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The Effect of Microbiological Parameters on Production of Pectin Lyase and Pectate Lyase by *Debaryomyces nepalensis*: A Statistical Approach

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Abstract: Response surface methodology was used to study the effect of microbiological parameters, viz., plate age, inoculum age and amount of inoculum on production of pectin lyase and pectate lyase. Individual optimum values of plate age, inoculum age and amount of inoculum were found to be 31.5 h, 12 h and 5.3% (v/v), respectively for PL and 27 h, 14 h and 4.2% (v/v), respectively for PGL. At optimal conditions of PL 12.7 and 10.5 U mL^{-1} of PL and PGL was produced and at optimal conditions of PGL 12.2 and 10.4 U mL^{-1} of PL and PGL was obtained. Since, there is no much difference in PL and PGL production at two different optimal conditions, it can be concluded that enzyme production is not affected in the range of optimal conditions obtained in this study. Hence, the individual optimal values of microbiological parameters for PL or PGL can be used to develop seed culture for maximum production of both PL and PGL. At optimal conditions the production of PL and PGL was increased by 1.2 fold, respectively.

Key words: Pectin lyase, pectate lyase, *Debaryomyces nepalensis*, microbiological parameters, plate age, inoculum age, inoculum level

Introduction

Pectinases degrade pectic compounds which are multiple forms due to the complex nature of the substrate. Pectic transeliminases or pectic lyases degrade pectic substances by transelimination mechanism yielding unsaturated oligogalacturonates. Pectate lyase (PGL), acting on polygalacturonic acid and pectin lyase (PL), acting on pectin are the two important transeliminases. Pectic lyases have been extensively used in fruit juice industries, in the degumming of ramie, hemp, flax and jute fibers, bioscouring of cotton fiber and also in coffee and tea fermentation (Whitaker, 1991; Naidu and Panda, 1998; Gummadi and Kumar, 2005; Hoondal *et al*., 2002; Carr, 1985). Since new applications for pectic transeliminases are emerging, the demand for production of these enzymes is increasing. It has been reported that PL is mostly produced by fungi whereas pathogenic bacteria are predominant producers of PGL (Gummadi and Kumar, 2005). Very few strains are available in the literature, which are capable of producing both PL and PGL in appreciable levels. With this in view, we previously isolated *Debaryomyces nepalensis* capable of producing both PL and PGL (Gummadi and Kumar, 2006a) and the medium components were optimized for maximum production of PL and PGL (Gummadi and Kumar, 2006b).

The development of best seed culture conditions is very important for enzyme production in bioreactors since it reduces time of fermentation and increases the production, which in turn reduces the cost of production. Therefore it becomes necessary to optimize important smicrobiological parameters such as slant or plate age, inoculum age and amount of inoculum to develop

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an effective seed culture for maximizing enzyme production in bioreactors. Panda and co-authors optimized the microbiological parameters for optimum production of pectolytic enzymes like polymethylgalacturonase, polygalacturonase and PL, using response surface methodology (Panda *et al*., 1999). Under the optimized conditions, the fermentation time was reduced from 144 to 120 h. However, isolate used in this study (*Debaryomyces nepalensis*) produces maximum amount of PL and PGL in 48 h (Gummadi and Kumar, 2006b). Production of PL and PGL by this yeast strain will be better alternative to fungal strains. Hence, optimization of microbiological conditions for *Debaryomyces nepalensis* would enhance enzyme production in much shorter period. Friedrich *et al*. (1990) studied the effect of inoculum size on PL production by conventional one variable at a time approach. The major disadvantage of this single variable optimization is that it does not include interactive effects among the variables; thus, it does not depict the net effects of various parameters on enzyme production. In this study, we have optimized the microbiological parameters, viz., plate age, inoculum age and amount of inoculum for maximum production of PL and PGL from a novel pectolytic strain *Debaryomyces nepalensis* using response surface methodology (RSM).

Materials and Methods

All the experiments were conducted during 2005-2006 at Department of Biotechnology, IIT Madras, Chennai, India. All the chemicals were of analytical grade procured in India. Pectin and polygalacturonic acid, substrates for PL and PGL was purchased from Sigma.

Microorganism

Debaryomyces nepalensis was used for all experiments and is deposited in National Collection of Yeast Cultures (NCYC), Norwich, UK with accession number D3893 (Gummadi and Kumar, 2006a). The isolate was maintained on YEPD agar plate at 4°C and subcultured every two weeks.

Enzyme Production

A loopful of the strain from YEPD agar plates was transferred to 5 mL sterile YEPD medium and incubated on rotary shaker at 180 rpm and 31.5°C. After the required time of incubation, appropriate amount (% v/v) of seed culture was transferred into a 100 mL Erlenmeyer flask containing 25 mL of production medium and incubated at 180 rpm and 31°C. The production medium had the following composition (g L^{-1}): galactose 9.6, yeast extract 1.9, lemon peel 2.4, Na_2HPO_4 6.0, K_2HPO_4 3.0, NaCl 5.0, $MgSO_4$ 0.1 and $FeCl_3$ 1.0 (Gummadi and Kumar, 2006b). The initial pH was adjusted to 7.0 before sterilization. The plate age (h), inoculum age (h) and the amount of inoculum (% v/v) was varied according to the experimental requirement (Table 1). At regular intervals, samples were collected and analyzed for PL and PGL activity. Enzyme activities at 48 h were found to be maximum and considered as response for analysis.

Enzyme Assay

Supernatant was used as the source for enzyme assay. PL and PGL activity was assayed by measuring the formation of unsaturated oligogalacturonates at 235 nm. The reaction mixture contained 0.19% (w/v) pectin in 100 mM citrate phosphate buffer (pH 6.4) and suitably diluted enzyme. To test PGL activity, pectin was replaced by 0.15% (w/v) polygalacturonic acid in 75 mM Tris-HCl buffer (pH-7.5) with 1 mM $CaCl_2$ (Gummadi and Kumar, 2006c). One unit of enzyme activity was defined as an increase of 1.0 unit of absorbance at 235 nm of the reaction mixture per minute per mL of enzyme solution.

Experimental Design and Data Analysis

Microbiological parameters were optimized by statistical experimental design technique called RSM and the design used was Central Composite Design (CCD). According to this design, the total number of treatment combinations is 2^k+2k+n_0, where k is the number of independent variables and n_o is the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$x_i = (X_i - X_0) / \delta X, \tag{1}$$

where: x_i is dimensionless coded value of the variable X_i, X_o is the value of the X_i at the center point and δX is the step change. The coded and uncoded values of the variables at various levels are given in Table 1. The number of center point replications can also be chosen to verify any change in the estimation procedure, which will also be a measure of precision described by the following equation:

$$n_o = \lambda_4(\sqrt{F} + 2)^2 - F - 2k, \tag{2}$$

where F is the number of points in factorial portion, i.e., first four experiments in experimental design and λ_4 is the mixed fourth order moment (Box and Wilson, 1951).
The behavior of the system was explained by the following quadratic equation:

$$Y= \beta_0 + \sum\beta_i x_i + \sum\beta_{ii} x_i^2 + \sum\beta_{ij} x_i x_j, \tag{3}$$

where Y is the predicted response, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect and β_{ij} is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using MATLAB® version 7.0 (Mathworks Inc., Natick, Massachusetts, USA).

Results and Discussion

Optimization of Microbiological Parameters

The present study was aimed to find out the best combination of microbiological parameters for maximizing the PL and PGL production. In order to optimize the microbiological parameters, viz., plate age, inoculum age and % inoculum, experiments were performed as shown in Table 1. Enzyme activities were found to be maximum at 48 h for all the design points, hence the activities at 48 h were considered for further analysis (Table 1). By applying multiple regression analysis on the experimental data, the following second order polynomial equations were obtained for PL and PGL production.

$$\begin{aligned}\text{PL production} = {} & 10.95 + 0.227x_1 - 1.325x_2 - 0.261x_3 - 0.857x_1^2 - 0.837x_2^2 \\ & - 0.841x_3^2 - 0.469x_1x_2 - 0.569\, x_1x_3 + 0.131\, x_2x_3\end{aligned} \tag{4}$$

$$\begin{aligned}\text{PGL production} = {} & 9.3 - 0.225x_1 - 0.826x_2 - 0.69x_3 - 0.956x_1^2 - 1.403x_2^2 \\ & - 0.410x_3^2 - 0.145x_1x_2 - 0.175\, x_1x_3 + 0.113\, x_2x_3\end{aligned} \tag{5}$$

where x_1 is the coded value of X_1 (plate age), x_2 is the coded value of X_2 (inoculum age), x_3 is the coded value of X_3 (amount of inoculum).

ANOVA for PL and PGL (Table 2 and 3) suggested that the model is very significant as indicated by a low probability value for PL [$(P_{model}>F) = 0.0016$] and for PGL [$(P_{model}>F) = 0.0818$] by Fisher's statistical test. In general the calculated F value should be several times greater than the tabulated value

Table 1: The effect of microbiological parameters on PL and PGL production by *Debaryomyces nepalensis*: experimental design with experimental and predicted activity values of PL and PGL by central composite design (CCD)

Design No.1	x_1 (= X_1)	x_2 (= X_2)	x_3 (= X_3)	PL ($U\ mL^{-1}$) model	PL Experimental	PGL ($U\ mL^{-1}$) model	PGL Experimental
1	-1.0(= 18)	-1.0(= 12)	-1.0(= 4)	8.5	8.8	8.2	7.8
2	+1.0(= 36)	-1.0(= 12)	+1.0(= 8)	9.1	9.7	6.5	7.4
3	-1.0(= 18)	+1.0(= 18)	+1.0(= 8)	7.4	6.8	5.8	6.2
4	+1.0(= 36)	+1.0(= 18)	-1.0(= 4)	7.2	6.9	6.25	5.6
5	0.0(= 27)	0.0(= 15)	0.0(= 6)	10.6	10.5	9.5	9.0
6	0.0(= 27)	0.0(= 15)	0.0(= 6)	10.6	10.6	9.5	9.2
7	-1.0(= 18)	-1.0(= 12)	+1.0(= 8)	9.54	10.0	8.1	9.4
8	+1.0(= 36)	-1.0(= 12)	-1.0(= 4)	11.7	12.5	9.5	9.9
9	-1.0(= 18)	+1.0(= 18)	-1.0(= 4)	7.2	6.9	7.7	7.5
10	+1.0(= 36)	+1.0(= 18)	+1.0(= 8)	6.5	6.4	5.9	6.9
11	0.0(= 27)	0.0(= 15)	0.0(= 6)	11.3	10.9	10.6	9.5
12	0.0(= 27)	0.0(= 15)	0.0(= 6)	11.3	10.8	10.6	9.5
13	-1.7(= 11.8)	0.0(= 15)	0.0(= 6)	8.2	8.4	5.5	5.2
14	+1.7(= 42.1)	0.0(= 15)	0.0(= 6)	8.9	8.4	4.8	4.1
15	0.0(= 27)	-1.7(= 9.9)	0.0(= 6)	10.8	9.7	5.3	4.3
16	0.0(= 27)	+1.7(= 20.1)	0.0(= 6)	6.4	7.2	2.5	2.5
17	0.0(= 27)	0.0(= 15)	-1.7(= 2.6)	9.0	8.8	7.8	8.7
18	0.0(= 27)	0.0(= 15)	+1.7(= 9.4)	8.1	8.0	5.5	3.7
19	0.0(= 27)	0.0(= 15)	0.0(= 6)	11.0	11.5	7.8	9.5
20	0.0(= 27)	0.0(= 15)	0.0(= 6)	11.0	11.4	7.8	9.4

X_1 = Plate age (h), X_2= Inoculum age (h), X_3= % inoculum (v/v). Values in the parenthesis are the decoded values. Experimental values are average of duplicates within ± 1 to ± 9%. For PL, R^2 = 0.93; R = 0.96; For PGL, R^2 = 0.826; R = 0.91;

Table 2: ANOVA table for PL production by *Debaryomyces nepalensis*

Source	Sum of Squares (SS)	Degree of Freedom (DF)	Mean square (MS = SS/DF)	F = MSR/MSE	p>F
Blocks	1.40733	2			
Model	55.83225	9	6.203584	10.35	0.0016
Error	4.79707	8	0.599634		
Total	62.03666	19			

Table 3: ANOVA table for PGL production by *Debaryomyces nepalensis*

Source	Sum of Squares (SS)	Degree of Freedom (DF)	Mean square (MS = SS/DF)	F = MSR/MSE	p>F
Blocks	28.7876	2			
Model	55.6035	9	6.17817	2.788	0.0818
Error	17.7286	8	2.21607		
Total	102.1197	19			

for a good model. If the F value is greater than tabulated F the null hypothesis is rejected at the α level of significance and infers the variation accounted for by the model is significantly greater than the unexplained variation. The value of R (coefficient of linear regression) for PL and PGL were found to be 0.96 and 0.93 suggesting that the experimental values are in good agreement with the model predicted values. The model equation was solved using MATLAB software to generate the optimal values for the above microbiological parameters. The optimal values of plate age (h), inoculum age (h) and amount of inoculum (% v/v) for PL production were found to be 31.5 h, 12 h and 5.3% v/v of inoculum, respectively. Similarly, for maximum production of PGL the optimal conditions of plate age, inoculum age and amount of inoculum were found to be 27 h, 14 h and 4.2% (v/v), respectively. Response surface plots showing the dependence of PL and PGL production on the variables considered for optimization (Fig. 1 and 2). Maximum activities of PL and PGL were indicated by the peak of the response surface plots. PL and PGL production were carried out at their respective model optimum conditions. Maximum activities of PL and PGL were found to be 12.7 and 10.5 $U\ mL^{-1}$, respectively when the isolate was grown in optimized conditions for PL. Similarly a maximum of 12.2 and 10.4 $U\ mL^{-1}$ of PL and PGL were obtained when grown at optimal conditions for PGL. Since, there

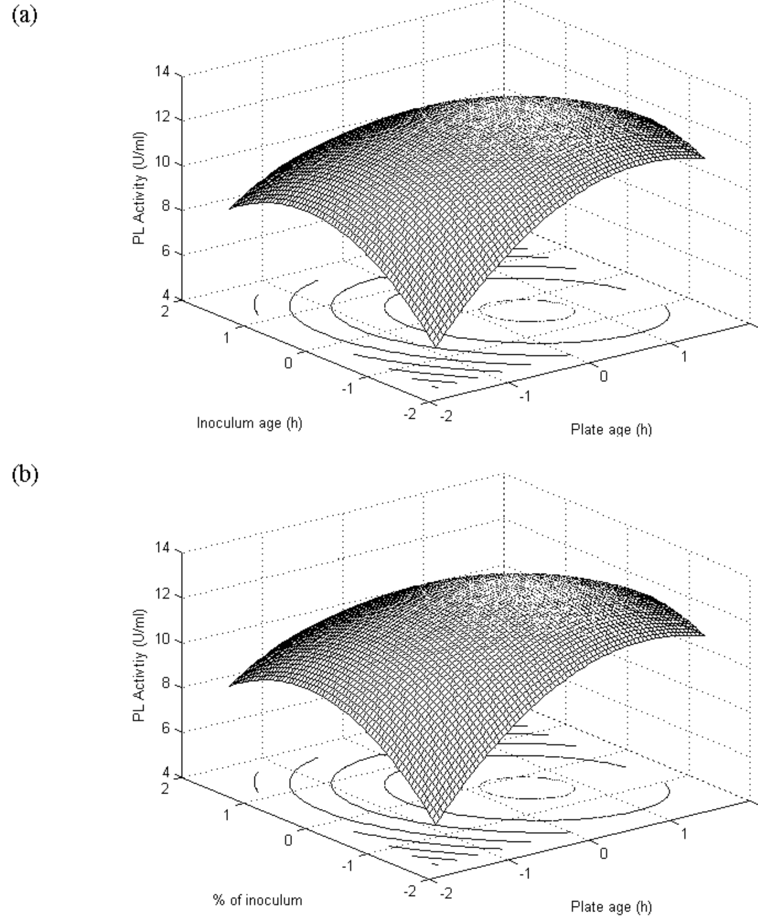

Fig. 1: Response surface plot (a) PL activity at different levels of inoculum age (h) and slant age (h) at optimum model predicted amount of inoculum (% v/v), (b) PL activity at different levels of amount of inoculum (% v/v) and slant age (h) at optimum model predicted inoculum age (h)

is no much difference in PL and PGL production at two different optimal conditions, it can be concluded that enzyme production is not affected in the range of optimal conditions obtained in this study. Hence, the individual optimal values of microbiological parameters for PL or PGL can be used to develop seed culture for maximum production of both PL and PGL. The model predicted values of PL and PGL at optimal conditions was 11.7 and 10.9 U mL^{-1}, respectively (from Eq. 4 and 5). The model predicted values are in very good agreement with experimental values of PL and PGL obtained under optimal conditions. At optimal conditions the production of PL and PGL was increased by 1.2 fold, respectively.

In order to verify whether the optimal conditions determined in this study enhances the enzyme production, experiments were performed in bioreactors. Under the optimized conditions of plate age, inoculum age and amount of inoculum experiments were performed in bioreactor and it was found that maximum of 13.5 U mL^{-1} of PL and 12.5 U mL^{-1} of PGL was produced in 24 h. These results showed

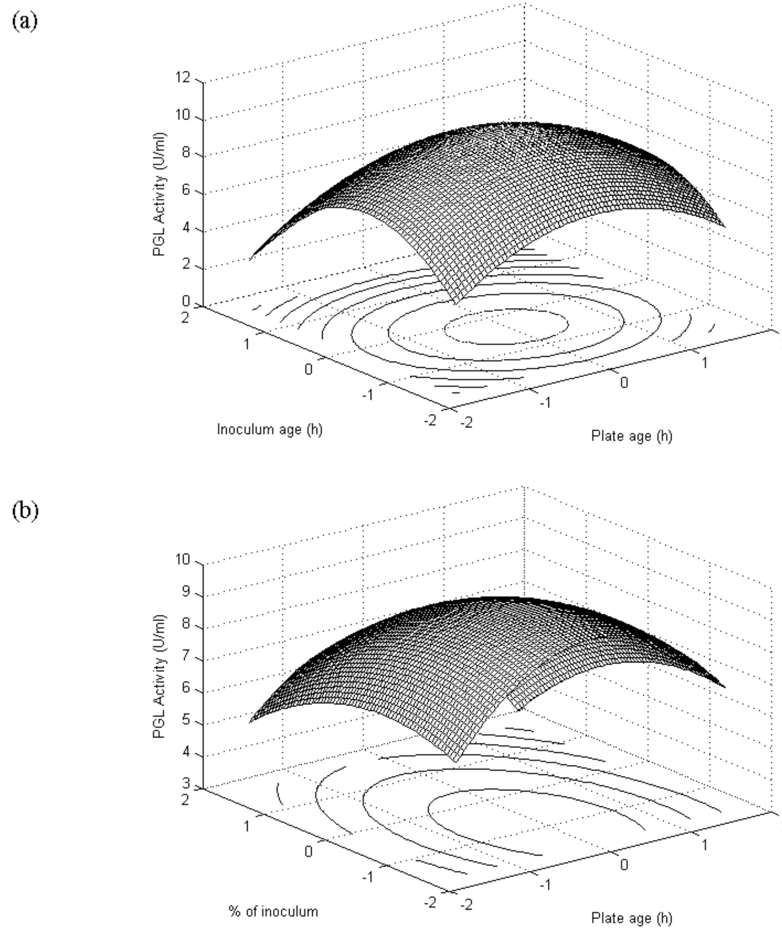

Fig. 2: Response surface plot (a) PGL activity at different levels of inoculum age (h) and slant age (h) at optimum model predicted amount of inoculum (% v/v), (b) PGL activity at different levels of amount of inoculum (% v/v) and slant age (h) at optimum model predicted inoculum age (h)

that enzyme production was enhanced and the time of fermentation was reduced from 48 to 24 h suggesting that response surface methodology (statistical experimental design) can be successfully used to develop best seed culture conditions for maximum enzyme production in bioreactors. Similarly, Panda and coworkers optimized the microbiological parameters for production of pectinases by *Aspergillus niger* and the fermentation time was reduced from 144 to 120 h. However, the isolated used in this study produced maximum PL and PGL in 24 h and the isolate can be an alternative to fungal strains for pectinase production. As there are very scanty reports available on microbiological parameters optimization for production of enzymes and other metabolites using response surface methodology, lot of attention need to be made on this aspect for enhanced production of industrial metabolites.

Apart for using RSM to determine the optimal conditions of microbiological parameters for maximum enzyme production, RSM can also be used to study the interaction effect between the

variables (Table 1). The highest value for PL (12.5 U mL^{-1}) production was noted in Run # 8 where plate age was at +1 level and inoculum age and amount of inoculum at -1 level. Consequently the lowest rate of enzyme production (6.4 U mL^{-1}) is noted in Run # 10 where all the factors were at +1 levels, which shows strong interaction between the different variables. The value of coefficients obtained by the model equation reflects the importance of the chosen parameters in relation to enzyme production. For e.g., the coefficient of x_2 is the highest suggests that the inoculum age has a significant role on PL production (Eq. 4). Similarly the coefficient of interaction between plate age and amount of inoculum (x_1x_3) is greater than the interaction between plate age and inoculum age (x_1x_2) or inoculum age and amount of inoculum (x_2x_3) suggesting that the interaction between plate age and amount of inoculum is very significant for PL production and can not be neglected. Such information will not be obtained when one factor at a time approach is used to optimization. In addition, the coefficient of squared term is very high and can not be neglected.

Similarly for PGL production, the interaction between the variables is clearly observed in Table 1. The highest value for PGL (9.5 U mL^{-1}) production was noted in Run # 8 where plate age was at +1 level and inoculum age and amount of inoculum at -1 level. The lowest rate of enzyme production (2.5 U mL↑$^{-1}$) is noted in Run # 16 where the inoculum age is at +1.7, which shows that changes in these variables effects enzyme production. Similarly, the interaction effect between the variables for PGL production can also be studied by examining the coefficients of Eq. 5. For e.g., the effect of squared term for x_2 is the highest suggests that inoculum age plays a significant role on PGL production. Similar to PL production the coefficient of interaction between plate age and amount of inoculum (x_1x_3) is greater than other interaction terms for PGL production suggesting that the interaction between plate age and amount of inoculum also plays a significant role for both PL and PGL production. The statistical analysis in the present study showed there strong interaction effect among the microbiological parameters is very important for enzyme production and can not be neglected. These interaction effects can not be studied by conventional one variable at a time approach. Hence, RSM can be successfully used to optimize the microbiological conditions for maximum production of PL and PGL by *Debaryomyces nepalensis* and also used to study the interaction effect among microbiological parameters.

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References

Box, G.E.P. and K.B. Wilson, 1951. On the experimental designs for exploring response surfaces. Ann. Math. Stat., 13: 1-45.

Carr, J.G., 1985. Tea, Coffee and Cocoa. In: Wood, B.J.B. (Eds.)., Microbiology of Fermented Food, Vol. 2. London, Elsevier, pp: 133-154.

Friedrich, J., A. Cimermenm and W. Steiner, 1990. Production of pectolytic enzymes by *Aspergillus niger*: Effect of inoculum size and potassium hexacyanoferrate II-trihydrate. Applied Microbiol. Biotechnol., 33: 377-381.

Gummadi, S.N. and D.S. Kumar, 2005. Microbial pectic transeliminases. Biotech. Lett., 27: 451-458.

Gummadi, S.N. and D.S. Kumar, 2006a. Pectin lyase and pectate lyase from *Debaryomyces nepalensis* isolated from apple. Res. J. Microbiol., 1: 152-159.

Gummadi, S.N. and D.S. Kumar, 2006b. Enhanced production of pectin lyase and pectate lyase by *Debaryomyces nepalensis* in submerged fermentation by statistical methods. Am. J. Food Technol., 1: 19-33.

Gummadi, S.N. and D.S. Kumar, 2006c. Optimization of chemical and physical parameters affecting the activity of pectin lyase and pectate lyase from *Candida* sp.: A statistical approach. Biochem. Eng. J., In Press.

Hoondal, G.S., R.P. Tewari, R. Tewari, N. Dahiya and Q.K. Beg, 2002. Microbial alkaline pectinases and their industrial applications a review. Applied Microbiol. Biotechnol., 59: 409-418.

Naidu, G.S.N. and T. Panda, 1998. Production of pectolytic enzymes - A review. Bioproc. Eng., 19: 355-361.

Panda, T., G.S.N. Naidu and J. Sinha, 1999. Multiresponse analysis of microbiological parameters affecting the production of pectolytic enzymes by *Aspergillus niger*: A statistical view. Proc. Biochem., 35: 187-195.

Whitaker, J.R., 1991. Microbial Pectolytic Enzymes. In: Fogarty, W.M. and C.T. Kelly (Eds.), Microbial. Enz. Biotechnol. London, Elsevier, pp: 133-176.