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UTILIZATION OF WHEAT BRAN FOR ENHANCED PRODUCTION OF EXO-POLYGALACTURONASE BY *Penicillium notatum* USING RESPONSE SURFACE METHODOLOGY

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Response surface methodology (RSM) has been one of the most commonly used optimization method in the recent years. Especially this technique is frequently used in the enzyme biotechnology. In this context *Penicillium notatum* was evaluated for enhanced biosynthesis of exo-polygalacturonse using statistical approach under solid state fermentation (SSF). Nine parameters were assessed for maximum biosynthesis of the enzyme using classical method (data not shown). Four factors (3 numerical and 1 categorical factor) were evaluated as the best having the influential effect on enzyme yield. These factors were incubation time (days), temperature (°C), pH, and nitrogen sources. Nitrogen sources like glycine and ammonium chloride, were selected in RSM optimization as they were found to be the most promising in multiplying the exo-polygalacturonse activity during the classical approach. Maximum activity of the enzyme achieved was 1129.62 U/gds with incubation period 3 days, pH 1.318, temperature 30 °C and using ammonium chloride as nitrogen source. All the experiments were performed at 40 % (V/W) moisture level using 10 g of wheat bran as substrate. An increase of 5.5 folds of activity was observed using this technique as compared to classical method. The results indicated that RSM could be used to get enhanced activities of exo-polygalacturonse under SSF.

Keywords: Exo-polygalacturonase, response surface methodology, *Penicillium notatum*, solid state fermentation
***Abbreviations*:** PL: Pectinlyase, PG: Polygalacturonase

INTRODUCTION

Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. The degradation of the pectin molecule is done through a synergistic and coordinated action of pectinolytic enzymes, including pectin methylesterase, polygalacturonases, pectatelyases and pectin lyases. The polygalacturonase (EC. 3.2.1.15) enzymes are of great relevance for the process of depolymerization of pectin, acting in the cleavage of the α-1,4-glycosidic bonds of pectin and pectic acids (de Vries and Visser, 2001; Gummadi and Kumar, 2005).

Pectinolytic enzymes are widely distributed in higher plants and microorganisms. They are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage. They also aid in maintaining ecological balance by causing decomposition and recycling of waste plant materials. Plant pathogenicity and spoilage of fruits and vegetables by rotting are some other major manifestations of pectinolytic enzymes (Jayani *et al*., 2005).

Microbial pectinases have tremendous potential to offer mankind because these pectic enzymes are used most frequently in the food industry. Most pectic enzyme preparations are used in the fruit processing industry and pectic enzymes alone account for about one quarter of the world's food enzyme production. Fungal pectinases are among the most important industrial enzymes and are of great significance with wide range applications in textile processing, degumming of plant blast fibers, treatment of pectic wastewaters, papermaking, coffee and tea fermentations. The degradative process by depolymerases plays an important role in food technology, due to reduction in time of filtration and to volume increase, and juice clarification. This process leads to more stable and concentrated product (Teixeira *et al*., 2000). For this reason, these enzymes have been the targets of studies that aim at obtaining expression systems on a large scale, especially for utilizing filamentous fungi (Cardoso *et al*., 2008).

RSM is advantageous over conventional methods available and it includes less experiment numbers, suitability for multiple factor experiments and search for common relationship between various factors towards finding the most suitable production conditions for the bioprocess and forecast response (Box and Wilson, 1951). RSM has been extensively utilized to optimize chemical and biochemical processes, such as production of enzymes (Goncalves *et al*., 2012; Amin *et al*., 2011; Barbosa *et al*., 2010; Hajji *et al*., 2008; Gupta *et al*., 2008), composition of cultivation media (Kunamneni and Singh, 2005), conditions of enzymatic hydrolysis (Shieh and Lai, 2000), parameters for juice

processing etc. (Abdullah *et al*., 2007).

Due to the significance of these important enzymes, the present project was aimed to apply RSM in the isolation of exo-polygalacturonase from the fungus *Penicillium notatum* using different parameter screened by the classical methods of optimization (data not shown here). A narrow range of levels of the most influential factors were used in order to get the best response. The experiments were conducted under solid state fermentation technique. The agricultural waste wheat bran and moisture contents were fixed at one level i.e., 10 g and 40 %, respectively, in the scale up of the RSM optimization under solid state fermentation i.e., 10g and 40% (V/W) respectively. Four factors (3 numerical and 1 categorical factor) were evaluated in classical approach as the best having the influential effect on enzyme yield. These factors were incubation time (days), temperature (°C), pH, and nitrogen sources. The minimum and maximum levels of the factors chosen were incubation time (2-4 days), pH(2-4), temperature(25–35°C) and categorical factor nitrogen sources (glycine and ammonium chloride). The main purpose of the RSM optimization study was to get the maximum enzyme titers with the help of above mentioned parameters screened during classical study, thus envisaging their applications in operations that are characteristic of the fruit juice and beverage industry.

MATERIALS AND METHODS

***Micro-organism and substrate*:** The microbial culture of *P. notatum* was obtained from the Department of Plant Pathology, University of Agriculture Faisalabad Pakistan. The microbial culture of *P. notatum* was maintained on potato-dextrose agar medium (OXOID) in slants and petri dishes at 30 °C for five to seven days in incubator. The slants were then preserved at 4°C in the refrigerator. The inoculum was obtained from a conidial suspension (1×10^8 spores/mL) obtained from the surface of slants/petri plate in a sterile solution of Kirk basal medium (Tien and Kirk, 1988). Agricultural waste wheat bran was selected as the natural substrate for the production of the exo-polygalaturonase using *P. notatum*. It was collected from the local market of Faisalabad Pakistan, dried in an oven at 60°C till constant weight and stored in the airtight container until used for SSF for the required enzyme production.

***Cultivation and harvest of the strain in solid state fermentation*:** Strain was cultivated in 250 mL Erlenmeyer flasks containing moist wheat bran as substrate under solid state fermentation inoculated with the inoculum having 1×10^8 spores/mL. Inoculum size of 2 mL was used for each experiment. Wheat bran (10 g) and 40 % moisture level were used during the whole study. The cultivated growth medium under SSF was harvested with 5 0mM phosphate buffer (pH 7) following by shaking at 120 rpm at 30°C for 30-45 minutes. The culture filtrate obtained after the SSF was filtered first through muslin cloth, then using Whatman filter paper No.1 by ultrafiltration using suction pump and finally assessed for the enzyme activity as described by Miller (1959).

***Experimental planning*:** Different parameters for enhanced production of exo-polygalacturonase were optimized by applying RSM using Central Composite Design (CCD). The first step in this study was the identification of parameters likely to be effective on the response i.e., enzyme production/isolation. Therefore, screening experiments of various factors using classical approach were performed to better facilitate the optimization of independent factors level (data not shown here). Using this classical approach incubation time, temperature, pH and nitrogen sources were selected as successful independent variables to be used for the maximum production of exo-polygalacturonase using RSM.A narrow range of the levels of the factors were selected for this optimization to get the optimized response. The minimum and maximum levels of the factors chosen were incubation time (2-4 days), pH(2-4), temperature(25–35°C) and categorical factor nitrogen sources (glycine and ammonium chloride). These factors were fed into the DOE statease software version-7 following the central composite design resulting in 40 experimental units. The design was comprised of the 6 central, 26 factorial and 8 axial points. The response variable was defined as the production of exo-polygalacturonase (U/gds). 6 central points were defined by the software in which the triplicates were carried out to better estimate the experimental error.

The following regression equation describes well the quadratic modeling utilized in the experimental planning, including the interaction effect.

$$R = 819.91-31.26[\mathbf{A}]-26.30038441[\mathbf{B}]-54.02[\mathbf{C}] + 40.96[\mathbf{D}] +70.48[\mathbf{AB}] + 138.41[\mathbf{AC}] + 13.23[\mathbf{AD}] + 29.32[\mathbf{BC}] + 9.18[\mathbf{BD}] + 4.34[\mathbf{CD}] -164.85[\mathbf{A^2}] + 8.28[\mathbf{B^2}] -109.51[\mathbf{C^2}] \quad \textbf{Equation 1}$$

The student *F*-test for the analysis of variance (ANOVA) was utilized to determine the significance of the regression model. The numerical and graphical analysis was carried out for the analysis of the optimization studies.

***Validation of the model*:** The validation of the second order quadratic model was carried out using the numerical and graphical optimization strategy of the software by varying the independent variables within the design space.

***Enzyme assay*:** Exopectinase (exo-polygalacuturonase) activity was determined by measuring the reducing groups released from the pectin solution using 3, 5-dinitrosalicylic acid (DNS) method as described by Miller (1959). D-Galacturonic acid monohydrate was used as the standard. A suitably diluted enzyme (0.1 mL) was added to reaction mixture containing 1% pectin solution and 50 mM acetate buffer (pH 5.5). The reaction mixture was incubated at 40°C for 30 minutes and terminated by adding 1 mL of 3,5-dinitrosalicylic acid reagent followed by heating in boiling

water bath for 5 min. The reducing sugars formed in the solution were measured at 535 nm. One unit (U) of exopectinase was defined as the quantity of enzyme that liberates one micromole of galacturonic acid per minute at 40°C and pH 5.5. Enzyme production in solid state fermentation was expressed in units per gram of dry substrate (U/gds).

RESULTS

***Response surface methodology applied to the optimization of exo-polygalacturonase production by Penicillium notatum*:** The key variables that have the maximum influence on the final response (exo-polygalacturonase activity) of the system were identified by classical approach and the optimization and interactions of various selected factors (3 numerical and 1 categorical) on exo-polygalacturonase production (incubation time, pH, temperature and nitrogen sources) were examined through RSM following central composite design (CCD). Responses obtained after running 40 experimental units were fed to Design of Expert Software version-7 and are presented in Table 1. The results were then analyzed by standard analysis of variance (ANOVA) which gave regression equation (equation 1 in terms of coded factors). Linear terms, interactive effects and *p*-values are shown in ANOVA Table 2.

Table1. **Experimental design and result of CCD of response surface methodology**

Run #	Incubation time(days)	PH	Temp. (°C)	Nitrogen source	Exo-polygalacturonase yield (U/gds)	
					Predicted	Experimental
1	3	1.31	30	NH_4Cl	912.54	1129.62
2	2	2	35	NH_4Cl	482.95	596.03
3	4	2	35	Glycine	483.39	533.58
4	3	3	30	NH_4Cl	860.78	859.27
5	3	4.6	30	Glycine	743.12	764.80
6	4	2	25	Glycine	381.96	214.22
7	3	3	30	Glycine	778.85	783.78
8	3	3	38.4	NH_4Cl	468.23	500.78
9	2	4	25	Glycine	619.47	570.74
10	3	4.68	130	NH_4Cl	855.90	871.13
11	3	3	30	Glycine	778.85	796.03
12	4	2	35	NH_4Cl	582.13	511.45
13	3	3	30	Glycine	778.85	747.02
14	4	4	35	NH_4Cl	747.97	529.23
15	4.68	3	30	Glycine	238.21	373.11
16	3	3	30	Glycine	778.85	720.54
17	4	2	25	NH_4Cl	463.30	251.77
18	3	3	22	NH_4Cl	659.91	722.51
19	2	2	25	NH_4Cl	917.77	951.76
20	3	3	30	NH_4Cl	860.78	706.31
21	2	2	25	Glycine	889.36	933.58
22	1.31	3	30	Glycine	387.92	258.88
23	1.31	3	30	NH_4Cl	425.34	280.23
24	3	3	30	NH_4Cl	860.78	711.05
25	2	2	35	Glycine	437.14	276.27
26	4	4	35	Glycine	612.51	521.73
27	3	3	22	Glycine	591.89	599.19
28	3	3	30	NH_4Cl	860.78	918.56
29	4	4	25	Glycine	393.79	279.44
30	2	4	35	Glycine	284.55	433.19
31	4	4	25	NH_4Cl	511.85	582.59
32	2	4	35	NH_4Cl	367.08	506.31
33	2	4	25	NH_4Cl	684.61	662.43
34	3	3	30	Glycine	778.85	793.26
35	3	3	38.4	Glycine	371.69	347.02
36	3	3	30	NH_4Cl	860.78	954.92
37	4.68	3	30	NH_4Cl	364.61	631.21
38	3	3	30	NH_4Cl	860.78	844.64
39	3	1.31	30	Glycine	861.49	876.66
40	3	3	30	Glycine	778.85	980.22

Table 2. ANOVA for Response Surface Quadratic Model

Source of variation	Sum of Squares	df	Mean Square	F. Value	p-value Prob> F	
Model	1667830	13	128294.6	6.38	<0.0001	Significant
A-Incubation time	27319.85	1	27319.85	1.36	0.25	
B-pH	18479.74	1	18479.74	0.92	0.34	
C-Temperature	79722.04	1	79722.04	3.97	0.05	
D-Nitrogen source	67134.86	1	67134.86	3.34	0.07	
AB	79362.78	1	79362.78	3.95	0.05	
AC	306524.9	1	306524.9	15.26	0.00	Significant
AD	4782.26	1	4782.26	0.23	0.62	
BC	13757.15	1	13757.15	0.68	0.41	
BD	2302.01	1	2302.01	0.11	0.73	
CD	516.32	1	516.32	0.02	0.87	
A^2	783274.5	1	783274.5	39.00	< 0.00	
B^2	1980.66	1	1980.66	0.09	0.75	
C^2	345675.7	1	345675.7	17.21	0.0003	
Residual	522073.6	26	20079.75			
Lack of Fit	426291.4	16	26643.21	2.78	0.0526	not significant
Pure Error	95782.19	10	9578.21			
Cor Total	2189904	39				

The *p*-values indicate the significance of each of the regression coefficients. Values lower than 0.05 are considered significant. In this case AC, A^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The significant effect marks the performance of the response. When a factor has a remarkable effect, the response is higher at higher level, and when a factor has a negative effect the response is low at high levels (Haaland, 1989). It was possible to observe that the regression model was significant. The regression equation obtained indicated R^2 (coefficient of determination) values of 0.7616 for exo-polygalacturonase isolation and thus the models could explain 22.20% of the variability in the response. The model F-value of 6.38 implies the model is significant. A signal to noise ratio greater than 4.0 is desirable.

RSM helps in evaluating the relationship between dependent and independent variables and in the last decade it is being used extensively in the optimization studies in spite of having some drawbacks as reported by Goncalves *et al*. (2012). The equation1 represents the enzyme yield as a function of incubation time, pH, temperature and nitrogen source, where R represents the response variable i.e., enzyme yield in U/gds. The model for the variable response of exo-PG production, the estimated parameters and the corresponding *p*-values show that the linear effect of independent variables had a non-significant effect on Exo-PG production. However, the interaction effect of temperature and incubation time was proved to be highly significant.

The model for Exo-PG production presented a non-significant lack of adjustment (*p*-values of 0.0526), indicating that they are adequate to estimate the variable response.

The statistical significance of Eq. (1) was verified by the ANOVA F-test and the analysis of variance for the quadratic model of the response surface is shown in Table 2. At a 95% confidence level, the model for exo-polygalacturonase production presents a calculated F-value higher than the tabulated value (Box and Wilson, 1951).

***Response surfaces and contour plots*:** Response surface and contour plot figures obtained by the analysis of the experimental data of CCD showed a relationship between two variables at time while maintaining other two variables at a fixed level. These figures are effective in understanding the optimization of the independent variables in order to obtain the maximum enzyme yield and as well as effective in the interpretation of both linear and interaction effects of two variables at a time. The surfaces of response described by the regression models are presented in figures. Fig.1 shows that Response Surface plot explaining the effect of the incubation time and pH while keeping temperature at 30°C and glycine as nitrogen source. The plot revealed that the Exo-PG production was low and production of Exo-PG decreased at high values of pH and incubation time.

Figure 2 helps in elucidating the optimum response when the incubation time and pH are interacting with each other while glycine and temperature were fixed at one level. At this point the maximum enzyme units were 783.37 U/gds located at level between 2.25–3.0 days of incubation within pH of 2 to 3.

The data observed by the varying temperature (°C) and incubation time (days) at constant value of pH 3 and nitrogen source (glycine) was plotted in Fig.3. It shows that an initial increase in incubation time with simultaneous increase in temperature resulted in a drastic increase in Exo-

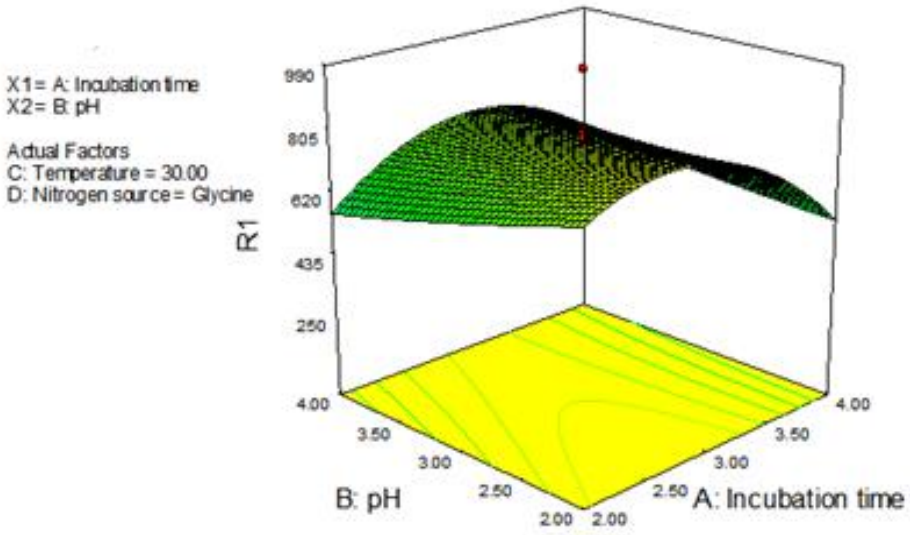

Figure 1. 3-Dimensional response surface plot for the interaction effect of incubation time and pH while other factors i.e., temperature and nitrogen source were fixed at one level.

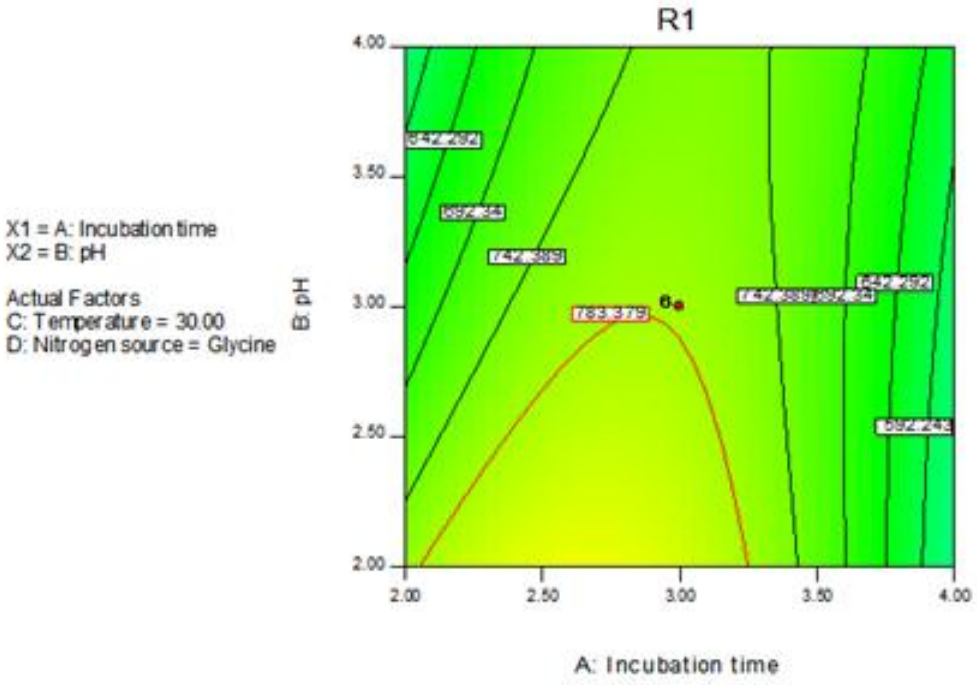

Figure 2. Contour plot for the interaction of incubation time in days and pH while other factors were fixed at one level. The figure depicts the maximum enzyme yield in U/gds obtained by the mutual interaction of these two factors.

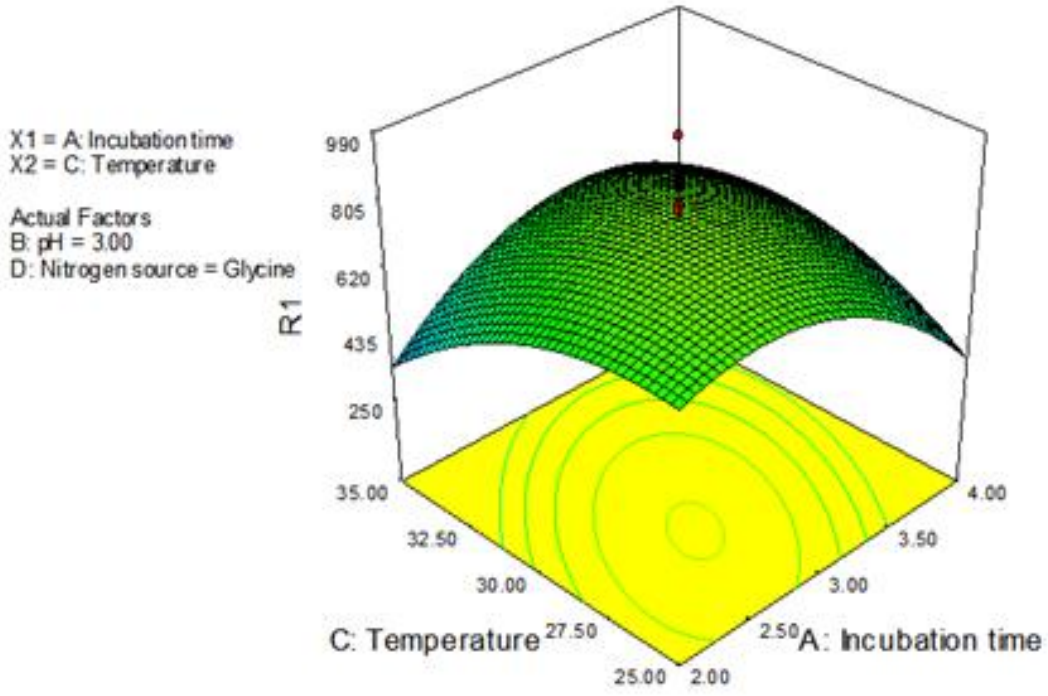

Figure 3. 3-Dimensional response surface plot for the interaction effect of incubation time and temperature while other factors i.e., pH and nitrogen source were fixed at one level.

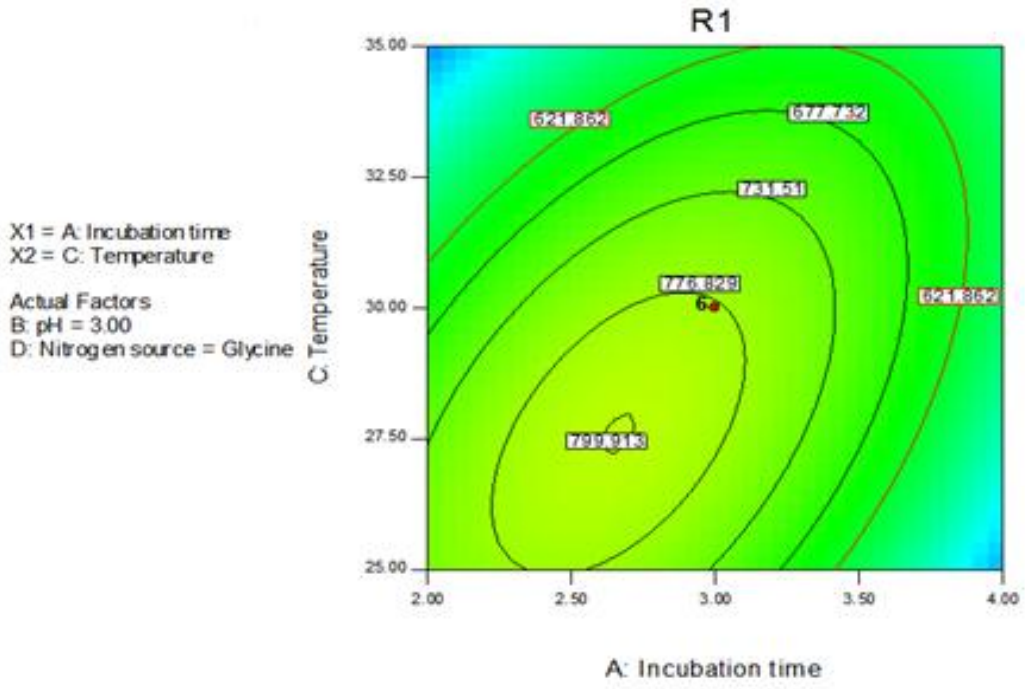

Figure 4. Contour plot for the interaction of incubation time in days and temperature while other factors were fixed at one level. The figure depicts the maximum enzyme yield in U/gds obtained by the mutual interaction of these two factors.

PG production. However, increase in temperature and incubation period beyond a certain limit has negatively affected the Exo-PG production. Fig.4 shows evidence that the model encompassed the optimum region of cultivation time and temperature for Exo-PG production, which decreased in the extreme values of both the variables. The maximum production time was located in the region between 2.25 and 3.0 days (Fig. 4). The maximum estimated PG production occurred at temperature 27°C. In this condition, the estimated PG activity was of 799.913U/gds.

DISCUSSION

***RSM analysis for the maximum production of Exo-PG*:** In the present study we have examined the different independent variables significant for the production of exo-polygalacturonase by the fungus *P. notatum* under solid state fermentation technique. The analysis of variance showed significant result for one interaction effect and RSM optimization resulted in 5.5 folds increase in the enzyme yield as compared to the classical study (data not shown). Main objective of the study was to determine the optimum parameters within the given parameters range. A narrow range of levels of the selected factors was chosen in order to maximize the optimization to get the better response (enzyme yield).In this study high similarity was observed between the predicted and experimentally calculated response which shows that the RSM modeling is adequate and accurate to determine the optimum points of the variables to have the maximum enzyme yields under solid state fermentation. The maximum enzyme units obtained by applying the RSM technique were 1129.62 U/gds. These units were attained after the 3days of incubation at 30 °C keeping the pH of the medium at 1.318 using ammonium chloride as (at 0.04% level) nitrogen source. The enzyme production declined after 3days of incubation. It may be due to the loss of moisture contents in the SSF system due to prolonged incubation in the incubator and/interaction with medium components (Gangadharan *et al*., 2006). A moisture level lower than optimum leads to higher water tension, a lower degree of swelling and a reduced solubility and accessibility of nutrients, present in the solid substrate (Matsumoto *et al.*, 2004).

Among physico-chemical parameters, pH plays an important role including morphological changes on organism in enzyme production. In this study, maximum PG production was achieved at pH 1.318 which is highly acidic. With further increase in pH, a slight reduction in enzyme production was observed. However, all in all this strain was capable of producing significant amounts of enzyme titersat whole range of pH used in the regression model.

The optimum conditions predicted by the numerical optimization strategy of the regression model suggested maximum enzyme yield of 720.38 U/gds after 2.18 days of incubation period at temperature 33 °C adjusting the pH of the solid state medium at 2 using ammonium chloride as nitrogen source (at 0.04 % level). Validation of the regression model was confirmed experimentally by taking the above combination of independent variables under conditions stated by the model. The maximum enzyme titers predicted by the model were proved to be in reasonable agreement with the experimentally calculated units i.e., 696.98 U/gds thus validating the second order quadratic model. The goals defined in the numerical optimization are outline in Table 3.

Goncalves *et al.* (2012) recently used RSM strategies for the optimization of Pectin lyase and PG through recombinant strain *Penicilliumgriseoroseum*T20. They successfully optimized the laboratory scale and fermenter scale fermentation of both enzymes using RSM strategies. The regression model for the response surface showed R^2 values of 0.4437, 0.2984 explaining that the model is only able to explain 44.37 % and 29.84 % of the data in spite of the fact that it resulted in significant titres of PL and PG. They finally concluded their study in discussing some drawbacks of the RSM modeling. However, they successfully utilized this strategy in their optimization studies. At the same time Shankar and Isaiarasu (2012) also carried out the cellulase optimization by using the RSM technique based on central composite design. The R^2 values of 0.7943 suggested that the models were able to explain the variation observed in the experimental results. They reported the 0.5751 IU/mL of cellulose production observed by the model by optimizing with coded factor. Amin *et al.* (2011) had successfully carried out the lipase production in batch fermentation studies in solid state media. They reported an R^2 of 0.994 suggesting that the RSM model is sufficient to explain the optimization of high enzyme titers as compared to the classical approach. Kammoun *et al*. (2008) reported the production of alpha-amylase by *Aspergillus oryzae* CBS

Table 3. Optimization criteria used in this study

Name	Goal	Lower limit	Upper limit	Importance
Incubation time	minimize	2	4	3
pH	minimize	2	4	3
Temperature	maximize	25	35	3
Nitrogen source	in range	Glycine	NH_4Cl	3
Response	maximize	214.2255	1129.625	3

819.72 optimized by using RSM through different experimental plans, such as the full factorial, the Box–Wilson and the rotatable central composite design. The adjusted model proved significant and the R^2 value of 0.957 indicated that model was able to explain the 95.7% of the data. The low CV value of 2.22% indicated high precision and experimental reproducibility.

***Conclusion*:** The RSM optimization has proved to be very accurate and valid method to be used in the optimization studies during the last decade. It encompasses many drawbacks in that not efficient many times in explaining the variability of the model but still successfully helpful in estimating not only the maximizing the response but also determine the relationship between the dependent and independent variables. The present study resulted in 5.5 fold (1129.62 U/gds) increase in the response variable, as compared to completely randomized design, from mesophilic fungus *P. notatum* using the agricultural waste wheat bran as solid substrate under solid state fermentation using optimizing conditions suggested by RSM. The exo-polygalacturonase isolated from these conditions is acidic in nature (optimum pH 5.5). The regression equation obtained during this study indicated an R^2 (coefficient of determination) values of 0.7616 for exo-polygalacturonase isolation and thus the models could explain 22.20% of the variability in the response. The predicted responses calculated using the regression equation suggested by the model are in much closer agreement with the experimental values indicating that the model is able to explain the variability in the responses calculated experimentally. This type of optimization study is helpful to propose the high production of the Exo-PG enzyme at industrial scale as it has broad applications in the food, animal feed, and beverage industry.

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