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PRODUCTION OF CHITINASE BY SOLID STATE FERMENTATION FROM SUGARCANE BAGASSE

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ABSTRACT

Statistics based experimental design on 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* 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 inoculum size were found to contribute to the response at a significant level. After optimization, the maximum enzyme yield was 34 U/mL from agroindustrial waste sugarcane bagasse.

Key words: Chitinase, *Trichoderma harzianum*, Optimization, Sugarcane bagasse.

INTRODUCTION

Chitin, α-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

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production[7]. 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 enzyme activity[8] and optimization of enzyme production[9]. 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[10]. Different types of substrates, which contain chitin, have been tried for the production of chitinase, which included fungal cell walls, crab and shrimp shells and agricultural residues. 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[11]. This study is an attempt to evaluate the effects of several factors on the production of an industrially important enzyme, chitinase. Screening of medium components was evaluated using Plackett-Burman statistical design and 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).

EXPERIMENTAL

Materials and methods

Micro-organism and inoculum preparation

A fungal isolate, *T. harzianum* 792 obtained from the MTCC, Chandigarh was used in the present study. The culture was maintained on malt extract agar medium and subcultured every thirty days. Slants were incubated for 2 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 2 mL reaction mixture contained 0.5 mL of 0.5% colloidal chitin in phosphate buffer (pH 5.5), 0.5 mL crude enzyme extract and 1 mL 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 3 mL 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 µmol 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. This model describes no interaction among factors and it is used to screen and evaluate the important factors that influence enzyme production. The factors that have confidence level above 95% are considered the most significant factors that affect the enzyme production. The main effect of the medium components, regression coefficient, F values and P values of the factors was 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*

Medium	High level	Low level
Peptone	2	1
$(NH_4)_2\ SO_4$	6	3
NaH_2PO_4	10	5
KH_2PO_4	2.5	1.5
$MgSO_4.\ 7H_2O$	0.4	0.2
Citric acid monohydrate	11	9
Urea	0.5	0.25
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:

(i) 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 and no center points ($n_o \geqslant 1$).

(ii) Two axial points on the axis of the design variable at a distance of $\pm a$ 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, $K = 4$; $\alpha = 2 \times ^{4/4}$; $\alpha = 2$

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

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 34 U/mL for sugarcane bagasse 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[16]. 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. On analysis of regression coefficient of eight medium components, peptone, $(NH_4)_2SO_4$, NaH_2PO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, citric acid monohydrate, urea and malt extract, among these $(NH_4)_2SO_4$, NaH_2PO_4, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ showed negative effect biomass production, where as, peptone, citric acid monohydrate, urea and malt extract showed positive effect in the tested range of concentration as shown in Pareto chart (Fig. 1).

Table 3: Observed and predicted responses for the experiments performed using Plackett-Burman design matrix to optimize cell growth of *Trichoderma harzianum*

Medium code	Peptone (A)	$(NH_4)_2SO_4$ (B)	NaH_2PO_4 (C)	KH_2PO_4 (D)	$MgSO_4.7H_2O$ (E)	Citric acid mono-hydrate (F)	Urea (G)	Malt extract (H)	Biomass production (g/L)
									Experimental (g/L)
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

Where A = Peptone, B = $(NH_4)_2SO_4$, C = NaH_2PO_4, D = KH_2PO_4, E = $MgSO_4 \cdot 7H_2O$, F = Citric acid monohydrate, G = Urea, H = Malt agar

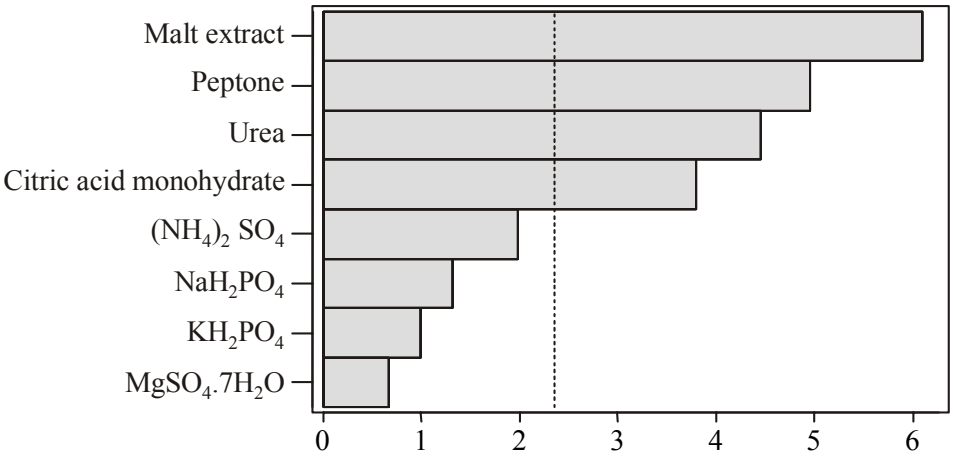

Fig. 1: Pareto-Plot for Plackett-Burman parameter estimates for twelve medium components

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, NaH_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. ANOVA consists of classifying and cross classifying statistical results and testing, whether the means of a specified classification differ significantly. This was carried by Fisher's statistical test for the analysis of variance. 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. The model equation fitted by regression analysis is given by -

$$Y = 342.6 + 7.42A - 24.23B + 14.66C - 37.76D - 8.87E - 16.26F + 4.51G - 6.89H \quad \ldots(1)$$

The graphical representations of the regression equation, called the surface, were obtained using the Minitab 14 software package. The second-degree polynomial regression equation (1) was solved by the sequential quadratic programming using MATLAB 7. The optimum values of test variables and the corresponding maximum biomass production (34 g/L) in coded units are A = 0.1949, B = 0.6378, C = and D = 0.3856, and these were converted to encoded units for the actual values. 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 was a significant model.

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

Model term	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

Optimization of process parameters for chitinase production using sugarcane bagasse as substrate

In this study, sugarcane bagasse was used as main substrate under solid state fermentation. For one thing, the use of purified chitin enhanced the cost of enzyme production and was a major limitation to the economic feasible of bioconversion and utilization of ignocellulosic materials. For another, agricultural residue was not only inexpensive, but it was also abundant and easily available, supplying the microorganism, a better nutrition. In order to obtain optimum levels of chitinase by *Trichoderma harzianum*[18], optimization of cultivation conditions variables, that had a significant impact on chitinase production, was necessary. It can be seen from Table 5 that there was a considerable variation in the chitinase production depending on the four chosen variables. The maximum chitinase production (34 $U \cdot mL^{-1}$) was achieved in run number **27**, while the minimum chitinase production (12.5 $U \cdot mL^{-1}$) was observed in run number **11**. The former was much higher than the latter, which adequately indicated that choosing appropriate cultivation conditions could evidently enhance the yield of chitinase. In order to estimate the error, the centre point in the design was repeatedly carried out for three times.

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 in equation (2).

$$Y = 26 + 3.0693A + 4.0355B – 0.5803C + 5.3113D – 2.7804A^2 - 2.4982B^2 + 1.3321C^2\text{--}1.5736D^2 + 0.9288AB\text{-}1.6123AC\text{-}2.8253AD + 5.2282BC - 4.6636BD + 0.9722CD \quad \ldots(2)$$

Where Y is the chitinase activity (U/mL), Where A = pH, B = Temperature, C = inoculums size and D = Substrate concentration

Table 5: Observed and predicted responses for the experiments performed using CCD design for Sugar cane bagasse

Run	pH	Temp.	Inoculum size	Substrate concentration	Chitinase production (U/mL)	
					Experimental	Predicated
1	1.2(-1)	30(-1)	4(-1)	1(-1)	5.5	5
2	1.2(-1)	40(1)	4(-1)	2(1)	18.5	18
3	1.2(-1)	40(1)	6(1)	2(1)	15.5	16
4	1.8(0)	35(0)	5(0)	2.5(2)	31	32
5	2.4(1)	40(1)	6(1)	2(1)	32	33
6	1.2(-1)	30(-1)	6(1)	1(-1)	28.5	29
7	1.8(0)	35(0)	5(0)	1.5(0)	26.5	37
8	1.8(0)	35(0)	5(0)	1.5(0)	26.5	38
9	2.4(1)	30(-1)	4(-1)	1(-1)	22	21
10	1.8(0)	35(0)	5(0)	0.5(-2)	29.5	30
11	1.2(-1)	40(1)	6(1)	1(-1)	12.5	20
12	2.4(1)	30(-1)	4(-1)	2(1)	32.5	34
13	1.8(0)	35(0)	5(0)	1.5(0)	26.5	27
14	1.8(0)	35(0)	5(0)	1.5(0)	26.5	27
15	1.2(-1)	30(-1)	4(-1)	2(1)	32.5	33
16	1.8(0)	35(0)	5(0)	1.5(0)	26.5	27
17	1.8(0)	35(0)	7(2)	1.5(0)	34	33

Cont…

Run	pH	Temp.	Inoculum size	Substrate concentration	Chitinase production (U/mL)	
					Experimental	Predicted
18	1.8(0)	35(0)	3(-2)	1.5(0)	26	31
19	2.4(1)	40(1)	4(-1)	2(1)	24.5	25
20	1.8(0)	35(0)	5(0)	1.5(0)	26.5	27
21	2.4(1)	40(1)	6(1)	1(-1)	20	19
22	1.8(0)	35(0)	5(0)	1.5(0)	26.5	27
23	2.4(1)	40(1)	4(-1)	1(-1)	33.5	34
24	2.4(1)	30(-1)	6(1)	1(-1)	25	26
25	0.6(-2)	35(0)	5(0)	1.5(0)	5	5.8
26	1.8(0)	45(2)	5(0)	1.5(0)	24	25
27	2.4(1)	30(-1)	6(1)	2(1)	34	22
28	1.8(0)	45(2)	5(0)	1.5(0)	24	25
29	1.2(-1)	40(1)	4(-1)	1(-1)	20	21
30	1.2(-1)	30(-1)	6(1)	2(1)	24.5	23
31	3.0(2)	35(0)	5(0)	1.5(0)	24.6	25

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^2 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. Joglekar and May (1987) have 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 sugarcane bagasse 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 is the 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. (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% (Joglekar and May, 1987). Usually, the higher is the 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 (Henika, 1972). Linear and quadratic terms were significant at the 1% level. Therefore, the quadratic model was selected in this optimization study.

Table 6: Regression coefficients and their significances from the results of central composite experimental design for chitinase production in solid state fermentation using sugarcane bagasse

Term	Coefficient	S. E. Coefficient	T	P
Constant	26.5	1.65677	15.995	0.00
A	3.0693	1.0589	2.898	0.010
B	4.0355	1.5400	2.620	0.019
C	-0.5803	1.1971	-0.485	0.634
D	5.3113	1.9621	2.707	0.016
A*A	-2.7804	0.8349	-3.330	0.004
B*B	-2.4982	1.0458	-2.389	0.030
C*C	1.3321	0.8349	1.595	0.130
D*D	-1.5736	1.2176	-1.292	0.215
A*B	0.9288	1.3326	0.697	0.496
A*C	-1.6123	1.4364	-1.122	0.278
A*D	-2.8253	1.3084	-2.159	0.046
B*C	5.2282	1.6041	3.259	0.005
B*D	-4.6636	1.305	-3.403	0.004
C*D	0.9722	2.0413	0.476	0.640

The Student *T*-distribution and the corresponding *P*-value, along with the parameter estimate, are given in Table 7. 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 (Sugarcane bagasse) 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 (Sugarcane bagasse) revealed the opposite effect. It was concluded that X_2 (pH) was the key factor influencing chitinase production, due to its largest t-value among the four variables. The quadric term of these four variables also had a significant effect. As could be seen, evident interactions existed in X_2 and X_3, but no interactions between the other variable pairs were found to contribute to the response at a significant level. It also could be seen from the P values in Table 7. 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, it can be seen that interactions between the AD, BC and CD should be more significant as compared to other interactions. It is evident from the counter plots Figs. 2 and 3 i.e. Temperature vs. Innoculam size and Temperature vs. pH.

Table 7: Analysis of variance (ANOVA) for the quadratic polynomial model of chitinase production for Sugarcane bagasse

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Regression	14	1270.6	1270.6	90.76	4.72	0.002
Linear	4	338.8	510.4	127.60	6.64	0.002
Square	4	361.5	413.3	103.32	5.38	0.006
Interaction	6	570.3	570.3	95.05	4.95	0.005
Residual error	16	307.4	307.4	19.21		
Lack-of-fit	4	157.5	157.5	39.39	3.15	0.055
Pure error	12	149.9	149.9	12.49		
Total	30	1578.0				

Three-dimensional response plots and their corresponding contour plots for the chitinase production using sugarcane bagasse by the above model are shown in Figs. 4 and 5. 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.

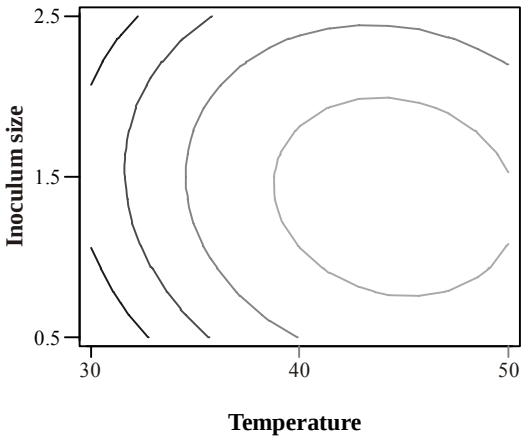

Fig. 2: Contour plot for chitinase production showing the interactive effect of temperature and pH

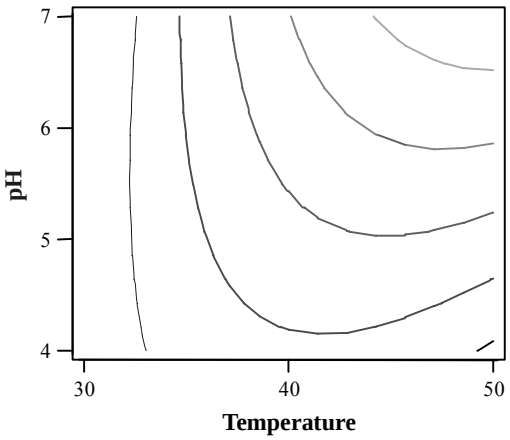

Fig. 3: Contour plot on chitinase production showing the interactive effect of temperature and inoculum size

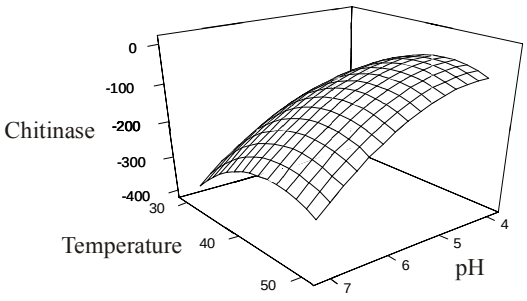

Fig. 4: Three dimensional response plot for chitinase production showing the interactive effects of temperature and pH

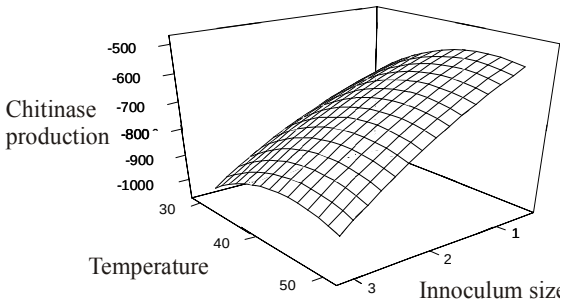

Fig. 5: Three dimensional response plot for chitinase production showing the interactive effects of temperature and inoculum size

CONCLUSION

The evaluation of the medium components for chitinase production was done using the Plackett-Burman statistical method. The effect of eight medium components were studied and among them peptone, malt extract, citric acid and urea were found to be the

significant variables for cell mass by *Trichoderma harzianum* as the percentage confidence level was more than 95%. Response surface methodology was proved to be a powerful tool for optimization of process parameters. Central composite design was employed to evaluate the effects of temperature, pH, inoculum size and substrate concentration on production of chitinase by *Trichoderma harzianum.* Using the above optimized nutrient solution, maximum chitinase activity of 34 U mL^{-1} was obtained at the 30ºC, initial pH of 6, inoculum size of 2.4 % and substrate concentration of 2.0 g/L for sugarcane bagasse. The statistical design of experiment offers an efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for chitinase production by microorganism. *Trichoderma harzianum* chitinase is active over a wide range of operating and environmental conditions and hence, it is designated as one of the best organism to study the production as well biochemical aspects of chitinase. In short, understanding more about the various chitinolytic enzymes such as the standardizations of suitable process parameters for its production and method of estimation will make them more useful in a variety of processes in near future.

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