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EXPERIMENTAL ARTICLES

Studies on pH and Thermal Stability of Novel Purified L-Asparaginase from *Pectobacterium Carotovorum* MTCC 1428[1]

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Abstract—Glutaminase free L-asparaginase is known to be an excellent anticancer agent. In the present study, the combined effect of pH and temperature on the performance of purified novel L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 was studied under assay conditions using response surface methodology (RSM). Deactivation studies and thermodynamic parameters of this therapeutically important enzyme were also investigated. The optimum pH and temperature of the purified L-asparaginase were found to be 8.49 and 39.3°C, respectively. The minimum deactivation rate constant (k_d) and maximum half life ($t_{1/2}$) were found to be 0.041 min^{-1} and 16.9 h, respectively at pH of 8.6 and 40°C. Thermodynamic parameters (ΔG, ΔH, ΔS, and activation energies) were also evaluated for purified L-asparaginase. The probable mechanism of deactivation of purified L-asparaginase was explained to an extent on the basis of deactivation studies and thermodynamic parameters.

Keywords: L-asparaginase, Anti-leukaemic enzyme, Deactivation kinetics, Thermodynamic parameters, Response surface methodology.

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L-asparaginase (L-asparagine amidohydrolase EC.3.5.1.1) is an enzyme of high therapeutic importance due to its antilymphomic and antineoplastic activities, and use in certain kinds of cancer therapies, mainly in acute lymphoblastic leukemia (ALL) [1, 2]. It is also used in food industry for the production of acrylamide free food, model enzyme for the development of new drug delivery system and L-asparagine biosensor for leukemia [3–5].

Studies on the molecular structure, catalysis, clinical aspects, genetic determinants involved in regulation and stabilization to enhance biological half-life of L-asparaginase have been reported [1, 6, 7] However, very few studies have been reported on thermodynamic aspects of this enzyme [8, 9]. Thermal stability studies would help to understand the relation between structure and function of a particular enzyme [10]. L-Asparaginase deactivation plays a vital role in cancer therapy [1]. Rapid inactivation may constrain the efficiency of therapeutic process. An improved knowledge of enzyme deactivation kinetics is needed to enhance the feasibility of therapeutic use. The deactivation studies would provide valuable physical insights into the structure and function of the enzyme. Deactivation is defined as a process where the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds [11]. The ability of enzyme is a measure of its ability to catalyze a reaction while the stability of the enzyme is judged by its residual activity. Both of these properties are modified to a large extend by temperature, pH and modifiers such as activators, inhibitors etc. The examination of relationships between enzyme properties and environmental conditions plays a vital role to predict, manipulate and engineer the protein structure.

It is well known that the activity and stability of an enzyme is strongly influenced by pH and temperature. Optimization of process variables using one variable at time is very tedious, labor intensive and lacks in explaining the interactive effects among the variables. Thus, it does not depict the net effects of various parameters on enzyme activity.

The aim of communication is to study the combined effect of pH and temperature on purified L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 under assay conditions using RSM, to demonstrate the deactivation kinetics of purified L-asparaginase, and to determine thermodynamic parameters.

MATERIAL AND METHODS

Bacterial strain and culture conditions. The bacterial strain, *P. carotovorum* MTCC 1428 used in the present investigation was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. The pure culture of this microbial isolate was

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grown on a medium containing (g/l): beef extract 1.0, yeast extract 2.0, NaCl 5.0, peptone 5.0 and agar 15.0 (pH 7.0), incubated at 30°C for 24 h. The culture was maintained at 4°C by sub-culturing every month.

Production of L-asparaginase and purification. The production of L-asparaginase was performed in the optimized semisynthetic medium containing (g/l): glucose 5.202; L-asparaginase 2.076; $Na_2HPO_4 \cdot 2H_2O$, 6.0; KH_2PO_4 1.772, NaCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.373; $CaCl_2 \cdot 2H_2O$ 0.015; yeast extract 1.0 and peptone 1.0 at initial pH of 6.5 [12]. The inoculum was prepared by adding a loop full of freshly prepared pure culture from the slant into 80 ml of sterile medium mentioned above containing glucose as a sole carbon source in a 500 ml Erlenmeyer flask. The culture flask was incubated at 30°C and 180 rpm in an orbital shaking incubator for 10–12 h. The culture was transferred (2% v/v, at mid-logarithmic phase (OD at 600 nm = 0.6–0.8)) in to a stirred tank 7 L bioreactor (Sartorius Biostat B plus, Germany) with working volume of 4 L. The bioreactor was operated at 30°C, 200 rpm, and 1.5 vvm with uncontrolled pH. The samples were drawn at regular intervals of time, and specific activity was. measured in triplicates.

Intracellular L-asparaginase was isolated from *P. carotovorum* MTCC 1428 as described by Kumar et al. [13]. In this study, we have used high purity of the enzyme preparation, which was obtained by precipitation of proteins with ammonium sulfate followed by Anion-exchange chromatography (DEAE cellulose) and gel filtration chromatography (Sephadex G-100) [14]. The protein concentration was determined according to the method described by Lowry et al. [15] using BSA as a standard. All chromatographic experiments were monitored for protein at 280 nm.

Enzyme assay. L-asparaginase activity was performed by modified method as described by Kumar et al. [13]. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μM of ammonia per minute at 37°C.

Experimental design to evaluate the effect of pH and temperature on purified L-asparaginase under assay conditions. The central composite design has been applied to optimize the levels and explain the combined effect of physical parameters viz., pH and temperature for purified L-asparaginase from *P. carotovorum* MTCC 1428 [16]. Each variable was assessed at five coded levels (−α, −1, 0, +1, and +α). According to this design, the total number of treatment combinations was 14 (= $2^k + 2k + 6$), where k is the number of independent variables [17]. Central composite design with four axial points (α = 2) and six replicates at the center point with a total number of 14 experiments were employed to evaluate the pure error. The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form is provided in the Table 1. The quadratic model for predicting the optimal levels was expressed according to the Eq. (1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j. \quad (1)$$

Where, Y is the predicted response, k is the number of variables. X is the coded levels of the independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. The statistical software package, MINITAB® Release 15.1, PA, USA was used for the regression analysis of the experimental data, and also to plot the response surface graphs. All the experiments were performed in triplicates and averages of the results were taken as the response.

Deactivation studies. Experiments were conducted to study the thermal stability of L-asparaginase. The culture filtrate was incubated at different combinations of pH and temperature. The levels of pH and range of temperature selected to study the deactivation of L-asparaginase were 7.6, 8.6 and 9.6 and 30–50, 40–60 and 30–50°C, respectively. The enzyme samples were deactivated at various combinations of pH and temperature as mentioned above and aliquots of samples were collected at different intervals of time. The pH of the samples was adjusted to standard assay conditions and measured the residual activity of L-asparaginase.

Estimation of deactivation rate constant (k_d) and half-life time ($t_{1/2}$). Since the deactivation of L-asparaginase is one of the major constraints in the efficiency of chemotherapy, a better understanding of the mechanism of deactivation is very important. The deactivation of L-asparaginase is assumed to follow first-order kinetics. This is called single step two-stage theory [10]. The two-state mechanism is as follows:

$$E \longrightarrow E_d \quad (2)$$

The assumption in the mechanism is that the active enzyme state E directly converts to inactive state E_d without providing any significant amount of intermediates. The first-order deactivation is represented as Eq. (3).

$$\frac{dE}{dt} = -k_d[E] \quad (3)$$

or,

$$\alpha = \exp(-k_d t) \quad (4)$$

where,

$$\alpha = \frac{E_d}{E}$$

From the plot of $\ln(\alpha)$ versus t, the slope gives the value of deactivation rate constant k_d.

Table 1. Experimental design and results for the activity of L-asparaginase from *P. carotovorum* MTCC 1428 at various combinations of pH and temperature

Run order	Uncoded and coded levels		Enzyme activity (U/ml)	
	pH	Temperature (°C)	Observed	Predicted
1	8.0(−1)	37(−1)	6244 ± 14	6229
2	9.6(+1)	37(−1)	5759 ± 20	5927
3	8.0(−1)	43(+1)	6069 ± 20	6078
4	9.6(+1)	43(+1)	5669 ± 25	5860
5	8.6(0)	40(0)	6598 ± 09	6485
6	8.6(0)	40(0)	6604 ± 07	6485
7	8.6(0)	40(0)	6607 ± 15	6485
8	7.4(−2)	40(0)	5574 ± 13	5621
9	9.8(+2)	40(0)	5237 ± 11	5101
10	8.6(0)	34(−α)	6331 ± 13	6298
11	8.6(0)	46(+α)	6137 ± 14	6081
12	8.6(0)	40(0)	6645 ± 14	6697
13	8.6(0)	40(0)	6635 ± 21	6697
14	8.6(0)	40(0)	6634 ± 21	6697

Note: Enzyme activity = Mean value ± S.D.

The half-life of an enzyme is defined as the time required by the enzyme to loose half of its initial activity, which is given by:

$$t_{1/2} = \ln(2)/k_d. \quad (5)$$

Estimation of thermodynamic parameters. The energies and entropies of deactivation can be estimated by making use of absolute reaction rates [18]. The temperature dependency of deactivation rate constant can be expressed as:

$$k_d = \frac{\kappa T}{h} \exp\frac{\Delta S^*}{R} \exp\frac{-\Delta H^*}{RT} \quad (6)$$

or,

$$\ln\frac{k_d}{T} = \ln\frac{\kappa}{h} + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT}. \quad (7)$$

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

$$\Delta G^* = \Delta H^* - T\Delta S^*. \quad (8)$$

The activation energy is calculated from the Arrhenius Eq. as:

$$k_d = k_0 \exp\frac{-E}{RT} \quad (9)$$

or,

$$\ln(k_d) = \ln(k_0) - \frac{E}{RT}. \quad (10)$$

The values of E and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$, respectively.

Table 2. Analysis of variance (ANOVA) for L-asparaginase activity: effect of pH and temperature

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	5492844	5492844	1098569	79.81	0.0001
Linear	2	476299	2657906	1328953	96.55	0.0001
Square	2	5012974	5012974	2506487	182.10	0.0004
Interaction	1	3570	3570	3570	0.26	0.0616
Residual Error	21	289046	289046	13764		
Lack-of-Fit	3	285089	285089	95030	432.35	0.0005
Pure Error	18	3956	3956	220		
Total	27	5799995				

Note: $R^2 = 95.02\%$; R^2(adj) = 93.59%; DF – degree of freedom; Seq SS – sequential sum of square; Adj SS – adjusted sum of square; Adj MS – adjusted square of mean; F – *F* value ; P – *P* value.

RESULTS AND DISCUSSION

Production of L-asparaginase and purification. In our previous paper [14] it was shown that the biomass yield and specific activity at the end of cultivation in bioreactor were found to be 0.97 ± 0.03 g/l and 28.87 ± 0.37 U/mg, respectively. Glutaminase-free L-asparaginase was purified to homogeneity after Sephadex G-100 chromatography with 42.02% of yield. The enzyme was purified approximately 72-fold with a specific activity of 2020.91 U/mg [14]. The active fractions of final purification step were pooled, concentrated using lyophilizer and dialyzed against the *Tris*–HCl buffer (50 mM, pH 8.6). The concentrated fraction was stored at –20°C for further analysis of experiments. L-asparaginase purity and molecular weight was confirmed by the SDS–PAGE, Native PAGE and gel-filtration chromatography [14]. The molecular weight of the purified homotetramer L-asparaginase and subunits was found 144.42 and 36.10 kDa, respectively by MADI-TOF MS [14].

Optimization of combined effect of pH and temperature on the performance of L-asparaginase under assay condition. The most important physical factors, which influence the enzymatic reaction rate, are pH and the temperature of incubation with the substrate. Each enzyme has a characteristic pH and temperature optima beyond which the reduction in activity was observed. The suitable temperature and pH for L-asparagine-L-asparaginase system was determined using statistical experimental design. In order to determine the optimal conditions, preliminary experiments were performed to study the effect of pH on the activity of L-asparaginase (keeping at constant temperature). The results clearly showed that the maximum activity was observed when the pH was varied from 8.0 to 9.0 [14]. Similarly, experiments were performed to study the effect of temperature on the activity of L-asparaginase and found that the maximum activity was obtained when the temperature was varied between 35 and 45°C [14]. Hence, experiments were performed at various combinations of pH and temperature. The design matrix and the corresponding results of observed and predicted responses (L-asparaginase activity) are shown in Table 1. The enzyme activity varies from 5237 to 6645 U/ml. By applying the multiple regression analysis on the experimental data, the following second-order polynomial equation was found to explain the dependence of L-asparaginase activity on pH and temperature of incubation.

$$Y_{EA} = -77954.2 + 15269.8A + 1008.9B - 927.7A^2 - 14.1B^2 + 11.7AB. \quad (11)$$

where, A is pH and B is temperature.

The results were analyzed using the ANOVA as appropriate to the experimental design used (Table 2). According to the ANOVA of the quadratic regression model, the model is highly significant, as is evident from the Fisher F test (mean square regression: mean square residual is 79.81) with a very low probability value ($P_{\text{model}} > F$ is 0.0001). This indicates that the combined effects of pH and temperature significantly contributed to maximizing the L-asparaginase activity. The goodness of the model was checked by coefficient of determination, R^2, which implies that the sample variation of 95.02 for the dependence of L-asparagi-

Table 3. Model coefficient estimated by multiple linear regressions

Term	Coef	SE Coef	T	P
Constant	−77954.2	9610.25	−8.112	0.0001
pH	15269.8	1246.95	12.246	0.0001
Temperature	1008.9	252.38	3.998	0.0001
pH*pH	−927.7	48.77	−19.021	0.0004
Temperature*Temperature	−14.1	1.95	−7.228	0.0004
pH*Temperature	11.7	23.04	0.509	0.0616

Note: Coef – coefficient ; SE Coef – standard error of coefficient; T – *t* value; P – *P* value.

nase activity is attributed to pH and temperature of incubation and also only 4.98% of the total variation is not explained by the model.

The Student's *t* distribution and the corresponding *P* values, along with the parameter estimate are shown in Table 3. The *P* values of all linear and quadratic relationships between process parameters and L-asparaginase activity under assay conditions suggest that they are highly significant ($P < 0.0004$). However, the interaction effect between pH and temperature is lower than linear effect ($P < 0.0616$), but accounted appreciably. In addition, it was also found that the coefficient for pH is very much larger than coefficient for temperature. This suggests that any change in pH has large effect on enzyme activity of L-asparaginase than temperature in the range studied.

The 2D contour plot explains the behavior of the system and enzymatic activity over independent variables, pH and temperature are shown in Fig. 1. The enzyme activity is higher at a range of temperature (36–41°C) and moderate pH (8.4–8.6). This equation was optimized and solved by MINITAB optimizer. The optimum levels of temperature and pH were found to be 39.3°C and 8.49, respectively. In order to verify the optimal conditions, the experiments were performed at central point and optimal levels of variables. These conditions have showed higher enzyme activity (6856 U/ml) value as compared to that used presently for the analysis of enzyme activity. After optimization, L-asparaginase activity was increased by 200 U/ml in purified sample.

Deactivation studies. L-Asparaginase has been deactivated under various combinations of pH and temperature as discussed in methods section. The extent of deactivation is measured by the deactivation rate. The deactivation rate is proportional to the active enzyme concentration, and k_d (deactivation rate constant) is the proportional constant. The deactivation process is modeled as first-order kinetics and the deactivation rate constant was evaluated. The effect of temperature on deactivation has been studied and the results are shown in Figs. 2a–2c. The minimum value of k_d observed for L-asparaginase was 0.041 min^{-1} (Table 4). The combinations of pH and temperature at which the above mentioned minimum deactivation rate constant have been observed to be 8.6 and 40°C, respectively.

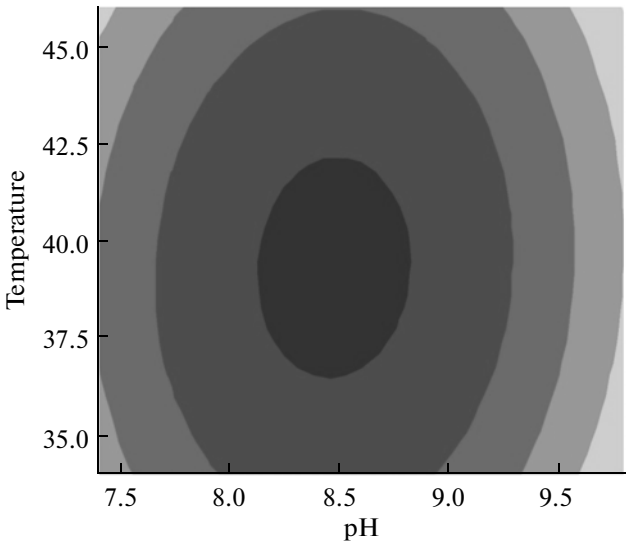

Fig. 1. 2D contour plot of L-asparaginase activity at different levels of pH and temperature of incubation. Enzyme activity (U/ml): < 5000; 5000–5500; 5500–6000; 6000–6500; >6500.

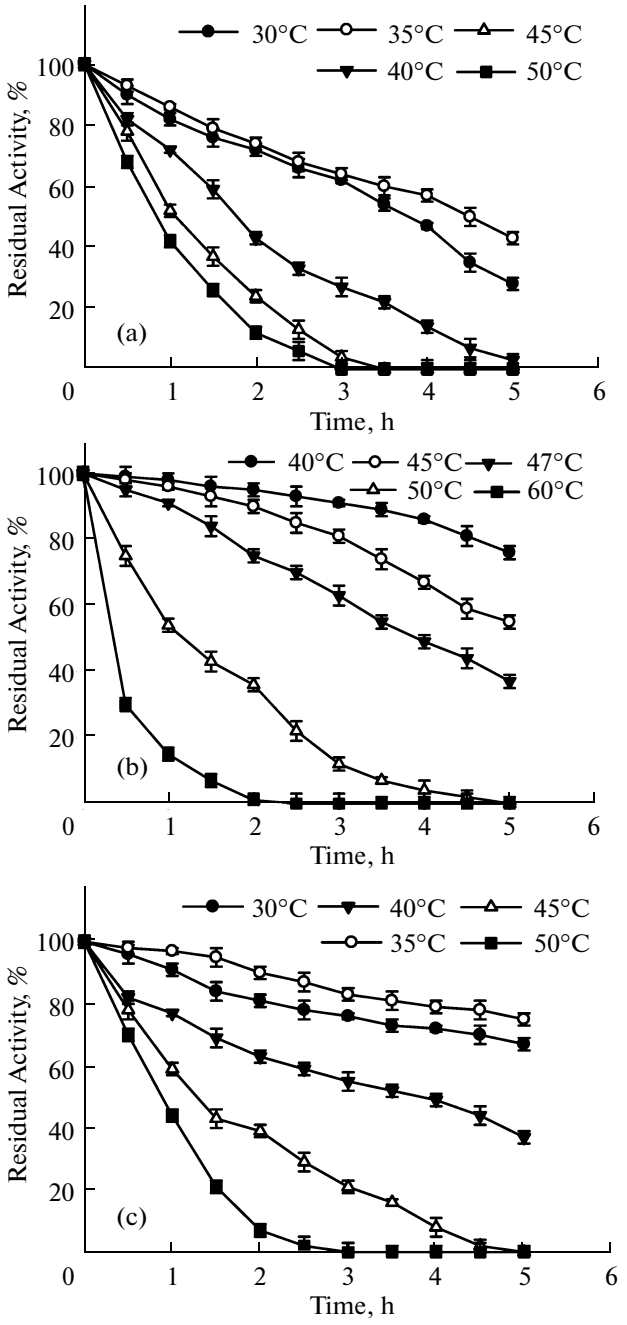

Fig. 2. Thermal stability of L-asparaginase at (a) pH 7.6 (b) pH 8.6 and (c) pH 9.6.

The deactivation process was found to be faster at pH 7.6 than at alkaline pH (8.6, 9.6) for L-asparaginase. Naidu and Panda [19] observed that the similar effect of pH on deactivation rate constant as reported in this study. This is possibly due to disulfide exchange, which usually occurs at near neutral and alkaline conditions [20]. Furthermore, the observation of interrelationship between conformational stability and enzyme activity suggested that in naturally occurring enzymes one cannot expect to find stability at temperatures far above than that of growth of an organism [21]. The results obtained in the present study also indicate that the optimum pH and temperature lie near that of the growth condition.

Estimation of thermodynamic parameters. The change in enthalpy and entropy has been calculated by transition state theory according to the Eqs. (7) and (8). The results are shown in Table 5 for purified L-asparaginase from *P. carotovorum* MTCC 1428. The entropy value was found to be negative for L-asparaginase at pH 7.6. The negative values of entropy of deactivation of L-asparaginase suggested that the denaturation may be due to the compactation of the reacting enzyme molecule and the ordering of solvent molecules [22]. Solvent and structural effects are the two major factors, which influence the numerical values of ΔH^* and ΔS^*. Increase in entropy and enthalpy values were observed when incubated at higher levels of pH. The probable reason is that the enzyme gets unfolded during deactivation and the negative sign is due to the formation of charged particles around the enzyme molecule with increase in pH or it may be due to the ordering of solvent molecules. The increase in ΔS^* indicates an increase in the number of protein molecules in a transition activated state, which in turn, gives lower values of ΔG^*. The values of ΔG^* (calculated from Eq. (8)) are given in Table 5 for the enzyme. The decrease in entropy and enthalpy values was observed with increase in pH (Table 5). Probably, at higher pH, the stable three dimensional structure of enzyme gets compressed, resulted in decrease in residual activity. To gain a deeper insight into the mechanism and specificity of L-asparaginase, the temperature-dependence of the catalytic activity was investigated. The temperature dependency of first-order deactivation rate constant was studied by Arrhenius Eq. (Eq. (9)). The activation energy E and frequency factor k_0 were estimated from Eq. (9) and they are shown in Table 5. It was found that the deactivation energy maximum at optimum pH for L-asparaginase and starts decreasing at higher range of pH. Naidu and Panda [19, 23] also found that the similar observation on temperature dependency of deactivation rate constant for thermal deactivation of chitinase from *Trichoderma harzianum* and pectolytic enzymes from *Aspergillus niger*. For L-asparaginase, the deactivation energy increases at higher temperature suggesting that L-asparaginase require more amount of energy to deactivate. This is in agreement with the result that the L-asparaginase is more stable at pH 8.6 with lower temperature rather than pH 9.6 and 7.6 at higher temperature.

In this study, RSM has been successfully applied to study the combined effect of pH and temperature of purified L-asparaginase from *P. carotovorum* MTCC 1428 under assay conditions. Purified L-asparaginase is more stable at pH of 8.6 with lower temperature rather than pH of 9.6 and 7.6 at higher temperature. At the optimal pH of 8.6, the rate of deactivation of was

Table 4. Effect of temperature on deactivation constant (k_d) and half life time ($t_{1/2}$) of the purified enzyme

pH	Temperature °C	k_d (h^{-1})	$t_{1/2}$ (h)
7.6	30	0.20	3.33
	35	0.15	4.50
	40	0.54	1.27
	45	0.87	0.79
	50	1.04	0.66
8.6	40	0.04	16.9
	45	0.09	7.07
	47	0.17	3.96
	50	0.75	0.91
	60	2.09	0.33
9.6	30	0.08	8.15
	35	0.05	12.37
	40	0.19	3.57
	45	0.64	1.08
	50	1.34	0.51

Note: R^2 of plot of $\ln(E_d/E)$ versus t is 0.979.

found to be least for different temperatures. The negative values of entropy of deactivation of L-asparaginase at pH 7.6 suggested that the denaturation may be due to the compactation of the reacting enzyme molecule.

REFERENCES

1. Narta, U.K., Kanwar, S.S., and Azmi, W., Pharmacological and Clinical Evaluation of L-Asparaginase in the Treatment of Leukemia, *Crit. Rev. Oncol./Hematol.*, 2007, vol. 61, pp. 208–221.
2. Kotzia, G.A. and Labrou, N.E., L-Asparaginase from *Erwinia chrysanthemi* 3937: Cloning, Expression and Characterization, *J. Biotechnol.*, 2007, vol. 127, pp. 657–669.
3. Pedreschi F., Kaack K., and Granby, K., The Effect of Asparaginase on Acrylamide Formation in French Fries, *Food Chem.*, 2008, vol. 109, pp. 386–392.
4. Teodor, E., Litescu, S.C., Lazar, V., and Somoghi, R., Hydrogel-Magnetic Nanoparticles with Immobilized L-Asparaginase for Biomedical Applications, *J. Mater. Sci. Mater. Med.*, 2009, vol. 20, pp. 1307–1314.
5. Verma, N., Kumar, K., Kaur, G., and Anand, S., *E. coli* K-12 Asparaginase-Based Asparagines Biosensor for Leukemia, *Artif. Cells Blood Substit. Immobil. Biotechnol.*, 2007, vol. 35, pp. 449–456.
6. Kelo, E., Noronkoski, T., Stoineva, I.B., Petkov, D.D., and Mononen, I. β-Aspartylpeptides as Substrates of L-Asparaginases from *Escherichia coli* and *Erwinia chrysanthemi*, *FEBS Lett.*, 2002, vol. 528, pp. 130–132.
7. O'Fágáin, C., Enzyme Stabilization—Recent Experimental Progress, *Enzyme Microb. Technol.*, 2003, vol. 33, pp. 137–149.

Table 5. Estimated thermodynamic parameters during the thermal deactivation of purified L-asparaginase from *P. carotovorum* MTCC 1428[a]

pH	ΔH (KJ/mol)	ΔS (J/mol/K)	E (KJ/mol)	k_0 (hr^{-1})	ΔG (KJ/mol)
7.6	78.14	–70.66	80.75	1.0×10^6	99.56–100.97[b]
8.6	175.66	221.57	178.67	2.2×10^{21}	106.27–101.84[c]
9.6	126.35	78.41	128.87	6.1×10^{13}	102.58–101.01[d]

[a] R^2 of plot of $\ln(k_d/T)$ verus 1/T is 0.965; R^2 of plot of $\ln(k_d)$ versus 1/T is 0.952.
[b] The temperature range is 30–50°C.
[c] The temperature range is 40–50°C.
[d] The temperature range is 30–50°C.
ΔH – change in enthalpy; ΔS – change in entropy; E – activation energy; k_0 – Arrhenius equation constant; ΔG – Gibbs free energy.

8. Kotzia, G.A. and Labrou, N.E.. Cloning, Expression and Characterization of *Erwinia carotovora* L-Asparaginase, *J. Biotechnol.*, 2005, vol. 119, pp. 309–323.
9. Krasotkina, J., Borisova, A., Gervaziev, Y., and Sokolov, N., One-Step Purification and Kinetic Properties of the Recombinant l-Asparaginase from *Erwinia carotovora, Biotechnol. Appl. Biochem.*, 2004, vol. 39, pp. 215–221.
10. Sadana, A., *Biocatalysis: Fundamentals of Enzyme Deactivation Kinetics*, New Jersey: Prentice-Hall, 1991, pp. 68–131.
11. Joly, M., *Physico-Chemical Approach to the Denaturation of Proteins*, New York: Academic, 1965, pp. 191–260.
12. Kumar, S., Pakshirajan, K., and Venkata Dasu, V., Development of Medium for Enhanced Production of Glutaminase Free L-Asparaginase from *Pectobacterium carotovorum* MTCC 1428, *Appl. Microb. Biotechnol.*, 2009, vol. 84, pp. 477–486.
13. Kumar, S., Venkata Dasu, V., and Pakshirajan, K., Localization and Production of Novel L-Asparaginase from *Pectobacterium carotovorum* MTCC 1428, *Process Biochem.*, 2010, vol. 45, pp. 223–229.
14. Kumar, S., Venkata Dasu, V., and Pakshirajan, K., Purification and Characterization of Glutaminase-Free L-Asparaginase from *Pectobacterium carotovorum* MTCC 1428, *Bioresour Technol.*, 2010, doi:10.1016/j.biortech.2010.07.114.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, pp. 265–275.
16. Khuri, A.I. and Cornell, J.A., *Response Surfaces: Design and Analysis*, New York: Marcel Decker Inc., 1987.
17. Araujo, P.W. and Brereton, R.G., Experimental Design II. Optimization, *Trends Analyt. Chem.*, 1996, vol. 15, pp. 63–70.
18. Eyring, H., The Activated Complex in Chemical Reaction, *J. Chem. Phys.*, 1935, vol. 3, pp. 107–115.
19. Naidu, G.S.N. and Panda, T., Studies on pH and Thermal Deactivation of Pectolytic Enzymes from *Aspergillus niger, Biochem. Eng. J.*, 2003, vol. 16, pp. 57–67.
20. Munch, O. and Tritsch, D., Irreversible Thermoinactivation of Glucoamylase from *Aspergillus niger* and Thermoinactivation by Chemical Modification of Carboxyl Groups, *Biochim. Biophys. Acta*, 1990, vol. 1041, pp. 111–116.
21. Daniel, R.M., The Upper Limits of Enzyme Thermal Stability, *Enzyme Microb. Technol.*, 1996, vol. 19, pp. 74–79.
22. Foster, R.L., Modification of Enzyme Activity, in *The Nature of Enzymology*, Baron, P.J., Ed., London: Croom Helm, 1980, pp 91–161.
23. Naidu, G.S.N. and Panda, T., Behaviour of Pectinases under Process Conditions, *Bioprocess Eng.*, 1999, vol. 21, pp. 397–400.