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Research Article

Process optimization for chitinase production by *Trichoderma harzianum*

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Abstract

Statistics-based experimental design of chitinase production by *Trichoderma harzianum* was optimized in solid state fermentation using Plackett-Burman design and response surface methodology. The important medium components identified by initial screening method of Plackett-Burman were peptone, malt extract, citric acid and urea. Plackett-Burman Pareto chart illustrates the order of significance of the variables affecting the cellmass production. Central composite response surface methodology was performed to evaluate the effects of temperature, pH, inoculum size and substrate concentration on production of chitinase by *Trichoderma harzianum.* Production was studied using sugarcane bagasse under solid state fermentation. Statistical analysis of results showed that the linear and quadric terms of these four variables had significant effects and evident interactions existing between pH and substrate concentration were found to contribute to the response at a significant level. Under these conditions, namely temperature of 35ºC, pH of 6, inoculum size of 2.4 g/l and substrate concentration of 1.5%, the model predicted a chitinase activity of 64 U/ml.

Keywords: response surface methodology, Plackett-Burman, rice bran, SSF, India.

Introduction

Chitin, a _-1, 4-linked homopolymer of *N*-acetylglucosamine is the second most abundant polysaccharide in nature. It is insoluble in water, dilute and concentrated alkalis, alcohol and other organic solvents. It forms the major structural component in the shells and cuticles of arthropods, crustaceans and insects and in cell walls of fungi. The major contribution of chitin to nature is in the form of animal biomass. Chitinases, belonging to the family of glycosyl hydrolases [1], are the enzymes responsible for biological conversion of chitin. These enzymes find major applications in the field of agriculture [2], medicine [3], biotechnology [4], food technology, waste management [5] and industry [6]. Studies on optimization of chitinases have been reported earlier with effects of different media ingredients on its production. The concept of response surface methodology (RSM) has eased process development and has been of significant use at industrial level. At a basic biological level, recent studies have indicated the use of RSM for analyzing effects of different factors on proteolytic activity [8] and optimization of enzyme production [9]. This study is an attempt to evaluate the effects of several factors on the production of an industrially important enzyme, chitinase. Solid-state fermentation (SSF) has emerged as an appropriate technology for the management of agro-industrial residues and for their value addition. SSF is a promising technology for the development of several bioprocesses and products including production of industrial enzymes on large-scale [4, 5]. Different types of substrates, which contain chitin, have been tried for the production of chitinase[7], which included fungal cell walls, crab and shrimp shells and agricultural residues [6, 8]. The use of *Trichoderma* sp. in SSF for the production of lytic enzymes such as cellulose and chitinase has tremendous impact for an industrial scale production [9, 11].

Materials and Methods

Microorganism and inoculum preparation

A fungal isolate, *T. harzianum792* obtained from the MTCC, Chandegarh was used in the present study. The culture was maintained on potato-dextrose agar (PDA) medium and subcultured every fortnight. Slants were incubated for 8 days at 30˚C and stored at 4˚C. The spores of a fully sporulated slant were dispersed in 10 ml of 0.1% Tween 80 solution by dislodging them with a sterile loop under aseptic conditions. The spore suspension obtained was used as the inoculum. Viable spores present in the suspension were determined by serial dilution followed by plate count.

Chitinase assay

Chitinase activity was determined by a dinitrosalicylic acid (DNS) method [12]. This method works on the concentration of *N*-acetyl glucosamine (NAG), which is released as a result of enzymic action [13, 14]. The 2ml reaction mixture contained 0.5 ml of 0.5% colloidal chitin in phosphate buffer (pH 5.5), 0.5 ml crude enzyme extract and 1ml distilled water. The well vortexed mixture was incubated in a water bath shaker at 50˚C for 1 h. The reaction was arrested by the addition of 3ml DNS reagent followed by heating at 100˚C for 10 min with 40% Rochelle's salt solution. The coloured solution was centrifuged at 10,000 rotations per minute for 5 min and the absorption of the appropriately diluted test sample was measured at 530 nm using UV spectrophotometer (UV-160 A, Shimadzu, Japan) along with substrate and enzyme blanks. Colloidal chitin was prepared by the modified method of Roberts and Selitrenkoff [15]. One unit (U) of the chitinase activity is defined as the amount of enzyme that is required to release 1_mol of *N*-acetyl-_-d-glucosamine per minute from 0.5% of dry colloidal chitin solution under assay conditions.

Optimization of nutrient supplements

The medium components were evaluated using Plackett-Burman statistical design [16]. This is a fraction of a two-level factorial design and allows the investigation of '*n*-1' variables with at least '*n*' experiments. The main effect was calculated as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (−1) of each factor. Plackett-Burman experimental design is based on the first order model where *Y* is the response (Cell growth), β_0 is the model intercept and βi is the variable estimates. This model describes no interaction among factors and it is used to screen and evaluate the important factors that influence lipase production. The factors that have confidence level above 95% are considered the most significant factors that affect the lipase production. The effect of twelve medium components of the fermentation for chitinase production by *Trichoderma harzianum* was examined using Plackett-Burman statistical design. The main effect of the medium components, regression coefficient, F values and P values of the factors investigated in the present study. Table 1 shows selected experimental variables for conducting twelve experimental trials.

Table 1. Variables to be monitored in Plackett-Burman statistical design for cell growth of *Trichoderma harzianum*.

S.No	Medium	High Level	Low Level
1.	Peptone	2	1
2.	$(NH4)_2 SO_4$	6	3
3.	NaH_2PO_4	10	5
4.	KH_2PO_4	2.5	1.5
5.	$MgSO_4. 7H_2O$	0.4	0.2
6.	Citric acid monohydrate	11	9
7.	Urea	0.5	0.25
8.	Malt extract	11	9

Experimental designs

From the optimized nutrient composition for *Trichoderma harzianum* growth rate, the effect of the temperature, pH, inoculum size and substrate concentration level were studied using Central Composite Design (CCD)[17]. A Central Composite Design consists of:

1) A complete 2^K factorial design, where the factor levels are coded to the usual -1, +1 value. This is called the factorial portion of the design
2) No center points (n_o >_ 1)
3) Two axial points on the axis of the design variable at a distance of ±*a* from the - design center. This is called the axial portion of the design.

The total number of design points is thus equal to $\alpha = [2^k]^{1/4}$
For this investigation, temperature (X_1), pH (X_2), inoculum size (X_3) and substrate concentration (X_4) are the independent variables in a series of chitinase production experiment.
Thus K = 4 $\alpha = 2 \text{ x }^{4/4}$ $\alpha = 2$
A CCD with six star points (*a* = 2) and six replicates at the center point (no 6) with a total number of experiments (N) N = 31

The experiments was conducted by five different level o were employed simultaneously covering the spectrum of variables for the production of chitinase in the Central Composite Design. Table 2 indicates the range and levels of the independent variables selected for the production of chitinase. To understand the effects of the parameters temperature, pH, inoculum size, substrate concentration, and their interactions on the production of chitinase process, statistically designed experiments were used.

Table 2. Range and levels of the independent variables selected for the production of chitinase.

Parameters	-2	-1	0	1	2
Temperature	30	35	40	45	50
pH	3	4	5	6	7
Inoculum Size	0.6	1.2	1.8	2.4	3.0
Substrate Concentration	0.5	1	1.5	2	2.5

Results and Discussion

Chitinase activity

Trichoderma harzianum.792 gave maximum chitinase activity of 66.5 U/ml for rice bran after incubation for 6 days.

Screening of important media components

The effect of eight medium components of the fermentation for chitinase production by *Trichoderma harzianum* was examined using Plackett-Burman statistical design. Observed and predicted responses for the experiments performed using Plackett–Burman design the biomass production shown in Table 3. The main effect of the medium components, regression coefficient, F values and P values of the factors investigated in the present study is illustrated in Table 3. The cell growth of *Trichoderma harzianum* was found to vary from 0.3 to 34 g/l in the twelve experiments due to the strong influence of medium components. On analysis of regression coefficient of eight medium components, peptone, $(NH_4)2SO_4$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, citric acid monohydrate, urea and malt extract, among these $(NH_4)2SO_4$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ showed negative effect biomass production, whereas, citric acid monohydrate, urea and malt extract showed positive effect in the tested range of concentration as shown in Figure 1. The Pareto chart illustrates the order of significance of the variables affecting the cellmass production. The order of significance as indicated by Pareto chart is malt extract, peptone, citric acid monohydrate, urea $(NH_4)2SO_4$, KH_2PO_4, and $MgSO_4{\cdot}7H_2O$

The significant factors identified by Plackett-Burman design were considered for the next stage in the medium optimization using response surface optimization technique for the future study. The analysis of variance (ANOVA) was employed (shown in Table 4) for the determination of significant parameters. ANOVA consists of classifying and cross classifying statistical results and testing whether the means of a specified classification differ significantly. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model.

Table 3. Observed and predicted responses for the experiments performed using Plackett–Burman design.

MEDIUM CODE	Peptone A	$(NH_4)_2SO_4$ B	NaH_2PO_4 C	KH_2PO_4 D	$MgSO_4.7H_2O$ E	Citric acid monohydrate F	Urea G	Malt extract H	Biomass Production(g/l) Experimental
1	+	+	-	+	-	-	-	+	0.03
2	+	-	+	+	-	+	-	-	0.12
3	-	-	-	-	-	-	-	-	0.17
4	+	-	+	-	-	-	+	+	0.26
5	-	+	+	+	-	+	+	-	0.08
6	+	+	-	-	+	-	+	-	0.05
7	+	-	-	-	+	+	+	-	0.04
8	-	-	-	+	+	+	-	+	0.05
9	-	-	+	+	+	-	+	+	0.27
10	-	+	-	-	-	+	+	+	0.04
11	-	+	+	-	+	-	-	-	0.34
12	+	+	+	-	+	+	-	+	0.05

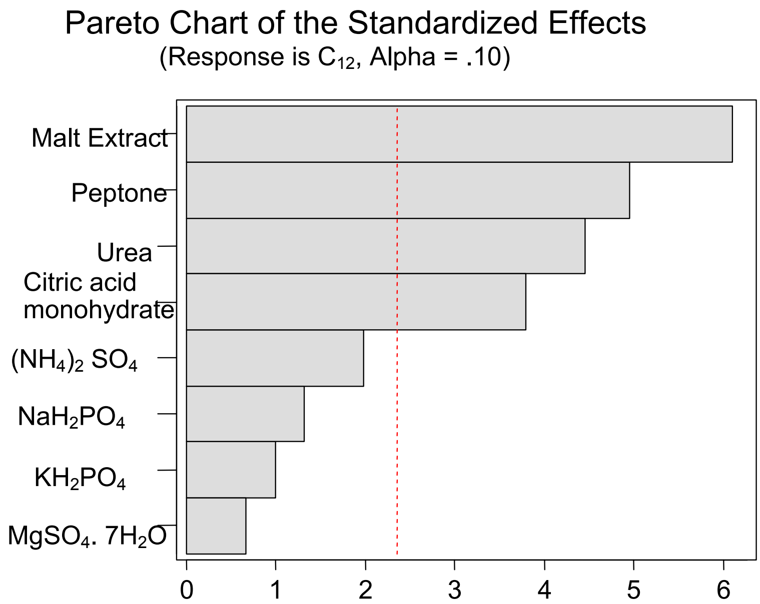

Figure 1. Pareto-Plot for Plackett-Burman parameter estimates for twelve medium components.

The model equation fitted by regression analysis is given by:

$$Y = 0.12583 + 00083A - .00750B - 03250C - 0.06250D - 0.04417E + 0.000417F + 0.019171G \quad \text{----Eq(3.1)}$$

Where A = Peptone, B= $(NH_4)2SO_4$, C= KH_2PO_4, D = $MgSO_4 \cdot 7H_2O$, E = Citric acid monohydrate, F= Urea G = Malt extract.

Table 4. Analysis of variance (ANOVA) for quadratic model for biomass production.

Model term	Parameter estimate (coefficients)	T	P
Constant	342.66	23.374	0.000
A	7.42	0.764	0.467
B	-24.23	-2.493	0.037
C	14.66	1.508	0.170
D	-37.76	-3.989	0.004
E	-8.87	-0.937	0.376
F	-16.26	-1.718	0.124
G	4.51	0.355	0.732
H	-6.89	-0.542	0.602

The graphical representations of the regression equation called the surface were obtained using the Minitab 14 software package. The second-degree polynomial regression equation was solved by the sequential quadratic programming using MATLAB 6.5 (Appendix 3). The optimum values of test variables and the corresponding maximum biomass production (34 g/l) in coded units as A =0.1949, B = 0.6378, C= and D = 0.3856, and they were converted to encoded units. The model F-value of 29.37, and values of prob > F (<0.05) indicated that the model terms are significant. For biomass production, A, B, C and D were significant models.

Table 5. Statistical analysis of model for biomass production.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Regression	9	36271.7	4030.19	3.12	0.062
Linear	3	11707.0	3902.34	3.02	0.094
Square	3	23025.4	7675.15	5.95	0.020
Interaction	3	1539.2	513.07	0.40	0.758
Residual Error	8	10321.4	1290.17		
Total	19	51569.6			

Central composite design

Response surface methodology was used to optimize the levels of the significant variables identified by the 2-level fractional factorial design. A CCD matrix was developed for each isolate depending on the number of factors considered for optimization. Based on the identification of variables by the 2-level fractional factorial, a central composite design was developed for variables significantly affecting chitinase production in each isolate. All the non-significant factors were maintained at central points ('0' coded level) of the levels used in the 2-level fractional factorial design.

Table 2 shows the five levels of variables chosen for trials in CCD. For response surface methodology (RSM) based on the Box-Wilson, which was used to optimize cultivation conditions for chitinase production, 31 experimental runs with different combinations of four factors and five levels were carried out (Table 5). The variables used for the factorial analysis were temperature, pH, inoculum size and substrate concentration, named X_1, X_2, X_3 and X_4 this design, respectively. The effects of the four independent variables on chitinase production and the experimental response along with the predicted response obtained from the regression equation for each run are shown in Table 5. It can be seen from Table 5, there was a considerable variation in the chitinase production depending on the four chosen variables. The maximum chitinase production (66.5 U·mL-1) was achieved in run number 20, while the minimum chitinase production (32 U·mL-1) was observed in run number 9 for rice bran. The former was much higher than the latter, which adequately indicated that choosing appropriate cultivation conditions could evidently enhance the yield of chitinase. By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the chitinase production by only considering the significant terms and was shown as below:

$$Y = 55.871 + 705B - 0.8609C - 2.2843D - 0.4308A^2 - 1.1636D^2 + 2.4156AB + 0.1548\,AC - 0.0133AD - 2.0817\,BC + 1.8005BC - 5.8709CD \qquad \text{Eq--- (3.2)}$$

Where A = pH, B = Temperature, C = Inoculums Size and D = Substrate Concentration

The independent variables were fitted to the second order model equation and examined for the goodness of fit. Several indicators were used to evaluate the adequacy of the fitted model and the results are shown in Table 6. The determination coefficient R^2 value, correlation coefficient R value, coefficients of variation (CV) and model significance (F-value) were used to judge the adequacy of the model. R^2, or coefficient of determination, is the proportion of variation in the response attributed to the model rather than to random error, Suggested for a good fit of a model, R^2 should be at least 80%. The determination coefficient (R^2) implies that the sample variation of 99.59% for chitinase production using rice bran as substrate is attributed to the independent variables, and only about 0.4% of the total variation can not be explained by the model. The closer value of R (correlation coefficient) to 1, the better is the correlation between the experimental and predicted values. Here the value of R (0.9979) for Eq. (3.2) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response, expressed as a percentage. A model can be considered reasonably reproducible if the CV is not greater than 10%. Usually, the higher value of CV, the lower is the reliability of experiment. Here, a lower value of CV (9.02) indicated a greater reliability of the experiments performed. The model significance (F-value) indicates the level of confidence that the selected model can not be due to experimental error. Linear and quadratic terms were significant at the 1% level. Therefore, the quadratic model was selected in this optimization study. The Student T-distribution and the corresponding P value, along with the parameter estimate, are given in Table 6. The P-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The parameter estimates and the corresponding *P*-values showed that among the independent variables, X_1 (Temperature), X_2 (pH), X_3 (Inoculum Size) and X_4 (Rice bran) had a significant effect on chitinase production. Positive coefficients for X_1 and X_3 indicated a linear effect to increase chitinase production, while negative coefficient of X_4 revealed the opposite effect. It was included that X_2 (pH) was the key factor influencing chitinase production, due to its largest t-value among the four variables. So, compared with the traditional 'one- ariableat- a-time' approach which is unable to detect the frequent interactions occurring between two or more factors although they often do occur, RSM has immeasurable effects and tremendous advantages. From Table 6 interaction between the AD, BC and CD should be more significant compared to other interaction. It is evident from the counter plot Figure 2, temperature Vs pH.

Table 6. Observed and predicted responses for the experiments performed using CCD design for rice bran.

Run	Temperature	pH	Inoculum size	Substrate Concentration	Chitinase Production (U/ml)	
					Experimental	Predicated
1	30	4	1.2	1	54.5	54.1
2	40	4	1.2	2	64	63.89
3	40	6	1.2	2	59	58.7
4	35	5	1.8	2.5	56	55
5	40	6	2.4	2	51.5	50
6	30	6	1.2	1	47.5	48
7	35	5	1.8	1.5	58	59.1
8	35	5	1.8	1.5	58	57
9	30	4	2.4	1	49.5	49
10	35	5	1.8	0.5	32	31.4
11	40	6	1.2	1	56	55
12	30	4	2.4	2	56	54
13	35	5	1.8	1.5	52	51
14	35	5	1.8	1.5	58	57
15	30	4	1.2	2	58	57
16	35	5	1.8	1.5	48.5	49
17	35	7	1.8	1.5	58	59
18	35	3	1.8	1.5	61.4	61.2
19	40	4	2.4	2	63.5	62.7
20	35	6	2.4	1.5	66.5	64
21	40	5	1.8	1	58	59
22	35	5	1.8	1.5	52	51
23	40	4	2.4	1	58	57
24	30	6	2.4	1	56.5	56.4
25	35	5	0.6	1.5	56.5	56.4
26	45	5	1.8	1.5	43.5	43
27	30	6	2.4	2	39.2	38.9
28	45	5	1.8	1.5	43.5	43.1
29	40	4	1.2	1	40.5	40
30	30	6	1.2	2	44.3	44
31	35	5	3.0	1.5	54.5	54.1

Three-dimensional response plots and their corresponding contour plots for the chitinase production using rice bran model were shown in Figure 2. The contour plots affirm that the objective function is unimodal in nature which shows an optimum in the boundaries. The boundary optimum point was evaluated using gradient method in the direction of steepest ascent. The graphical representation provides a method to visualize the relation between the response and experimental levels of each variable, and the type of interactions between test variable in order to deduce the optimum conditions. Figure 3 depicts the three-dimensional plot and Figure 2 its respective contour plot showing the effects of temperature vs. pH on chitinase production.

Table 7. Regression coefficients and their significances for chitinase production from the results of Central Composite design for chitinase production in SSF.

Term	Coefficient	S.E Coefficient	T	P
Constant	55.8571	3.003	18.599	0.00
A	0.1065	1.920	0.055	0.010
B	-1.0705	2.792	-0.383	0.019
C	-0.8609	2.170	-0.397	0.634
D	-2.2843	3.557	-0.642	0.016
A*A	-0.4308	1.513	-0.285	0.004
B*B	-1.8955	1.896	-1.000	0.030
C*C	1.5692	1.513	1.037	0.130
D*D	-1.1636	2.207	-0.527	0.215
A*B	2.4156	2.416	1.000	0.496
A*C	0.1548	2.604	0.059	0.278
A*D	-0.0133	2.372	-0.006	0.046
B*C	-2.0817	2.908	-0.716	0.005
B*D	1.8005	2.484	0.725	0.004
C*D	-5.8709	3.700	-1.587	0.640

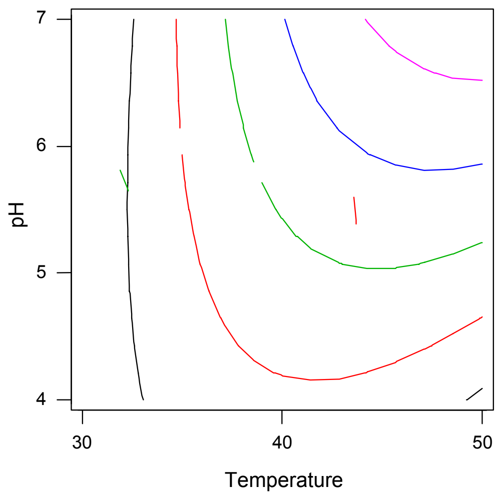

Figure 2. Counter plot for chitinase production showing the interactive effects of temperature and pH for rice bran.

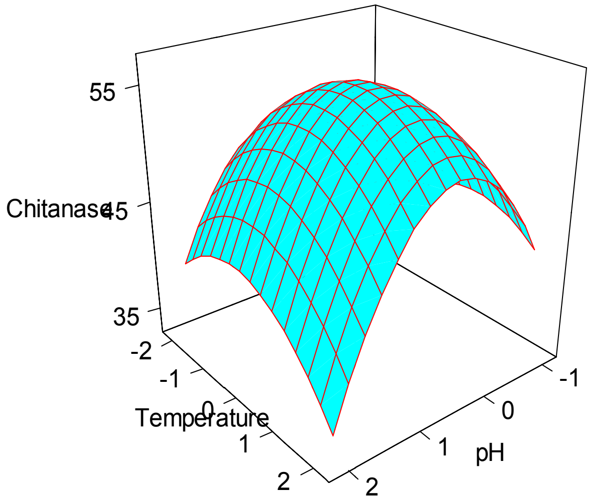

Figure 3. Three-dimensional response surface plot for chitinase production showing the interactive effects of temperature and pH for rice bran.

Conclusions

The conventional method (i.e., change-one-factor-at-a-time) traditionally used for optimization of multifactor experimental design had limitations because (i) it generates large quantities of data which are often difficult to interpret (ii) it is time consuming and expensive (iii) ignores the effect of interactions among factors which have a great bearing on the response. To overcome these problems, a central composite design (CCD) and RSM were applied to determine the optimal levels of process variables on chitinase enzyme production. Only 31 experiments were necessary and the obtained model was adequate ($P < 0.001$). By solving the regression equation, the optimum process conditions were determined; substrate concentration 1.8g/l of rice bran, initial pH 6.5, fermentation temperature 33°C and inoculum size 2.4%. A maximum chitinase yield of 66.5U/ml was obtained at the optimized process conditions. The research results indicated that RSM not only helps us locate the optimum conditions of the process variables in order to enhance the maximum chitinase enzyme production, but also proves to be well suited to evaluating the main and interaction effects of the process variables on chitinase production from waste agricultural residues.

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References

1. Henrissat, B. and Bairoch, A. (1993). New Families In The Classification of Glycosyl Hydrolases Based on Amino Acid Sequence Similarities. ***Biochemistry Journal,*** 293, 781–788**.**

2. Lorito, M., diPietro, A., Hayes, C.K., Woo S.L. and Harman, G.E. (1993). Antifungal, Synergistic Interaction between Chitinolytic Enzymes from *Serratia marcescens* and *Enterobacter cloacae*. ***Phytopathology,*** 83, 721–728.

3. Fenton, D.M. and Eveleigh, D.E. (1981). Purification and Mode of Action of a Chitinase from *Penicillium islandicus*. ***Journal of General Microbiology*****,** 126, 151–154.

4. Rose, M.D., Winston, F. and Hieter, P. (1990). Methods in Yeast Genetics: A Laboratory Course Manual, New York: Cold Spring Harbor Laboratory Press.

5. Aloise, P.A., Lumme, M. and Aynes, C.A. (1996). *N*-Acetyl-d-Glucosamine Production from Chitin-Waste Using Chitinases from *Serratia marcescens*, In: Muzzarelli RAA, editor. *Chitin Enzymology*, Vol. 2. Italy: Ncona, 581–594.

6. Usai, T., Hayashi, Y., Nanjo, F., Sakai, K. and Ishido, Y. (1987). Transglycosylation reaction of a chitinase purified from *Nocardia orientails*. ***Biochemica Biophysica Acta,*** 923, 302- 309.

7. K. Madhavan Nampoothiri, T.V. Baiju, C. Sandhya and Ashok Pandey (2004). Process optimization for antifungal chitinase production by *Serratia marcescens*. ***Process Biochemistry,*** 39, 1583-1590.

8. Nawani, N.N. and Kapadnis, B.P. (2005). Optimization of chitinase production using statistics based experimental designs*.* ***Process Biochemistry,*** 40, 651-660.

9. Souza, M., Roberto, I.C. and Milagres, A.M.F. (1999). Solid-State fermentation for xylanase production by *Thermoascus aurantiacus* using response surface methodology. ***Applied Microbiology and Biotechnology,*** 52, 768–772.

10. Chahal, D.S. (1985). Solid State fermentation in *Trichoderma reesie* for cellulose production. ***Applied Environmental Microbiology,*** 499, 205–10.

11. Sim, T. and Oh, J.C.S. (1990). Spent brewery as substrate for the production of cellulase by *Trichoderma reesei* QM 94114. ***Journal of Industrial Microbiology*****,** 5, 153–8.

12. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. ***Analytical Chemistry,*** 31, 426–8.

13. Massimiliano, F, Jean-Louis, L. and Federici, F. (1998). Chitinolytic enzymes activity of *Penicillium janthinellum* P9 in bench top bioreactor. ***Journal of Fermentation and Bioengineering*****,** 86, 620–2.

14. Ulhoa, C.J. and Peberdy, J.F. (1991). Regulation of chitinase synthesis in *Serratia marcescens*. ***Journal of General Microbiology*,** 14, 2163–9.

15. Roberts, A. and Selitrenkoff, N. (1985). Colloidal chitin preparations. ***Journal of General Microbiology,*** 134, 169–76.

16. Rajiv Vaidya, Pranav Vyas, Chhatpar H.S. (2003). Statistical optimization of medium components for the production of chitinase, by *Alcaligenes xylosoxydans*. ***Enzyme and Microbial Technology,*** 33, 92-96.

17. Montgomery, D.C. (1997). Design and analysis of experiments, 4th ed. New York: John Wiley and Sons.