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Application of response surface methodology for optimization of polygalacturonase production by *Aspergillus niger*

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Abstract

Polygalacturonase (PG) degrades pectin into D-galacturonic acid monomers and is used widely in food industry especially for juice clarification. In the present study, fermentation conditions for polygalacturonase production by *Asgergillus niger* NAIMCCF-02958, using mango peel as substrate, were optimized using the 2^3 factorial design with central composite rotatable experimental design (CCRD) of response surface methodology (RSM). The maximum PG activity 723.66 U g^{-1} was achieved under pH 4.0, temperature 30 ºC and 2% inoculum by response surface curve. The experimental value of PG activity was higher 607.65 U g^{-1} than the predicted value 511.75 U g^{-1}. Under the proposed optimized conditions, the determination coefficient (R^2) was equal to 0.66 indicating that the model could explain 66% of the total variation as well as establish the relationship between the variables and the responses. ANOVA analysis and the three dimensional plots also confirmed interactions among the parameters.

Key words

Aspergillus niger, Enzyme production, Mango peel, Polygalacturonase production, Response surface methodology

Introduction

Polygalacturonase constitutes a heterogeneous group of enzymes that degrade pectin (Gummadi and Panda, 2003). These enzymes are utilized in various industrial sector segments, for the clarification of fruit juice and wine, manufacturing of hydrolyzed pectin products, extraction of oil from oleaginous seeds and pigments, degumming of natural fibers, etc. (Kashyap *et al.,* 2001; Rai *et al.*, 2004). The production of pectinolytic enzymes has been widely reported in bacteria and filamentous fungi (Pedrolli *et. al.,* 2008). Fungal polygalacturonases are widely used for fruit juices and wine clarification (Padma *et. al.,* 2012). Various agricultural residues and fruit wastes including apple peel, banana peel, orange peel, jackfruit rind, mango peel and pine apple peel have been reported to be used as substrate for enzyme production. (Castilho *et al.,*2000; Padma *et. al.,* 2012, Kumar *et. al.,* 2012). Mango peel is a rich source of pectin and has been reported for pectinase production a by number of workers (Srirangarajan and Shrikhande, 1976; Berardini *et al*., 2005; Garg and Ashfaque, 2010). Statistical technique is often used for predicting the optimum process conditions for microbial enzyme production (Mullai *et al*., 2010; Zambare, 2010). Recently, response surface methodology has been extensively used for the process and media optimization studies by microorganisms for enzyme production (Kawaguti *et al.,* 2005; Palaniyappan *et al.,* 2009; Songpim *et al.,* 2010). This method represents a set of statistical techniques for executing planning and evaluating the effect of independent variables on the desired variable response (Mohandas *et al*., 2010). The present study reports the application of RSM for polygalacturonase production by *A. niger* NAIMCC-F-02958 using mango peel as substrate under aerated condition.

Materials and Methods

Mature mango peel was dried (until 5-7% moisture content) and powdered to 50 mesh size particles. Pectin was extracted from dried mango peel by the method of Tandon *et al.* (1995) and the pectin extract was used as substrate for PG production.

The microorganisms isolated from pectin rich waste were screened for PG activity (Garg and Ashfaque, 2010). A fungal isolate, isolated from bio-dynamic preparation, identified as *A. niger* NAIMCC-F-02958 was maintained as pure culture on potato dextrose agar (PDA) slants.

Experimental design and optimization by RSM : All treatment combinations were performed in 250 ml pectin extract from mango peel. A central composite rotatable experimental design (CCRD) for three independent variables was used. Coding of the variables was done according to the following equation:

$$x_i = \frac{X_i - X_{cp}}{\Delta Xi} \qquad i = 1, 2, 3 \ldots\ldots k \qquad (1)$$

Where *xi* dimensionless value of an independent variable; *Xi*, real value of an independent variable; *Xcp*, real value of an independent variable at the center point; and *ΔXi*, step change of real value of the variable *i* corresponding to a variation of a unit for the dimensionless value of the variable *I* (Table 1).

Each factor in the CCRD was studied at three different levels (-1, 0, +1). The minimum and maximum ranges of variables were investigated with respect to their values in actual and coded form (Table 1), X_1 = (pH -5)/1, X_2= (temperature-40)/10°C, X_3 = Inoculum size-3)/2%, ml. According to this design, from the experimental data a second order polynomial regression model was selected which is as follows (Table 2).

$$Y = \beta_0 + \sum_{I=1}^{k} b\,iXi + \sum_{i=1}^{k} b\,iiXiXi + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta ijXiXj \qquad (2)$$

Y is the predicted response; β_0 a constant; *βi* the linear coefficient; *βii* the squared coefficient; and *βij* the cross-product coefficient, *k* the number of factors.

To optimize the conditions for PG production, SAS 9.3 software was used. A 2^3 factorial CCRD with three factors leading to a total of 20 sets per experiment was formulated to optimize the process parameters. This experiment included 8 factorial design, 6 star and 6 central points. All the variables *i.e.* pH, temperature and inoculum size were taken at a central coded value and considered as zero. The conditions of these environmental factors for the production medium varied according to the experimental design (Table 2). All the experiments were carried out in three replications.

Based on the regression analysis, a second order polynomial model was used to describe the relationship between the independent variables and PG activity and a model was developed. When the value of composite desirability was close to 1, the predicted values and model was well fitted with the experimental results.

Polygalacturonase assay: PG activity was determined by the methodology as described by Miller (1959). One unit of PG activity was defined as the quantity of the enzyme necessary to produce one micro mole (µ mole) of galacturonic acid per minute.

Results and Discussion

Central Composite Design (CCD) experiments using response surface methodology (RSM) have proved to be an optimal tool for optimization of medium parameters for PG production. Palaniyappan *et al.* (2009) used RSM method for PG production by *Aspergillus fumigatus* MTCC 870 in submerged fermentation.

In the present study, data was analyzed using SAS 9.3 software and the following regression equation was obtained which fitted in the model for PG production.

371.2654-3.3545X1- 68.6902X2+122.8244X3+82.74X12+69.44 X13 27.5925 X23+36.2247 X11 -117.2847 X22 -14.2115X33 .. (3)

Using predicted values (obtained from equation 3) and the experimental values of PG production, the parity plot drawn indicated a satisfactory association (Fig. 1). The results indicate good agreement between experimental and predicted vlaues of PG production with R^2 (Coefficient of determination) 85%. The adj. R^2 (adjusted coefficient of determination) obtained was 0.37, which was also high and indicated a significance of the model with p value < 0.05 (Olivera *et al.,* 2004 ; Trupkin *et al.,* 2003).

Table 1 : Experimental range and levels of three independent variables used in RSM in terms of actual and coded factors for optimization of PG production

Variable	Unit	Range of levels					
		Actual	Coded	Actual	Coded	Actual	Coded
pH	--	4	-1	5	0	6	+1
Temperature	ºC	30	-1	40	0	50	+1
Inoculum size	ml	1	-1	3	0	5	+1

X_1= (pH -5)/1, X_2= (Temperature-40)/10 and X_3= (Inoculum size-3)/2

Table 2 : Design matrix of full-factorial CCD and observed effects for PG production

Standard Order	Parameters (coded variables)[a]			PG production (U g^{-1})[b]		Residual standard deviation
	pH X_1	Temperature (°C) X_2	Inoculum (%, ml) X_3	Experimental	Predicted	
1	4 (-1)	30 (-1)	1 (-1)	345.93	349.8	-3.8
2	6 (1)	30 (-1)	1 (-1)	79.32	38.73	40.58
3	4 (-1)	50 (1)	1 (-1)	89.45	102.12	-12.67
4	6 (1)	50 (1)	1 (-1)	24.34	122.01	-97.67
5	4 (-1)	30 (1)	5 (1)	607.65	511.75	95.89
6	6 (1)	30 (-1)	5 (1)	489.34	478.44	10.89
7	4 (-1)	50 (1)	5 (1)	111.34	153.71	-42.37
8	6 (1)	50 (1)	5 (1)	453.45	451.36	2.08
9	3.32 (-1.68)	40 (0.00)	3 (0.00)	456.52	479.36	-22.84
10	6.68 (1.68)	40 (0.00)	3 (0.00)	493.45	468.08	25.36
11	5 (0.00)	23.18 (-1.68)	3 (0.00)	68.87	155.05	-86.18
12	5 (0.00)	56.82 (1.68)	3 (0.00)	12.72	-75.98	88.74
13	5 (0.00)	40 (0.00)	-0.36 (-1.68)	167.43	124.55	2.92
14	5 (0.00)	40 (0.00)	6.36 (1.68)	497.23	37.63	-40.41
15	5 (0)	40 (0)	3 (0)	489.68	371.26	18.41
16	5 (0)	40 (0)	3 (0)	493.29	371.26	122.02
17	5 (0)	40 (0)	3 (0)	584.34	371.26	213.07
18	5 (0)	40 (0)	3 (0)	123.43	371.26	-247.83
19	5 (0)	40 (0)	3 (0)	45.78	371.26	-325.48
20	5 (0)	40 (0)	3 (0)	490.64	371.26	119.37

[a] Values within the parentheses are coded variables. Values X_1, X_2 and X_3 are actual values; (X_1 = (pH -5)/1.0, X_2 = (Temperature-40)/10 and X_3 = (Inoculum - 3)/2 ;[b]represents readings average of three determinations or determinants.

The experimental value of optimum PG production was higher (607.65 U g^{-1}) in mango peel at pH 4, temperature 30 °C and 2% of inoculums than the predicted value (511.75 U g^{-1}) Table 2). The experiment was validated on large scale (1 l substrate per replicate) using optimized conditions. Fig. 2 represents the three dimensional plot as a function of temperature, pH and inoculum size on PG production. The maximum production of PG (723.66 U g^{-1}) was observed under optimized conditions by response surface curve. Further, increase or decrease in the temperature or pH led to decrease in enzyme production as evident from the results (Fig 2 A, B and C, Table 2).

Generally, the optimum temperature for PG production corresponds with the growth temperature of the respective microorganism (Sharma *et al.,* 2002). For extracellular enzymes, temperature influences their secretion, possibly by changing the physical properties of the cell membrane (Rahman *et al.,* 2005). On the other hand, though higher temperature causes higher reaction rates and higher solubility of substrate and products, yet oxygen solubility is usually decreased. At a suitable inoculum size, the nutrient and oxygen levels are enough for sufficient growth of fungi and therefore, enhanced PG production. If the inoculum size is too small, insufficient fungal counts will lead to reduced amount of secreted PG. High inoculum size can result in the lack of oxygen and nutrient depletion in the culture media (Rahman *et al*., 2005 and Shafee *et al.,* 2005). The experimental production of PG, supported the model tested and indicated that RSM can be successfully used for enhancing the yield of PG production. This finding is in consensus with the finding of other workers who used RSM for increasing enzyme production (Martos *et al.,* 2009; Goncalves *et al.,* 2012; Bhattacharya *et al.,* 2010).

The statistical approach for optimizing PG production by *A. niger* NAIMCC-F-02958 using mango peel pectin extract as

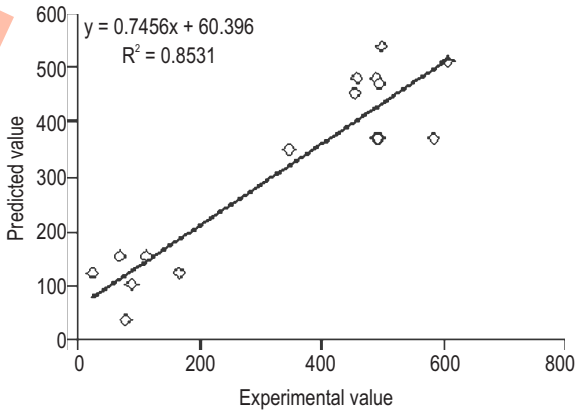

Fig. 1 : Parity plot showing the distribution of experimental vs. predicted values of PG production

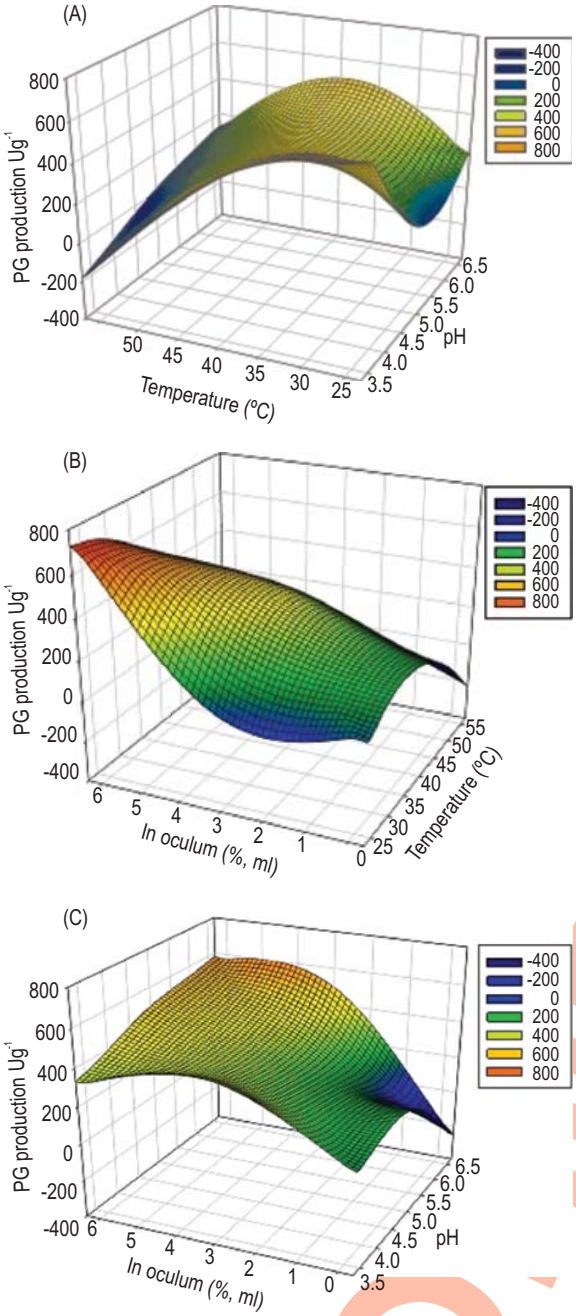

Fig. 2 : Three dimensional plots showing the effect of temperature and pH (2A) inoculum size and temperature (2B) inoculum size and pH (2C) for optimum PG production by A. niger NAIMCC-F-02958 using mango peel pectin extracted as substrate

substrate resulted in the production of 607.65 U PG /g. This methodology may therefore, be effectively used to study the large scale production of PG at industrial level.

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