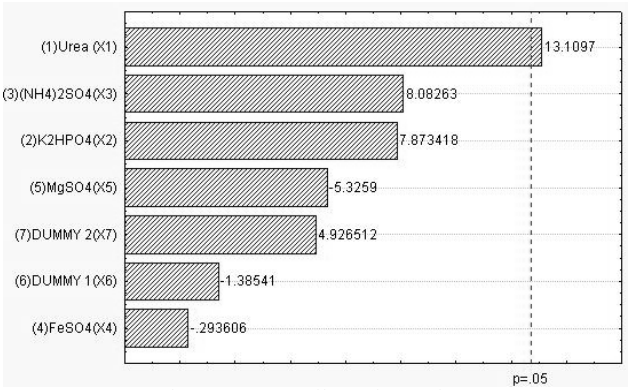

Fig 1: Pareto chart of standardadized effects of seven factors screening design for the production of endopolygalacturonase

with p-value of significance<0.05, apple pomace and glucose for the production of endo-polygalacturonase. Doehlert experimental design (Table 2) is employed to optimize their individual concentrations. On regression analysis of the experimental data, the following second order polynomial equation is shown to account for endo-polygalacturonase production.

$$Y = -3.726 + 2.566X_1 + 0.3916X_2 + 0.776X_3 - 1.428X_1^2 - 0.0168X_2^2 - 0.0274X_3^2 - 0.0217X_1X_2 - 0.09364X_1X_3 + 0.00099X_2X_3$$

Where Y represents the response variable and X_1, X_2 and X_3 represent the uncoded values of urea, apple pomace and glucose

Table 3: Analysis of variance (ANOVA)

	Sum of Squares	Degree of freedom	Mean Squares	F	p-value
Urea (X_1)	2.2789	1	2.278963	274.3651	0.038387
K_2HPO_4 (X_2)	0.7937	1	0.793712	95.5552	0.064900
$(NH_4)_2SO_4$ (X_3)	0.8380	1	0.838033	100.8911	0.063172
$FeSO_4$ (X_4)	0.0018	1	0.001844	0.2220	0.719681
$MgSO_4$ (X_5)	0.4091	1	0.409178	49.2610	0.090098
Dummy 1 (X_6)	0.0324	1	0.032469	3.9090	0.298108
Dummy 2 (X_7)	0.2940	1	0.294014	35.3965	0.106013
Error	0.0083	1	0.008306		
Total SS	4.6565	8			

respectively. From the regression equation the optimized values are calculated by partial differentiating the above equation with respect to X_1, X_2 and X_3 and equating to zero. Solving the three equations from partial differentiation results in the optimal medium composition. The final optimal solid-state fermentation medium consists g/100g: urea, 0.3775; $(NH_4)_2SO_4$ 1.26, K_2HPO_4 0.65, $MgSO_4$ 0.02, $FeSO_4$ 0.029, apple pomace 11.8011, sugar cane bagasse (SCB) 23.1 and 70 ml distilled water. The final concentration of the fermentation medium was maintained with glucose 13.76 g/l.

The contour plot (Figure 2) shows the relative effects of urea, apple pomace and glucose. The optimal values obtained from the contour plot are almost equal to the results obtained by optimizing the regression equation. To test the goodness of fit of the regression equation, the multiple coefficients of correlation R and the determination coefficient R^2 are evaluated. The coefficient of determination, R^2 = 0.9915, which indicates a good agreement between the experimental and predicted values (Chun-Ping Xu et al. 2002). The experiment with three replicates is conducted with the optimal values of urea, apple pomace and glucose.

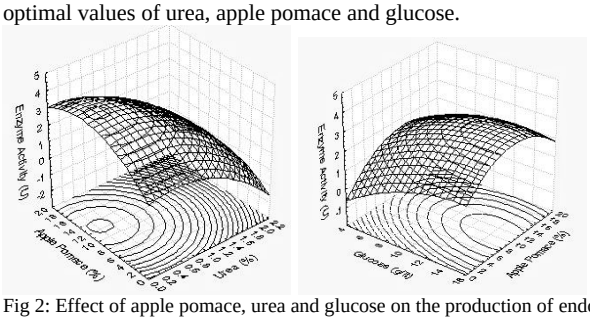

Fig 2: Effect of apple pomace, urea and glucose on the production of endo-polygalacturonase enzyme

The enzyme activity obtained is 4.266 U, which is near to the theoretical value 4.3894 U conforming the fitness and adequacy of the experiment.

Conclusion

Unlike the classical method of optimizing medium components, statistical techniques were performed, where the levels of variables were changed simultaneously to study their collective effect on endo-polygalacturonase production. In the present study, the Plackett-Burman design and Doehlert design were proved to be the

potent tools in optimizing medium composition for endo-polygalacturonase production by *Aspergillus niger* GHRM5.

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Reference

Berovic M, Ostroversnik H (1997) Production of Aspergillus niger pectolytic enzymes by solid state bioprocessing of apple pomace. J. Biotechnol. 53: 47-53

Chun-ping Xu, Sang WK, Hwang HJ, Yan JW (2002) Application of statistically based designs for the optimization of exo-polysaccharide production by Cordyceps militaris NG3. Biotechnol. Appl. Biochem. 36:127-131

Dhillon SS, Gill RK, Gill SS, Malkiat Singh (2004) Studies on the utilization of citrus peel for pectinase production using fungus Aspergillus Niger. Intern. J. Environ. Studies. 61(2):199–210.

Hang YD, Lee CY, Woodams EE (1982) A solid state fermentation system for the production of ethanol from apple pomace. J. Food Sci. 47:1851-1852

Joshi C, Joshi VK (1990) Food processing waste management technology, the need for an integrated approach. Indian Food Packer. 44:56-67

Maldonado MC, Saad AMSD (1998) Production of pectinesterase and polygalacturonase by Aspergillus niger in submerged and solid state systems, J. Ind. Microbiol. Biot. 20:34-38

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426–428

Montgomery DC (1997), Design and analysis of experiments, 4th edn. Wiley, New York

Nair SR Panda T (1997) Statistical optimization of medium components for improved synthesis of pectinase by Aspergillus niger. Bioprocess Eng. 16:169–173

Smock RM, Neubert AM (1950) Apple and apple products, Interscience, New York

Taragano VM, Pilosof AMR (1999) Application fo Doehlert designs for water activity, pH and fermentation time optimization for Aspergillus niger pectolytic activities production in solid-state and submerged fermentation, Enzyme Microb. Tech. 25:411-419