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pH and thermal stability studies of chitinase from Trichoderma harzianum: A thermodynamic consideration

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Abstract The combined effect of pH and temperature on chitinase was investigated using response surface methodology. A central composite design for two variables was employed. The optimal pH and temperature for the least degree of deactivation were found out to be 5.4 and 24°C respectively. The deactivation rate constants and the half life of chitinase were estimated at different pH and temperature combinations. At the optimal pH of 5.4, the rate of the deactivation was found to be the least. Thermodynamic parameters, viz., ΔH^*, ΔS^*, ΔG^* and activation energy of thermal deactivation of chitinase were calculated in the temperature range from 50°C to 60°C.

1 Introduction

The objective of enzyme technology is to attain the capacity to tailor enzyme characteristics to suit the requirements of the process under consideration. Enzyme inactivation plays a significant role in biotechnological process [1]. Rapid inactivation may constrain the efficiency of the process. The desirable features of the enzyme, which include good catalytic ability and stability, have to be strengthened. An improved knowledge of enzyme deactivation kinetics is needed to enhance the feasibility of biotechnological process. Besides, a better understanding of enzyme deactivation process would provide valuable physical insights into the structure and function of enzymes.

The ability of the enzyme is a measure of its ability to catalyze a process while the stability of the enzyme is judged by its residual activity. The term denotes the activity retained by the enzyme after the inactivation process has attained completion. Both of these properties are modified to a large extent by temperature, pH and modifiers such as activators, inhibitors etc. Thus, the examination of the relationships between enzyme properties and the environment is vital in order to achieve the ability to predict, manipulate and engineer protein structure. Chitinase (E.C. 3.2.1.14), which hydrolyses chitin, a biopolymer of N-acetyl-D-glucosamine is widely used in biological and agricultural research [2]. It has got industrial applications as well [3]. Immobilization of chitinase for the degradation of chitin has been reported [4]. In order to develop a suitable process for large scale production and purification of chitinase, it is necessary to investigate the nature of enzyme. A novel approach has been taken to reduce the combined denaturing effect of pH and temperature on chitinase using response surface methodology. The thermodynamic parameters for chitinase deactivation have also been evaluated.

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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 105 hours.

2.2 Production of chitinase

The spores as the inoculum from 105 h old slant were suspended in 10 ml sterile distilled water (1.0×10^5 spores per cm^3) and this suspension was added aseptically to 500-cm^3 Erlenmeyer flask containing 100 cm^3 modified growth medium [5]. The culture was incubated on a temperature controlled shaker at 30°C and at 160 rpm for 43 hours. 10% v/v (2.62 kg/m^3 dry mycelial weight equivalent) of inoculum was transferred to 100 cm^3 enzyme production medium. The enzyme production medium contained (kg/m^3): chitin, 12.5; $(NH_4)_2SO_4$, 4.2; KH_2PO_4, 2.0; NaH_2PO_4, 6.9; $MgSO_4.7H_2O$, 0.03; Tween 80, 0.2; $ZnSO_4.7H_2O$, 0.0014, $CaCl_2.2H_2O$, 0.002, $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.0016 [6]. The culture was incubated at 30°C for 6 days on a rotary shaker maintained at 160 rpm. The culture was harvested on 6th day, centrifuged and the supernatant was stored in sterile container at 10°C.

2.3 Assay of 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 [7]. The reaction mixture contained 0.55 cm^3 of 5 kg/m^3 swollen chitin

(suspended in 50 mM of acetate buffer, pH 4.75), 0.15 cm^3 of culture filtrate and 0.3 cm^3 acetate buffer (50 mM, pH 4.75). It was incubated for 1 h at 47°C [8]. After the incubation, products released from the hydrolysis of chitin were estimated as reducing sugar using N-acetyl-D-glucosamine as the standard for Miller's method [9].

The enzyme activity was expressed in terms of unit (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.

2.4
Optimization of pH and temperature for the stability of chitinase

The pH of culture filtrate (containing the enzyme) and the temperature of the incubation were chosen as the independent variables in a central composite design [10]. Using this method, the total number of treatment combinations was $2^k + k + n_0$ where k is the number of variable and n_0 is the number of repetition of the experiment at the center point. For statistical calculations, the variables X_i were coded as x_i according to the following equation:

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

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

The behavior of the system was explained by the following second degree polynomial equation;

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

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

The above equation was solved using the Design Expert (Stat-Ease Inc, Minneapolis, MN) to estimate the response of the dependent variable. All experiments were performed in duplicate. To obtain the optimum values of the independent variables, the regression equation was optimized following an iterative method [11].

The relative activity (the ratio of the residual activity to the initial activity of the enzyme) was taken as the dependent output variable. A 2^4-factorial experimental design with four axial points (α = 1.414) and six replicates at the center point with total number of 14 experiments was employed. The coded values of the variables are given in Table 1.

Table 1. Coded values of the independent variables in central composite design for two variables

Variable	Parameter	Level				
		−1.414	−1	0	1	1.414
X_1	pH	3.34	3.75	4.75	5.75	6.16
X_2	Temperature (°C)	23	30	45	60	66

x_i = coded value of the variable X_i
x_1 = (pH − 4.75)/1
x_2 = (Temperature − 45)/15

The pH of the culture filtrate was adjusted with acetic acid (1 M) or sodium acetate solution (1 M) according to the experimental plan given in Table 2. The culture filtrate was then incubated at a definite temperature (Table 2) for 50 minutes. The enzyme sample was taken every five minutes. The temperature of the sample was adjusted to 30°C and then assayed for the residual activity following the standard procedure. The relative activity was calculated by dividing the residual activity at definite period of incubation by the enzymatic activity at 0 minute of incubation.

2.5
Studies on the deactivation kinetics

Experiments were conducted in order to study the thermal stability of chitinase. The enzyme was incubated at three different temperatures at 5°C intervals between 50°C and 60°C. The pH of the culture filtrate was adjusted to three different values, viz., 3.0, 5.4 and 6.0. Aliquot of samples were taken at an interval of 5 mins, cooled to 30°C and assayed for the enzyme activity as described earlier.

2.6
Estimation of deactivation rate constant

The following first order expression was used to account for the zero activity at a particular temperature and at specified incubation time:

Table 2. Experimental and predicted relative activity of chitinase after 50 minutes of incubation at defined pH and temperature. The values are the result of duplicate experiments with a variation of ±3.92%

Experiment Number	pH	Temperature (°C)	Chitinase (relative activity) Experimental			Chitinase (relative activity) Predicted
			Set 1	Set 2	Average	
1	3.75	30	0.401	0.401	0.401	0.404
2	5.75	30	0.758	0.760	0.759	0.829
3	3.75	60	0	0	0	0
4	5.75	60	0	0	0	0.021
5	4.75	45	0.338	0.332	0.335	0.392
6	4.75	45	0.476	0.475	0.475	0.392
7	4.75	45	0.412	0.413	0.413	0.392
8	3.34	45	0.012	0.011	0.012	0.046
9	6.16	45	0.452	0.453	0.453	0.394
10	4.75	23	0.957	0.956	0.956	0.910
11	4.75	66	0	0	0	0.022
12	4.75	45	0.414	0.414	0.414	0.432
13	4.75	45	0.418	0.418	0.418	0.432
14	4.75	45	0.415	0.416	0.416	0.432

$$\frac{dE}{dt} = -k_d E \,, \tag{3}$$

so that,

$$\ln\left[\frac{E_t}{E_0}\right] = -k_d t \,, \tag{4}$$

The k_d (deactivation rate constant) values were calculated from the plot of $\ln [E_t/E_0]$ vs. t at a particular temperature.

2.7 Estimation of thermodynamic parameters for chitinase deactivation

In order to obtain the energies and entropies of deactivation, it is necessary to make use of the theory of absolute reaction rates [12]. The central point of this theory is that the rate of any reaction at a given temperature depends only on the concentration of an energy-rich activated complex which is in equilibrium with the inactivated reactants. The deactivation constant is expressed by the following equation:

$$k_d = \frac{\kappa T}{h} \cdot e^{\frac{\Delta S^*}{R}} \cdot e^{\frac{-\Delta H^*}{RT}} \,, \tag{5}$$

where κ = Boltzman's constant, h = Planck's constant, T = temperature in K, R = gas constant, ΔS^* = change in entropy, ΔS^* = change in enthalpy,
so that:

$$\ln\left[\frac{k_d}{T}\right] = \left(\ln\left(\frac{\kappa}{h}\right) + \frac{\Delta S^*}{R}\right) - \frac{\Delta H^*}{R} \cdot \frac{1}{T} \,. \tag{6}$$

The ΔH^* and ΔS^* values were calculated from the slope and the intercept of the plot of $\ln [k_d/T]$ vs. $1/T$ respectively. ΔG^* was estimated from the following relationship:

$$\Delta G^* = \Delta H^* - T \cdot \Delta S^* \,. \tag{7}$$

The energy of the deactivation was estimated using Arrhenius equation:

$$k_d = A\ e^{\frac{-E}{RT}} \,. \tag{8}$$

The energy involved in this process was calculated from the slope of the linear plot of $1/T$ vs. $\ln (k_d)$.

3 Results and discussion

The most important physical factors which affect the stability of an enzyme are pH and temperature. In order to study the combined effect of these factors, experiments were performed at different pH and temperature combinations and a suitable combination was determined using statistical experimental design to enhance the stability of the enzyme. The details of the experimental plan is given in Table 2. Using the results of the experiments, the following equation giving the relative enzyme activity as the function of pH and the temperature was obtained:

$$y = 0.411836 + 0.122776x_1 - 0.314066x_2 - 0.106157x_1^2 + 0.016881x_2^2 - 0.08955x_1x_2 \,,$$

where $x_1 = pH$ and x_2 = temperature (°C)

The squared regression was significant at the level of 99%. The predicted relative activity at each experimental point are shown in Table 2 and the summary of the ANOVA is shown in Table 3. The contour plot (Fig. 1) explains the behavior of the system. The values obtained after optimizing the regression equation following an iterative method are 5.4 and 24°C for pH and temperature respectively. The optimum values were verified experimentally which resulted minimum deactivation of chitinase in a definite period of time.

Thermal stability of chitinase was examined at relatively higher temperature from 50°C to 60°C. It was observed that chitinase was completely deactivated in this temperature range. The time needed for complete denaturation is, however, dependent on pH of the enzyme solution (Table 4). Among three values of pH examined, viz., pH 3.0, 5.4 (optimum) and 6.0, time taken for complete deactivation was the highest in case of pH 5.4.

Table 3. Regression analysis for the relative enzyme activity: effect of pH and temperature on the stability of chitinase
Quadratic response surface model fitting

Source	Sum of Squares	Degree of freedom	Mean Square	F ($P < 0.01$)
Blocks	0.005818	1		
Model	1.029465	5	0.205893	
Error	0.026183	7	0.003740	55.05
Total	1.061466	13		

Root mean square error = 0.061159
R = 0.9875, R^2 = 0.9753
R = Coefficient of correlation, R^2 = Coefficient of determination

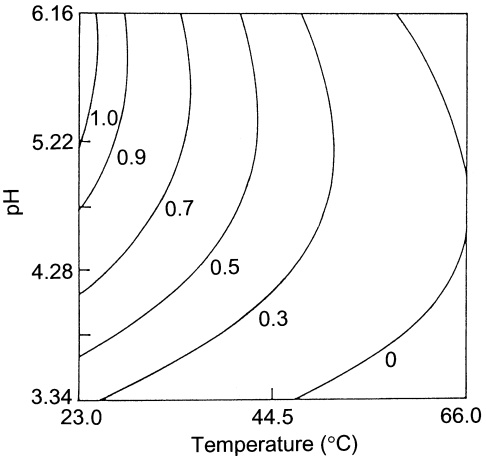

Fig. 1. Contour plot of relative activity of chitinase: Effect of pH and temperature

Table 4. Time taken for complete deactivation of chitinase at different pH and temperature

pH	Time (min) 50°C	55°C	60°C
3.0	45	25	20
5.4	50	40	30
6.0	50	30	30

The deactivation rate constant was calculated at each pH and temperature combination (Table 5). The dependence of k_d on pH and temperature is represented in Fig. 2. The half life of enzyme at different pH and temperature was also calculated (Table 5). The rate of the deactivation of the enzyme was minimum at pH 5.4 of the reaction considering a particular temperature as it is reflected in the values of half life of the enzyme.

The change in enthalpy and entropy were calculated within the temperature range 50°C–60°C (Table 6). In all cases of different pH, entropy change was found to be negative. This is consistent with a compaction of the reacting enzyme molecule, but equally such changes could arise from the formation of charged particles and the associated gain and ordering of solvent molecules [13]. The ΔG^* values were also calculated for different pH and at different temperature (Table 7). The standard free energy change increased with the increase in temperature though the increase is marginal. The energy of chitinase deactivation was calculated from Arrhenius plot. At higher pH, the activation energy was found to be the lowest (Table 8).

Table 5. k_d and $t_{1/2}$ values of chitinase deactivation at different pH and temperature

Temperature (°C)	k_d (min^{-1})			$t_{1/2}$ (min)		
	pH 3.0	pH 5.4	pH 6.0	pH 3.0	pH 5.4	pH 6.0
50	0.0689	0.0608	0.0687	10.06	11.41	10.08
55	0.1199	0.0905	0.1076	5.78	7.66	6.44
60	0.1664	0.1397	0.1551	4.17	4.96	4.47

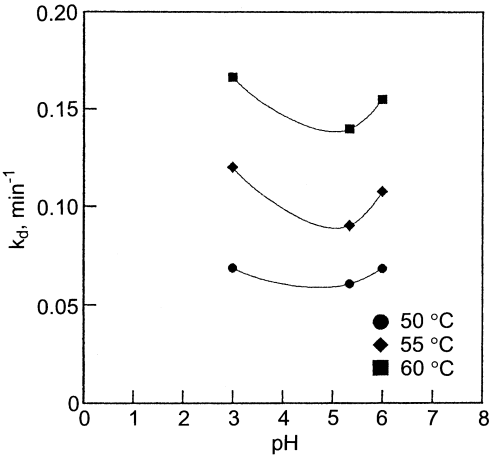

Fig. 2. Effect of pH on k_d values of chitinase deactivation at different temperature

Table 6. Values of ΔH^* and ΔS^* for chitinase deactivation (temperature range from 50°C to 60°C)

pH	ΔH^* (kJ/mol)	ΔS^*(J/mol.K)
3.0	76.34	−65.32
5.4	71.68	−81.13
6.0	70.01	−85.12

Table 7. Values of ΔG^* for chitinase deactivation (temperature range from 50°C to 60°C)

pH	ΔG^* (kJ/mol)		
	50°C (323.15 K)	55°C (328.15 K)	60°C (333.15 K)
3.0	97.45	97.78	98.10
5.4	97.89	98.30	98.71
6.0	97.51	97.94	98.37

Table 8. Values of activation energy at different pH (temperature range from 50°C to 60°C)

pH	E (kJ/mol)	A
3.0	79.04	4.2697×10^{11}
5.4	74.31	6.2223×10^{10}
6.0	72.83	4.1129×10^{10}

4 Conclusion

The combined effect of pH and temperature on chitinase has been investigated using response surface methodology. The denaturation of the enzyme was found to be the lowest at the optimal values of 24°C and 5.4 for temperature and pH respectively. The negative values of entropies of deactivation of chitinase suggest the denaturation may be due to the compactation of the reacting enzyme molecule.

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