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# Optimization of process conditions and evaluation of pH & thermal stability of recombinant L-Asparaginase II of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli*

Rachna Goswami [a,*], Venkata Dasu Veeranki [b], Vijay Kumar Mishra [a]

[a] Department of Biosciences, AP IIIT Nuzvid, Rajiv Gandhi University of Knowledge of Technology, Nuzvid, Kishna, Andhra Pradesh, 521202, India
[b] Biochemical Engineering Laboratory, Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, 781039, Assam, India

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ABSTRACT

L-asparaginase (EC 3.5.1.1) is an important antineoplastic agent, used in the acute lymphoblastic leukemia chemotherapy and well known as food processing aid for reducing the acrylamide formation in starch rich foods. In the present study, we have cloned, expressed and purified recombinant L-asparaginase II of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli*. To study the best condition for performance of recombinant enzymes, pH and temperature of process conditions were optimized using response surface methodology. Optimal pH and temperature for enzyme were found to be 7.79 and 47.21 °C. Furthermore, the purified recombinant enzyme was subjected to thermal deactivation studies. pH and thermal stability of enzyme was studied at various combinations of pH and temperature. In addition, thermodynamic parameters of recombinant L-asparaginase II were evaluated. It was observed that recombinant L-asparaginase II enzyme is more stable at pH 8.5 at 35 °C relatively pH 6.5, 7.5 and 9.5. It was also observed that recombinant L-asparaginase II is more stable at pH 8.5 than pH 9.6 and 7.6 at higher temperature.

## 1. Introduction

L-asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) hydrolyses L-asparagine to L-aspartic acid and ammonia. It has been used as an anti tumor agent for the effective treatment of acute lymphoblastic leukemia (ALL) and lymphosarcoma (Narta et al., 2007). The asparagine deficiency rapidly impairs the protein synthesis and leads to a delayed inhibition in DNA and RNA synthesis and hence to an impairment of cellular function and cell death of tumor cell (Muller and Boos, 1998). Apart from this, asparaginases are also used as a processing aid in the manufacture of food. It is marketed under the brand names Acrylaway and PreventASe, used to reduce the formation of acrylamide, a suspected carcinogen, in starchy food products such as potato chips and biscuits. During heating the amino acid asparagine, naturally present in starchy foods, is converted into acrylamide in a procedure called the Maillard reaction. By addition of asparaginase prior to baking or frying the food, asparagine is converted into another common amino acid, aspartic acid, and ammonium consequently, unable to take part in the Maillard reaction. As a food processing aid, asparaginases can effectively reduce the level of acrylamide up to 90% in a range of starchy foods without changing the taste and appearance of the end product (Hendriksen et al., 2009).

L-asparaginases produced from *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA) has been used in medical purposes (Bansal et al., 2010; Kravchenko et al., 2008) but L-asparaginase isolated from microorganisms have glutaminase activity (Narta et al., 2007; Imada et al., 1973). They hydrolyzes glutamine present in treated patients of blood to monosodium glutamate. which is the reason for wide range of toxicity, immuno-suppression, thromboembolysis (Kravchenko et al., 2008). Recently, Einsfeldt et al. (2016), Sindhu and Manonmani (2018), Goswami et al. (2015) have developed the recombinant L-asparaginase of *Zymomonas mobilis, Pseudomonas fluorescens* and *E. carotovora*. There was no L-glutaminase activity in L-asparaginase of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 was reported therefore, it was selected for further study.

As it is well acknowledged, enzyme activity is largely dependent on process conditions like pH and temperature (Kumar et al., 2011; Goswami et al., 2013). The main drawback of single variable optimization (altering one parameter while keeping the other at constant level) is that it does not consist of the interactive effects among the factors as a result

* Corresponding author.
*E-mail addresses:* rachna@rguktn.ac.in (R. Goswami), vijay@rguktn.ac.in (V.K. Mishra).

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does not describe the net effects of various parameters on enzyme activity. Therefore, to study the optimum conditions for higher recombinant L-asparaginase II activity of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli*, statistical methodologies is preferred due to their advantages of reducing the number of experiments and error in analyzing the combined effect of factors in a cost-effective manner. To study the best condition for performance of recombinant enzymes, pH and temperature of process conditions were optimized using response surface methodology.

We haves studied the pH and thermal stability of enzyme at different combinations of pH and temperature. Furthermore, thermodynamic parameters for recombinant L-asparaginase II were evaluated. To the best of our knowledge this is the first report on optimization of pH and temperature under process condition for better performance of recombinant L-asparaginase II and deactivation study of recombinant L-asparaginase II enzyme of *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli*. This is first study with recombinant L-asparaginase activity optimized by statistical method. However, similar study is carried out by Kumar et al. (2011).

## 2. Materials and methods

### 2.1. Chemicals

Isopropyl- β-D-thiogalactopyranoside (IPTG) and ampicillin were purchased from sigma. Culture media and their constituents, L-asparagine, ammonium sulfate, were purchased from Hi-Media, India. Nessler's reagent's was purchased from Loba Company, India. All chemicals were purchased from Sigma unless otherwise stated and were of the highest quality available.

### 2.2. Bacterial strain and plasmid

*E. carotovora* subsp. *atroseptica* SCRI 1043 was kindly provided by Paul Birch, Scottish Crop.

Research Institute, Scotland, United Kingdom. *E.coli* strain BL21 (DE3) and plasmid pET22b (+) were obtained from Novagen, USA. *E. coli* (DH5α) was obtained from Microbial Type Culture Collection, Institute of Microbial Technology (MTCC), Chandigarh, India.

### 2.3. Cloning and expression of L-asparaginase II gene

Cloning and expression was carried out as described by Goswami et al. (2015).

### 2.4. Growth medium and expression condition

The inoculum was prepared by streaking on a Luria-Bertani (LB) plate (yeast extract 5 g/l, tryptone 10 g/l, and NaCl 10 g/l, 2% agar, pH 7.0), supplemented with ampicillin (100 μg/ml) of frozen glycerol stock of recombinant strain (kept at −80 °C) and incubated at 37 °C for 12 h. A single isolated colony was then transferred to test tubes with 5 ml of LB-medium containing ampicillin, (100 μg/ml) on a rotary shaker at 37 °C and 200 rpm for 10h. This pre-inoculum was transferred at a rate of 2.3% (v/v) to 100 ml of production medium (tryptone: 14.73 g/l, yeast extract: 5.30 g/l and NaCl 4.03 g/l, Initial pH: 7.4) supplemented with ampicillin (100 μg/ml), in 500 ml conical flasks at 37 °C with shaking at 216 rpm. When the cell OD at $Ab_{600nm}$–0.8–1.0, the expression of L-asparaginase was induced with 1 mM IPTG at 30 °C under shaking at 212 rpm and cells were harvested after 6h of post-induction by centrifugation.

### 2.5. Purification of recombinant L-asparaginase-II

The cells were harvested by centrifugation and the pellet was suspended in 10 ml of 50 mM sodium phosphate buffer, pH 7.0, 500 mM NaCl, 10 mM imidazole, sonicated and centrifuged (20,000×*g*, 20 min). The supernatant was loaded onto a 2 mL Ni affinity column (Ni-NTA-Sigma), equilibrated in 50 mM sodium phosphate buffer pH 8.0, 500 mM NaCl, 10 mM imidazole. After 30 min, the column was washed with 20 mL of the same buffer, containing 20 mM imidazole. The protein was eluted with 200 mM imidazole and dialyzed in 50 mM Tris –HCl buffer for 24 hour. After dialysis enzyme activity and protein concentration of the purified L-asparaginase was determined.

### 2.6. Assays for L-Asparaginase and L-Glutaminase activity

L-asparaginase activity was calculated in terms of hydrolysis rate of L-asparagine by measuring the quantity of ammonia released in the reaction assay of L-asparaginase. Assay for L-asparaginase and glutaminase was done as described by Goswami et al. (2015). The ammonia produced in the reaction was determined based on a standard curve obtained with ammonium sulfate as standard. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of ammonia per minute at 37 °C. Specific activity is expressed as units per milligram of protein.

### 2.7. Protein determination

The total protein contents of the samples were determined according to the method described by Lowry et al., (1951) using bovine serum albumin (Sigma) as standard.

### 2.8. Optimization of combined effect of pH and temperature under process conditions

A statistical design was used for optimization of process parameters. The central composite design (Khuri and Cornell, 1987) has been employed to optimize the levels and analysis of the combined effect of the pH and temperature. Enzyme sp. activity was considered as the dependent output variable while pH of the buffer and incubation temperature were considered as independent variable. The center point and the different levels considered in central composite design for recombinant enzymes are given in Table 1. According to the central composite design, reaction was performed at various combinations of pH and temperature. The lower and higher ranges of the variables for physical process parameters used and the full experimental plan with regard to their values in actual and coded form is provided in Table 1. The optimization of process parameters was designed based on $2^2$ full factorial center composite designs (CCD) with 6 axial points, resulting in a total of fourteen experiments. Each variable (pH and temperature) was assessed at five coded levels (−2, −1, 0, +1, and +2) with 14 ($=2^k+2k+6$) treatment combinations for optimization where k is the number of

**Table 1**
Experimental design and results for the activity of the recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 at various combinations of pH and temperature.

| Run No | Uncoded and coded levels | | Specific activity | |
|---|---|---|---|---|
| | pH | Temperature | Observed Value | Predicted Value |
| 1 | 6.5 (−1) | 40 (−1) | 335.13 | 334.07 |
| 2 | 8.5 (+1) | 40 (−1) | 390.25 | 387.85 |
| 3 | 6.5 (−1) | 50(+1) | 389.30 | 391.53 |
| 4 | 8.5(+1) | 50(+1) | 480.52 | 481.42 |
| 5 | 7.5 (0) | 45 (0) | 509.52 | 510.54 |
| 6 | 7.5 (0) | 45 (0) | 509.96 | 510.54 |
| 7 | 7.5 (0) | 45 (0) | 511.79 | 510.54 |
| 8 | 6.08(-2) | 45 (0) | 345.64 | 344.78 |
| 9 | 8.91(+2) | 45 (0) | 445.34 | 446.36 |
| 10 | 7.5 (0) | 37.93(-2) | 378.04 | 380.46 |
| 11 | 7.5 (0) | 52.07(+2) | 489.505 | 487.26 |
| 12 | 7.5 (0) | 45 (0) | 527.25 | 526.53 |
| 13 | 7.5 (0) | 45 (0) | 523.49 | 526.53 |
| 14 | 7.5 (0) | 45 (0) | 529.18 | 526.53 |

independent variables (Araujo and Brereton, 1996). The specific activity of L-asparaginase was collected as the response. For statistical calculations, the relation between the coded values and real values were as described in the following Eq. (1):

$$xi = \frac{Xi - X0}{\Delta Xi}, i = 1, 2, 3.....k \quad (1)$$

Where, $x_i$ is the independent variable coded value, $X_i$ the real value of the independent variable, $X_0$ the real value of the independent variable on the center point, $\Delta Xi$ the step change and the central point was set with a α of 1.414 for optimization of physical process parameter. Adequacy of the method developed was further analyzed. The quadratic model for predicting the optimal levels was expressed according to Eq. (2):

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

Where, $Y$ is the predicted response (Sp. activity), k is the number of factor variables. $X_i$ *and* $X_j$ are independent variables, $\beta_0$ is the offset term, $\beta_i$ is the *i*th linear coefficient, $\beta_{ii}$ is the *i*th quadratic coefficient, and $\beta_{ij}$ is the *ij*th interaction coefficient. The statistical software package MINITAB® Release 15.1, PA, USA was used for analysis of the significance of each coefficient (linear or quadratic), regression analysis of the experimental data and also to plot the response surface graphs. *P*-value (Probability > *F*) less than 0.05 indicated that the model terms are significant. Adequacy of the method developed was further analyzed.

### 2.9. Deactivation studies

In order to study the thermal stability of recombinant L-asparaginase II, the enzyme was incubated at five different temperatures of 5 °C intervals between 35 °C and 55 °C at different pH (6.5, 7.5, 8.5 and 9.5). 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 activity of L-asparaginase was measured under standard assay conditions and residual activity was determined (The % residual activity = L-asparaginase activity at different pH and temperature/Maximum L-asparaginase activity at pH 8.5 and 37 °C *100).

### 2.10. Estimation of deactivation rate constant ($k_d$) and half-life time ($t_{1/2}$)

Since the enzyme deactivation is one of the major restrictions in the development of biotechnological processes, a better understanding of the mechanism of deactivation becomes imperative. The deactivation of free and immobilized recombinant L-asparaginase is presumed to follow first-order kinetics, of the single step two-stage theory (Sadana, 1995). The mechanism of the two stage theory as follows:

$$E \rightarrow E_d \quad (3)$$

In this mechanism, it is assumed that the active enzyme state $E$ directly converts to inactive state $E_d$ without providing any considerable amount of intermediates. The first-order deactivation is represented as:

$$\frac{dE}{dt} = -kd(E) \quad (4)$$

Integration of Eq. (2) leads to:

$$\alpha = \exp(-k_d t) \quad (5)$$

Where

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

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

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:

$$t1 \Big/ 2 = \ln \frac{2}{kd} \quad (6)$$

### 2.11. Estimation of thermodynamic parameters

The energies and entropies of deactivation can be estimated by making use of absolute reaction rates (Eyring, 1935). The temperature dependency of deactivation rate constant can be expressed as:

$$kd = \left(k\frac{T}{h}\right)\exp\left(\frac{\Delta S^*}{R}\right)\exp\left(\frac{-\Delta H^*}{RT}\right) \quad (7)$$

or

$$\ln\left(\frac{kd}{T}\right) = \ln\left(\frac{k}{h}\right) + \left(\frac{\Delta S^*}{R}\right) - \left(\frac{\Delta H}{R}\right)\left(\frac{1}{T}\right) \quad (8)$$

Where, $k_d$ is enzyme deactivation rate constant ($hr^{-1}$); κ is Boltzmann constant ($1.38 \times 10^{-23}$ J $K^{-1}$); h is Plank's constant ($6.626 \times 10^{-34}$J.s) $\Delta H^*$ is change in enthalpy ($^{J.mol-1}$); $\Delta S^*$ is change in entropy ($^{J.mol-1.K-1}$); R is gas constant (8.314 J $M^{-1}$·$K^{-1}$) and T is temperature (K). The values of $\Delta H^*$ and $\Delta S^*$ were calculated from the slope and intercept of the plot of ln ($k_d/T$) versus $1/T$, respectively. Values of change in free energy ($\Delta G^*$) were further estimated by the following relationship:

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (9)$$

Where, $\Delta G^*$ is change in free energy ($^{J\ mol-1}$);

The activation energy ($E_A$) was calculated from the Arrhenius equation as:

$$k_d = k_0 \exp\left(-\frac{E_A}{RT}\right) \quad (10)$$

or

$$\ln k_d = \ln k_0 - \left(\frac{E_A}{R}\right)\left(\frac{1}{T}\right) \quad (11)$$

Where, $E_d$ deactivation energy (J $mol^{-1}$) and $k_0$ is frequency factor ($hr^{-1}$)The values of $E_d$ and $k_0$ were estimated from the slope and intercept of the plot of ln($k_d$) versus $1/T$, respectively.

## 3. Results and discussion

### 3.1. Cloning and expression of genes encoding lasparaginase II (ansB2) of Erwinia carotovora subsp. Atroseptica SCRI 1043

BLAST result of the cloned gene sequence of *ansB2* displayed 100% similarity with *ansB2* (NCBI accession no. BX950851.1) gene sequence of *E. carotovora* subsp. *atroseptica* SCRI 1043, 100% similarity with *ansB2* gene sequence of *Pectobacterium carotovorum* culture-collection MTCC:1428 (NCBI accession no. JN6388851.1) and 89.71% similarity with L-asparaginase *Pectobacterium carotovorum* subsp. *carotovorum* PC1 gene sequence of available in NCBI data base. The best temperature for expression of recombinant L- Lasparaginase II was found to be 30 °C (data not shown) with 1 mM IPTG.

### 3.2. Purification of enzyme

The enzyme was purified approximately 4.2 fold with an overall yield of 82% and specific activity of 282.45 U/mg was achieved. SDS-PAGE and native PAGE of the purified enzyme revealed that

molecular mass of the subunits is approximately 37.5 kDa and 150 kDa, respectively (Fig. 1) (Goswami et al., 2015). Alrumman et al. (2019) have reported a final specific activity of 36.08 U/mg, and the molecular weight 37 kDa by SDS-PAGE analysis for purified L-asparaginase from *Bacillus licheniformis*. Wink et al., (2010) have also cloned and expressed L-asparaginase II of *E. carotovora* subsp. *atroseptica* with (AspSP) and without the signal peptide (AspMP) and both enzymes presented similar high specific activities: 208.1 and 237.6 U mg-1, respectively. Characterization of purified enzyme was carried out in our previous study (Goswami et al., 2015).

### 3.3. Optimization of combined effect of pH and temperature on the performance of recombinant L-asparaginase II under assay conditions

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 further than the reduction in activity is usually observed. Therefore, determination of most favorable combination of temperature and pH for recombinant L-asparaginase II is essential. In order to determine the optimal conditions, preliminary experiments were performed to study the effect of pH on the activity of recombinant L-asparaginase II (at constant temperature). The results clearly showed that the maximum activity was observed when the pH was varied from 7.5 to 8.5 for recombinant L-asparaginase II (data not shown). Similarly, experiments were performed to study the effect of temperature on the activity of L-asparaginase and found that the maximum enzymes activity was obtained when the temperature was varied between 47 and 52 °C (data not shown). Experiments were performed at various combinations of pH and temperature according to the design matrix shown in Table 1. The effects of temperature and pH on the activity of recombinant L-asparaginase II are shown in Table 1. By applying the multiple regression analysis on the experimental data, the following second-order polynomial equation was found to explain the maximum response of recombinant L-asparaginase II as given by equation (12).

$$Y = -6917.98 + 936.90X_1 + 160.82\,X_2 - 65.48\,X_1^2 - 1.85X_2^2 + 1.81\,X_1X_2 \quad (12)$$

Where, $Y$ is enzyme sp. activity, $X_1$ is pH and $X_2$ is temperature.

The results revealed that pH ($X_1$) had a significant effect on recombinant L-asparaginase II activity as it had largest coefficient ( 936.90, $P = 0$. The much higher coefficient of pH than temperature suggests that pH is having greater effect on the process condition than temperature within the experimental range. Positive coefficients of $X_1$ and $X_2$ variables indicated a linear effect for the increase in recombinant L-asparaginase II activity. Negative coefficients were observed for quadratic terms of both variables. Low $P$-value of $X_1$ and $X_2$ variables for quadratic terms of enzyme indicated that they are significant. Among the interactions $X_1X_2$ ($P = 0.0001$ had positive coefficients (Table 2). A positive sign indicates that a higher-level variable setting consequences in a higher response than the lower-level variable setting while a negative sign indicates that the lower-level variable setting results in a higher response than the high-level variable setting. This suggests that the combined effects of pH and temperature significantly attributed for maximizing the performance of recombinant L-asparaginase II. The results were analyzed using the ANOVA (Table 3). According to the ANOVA of the quadratic regression model, the model is highly significant, as is evident from the Fisher $F$ test with a very low probability value ($P_{model=}0.0001$). The $F$ value is the ratio of the mean square due to regression to the mean square due to error. To assess the fit of the model equation, the regression based determination coefficient $R^2$ was evaluated (Haider and Pakshirajan, 2007). The goodness of the model was confirmed by coefficient of determination, $R^2$, which implied that the model can explain the variation of 99%. The Student's $t$ distribution, $P$ values, and the estimated parameters are shown in Table 2. The $P$ values of all linear, quadratic and interaction relationships between process parameters under assay condition and recombinant L-asparaginase II activity are highly significant ($P < 0.05$). The closer the values of $R^2$ to 1, the model would explain better for variability of experimental values to the predicted values (Sayyad et al., 2007). Significance of the model is revealed by results, and is evident from Fischer's $F$ test with a very low probability value ($P < 0.05$). Moreover, $P$ value for 'lack of fit' was greater than 0.05 indicated that the 'lack of fit' of model was insignificant for free and immobilized enzyme. The $F$ statistic was found to be 2064.08 corresponding to $P$-value of 0.0002 (the confidence interval is 0.05) indicating that the model was adequate for enzyme. In other terms, the model was fit with the responses data collected. This suggests that the combined effects of pH and temperature significantly attributed for maximizing the performance of recombinant L-asparaginase II (Table 3). Contour plot described by Eq. (12) represented in Fig. 2, which is the graphical description of regression equation for visualizing the relationship between the response and variable and the interaction between the variables for presuming the most favorable conditions. 2D contour plot elucidates the behavior of the system and enzymatic activity over independent variables, pH and temperature in Fig. 2. The plot is having elliptical shape, which suggests the high significance of the interaction between the two parameters. Maximization of the regression equation (Eq. (12)) was carried out using an iterative technique to acquire optimum levels of parameters by substituting the corresponding coded concentration levels of the factors into the regression equation by using 'response optimizer' in Minitab software. For recombinant L-asparaginase II enzyme, the optimum levels of pH and temperature were determined to be 7.80 and 47.21 °C. The maximum value of enzyme sp. activity calculated from the model according to the predicted optimum production condition. To verify the adequacy of the model, verification experiments were carried out using the predicted optimum pH and temperature. Enzyme activity of recombinant L-asparaginase II was enhanced under the optimum conditions. Under optimized condition enzyme has shown higher enzyme sp. activity of

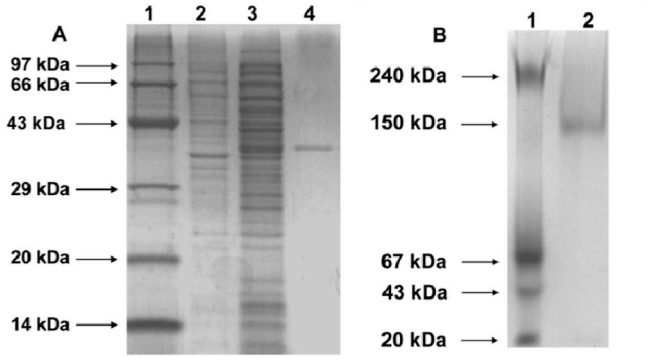


**Fig. 1.** Purification of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 (A: SDS Polyacrylamide gel electrophoresis (12.5%) of purified recombinant L-asparaginase II (Lane 1: Marker, Lane 2: pET 22b(+) in *E. coli* BL21 (DE3) without any insert, Lane 3: Crude extract and Lane 4: Purified recombinant L-asparaginase II. B: Native PAGE (7.5%) of L-asparaginase (Lane 1: Marker and Lane 2: Purified recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043).

**Table 2**
Estimated regression coefficients for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043.

| S. No. | Term | Coef | SE Coef | $T$ | $P$ |
|---|---|---|---|---|---|
| 1 | Constant | −6917.98 | 127.957 | −54.065 | 0.0001 |
| 2 | Block | −8.00 | 0.678 | −11.800 | 0.0002 |
| 3 | $X_1$ | 936.90 | 18.085 | 51.807 | 0.0002 |
| 4 | $X_2$ | 160.82 | 3.865 | 41.609 | 0.0003 |
| 5 | $X_1$*$X_1$ | −65.48 | 0.933 | −70.160 | 0.0002 |
| 6 | $X_2$*$X_2$ | −1.85 | 0.037 | −49.648 | 0.0004 |
| 7 | $X_1$*$X_2$ | 1.81 | 0.254 | 7.118 | 0.0001 |

**Table 3**
Analysis of variance for recombinant L-asparaginase II of E. carotovora subsp. atroseptica SCRI 1043.

| S. No. | Source | DF | Seq SS | Adj SS | Adj MS | *F* | *P* |
|---|---|---|---|---|---|---|---|
| 1 | Block | 1 | 895.6 | 895.6 | 895.6 | 139.23 | 0.0002 |
| 2 | Regression | 5 | 66385.3 | 66385.3 | 13277.1 | 2064.08 | 0.0002 |
| 3 | Linear | 2 | 21725.6 | 21725.6 | 10563.0 | 1642.14 | 0.0001 |
| 4 | Square | 2 | 44333.9 | 44333.9 | 22166.9 | 3446.11 | 0.0003 |
| 5 | Interaction | 1 | 325.9 | 325.9 | 325.9 | 50.66 | 0.0003 |
| 6 | Residual error | 7 | 45.0 | 45.0 | 6.4 | | |
| 7 | Lack of fit | 3 | 25.4 | 25.4 | 8.5 | 1.73 | 0.298 |
| 8 | Pure Error | 4 | 19.6 | 19.6 | 4.9 | | |
| | Total | 13 | 67325.9 | | | | |

R-Sq = 99.98%, R-Sq(adj) = 99.88%.

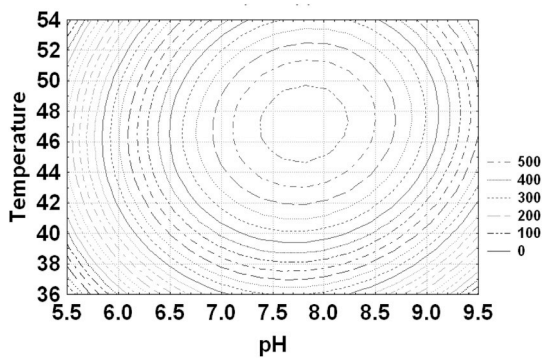


**Fig. 2.** Contour plots showing effect of different levels of pH and incubation temperature on recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043.

533.29 U/mg. After optimization, recombinant L-asparaginase II activity was increased by 1.88 folds. It is in very good agreement with the value predicted by the model, 532.25 U/mg of protein for. It should be noted that this L-asparaginase activity was higher than the central point as well as any of those in the 14 experiments indicating that the optimization was quite successful. The optimum pH and temperature of the purified enzyme from marine *Aspergillus terreus* were 5.8 and 40 °C, respectively. It was onserved that enzyme was stable from pH 4 to 5.8 and stable up to 70 °C (Hassan et al., 2018). The enzyme of *Streptomyces brollosae* NEAE-115 showed maximum activity at pH 8.5, optimum temperature of 37 °C (Noura El-Ahmady et al. (2018). For L-asparaginase of *P. carotovorum* MTCC 1428 Kumar et al. (2011) reported the optimum pH and temperature of the purified L-asparaginase were found to be 8.49 and 39.3° C, respectively. The optimum pH and temperatures for recombinant enzyme from *E-coli* were 7.5 and 37 °C respectively. L-Asparaginase from *Cornyebacterium glutamicum* has displayed pH 7 and at temperature 40 °C. The majority of L-asparaginases from *Erwinia* sp. showed alkaline pH optima (8.0–9.0) whereas the enzyme from *E. coli* exhibited an acidic pH optimum of 5.0–6.0 (Mesas et al., 1990). We did not get any report for optimization of process parameters for recombinant L-asparaginase by RSM, only Kumar et al. (2011) has done similar experiment with L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. They have reported optimum pH and temperature of the purified L-asparaginase 8.49 and 39.3 °C, respectively.

### 3.4. Enzyme deactivation studies

Thermo stability represents the capability of an enzyme molecule to resist against thermal unfolding in the absence of substrate. Recombinant L-asparaginase II has been deactivated under various combinations of pH and temperature. 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). The deactivation process is modeled as first-order kinetics and the deactivation rate constant was evaluated. The effect of temperature on half-life time has been studied and the results are shown in Table 4. The effect of temperature on half-life time of enzyme was studied and the results are shown in Table 4. The minimum value of $k_d$ observed for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 was 0.022 $h^{-1}$ (Table 4). The combination of pH and temperature at which the above mentioned minimum deactivation rate constant was observed at pH of 8.5 and 35 °C. For recombinant L-asparaginase II the deactivation process was found to be faster at pH 6.5 and 9.5 than pH 7.5 and pH 8.5 (Fig. 3). The maximum values of $k_d$ observed at pH 9.5 for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 were 1.190 at 55 °C. Similarly, Kumar et al. (2011) has reported 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 from *Pectobacterium carotovorum* MTCC 1428. The L-asparaginase by *Streptomyces griseoplanus* strain, have achieved the maximum activity at 45 °C. The half-lives of the free enzyme were calculated to be 521 min (8.5 h) at 50 °C, 312.6 min (5.2 h) at 55 °C, and 195.2 min (3.25 h), at 60 °C (El-Hadi et al., 2019). The enzyme of *Penicillium brevicompactum* 3 NRC 829 was more stable at alkaline pH than the acidic one and thermally stable up to 60 min at 50–60 °C and the purified L-asparaginase displayed its maximal activity against L-asparagine when incubated at pH 8.0 at 37 °C for 30 min (El-shafei et al., 2012). The thermal stability of purified L-asparaginase of *Fusarium culmorum* ASP-87 was studied it revealed that the enzyme was highly stable for 120 min at 30 °C–40 °C however, it is inactivated at temperature above 40 °C while retaining 50% activity at 60 °C for 1 hour (Meghavarnam and Janakiraman, 2015). The temperature impact on L-asparaginase

**Table 4**
Effect of process conditions on half life time ($^{t1/2}$) and deactivation constant ($^{kd}$) of the purified recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043.

| pH | Temp (°C) | $K_d$ ($h^{-1}$) | $t^{1/2}$ (h) |
|---|---|---|---|
| 6.5 | 35 | 0.071 | 9.705 |
| | 40 | 0.091 | 7.615 |
| | 45 | 0.260 | 2.658 |
| | 50 | 0.524 | 1.320 |
| | 55 | 0.936 | 0.740 |
| 7.5 | 35 | 0.042 | 16.500 |
| | 40 | 0.065 | 10.660 |
| | 45 | 0.181 | 3.828 |
| | 50 | 0.351 | 1.974 |
| | 55 | 0.801 | 0.865 |
| 8.5 | 35 | 0.022 | 31.935 |
| | 40 | 0.050 | 13.887 |
| | 45 | 0.126 | 5.500 |
| | 50 | 0.203 | 3.410 |
| | 55 | 0.711 | 0.974 |
| 9.5 | 35 | 0.076 | 9.118 |
| | 40 | 0.095 | 7.294 |
| | 45 | 0.280 | 2.475 |
| | 50 | 0.620 | 1.117 |
| | 55 | 1.190 | 0.582 |

$R^2$ of plot of $\ln(E_d/E)$ verus *t* is 0.98.

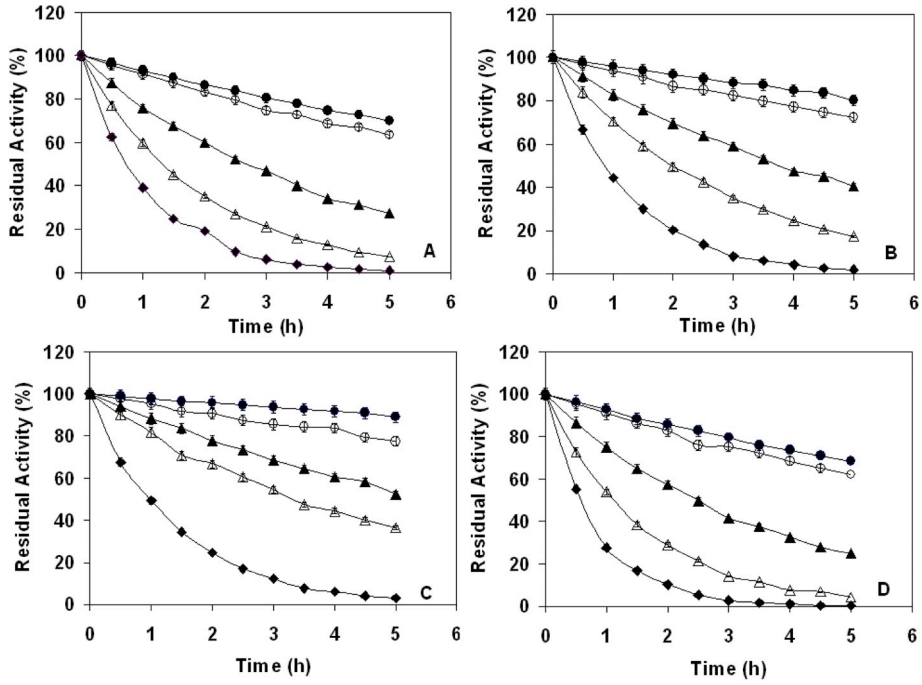


**Fig. 3.** Thermal stability of the recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 at (A) pH 6.5 (B) pH 7.5 and (C) pH 8.5 and (D) pH 9.5(–●– 35 °C, –○– 40 °C, –▲– 45 °C, –△– 50 °C, and –◆– 55 °C).

stability of *Streptomyces brollosae* NEAE-115 was studied by Noura El-Ahmady et al. (2018) and they observed maximum L-asparaginase stability at 40 °C with retained enzyme activity of 97.44% from initial activity after incubation time of 20 min. However, enzyme exposure to higher temperature and longer incubation time of 50 °C and 80 °C for 90 min led to observed rapid decrease in L-asparaginase activity with residual activity of 31.84 and 7.69%; respectively. The maximum activity and stability of the purified L-asparaghinase of from *Bacillus licheniformis* Isolated from the Red Sea occurred at pH values of 7.5 and 8.5, respectively, with maximum activity at 37 °C and complete thermal stability at 70 °C for 1 h (Alrumman et al., 2019). El-Hadi et al. (2019) reported the maximum relative activity of native L-asparaginase of *streptomyces* spp was at pH 8. only 32.5% and 15.48% of its relative activity was retained at pH 8.5 and 9, respectively. Dhevagi and Poorani (2006) observed that pH 8 and pH 8.5 were the best pHs for L-asparaginase activity of *Streptomyces* spp. PDK7 and pH 9 was the optimum for L-asparaginase activity isolated from *S. gulbargensis*. El-shafei et al. (2012), observed that pH 8.0 was the optimal for L-asparaginase from *Penicillium brevicompactum* NRC 829. Ohshima et al. (1976) reported that, in general, L-asparaginase is active at neutral and alkaline pHs. In Basha et al., 2009, reported that maximum activity of asparaginase by marine actinomycetes between pH 7.0 and 8.0 with 80% activity at physiological pH.

Enzyme stability is controlled by its three-dimensional configuration, which is determined by genetic (primary structure) and environmental (temperatures, pHs). Temperature, however, it reveals opposed effects on enzyme activity and stability and is therefore a key variable in any biocatalytic process (Illanes, 1999). Naidu and Panda (2003) reported the similar effect of pH on deactivation rate constant as observed in this study. The change in enthalpy and entropy was calculated by transition state theory (Eqs. (7) and (8)). The thermal inactivation of enzymes is accompanied by the disruption of lots of non-covalent linkages including hydrophobic interactions with concomitant increase in the enthalpy of activation for recombinant L-asparaginase II enzyme. Solvent and structural effects are the two major factors, which influence the numerical values of $\Delta H^*$ and $\Delta S^*$. Increase in entropy and enthalpy values was observed when pH increases from 7.5 to 8.5. Below pH 7.5 and above pH 8.5, there is reduction in the values of the entropy and enthalpy was observed. Probably, at lower (pH 6.5) and higher pH (9.5), the stable three dimensional structure of active site of the enzyme gets distorted, resulted in decrease in residual activity. Positive value of $\Delta S^*$ indicates that the protein has become more disordered as it deactivated by temperature. The increase in $\Delta S^*$ demonstrates an increase in the number of protein molecules in a transition activated state, which in turn, gives lower values of $\Delta G^*$ (Naidu and Panda, 2003). The values of $\Delta G^*$ (calculated from Eq. (9)) are given in Table 5. To gain a deeper insight into the mechanism and specificity of L-asparaginase II enzyme, the temperature-dependence of the catalytic activity was investigated. The temperature dependency of first-order deactivation rate constant was studied by Arrhenius equation (Eq. (10)). The activation energy $E_A$ and frequency factor $k_0$ were estimated from equation (Eq. (11)) and they are shown in Table 5. Maximum deactivation energies for free were observed at optimum pH and further increase in pH, consequences in decrease of activation energy. Kapat and Panda 1997 and Naidu and Panda 2003 also reported the similar observation on temperature dependency of deactivation rate constant for thermal deactivation of chitinase from *Trichoderma harzianum* and pectolytic enzymes from *Aspergillus niger*, respectively. For recombinant L-asparaginase II, the deactivation energy increased at optimum pH and temperature suggested that recombinant L-asparaginase II require more amount of energy to deactivate at optimum pH. This is in agreement with the result that recombinant L-asparaginase II enzyme are more stable at pH 8.5 at 35 °C relatively pH 6.5,7.5 and 9.5 at higher temperature, respectively.

**Table 5**
Estimated thermodynamic parameters during the thermal deactivation of recombinant L-asparaginase II[a] of *E. carotovora* subsp. *atroseptica* SCRI 1043.

| S. No. | pH | ΔH (KJ/Mol) | ΔS (J/Mol/K) | ΔG (KJ/Mol) | E (KJ/Mol) | $K_o$ ($Min^{-1}$) |
|---|---|---|---|---|---|---|
| 1 | 6.5 | 113.120 | 98.61 | 82.74–80.28 | 115.756 | $2.5 \times 10^{18}$ |
| 2 | 7.5 | 124.602 | 131.81 | 84.00–80.71 | 127.237 | $1.4 \times 10^{20}$ |
| 3 | 8.5 | 138.029 | 170.82 | 85.41–81.14 | 135.435 | $1.5 \times 10^{22}$ |
| 4 | 9.5 | 121.110 | 124.78 | 82.67–79.56 | 123.754 | $5.9 \times 10^{20}$ |

$R^2$ of plot of $\ln(k_d/T)$ verus 1/T is 0.97. The temperature range is 35–55 °C.

Noura et al., (2018) have observed that maximum activity at pH 8.5, optimum temperature of 37 °C.

## 4. Conclusions

The recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 was cloned, epressed and purified. After optimizing the process condition (pH and temperature) of the recombinant L-asparaginase II activity of enzyme was increased by 1.88 folds, as compared to un-optimized conditions. The estimated thermodynamic parameters i.e. positive values of entropy of recombinant L-asparaginase II deactivation suggest the verity that deactivation of enzyme is due to the disorder of the protein molecules at the higher temperature.

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