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Production, characterization, and applications of a novel thermo-acidophilic L-asparaginase of *Pseudomonas aeruginosa* CSPS4

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Pseudomonas aeruginosa

Thermo-acidophilic enzyme

Acrylamide reduction

Food industry

ABSTRACT

In present investigation, a potential L-asparaginase-producing bacterial isolate, *Pseudomonas aeruginosa* CSPS4, has been explored to enhance the production and purification of the asparaginase enzyme. Production of L-asparaginase is enhanced using the 'one variable at a time approach (OVAT)'. In Placket Burman (PB) analysis, pH, sucrose, and temperature significantly influence L-asparaginase production. Thereafter, L-asparaginase enzyme was recovered from culture broth using fractional precipitation with chilled acetone. The partially purified L-asparaginase showed a molecular weight of ~35 KDa on SDS-PAGE. L-asparaginase was characterized as a thermo-acidophilic enzyme exhibiting optimum pH and temperature of 6.0 and 60 °C, respectively. These characteristics render this enzyme novel from other available asparaginases of *Pseudomonas* spp. L-asparaginase activity remained unaffected by different modulators. L-asparaginase of this investigation was successfully employed for acrylamide degradation in commercial fried potato chips, establishing its applicability in food industries.

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1 Introduction

L-asparaginase (EC 3.5.1.1) hydrolyzes the amide group of L-asparagine and liberates aspartic acid and ammonia (Qeshmi et al. 2022; Zhou et al. 2022). The enzyme finds several biotechnological applications related to human health in the food and pharmaceutical industries (Batool et al. 2016; Brumano et al. 2019). L-asparaginases grabbed special attention due to their major role as an antitumor agent (Shrivastava et al. 2016; Luo et al. 2018; Osama et al. 2023). Tumor cells cannot synthesize asparagine due to the lack of an asparagine synthase enzyme, which leads to inhibition of translation followed by complete cell cycle arrest (Povlova et al. 2018). By taking advantage of this fact, asparaginase was employed as an antitumor agent for diminishing the essential L-asparagine for the growth of leukemic cells (Mahajan et al. 2014; Ali et al. 2016; Osama et al. 2023). Several commercial L-asparaginases (Bionase®, Spectrila®, Erwinase®, and Oncaspar®) are available in the market that are chiefly sourced from either *Escherichia coli* or *Erwinia chrysanthemi* (Kataria et al. 2015; Vimal and Kumar 2022). Besides, using L-asparaginase has also proven acrylamide degradation (Xu et al. 2016). Therefore, this enzyme exhibits a significant role in the starch-based food industry, where it reduces the concentration of acrylamide, which is generated due to the Maillard reaction between sugars and L-asparagine at high temperatures (Stadler et al. 2002; Kukurova et al. 2009; Jia et al. 2021). Despite many available L-asparaginases from various sources, industries compromise with the enzyme characteristics. For example, weak thermostability at higher temperatures makes this enzyme unsuitable for food and baking industries (Li et al. 2019; Qeshmi et al. 2022; Muzuni et al. 2023). Similarly, glutaminase-free L-asparaginases are the pre-requisite of pharmaceutical industries to minimize the immunogenic response while treating acute lymphoblastic leukaemia (ALL). Therefore, further research is required to achieve the L-asparaginase with desired properties. In the present investigation, wild-type L-asparaginase of *Pseudomonas aeruginosa* CSPS4 is studied for the production, characterization, and application. The enzyme finds applicability and could be a candidate in the starch-based food industry due to the degradation of acrylamide.

2 Materials and Methods

2.1 Qualitative L-asparaginase assay for screening of L-asparaginase

Soil samples from a slaughter shop market in Lucknow, Uttar Pradesh, India, were collected in sterile polybags. Samples were stored at 4 ℃ in a refrigerator till their use. The serial dilution was performed to isolate bacteria from the samples (Yan et al. 2015). An appropriate dilution of 10^{-5} was spread on the C-Dox agar medium from 1.0 gm of soil suspension made in 10 ml of dH_2O. The composition of 1L modified C-DOX agar medium was [$Na_2HPO_4.2H_2O$ (6 gm), KH_2PO_4 (3 gm), NaCl (0.5 gm), L-asparagine (5 gm), 1M $MgSO_4.7H_2O$ (2 ml), 0.1M $CaCl_2.2H_2O$ (1 ml), 20% Glucose stock solution (10 ml), Agar (20 gm), 2.5% phenol red indicator (0.04-0.36 ml), and pH 7.0] as described by Mahajan et al. (2012) along with 0.5 (w/v) % of L-asparagine as substrate (Mahajan et al. 2012; Doriya and Kumar 2016). The colonies that were able to turn the orange-yellow colour of the media into pink-red color due to hydrolysis of L-asparagine into L-aspartate and ammonia (NH^{4+}) were picked as L-asparaginase-producers (Doriya and Kumar 2016).

2.2 Thermal screening of the L-asparaginase enzyme

Bacterial isolates were further screened at 50 ℃ to obtain a thermostable L-asparaginase. For this, 50 ml C-Dox medium was inoculated with 1% (v/v) overnight grown inoculum of bacterial isolates. The culture broth was collected at 24 and 48 hrs to assess the L-asparaginase activity at 50°C. A bacterium exhibiting the highest L-asparaginase activity was selected for further investigation.

2.3 L-asparaginase and L-glutaminase assay

L-asparaginase and L-glutaminase activity was measured by assaying the cell-free supernatant of overnight grown culture (Shirfrin et al.1974). The supernatant (L-asparaginase) was mixed with an equal volume of 1 % of respective substrates, either L-asparagine or L-glutamine solutions (dissolved in 0.1 M tris-HCl; pH 6.0) for hydrolysis of these substrates followed by measurement of ammonia using Nessler's reagent (Simas et al. 2021).

One international unit (IU) of L-asparaginase or L-glutaminase is defined as the amount of enzyme required for liberating 1 nanomole of ammonia at pH 6.0 and temperature 60 ℃ from these substrates. For the standard curve, varying concentrations (10-100 µM) of ammonium sulfate were prepared, where ammonia was assayed using Nessler's reagent.

2.4 Morphological, biochemical, and molecular characterization of isolated strain CSPS4

The isolated bacterial isolate was characterized for its morphology and biochemical properties according to Bergey's Manual of Determinative Bacteriology (Williams et al. 1989). Colour, shape, margins, texture, opacity, and odour were observed for morphological characterization. Besides, Gram staining was also performed to determine the characteristics of the bacteria (Dipali and Ajit 2012; Tripathi and Sapra 2023). For molecular-level identification of the bacterium, 3.0 ml of overnight-grown bacterial broth was sedimented at 10,000 rpm to collect the bacterial pellet. The pellet was homogenized in 1 ml of 1% extraction buffer (*N*-,*N*-

,*N*-,*N*-cetyltrimethylammonium bromide (CTAB), 2% polyvinylpolypyrrolidone (PVPP), 1.5 M NaCl, 100 mM EDTA, 0.1 M TE buffer (pH 8.0), 0.1 M sodium phosphate buffer (pH 8.0), and 100 µL RNaseA and treated with 5 µL of lysozyme (10 mg/ml) and proteinase-K (10 mg/ml) (Verma and Satyanarayana 2011). The suspension was kept at 37 ℃ for 1 hr, followed by adding 100 µL of 10% (w/v) SDS solution. The bacteria were further lysed at 60 °C for 1 hr. The cell debris was separated by centrifuging the suspension at 10,000 rpm for 5 min. The supernatant was collected and treated with an equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) solution to remove the proteins (Simpson and Beynon 2010; Verma and Satyanarayana 2011). The aqueous phase was collected by centrifugation at 10,000 rpm and treated with 0.7 V of isopropanol to precipitate the DNA. The precipitated DNA pellet was obtained after high-speed centrifugation of 10,000 rpm for 10 min. The pellet was washed with 70% (v/v) ethanol and dried at room temperature for 2 hrs. The pellet was suspended in 50 µL sterile TE buffer (0.1 M; pH 8.0). Thus, obtained genomic DNA was used for the amplification of bacterial-specific 16S rRNA gene using bacterial-specific primers (Forward: 5'*AGAGTTTGATCMTGGCTCAG* 3'; Reverse: 5'*AAGGAGGTGATCCANCCRCA*3') to identify the bacterial species. A PCR reaction was set up at initial denaturation of 95 °C followed by 29 cycles of denaturation (95 °C for 1 min), annealing (56 °C for 1 min), and extension (72 °C for 1 min) was carried out in a thermal cycler (Bio-Rad, China) with a final extension of 10 min at 72 °C. The amplified DNA was sequenced, followed by BLASTn analysis to determine the % identity for bacterial identification.

2.5 Production of the L-asparaginase from *P. aeruginosa* CSPS4

Asparaginase production from *P. aeruginosa* CSPS4 was optimized using the C-Dox medium as a base medium by the 'one variable at a time' (OVAT) approach. Various physical (pH, temperature, inoculum age, inoculum size, agitation rate, and flask volume) and nutritional (carbon and nitrogen) parameters, along with detergent levels, were optimized to enhance the asparaginase production by the bacterial isolate.

2.6 Statistical optimization for L-asparaginase production using Plackett-Burman Design

The selected parameters obtained by OVAT analysis were further used to generate the Plackett-Burman (PB) factorial design for identifying the significant factors/culture variables for the L-asparaginase enzyme production. Nine variables (pH, temperature, incubation time, inoculum age, inoculum size, agitation rate, flask volume, sucrose, and peptone) were selected for PB design by considering higher (+) and lower (-) levels (Table 1). Statistical tool Design Expert Software 6.1.10 (https://www.statease.com/software/design-expert/) was used to design the PB model for analyzing the predicted enzyme production under the given conditions of the model. The experiment was performed in triplicates to calculate the standard deviations in each set of experiments. The experimental design was the estimated mean of L-asparaginase production that followed the first-order model with the following equation (El-Naggar et al. 2019).

Table 1 Statistical optimization for L-asparaginase production from *P. aeruginosa* CSPS4 using Plackett-Burman design

Run	Block 1	Incubation time (hrs.)	pH	Temp (°C)	Inoculum size (%)	Inoculum age (hrs)	RPM	Flask volume (ml)	Sucrose (%)	Peptone (%)	Enzyme Activity (nM/min)
1.	Block 1	72	8	25	6	12	100	10	4	4	249.77±2.03
2.	Block 1	24	8	45	1	24	100	10	0.5	4	70.22±3.35
3.	Block 1	72	8	45	1	24	250	10	4	0.5	498.66 ±3.35
4.	Block 1	24	4	25	6	24	250	10	4	4	19.55±2.77
5.	Block 1	24	4	45	6	24	100	200	4	0.5	56.88±0.76
6.	Block 1	24	8	25	1	12	250	200	4	0.5	453.33±4.07
7.	Block 1	72	4	25	1	24	250	200	0.5	4	101.77±2.03
8.	Block 1	24	4	25	1	12	100	10	0.5	0.5	131.55±3.35
9.	Block 1	24	8	45	6	12	250	200	0.5	4	204.88±4.33
10.	Block 1	72	8	25	6	24	100	200	0.5	0.5	521.33±4.07
11.	Block 1	72	4	45	1	12	100	200	4	4	159.11±3.33
12.	Block 1	72	4	45	6	12	250	10	0.5	0.5	161.77±2.03

Here ± indicates standard deviation (SD); Experiments were carried out in triplicates to calculate SD

$$Y = \beta_0 + \Sigma\beta_i x_i \quad \text{Eq. 1}$$

Where Y depicts L-asparaginase activity response, βi is a linear variable coefficient, β_0 is the model intercept, xi is the level independent variable, and i is the variable number. Design Expert 6.1.10 software was used to scan the variables. Nessler's reagent was used to measure the L-asparaginase activity (Table 1).

2.7 Purification of L-asparaginase

Extracellular L-asparaginase was purified by the acetone precipitation method (Simpson and Beynon 2010). Briefly, 1 L C-Dox medium was inoculated with 1% (v/v) overnight grown culture of *P. aeruginosa* CSPS4. L-asparaginase production was achieved by incubating the bacteria at 150 rpm at 37 °C for 48 hrs. The culture was centrifugated at 10000 rpm, and the culture supernatant was harvested to recover extracellular L-asparaginase. The total protein was precipitated from one liter of supernatant by gradually adding 6V of prechilled acetone (-20 °C) into one liter of cell-free supernatant on a continuous stirrer at 4 °C and the precipitated protein was collected using centrifugation followed by complete evaporation of acetone. The crude protein was suspended in 5.0 ml of Tris-Cl buffer (0.1M; pH 6.0). This suspension was further used for collecting various fractions of precipitated proteins to achieve the purified L-asparaginase. Acetone was added to 5.0 ml of suspended protein under varying concentrations. The first protein fraction was precipitated by adding 10% (v/v) prechilled acetone into 5.0 ml of suspended protein through the tube wall and incubated for 1 hr at low temperature. Thereafter, the precipitated crude protein was collected using centrifugation followed by complete evaporation of acetone. The crude protein was dissolved in 5.0 ml of Tris-HCl buffer (0.1M; pH 6.0). The supernatant was further used to precipitate the remaining protein in another cycle. It was carried out by sequentially adding chilled acetone by using 20%, 40%, 60%, 80%, and 100%) (v/v) into the supernatant obtained from each cycle. The precipitated protein obtained from each cycle was dried at 4 °C by uncapping the respective tubes at 4 °C for 6 hrs and solubilized into 0.5 ml of 0.1 M Tris-HCl buffer of pH 6.0. Each precipitated protein fraction was subjected to protein estimation (Kruger 1994). The fractions of precipitated proteins were checked for L-asparaginase activity and also on SDS-PAGE. The purified L-asparaginase was further analyzed using high-pressure liquid chromatography (HPLC).

2.8 Biophysical and biochemical characterization of L-asparaginase

Various buffers (pH 3-12) were used for determining the optimum pH of L-asparaginase. Similarly, the optimum temperature of L-asparaginase was obtained by assaying the enzyme at different temperatures (40-80 °C). The stability of the enzyme was characterized by incubating the purified enzymes at varying pH and temperatures. The samples were collected at different time intervals and assayed for residual enzyme activity. Biochemical characterization was done by incubating the purified enzyme with different modulators (Tweens, SDS, and EDTA) followed by an L-asparaginase assay. The substrate specificity was also assessed in the presence of L-glutamine to determine the glutaminase activity in the L-asparaginase enzyme. Activity on other protein-based substrates like casein and keratin was also evaluated.

2.9 Applications of L-asparaginase

2.9.1 inhibition of acrylamide gel polymerization

Inhibition of acrylamide polymerization was checked by using 20% and 30% acrylamide solutions. The reaction was set by mixing different concentrations of L-asparaginase into 5.0 ml of 20% and 30% acrylamide solution along with 2.5 ml dH_2O and kept at 60 °C for 30 min for enzymatic catalysis of acrylamide. To check the polymerization, polymerization of the acrylamide gel was initiated by adding 200 µL of 10% APS (ammonium persulfate) along with 20 µL of TEMED (tetra-methyl-ethylene-diamine) solution into the reaction tubes. The inhibition of polymerization was observed by recording the solidification time in respective tubes. The gel flow was also observed in similar reactions to determine the inhibition of polyacrylamide gel formation.

2.9.2 Acrylamide degradation in potato chips

10 gm commercial potato chips were soaked into 10 ml of MilliQ water and vortexed to solubilize the acrylamide present in the chips. The supernatant was collected using high-speed centrifugation, and 2.0 ml of supernatant was treated with ~30 IU of L-asparaginase and incubated at 60 °C for 30 min. 200 µL of the sample was collected at varying time intervals and assessed for the presence of ammonia using Nessler's reagent (Simas et al. 2021). The control samples were also collected similarly from another set of 2.0 ml supernatant without adding L-asparaginase.

2.10 Statistical tool

For PB analysis, Design-Expert version 9.0 (Stat-Ease Inc., Minneapolis, USA) was used. All other experiments were carried out in triplicates to determine the mean values and their standard deviation wherever required.

3 Results

3.1 Thermal screening and selection of L-asparaginase-producing bacteria

Of the seven potential isolates (CGRS, CSPS4, RNF, GSS, GSPS, NBBD1, and NBBD2) selected through qualitative screening, one isolate (CSPS4) showed the maximum production at 50 °C after 24

hrs of incubation as compared to other L-asparaginase-producing bacteria (Figure 1A). Study of colony characteristics showed that isolated bacterium formed greenish colour circular colonies with rough margins and umbonate surface after 24 hrs of incubation on nutrient agar at 37 ℃ (Figure 1B). The bacterium CSPS4 was identified as a Gram-negative stain (Figure 1C). BLASTn analysis of 16S rRNA sequence showed the identity with various strains of *P. aeruginosa*. The maximum identity was observed with *P. aeruginosa* strain OLB-1 (99.87%; LR130528.1), strain JADE-X (99.80%; CP114374.1), and strain M6A146 (99.80%; accession no. CP113974.1) (Figure 1D).

3.2 Production of L-asparaginase from *Pseudomonas aeruginosa* CSPS4

The OVAT approach for media optimization resulted in maximum L-asparaginase production of 512±16.87 nM/min at pH 6.0, temperature 37 ℃ and incubation time of 48 hrs (Table 2; Figure 2A and 2B). Other optimized physical conditions were inoculum size (3%), inoculum age (18 hrs.), aeration (150 rpm), and flask volume (250 ml) (Figure 2C, 2D, 2E, and 2F). Whereas optimized nutrient parameters were carbon (1% sucrose) and nitrogen sources (1% peptone) (Figure 3A, 3B, 3C, and 3D).

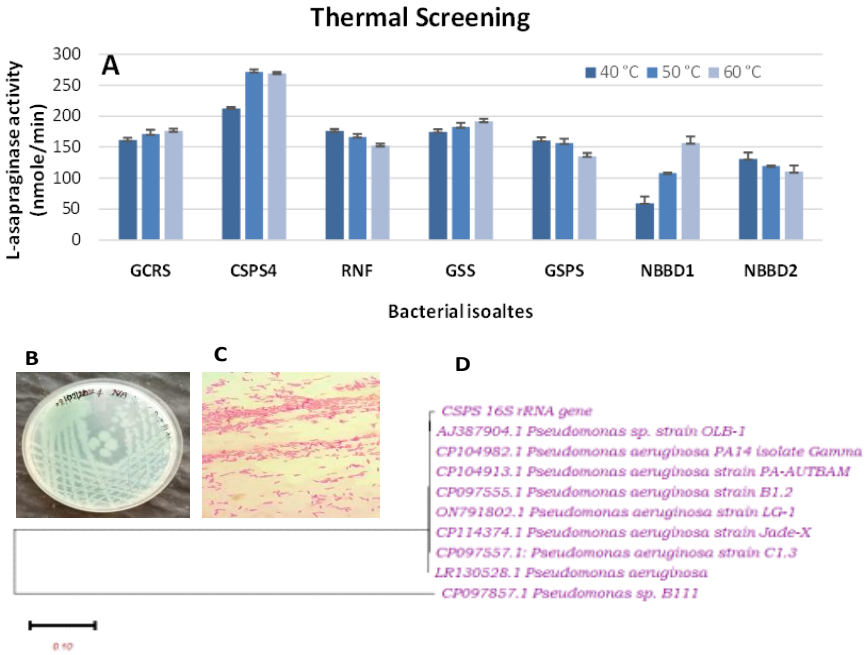


Figure 1 Thermal screening of the (A) bacterial isolates, (B) Colony morphology, (C) Gram-staining, and (D) Phylogenetic tree of the strain CSPS4. The phylogenetic tree was constructed using MEGA11 software by picking Neighbor-joining (NJ) method.

Table 2 Various parameters for L-asparaginase production using OVAT approach

S. No.	Parameters	Optimized condition	Optimum activity (nM/min)
1.	Incubation time	48	268±10.65
2.	pH	6.0	318±8.76
3.	Incubation temperature	37	332±11.32
4.	Inoculum size-3%	3	438±10.14
5.	Inoculum age-18 hrs	18	447±15.90
6.	RPM-150	150	468±14.32
7.	Flask volume-50ml	250	469±13.23
8.	Carbon source	1% (Sucrose)	480±12.22
9.	Sucrose	3%	504±13.32
10.	Nitrogen source	1% (Peptone)	474±13.22
11.	Optimized parameters		512±16.87

Here ± indicates standard deviation (SD); Experiments were carried out in triplicates to calculate SD

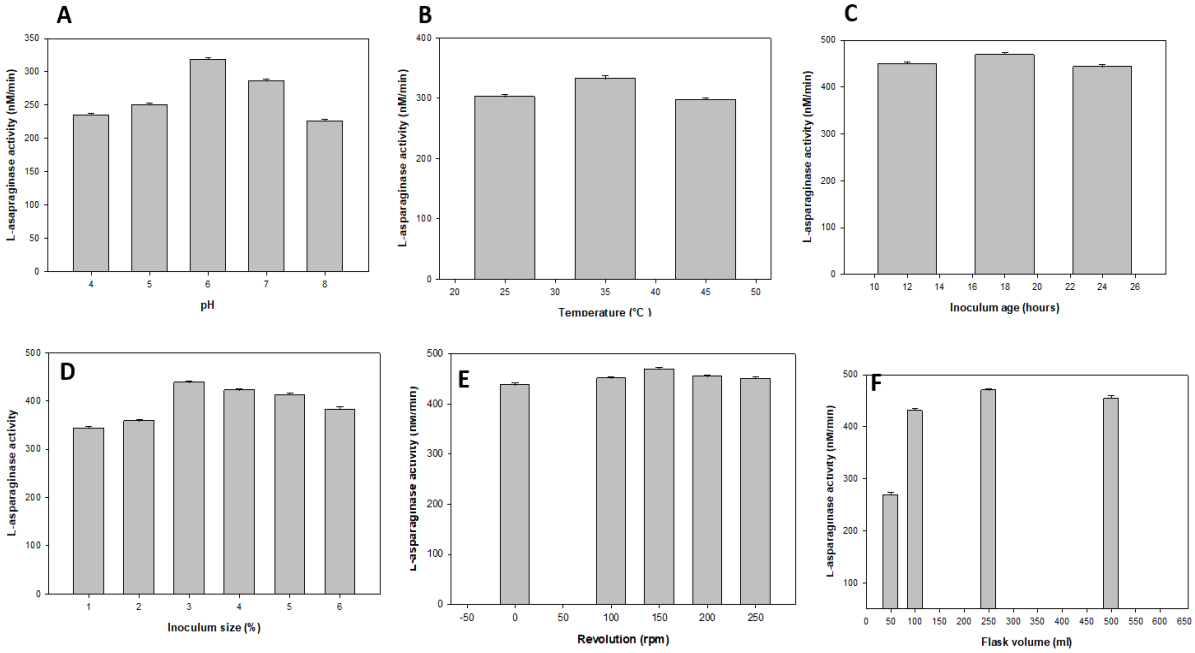


Figure 2 Optimization of physical parameters such as (A) pH, (B) temperature, (C) inoculum age, (D) inoculum size, (E) agitation rate, and (F) aeration conditions to produce L-asparaginase from *P. aeruginosa* CSPS4.

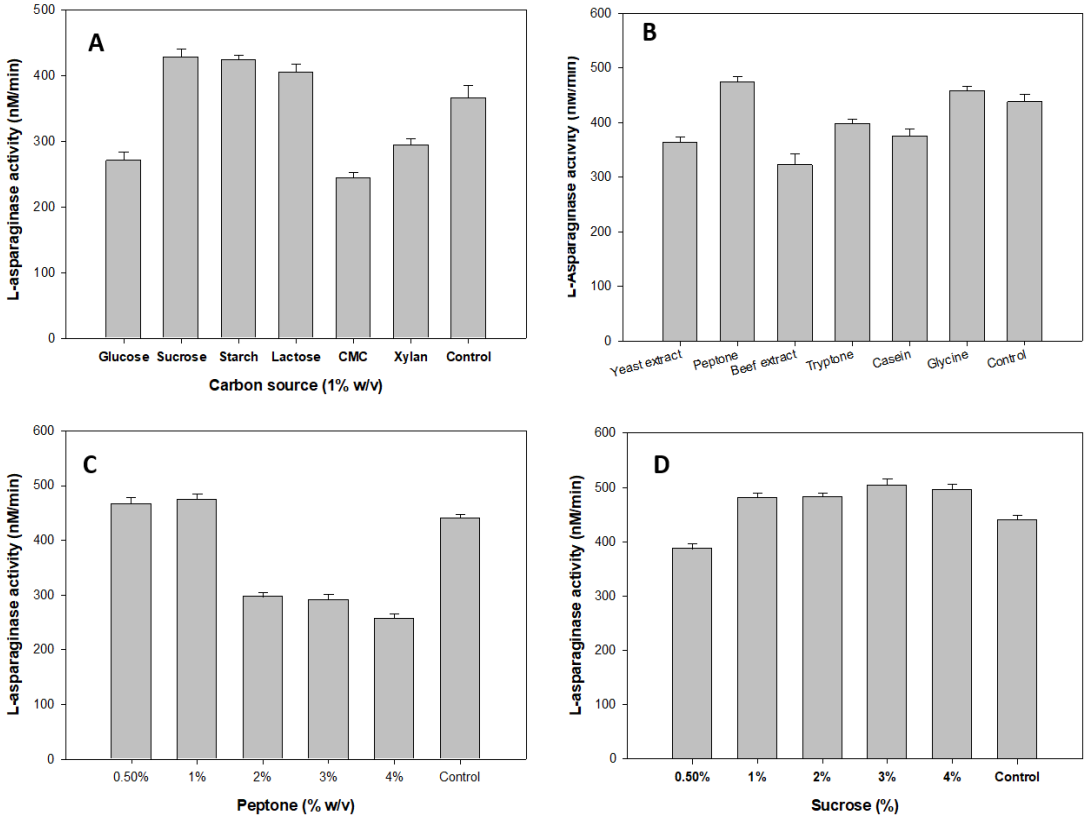


Figure 3 Optimization of nutrient components such as (A) carbon source, (B) nitrogen source, (C) peptone, and (D) sucrose to produce L-asparaginase from *P. aeruginosa* CSPS4.

Table 3 ANOVA analysis

	Source	Sum of Squares	Df	Mean Square	*f-value*	*p-value* Prob>F	Significant/Non-significant
	Model	2.28	9	0.25	83.99	0.0118	Significant
A	Incubation time	0.024	1	0.024	8.13	0.1041	Non-Significant
B	pH	1.43	1	1.43	475.38	0.0021	Significant
E	Inoculum age	2.407E-003	1	2.407E-003	0.80	0.4656	Non-Significant
F	Agitation (rpm)	0.023	1	0.023	7.53	0.1111	Non-Significant
G	Flask volume	0.027	1	0.027	8.91	0.0963	Non-Significant
H	Sucrose	0.12	1	0.12	38.44	0.0250	Significant
J	Peptone	0.58	1	0.58	193.32	0.0051	Significant
K	Dummy1	0.021	1	0.021	6.98	0.1183	Non-Significant
L	Dummy2	0.049	1	0.049	16.40	0.0559	Significant
Residual	Residual	6.024E-003	2	3.012E-003			
Cor Total	Cor Total	2.28	11				

3.3 Statistical optimization for L-asparaginase production using Plackett-Burman Design

Nine factors were assessed to identify the significant variables for achieving maximum L-asparaginase production. The model provided 12 runs, where columns and rows correspond to the variables and experimental conditions. During the trials, L-asparaginase production ranged from 19.55±2.77 nM/min (4^{th} run) to 521.33±4.07 nM/min units (10^{th} run). Multiple linear regression analysis was performed to determine the *F*- and *p*-value of respective components based on the PB design. First order linear model showed the effect of independent variables on L-asparaginase production [Eqn. 2 (coded factors) and Eqn. 3 (actual factors)].

3.3.1 Final equation in terms of coded factors

Y (L-asparaginase) =Log_{10}(Response 1) = +1.81 +0.045 * A +0.35 * B +0.014 * E -0.043 * F +0.047 * G +0.098 * H -0.22 * J -0.042 * K +0.064 * L (Eq. 2)

3.3.2 Final equation in terms of actual factors

Y (L-asparaginase) = Log_{10}(Response 1) = +0.84449 +1.88230E-003 * Incubation time + 0.17271* pH +2.36062E-003 * Inoculum age -5.79674E-004 * RPM +4.97897E-004 * Flask volume +0.056129 * Sucrose -0.12587 * Peptone -0.041861 * Dummy 1 +0.064154 * Dummy 2 (Eq. 3)

$$Y = \beta 0 + \Sigma \beta i\, xi$$

Where Y is the response of L-asparaginase enzyme activity, β0 is the model intercept, βi is a linear variable coefficient, i is the variable number, and xi is the level independent variable. Analysis of variance (ANOVA) (root mean squares) identified the significant model terms affecting the production of L-asparaginase (Table 3). B, H, and J were identified as significant model terms in this case.

In this case, pH (model term B) and peptone (model term J) were the only significant model terms. Values >0.1000 indicate that the model terms are non-significant. Overall, the model was significant, having a *p-value* of 0.011. Moreover, the model *F-value* of 83.99 implies that the model is significant. There is only a 1.18% chance that a "Model *F-value*" this large could occur due to noise. Moreover, the R-squared value and Adj R-squared values were 0.9974 and 0.9855, with a predicted R-squared value of 0.9050. Thus, the model can explain more than 99% of the variation. Here, the 'adeq-precision' ratio was 28.551, which indicates an adequate signal and can be used to navigate the design space.

3.4 Purification of L-asparaginase

During fractional precipitation, fraction number four (F4) showed the maximum L-asparaginase activity (Table 4). The purified enzyme exhibited a molecular weight of ~35 KDa on SDS-PAGE gel (Figure 4A). The purified L-asparaginase was also identified as a single peak during HPLC analysis (Figure 4B) that showed the pink/red zone on plate assay of L-asparaginase (Figure 4C).

3.5 Biophysical and Biochemical Characterization of L-asparaginase

Purified L-asparaginase exhibited an optimum temperature of 60 °C (Figure 5A) under an acidic condition of pH 6.0 (Opt. pH) (Figure

Table 4 Fractional precipitation and purification of L-asparaginase.

Fractions (F)	Acetone fractions (%)	L-asparaginase activity (nM/min)	Protein concentration (mg)	Specific L-asparaginase activity (nM/min/mg)
Total soluble protein (TSP)	Nil	842.22 ±1.31	3.8	221.63 ±1.23
F1.	0-20%	102.22 ±2.13	0.2	511.11 ±2.01
F2.	20-40%	468.00±1.85	1.1	425.45 ±.1.87
F3.	40-60%	351.11±1.63	0.48	731.48 ±1.66
F4.	60-80%	796.89±1.87	0.68	1171.89±1.98
F5.	80-100%	66.67 ±1.54	0.43	155.04 ±1.55

Here ± indicates standard deviation (SD); Experiments were carried out in triplicates to calculate SD

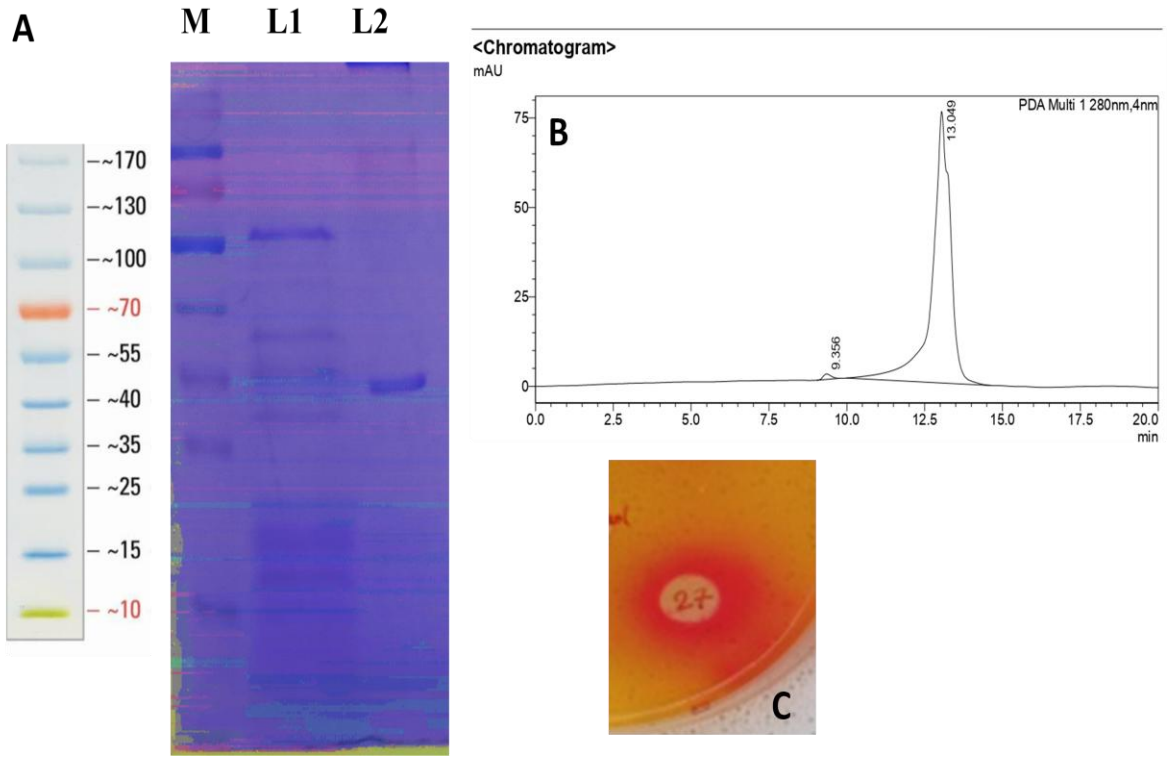


Figure 4 (A) SDS-PAGE analysis of extracellular L-asparaginase of *P. aeruginosa* CSPS4, where M, L1, and L2 can be defined as protein ladder (Thermo Scientific™ PageRuler™), total precipitated protein, and purified L-asparaginase enzyme, (B) HPLC analysis of the purified L-asparaginase enzyme, (C) Confirmation of purified L-asparaginase enzyme activity on L-asparagine containing agar plate

5B). The enzyme exhibited adequate stability at 50°, 60°, and 70 °C for 180 min (Figure 5C). The enzyme retained almost 50% activity at 80 °C after 3 hrs. Similarly, the purified L-asparaginase was stable at a broader pH scale from 4 to 8. The enzyme could hold greater than 60% activity even after 40 hours under pH 4.0 and 5.0 acidic conditions. However, at neutral pH, the L-asparaginase enzyme lost 50% activity at 40 hrs. In alkaline pH, the enzyme was comparatively less stable over the neutral and acidic conditions (Figure 5D). The enzymes retained > 90% activity in the presence of detergents and were slightly stimulated by adding 10% SDS. Negligible inhibition was observed in L-asparaginase activity in the presence of EDTA (Table 5).

3.6 Glutaminase activity

The enzyme also exhibited significant glutaminase (419.5±1.55 nM/min; 81.20%) under assay conditions of pH 6.0 and temperature of 60 °C. Other protein-based substrates, such as casein and keratin, showed no activity towards L-asparaginase (Table 6).

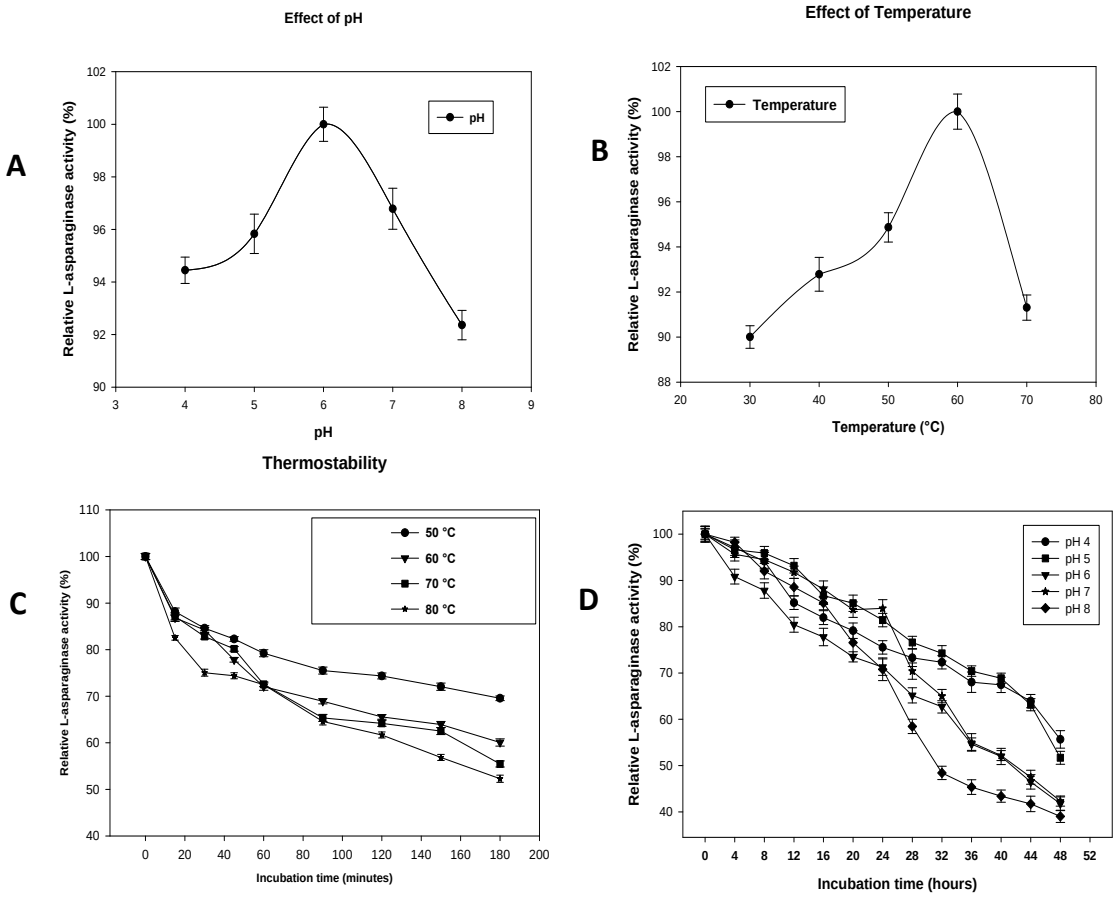


Figure 5 Biophysical characterization of L-asparaginase enzyme for determining optimum (A) pH, (B) optimum temperature, (C) thermostability, and (D) pH stability

Table 5 Effect of modulators on L-asparaginase activity.

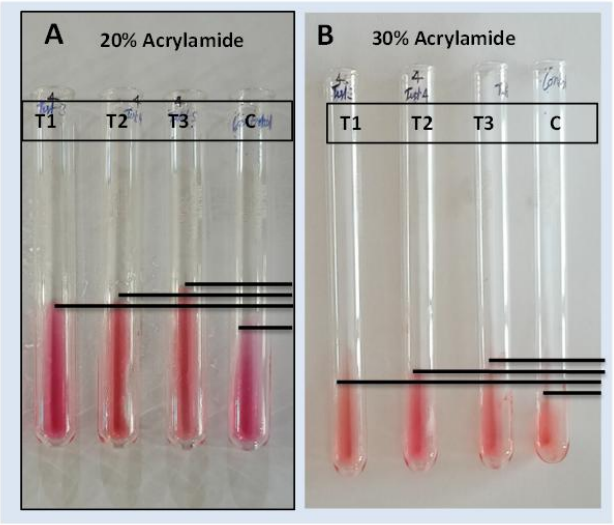
S. No.	Modulators	Residual Enzyme activity (%)
1.	Control	100
2.	1%Tween 20	93.13 ± 1.73
3.	1% Tween 80	91.55 ± 1.89
4.	5% SDS	99.07 ± 2.32
5.	10% SDS	104.57 ± 1.45
6.	1mM EDTA	95.15 ± 1.03

Here ± indicates standard deviation (SD); Experiments were carried out in triplicates to calculate SD

Table 6 Effect of substrates on L-asparaginase activity.

S. No.	Modulators	Residual Enzyme activity (%)
1.	L-asparagine	100 ±2.02
2.	Glutamine	81.20 ±1.55
3.	Casein	0
4.	Keratin	0

Here ± indicates standard deviation (SD); Experiments were carried out in triplicates to calculate SD



C

	Ammonium (NH_4^+) liberation					
Incubation time (min)	Control	0	15	30	45	60
(NH_4^+) (nM/min)	18 ± 0.5	232 ± 1.32	444 ± 1.42	464 ± 1.23	447 ± 1.54	427 ± 1.39

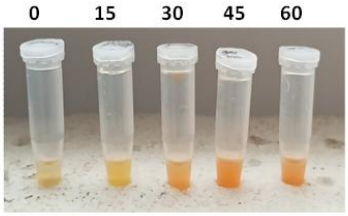


Figure 6 A and B Applications of L-asparaginase of *P. aeruginosa* CSPS4 in inhibition of acrylamide gel polymerization and (C) acrylamide degradation in fried-potato chips

3.7 Applications of L-asparaginases

3.7.1 inhibition of acrylamide polymerization

The purified L-asparaginase showed inhibition in gel polymerization at both the concentrations of acrylamide (20% and 30%) tested. It was observed by visualizing the enhanced flow of gel at varying concentrations of L-asparaginase (Figure 6A and 6B).

3.7.2 Acrylamide degradation in potato chips

The liberation of ammonia was detected in the potato chips in the presence of L-asparaginase over the control groups (Figure 6C). L-asparaginase showed a maximum liberation of 464 ± 1.23 nM/min after 30 min in potato chips.

4 Discussion

L-asparaginase belongs to one of the important biotechnological groups of therapeutic and industrial enzymes that share 40% of global sales of enzymes (Qeshmi et al. 2022). Due to the potential to hydrolyze L-asparagine (an essential amino acid), the enzyme has been successfully employed for destroying leukemia cells (Osama et al. 2023). Recent investigations have also reported the role of the enzyme in treating human neoplastic cells (Alrumman et al. 2019). However, most of the available L-asparaginase also exhibits glutaminase activity along with L-asparaginase activity (EI-Bessoumy et al. 2004; EI-Sharkawy et al. 2016). Glutaminase has several side effects, such as enhanced hypersensitivity, immunogenic response, and coagulation abnormalities (Duval et al. 2002; Mahajan et al. 2014). Therefore, pharmaceutical industries demand glutaminase-free L-asparaginase enzymes. *Streptomyces olivaceus* NEAE-119 (EI-Naggar et al. 2015), *Erwinia carotovora* subsp. *Atroseptica* SCRI 1043 (Goswami et al. 2015), *Pectobacterium carotovorum* MTCC 1428 (Kumar et al. 2017) and *Bacillus paralicheniformis* (Mahajan et al. 2014) are the names of a few bacterial sources that show glutaminase free L-asparaginase activity. Recently, *Pseudomonas* spp. has gained attention for harnessing L-asparaginase activity (Badoei-Dalfard 2015; Fatima et al. 2019; Kumar et al. 2022). In the present investigation, a strain of *P. aeruginosa* was isolated from soil samples of poultry slaughter regions exhibiting L-asparaginase activity. Amany et al. (2021) reported several L-asparaginase producers from fish, meat, and egg sources (Amany et al. 2021). Similarly, a potential anticancer activity was reported from L-asparaginase of the *P. aeruginosa* strain. Several Gram-negative bacteria have been shown to produce L-asparaginase activity (Kamble et al. 2012; Managamuri et al. 2017; Brumano et al. 2019). However, most of the *Pseudomonas*-derived L-asparaginase also exhibit L-glutaminase activity (Brumano et al. 2019; Kumar et al. 2022). This was consistent with our findings too, where reduced L-glutaminase activity was observed in the asparaginase of *P. aeruginosa* CSPS4 strain. Moreover, these strains were reported to produce extracellular L-asparaginase, followed by their OVAT approach production. The present study enhanced the extracellular enzyme using the OVAT approach, where a ~2-fold increase was observed. Nutrient source Sucrose (3%) and peptone (1%) showed maximum L-asparaginase production at 37 °C under acidic pH 6.0 after 48 hrs of incubation (Table 1). Very similar parameters were reported to produce L-asparaginase from another *P. aeruginosa* strain, where 1% (w/v) asparagine and glucose were reported for the maximum productivity after 48 hrs incubation at 35 °C (Amany et al. 2021). Statistical optimization using PB and Response Surface Methodology (RSM) is always beneficial to

understand the interaction among the physical and chemical parameters for enhancing the production of enzymes (Sharma and Satyanarayana 2011; Joshi and Satyanarayana 2015; Talluri et al. 2019). Here, the results of this study observed maximum L-asparaginase production from 19.55±2.77 nM/min (4^{th} run) to 521.33±4.07 nM/min units (10^{th} run) during the PB analysis. Further, the study's results revealed that pH, sucrose, and peptone significantly interacted significantly in enhanced L-asparaginase production. It was also found that the OVAT approach was comparable in L-asparaginase activity to the PB design in this case. It may be due to the temperature (25 °C) and pH (8.0) that do not support the maximal production of the enzyme as optimized through the OVAT approach. However, a significant difference in the range of L-asparaginase production under varying conditions (runs) suggests the influence of physicochemical parameters on enzyme production. Sharma and Satyanarayana (2011) successfully used statistical optimization to enhance the production of acidic amylase from *Bacillus acidicola* PB analysis showed the influence of media components and physical parameters on L-asparaginase production from *Acinetobacter baumannii* ZAS1 (Abhini et al. 2022).

Purified L-asparaginase from *P. aeruginosa* CSPS4 strain showed a molecular weight of ~35 kDa. Qeshmi et al. (2022) reported a similar molecular weight of ~35 kDa of a recombinant L-asparaginase from *P. aeruginosa* HR03 (Qeshmi et al. 2022). It was consistent with the recombinant L-asparaginase of P. *aeruginosa* CSPS4 (Kumar et al. 2024). However, L-asparaginase of *P. aeruginosa* WCHPA075019 (Amany et al. 2021) and *P. aeruginosa* 50071 (EI-Bessoumy et al. 2004) showed a higher molecular weight L-asparaginases of 123 kDa and 160 kDa, respectively. Kumar et al. (2022) also reported a high molecular mass L-asparaginase of 148.0 kDa from *Pseudomonas* sp. PCH44 (Kumar et al. 2022), however, it was unveiled as a homo-tetrameric protein of molecular weight of 37 kDa. In another report, a homo-hexamer of L-asparaginase of ~205 kDa was observed on native PAGE that was identified as a monomeric protein of ~34 kDa on SDS-PAGE (Husain et al. 2016). It suggests that *Pseudomonas* spp. harbor a wide variety of L-asparaginases that may exhibit homomeric forms. On the physiological characterization of purified L-asparaginase from *P. aeruginosa* CSPS4, the optimum pH and temperature of the enzyme were 6.0 and 60 °C. These properties render this enzyme unique from the other available L-asparaginases from *Pseudomonas* spp. and categorize it as a thermo-acidophilic L-asparaginase. It was further confirmed at the molecular level by cloning and expressing the L-asparaginase encoding gene (*rAsn_PA*) of *P. aeruginosa* CSPS4 (Kumar et al. 2024). To the best of our information, only one thermo-acidophilic L-asparaginase has been reported from *P. aeruginosa* that exhibits optimum pH and temperature at an acidic pH of 5.0 and temperature of 50 °C (Badoei-Dalfard 2015). Comparable properties in another L-asparaginase (T_{opt}: 60 °C and pH_{opt} : 5.6) were also observed; however, it was from a fungal strain *Aspergillus tubingensis* SY1 (Yahya et al. 2016). Most of the *Pseudomonas*-derived L-asparaginases exhibit optimum activity at neutral pH (Fatima et al. 2019; Amany et al. 2021; Kambe et al. 2012). Alkaline L-asparaginases have also been reported from various species of *Pseudomonas* (Qeshmi et al. 2022; EI-Bessoumy et al. 2004; Kumar et al. 2022). Most of the L-asparaginase cluster was recorded at the optimum temperature of 37 °C (EI-Bessoumy et al. 2004; Fatima et al. 2019; Brumano et al. 2022). The enzyme reported in the present investigation showed the optimum temperature at 60 °C. L-asparaginase from *Pseudomonas* sp. PCH44 and *P. aeruginosa* HR03; however, they showed optimum activity at 45 °C and 40 °C, respectively. L-asparaginase from *P. aeruginosa* CSPS4 was stable at higher temperature ranges of 50 °C to 80 °C even after 3 hrs of incubation. These properties find the applicability of this enzyme in pharmaceutical and starch-based food industries. L-asparaginase of *P. aeruginosa* HR03 was also thermostable at 40-90 °C (Qeshmi et al. 2022). This enzyme could hold 50% of its activity at 90 °C after 10-min of incubation. Similarly, 76.53% of L-asparaginase activity was retained at 45 °C after 120 min of incubation from *Pseudomonas* sp. PCH44 (Kumar et al. 2022). In addition to thermostability, L-asparaginase from *P. aeruginosa* CSPS4 also showed stability at a broad pH range (4 to 8). The enzyme retained better stability under acidic pH compared to the alkaline conditions which makes this enzyme suitable for different biotechnological applications. Only a handful of reports are available that discuss the pH stability of L-asparaginase from *Pseudomonas* spp. (Qeshmi et al. 2022). For example, L-asparaginase of *Pseudomonas aeruginosa* HR03 retained 52% and 60% residual activity at pH 4.0 and 11.0, respectively (Qeshmi et al. 2022). Modulators showed a marginal effect on L-asparaginase activity in this investigation. The enzyme was able to retain 90% activity in the presence of detergents (SDS and Tween) which confirm the suitability of this enzyme in pharmaceutical industries. Besides, a non-significant inhibition was recorded in L-asparaginase activity in the presence of EDTA. *P. aeruginosa* WCHPA075019-derived L-asparaginase was also not inhibited by EDTA (Amany et al. 2021). It indicates that this L-asparaginase lacks metals in its structure and does not belong to the group of metalloenzymes. However, Ps44-ASNase II of *Pseudomonas* spp. PCH44 was strongly inhibited by EDTA (Kumar et al. 2022), suggesting diversity among L-asparaginase of *Pseudomonas* spp.

Due to the hydrolysis ability of amide groups, L-asparaginase of the present investigation was successfully employed to hydrolyze acrylamide. Here, we first report for assessing the L-asparaginase activity in acrylamide hydrolysis from a *P. aeruginosa* strain. A significant delay was observed in acrylamide gel polymerization as compared to the control, where immediate solidification was

observed. Mahajan et al. (2014) also reported a delayed polymerization of acrylamide gel by using L-asparaginase of *B. licheniformis* (Mahajan et al. 2014). The associated application of acrylamide reduction was also performed in fried potato chips. Here, acrylamide degradation was observed indirectly by estimating the liberation of ammonia in the enzyme-treated fried potato chips. L-asparaginase of *P. oryzihabitans* has also been reported to reduce the acrylamide levels in fried potato slices (Bhagat et al. 2016). The permissible limit of acrylamide in food ranges from 13.76-71.13 g for fried snack foods and 12.97- 93.88 gm for baked food items for a person of average weight of 57 Kg (WHO). However, acrylamide concentration in snack foods ranges from 371.56 µg/kg to 6391.73 µg per kg, which is quite high and keeps humans at high health risks. Therefore, pretreatment of starch-based fried food products with L-asparaginase must be strictly implemented.

Conclusion

The study resulted in identification of a novel thermo-acidophilic L-asparaginase from *P. aeruginosa* strains CSPS4. The optimum temperature and pH at 60 °C and 6.0, respectively, renders this enzyme novel from other L-asparaginases of *Pseudomonas* spp. Besides, L-asparaginase also exhibits stability under a broad pH range of 4 to 8, indicating its potential applicability in the pharmaceutical and food industries. The enzyme successfully inhibited acrylamide polymerization and degraded acrylamide in commercial fried potato chips. Because of its potential in acrylamide reduction in food products and to meet industrial demands, further studies are required to develop its recombinant version.

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Conflict of Interest

The authors have no financial competing interests to declare

Compliance with Ethical Standards

There is no participation of human and animals or their biological materials; therefore, no ethical approval is required.

Author's Contribution

DV conceived the idea and designed the experiments. VK performed the experimental work and analyzed the data. SJ assisted in media optimization experiments. BK assisted in protein purification and analysis. VK and DV wrote the manuscript. VK, SJ, and DV edited the manuscript. All the authors read and approved the final version of the manuscript.

References

Abhini, K.N., Rajan, A.B., Fathimathu, Z.K., & Sebastian, D. (2022). Response surface methodological optimization of L-asparaginase production from the medicinal plant endophyte *Acinetobacter baumannii* ZAS1. *Journal of Genetic Engineering and Biotechnology*, *20* (1), 1-13. doi: 10.1186/s43141-022-00309-4.

Ali, U., Naveed, M., Ullah, A., Ali, K., Shah, S.A., Fahad, S., & Mumtaz, A.S. (2016). L-asparaginase, as a critical component to combat acute lymphoblastic leukemia (ALL): A novel approach to target ALL. *European Journal of Pharmacol*ogy, *77* (1), 199-210. doi.org/10.1016/j.ejphar.2015.12.023.

Alrumman, S.A., Mostafa, Y.S., Al-Izran, K.A., Alfaifi, M.Y., Taha, T.H., Elbehairi, S.E. (2019). Production and anticancer activity of aL-Asparaginase from *Bacillus licheniformis* isolated from the red sea, Saudi Arabia. *Scientific Reports*, *6*, 3756. doi.org/10.1038/s41598-019-40512-x.

Amany, B., Abd El–Aziz, Wesam, A., Hassanein Zakaria, A., Mattar., Rabab, A., & El-Didamony (2021). Production of chemotherapeutic agent L-asparaginase from gamma-Irradiated *Pseudomonas aeruginosa* WCHPA075019. *Jordan journal of Biological Sciences*, *14*, 403-412. doi.org/10.54319/jjbs/140304.

Badoei-Dalfard, A. (2015). Purification and characterization of l-asparaginase from *Pseudomonas aeruginosa* strain SN004: production optimization by statistical methods. *Biocatalysis and Agricultural Biotechnology*, *4*, 388-397. doi.org/10.1016/ j.bcab.2015.06.007.

Batool, T., Makky E.A., Jalal, M., & Yusoff, M.M. (2016). A comprehensive review on L-asparaginase and its applications. *Applied biochemistry and biotechnology*, *178*, 900-923. doi:10.1007/s12010-015-1917-3.

Bhagat, J., Kaur, A., & Chadha, B.S. (2016). Single step purification of asparaginase from endophytic bacteria *Pseudomonas oryzihabitans* exhibiting high potential to reduce acrylamide in processed potato chips. *Food and Bioproducts Processing*, *99*, 222-230. doi.org/10.1016/j.fbp.2016.05.010.

Brumano, L.P., da Silva, F.V.S., Costa-Silva, T.A., Apolinário, A.C., Santos J.H.P.M., et al. (2019). Development of L-

Asparaginase Biobetters: current research status and review of the desirable quality profiles. *Frontiers in Bioengineering and Biotechnology, 6*, 212. doi.org/10.3389/fbioe.2018.00212.

Brumano, L.P., Moguel, I.S., Yamakawa, C.K., Pessoa, A.Jr., & Mussatto, S.I. (2022). Selection and optimization of medium components for the efficient production of L-asparaginase by *Leucosporidium scottii* L115—A Psychrotolerant Yeast. *Fermentation, 8*, 398. doi.org/10.3390/fermentation8080398.

Dipali, P., & Ajit, P. (2012). Characterization of amylase producing bacterial isolates. *Pharmaceutical Life Science, 1*, 42–47. doi.org/10.1155/2021/5592885.

Doriya, K., & Kumar, D.S. (2016) Isolation and screening of L-asparaginase free of glutaminase and urease from fungal sp. *3 Biotech, 6*, 239.

Duval, M., Suciu, S., Ferster, A., Rialland, X., Nelken, B., et al. (2002). Comparison of *Escherichia coli*-asparaginase with *Erwinia*-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organization for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trial. *Blood, 99*, 2734–2739. doi.org/10.1182/blood.V99.8.2734.

El-Bessoumy, A.A., Sarhan, M., & Mansour, J. (2004). Production, isolation, and purification of L-asparaginase from *Pseudomonas aeruginosa* 50071 using solid-state fermentation. *Korean Society for Biochemistry and Molecular Biology. BMB Reports, 37*, 387-393. doi.org/10.5483/BMBRep

El-Naggar, N.E.A., Moawad, H., El-Shweihy, N.M. et al (2019). Process development for scale-up production of a therapeutic L-asparaginase by *Streptomyces brollosae* NEAE-115 from shake flasks to bioreactor. *Scientific Reports, 9*, 13571.

El-Naggar, Nel-A., Moawad, H., El-Shweihy, N.M., & El-Ewasy, S.M. (2015). Optimization of culture conditions for production of the anti-leukemic glutaminase free L-asparaginase by newly isolated *Streptomyces olivaceus* NEAE-119 using response surface methodology. *BioMed Research International, 2015* (17). doi.org/10.1155/2015/627031.

El-Sharkawy, A.S., Farag, A.M., Embaby, A.M., Saeed H., & El-Shenawy, M. (2016) Cloning, expression and characterization of *Pseudomonas aeruginosa* EGYII *L-Asparaginase* from *Pseudomonas aeruginosa* strain EGYII DSM 101801 in *E. coli* BL21(DE3) pLysS. *Journal of Molecular Catalysis B: Enzymatic, 132*, 16-23. doi.org/10.1016/j.molcatb.2016.06.011.

Fatima, N., Khan, M.M., & Khan, I.A. (2019). L-asparaginase produced from soil isolates of *Pseudomonas aeruginosa* shows potent anticancer activity on HeLa cells. *Saudi Journal of Biological Sciences, 26*, 1146-1153. doi.org/10.1016/j.sjbs.2019.05.001.

Goswami, R., Hegde, K., & Veeranki, V.D. (2015). Production and characterization of novel glutaminase free recombinant L-asparaginase II of *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 in *E. coli* BL21 (*DE3*) *British Microbiology Research Journal*, *6*, 95-112. DOI: 10.9734/BMRJ/2015/13867

Husain, I., Sharma, A., Kumar, S., & Malik, F. (2016). Purification and characterization of glutaminase free asparaginase from *Enterobacter cloacae*: in-vitro evaluation of cytotoxic potential against human Myeloid Leukemia HL-60 cells. *PLoS One, 1811*, e0148877. doi.org/10.137/journal.pone.0148877.

Jia, R., Wan, X., Geng, X., Xue, D., Xie, Z., & Chen, C. (2021). Microbial L-asparaginase for application in acrylamide mitigation from food: Current research status and future perspectives. *Microorganisms, 9*, 1659.

Joshi, S., & Satyanarayana, T. (2015). Bioprocess for efficient production of recombinant *Pichia anomala* phytase and its applicability in dephytinizing chick feed and whole wheat flat Indian breads. *Journal of Industrial Microbiology and Biotechnology, 42*, 1389-1400 doi.org/10.1007/s10295-015-1670-1.

Kamble, K.D., Bidwe, P.R., Muley, V.Y., Kamble, L.H., Bhadange. D.G., & Musaddiq, M. (2012). Characterization of l-asparaginase producing bacteria from water, farm, and saline soil. *Bioscience discovery, 3*, 116-119.

Kataria, M., Kaur, N., Narula, R., Kumar, K., Kataria. S., & Verma, N. (2015). L-Asparaginase from novel source: *Solanum nigrum* and development of asparagine biosensor. *The Pharma Innovation Journal, 4*, 81.

Kruger, N.J. (1994). Detection of polypeptides on immunoblots using secondary antibodies or protein A. *Methods in Molecular Biology, 32*, 17–24.

Kukurova, K., Morales, F.J., Bednáriková, A., & Ciesarová, Z. (2009). Effect of l-asparaginase on acrylamide mitigation in a fried-dough pastry model. *Molecular Nutrition & Food Research, 53*, 1532-1539. doi: 10.1002/mnfr.200800600.

Kumar, S., Ashish, P.A., Venkata, D.V., & Pakshirajan, K. (2017). Kinetics of growth on dual substrates, production of novel glutaminase-free L-asparaginase and substrates utilization by *Pectobacterium carotovorum* MTCC 1428 in a batch bioreactor. *Korean Journal of Chemical Engineering, 34*, 118-126. doi: 10.1007/s11814-016-0216-1.

Kumar, S., Darnal, S., Patial, V., Kumar, V., Kumar, S., & Singh, D. (2022). Molecular cloning, characterization, and in-silico

analysis of L-asparaginase from Himalayan *Pseudomonas* sp. PCH44. *3 Biotech*, *12*, 162. doi.org/10.1007/s13205-022-03224-0

Kumar, V., Kumar, R., Sharma, S., Shah, A., Chaturvedi, C.P., & Verma, D. (2024) Cloning, expression, and, characterization of a novel thermo-acidophilic l-asparaginase of *Pseudomonas aeruginosa* CSPS4. *3 Biotech 14*, 54. doi.org/10.1007/s13205-024-03916-9.

Li, X., Zhang, X., Xu, S., Xu, M., Yang, T., et al. (2019). Insight into the thermostability of thermophilic L-asparaginase and non-thermophilic L-asparaginase II through bioinformatics and structural analysis. *Applied microbiology and biotechnology*, *103*, 7055-7070. doi.org/10.1007/s00253-019-09967-w.

Luo, M., Brooks, M., & Wicha, M.S. (2018). Asparagine and Glutamine: Co-conspirators Fueling Metastasis. *Cell Metabolism*, *27*, 947-949. doi: 10.1016/j.cmet.2018.04.012.

Mahajan, R.V., Kumar, V., Rajendran, V., Saran, S., Ghosh, P.C., & Saxena, R.K. (2014). Purification and characterization of a novel and robust L-asparaginase having low-glutaminase activity from *Bacillus licheniformis*: in vitro evaluation of anti-cancerous properties. *PLoS One*, *6*, 99037.

Mahajan, R.V., Saran, S., Kameswaran, K., Kumar, V., & Saxena, R.K. (2012). Efficient production of L-asparaginase from *Bacillus licheniformis* with low-glutaminase activity: optimization, scale up and acrylamide degradation studies. *Bioresource Technology*, *125*, 11-6. doi.org/10.1016/j.biortech.2012.08.086.

Managamuri, U., Vijayalakshmi, M., Ganduri, VSRK., Babu, S., & Poda, S. (2017). Optimization of culture conditions by response surface methodology and unstructured kinetic modeling for L-asparaginase production by *Pseudonocardia endophytica* VUK-10. *Journal of Applied Pharmaceutical Science*, *7*, 42–50. doi: 10.7324/JAPS.2017. 70106.

Muzuni, Aprilyani, R., Ardiansyah, S., Farij, M., & Gultom, M.T. (2023). Characterization of the type 2 l-asparaginase gene in thermo-halophilic bacterial from *Wawolesea* hot springs, Southeast Sulawesi, Indonesia. *Pakistan Journal of Biological Sciences 26*, 392-402. doi: 10.3923/pjbs.2023.392.402.

Osama, S., El-Sherei, M.M., Al-Mahdy, D.A., Bishr, M., Salama, O., & Raafat, M.M (2023). Optimization and characterization of antileukemic L-asparaginase produced by *Fusarium solani* endophyte. *AMB Express*, *13*, 96. doi: 10.1186/s13568-023-01602-2

Pavlova, N.N., Hui, S., Ghergurovich, J.M., Fan, J., Intlekofer, A.M., et al. (2018). As extracellular glutamine levels decline, asparagine becomes an essential amino acid. *Cell Metabolism*, *6*, 428-438.e5. doi.org/10.1016/j.cmet.2017.12.006.

Qeshmi, I.F., Homaei, A., Khajeh, K., Kamrani, E., & Fernandes, P. (2022). Production of a Novel Marine *Pseudomonas aeruginosa* recombinant L-asparaginase: Insight on the structure and biochemical characterization. *Marine Biotechnology*, *24*, 599-613. doi.org/10.1007/s10126-022-10129-9.

Sharma, A., & Satyanarayana, T. (2011). Optimization of medium components and cultural variables for enhanced production of acidic high maltose-forming and Ca2+-independent α-amylase by *Bacillus acidicola*. *Journal of Biosciences Bioengineering*, *111* (5), 550-3.

Shirfrin, S., Parrott, C.L., & Luborsky, S.W. (1974). Substrate binding and inter-subunit interactions in L-asparaginase. *Journal of Biological Chemistry*, *249*, 1335–1340.

Shrivastava, A., Khan, A.A., Khurshid, M., Kalam, M.A., Jain, S.K., & Singhal, P.K. (2016). Recent developments in L-asparaginase discovery and its potential as anticancer agent. *Critical reviews in oncology/hematology*, *100*, 1-10. doi: 10.1016/j.critrevonc.2015.01.002.

Simas, R.G., Krebs Kleingesinds, E., Pessoa Junior, A., & Long, P.F. (2021). An improved method for simple and accurate colorimetric determination of l-asparaginase Enzyme Activity Using Nessler's Reagent. *Journal of Chemical Technology & Biotechnology*, *96*, 1326–1332.

Simpson, D.M., & Beynon, R.J. (2010). Acetone precipitation of proteins and the modification of peptides. *Journal of Proteome Research*, *9*, 444-450. doi: 10.1021/pr900806x.

Stadler, R.H., Blank. I., Varga, N., Robert F, Hau J, et al. (2002). Acrylamide from Maillard reaction products. *Nature*, *3*,449-50. doi: 10.1038/419449a.

Talluri, P.V., Lanka, S.S., Rajagopal Saladi, V., & Avicenna, J. (2019). Statistical Optimization of Process Parameters by Central Composite Design (CCD) for an Enhanced Production of L-asparaginase by *Myroides gitamensis* BSH-3, a Novel Species. *Avicenna Journal of Medical Biotechnology*, *11*, 59-66.

Tripathi, N., & Sapra, A. (2023) Gram Staining. In: Stat-Pearls [Internet]. Treasure Island (FL): StatPearls Publishing.

Verma, D., & Satyanarayana, T. (2011). An improved protocol for DNA extraction from alkaline soil and sediment samples for constructing metagenomic libraries. *Applied Biochemistry and Biotechnology*, *165*, 454-64. doi: 10.1007/s12010-011-9264-5.

Vimal, A., & Kumar, A., (2022) L-asparaginase: need for an expedition from an enzymatic molecule to antimicrobial drug.

International Journal of Peptide Research and Therapeutics, *28*, 9. https://doi.org/10.1007/s10989-021-10312-x.

Williams, S.T., Sharpe, M.E., & Holt, J.G. (1989). *Bergey's Manual of Systematic Bacteriology*. 2nd. Baltimore, MD, USA: *Springer Science+Business Media, LLC*.

Xu, F., Oruna-Concha, M.J., & Elmore, J.S. (2016).The use of asparaginase to reduce acrylamide levels in cooked food. *Food chemistry*, *105*, 163–171. doi: 10.1016/j.foodchem.2016.04.105.

Yahya, S., Jahagir, S., Shaukat, S., Sohail, M., & Khan, S. (2016). Production optimization by using Plackett-Burman design and partial characterization of amylase from *Aspergillus tubingensis* SY1. *Pakistan Journal of Botany*, *48*,, 2557-2561.

Yan, Y., Kuramae, E.E., Klinkhamer, P.G., & van Veen, J.A. (2015). Revisiting the dilution procedure used to manipulate microbial biodiversity in terrestrial systems. *Applied Environmental Microbiology*, *81*, 4246-52.

Zhou, Y., Jiao, L., Shen, J., Chi, H., Lu, Z., et al. (2022). Enhancing the Catalytic Activity of Type II L-Asparaginase from *Bacillus licheniformis* through Semi-Rational Design. *International Journal of Molecular Sciences*, *23*, 9663. doi.org/10.3390/ijms23179663.