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Optimization of carbon and nitrogen sources in the medium and environmental factors for enhanced production of chitinase by Trichoderma harzianum

A. Kapat, S.K. Rakshit, T. Panda

Abstract Statistical design was used to determine the optimal levels of medium components, the optimal initial pH of the enzyme production medium, the temperature of fermentation, age of the organism in the slant growth and the age of the inoculum for the production of chitinase in shake flask fermentations. The use of high concentrations of chitin and ammonium sulphate and exclusion of peptone and urea from the medium resulted in the production of higher level of the enzyme. The optimal concentrations of the medium components were 12.5 kg/m^3 and 4.2 kg/m^3 for the chitin and ammonium sulphate respectively. The effect of the addition of peptone and urea to the optimized medium was studied. The optimal values of initial pH and temperature were 5.6 and 28 °C respectively. The optimal age of the slant and the inoculum were found to be 105 h and 43 h respectively. The highest level of chitinase before optimization of the above variables was 0.054 U which was maximized to the level of 0.197 U.

1 Introduction

Chitinases have been the focus of the recent research owing to its multifarious usages in different fields [1, 2]. These enzymes specifically degrades chitin to its monomer N-acetyl-D-glucosamine (GlcNAc) [3]. Chitinases have found extensive use in the preparation of protoplasts from fungi [4, 5]. Chitinase producing fungi have been used as an effective biocontrol agent against phytopathogenic fungi [6–9]. Attempts have also been made to clone the enzyme in plants to boost its resistance against fungal attack [10–12]. The use of chitinase has been suggested in the bioconversion of shellfish wastes to single cell protein [13–16] which is an effective process for the disposal of chitinous waste.

The objective of the present study was to optimize all the physicochemical parameters governing chitinase fermentation. This study involves identifying the most important medium components and their suitable concentrations in order to enhance the production of chitinase using Trichoderma harzianum, NCIM 1185. The fermentation process depends on the various physical factors, viz., initial pH of the enzyme production medium and the temperature of the fermentation. Additionally, for the production of the enzyme, the age of the organism is reported as an important criterion [17]. The determination of optimal conditions for the above mentioned parameters would require experiments with all possible combinations of parameter values. However, it is possible to undertake a rational study by using suitable experimental statistical designs which reduces the number of experiments and broadens the range of information about the system. Initial concentration of major carbon and nitrogen sources, the initial pH, the temperature of fermentation, the age of the organism in slant growth and the age of the inoculum have been considered as the critical variables. The central composite design [18, 19] was followed to optimize these variables. The whole optimization procedure was carried out in three successive steps. In the first stage, the concentrations of the medium constituents were optimized. Secondly, the physical parameters, viz., the initial pH of the enzyme production medium and the temperature of fermentation were optimized. Finally, the age of the organism in the slant growth and the age of the inoculum were studied as the variables. Optimization at each step was verified experimentally for the enhanced production of the enzyme by Trichoderma harzianum, NCIM 1185.

2 Materials and methods

2.1 Organism

Trichoderma harzianum, NCIM 1185, was obtained from the National Chemical Laboratory, Pune, India. It was maintained on potato-dextrose-agar slants containing (kg/m^3): Potato, 200; dextrose, 25; agar, 20. Slants were incubated at 30 °C for 72 h.

2.2 Cultivation and culture conditions

2.2.1 Growth medium

The modified growth medium [20] contained (kg/m^3): Glucose, 10.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4 \cdot 7H_2O$, 0.3; peptone, 1.0; citric acid monohydrate, 10.5. One hundred milliliter of growth medium was taken in a 500-cm^3 Erlenmeyer flask. The initial pH of the culture

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medium was adjusted to 5.0 by 1 M NaOH. The spores as the inoculum from a three days old slant were suspended in 10 cm^3 sterile distilled water (1.0×10^5 spores per cm^3) and this suspension was added to the seed development medium aseptically. The culture was grown at 30 °C for 36 h on a rotary shaker at 180 rev/min. The mycelial pellets formed after the incubation were used as the seed for the enzyme production.

2.2.2
Enzyme production medium (design medium)

This medium contained (kg/m^3): KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4 \cdot 7H_2O$, 0.3; Tween 80, 0.2; $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4 \cdot 7H_2O$, 0.0014; $CaCl_2 \cdot 2H_2O$, 0.002; plus the chitin, ammonium sulphate, peptone and urea at a level as per the requirement of the experimental design used. The initial pH of the design medium was adjusted to 6.0 by 1 M NaOH. 100 cm^3 of medium was dispensed in 500-cm^3 Erlenmeyer flasks and 10% v/v (2.62 kg/m^3 dry mycelial weight equivalent) inoculum was added to each flask. The cultures were incubated at 30 °C (unless mentioned otherwise) for 6 days on a rotary shaker maintained at 180 rev/min. Samples were withdrawn at regular intervals of 24 h and were analyzed for the enzyme activity.

2.3
Analytical methods

2.3.1
Enzyme assays

2.3.1.1
Chitinase

Swollen chitin (Sigma, USA) was used as the substrate for the enzymatic reaction. 1 g of chitin was added to 10 cm^3 of 85% orthophosphoric acid and was stirred at 0 °C for 24 h. The gelatinous mixture was then reprecipitated into an excess of cold (15 °C) distilled water [21]. The reaction mixture contained 0.55 cm^3 of 5 kg/m^3 swollen chitin (suspended in 50 mM acetate buffer, pH 4.75), 0.15 cm^3 of culture filtrate and 0.3 cm^3 of acetate buffer (50 mM, pH 4.75). It was incubated for 1 h at 47 °C [Kapat et al. unpublished work]. After the incubation, products released from the hydrolysis of chitin were estimated as reducing sugar using N-acetyl-D-glucosamine as the standard for the Miller's method [22].

The enzyme activity was expressed in terms of units (U). One unit of chitinase activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of N-acetyl-D-glucosamine in 1 minute per cm^3 of culture filtrate at 47 °C and at pH 4.75.

2.3.1.2
Protease

The reaction mixture contained 0.8 cm^3 of casein (6.0 kg/m^3 Hammersten casein, SRL, India, dissolved in 0.05 M disodium hydrogen orthophosphate buffer, pH 6.0) and 0.2 cm^3 of culture filtrate. It was incubated unstirred for 15 minutes at 37 °C. After the incubation, enzyme was deactivated by adding 2 cm^3 of trichloroacetic acid (50 kg/m^3). The mixture was centrifuged at 4000 rev/min and 1 cm^3 of the supernatent was taken for tyrosin assay using Folin-phenol method [23, 24].

One unit of protease activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of tryosine in 1 minute per cm^3 of culture filtrate at 37 °C and at pH 6.0.

2.3.2
Estimation of carbon and nitrogen content

The carbon and nitrogen content of chitin and peptone were analysed by CHN analyser (Heraeus CHN-O-rapid analyser, Germany).

2.3.3
Experimental design

Central composite design [18] was used for the optimization of all the variables. Using this method, the total number of treatment combinations was $2^k + 2k + n_0$, where k is the number of variables and n_0 is the number of repetition of the experiment at the centre point. For statistical calculations, the variables X_i were coded as x_i according to the following equation:

$$x_i = (X_i - X_0)/\Delta X, \quad i = 1, 2, 3, \ldots, k, \tag{1}$$

where x_i = coded (dimensionless) value of the variable X_i, X_0 = the value of X_i at the centre point and ΔX = the step change.

The behaviour of the system was explained by the following second degree polynomial equation:

$$y = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i^2 + \Sigma \beta_{ij} x_i x_j, \tag{2}$$

where y = predicted response, β_0 = offset term, β_i = linear effect, β_{ii} = squared effect, β_{ij} = interaction effect.

Equation (2) was solved using the Design Expert (Stat-Ease Inc., Minneapolis, MN,) to estimate the responses of the dependent variable. All experiments were performed in duplicate.

2.3.3.1
Optimization of the medium constituents

The initial concentrations of chitin (X_1, kg/m^3), ammonium sulphate (X_2, kg/m^3), peptone (X_3, kg/m^3) and urea (X_4, kg/m^3) were chosen as the independent variables in a series of separate batch fermentations. The chitinase activity (U) was taken as the dependent output variable. A 2^4-factorial-experimental-design, with eight axial points ($\alpha = 2.0$) and six replicates at the centre point with total number of 30 experiments were employed. The coded values of the variables are given in Table 1.

Table 1. Optimization of the major carbon and nitrogen sources: Independent variables in the 2^4-factorial experimental design

Variable	Component (kg/m^3)	Level				
		−2	−1	0	1	2
X_1	Chitin	0	2.50	5.0	7.50	10.0
X_2	Ammonium sulphate	0	0.70	1.4	2.10	2.8
X_3	Peptone	0	0.50	1.0	1.50	2.0
X_4	Urea	0	0.15	0.3	0.45	0.6

x_i = coded value of the variable X_i
$x_1 = (\text{chitin} - 5.0)/2.5$, $x_2 = (\text{ammonium sulphate} - 1.4)/0.7$,
$x_3 = (\text{peptone} - 1.0)/0.5$, $x_4 = (\text{urea} - 0.3)/0.15$

Table 2. Optimization of initial pH, temperature of fermentation, age of the organism in slant growth and the age of the inoculum: Independent variables in the 2^2-factorial experimental design

Variables	Parameter	Level				
		−1.414	−1	0	1	1.414
X_1	pH	4.59	5.0	6.0	7.0	7.41
X_2	Temperature (°C)	22.93	25.0	30.0	35.0	37.07
X_1	Age of the slant (h)	38.06	48.0	72.0	96.0	105.94
X_2	Age of the inoculum (h)	19.03	24.0	36.0	48.0	52.96

x_i = coded value of the variable X_i
$x_1 = (\text{pH} - 6.0)/1.0$, $x_2 = (\text{Temperature} - 30\,^\circ\text{C})/5\,^\circ\text{C}$
$x_1 = (\text{Age of the slant} - 72\text{ h})/24\text{ h}$, $x_2 = (\text{Age of the inoculum} - 36\text{ h})/12\text{ h}$

2.3.3.2 Optimization of initial pH and the temperature of fermentation

These experiments were performed after obtaining the optimal concentrations of the major carbon and nitrogen sources in the first step (that is, after obtaining the results from the experiments described as per section 2.3.3.1). In this case, a 2^2-factorial experimental design with axial points ($\alpha = 1.414$) and 6 replicates at the centre point with a total number of 14 experiments were employed. The coded values of the variables are given in Table 2. The age of the organism in slant growth and the age of the inoculum were 72 h and 36 h respectively. The experiments were conducted in a temperature controlled rotary shaker (REMI orbital incubator, India).

2.3.3.3 Optimization of the age of the slant and the age of the inoculum

Results obtained from the previous steps were taken into consideration while performing these experiments. A 2^2-factorial experimental design with axial points ($\alpha = 1.414$) and 6 replicates at the centre point with a total number of 14 experiments were employed. The coded values of these two variables are given in Table 2. The slants were grown at 30 °C in an incubator. The inoculum was grown at 30 °C in a temperature controlled incubator shaker.

3 Results and discussion

3.1 Optimization of the medium constituents

As the ratio of carbon and nitrogen in the medium plays an important role on the production of the enzyme [25], the level at which carbon and nitrogen sources had to be varied were determined using the statistical experimental design. The carbon and nitrogen content of the major nutrients used in the medium is given in Table 3. Medium constituents were optimized as per the experimental plan described in Table 2. Table 4 summarizes the response for each individual experiment. The regression equation obtained after analysis of variance gives the level of chitinase produced as a function of the initial concentrations of chitin (X_1, kg/m^3), ammonium sulphate (X_2, kg/m^3), peptone (X_3, kg/m^3) and urea (X_4, kg/m^3). All terms regardless of their significance are included

Table 3. Carbon and nitrogen contents of major nutrients

Nutrient	C (% wt/wt)	N (% wt/wt)
Chitin	43.01	04.08
Ammonium sulphate	–	21.21
Peptone	42.28	14.18
Urea	20.00	46.66

in the following equation:

$$y = 0.0542 + 0.006906x_1 - 0.000934x_2 - 0.012461x_3 - 0.009233x_4 - 0.000969x_1^2 + 0.001481x_2^2 + 0.002331x_3^2 + 0.001731x_4^2 - 0.00167x_1.x_2 - 0.00112x_1.x_3 - 0.000474x_1.x_4 - 0.002626x_2.x_3 - 0.001334x_2.x_4 + 0.004726x_3.x_4, \quad (3)$$

where y is the predicted response.
Linear regression was significant at the level of 99% while the square regression was significant at the level of 95%. The enzyme produced predicted from the model at each experimental point are summarized in Table 4 along with the experimentally observed values. The coefficients of equation 3 are calculated using Design Expert and their values are listed in Table 5. The summary of the analysis of variance (ANOVA) is shown in Table 6.

As evident from the experiments, the production of chitinase is dependent mainly on chitin as the carbon source and ammonium sulphate as the nitrogen source. The presence of either peptone or urea resulted in lesser production of chitinase. Fig. 1(a) corroborates the fact that the maximization of chitinase production is possible in the presence of higher concentrations of chitin and ammonium sulphate. Fig. 1(b) shows the effect of peptone on the production of chitinase. It indicates that the higher production of chitinase at different levels of chitin when peptone concentrations are low. Similarly the presence of urea also resulted in the decrease of the enzyme produced (Fig. 1c).

The experimental evidence was further supported by the results obtained after optimizing the regression Eq. (3). The optimization was carried out by an iterative procedure [26] which determine the point at which the function is maximized. The optimum values for chitin and ammonium sulphate were 12.5 kg/m^3, 4.2 kg/m^3 respectively with an optimal carbon to

Table 4. Effect of the medium constituents: Experimental plan with experimental and predicted values of extracellular chitinase (initial pH = 6.0, temperature of the fermentation = 30 °C, slant age = 72 h, inoculum age = 36 h, inoculum level = 10 % v/v (2.62 kg/m^3 dry mycelial weight equivalent), other constituents of the medium (kg/m^3): KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4.7H_2O$, 0.3, Tween 80, 0.2; $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4.7H_2O$, 0.0014; $CaCl_2.2H_2O$, 0.002). Experiments were performed in duplicate with a variation of ±3.2 %

Experiment Number	x_1	x_2	x_3	x_4	Chitinase (U) Experimental			Chitinase (U) predicted
					Set 1	Set 2	Average	
1	−1	−1	−1	1	0.0702	0.0650	0.0676	0.0572
2	1	−1	−1	−1	0.1000	0.1156	0.1078	0.1018
3	−1	1	−1	−1	0.0732	0.1256	0.0994	0.0908
4	1	1	−1	1	0.0559	0.0929	0.0744	0.0729
5	−1	−1	1	−1	0.0570	0.0554	0.0562	0.0545
6	1	−1	1	1	0.0572	0.0580	0.0576	0.0631
7	−1	1	1	1	0.0376	0.0420	0.0308	0.0427
8	1	1	1	−1	0.0558	0.0546	0.0552	0.0626
9	0	0	0	0	0.0566	0.0566	0.0566	0.0636
10	0	0	0	0	0.0566	0.0606	0.0586	0.0636
11	−1	−1	−1	−1	0.0814	0.0950	0.0882	0.0784
12	1	−1	−1	1	0.0764	0.0844	0.0806	0.0725
13	−1	1	−1	1	0.0612	0.0616	0.0614	0.0561
14	1	1	−1	−1	0.1044	0.1112	0.1078	0.1014
15	−1	−1	1	−1	0.0430	0.0430	0.0430	0.0461
16	1	−1	1	−1	0.0616	0.0732	0.0674	0.0673
17	−1	1	1	−1	0.0520	0.0392	0.0456	0.0504
18	1	1	1	1	0.0381	0.0428	0.0404	0.0469
19	0	0	0	0	0.0468	0.0476	0.0472	0.0606
20	0	0	0	0	0.0610	0.0610	0.0610	0.0606
21	−2	0	0	0	0.0118	0.0130	0.0124	0.0206
22	2	0	0	0	0.0530	0.0474	0.0502	0.0483
23	0	−2	0	0	0.0404	0.0308	0.0356	0.0461
24	0	2	0	0	0.0502	0.0430	0.0466	0.0423
25	0	0	−2	0	0.0532	0.0444	0.0488	0.0725
26	0	0	2	0	0.0402	0.0402	0.0402	0.0227
27	0	0	0	−2	0.0448	0.0688	0.0568	0.0637
28	0	0	0	2	0.0548	0.0294	0.0274	0.0267
29	0	0	0	0	0.0404	0.0404	0.0404	0.0383
30	0	0	0	0	0.0608	0.0620	0.0614	0.0383

Levels of the variables are defined in Table 1

Table 5. Coefficients of the regression equation (3)

Coefficient	Value	Coefficient	Value
β_0	0.05420	β_{44}	0.001731
β_1	0.006906	β_{12}	0.001731
β_2	−0.000934	β_{13}	−0.00112
β_3	−0.012461	β_{14}	−0.000474
β_4	−0.009233	β_{23}	−0.002626
β_{11}	−0.000969	β_{24}	−0.001334
β_{22}	0.001481	β_{34}	0.004726
β_{33}	0.002331		

Table 6. Regression analysis for the production of extracellular chitinase: Effect of carbon and nitrogen sources
Quadratic response surface model fitting

Source	Sum of squares	Degrees of freedom	Mean square	F ($P<0.05$)
Blocks	0.003629	2		
Model	0.007805	14	0.000558	2.833
Error	0.002514	13	0.000193	
Total	0.014148	29		

Root mean square error = 0.013906
R = 0.9066, R^2 = 0.8223
R = coefficient of correlation, R^2 = coefficient of determination

nitrogen ratio of 3.792. The optimized medium does not require peptone and urea. Thus, from the experimental finding as well as from the prediction made by the regression equation in optimization, it evidences that the production of chitinase is increased by the pressure of chitin alone which is not influenced by other organic carbon and nitrogen sources in the medium (Fig. 2).

To reconfirm this phenomenon, the medium suggested by Peberdy et al. [27] was examined. The medium which was suggested, contained (kg/m^3): glucose, 3.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4$, 0.3; $CaCl_2 \cdot 7H_2O$, 0.3; peptone, 1.0; urea, 0.3 and chitin, 5.0. T. harzianum, NCIM 1185, was grown in this medium where peptone and urea were present and also without the addition of these components. Chitinase levels were higher when the medium did not contain peptone and urea (Fig. 2).

Further it was also observed that in the presence of urea and peptone in the medium, protease production was more compared to the chitinase (Fig. 3). Protease is constitutively

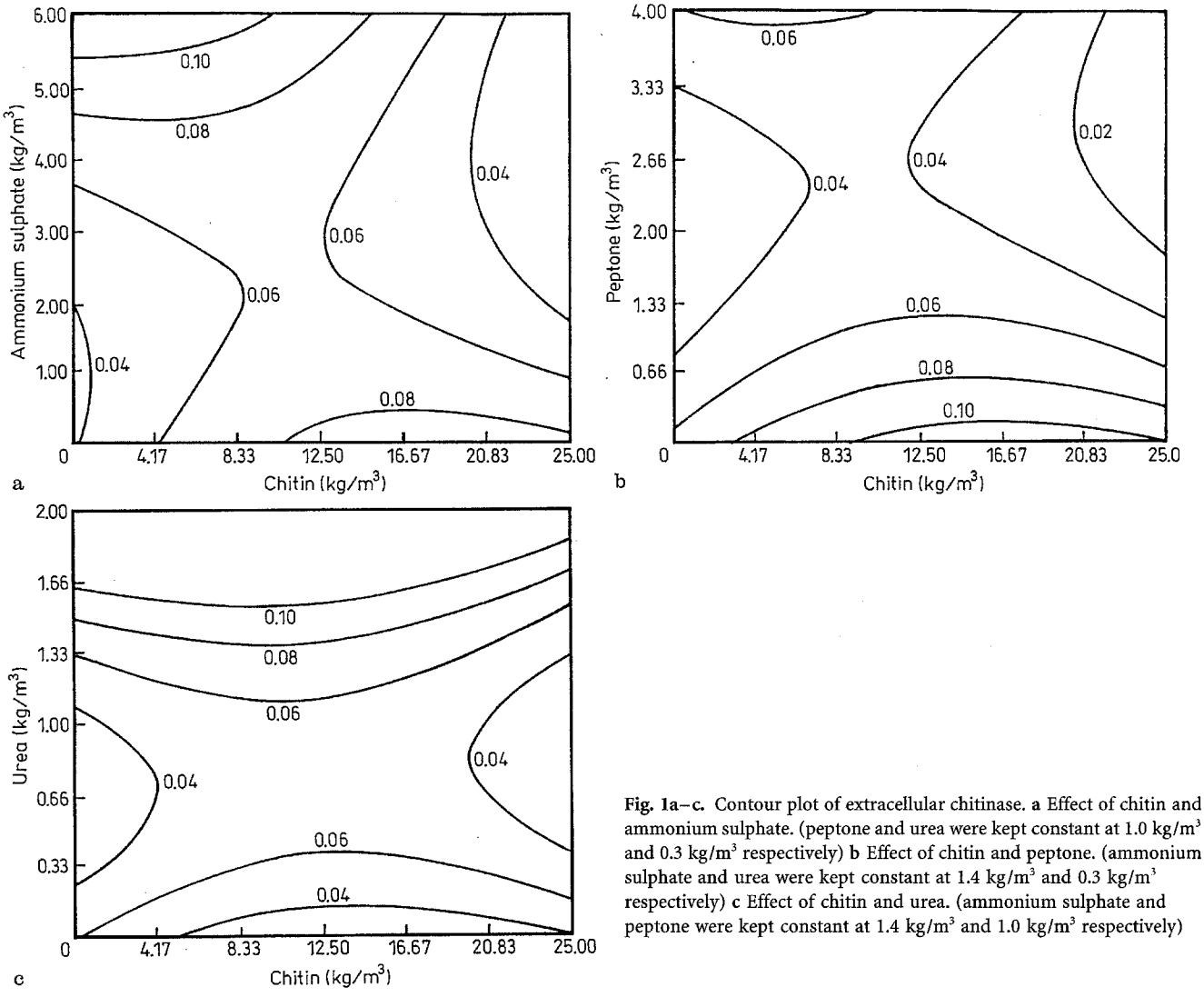

Fig. 1a–c. Contour plot of extracellular chitinase. **a** Effect of chitin and ammonium sulphate. (peptone and urea were kept constant at 1.0 kg/m³ and 0.3 kg/m³ respectively) **b** Effect of chitin and peptone. (ammonium sulphate and urea were kept constant at 1.4 kg/m³ and 0.3 kg/m³ respectively) **c** Effect of chitin and urea. (ammonium sulphate and peptone were kept constant at 1.4 kg/m³ and 1.0 kg/m³ respectively)

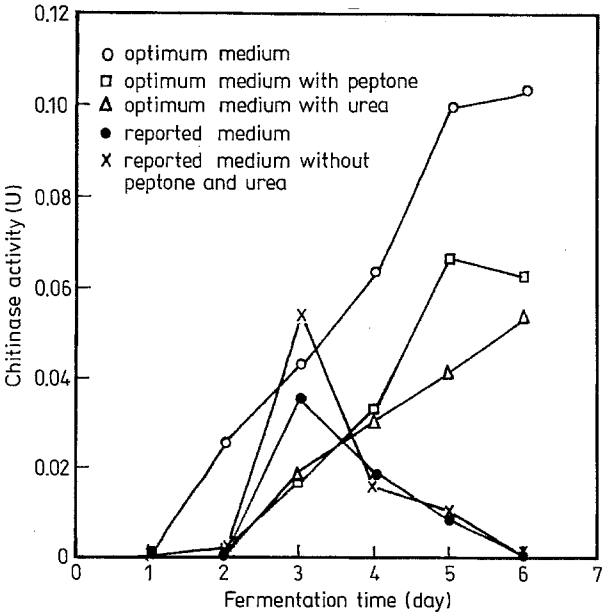

Fig. 2. Effect of the addition of peptone and/or urea to the optimized medium and to the reported medium of Peberdy et al. (initial pH of the medium = 6.0, temperature of incubation = 30 °C) on the production of chitinase

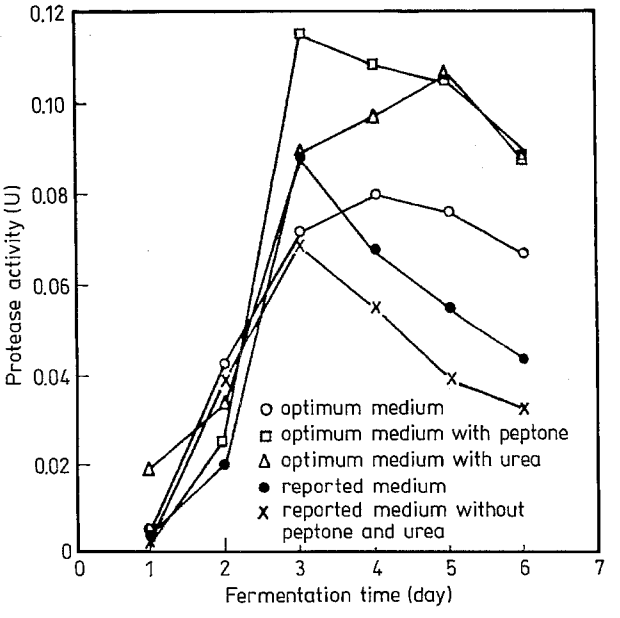

Fig. 3. Effect of the addition of peptone and/or urea to the optimized and to the reported medium of Peberdy et al. (initial pH of the medium = 6.0, temperature of incubation = 30 °C) on the production of protease

Table 7. Effect of pH and temperature: Experimental plan with experimental and the predicted values of extracellular chitinase (slant age = 72 h, inoculum age = 36 h, inoculum level = 10% v/v (2.62 kg/m³ dry mycelial weight equivalent), medium composition (kg/m³): chitin, 12.5; $(NH_4)_2SO_4$, 4.2; KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4 \cdot 7H_2O$, 0.3; Tween 80, 0.2; $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4 \cdot 7H_2O$, 0.0014; $CaCl_2 \cdot 2H_2O$, 0.002). Experiments were performed in duplicate with a variation of ±3.4%

Experiment Number	x_1	x_2	Chitinase (U) Experimental			Chitinase (U) predicted
			Set 1	Set 2	Average	
1	−1	−1	0.1235	0.0890	0.1062	0.1146
2	1	−1	0.0301	0.0337	0.0319	0.0375
3	−1	1	0	0	0	0.0119
4	1	1	0	0	0	0.0091
5	0	0	0.1542	0.1630	0.1586	0.1348
6	0	0	0.1366	0.1348	0.1357	0.1348
7	0	0	0.1532	0.1374	0.1453	0.1348
8	−1.41	0	0.1159	0.1119	0.1178	0.1071
9	1.41	0	0.0568	0.0580	0.0574	0.0506
10	0	−1.41	0.0832	0.0918	0.0875	0.0813
11	0	1.41	0	0	0	0
12	0	0	0.1446	0.1438	0.1442	0.1483
13	0	0	0.1312	0.1300	0.1306	0.1483
14	0	0	0.1356	0.1348	0.1352	0.1483

Levels of the variables are defined in Table 2

produced at a level of 0.08 U in the absence of urea and peptone. Protease synthesized by the organism might affect the chitinase production. Detailed analysis on the role of protease for the production of chitinase by Trichoderma harzianum is currently under investigation which will be reported in a separate communication.

3.2 Optimization of initial pH and temperature

The results of the experiments suggested by the experimental design are shown in Table 7. The regression equation obtained after analysis of variance gives the level of chitinase as the function of the initial pH (X_1) and the temperature of fermentation (X_2, °C):

$$y = 0.141567 - 0.019976\,x_1 - 0.032744\,x_2 - 0.034765\,x_1^2 - 0.056702\,x_2^2 + 0.01868\,x_1x_2, \quad (4)$$

where y is the predicted response.

The square regression was significant at the level of 99% indicating that the combined effect of the initial pH and the temperature of fermentation contributes significantly to the variation on the production of chitinase. There was a close agreement between the experimental and the theoretical values (Table 7) as indicated by the fact that the correlation coefficients for the production of chitinase were 0.9798. The result shows that the organism is unable to produce the enzyme above 35 °C. The coefficients of the equation 4 and their values are listed in Table 8. The summary of the analysis of variance is shown in Table 9.

Table 8. Coefficients of the regression equation (4)

Coefficient	Value	Coefficient	Value
β_0	0.141567	β_{11}	−0.034765
β_1	−0.019976	β_{22}	−0.056702
β_2	−0.032744	β_{12}	0.01868

Table 9. Regression analysis for the production of extracellular chitinase: Effect of the initial pH of the medium and the temperature of fermentation Quadratic response surface model fitting

Source	Sum of squares	Degrees of freedom	Mean square	F ($P<0.05$)
Blocks	0.000642	1		
Model	0.043748	5	0.008750	33.18
Error	0.001846	7	0.000264	
Total	0.046236	13		

Root mean square error = 0.016239
$R = 0.9798$, $R^2 = 0.9601$

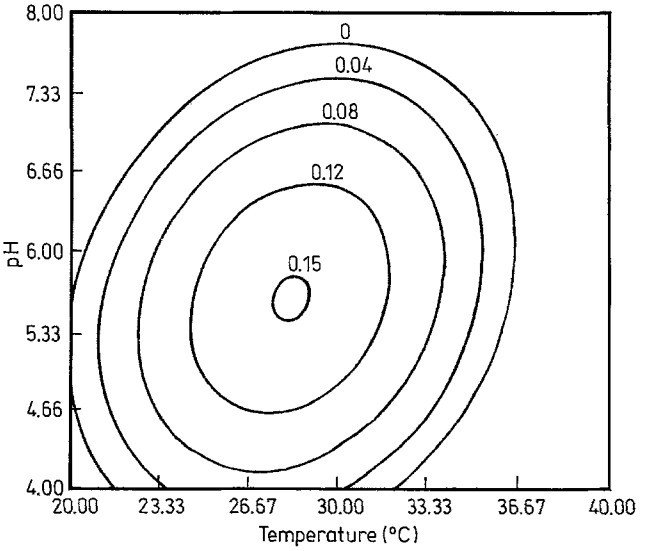

Fig. 4. Contour plot of extracellular chitinase: effect of the initial pH of the enzyme production medium and the temperature of fermentation

The contour plot is given in Figure 4. Both the study of contour plot and the optimization of regression equation indicated that the initial pH of 5.6 and the temperature of 28 °C to be optimum for the fermentation.

Table 10. Effect of the organism in slant growth and the age of the inoculum: Experimental plan with experimental and the predicted values of extracellular chitinase (The temperature of fermentation = 28 °C, the initial pH of the enzyme production medium = 5.6, inoculum level = 10 % v/v (2.62 kg/m^3 dry mycelial weight equivalent), medium composition (kg/m^3): chitin, 12.5; $(NH_4)_2SO_4$, 4.2; KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4 \cdot 7H_2O$, 0.3; Tween 80, 0.2; $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4 \cdot 7H_2O$, 0.0014; $CaCl_2 \cdot 2H_2O$, 0.002). Experiments were performed in duplicate with a variation of ±3.5 %

Experiment Number	x_1	x_2	Chitinase (U) Experimental			Chitinase (U) predicted
			Set 1	Set 2	Average	
1	−1	−1	0.1148	0.1158	0.1153	0.1012
2	1	−1	0.1296	0.1480	0.1388	0.1513
3	−1	1	0.1988	0.2164	0.2070	0.1743
4	1	1	0.1986	0.1970	0.1978	0.1912
5	0	0	0.1526	0.1498	0.1512	0.1615
6	0	0	0.1438	0.1542	0.1490	0.1615
7	0	0	0.1434	0.1422	0.1428	0.1615
8	−1.41	0	0.1022	0.0988	0.1005	0.1297
9	1.41	0	0.1936	0.1776	0.1856	0.1771
10	0	−1.41	0.0712	0.0760	0.0736	0.0705
11	0	1.41	0.1304	0.1224	0.1264	0.1503
12	0	0	0.1546	0.1586	0.1566	0.1389
13	0	0	0.1582	0.1594	0.1588	0.1389
14	0	0	0.1446	0.1416	0.1431	0.1389

Levels of the variables are defined in Table 2

3.2 Optimization of the organism in slant growth and the age of the inoculum

To study the effect of the organism in slant growth and the age of the inoculum on the production of chitinase, both were varied simultaneously and the result at each point of the experimental design are given in Table 10. The regression equation was obtained after the analysis of variance which expresses the chitinase level as a function of slant and the age of inoculum. The equation is as follows:

$$y = 0.150272 + 0.016757\,x_1 + 0.028247\,x_2 + 0.007228\,x_1^2 - 0.014299\,x_2^2 - 0.00832\,x_1x_2, \quad (5)$$

where y is the predicted response.

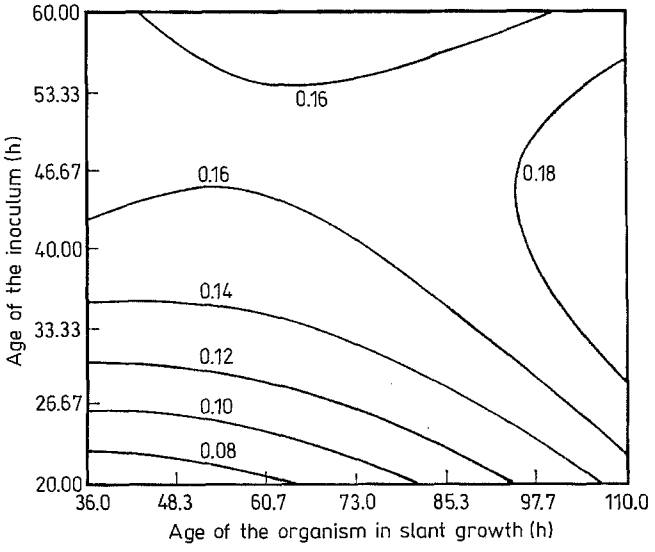

Fig. 5. Contour plot of extracellular chitinase: effect of the organism in slant growth and the age of inoculum

Table 11. Coefficients of the regression equation (5)

Coefficient	Value	Coefficient	Value
β_0	0.150272	β_{11}	0.007228
β_1	0.016757	β_{22}	−0.014299
β_2	0.028247	β_{12}	−0.00832

Table 12. Regression analysis for the production of extracellular chitinase: Effect of the age of the slant and the age of inoculum Quadratic response surface model fitting

Source	Sum of squares	Degrees of freedom	Mean squares	F ($p < 0.05$)
Blocks	0.001782	1		
Model	0.010929	5	0.002186	3.512
Error	0.004357	7	0.000622	
Total	0.017068	13		

Root mean square error = 0.024949
$R = 0.8629$, $R^2 = 0.7447$

The square regression was significant at the level of 95 %. The coefficients of the Eq. (5) and their values are listed in Table 11. The summary of the analysis of variance is given in Table 12. Figure 5 shows the contour plot showing the effect of age of the slant and inoculum. The optimum slant age and the inoculum age were found to be 105 h and 43 h respectively.

4 Conclusion

Chitin and ammonium sulphate were proved to be the most suitable constituents for the maximization of the enzyme production. Presence of peptone and urea in the medium resulted in the decrease in the chitinase level. The optimum temperature and the initial pH of the enzyme production medium were 28 °C and 5.6 respectively. The age of the inoculum has a remarkable effect on the extent of the production of the enzyme.

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