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RESEARCH ARTICLE

Lucrative pectinase production by novel strain *Pseudozyma* sp. SPJ with statistical optimization techniques using agro-industrial residues

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Abstract **Production of high titers of an alkaline, extracellular and thermo-tolerant pectinase by a newly isolated yeast *Pseudozyma* sp. SPJ was carried out under solid state fermentation. Citrus peel, the inexpensive agro-industrial residue used as substrate, was experienced to be unsurpassed. Response surface methodology was conducted to optimize the culture conditions for *Pseudozyma* sp. SPJ for hyper production of pectinase. Plackett Burman design was applied to identify the most effective culture variables. Out of nine variables studied, incubation time, moisture content and ammonium sulfate were detected as most important. A full factorial Central Composite Design was used to optimize the levels of these variables, which resulted in 17-fold increase (71.19 IU/g to 1215.66 IU/g dry substrate) in the enzyme yield. The results of analysis of variance and multiple regression analysis implies that the effect of incubation time ($p < 0.000$) and moisture content ($p < 0.018$) is more than ammonium sulfate. And also the interaction of moisture content with ammonium sulfate ($p < 0.002$) is more significant.**

Keywords pectinase, *Pseudozyma* sp. SPJ, response surface methodology, solid state fermentation

Introduction

Pectinases are the enzymes that breakdown the glycosidic bonds of the long chain of galacturonic acid residues of pectic substances (the structural polysaccharides of plant cell wall). Microbial pectinases contribute to almost 25% of the global food enzyme sales and are estimated to increase further. Pectinases are meant for extraction, liquefaction and clarification of fruit juices (Kashyap et al., 2001). In fabric industry, they are employed to ret plant fibers such as flax, hemp and jute (Hoondal et al., 2002; Sharma et al., 2011a). They are also applied to mechanical pulp to solve the retention problems in the paper industry (Reid and Ricard, 2000).

Sub-merged fermentation (SmF) is extensively used for the production of enzymes and for understanding the physiological aspects of enzyme synthesis while solid-state fermentation (SSF) is mainly advocated for the improvement in production level of enzymes (Patil and Dayanand, 2006). In SSF microbes grow and make products while sticking on the surface of solid substrates in near absence of free flowing water. Such system proves more efficient in production of several enzymes and metabolites by microbes as being closer to the microbes' natural habitat. This cultivation technique has acquired a special relevance in the field of biotechnology because it gives higher productivity per reactor volume, has lower operating costs, produces less wastewater, demands less energy and space, requires simpler equipments, gives higher product concentration, avoids foaming and lowers the risks of contamination (Pandey et al., 2000; Suryanarayan, 2003).

The cost of enzyme production is one of the main factors which determine the use of enzyme at industrial scale. To meet industrial demands, high yielding, extracellular pectinase producing organisms are suitable that can use agro-residues as substrate to produce the enzyme cost effectively. The citrus processing residues are attractive and potential feed stock for their biological conversion to value added products as they are rich in both soluble and insoluble carbohydrates. Citrus peel contains 30% (approx.) of pectin in its fresh weight. Citrus peel is also reported earlier (Garzon and Hours,

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1991; Ismail, 1996; Nabi et al., 2003; Dhillon et al., 2004; Sharma et al., 2012) as a good substrate for pectinase production as it consists of pectin with proteins (Dhillon et al., 2004).Use of these residues can help in solving the problem of pollution (Cauto and Sanroman, 2005), as they can increase the Biological Oxygen Demand (BOD) of water.

Optimization of culture conditions is very important as it improves the productivity, economy and practicability of the process (Souza and Roberto 1999; Chen et al., 2002). One variable at a time strategy is the conventional method used for this purpose. This method is not only time consuming, but being one dimensional it can also result in misinterpretation of optimum conditions (Box et al., 1978; Cochran and Cox, 1957). Response surface methodology (RSM) has recently attracted attention as an optimization technique in the field of numerical analysis. It is a powerful statistical technique (Box and Wilson, 1951), consuming less time and labor as fewer experiments are required to give even better results. Its most attractive feature is that it takes all the interactions between the independent variables into consideration and quantifies them (Haaland, 1989; Bogar et al., 2003; Vaidya et al., 2003; Wejse et al., 2003). A 3.5 fold increase in keratinase production using RSM has been reported (Tatineni et al., 2007). Sharma et al. (2011b) have reported 4.2 fold increase in pectinase production under submerged culture conditions using RSM. The aim of study was to achieve maximum pectinase yield from newly isolated yeast *Pseudozyma* sp. SPJ using an economic substrate by statistically optimizing its culture conditions under solid state fermentation.

Materials and methods

Microorganism and its cultivation

An alkaline pectinase producing yeast was isolated from fruit waste disposal site and identified as *Pseudozyma* sp. SPJ by Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, and given accession No. 9842. The microorganism was identified on the basis of its phenotypic characterization and results were further confirmed by 26S rRNA sequencing method. The culture was maintained on yeast extract peptone dextrose (YEPD) medium slants and stored at 4°C (sub-cultured every 3 months).

Cultivation under SSF

In a 250 mL Erlenmeyer flask, 10 g of solid substrate (citrus peel) and 25 mL mineral salt solution (in g/L: $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 0.4; pH 8.0) were taken; sterilized by autoclaving at 1.05 kg/cm^2 for 30 min; inoculated with 10% (v/w) inoculum of 16 h old yeast culture and incubated in a humidified incubator at 32°C for 72 h. The flasks were tapped at regular intervals in order to mix the contents.

Enzyme extraction

The enzyme was extracted twice using100 mL glycine-NaOH buffer (0.01 M, pH 9.0) for each flask of the cultured solid substrate. The contents were squeezed through a wet muslin cloth. The enzyme extract as centrifuged (10000 r/min, 30 min, 4°C) and the clear supernatant obtained was used as crude enzyme for enzymatic measurements.

Pectinase assay

Suitable dilution of crude enzyme (10 μL) was added to 490 μL of pectin (0.1%) and incubated at 65°C for 5 min. Then, 1.5 mL of dinitrosalicylic acid (DNSA) was added to the reaction mixture and heated in boiling water bath for 15 min. Absorbance was measured at 540 nm (Miller 1959). Three controls have been used in the enzyme assay; blank (500 μL buffer), substrate blank (10 μL buffer + 490 μL substrate) and enzyme blank (10 μL enzyme + 490 μL buffer).The absorbance values for the blanks are subtracted from the absorbance of the test reaction mixture. One unit of enzyme activity was defined as the amount of enzyme that released 1μmol galacturonic acid per min under assay conditions, using galacturonic acid as standard.

Identification of effective culture conditions

The Plackett Burman design (PBD) was used to find out the critical culture conditions (physical and chemical) for the pectinase production by *Pseudozyma* sp. SPJ. The software package *Minitab 16* was used to design and analyze the experiments. It designed 12 experiments for 9 variables (Table 1) out of which the inoculum size was taken as dummy variable. After performing the experiments, the pectinase activity was determined in each case by taking cell free supernatant as crude enzyme. The experiments were conducted in triplicates and the mean value of three responses (pectinase activity) was taken as result. The variables showing maximal effect were identified by performing *F*-test.

F value for each variable is calculated with Eq. (1):

$$F \text{ value} = \frac{\text{Factor mean square}}{\text{Error mean square}} \quad (1)$$

The factor mean square for variable A was calculated with Eq. (2):

$$\text{Factor mean square} = \frac{\left(\sum A(H) - \sum A(L)\right)^2}{12} \quad (2)$$

Here, *H* is the value of response of the experiments having higher values of the variable and *L* is the value of response of the experiments having lower values of the variable. The error mean square was the factor mean square for the dummy variable used in the design.

Table 1 Experimental Plackett Burman design and results of screening significant culture conditions for pectinase production by *Pseudozyma* sp. SPJ under SSF

Run	Temperature (°C)	pH	Time(h)	Inoculum size (%)	Moisture content (mL/g)	$MgSO_4$(%)	$(NH_4)_2SO_4$ (%)	KH_2PO_4 (%)	Yeast extract (%)	Response (IU/ g)
1	35	6	72	1	2	0.05	0.2	0.2	0.5	655.17
2	35	9	72	1	4	0.1	0.1	0.2	0.0	591.93
3	35	6	24	1	4	0.1	0.2	0.1	0.5	608.35
4	35	9	24	2	2	0.05	0.1	0.2	0.5	697.33
5	35	9	24	2	4	0.05	0.2	0.1	0.0	679.37
6	35	6	72	2	2	0.1	0.1	0.1	0.0	662.53
7	25	9	72	1	4	0.05	0.1	0.1	0.5	605.00
8	25	6	24	1	2	0.05	0.1	0.1	0.0	673.15
9	25	6	24	2	4	0.1	0.1	0.2	0.5	623.11
10	25	9	72	2	2	0.1	0.2	0.1	0.5	683.23
11	25	6	72	2	4	0.05	0.2	0.2	0.0	630.05
12	25	9	24	1	2	0.1	0.2	0.2	0.0	632.86
F value	0.22	0.14	3.73	1	2.46	1.88	2.05	0.65	1.21	

Optimization of most effective variables using Central Composite Design (CCD)

A full factorial CCD was designed, studying each variable at 5 levels i.e. incubation time (24–120 h), moisture content (1–5 mL/g dry substrate)and concentration of ammonium sulfate (0.1%–0.5%), with total 20 runs (8 cube points, 6 axial points and 6 central points) as shown in Table 2. Pectinase activity was measured for all the runs designed and multiple regression analysis and Analysis of Variance (ANOVA) were performed with the resulting data. The quadratic model for predicting the optimal response i.e. pectinase activity is given as Eq. (3):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (3)$$

where Y is the pectinase activity, β_0 is the intercept, β_1, β_2 and β_3 are linear coefficients; β_{11}, β_{22} and β_{33} are squared coefficients; and β_{12}, β_{13} and β_{23} are interaction coefficients.

Results and discussion

Nine variables were taken to identify the most important ones by using PBD for the production of pectinase and the results were measured in terms of pectinase activity (Table 1). The three variables: incubation time, moisture content and ammonium sulfate were found to be most effective by observing the results of *F*-test performed (Table 1). The three most effective variables were subjected to CCD (Chen et al., 2002) for their level optimization. The measured and predicted values for the pectinase activity are given in the Table 2. The results were further analyzed using actual (uncoded) values of the independent variables used for RSM. The estimated regression coefficients for pectinase activity were given in Table 3. By applying multiple regression analysis, the Eq. (4) is explaining the pectinase production.

$$Y = 1114.51 + 5.53x_1 - 66.71x_2 - 220.37x_3 - 0.03x_1^2 - 3.55x_2^2 - 91.19x_3^2 - 0.06x_1x_2 - 2.73x_1x_3 + 180.33x_2x_3 \quad (4)$$

The value of R^2 (95.86%), adjusted R^2 (92.14%) and insignificant ($p < 0.130$) Lack of Fit concluded the model as fit, suitable and significant (Box et al., 1978; Cochran and Cox, 1957; Akhnazarova and Kafarov, 1982). ANOVA was conducted and the significance of each coefficient was determined by student's *t*-test and *p* value (Table 4). The larger the magnitude of the *t* value and smaller the *p* value ($p < 0.05$ at 5% significance level), the more significant is the corresponding coefficient (Akhnazarova and Kafarov, 1982). This implies that the effect of incubation time ($p < 0.001$) and moisture content ($p < 0.018$) is more than other variables and also the interaction of moisture content with ammonium sulfate ($p < 0.002$) is more significant at 5% significance level. *p* values for linear (0.000), square (0.000) and interaction (0.010) coefficients found to be significant. The model *F* value 25.73 indicated model terms as significant. The results of statistical analysis were represented in the form of contour plots (Fig. 1). Each plot represents an infinite number of combinations of two test variables while keeping the third one at its respective constant value. A 3.5 fold increase in the exo-polygalacturonase production was attained using RSM (Gupta et al., 2008).

The *p* value had shown that the most important factor for pectinase production is the time of incubation.The maximum activity of enzyme was observed in the culture filtrate harvested after 72 h (1215.66 IU/g dry substrate). The enzyme yield increased with the time of incubation up to 72 h and decreased thereafter (Fig. 1A, 1B). This might be a result of variation in pH of the medium during fermentation. Microorganisms start hydrolyzing the enzymes for synthesis of biomass proteins after a certain time period (Nabi et al.,

Table 2 Experimental Central Composite Design and results obtained for optimization of pectinase production by *Pseudozyma* sp. SPJ under SSF

Run	Incubation time (h)	Moisture (mL/g dry substrate)	$(NH_4)_2SO_4$ (%)	Measured response (IU/g)	Predicted response (IU/g)
1	72	1	0.3	1215.66	1201.33
2	72	3	0.3	1140.53	1139.77
3	96	2	0.2	1178.37	1178.50
4	48	4	0.2	1048.74	1045.85
5	72	3	0.5	1161.77	1149.97
6	72	3	0.3	1135.01	1139.77
7	72	3	0.3	1130.61	1139.77
8	24	3	0.3	1073.24	1061.93
9	48	2	0.4	1132.35	1145.92
10	72	5	0.3	1045.14	1049.80
11	48	2	0.2	1142.98	1155.03
12	96	2	0.4	1130.61	1143.17
13	96	4	0.2	1067.92	1064.01
14	72	3	0.3	1155.97	1139.77
15	72	3	0.3	1144.68	1139.77
16	96	4	0.4	1103.21	1100.82
17	48	4	0.4	1099.33	1108.87
18	72	3	0.1	1120.13	1122.27
19	72	3	0.3	1141.46	1139.77
20	120	3	0.3	1075.69	1077.33

Table 3 Estimated regression coefficients for pectinase activity of *Pseudozyma* sp. SPJ under SSF

Term	Coefficient	SE[a]Coef	*t* value	Significance (*p* value)[b]
β_0	1114.51	81.00	13.760	0.000
β_1	5.53	0.99	5.588	0.000
β_2	−66.71	23.74	−2.810	0.018
β_3	−220.37	237.44	−0.928	0.375
β_{11}	−0.03	0.00	−7.165	0.000
β_{22}	−3.55	2.45	−1.451	0.178
β_{33}	−91.19	244.72	−0.373	0.717
β_{12}	−0.06	0.18	−0.305	0.767
β_{13}	−2.73	1.81	−1.511	0.162
β_{23}	180.33	43.38	4.157	0.002

a – standard error.
b –5% significance level.

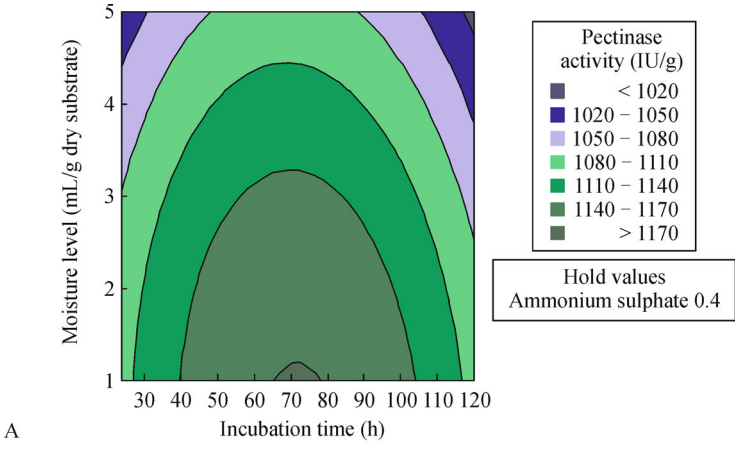
Table 4 Analysis of variance and regression analysis for the present RSM model

Source	df[a]	SeqSS[b]	Adj MS[c]	*F* value	Significance (*p* value)
Regression	9	34871.06	3874.56	25.73	0.000
Linear	3	23966.39	3189.17	21.18	0.000
Square	3	7945.11	2648.37	17.59	0.000
Interaction	3	2959.56	986.52	6.55	0.010
Residual error	10	1505.69	150.57		
Lack-of-fit	5	1124.53	224.91	2.95	0.130
Pure error	5	381.17	76.23		
Total	19	36376.75			

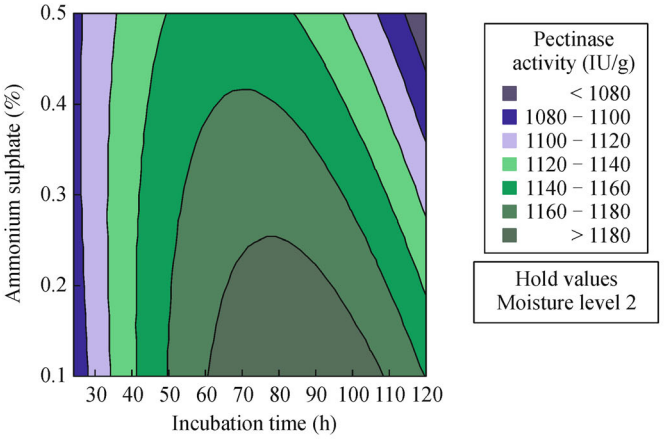
a– degree of freedom.
b– sum square.
c – mean square.

2003). Maximum pectinase was harvested after 96 h (Hours et al., 1998; Bayoumi et al., 2008) and after 65 h (Piccoli-Valle et al., 2001).

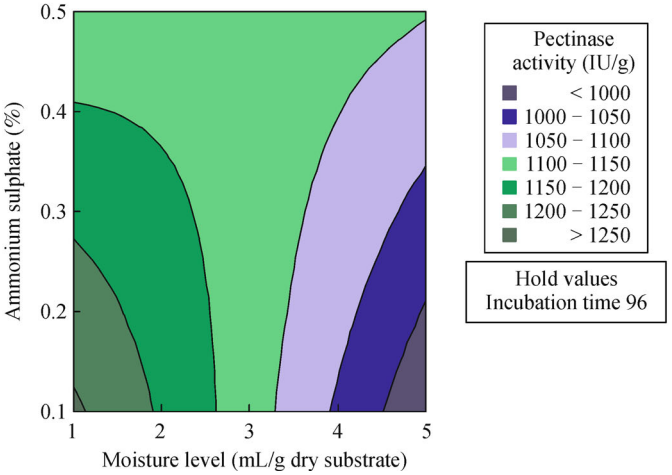
Moisture content is also found crucial for growth of the organism and enzyme production.The importance of moisture level in SSF medium and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate. *Pseudozyma* sp. SPJ produced maximum titer of pectinase when citrus peel was moistened with MA (g/L: $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 1.0) in a ratio of 1:1 to 1:2.5 (Fig. 1A) and decreased with any increase or decrease in the moisture level. The porosity and gas volume may reduce with increase in moisture level and when lower it is known to



A



B



C

Figure 1 Contour plots of interaction between different variables and their effect on pectinase activity produced by *Pseudozyma* sp. SPJ optimizing the culture conditions statistically under SSF.

decrease nutrient solubility and swelling of solid substrate. It also increases water tension (Raimbault and Alazard, 1980; Narahara et al., 1982). Maximum pectinase yield at substrate to moisture ratio of 1:5 has been reported for *Streptomyces* sp. RCK-SC (Kuhad et al., 2004).

The concentration of ammonium sulfate was not found as a significant variable individually; however its interaction with moisture content (Fig. 1C) had shown that requirement of ammonium sulfate increases with increase in the moisture level. Ammonium sulfate is a good inorganic source of nitrogen for growth of microbes.It has been optimized as 0.3% experimentally and up to 0.2% with graphical evaluation.

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Compliance with ethics guidelines

Sampriya Sharma, Jitender Sharma and Rishi Pal Mandhan declare that they have no conflict of interest. This article does not contain any studies with human and animal subjects performed by any of the authors.

References

Akhnazarova S, Kafarov V (1982). Experiment Optimization in Chemistry and chemical Engineering. Mir Publishers Moscow.

Bayoumi R A, Yassin H M, Swelim M A, Abdell-All E Z (2008). Production of bacterial pectinase(s) from agro-industrial wastes under solid state fermentation conditions. J ApplSci Res, 4(12): 1708–1721

Bogar B, Szakacs G, Pandey A, Abdulhameed S, Linden J C, Tengerdy R P (2003). Production of phytase by Mucor racemosus in solid-state fermentation. Biotechnol Prog, 19(2): 312–319

Box GEP, Hunter WG, Hunter JS (1978). Statistics for Experimenters, Wiley New York, 294–334

Box G E P, Wilson K B (1951). On the Experimental Attainment of Optimum Conditions. J R Stat Soc, B, 13: 1–45

Cauto S R, Sanroman M S (2005). Application of solid-state fermentation to food industry–a review. J Food Eng, 22: 211–219

Chen Q H, He G Q, Mokhtar A M A (2002). Optimization of medium composition for the production of elastase by *Bacillus* sp. EL31410 with response surface methodology. Enzyme Microb Technol, 30(5): 667–672

Cochran W G, Cox G M (1957). Experimental Designs. 2nd edn. Wiley New York, 346–354

Dhillon S S, Gill R K, Gill S S, Singh M (2004). Studies on the utilization of citrus peel for pectinase production using fungus *Aspergillus niger*. Int J Environ Stud, 61: 199–210

Garzon C G, Hours R A (1991). Citrus waste: an alternative substrate for pectinase production in solid state culture. Bioresour Technol, 39(1): 93–95

Gupta S, Kapoor M, Sharma K K, Nair L M, Kuhad R C (2008). Production and recovery of an alkaline exo-polygalacturonase from *Bacillus subtilis* RCK under solid-state fermentation using statistical approach. Bioresour Technol, 99(5): 937–945

Haaland PD (1989). Experimental Design in Biotechnology. Marcel Dekker New York, 1–18

Hoondal G S, Tiwari R P, Tewari R, Dahiya N, Beg Q K (2002). Microbial alkaline pectinases and their industrial applications: a review. Appl Microbiol Biotechnol, 59(4–5): 409–418

Hours R A, Voget C E, Ertola R J (1998). Some factors affecting pectinase production from apple pomace in solid state cultures. Biol Wastes, 24(2): 147–157

Ismail A S (1996). Utilization of orange peels for the production of multi enzyme complexes by some fungal strains. Process Biochem, 31(7): 645–650

Kashyap D R, Vohra P K, Chopra S, Tewari R (2001). Applications of pectinases in the commercial sector: a review. Bioresour Technol, 77 (3): 215–227

Kuhad R C, Kapoor M, Rustagi R (2004). Enhanced production of an alkaline pectinase from *Streptomyces* sp. RCK-SC by whole-cell immobilization and solid state cultivation. World J Microbiol Biotechnol, 20(3): 257–263

Miller L G (1959). Use of dinitrosalicylic acid reagent for the determination of reducing sugars. Anal Chem, 31(3): 426–428

Nabi N G, Asgher M, Shah A H, Sheikh M A, Asad M J (2003). Production of pectinase by *Trichoderma harzianum* in solid state fermentation of citrus peel. Pak J Agri Sci, 40: 3–4

Narahara H, Koyama Y, Yoshida T, Pichangukura S, Ueda R, Taguchi H (1982). Growth and enzyme production in a solid-state culture of *Aspergillus oryzae*. J Ferment Technol, 7: 258–265

Pandey A, Soccol C R, Nigam P, Soccol V T (2000). Biotechnological potential of agro-industrial residues: sugar-cane bagasse. Bioresour Technol, 74(1): 69–80

Patil S R, Dayanand A (2006). Exploration of regional agrowastes for the production of pectinase by *Aspergillusniger*. Food Technol Biotechnol, 44(2): 289–292

Piccoli-Valle R H, Brandi I V, Silva D O, Passos F J V (2001). Pectin lyase production by *Penicillium griseoroseum* grown in sugar cane juice in repeated batch cultures. World J Microbiol Biotechnol, 17(5): 433–437

Raimbault M, Alazard D (1980). Culture method to study fungal growth in solid fermentation. Eur J Appl Microbiol Biotechnol, 9(3): 199–209

Reid I, Ricard M (2000). Pectinase in papermaking: solving retention problems in mechanical pulps bleached with hydrogen peroxide. Enzyme Microb Technol, 26(2–4): 115–123

Sharma S, Mandhan R P, Sharma J (2011a). *Pseudozyma* sp. SPJ: an economic and eco-friendly approach for degumming of flax fibers. World J Microbiol Biotechnol, 27(11): 2697–2701

Sharma S, Mandhan R P, Sharma J (2011b). Statistical optimization of culture parameters for enhanced pectinase production by a novel strain *Pseudozyma* sp. SPJ. Inter J Appl Eng Res, 6(5): 792–798

Sharma S, Mandhan R P, Sharma J (2012). Utilization of agro-industrial residues for pectinase production by the novel strain *Pseudozyma* sp. SPJ under solid state cultivation. Ann Microbiol, 62(1): 169–176

Souza M O, Roberto I C (1999). Solid-state fermentation for xylanase production by *Thermoascus aurantiacus* using response surface methodology. Appl Microbiol Biotechnol, 52: 768–772

Suryanarayan S (2003). Current industrial practice in solid state fermentations for secondary metabolite production: the Biocon India experience. Biochem Eng J, 13(2–3): 189–195

Tatineni R, Doddapaneni K K, Potumarthi R C, Mangamoori L N (2007). Optimization of keratinase production and enzyme activity using response surface methodology with *Streptomyces* sp7. Appl Biochem Biotechnol, 141(2-3): 187–201

Vaidya R, Vyas P, Chhatpr H S (2003). Statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*. Enzyme Microb Technol, 33(1): 92–96

Wejse P L, Ingvorsen K, Mortensen K K (2003). Xylanase production by a novel halophilic bacterium increased 20 fold by response surface methodology. Enzyme Microb Technol, 32(6): 721–727