

## Chapter 5

### L-asparaginase purification and characterization from *Bacillus indicus*

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#### 5.1 Introduction

The purification of native enzymes can be successfully performed using a combination of precipitation and chromatography techniques. The preliminary or the first step in the purification of extracellular enzyme from the supernatant fraction of the production broth is often the salt fractionation using inorganic salts or the solvent precipitation methods (Wingfield, 1998). Salting out methods using the ammonium sulphate  $(\text{NH}_4)_2\text{SO}_4$  precipitation is an efficient way for enriching the proteins of interest by precipitating proteins from solution over a narrow scale of salt concentrations (Barros *et al.*, 2001). The added ions interact with water more vigorously, bringing about the protein molecules to precipitate. This concentration step is then followed by diverse chromatography techniques including size exclusion chromatography (SEC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and many others (Mhetras *et al.*, 2009). Ion-exchange chromatography is one of the widely utilized chromatography technique that offers high resolution during purification and the mixture of proteins are separated on the basis of charge properties. Similarly, gel filtration or size exclusion chromatography is another fractionation chromatography that separates proteins on the basis of size properties (Barth *et al.*, 1994; Burgess, 2018). The resolution achieved is lower than IEC during purification but can efficiently remove the proteins that are smaller or larger in size than the protein of interest. SEC offers the advantages of simplicity, low cost, ease of scale-up and being the mildest chromatography leading to minimum loss of the enzyme activity due to denaturation. The combination of IEC and SEC is a widely utilized method that can be performed in tandem for

recovery of the enzymes in the microbial production processes. Chapter 5 describes the purification methodology utilized and the characterization of a new L-asparaginase enzyme preparation from *Bacillus indicus* MTCC 4374 bacteria.

## **5.2 Materials and Methods**

### **5.2.1 Enzyme purification steps**

The crude extracellular asparaginase in the supernatant was obtained by centrifugation of the fermentation broth at 11000 rpm for 20 min at 4°C. The crude enzyme was further purified using a combination of ammonium salt precipitation followed by two different chromatography steps. All the purification procedures were performed under cold conditions at 4°C.

### **5.2.2 Ammonium sulfate precipitation**

The crude enzyme preparation was subjected to  $(\text{NH}_4)_2\text{SO}_4$  precipitation starting with 20% saturation with further increase up to 80% saturation level under constant stirring. After salt dissolution, the preparation was centrifuged at 15000 rpm for 30 min to obtain the precipitated enzyme pellet which was further redissolved in minimal amounts of 0.05 M Tris-HCl buffer (pH 8.6). The preparation was further dialyzed using a 10 kDa dialysis membrane (Himedia, India) against the same buffer under constant stirring with the change of buffer after every 4h (3 times) to eliminate the salt impurities. The dialyzed preparation was then assessed for L-asparaginase activity and total protein content.

### **5.2.3 Ion exchange chromatography (IEC)**

The dialyzed sample was then loaded on a DEAE cellulose column (GE Healthcare) which was pre-equilibrated with 0.05 M Tris-HCl buffer (pH 8.6). The column was washed two times with the same buffer and elution of the enzyme was performed using a linear gradient

of 0-1000 mM NaCl in Tris HCl buffer (pH 8.6) at a flow rate of 0.5 mL/min. The chromatographic runs were carried out on a Fast Protein Liquid chromatography (FPLC) apparatus (Akta Pure, GE healthcare, UK). Fractions collected were then analyzed for protein concentration and enzyme activity. Fractions containing asparaginase activity were pooled, dialyzed and concentrated using Amicon Ultracel centrifugal filter tubes (Millipore).

#### **5.2.4 Size exclusion chromatography (SEC)**

The samples obtained from previous runs were further subjected to gel filtration or size exclusion chromatography purification on HiLoad™ 16/600 Superdex™ 200 pg filtration column pre-equilibrated with Tris-HCl buffer (pH 8.6). The elution of the enzyme was carried out in the same buffer and fractions corresponding to the peak at 280 nm were collected and analyzed for L-asparaginase enzyme activity. The desired enzyme fractions possessing L-asparaginase activities were concentrated using an Amicon filter and utilized for further analysis.

#### **5.2.5 Assessment of molecular weight and purity**

The purity of each purification step and the monomeric molecular mass of the enzyme were assessed on SDS-PAGE containing resolving gel (12%) of pH 8.8 and a stacking gel (5%) of pH 6.8 (Laemmli, 1970). To determine the intact molecular weight and the oligomeric nature of the enzyme, the Native PAGE analysis was performed using 7.5% polyacrylamide gel in Tris-Glycine buffer at 50 V and  $4 \pm 1$  °C (Gallagher, 1995). The gels were stained with Coomassie blue G-250 dye and the molecular mass of the intact and monomeric form was determined using SDS-PAGE and Native PAGE markers respectively.

#### **5.2.6 Evaluation of kinetic parameters and substrate specificity**

The kinetic parameters including the Michaelis-Menten constant ( $K_m$ ), maximum velocity ( $V_{max}$ ), turnover number ( $k_{cat}$ ) and specificity constant ( $k_{cat}/K_m$ ) were evaluated. The values of

$K_m$  and  $V_{max}$  were calculated using the experimentally obtained steady-state data in Graph Pad prism 6.00 software. The calculation of turnover number and specificity constant was performed by considering one active site/subunit (~35 kDa). The binding affinity to various structurally similar amides was checked to ascertain the specificity of the purified L-asparaginase from *Bacillus indicus* MTCC 4374. Different amide compounds were taken at a concentration of 10 mM for the substrate specificity studies. The enzyme activity of the substrate L-asparagine was taken as the standard and the enzyme activities of other structural analogs were calculated in terms of relative enzyme activity (%).

### **5.2.7 Influence of temperature and pH on activity and pH stability of the purified L-asparaginase**

The effect of pH and temperature on the enzyme activity was evaluated at diverse pH and temperature ranges. The optimal pH showing the highest enzyme activity was determined in the pH range of 5.5-10.5 values using buffers using 0.05 M potassium phosphate buffer (pH 5.5-7.5) and 0.05 M Tris-HCl buffer (pH 8.0-10.5). The pH stability studies were carried out by incubating the enzyme at pH 5.5-10.5 for 24 h (4 °C) and measuring the residual enzyme activity under standard assay conditions (pH 8.6, 50 mM). The effect of the temperature on the enzyme activity was evaluated by conducting the enzyme assays in the temperature range of 25 °C - 60 °C.

### **5.2.8 Determination of the secondary structure of purified L-asparaginase**

The secondary structure elements present in the purified L-asparaginase enzyme were determined using Circular Dichroism spectroscopy performed on a JASCO J-1500 circular dichroism spectrophotometer (Jasco Corporation, Japan). The size exclusion chromatography purified enzyme fractions in 0.05 M Tris-HCl buffer (pH 8.6) were taken in a cylindrical 2 mm quartz cell and used as input. The CD spectra were recorded in the far-UV region

(wavelength range of 260-190 nm) at an enzyme concentration of 0.1 mg/mL at 37 °C using the Spectra manager 2 software.

## **5.3 Results and Discussion**

### **5.3.1 Purification of L-asparaginase enzyme**

The production broth from a 24 h batch culture of *Bacillus indicus* MTCC 4374 for the production of L-asparaginase enzyme was harvested and centrifuged to obtain a crude extract which was further subject to different purification steps. The purification steps were performed in tandem to minimize the losses in the enzyme activity.

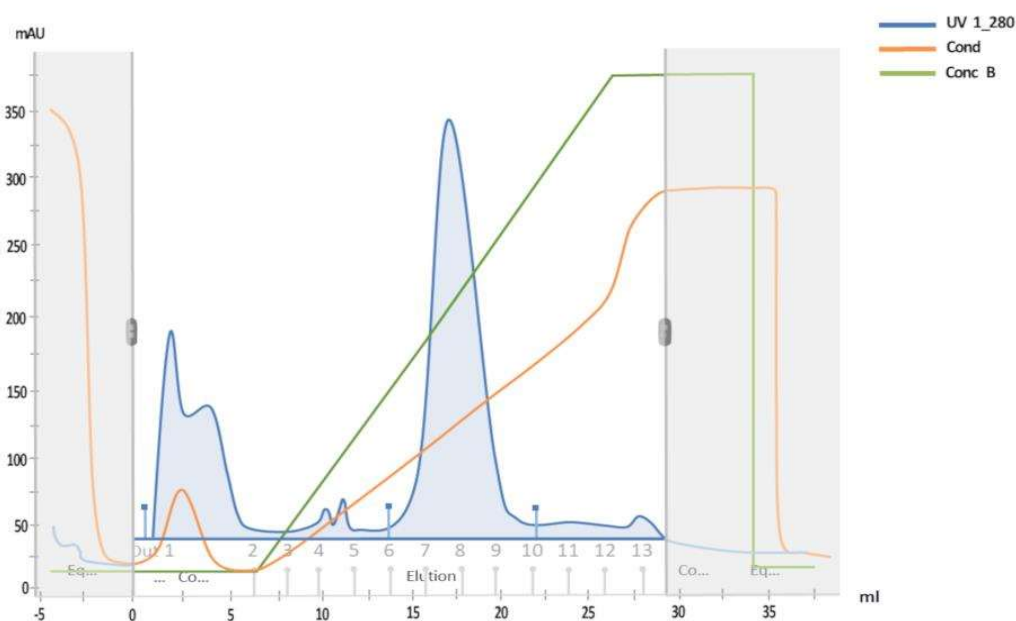
### **5.3.2 Ammonium sulfate precipitation**

Crude L-asparaginase present in the supernatant fraction was firstly subjected to salt precipitation. The precipitate fraction having 60-80% saturation showed maximal enzyme activity and was dialyzed in the Tris-HCl buffer (pH 8.6). Initially in the first fractionation precipitation was carried out using 30%  $(\text{NH}_4)_2\text{SO}_4$ . No L-asparaginase activity was detected in the precipitate. During the second step of fractionation, 30-60% solid ammonium sulfate was slowly added under ice-cold conditions and again no L-asparaginase activity was detected in the precipitated fraction. L-asparaginase activity was detected in the 60-80% ammonium sulfate saturation during the third phase of fractionation. The precipitated enzyme was then solubilized in minimal volume of 0.05M Tris-HCl buffer of pH 8.6. Further, dialysis was carried out to remove the ammonium sulfate and low molecular weight proteins using dialysis membrane of MWCO of 10 kDa.

### **5.3.3 Anion exchange chromatography**

The enzyme was further purified using a DEAE-cellulose ion-exchange chromatography column performed on the FPLC system. HiTrap DEAE FF (fast flow) column pre-equilibrated with 0.05 M Tris-HCl buffer of pH 8.6 was utilized. The resulting chromatogram

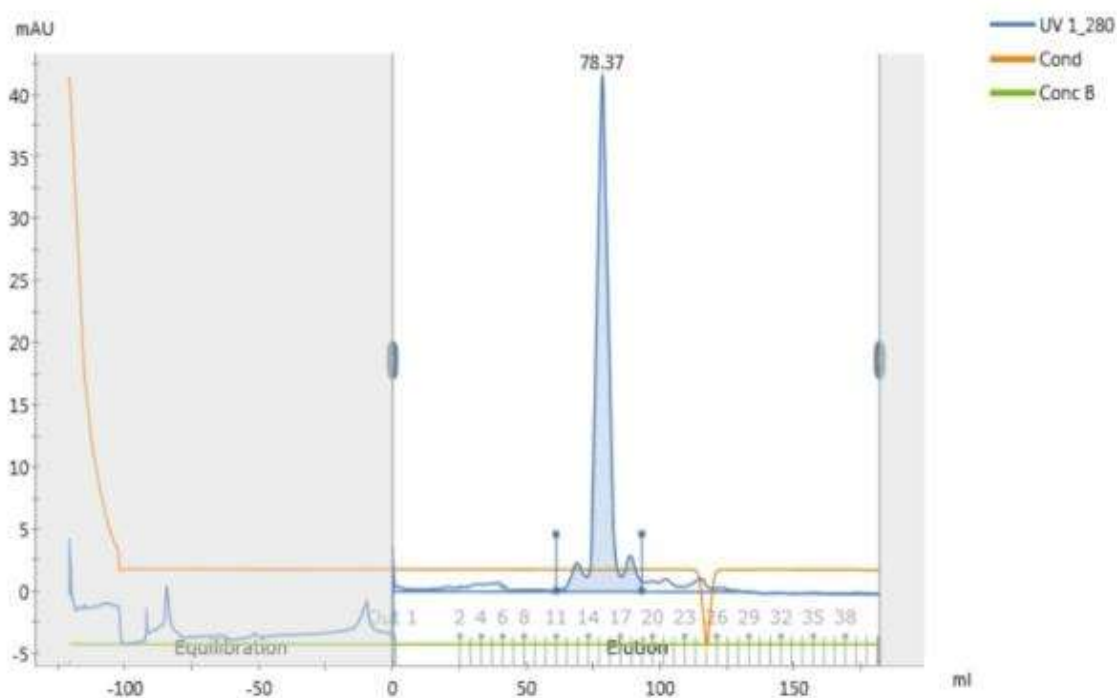
obtained using DEAE anion exchange chromatography was shown in **Figure 5.1**. The enzyme was eluted using a linear gradient of 0-1M NaCl. 3 major peaks can be seen during the analysis for protein content at a wavelength of 280 nm in the obtained chromatogram. The initial peaks obtained represented the unbound proteins that were unable to bind to the anion exchanger column and were washed away during the column wash step. These proteins were having the net positive charge as that of the beads of DEAE column. The peaks obtained during the linear gradient elution corresponded to the proteins that were able to bind to the anion exchange column as they were having the opposite (negative) charge to that of the DEAE beads. Fractions containing 2.0 ml volume were collected and the L-asparaginase activity was found in the eluted fractions 7-9 as depicted by the chromatogram. The eluted fractions showing the L-asparaginase activity were further pooled, dialyzed and concentrated.



**Figure 5.1:** Chromatogram depicting the elution profile obtained on ion-exchange chromatography (IEC) column for the purification of L-asparaginase.

### 5.3.4 Size-exclusion chromatography purification

Subsequently, gel filtration or size-exclusion chromatography was utilized for further purification on the basis of molecular sieving and resulting in a final yield recovery of 13.47 % and 18.3-fold protein purification as depicted in **Table 5.1**. The highly efficient pre-packed column consisting of a composite matrix of dextran and deeply cross-linked agarose was utilized for the purification. The chromatogram obtained using the size-exclusion chromatography performed on the Superdex column was shown in **Figure 5.2** and the eluted fractions 14-17 were collected, pooled, dialyzed and concentrated using Amicon centrifugal concentrators.



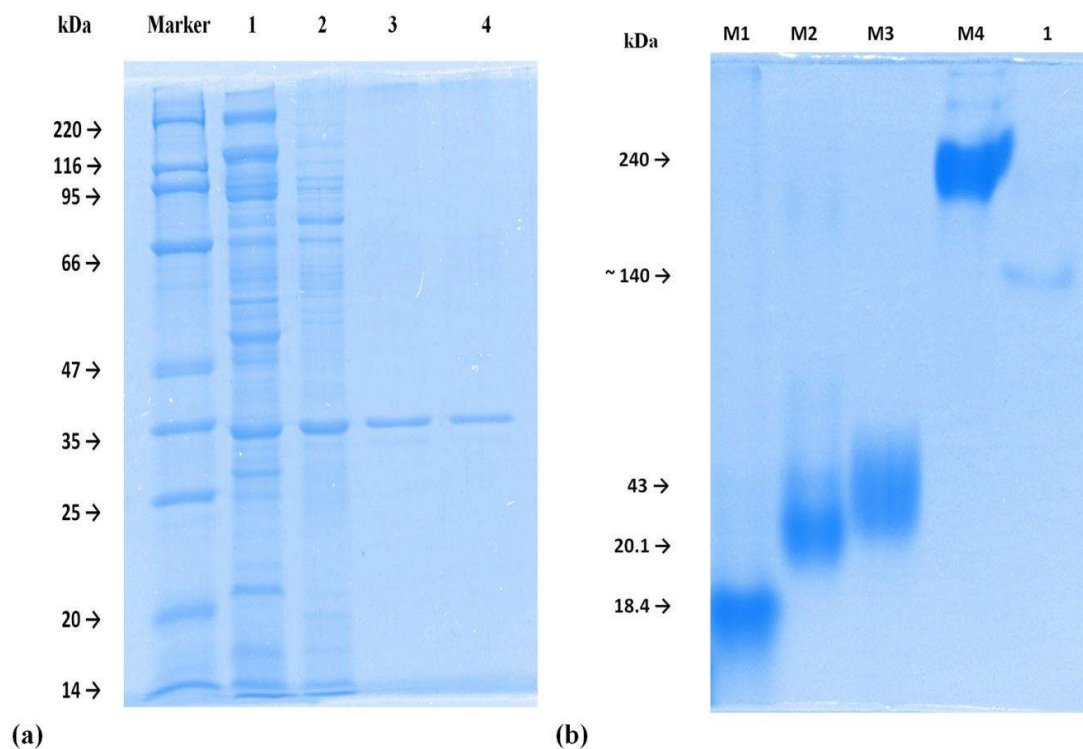
**Figure 5.2:** Chromatogram depicting the elution profile obtained on size-exclusion chromatography (SEC) column for the purification of L-asparaginase.

**Table 5.1:** Summary of the purification procedure employed for L-asparaginase purification from *Bacillus indicus* MTCC 4374.

Purification steps	Total Units (IU)	Total Protein (mg)	Specific activity (IU/mg)	Activity Yield (%)	Purification Fold
Crude Extract	41053	3825	10.73	100.00	-
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> salt precipitation	23326	1198.6	19.46	56.81	1.81
DEAE–cellulose anion exchange chromatography	9581	72.3	132.51	23.33	12.34
Sephadex – G 200 pg gel filtration chromatography	5531	28.1	196.83	13.47	18.34

### 5.3.5 Determination of molecular weight and purity

The purified enzyme on SDS-PAGE gel was seen as a single band with an approximate molecular mass of 35 kDa (**Figure 5.3a**). The experimental molecular weight obtained was confirmed using the in-silico analysis of predicted molecular weight for the L-asparaginase of *Metabacillus indicus* on the NCBI protein database. The producer organism *Bacillus indicus* MTCC 4374 or *Metabacillus indicus* is a type strain available in different culture collections with following equivalent numbers (Equivalent number: DSM 15820, LMG 22858) and can be searched through BacDive database (Bacterial diversity Metadatabase) (<https://bacdive.dsmz.de/strain/1233>). Similarly, Native PAGE analysis was performed to confirm the intact tetrameric nature of L-asparaginase from *Bacillus indicus* MTCC 4374 with a molecular weight of  $\sim 140.0 \pm 1.0$  kDa (**Figure 5.3b**). The tetrameric structure of the bacterial L-asparaginases has been reported in several studies while the fungal L-asparaginases were known to possess a trimeric native structure (Strzelczyk *et al.* 2020; Vala *et al.* 2018; Lubkowski and Wlodawer, 2021).

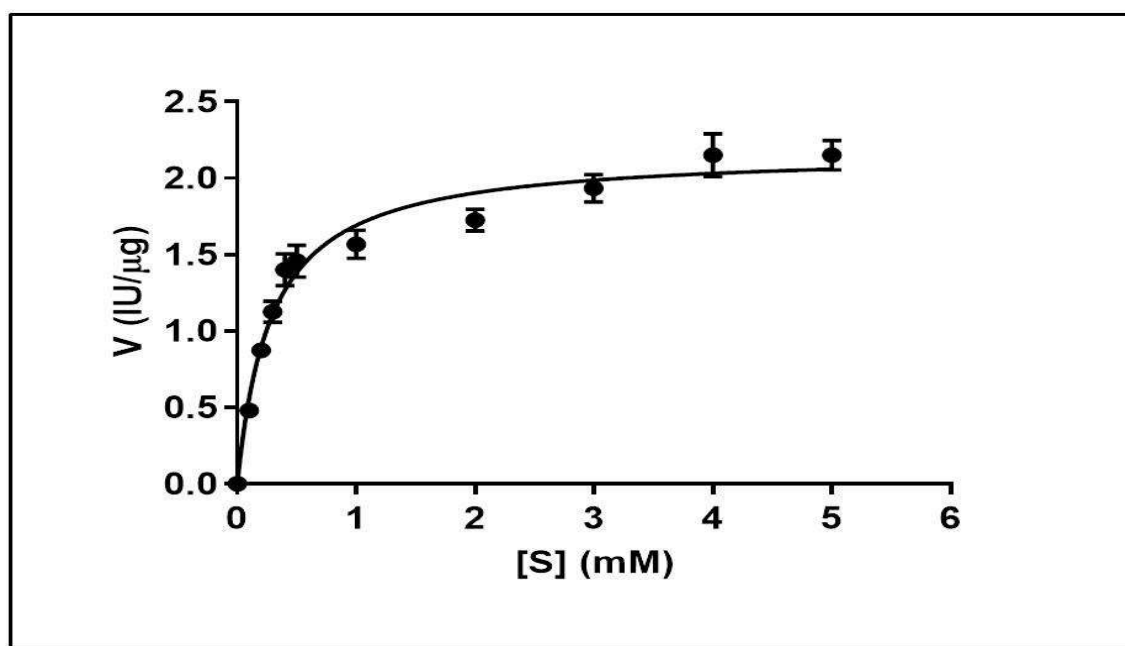


**Figure 5.3:** (a) Assessment of molecular weight of L-asparaginase by *Bacillus indicus* MTCC 4374 using a 12 % SDS-PAGE gel depicting standard marker lane, crude extract in lane 1, ammonium sulfate purified extract in lane 2, anion exchange chromatography purified extract in lane 3, followed by size exclusion chromatography purified extract in lane 4; and (b) 7.5 % Native PAGE gel depicting the marker proteins in lanes M1, M2, M3, M4 and the tetrameric nature of the purified L-asparaginase in lane 1 (Sharma and Mishra, 2023).

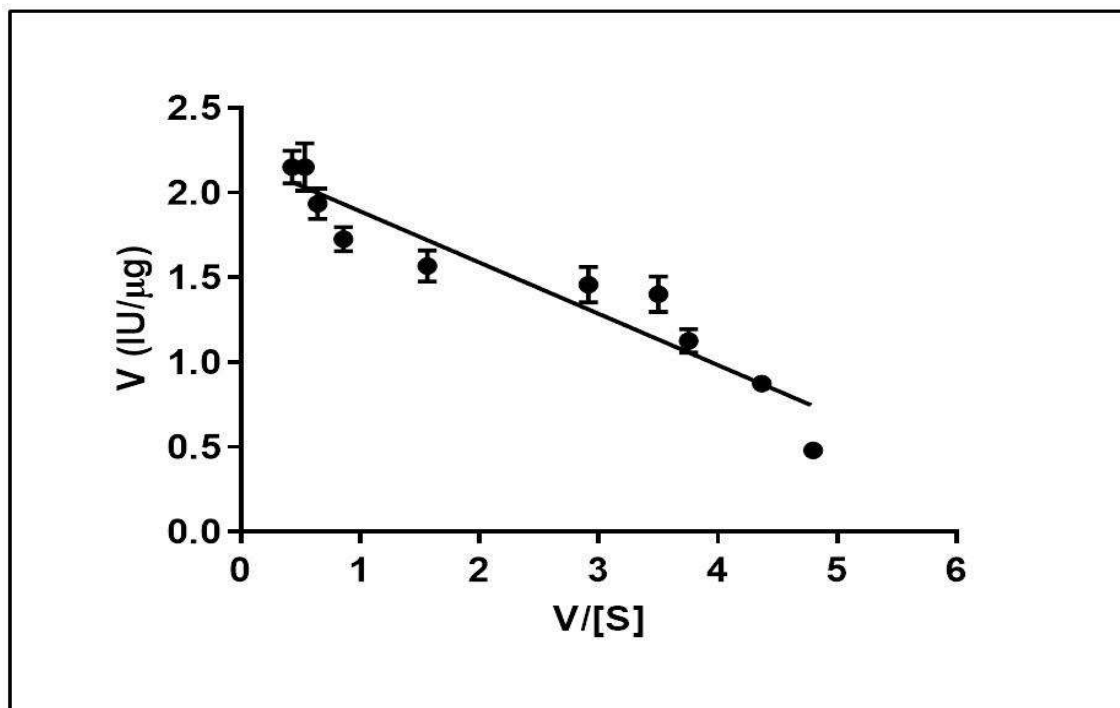
### 5.3.6 Evaluation of kinetic parameters and substrate specificity

The purified enzyme followed the classical Michaelis-Menten kinetics (**Figure 5.4**). The linearization of the Michaelis-Menten plot was depicted in the form of Eadie-Hofstee plot for the determination of kinetic parameters (**Figure 5.5**). The  $K_m$  and  $V_{max}$  kinetic parameters were 0.287 mM and 2.178 IU/ $\mu$ g respectively. The turnover number ( $k_{cat}$ ) and specificity constant ( $k_{cat}/K_m$ ) were evaluated to be 1263.24 s<sup>-1</sup> and 4.40 $\times$ 10<sup>3</sup> mM<sup>-1</sup> s<sup>-1</sup>. The low value of  $K_m$  obtained with the *Bacillus indicus* MTCC 4374 L-asparaginase is a favorable aspect as it

signifies the high binding affinity of the enzyme to the substrate L-asparagine and is also required for the specific removal of L-asparagine to target the leukemia cells. Earlier studies on the L-asparaginases from different sources reported the  $K_m$  values ranging from 0.074 mM to 3.5 mM (Yaacob *et al.* 2014). The  $K_m$  shown by the purified L-asparaginase was found to be significantly lower than the  $K_m$  values for different L-asparaginases, viz. 3.5 mM for L-asparaginase of *E. coli* (Willis and Woolfolk, 1974), 1.58 mM for *Enterobacter cloacae* (Husain *et al.*, 2016), 9.74 mM for *Sarocladium strictum* (Golbabaie *et al.*, 2020) and  $0.559 \pm 0.12$  mM for *Pseudomonas* sp. PCH44 (Kumar *et al.*, 2022). Also, the  $V_{max}$  obtained in the present study was within the range of  $V_{max}$  reported for other cytotoxic L-asparaginases in previous studies. The L-asparaginases from *Bacillus aryabhatai*, *Enterobacter cloacae*, and *Pseudomonas* sp. PCH44 reported a  $V_{max}$  of 1.537 IU/ $\mu$ g, 2.22 IU/ $\mu$ g, and 29.41 U/mg, respectively (Singh *et al.*, 2013; Husain *et al.*, 2016; Kumar *et al.*, 2022).



**Figure 5.4:** Michaelis-Menten kinetics plot between the reaction velocity ( $V$ ) versus the substrate concentration  $[S]$  of the purified L-asparaginase ( $R^2 = 96.71$ ) (Sharma and Mishra, 2023).



**Figure 5.5:** Linearization of the Michaelis-Menten kinetics plot in the form of Eadie-Hofstee plot for the purified L-asparaginase.

In terms of substrate specificity, the relative activities shown towards the diverse substrates by the purified asparaginase are depicted in **Table 5.2**. The enzyme displayed the highest activity for the substrate L-asparagine and an extremely low/negligible level of activity towards L-glutamine (<1). Thus, the enzyme fulfills the potential of high specificity toward the hydrolysis of the amino acid L-asparagine, which is an essential requirement for the L-asparaginase to show minimal side effects. No other catalytic activity towards the other substrates was observed.

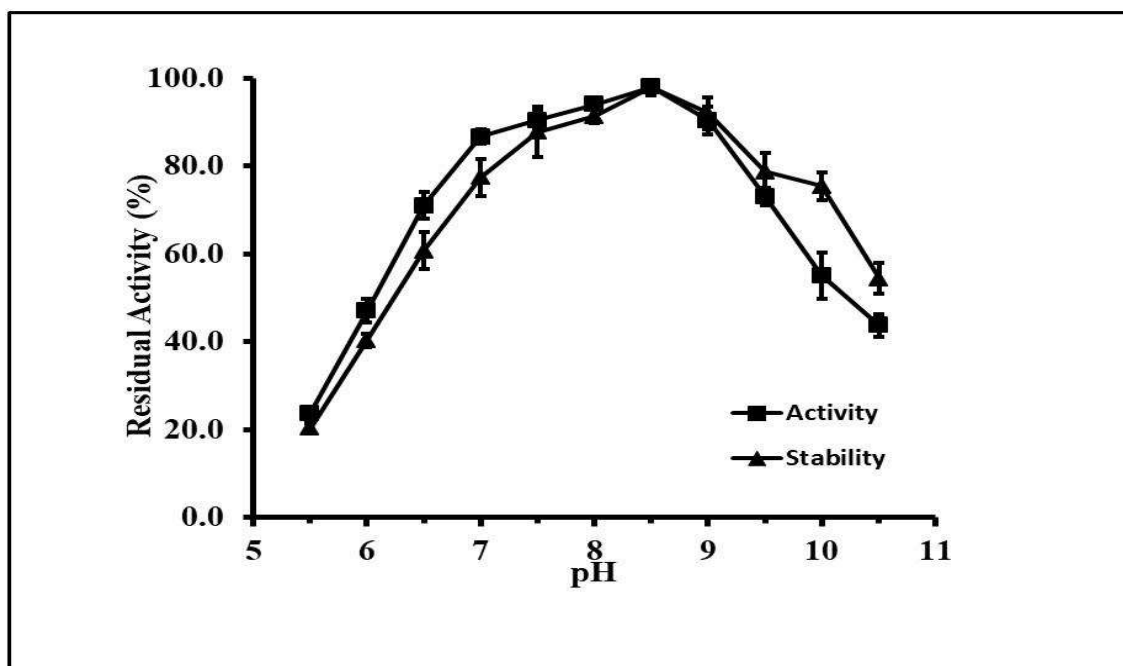
**Table 5.2:** Substrate specificity shown by the purified L-asparaginase from *Bacillus indicus* MTCC 4374.

Substrate	Concentration (mM)	Relative Activity (%) <sup>a</sup>
L-asparagine	10	100.0
D-asparagine	10	N.D.
L-glutamine	10	0.8 ± 0.12
D-glutamine	10	N.D.
L-ornithine	10	N.D.
Urea	10	N.D.

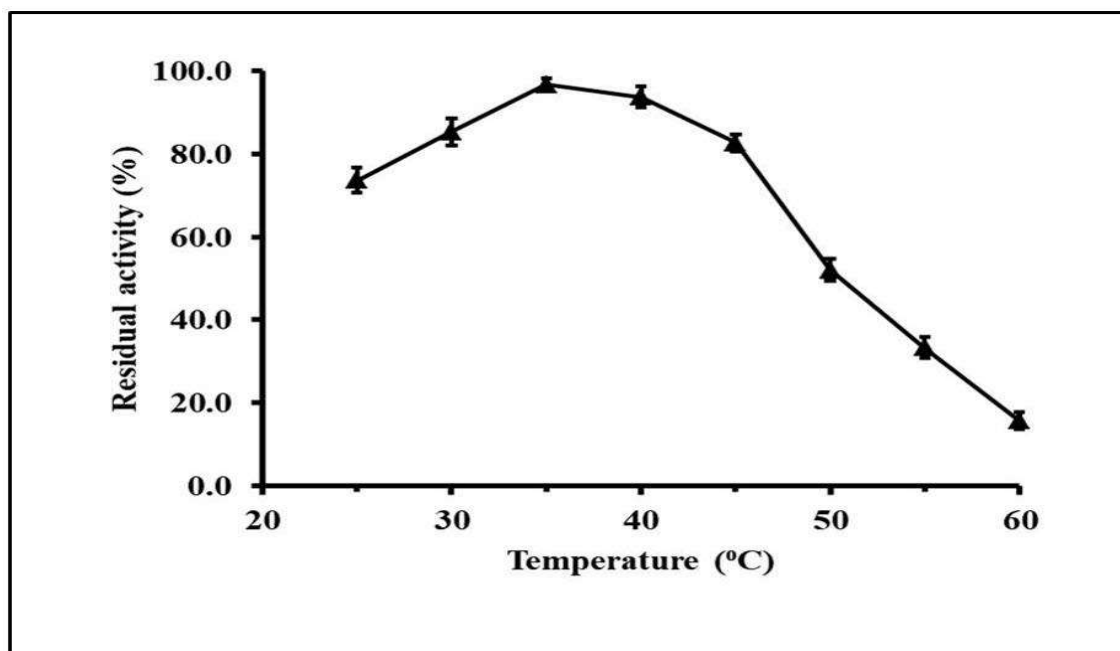
<sup>a</sup> Mean values (n=3) ± standard deviation

### 5.3.7 pH and temperature effects on the purified L-asparaginase

The purified L-asparaginase enzyme from *Bacillus indicus* MTCC 4374 showed significant enzyme activity in the pH range of 6-10 with more than 90 % of activity obtained near physiological pH (**Figure 5.6**). Moreover, in terms of pH stability, the enzyme was found to be active in the pH values of 6.5-8.5 over the period time of 24 hours (**Figure 5.6**). The enzyme was highly active in the 30° - 40°C temperature range (optimum) and a decline in activity was seen on either side of the range (**Figure 5.7**).



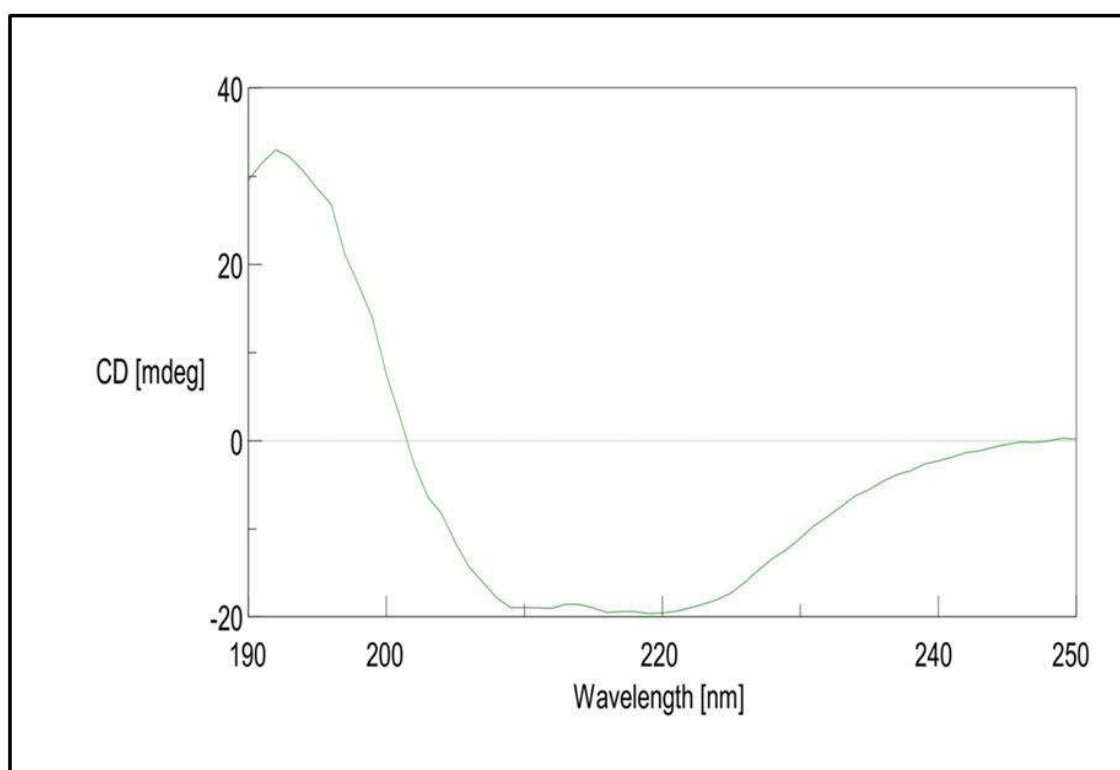
**Figure 5.6:** pH effects on the enzyme activity and enzyme stability of L-asparaginase from *Bacillus indicus* MTCC 4374 (Sharma and Mishra, 2023).



**Figure 5.7:** Temperature effects on the enzyme activity of L-asparaginase from *Bacillus indicus* MTCC 4374 (Sharma and Mishra, 2023)

### 5.3.8 Secondary structure evaluation

The secondary structure analysis of the purified L-asparaginase was performed in the wavelength range of 260-190 nm in far UV region. The CD spectra converged into two negative ellipticities at 222 nm and 208 nm wavelengths and a higher ellipticity was observed at a later wavelength (**Figure 5.8**). Further analysis demonstrated that the purified enzyme belongs to the mixed ( $\alpha + \beta$ ) class of proteins as it contains 38.7 % alpha helix region and 27.4 % beta sheets region.



**Figure 5.8:** Circular dichroism spectroscopy spectrum of the purified L-asparaginase from *Bacillus indicus* obtained at an enzyme concentration of 0.1 mg/mL at 37 °C.

### 5.4 Conclusion

The successful purification of the L-asparaginase enzyme preparations is highly dependent on the combinatorial utilization of efficient precipitation and chromatography procedures. The present study reports the successful purification of the enzyme to homogeneity using a

tandem combination of ion-exchange chromatography (IEC) and size exclusion chromatography (SEC) techniques. The purified enzyme was found out to be a homotetramer on Native PAGE analysis. The molecular weight of each monomeric subunit was found to be 35 kDa. The secondary structure elucidation using circular dichroism (CD) spectroscopy revealed the ( $\alpha + \beta$ ) mixed type of secondary structure for the purified L-asparaginase from *Bacillus indicus* MTCC 4374. The enzyme preparation was highly specific towards the substrate L-asparagine by possessing significant L-asparaginase activity. The purified L-asparaginase exhibited a very low  $K_m$  value. Hence, indicating a strong binding affinity towards the L-asparagine (substrate) which is essentially required for the specific removal of L-asparagine to target the cancer cells. The enzyme preparation was found to be optimally active at physiological pH and temperature making it highly suitable as a new enzyme preparation for the therapeutic and food processing applications.