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Production and Characterization of Novel Glutaminase Free Recombinant L-asparaginase II of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3)

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Authors' contributions

This work was carried out under the guidance of author VDV. Author RG designed the study, performed the experiments, authors RG and KH wrote the manuscript and managed literature searches. All the authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To clone and express, the gene encoding L-asparaginase II (*ansB2*) from *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3). Further, the work is also comprised of purification and detailed biochemical characterization of L-asparaginase II.
Place and Duration of Study: Biochemical Engineering laboratory, Department of Biotechnology, Indian Institute of Technology Guwahati, Assam India. Experiments conducted as a part of project and a PhD thesis from December 2010 to January 2014.
Methodology: The gene encoding L-asparaginase II (*ansB2*) from *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 was cloned and expressed in *E. coli* BL21 (DE3). Affinity chromatography was employed to purify the enzyme to homogeneity. Detailed biochemical characterization, such as substrate specificity, operational stability in various effect or molecules, effect of pH and temperature, kinetic parameters were investigated.
Results: The enzyme was found to be highly specific towards its natural substrate, L-asparagine.

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The activity of recombinant L-asparaginase II was activated by various effect or compounds, such as mono cations, L-cystine, L-histidine, 2-mercaptoethanol and glutathione. However, considerable inhibitory effect was observed with divalent cations and iodoactamide. Kinetic parameters (V_{max}, K_m, k_{cat} and K_{cat}/K_m) of purified recombinant L-asparaginase II were found to be 0.656 mM, 312.50 IU mg^{-1}, 1.38×10^{2} s^{-1} and 2.11×10^{5} $M^{-1}s^{-1}$, respectively. Optimum range of pH and temperature for the hydrolysis of L-asparagine by purified recombinant L-asparaginase II were found to be 6.5-9.5 and 47-52ºC, respectively. Under optimal levels of medium components and physical process parameters (pH, inoculum size, agitation), the production of recombinant L-asparaginase II was increased by 1.95 fold. The purified recombinant L-asparaginase II has shown no glutaminase activity.

Conclusion: The present characterization experiments of the L-asparaginase II from *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 showed very high specificity for its natural substrate, L-asparagine and shown no glutaminase activity which makes it a better alternative in therapeutic applications like an anticancer drug.

Keywords: Recombinant L-asparaginase II; Erwinia carotovora subsp. atroseptica SCRI 1043; central composite design.

1. INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) is used in chemotherapy regimens for the treatment of acute lymhoblastic leukemia and non-Hodgkin's lymphoma [1]. The enzyme has also been used to develop L-asparagine biosensor for leukemia [2] and in food industry for the production of acrylamide free food [3]. The application of L-asparaginase in anti-cancer therapy largely depends on its capability to cleave L-asparagine to ammonia and L-aspartic acid. In view of the fact that, lymphoblasts are not able to produce endogenous L-asparagine, starvation of this amino acid causes death of these cells [4,5].

Although L-asparaginase is produced by various microorganisms [2], L-asparaginase from *Erwinia chrysanthemi* and *Escherichia coli* are used for clinical purpose [6.7]. L-asparaginases from *E. coli* and *Erwinia* show different immunological specificities [8] and comparison of the two enzymes has revealed that *E. coli*-asparaginase can be prescribed to the patient for therapy, reserving *Erwinia*-asparaginase for allergic patients, because most patients allergic to the *E. coli* asparaginase are not immediately allergic to the *Erwinia*-asparaginase, which is reported to be less toxic [7,9]. However, the occurrence of L-glutaminase activity in L-asparaginase is known to be the major cause of the various side effects associated with this drug [10,11]. In addition, presence of glutaminase activity is one of the causes of toxicity [12,13]. Unfortunately, a therapeutic response by patients rarely occurs without some evidence of toxicity [1]. *Erwinia* asparaginase shows lower glutaminase activity [4]. The development of recombinant strain for higher production of glutaminase free L-asparaginase II plays a vital role to commercialize the product because L-asparaginases with high specificity for L-asparagine and low-to-negligible activity for L-glutamine are found to be less troublesome during the course of anti-cancer therapy [13]. Therefore, there is a need to find novel sources for higher production of L-asparaginase that is free of glutaminase. There are two types of L-asparaginases found in bacteria (type I and type II). Type II L-asparaginase has higher affinity towards L-asparagine than type I. Hence, L-asparaginase II is used as anticancer agent [14].

The increase in productivity of any metabolite would be attained through manipulation of nutritional and physical parameters. Often, conventional optimization studies involving one-factor-at-time strategy which is tiresome and has a tendency to overlook the effects of interaction between and among the factors and might guide to misinterpretation of results. In contrast, statistically designed experiments reduce the error in determining the effect of factor in an economical manner. Response surface methodology (RSM) is an efficient statistical tool for optimization of multiple variables to find out the best conditions using a minimum number of experiments. Therefore, it is superior to the traditional approach of studying one variable at a time [15]. The economic feasibility of commercialization of recombinant L-asparaginase also depends on various other parameters such as stability during isolation, easy purification using affinity tags [16], storage

as well as its robustness under environmental conditions.

There are three genes' sequences available for L-asparaginase (L-asparaginase, L-asparaginase I and L-asparaginase II) of *E. carotovora* subsp. *atroseptica* SCRI 1043 in NCBI gene bank. To know whether all L-asparaginase genes encode functional L-asparaginase proteins or not, in the present study L-asparaginase genes encoding L-asparaginase (L-asparaginase, L-asparaginase I and L-asparaginase II) from *E. carotovora* subsp. *atroseptica* SCRI 1043 were cloned separately into pET22b(+) and expressed with and without C-terminal His-tag in *E. coli* BL21 (DE3). Since, type II L-asparaginase has higher affinity towards L-asparagine and therapeutic importance than type I therefore, L-aspargainase II was selected for further study. The production, purification and characterization of recombinant L-asparaginase II (with His-tag) was performed as L-asparaginase II is used as anticancer agent. The recombinant L-asparaginase II was purified using Ni-NTA column. The effect of pH, ionic strength of Tris-HCl buffer and NaCl, temperature, incubation time, various metals ions and inhibitors on the activity of L-asparaginase II was studied. In addition, substrate specificity of L-asparaginase with various substrate analogues has been studied and kinetic parameters were also determined [17]. The optimal levels of medium components and physical parameters (pH, inoculum size, agitation) were determined for enhanced production of recombinant L-asparaginase II (with His-tag) in *E. coli*.

2. MATERIALS AND METHODS

2.1 Chemicals

All restriction enzymes, *Phusion* DNA polymerase, T_4 DNA ligase were purchased from New England Biolabs (NEB), USA. Isopropyl- β-d-thiogalactopyranoside (IPTG), ampicillin and agarose were purchased from Sigma, Bangalore, India. L-asparagine and ammonium sulfate were purchased from Hi-Media, Mumbai, India. Nessler's reagent was purchased from Loba Company, Mumbai, India. All other chemicals were purchased from Sigma and were of analytical grade unless otherwise stated.

2.2 Strains and Plasmids

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 of L-asparaginase Genes

Genomic DNA was isolated using genomic DNA isolation kit (Sigma) from *E. carotovora* subsp. *atroseptica* SCRI 1043. The segments encoding the L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) were amplified by PCR using gene specific primers, designed according to the sequence of the asparaginase genes of *E. carotovora subsp. atroseptica* SCRI 1043 (NCBI accession No: BX950851). Each gene was cloned with and without stop codon for expressing the protein without and with C-terminal His-tag, respectively. Amplification of L-asparaginase was carried out using forward primer 5′-ggaattcggatccaatgacgaaacccgtgattgtg-3′ (*Bam*HI site underlined) and reverse primer 5′-gaagcttctcgagacgatagatatcggcgacggg-3′ (*Xho*I site underlined) or with same forward and reverse primer with stop codon to express with or without C-terminal His-tag fusion. Amplification of L-asparaginase I was carried out using forward primer 5′-ggagctcggatccaatgcaaaagaaatccat-3′ (*Bam*HI site underlined) and reverse primer 5′-ggaagcttctcgagatctttatcgctcaattc-3′ (*Xho*I site underlined) or with above mentioned forward and reverse primer with stop codon to express with or without C-terminal His-tag fusion. Amplification of L-asparaginase II was carried out using forward primer 5′-ggaattcggatccaatgcaactctcatttatcgcc-3′ (*Bam*HI site underlined) and reverse primer 5′-gaagcttctcgagctgctcgaaataggtacggatt-3′ (*Xho*I site underlined) or with same forward and reverse primer with stop codon to express with or without C-terminal His-tag fusion. The PCR reaction was carried out in a total volume of 50 µl contained 0.5 mM of each primer, 0.2 mM of each dNTP, 10 µl of 5× Phusion Taq buffer and 2 units of Phusion Taq DNA polymerase (NEB, USA). 1.0, 1.5 ng and 2.0 ng template genomic DNA was used for amplification of L-asparaginase, L-asparaginase I and L-asparaginase II, respectively. The PCR procedure comprised 30 cycles of 30 s at 96ºC, 45 s at 55ºC and 60 s at 72ºC have used for amplification of genes. A final extension time at 72ºC for 10 min was performed after the 30 cycles. The amplified DNA fragment was ligated into the T7 expression vector pET22b (+) in-frame with pel B leader sequence between

*Bam*HI and *Xho*I restriction sites. From this ligation mixture, ~10 ng of DNA was transformed aseptically in to competent cells of *E. coli* (DH5α) by heat shock method [15]. Clones were analyzed by restriction digestion with *Bam*HI and *Xho*I, further confirmed by sequencing and transformed into expression host *E. coli* BL21 (DE3). The gene cassette was expressed in frame with pelB signal sequence for periplasmic localization under IPTG inducible T7 promoter [18].

2.4 Expression of L-asparaginase Genes

The active culture was prepared by streaking frozen glycerol stock of recombinant strain (kept at -80ºC) on a LB agar plate (yeast extract 5 g l^{-1}, tryptone 10 g l^{-1}, NaCl 10 g l^{-1}, 2% agar, pH 7.0), supplemented with ampicillin (100 µg ml^{-1}) and incubated at 37ºC for 12 h. A single isolated colony was transferred in to 20 ml of LB medium containing ampicillin (100 µg ml^{-1}) and incubated on a rotary shaking incubator at 37ºC and 200 rpm for 10 h. This pre-inoculum was transferred (2% vv^{-1}) in to 50 ml of LB medium supplemented with ampicillin (100 µg ml^{-1}) in 250 ml conical flasks and incubated in a rotatory shaking incubator at 37°C and 200 rpm. When cell OD at 600 nm was reached to ~0.8, the expression of recombinant proteins (L-asparaginase, L-asparaginase I and L-asparaginase II) were induced by adding 1 mM IPTG, at different temperatures (25ºC, 30ºC and 37ºC). Samples were collected at different time intervals of post-induction and expression profile of recombinant protein was analysed. All the experiments were conducted in duplicates unless otherwise mentioned. The SDS–PAGE of recombinant proteins was performed in 12.5% polyacrylamide gel under reducing conditions [14]. Proteins were reduced by treatment with 10% of 2-mercaptoethanol at 95°C for 10 min and stained with Coomassie brilliant blue R-250 [19].

2.5 Assay of L-asparaginase and L-glutaminase

Some of the common assay methods used for measuring L-asparaginase activity include the Nesslerization reaction and Indooxine method [20], a coupled enzyme assay with excess glutamic-oxaloacetate transaminase and malic dehydrogenase [21], and fluorometric assay using L-aspartic acid β-(7-amido-4-methlcoumarin) as a substrate [22]. However, the assay mentioned latter show some drawbacks, such as multistep operation, requirement for the highlytoxic reagent and inapplicability for real time. Therefore, in the present study, Nesslerization method was used because it is economical and faster method. For asparaginase assay, samples were centrifuged at 10,000 *g* for 10 min at 4±1ºC and washed twice with 0.05 M Tris-HCl buffer (pH 8.5) and ultrasonicated (VC 505 Microprocessor based cell Ultrasonic processor, Sonics & Materials Inc, CT, USA) at 20 MHz, 35% amplitude, 4 cycles (2 min per cycles with 3 s on and 1 s off). The contents were centrifuged at 20,000 *g* for 15 min (4±1ºC) and the supernatant was analyzed for intracellular L-asparaginase activity as described by Kumar et al. [23]. L-asparaginase catalyzes the conversion of L-asparagine to L-aspartic acid and ammonia. Ammonia would react with the Nessler's reagent to produce an orange product. The enzyme assay mixture consisted of 900 µl of L-asparagine (10 mM) in Tris HCl buffer (pH 8.5) and 100 µl of crude extract of the enzyme. The reaction mixture was incubated at 37ºC for 30 min and 100 µl of 15% trichloroacetic acid (TCA) was added to stop the reaction. The reaction mixture was centrifuged at 10,000 *g* for 5 min at room temperature to remove the precipitates. Ammonia released in the supernatant was determined colorimetrically by adding 100 µl Nessler reagent into sample containing 100 µl of supernatant of above reaction mixture and 800 µl distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min and OD at 425 nm was measured against the blank that received TCA before the addition of enzyme. The ammonia produced in the reaction was determined based on a standard curve obtained with ammonium sulfate as standard. Standard curve was prepared by making different concentration of $(NH_4)_2SO_4$ solution in 50 mM Tris-HCl buffer of pH 8.5.

2.6 Calculation of L-asparaginase Activity

L-asparaginase activity in the test sample was calculated by the following equation.

$$\text{L-asparaginase activity (U ml}^{-1}) = \frac{A_C \times V_T}{V_R \times T \times V_C} \quad \text{(Eq. 1)}$$

Where, A_c = amount of ammonia released during reaction in µmol (test sample absorbance at 425 nm (Abs_{425})× 3.851 µmol), V_T = total volume of reaction (1.1 ml), V_R= volume of reaction mixture used in step 2 (0.1 ml of reaction mixture), T = time of assay (30 min), V_C = volume of crude enzyme (0.1 ml).

After substituting the all values in Eq. 1, it simplify into Eq. 2.

L-asparaginase activity (U ml^{-1}) = Abs_{425} × 14.12 (Eq. 2)

Where, Abs_{425} = test sample absorbance at 425 nm against appropriate blank.

L-glutaminase activity was determined as described above for L-asparaginase activity using modified method of Mashburn and Wriston [23,24]. L-asparaginase activity (IU ml^{-1}) was defined as the micromoles of ammonia released in one minute by one ml of enzyme and specific activity is expressed as units per milligram of protein.

2.7 Protein Estimation

The total protein contents of the samples were determined according to the method described by Lowry et al. [25] using bovine serum albumin (Sigma) as standard.

2.8 Purification of Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL21 (DE3)

After 6 h of post induction, cells were separated from culture broth by centrifugation and washed twice with Tris-HCl buffer (50 mM, pH 8.5) and the pellet was suspended in 50 mM sodium phosphate buffer (pH 7.0, 10 mM imidazole, 500 mM NaCl,), and suspended cells in above buffer was ultrasonicated on ice. The contents were centrifuged at 20,000 *g* for 15 min (4±1°C) after ultrasonication. After separation of pellet, the supernatant was loaded onto a Ni affinity column, which was pre-equilibrated with 50 mM sodium phosphate buffer (pH 8.0, 500 mM NaCl and 10 mM imidazole). The column was washed with 10 times volume of buffer containing 20 mM imidazole after 30 min. Elution of the protein was carried out with 200 mM imidazole and dialyzed in Tris–HCl buffer (50 mM) for 24 h. The enzyme activity and protein concentration of the purified enzyme was calculated according to the method described in the section 2.5 and 2.6, respectively [26].

2.9 Characterization of Recombinant L-asparaginase II

2.9.1 Effect of pH, incubation temperature and time on activity of purified enzyme

The effect of pH on the activity of L-asparaginase II was evaluated under assay conditions at different levels of pH. For pH stability experiments, enzyme preparations were incubated at a pH range of 5.5-10.5 for 24 h at 4±1°C and residual activity was determined as mentioned in section 2.5. To evaluate the effect of temperature on the activity of L-asparaginase II, assay was carried out at a temperatures ranging from 27 to 57°C. To find out the optimum incubation time, enzyme was incubated with substrate for 15- 90 minutes under the standard conditions and then enzyme activity was measured [17,27].

2.9.2 Effect of ionic strength on L-asparaginase activity

To determine the effect of ionic strength on the activity of L-asparaginase II, the activity of the enzyme was evaluated at different levels of ionic strength of Tris-HCl and NaCl (5 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM) [17,27].

2.9.3 Effect of various effectors on L-asparaginase activity

After 30 min exposure to the different effectors at their concentrations as shown in Table 4, the enzyme activity was evaluated. The most favorable concentration of different effectors mentioned by various researchers for characterization of L-asparaginase has been selected in this study [12,23,28]. The relative activity was denoted as the percentage ratio of the activity of the enzyme with the effectors to that of the untreated enzyme.

2.9.4 Substrate specificity

Activity was determined with L-asparagine, D-asparagine, DL-asparagine, L-glutamine, L-aspartic acid, D-aspartic acid, DL-aspartic acid, L-aspartic acid amide, L-glutamic acid, succinamic acid, L-asparagine-t-butyl ester HCl, BOC-L-asparagine and N-a-acetyl-L-asparagine as substrate at a final concentration of 10 mM. Method used for L-asparaginase activity was used for determination of enzyme activity with other substrates. Activity of enzyme for other substrate was determined by same method. The

relative activity was expressed as the percentage ratio of the enzyme activity determined against different structure analogs of L-asparagine to enzyme activity with L-asparagine [12,23].

2.9.5 Determination of kinetic parameters

The Michaelis constant (K_m), turnover numbers (k_{cat}) and maximal velocity (V_{max}) of the purified recombinant L-asparaginase II was evaluated using L-asparagine as substrate in the range of 0.05 to 2.5 mM. The kinetic parameters K_m,V_{max} and k_{cat} were calculated as reported earlier by Kumar et al. [23]. Turnover numbers were determined on the basis of one active site per 37.5 kDa subunit by SDS-PAGE.

2.9.6 Electrophoresis

Native PAGE of the purified L-asparaginase II was carried out using 7.5% polyacrylamide gel in glycine buffer (pH 10.0) at 5±1ºC [29]. SDS-PAGE was done by following the modified method of Laemmli (1970)with a 12.5 and 5 % acrylamide gel (pH 8.8) and stacking gel (pH 6.8) respectively, containing 0.1% SDS. The gel was stained with Coomassie brilliant blue R-250 and subunit molecular weight and intact molecular weight of L-asparaginase were identified using standard markers in the SDS–PAGE and native PAGE, correspondingly.

2.10 Experimental Design and Optimization

To find out the best medium for the maximum production of recombinant L-asparaginase II, six media [Luria Burtani (LB) medium, 2x Yeast extract and Tryptone (2x YT) medium, Terrific broth (TB) medium, Super broth (SB) medium, M9 Minimal medium, and Reseinberg medium)] were screened. Highest expression of recombinant L-asparaginase II (with His-tag) of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* was observed in the LB medium as compared to other media (data not shown). Therefore, LB medium was selected for further optimization study. The central composite design [30] was applied to optimize the levels and analysis of the combined effect of the medium constituents (tryptone, yeast extract and NaCl) and physical process parameters (pH, agitation, and inoculum size (%) on the production of recombinant L-asparaginase II. Each variable (medium component and physical variables) was assessed at five coded levels (−1.682, −1, 0, +1, and +1.682). The optimization of chemical and physical parameters was performed based on 2^3 full factorial central composite designs (CCD) with 6 axial points, resulting in a total of twenty experiments [31]. The full experimental plan with regard to their values in actual and coded form is provided in Tables 1 and 2, for optimization of medium components and physical parameters, respectively. All experiments were performed in duplicates and the specific activity of recombinant L-asparaginase II was considered as response. For statistical calculations, the relation between the coded values and real values were described in the following Eq. 3:

$$\chi_i = \frac{X_i - X_0}{\Delta X_i}, i = 1,2,3,.....k \quad (3)$$

Where, χ_i is the independent variable coded value, X_i is the real value of the independent variable, X_o is the real value of the independent variable on the center point, $\Delta X_i$is the step change and the central point was set with a α of 1.682 for optimization of medium components and physical parameters. The quadratic model for predicting the optimal levels was expressed according to the Eq. 4:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (4)$$

Where, Y is the predicted response (Sp. activity), k is the number of factor variables. X_i and $X_j$are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the i^{th} quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. Statistical analysis of the data was performed by statistical software package MINITAB® Release 15.1, PA, USA. *P*-value less than 0.05 indicated that the model terms are significant and adequacy of the method developed was further analyzed. The significance of the model equation and model terms was estimated by *F* test. The quality of the polynomial model equation was expressed by determination of R^2, adjusted R^2 and adequate precision. Analysis of variance (ANOVA) was carried out to evaluate the statistical significance of the model. By solving the regression equation, the most favorable combination of parameters was obtained.

In order to verify validity of the model, experiments were carried out at optimal levels of variables in a 250 ml Erlenmeyer flask with 50 ml of medium. All experiments were conducted in duplicates and averages of the results were

taken as response. The samples were drawn at regular interval of time and specific activity was measured in duplicates.

3. RESULTS AND DISCUSSION

3.1 Cloning and Expression of Genes Encoding L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*) of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043

BLAST result of the cloned gene sequences of *ans, ansA* and *ansB2* showed 100% similarity with *ans* (NCBI accession no. BX950851; Region: 152758-153705), *ansA* (NCBI accession no. BX950851; Region:2651794-2652813) and *ansB2* (NCBI accession no. BX950851; Region: 4603858-4604907) gene sequence of *E. carotovora* subsp. *atroseptica* SCRI 1043 available in NCBI data base. The optimum temperature for recombinant L-asparaginase (with and without 6xHis-tag) and L-asparaginase I (with and without His-tag) of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* was found to be 25°C, whereas the optimum temperature for expression of recombinant L-asparaginase II (with and without 6xHis-tag) of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* was found to be 30°C (Fig. 1). All the recombinant proteins (L-asparaginase, L-asparaginase I and L-asparaginase II) with and without 6xHis-tag, were expressed as intracellular proteins in soluble form (Fig. 2).

There are several reports on positive as well as negative effect of His-tag on recombinant protein expression and activity [32-34]. In the present investigation, higher expression was observed in presence of His-tag for L-asparaginase I and II. L-asparaginase (*ans*), L-asparaginase I and L-asparaginase II with His-tag showed activity of 3.79 U mg^{-1}, 72.23 U mg^{-1} and 34.49 U mg^{-1}, respectively, whereas L-asparaginase (*ans*), L-asparaginase I and L-asparaginase II without His-tag showed the activity of 6.46 U mg^{-1} 68.76 U $mg^{-1}$and 30.60 U mg^{-1}, respectively, which might be due to improved production of tagged protein [35-36]. The positive effect of the His-tag on the expression of recombinant protein might be due to the hydrophilic nature of the His-tag as generating a more hydrophilic protein, which is more compatible with the host cell [34]. Furthermore, the presence of affinity tag helps in improving the solubility and stability of the recombinant protein [32,33]. In contrast, Khushoo et al. [37] observed no effect on the expression due to C-terminal His-tag which shows that the effect of His-tag on expression varies with nature and structure of protein.

The presence of glutaminase activity would lead to the allergic reactions during therapy by L-asparaginase hence, glutaminase free L-asparaginase are preferred for therapeutic purpose [12]. The presence of L-glutaminase activity in all the recombinant proteins (L-asparaginase, L-asparaginase I and L-asparaginase II with/without His-tag) was analyzed and none of them shown L-glutaminase activity. Among all the three L-asparaginases studied, only L-asparaginase II is used as anticancer agent [14]. Therefore, all further studies were carried out with recombinant L-asparaginase II fused with C-terminal His-tag.

3.2 Purification of Recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL21 (DE3)

The purification of the recombinant L-asparaginase II was carried out according to the method described in the 'Materials and Methods'. After purification, specific activity of recombinant asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (DE3) increased from 67.26 to 282.45 U mg^{-1}. The recombinant L-asparaginase II was purified by 4.20 fold with 82 % of yield. SDS–PAGE and native PAGE analysis of the purified recombinant L-asparaginase II have shown single protein band of ~37.5 kDa and ~150.0 kDa, respectively (Fig. 3) which confirmed that recombinant L-asparaginase II is a tetramer.

3.3 Characterization of Purified Recombinant L-asparaginase II

3.3.1 Effect of pH, temperature and incubation time on enzyme activity

Recombinant L-asparaginase II showed a wide range of activity between pH 7.5- 9.5 with optimum pH of 8.5. In acidic pH, L-aspartic acid has a greater affinity towards active site of the enzyme and becomes a competitive inhibitor [38]. In alkaline pH, the equilibrium was shifted towards the aspartate, which has lower affinity to the active site [39]. At pH above 9.5 and below 6.5, the enzyme lost 45 and 65% activity, respectively (Fig. 4A). Though maximum activity at a physiological pH is one of the prerequisites for antitumor activity of L-asparaginase, due its broad

pH activity profile, ~85% of the enzyme activity was retained at pH 7.5. The enzyme displayed considerable stability at alkaline pH range (pH 7.5–9.5) with retention of ~80% of its original activity after incubation for 24 h.Most of the L-asparaginases from *Erwinia* species are reported to have optimal pH in the alkaline range (8.0-9.0). However, L-asparaginase from *E. coli* has shown optimum pH in the acidic range from 5.0-6.0 [10]. The purified enzyme showed maximum activity at 47-52ºC and pH of 8.6. The activity of enzyme was lost by 75% at 57ºC (Fig. 4B). Maladkar et al. [40] have also observed that the optimum temperature for *Erwinia* L-asparaginase at 50ºC. At lower temperature, the activity of the enzyme was found to be less due to slow reaction rate. Incubation time shows inverse effect on activity of enzyme. Maximum enzyme activity was observed after 30 minutes of incubation (Fig. 4C). It may be due to product inhibition. Products of enzymatic reactions are reversible inhibitors of the enzymes. A decrease in the rate of an enzyme caused by the accumulation of its own product plays an important role in the balance and most economic usage of metabolic pathways. It prevents one enzyme in a sequence of reactions from generating a new product more than the capacity of the next enzyme in that sequence, e.g., inhibition of hexokinase by accumulating glucose 6-phosphate [41]. Goswami et al. [17,27] and El-Sayed et al. [42] have also observed the decrease in L-asparaginase activity after incubating for 90 minutes.

3.3.2. Effect of ionic strength of buffer

As the activity of an enzyme would vary with ionic strength of buffer in the assay mixture, the assay of recombinant L-asparaginase II was performed at different ionic strengths of buffers. Ionic strength of buffer might affect activity of enzyme as inorganic ions of buffer may bind to some of the ionic side chains of a protein. Although this kind of interaction was not affecting the three dimensional configuration of the enzyme in a considerable manner, it could increase substrate binding to the active site of the enzyme [43]. Minimum and maximum L-asparagine hydrolysis was observed at concentration of 5 and 50 mM, respectively (Fig. 4D). Drop in the enzyme activity at lower and higher ionic strength of buffer was due to the inability of enzyme to form non-covalent interaction with the substrate [43]. We could not compare the effect of NaCl result as assay of L-asparaginase was performed by various researchers at a constant ionic strength of 50 mM Tris-HCl [4,23,26,38]. However, Goswami et al. [17,27] have observed similar type of effect of Tris-HCl on enzyme activity.

3.3.3 Effect of various effectors and substrate specificity

Activity analysis of L-asparaginase II was performed with various effector molecules as given in the Table 3. Considerable loss of activity was observed with Hg^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} Cu^{2+}, Fe^{3+}, Mg^{2+} and Zn^{2+}, whereas Na^{+} and K^{+} enhanced the activity of L-asparaginase II. Unaffected activity profile in presence of metal chelator (EDTA), indicated that it was not a metalloprotein. Inhibition of enzyme activity in presence of Hg^{2+}, Cd^{2+}, and Zn^{2+}indicated that presence sulfhydryl group(s) is essential for catalysis. The role of sulfhydryl groups in the catalytic activity of the enzyme was also evaluated by the stimulation of activity by the reducing source *viz.*, 2-mercaptoethanol and glutathione, and inhibition by thiol group blocking agent, iodoactamide. L-cysteine and L-histidine were observed to be stimulators for L-asparaginase II activity. The L-asparaginase II has lost 38% of its activity with 2.5 M urea and 17% of activity was remained at 2.5 M sodium dodecyl sulfate (SDS) indicating the fact that L-asparaginase has the thiol group binding domain with high affinity towards free-SH group containing effectors and these effectors change the asparaginase from one conformation to other to favor catalytic activity. The L-asparaginase activation stimulated by GSH and Cys supports the hypothesis that all thiol group having compounds and amino acids may interact with the same activator site on L-asparaginase. Thiol reactivity has been mentioned with the purified L-asparaginase from *P. carotovorum* MTCC 1428 and *E. carotovora* [23,28]. The substrate specificity of the recombinant L-asparaginase II is given in the Table 3. No hydrolysis was observed when L-glutamine, D-aspartic acid, DL-aspartic acid, L-aspartic acid, and L-glutamic acid were used individually as substrates. After purification of recombinant L-asparaginase II, no glutaminase activity was observed. Therefore, it is predicted that recombinant L-asparaginase II will have no secondary glutaminase activity. The lack of glutaminase activity would minimize the risk factor for successful clinical studies [8]. This typical quality makes the recombinant L-asparaginase II highly suitable for remedial applications.

3.3.4 Determination of kinetic parameters

The K_m, V_{max}, turnover number (k_{cat}) and specificity constants (k_{cat}/K_m) of purified recombinant L-asparaginase II were found to be 0.656 mM, 312.50 IU mg^{-1}, 1.38×10^2 s^{-1} and 2.11×10^5 $M^{-1}s^{-1}$, respectively (Fig 5, Table 4). Higher K_m values of 2.5 mM and 3.5 mM have been reported for L-asparaginase from *C. glueamicum* and cytoplasmic L-asparaginase (Type1) of *E. coli,* respective [38]. The K_m value of L-asparaginase from *Pseudornonasstutzeri*MB-405A was reported to be 0.145 mM [12]. The lower $K_m$value of 0.074 mM and 0.09 mM were reported for L-asparaginase from *V. succinogenes* and *E. carotovora*, respectively [28,44].K_m, V_{max}, turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) of purified L-asparaginase of *P. carotovorum* MTCC 1428 were reported to be 0.657 mM, 4.45 $U\mu g^{-1}$, $12.751 \times 10^3 s^{-1}$ and 4.187×10^6 $M^{-1}s^{-1}$, respectively [23]. The kinetic parameters determined in this study were comparable with those reported for many bacterial recombinant L-asparaginases [4,17,44,45].

3.4 Optimization of Medium Components Using Central Composite Design (CCD)

Among the media tested, the maximum production of recombinant L-asparaginase II from *E. coli* BL21 (DE3) was achieved in the LB medium (data not shown). Hence, experiments were performed to optimize the levels of medium components (tryptone, yeast extract and NaCl) using central composite design (CCD). The observed and predicted responses (production 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 (3) was found to explain the production of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 from *E. coli* BL 21 (DE3).

$$Y_{\text{specific activity}} = 24.9880 + 3.7113\,X_1 + 0.7158\,X_2 + 0.5537\,X_3 - 0.1296\,X_1^2 - 0.0833\,X_2^2 - 0.0527\,X_3^2 + 0.0047\,X_1X_2 - 0.0178\,X_1X_3 + 0.0237\,X_2X_3 \quad (5)$$

Where, X_1 is tryptone, X_2 is yeast extract and X_3 is NaCl.

The results inferred that tryptone (X_1) has shown maximum effect on recombinant L-asparaginase II production as it had largest coefficient (3.7113) followed by yeast extract (X_2) (0.7158) and NaCl (X_3) (0.5537). Positive coefficients of X_1, X_2 and X_3 indicated a linear effect on increase in recombinant L-asparaginase II production. Negative coefficients were observed for quadratic terms of all the three variables. Among the interactions, X_1X_2 and X_2X_3 had positive coefficients, while, X_1X_3 had negative coefficient. 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. According to the ANOVA of the quadratic regression model, it was highly significant, as evident from the Fisher, *F*-test (The *F* value is the ratio of the mean square due to regression to the mean square due to error). The *F* values for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 589.49 (the confidence interval is 0.05), indicating that the model was adequate (Table 5). For recombinant L-asparaginase II production, *P*-value for 'lack of fit' was greater than 0.05 (*P*= 0.739). In other words, the model was fit with the responses data collected and R^2 value was found to be 99.81%. This implies that more than 99.8% for recombinant L-asparaginase II production is attributed to the chemical parameters and 0.2% of the total variation is not explained by the model. The maximum predictable response for the production of recombinant L-asparaginase II was calculated by applying the regression analysis to the Eq. 5 using 'response optimizer' in Minitab software. The optimal levels of tryptone, yeast extract and NaCl for recombinant L-asparaginase II production were found to be 14.50 g l^{-1}, 5.30 g l^{-1} and 4.03 g l^{-1}, respectively.

Though, yeast extract is a complex nitrogen source, there was no remarkable difference in production of recombinant L-asparaginase due to difference in concentration of yeast extract in the medium (Table 1). There was a steep increase in the production of recombinant L-asparaginase II with an increase in tryptone concentration. It is well known that nitrogen source is mainly utilized to synthesize protein, nucleic acid and metabolites of nitrogen and supports enzyme production [46]. Relatively lower concentration of nutrient is the limited factor for cell growth and protein synthesis [47]. Since, osmoregulation is a significant biological process by which it can avert the cells from dehydration, studies on the osmotic regulation is significant for cell growth [48] and production of proteins [49].

Table 1. A 2^3 full-factorial central composite circumscribed design matrix in uncoded units and coded values (in parenthesis) with experimental and predicted values of recombinant L-asparaginase II production for chemical parameters optimization

Run order	Tryptone (X_1) (g l^{-1})	Yeast Extract (X_2) (g l^{-1})	NaCl (X_3) (g l^{-1})	Sp. Activity (U mg^{-1})		Activity (U ml^{-1})	Dry cell weight (g l^{-1})
				Observed	Predicted		
1	5.00 (-1)	3.00 (-1)	5.00 (-1)	43.198±0.023	43.203	8.844±0.038	0.82±0.02
2	19.00 (+1)	3.00 (-1)	5.00 (-1)	51.129±0.103	51.468	10.051±0.033	1.18±0.05
3	5.00 (-1)	11.00(+1)	5.00 (-1)	40.671±0.009	40.736	8.282±0.012	0.92±0.02
4	19.00 (+1)	11.00 (+1)	5.00 (-1)	49.877±0.210	49.528	11.189±0.026	1.29±0.05
5	5.00 (-1)	3.00 (-1)	19.00(+1)	32.686±0.018	33.011	4.858±0.014	0.70±0.03
6	19.00 (+1)	3.00 (-1)	19.00(+1)	37.882±0.023	37.794	7.258±0.312	1.16±0.05
7	5.00 (-1)	11.00 (+1)	19.00(+1)	33.556±.0.053	33.193	4.098±0.078	0.82±0.01
8	19.00 (+1)	11.00 (+1)	19.00(+1)	38.531±0.320	38.503	9.783±0.052	1.13±0.02
9	0.23 (-2)	7.00 (0)	12.00 (0)	27.793±0.051	27.763	4.002±0.192	0.57±0.02
10	23.78 (+2)	7.00 (0)	12.00 (0)	39.115±0.056	39.179	9.400±0.231	1.24±0.08
11	12.00 (0)	0.27 (-2)	12.00 (0)	48.388±0.036	48.031	9.406±0.065	0.97±0.07
12	12.00 (0)	13.73 (+2)	12.00 (0)	46.163±0.019	46.553	7.743±0.123	1.33±0.07
13	12.00 (0)	7.00 (0)	0.23 (-2)	52.731±0.011	52.684	9.758±0.232	1.02±0.05
14	12.00 (0)	7.00 (0)	23.77(+2)	34.762±0.002	34.843	8.070±0.212	0.92±0.04
15	12.00 (0)	7.00 (0)	12.00 (0)	50.752±0.235	51.061	11.25±0.0309	1.18±0.09
16	12.00 (0)	7.00 (0)	12.00 (0)	51.639±0.163	51.061	11.101±0.052	1.15±0.06
17	12.00 (0)	7.00 (0)	12.00 (0)	50.516±0.142	51.061	11.253±0.068	1.17±0.04
18	12.00 (0)	7.00 (0)	12.00 (0)	50.839±0.020	51.061	12.044±0.045	1.19±0.08
19	12.00 (0)	7.00 (0)	12.00 (0)	51.623±0.015	51.061	11.492±0.087	1.20±0.05
20	12.00 (0)	7.00 (0)	12.00 (0)	50.805±0231	51.061	12.520±0.099	1.16±0.07

Table 2. A 2^3 full-factorial central composite circumscribed design matrix in uncoded units and coded values (in parenthesis) with experimental and predicted values of recombinant L-asparaginase II production for physical parameters optimization

Run Order	pH (X_1)	Inoculum(%)(X_2)	Agitation (RPM) (X_3)	Sp. activity (U mg^{-1})		Activity (U ml^{-1})	Dry cell weight (g l^{-1})
				Observed	Predicted		
1	6.50 (-1)	2.00 (-1)	160.00 (-1)	43.030±0.230	42.735	10.840± 0.186	1.03±0.01
2	7.50 (+1)	2.00 (-1)	160.00 (-1)	52.930±0.120	52.639	10.220± 0.162	0.92±0.15
3	6.50 (-1)	4.00 (+1)	160.00 (-1)	58.490±0.009	58.358	11.370± 0.236	1.10±0.29
4	7.50 (+1)	4.00 (+1)	160.00 (-1)	58.806±0.015	58.644	9.619± 0.012	1.03±0.18
5	6.50 (-1)	2.00 (-1)	240.00 (+1)	40.701±0.320	40.383	9.523± 0.288	1.06±0.39
6	7.50 (+1)	2.00 (-1)	240.00 (+1)	46.884±0.119	46.538	10.175± 0.071	1.01±0.12
7	6.50 (-1)	4.00 (+1)	240.00 (+1)	51.013±0.089	50.830	11.094± 0.043	1.20±0.14
8	7.50 (+1)	4.00 (+1)	240.00 (+1)	47.551±0.032	47.367	11.258± 0.092	1.14±0.01
9	6.16 (-2)	3.00 (0)	200.00 (0)	52.601±0.045	50.922	11.620± 0.076	1.12±0.13
10	7.84 (+2)	3.00 (0)	200.00 (0)	55.981±0.026	56.338	11.168± 0.117	0.99±0.02
11	7.00 (0)	1.32 (-2)	200.00 (0)	36.122±0.302	36.637	8.047± 0.215	1.06±0.09
12	7.00 (0)	4.68 (+2)	200.00 (0)	50.309±0.212	50.471	14.521± 0.022	1.33±0.09
13	7.00 (0)	3.00 (0)	132.73(-2)	59.540±0.142	59.835	14.021±0.271	1.20±0.05
14	7.00 (0)	3.00 (0)	267.27(+2)	47.992±0.120	48.374	13.050± 0.159	1.26±0.05
15	7.00 (0)	3.00 (0)	200.00 (0)	63.288±0.087	62.675	13.697± 0.088	1.30±0.03
16	7.00 (0)	3.00 (0)	200.00 (0)	62.542±0.240	62.675	14.304± 0.234	1.32±0.01
17	7.00 (0)	3.00 (0)	200.00 (0)	62.488±0.055	62.675	14.624± 0.334	1.36±0.02
18	7.00 (0)	3.00 (0)	200.00 (0)	62.686±0.263	62.675	14.364± 0.034	1.33±0.01
19	7.00 (0)	3.00 (0)	200.00 (0)	62.888±0.201	62.675	14.512± 0.120	1.31±0.06
20	7.00 (0)	3.00 (0)	200.00 (0)	62.275±0.012	62.675	14.590± 0.012	1.35±0.08

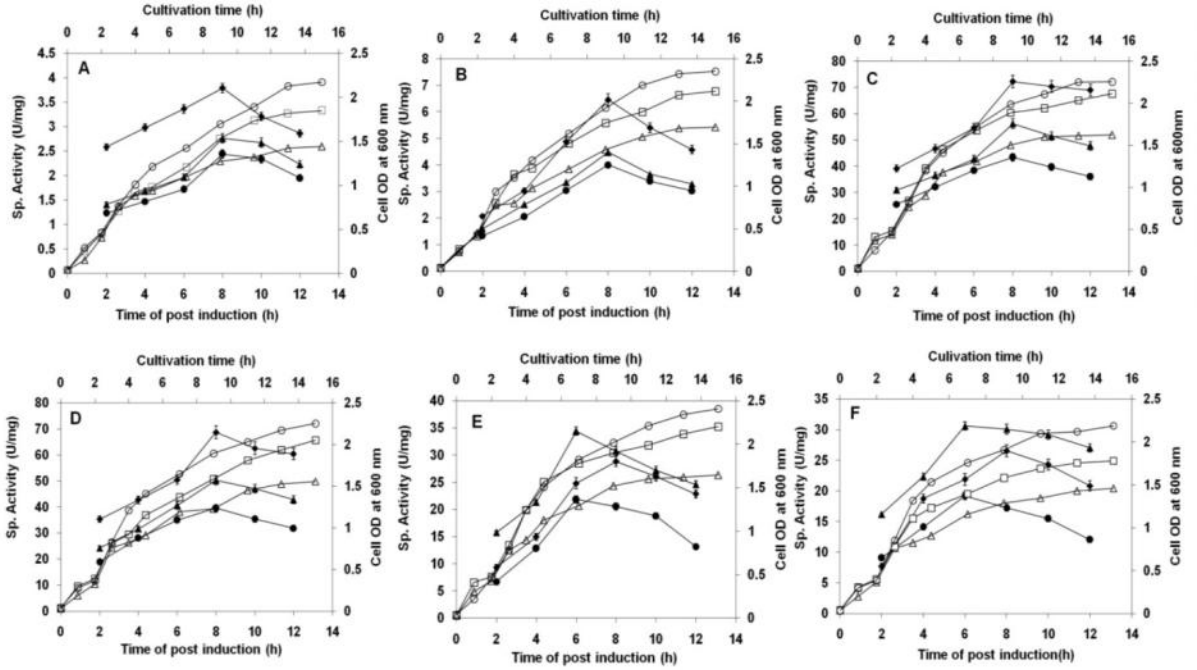

Fig. 1. Production of recombinant L-asparaginases of *E. carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* (A and B: recombinant L-asparaginase with and without his-tag, respectively, C and D: recombinant L-asparaginase I with and without his-tag, respectively and E and F: recombinant L-asparaginase II with and without his-tag, respectively) (—△— Cell growth at 25°C, , —□— Cell growth at 30°C, —○— Cell growth at 37°C, —◆— expression at 25°C, —▲— expression at 30°C, —●—expression at 37°C)

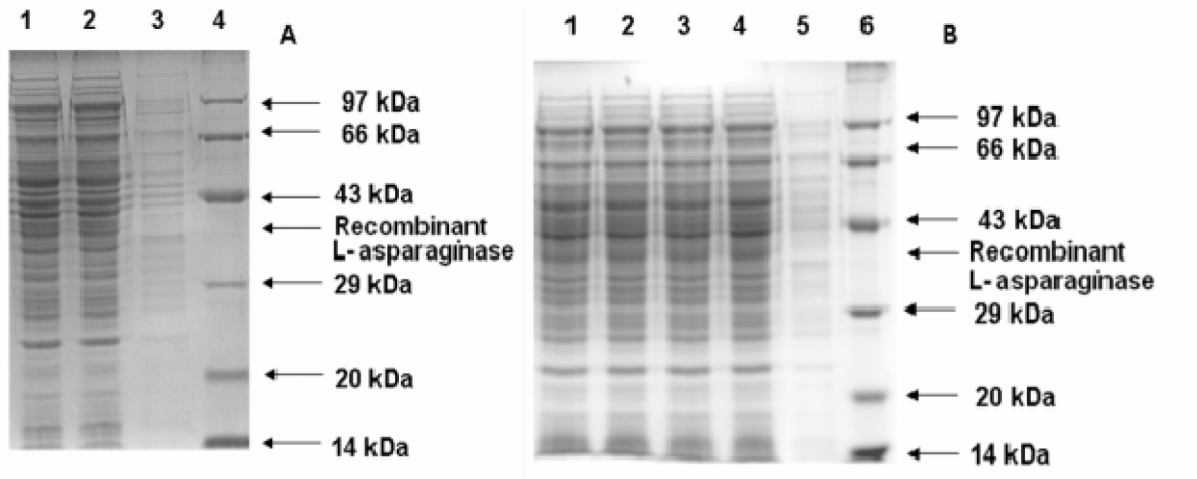

Fig. 2. SDS PAGE of recombinant L-asparaginases of *E. carotovora* subsp. *atroseptica* SCRI 1043 (A: Lane 1 and 2: recombinant L-asparaginase with and without His. Tag, respectively; Lane 3: pET 22b(+) (without any insert) in *E. coli* BL21 (DE3) and Lane 4: Marker); B: SDS PAGE of recombinant protein of *E. carotovora* subsp. *atroseptica* SCRI 1043 (Lane 1 and 2: recombinant L-asparaginase I with and without His-tag, respectively; Lane 3 and 4: recombinant L-asparaginase II with and without His-tag, respectively; Lane 5: pET 22b(+) (without any insert) in *E. coli* BL21 (DE3) and Lane 6: Marker)

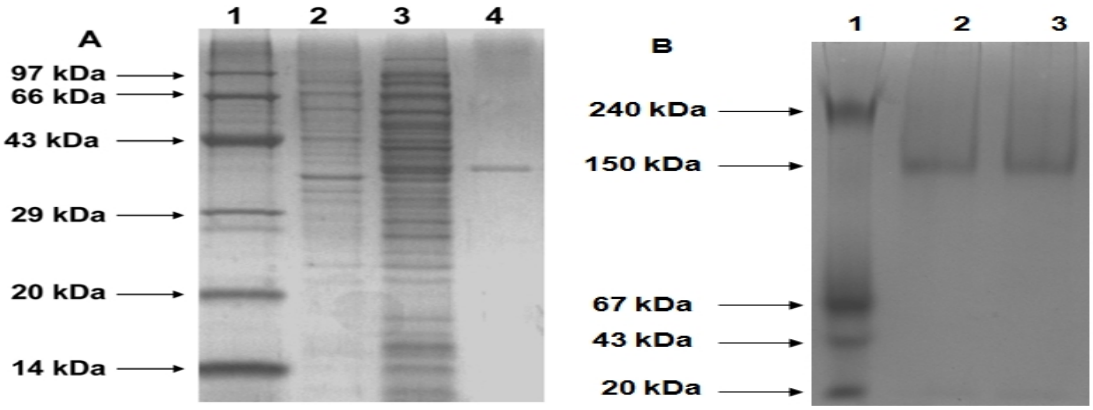

Fig. 3. Purification of recombinant L-asparaginase II of *E. carotovora*subsp. *atroseptica* SCRI 1043 (A: SDS Polyacrylamide gel electrophoresis (12.5%) of purified recombinant L-asparaginase II (Lane 1: Marker, Lane 2: pET22b (+) in *E. coli* BL21 (DE3) without any insert, Lane 3: Crude extract and Lane 4: Purified recombinant L-asparaginase II. B: Native PAGE (7.5%) of L-asparaginase (Lane 1: Marker and Lane 2 and 3: Purified recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043)

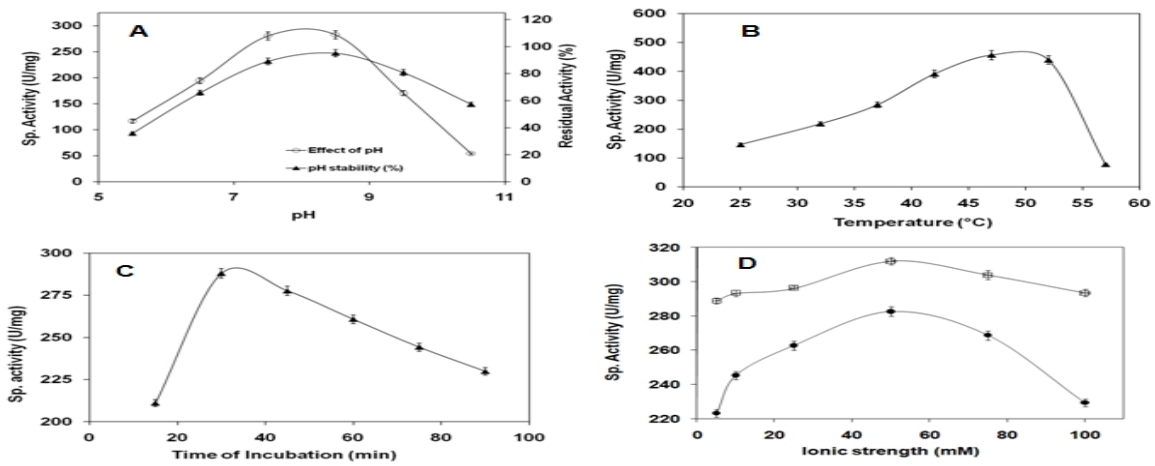

Fig. 4. Effect of pH (A), incubation temperature (B), incubation time (C) and ionic strength of Tris-HCl and NaCl (D) on activity of recombinant L-asparaginase II

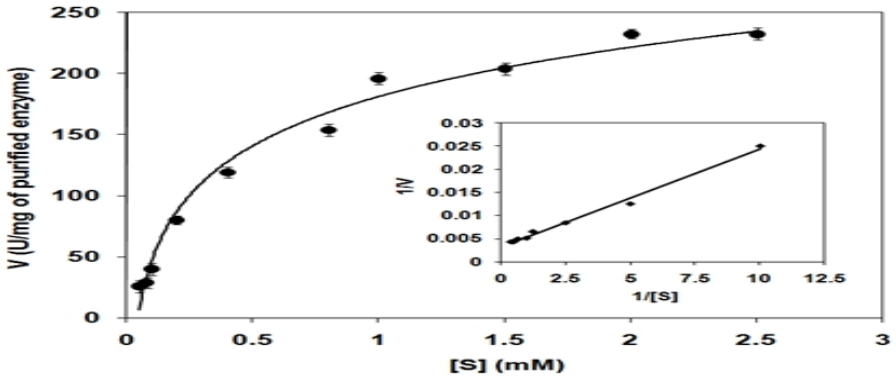

Fig. 5. Plot of the reaction velocities (V) vs. substrate concentration (S: 0.05-2.5 mM) fitted to the Michaelis–Menten equation and determination of K_m and V_{max} of purified recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043

Table 3. Influence of different effectors on enzyme activity and substrate specificity of purified recombinant L-asparaginase II

Addition		Conc. (mM)	Relative activity (%)
Effect of different effectors	No addition	-	100
	Na^+ (NaCl)	50.0	109±0.71
	K^+ (KCl)	150.0	114±0.35
	Mg^{2+} ($MgCl_2$)	40.0	65±0.71
	Ca^{2+} ($CaCl_2$)	150.0	32±0.42
	Mn^{2+} ($MnCl_2$)	100.0	3±0.28
	Zn^{2+} ($ZnCl_2$)	100.0	14±0.35
	Fe^{3+} ($FeCl_3$)	100.0	6±0.49
	Ni^{2+} ($NiCl_2$)	10.0	43±0.68
	Co ($CoCl_2$)	10.0	39±0.57
	Cu^{2+} ($CuCl_2$)	10.0	22±0.31
	Hg^{2+}($HgCl_2$)	10.0	43±0.50
	2-mercaptoethanol	0.5	132±0.17
	SDS	2.5	17±0.93
	Urea	2.5	62±0.30
	EDTA	5.0	112±0.11
	Iodoacetamide	5.0	72±0.42
	L-cysteine	25.0	107±0.78
	L-histidine	25.0	116±0.62
	Glutathione reduced	0.5	112±0.42
Substrate specificity	L-asparagine	10	100
	D-asparagine	10	68.±0.14
	DL-asparagine	10	80.±0.28
	L-glutamine	10	N. D.
	L-aspartic acid	10	N. D.
	D-asparatic acid	10	N. D.
	DL-asparatic acid	10	N. D.
	L-aspartic acid amide	10	75±0.42
	L-glutamic acid	10	N. D.
	Succinamic acid	10	84±0.93
	L-asparagine-t-butyl ester HCL	10	70±0.99
	BOC-L-asparagine	10	89±0.57
	N-alfa-acetyl L-asparagine	10	17±0.78

Table 4. Kinetic parameters of glutaminase free recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043

Kinetic parameters	Values
Michaelis constant (K_m)	0.656 mM
Maximum velocity (V_{max})	312.50 IU mg^{-1}
Turnover number (K_{cat})	1.38×10^2 s^{-1}
Specificity constant (K_{cat}/K_m)	2.11×10^5 $M^{-1}s^{-1}$

NaCl is a commonly used osmoticum. Therefore, NaCl was chosen as variable for medium optimization. The concentration of NaCl is lower in the optimized medium than un-optimized medium. Probably, higher NaCl concentration cause inhibitory effect for cell growth [48] and enzyme production [50].

To verify validity of the model, experiments were performed at optimal levels of medium components. The observed production (specific activity) of recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 57.06 U mg^{-1} is in good agreement with the value predicted by the model, 54.77 U mg^{-1}. After optimization of medium components, 1.65 fold higher production of recombinant L-asparaginase II was achieved as compared to un-optimized medium (34.49 U mg^{-1}).

Table 5. ANOVA for quadratic model for chemical parameter and physical process parameter optimization

Parameters	Source	DF	Seq SS	Adj SS	MS	*F*	*P*
For chemical parameters	Regression	9	1171.62	1171.617	130.180	589.49	0.0001
	Linear	3	544.18	427.949	142.650	645.96	0.0001
	Square	3	617.72	617.719	205.906	932.40	0.0001
	Interaction	3	9.71	93713	3.238	14.66	0.001
	Residual error	10	2.21	2.208	0.221	-	-
	Lack of fit	5	0.78	0.780	0.156	0.55	0.739
	Pure Error	5	1.43	1.428	0.286	-	-
	Total	19	1173.82				
For physical process parameters	Regression	9	1309.81	1309.815	145.535	767.99	0.0001
	Linear	3	424.98	422.906	140.969	743.89	0.0001
	square	3	818.16	818.155	272.718	1439.13	0.0001
	Interaction	3	66.98	66.675	22.225	117.28	0.0001
	Residual error	10	1.90	1.895	0.190	-	-
	Lack of fit	5	1.26	1.263	0.253	2.00	0.232
	Pure Error	5	0.63	0.632	0.126	-	-
	Total	19	1311.71				

For chemical parameters: R-Sq = 99.81% R-Sq(pred) = 99.28% R-Sq(adj) = 99.64%
For physical process parameters: R-Sq = 99.86% R-Sq(pred) = 99.20% R-Sq(adj) = 99.73%
DF degrees of freedom, SS sum of squares, MS mean square

3.5 Optimization of Physical Process Parameters Using CCD

In order to optimize the physical process parameters (initial pH of the medium, rpm of the shaking incubator and inoculum size) for enhanced production of recombinant L-asparaginase II, experiments were performed according to the CCD (Table 2). By applying the multiple regression analysis on the experimental data, the following second-order polynomial equation was found to explain the production of recombinant L-asparaginase II.

$$Y_{\text{specific activity}} = -917.896 + 207.171\,X_1 + 1.915\,X_2 + 33.074\,X_3 - 12.792\,X_1^2 - 0.003X_2^2 - 3.030X_3^2 - 0.096X_1X_2 - 1.875X_1X_3 - 0.026X_2X_3 \quad (6)$$

Where X_1 is pH, X_2 is inoculum size (%) and X_3 is agitation (rpm). According to the ANOVA of the quadratic regression model, the model is highly significant, as evident from the Fisher *F* test. *F* value was determined to be 767.99, corresponding to very low probability value ($P < 0.05$). R^2 values for recombinant L-asparaginase II of *E. carotovora* subsp. *atroseptica* SCRI 1043 was found to be 99.86%. Both, lower and higher agitation (rpm) was not found to be favorable for cell growth and enzyme production. Oxygen limitation has been found to influence the cell growth and expression of heterologous proteins in *E. coli* and oxygen supply is directly proportional to shaking speed [51,52]. Due to this, lower agitation was not found to be favorable. Probably, higher agitation is causing shear effect on bacterial cells therefore, 240 rpm was not found to be optimal for higher production of recombinant L-aspraginase II. The best combination of pH, agitation, inoculum size for the maximum production of recombinant L-asparaginase II was found to be 7.1, 216 rpm and 2.3%, respectively.

The model was validated by performing the experiments under the optimal levels of pH, agitation and inoculum size. The maximum production of recombinant L-asparaginase II was found to be 67.26U mg^{-1} (16.05 U ml^{-1}) with productivity of 2675.00 U l^{-1} h^{-1}, resulting an overall 1.95 fold increase in the production as compared to the un-optimized production conditions (34.49 U mg^{-1}). After optimization of medium component and process conditions, production of recombinant L-asparaginase II was 6.84 folds higher than L-asparaginase produced by *Erwinia carotovora* subsp. *atroceptica* SCRI (9.84 U mg^{-1}).

These parameters are optimized at shake flask level where pH was not controlled. Under controlled pH condition, expression of recombinant L-asparaginase was increased from 16.05 U ml^{-1} to 24.57 U ml^{-1} (unpublished data).

Pectobacterium carotovorum MTCC 1428[17] and *Vibrio succinogenes* [53] are found to be glutaminase free but scientists have used genetic

engineering [4,26,37,44,54,55] and enzyme engineering [55,56]to develop a novel L-asparaginase with reduced or negligible glutaminase activity. L-asparaginases from *E. coli, E. carotovora, Erwinia crysanthemi, Helicobacter pylori* and *S. cerevisiae* have been cloned and successfully expressed in bacterial and yeast expression systems [4,26,37,44, 54,55]. According to several literature reports the specific activity of recombinant L-asparaginases (production) varied in a wide-range from 0.72 to 95.7 U mg^{-1} of protein in crude extract [26,37,44,54]. Derst et al. [56] has engineered the substrate specificity of *E. coli* asparaginase II by replacing the asparaginse at 248 position (Asp248) with some other residue and after replacement observed the reduced glutaminse activity. Offman et al. [57] engineered L-asparaginase of *E. coli* for resisting proteolytic cleavage, for improving activity and for reducing glutaminase activity. L-asparaginase from Recombinant L-asparaginase II developed in present study is having high sp. activity 67.26U mg^{-1} after optimizing the medium and process condition and not shows any glutaminase activity. Therefore, it might be effective for treatment of leukemia patients and expected not to cause any toxic effects associated with glutaminse activity of L-asparaginase of *E. coli* [58]. Devi and Azmi [59] have purified glutaminase free L-asparaginase from *E. carotovora* MTCC 1428 and reported better performance than commercial L-asparaginase obtained from *E. coli*. In the present study purified recombinant L-asparaginase II reported is owing no glutaminase activity and sequence of asparaginase II gene has shown 100 % similarity with L-asparaginase II gene of *Erwinia carotovora* MTCC 1428. Therefore, it is expected that recombinant L-asparaginase II will have no secondary glutaminase activity and would be better choice for therapeutic purpose.

4. CONCLUSION

In the present study, the genes encoding L-asparaginase (L-asparaginase (*ans*), L-asparaginase I (*ansA*) and L-asparaginase II (*ansB2*)) of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 were cloned and expressed in *E. coli* BL21 (DE3). The production optimization including medium components and physical process parameters increased the recombinant L-asparaginase II yield by 1.95 fold as compared to unoptimized conditions. The biochemical characterization showed that the optimum range of pH and temperature for the hydrolysis of purified recombinant L-asparaginase II was 6.5-9.5 and 47-52ºC, respectively. The activity of recombinant L-asparaginase II was found to be activated by mono cations and inhibited by divalent cations and iodoactamide. The enzyme was found to be very specific for its natural substrate, L-asparagine and shown no glutaminase activity which makes it a better alternative in therapeutic applications like an anticancer drug.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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