CHAPTER 7

Purification and Characterization of L-Glutaminase

7.1. Introduction

Most of the microbial glutaminase are extracellular and the fermentation process is usually followed by removal of cells from the culture broth, either by centrifugation or filtration. The cell-free culture broth is then concentrated by ultra filtration, ammonium sulphate precipitation or extraction with organic solvents (ethanol, acetone). Most of the purification schemes are attempted with precipitation of 60-80% ammonium sulphate followed by a combination of several chromatographic methods such as ion exchange, gel filtration and affinity chromatography (Pabai et al., 1995). Most of the time, a single chromatographic step is not sufficient to get the required level of purity, and hence, a combination of chromatographic steps is required. Ion exchange chromatography as most common chromatographic method is used for purification of microbial glutaminase. Diethylaminoethyl (DEAE) group in anion-exchange and carboxymethyl (CM) in cation-exchange are most frequently employed ion-exchangers. Gel filtration and affinity chromatography methods are also employed for high degree of purification.

Various reports are available for purification and characterization of glutaminase from different microbes like *Aspergillus oryzae* (Yano et al., 1988), *Debaryomyces* sp (Dura et al., 2002), *Saccharomyces cerevisiae* (Soberon and Gonzalez, 1987), *Pseudomonas* sp.(Imada et al., 1973; Ohshima et al., 1976; Soda et al., 1972; Tachiki et al., 1996), *Micrococcus luteus* (Moriguchi et al., 1994), *Escherichia coli* (Hartman, 1968; Hartman and McGrath, 1973; Hartman and Stochaj, 1973), *Clostridium welchii* (Kozlov et al., 1972), *Bacillus pasteurii* (Klein et al., 2002); *Achromobacteraceae* (Roberts et al., 1972).

Glutaminase produced by different microorganism showed different optimum temperature and pH for its activity which indicates the suitability of their use in food or pharmaceutical industry (Wade et al., 1971; Iwasa et al., 1987; Klein et al., 2002; Dura et al., 2002; Sinsuwan et al., 2012; Ito et al., 2013).

In food industry, microbial glutaminases are commonly used as flavor enhancing agent by contributing sharp taste in soy products. Some microbial glutaminases are inhibited by high salt concentration in the soy sauce fermentation process (Yano et al., 1988) and hence salt-tolerant glutaminases (Moriguchi et al., 1994; Nakadai and Nasuno, 1989) are preferred for this fermentation process. Microbial glutaminases with high activity at physiologic pH-7.4, optimum temperature-37˚C and low K_m values for L-glutamine substrate (10^{-6} to 10^{-3}M) are reported to be suitable for therapeutic use (Roberts et al., 1970; Wade et al., 1971; Roberts et al., 2001). Metal ions participate as cofactor in catalytic activity of enzyme. These metal ions effect the ionic interaction of enzyme substrate complex and results stimulation or inhibition of activity of enzyme. Some glutaminases showed higher activity in presence of metal ions (Dura et al., 2002; Abe et al., 1974; Hartman, 1968; Holcenberg et al., 1978). L-glutaminases produced by *Debaryomyces* sp. (Dura et al., 2002), *Pseudomonas* sp. (Abe et al., 1974), *Escherichia coli* (Hartman, 1968), and *Acinetobacter* (Holcenberg et al., 1978) were reported as metalloenzymes which showed lower activity in presence of EDTA. Effect of some thiol-binding agents like, p-chloro mercuric benzoic acid, iodoacetamide and reducing agents like β-mercaptoethanol, glutathione, L-ascorbic acid, cysteine and dithiothreitol on the activity of purified glutaminase was studied to estimate the presence of sulfhydryl groups in their catalytic site (Kvamme

et al., 2000; Dura et al., 2002; Jeon et al., 2010; Davidson et al., 1977; Penninckx and Jaspers, 1985).

In this study, L-glutaminase from *Bacillus cereus* MTCC 1305 was purified to homogeneity using different purification steps like ammonium sulphate precipitation, dialysis, DEAE cellulose chromatography, SDS-PAGE and native-PAGE. The optimum pH and temperature of the purified L-glutaminase was determined to find out the interference of the different metal salts and other substances which may be present in the fermented broth. Thermo stability and thermal deactivation kinetic of the purified L-glutaminase at different temperature was further studied. In addition, the substrate specificity of the purified enzyme, its kinetic parameters and effect of different metal salts, EDTA, reducing agents, thiol binding agents on its activity were studied. The thermal deactivation kinetic of L-glutaminase was studied at temperature 35, 45, 50, and 60˚C.

7.2. Materials and methods

7.2.1. Chemicals

L-glutamine, D-glutamine, L-asparagine, D-asparagine, L-glutamic acid, L-γ glutamyl para-nitroanilide, $MgSO_4$, $MnCl_2$, $ZnSO_4$, $PbCl_2$, $CaCl_2$, $CoCl_2$, $HgCl_2$, $CdCl_2$, $CuCl_2$, NaCl, KCl, EDTA, iodoacetamide, L-cysteine, L-histidine, glutathione, 2-mercaptoethanol, p-chloro mercuric benzoic acid, sodium dodecyl sulphate, urea and coomassie brilliant blue R-250 stain were procured from Hi Media, India. Standard molecular weight marker (10-250KD) used in native PAGE was obtained from Bio-

Rad, India and marker (6-205KD) used in SDS–PAGE was obtained from Genei, India. All chemicals used in this study were of AR grade.

7.2.2. Production of L-glutaminase

The growth media and culture conditions for *Bacillus cereus* MTCC 1305 was already mentioned in Chapter3. The fermentation process of L-glutaminase was carried out with inoculation of 2litre production media (pH-7.5) containing sucrose (2.5g/l), peptone (2.5g/l), L-glutamine (5.0g/l), Na_2HPO_4 (6.0g/l), NaCl (0.5g/l) and $MgSO_4$ (0.5g/l) with 40ml inoculum in 5litre fermenter at optimum agitation speed (300rpm), aeration rate (2vvm) and dissolved oxygen concentration (≥20%) for 40hrs. Cell free broth was collected after centrifugation at 10,000×*g* at 4±1˚C for 10minutes and used as an enzyme source.

7.2.3. Enzyme purification and quantification

L-glutaminase from *Bacillus cereus* MTCC 1305 was purified from cell free fermented broth by the following methods:

7.2.3.1. Ammonium sulfate precipitation

Finely powdered ammonium sulfate was added to the crude extract of enzyme with constant stirring and kept for overnight. Maximum L-glutaminase activity was obtained with the fraction precipitated at 60–90% saturation. The precipitate was collected by centrifugation at 10000×g for 30min and dissolved in a minimal amount of 50mM Tris–HCl buffer (pH-7.2) and dialyzed against the same buffer for 24hrs using dialysis membrane-60 (Hi-media).

7.2.3.2. DEAE cellulose chromatography

The partially purified enzyme was further loaded on DEAE-cellulose column. Before loading the sample, the column was pre equilibrated with 50mM Tris–HCl (pH-7.2) at a flow rate of 3.5mlmin^{-1}. The column was washed with two volume of the above buffer and the adsorbed enzyme was eluted using a linear gradient of NaCl (0–0.6M) in 50mM Tris–HCl (pH-7.2). Different fractions of the purified enzyme were collected using fraction collector (LKB Bromma-21110 Multitrac, India). Purified fractions containing L-glutaminase activity were pooled, dialyzed with Tris–HCl (50mM and pH-7.2) and concentrated with amicon ultra filter unit (Merck Millipore).

7.2.3.3. Electrophoretic analyses

The molecular weight of native L-glutaminase and its subunits were determined using native-PAGE and SDS-PAGE electrophoresis respectively. Native PAGE of the purified L-glutaminase was performed on 7.5% polyacrylamide gel (pH-8.8) and 5% stacking gel (Tris-HCl buffer, pH-6.8, 30% acrylamide, 0.8% bisacrylamide, TEMED and 10% ammonium persulphate) at 4±1˚C as described by Gallagher (1995). SDS–PAGE was performed by the modified method of Laemmli (1970) with a 12.5% separating acrylamide gel (pH-8.8) and 5% stacking gel (Tris-HCl buffer, pH-6.8, 30% acrylamide, 0.8% bisacrylamide, TEMED, 10% SDS and 10% ammonium persulphate). Electrophoresis was performed at 120V for 3hrs at room temperature and proteins in the gel were stained with coomassie brilliant blue R-250. The molecular weight of intact enzyme and its subunit was determined using standard molecular weight protein marker in Native PAGE and SDS–PAGE, respectively.

7.2.3.4. Estimation of Isoelectric point

Isoelectric focusing of polyacrylamide gel electrophoresis (IEF–PAGE) was performed in a vertical gel apparatus using ampholytes (Bio-Rad, India) of pH 3–10. 2µg purified L-glutaminase was loaded to the gel and kept at 200–400V for a period of 4–5hrs. Protein with specific pI was visualized with coomassie brilliant blue R-250 stain on gel band (Deutscher, 1990).

7.2.4. Characterization of purified L-glutaminase

7.2.4.1. Effect of pH on activity of L-glutaminase

Effect of different pH (5.5-9.5) on the activity of purified L-glutaminase was studied using 100mM sodium acetate buffer for pH 5, and 5.5; 100mM Tris-maleate buffer for pH 6, and 6.5; 100mM Tri–HC1 buffer for pH 7.0, 7.5, 8, and 8.5 and 100mM glycine -NaOH buffer for pH 9, and 9.5.

7.2.4.2. Effect of temperature on activity and stability of L-glutaminase

The optimal temperature for activity of L-glutaminase was measured by incubating enzyme sample with L-glutamine as substrate at different temperatures (20-70˚C) in 100mM Tris-HCl buffer (pH-7.5). Thermostability of L-gutaminase was investigated by pre incubating the purified enzyme sample in 100mM Tris-HCl buffer (pH-7.5) without L-glutamine as substrate at different temperature (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70˚C) for 30minutes.

7.2.4.3. Effect of different effectors on the activity of purified L-glutaminase

Effect of various metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Ca^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, Na^{+} and K^{+}), phosphate ions, and chelator (EDTA), reducing agents (β-mercaptoethanol,

glutathione, L-ascorbic acid, cysteine and dithiothreitol) and thiol binding agents (p-Chloro mercuric benzoic acid (p-CMBA), iodoactamide) on activity of L-glutaminase was determined after adding them in the standard assay mixture with the concentration ranging from 5-100mM. The activity was expressed as relative activity compared to control.

7.2.4.4 Substrate specificity tests for L-glutaminase

Substrate specificity for L-glutaminase was determined by using different substrates (40mM) like D-asparagine, L-asparagine, L-glutamine, D-glutamine, D-aspartic acid, L-aspartic acid, and L-glutamic acid.

7.2.4.5. Estimation of kinetic parameters

Kinetic parameters such as Michaelis constant (K_m), and maximum reaction velocity (V_{max}) were determined using Lineweaver burk plot at different concentration of L-glutamine (1, 4, 8, 16, 20, 24, 40, 50mM). The hydrolysis of maximum number of substrate molecules into product per enzyme molecule per second was calculated in term of turnover number by using the formulae:

$$K_{cat} = V_{max}/E_t \quad \text{.....(7.1)}$$

Where, $K_{(cat)}$=Turnover number, E_t= Total enzyme concentration, $V_{(max)}$=Maximum reaction velocity

Catalytic efficiency of enzyme is a measure of how efficiently an enzyme converts a substrate into product and it was determined using following formulae:

$$\text{Catalytic efficiency} = K_{cat}/K_m \quad \text{.....(7.2)}$$

7.2.4.6. Thermal deactivation studies of L-glutaminase

Enzyme deactivation is one of the major constraints in the development of biotechnological processes. Experiments were conducted to study the thermal deactivation of purified L-glutaminase. The partially purified enzyme was incubated at different temperature (35, 45, 50, and 60˚C) for different intervals of time (30, 60, 90, 120, 150, 180, 210 and 240minutes). The deactivation of L-glutaminase enzymes is assumed to be first-order kinetic and followed reaction mechanism as proposed by Anthon and Barret (2002):

$$A_0 \longrightarrow A \ldots\ldots\ldots\ldots\ldots\ldots (7.3)$$

The assumption in the mechanism is that the active enzyme (A_0) without providing any significant amount of intermediates directly converts to inactive state (A):

$$d[A]/dt = -K_d[A_0] \ldots\ldots\ldots\ldots\ldots (7.4)$$

The following equation was obtained on integration of equation 7.4:

$$Ln[A] = ln[A_0] - K_d\, t \ldots\ldots\ldots\ldots\ldots (7.5)$$

Deactivation rate constant (K_d) was estimated from slope of graph plotted for Ln[A] versus time (t). The half-life of an enzyme (time required by the enzyme to lose half of its initial activity) was determined using following formulae:

$$\text{Half life } (t_{1/2}) = Ln2/ K_d \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7.6)$$

The conformation deactivation energy (E_d) was calculated from slope of plot between Ln(K_d) and reciprocal of absolute temperature using the following the Arrhenius equation:

$$Ln(K_d) = Ln(K_0) – E_d/RT……(7.7)$$

Where, R is gas constant ($8.314 mol^{-1}K^{-1}$) and T is the temperature in Kelvin.

The change in enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) of the enzyme deactivation were determined using the following equations:

$$\Delta H = E_d - RT……(7.8)$$

$$\Delta S = R\ [Ln(K_0) – Ln(KB/h_p) - LnT]……... (7.9)$$

$$\Delta G = \Delta H - T\Delta S……..…(7.10)$$

Where, KB=Boltzmann constant ($1.3807x10^{-23}$J/K), h_p=Planks constant ($6.626\ x10^{-34}m^2kg/s$), R=Gas constant ($8.314 Jmol^{-1}K^{-1}$), and T=Temperature (Kelvin)

7.3. Results and discussion

7.3.1. Purification and quantification of L-glutaminase

L-glutaminase from *Bacillus cereus* MTCC 1305 was partially purified after 60-90% ammonium sulfate fractionation. The enzyme was further purified to homogeneity after loading on DEAE-Cellulose column in anion exchange chromatography. The bound proteins were eluted with a linear salt gradient of NaCl (0–0.6M) in 50mM Tris–HCl (pH-7.2) and total 160 fractions were collected at a flow rate of 3.5ml/min. All fractions were assayed for protein concentration and L-glutaminase activity. The elution profile was resolved into two peaks and maximum L-glutaminase activity was obtained for fraction 55-100 within peak II (Fig7.1). These fractions were found to be homogeneous and resulted in a single band after electrophoresis. L-glutaminase was purified with specific activity of $7.44 Umg^{-1}$, 14.8fold purification and 54.32% yield as shown in Table7.1.

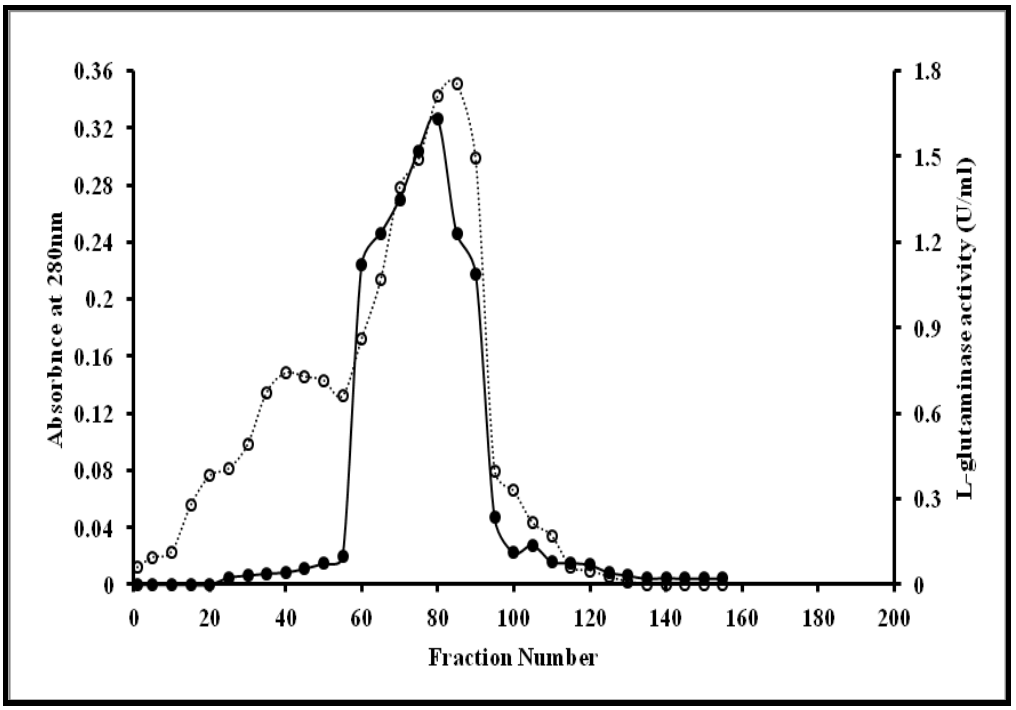

Fig.7.1. Elution profile of L-glutaminase by anion exchange chromatography on a DEAE-cellulose column with protein concentration (⋯○⋯) and L-glutaminase activity (—●—)

Table7.1. Purification of L-glutaminase from *Bacillus cereus* MTCC 1305

Purification steps	Total Activity (Unit)	Total protein (mg)	Specific Activity (Unit/mg)	Yield (%)	Purification fold
Crude Extract	3260	6495	0.502	100	1
Ammonium sulfate precipitation	2832	3972	0.713	86.87	1.42
DEAE-Cellulose chromatography	1771	238	7.44	54.32	14.8

The fractions having high activity of L-glutaminase were pooled, concentrated and stored at 4˚C for further use. The purified L-glutaminase showed one band on SDS-PAGE (Fig7.2a) corresponding to molecular weight of its subunits as approximately 36KD. The molecular mass of native enzyme was determined as approximately 144KD on native-PAGE (Fig7.2b). These results showed that the native L-glutaminase was of homotetrameric nature with four identical subunits. L-glutaminase from *Bacillus cereus* (Ivanova et al., 2003), *Escherichia coli* YbaS (Brown et al., 2008), *Bacillus subtilis* 168 YbgJ (Brown et al., 2008), *Pseudomonas acidovorans* (Davidson et al., 1977) were reported as homotetramer. Some microorganisms like *Bacillus licheniformis* GlsA (Sinsuwan et al., 2012), *Pseudomonas nitroreducens* IFO12694 (Tachiki et al.,1996), *Stenotrophomonas maltophilia* NYW-81 (Wakayama et al., 2005), and C*ryptococcus albidus* (Iwasa et al., 1987) were reported to produce monomeric L-glutaminase. The purified L-glutaminase from *Micrococcus luteus* K-3 (Moriguchi et al., 1994), *Bacillus pasteurii* (Klein et al., 2002), *Escherichia coli* YneH (Brown et al., 2008), *Bacillus subtilis* 168 YlaM (Brown et al., 2008), *Lactobacillus reuteri* KCTC3594 (Jeon et al., 2010), *Aspergillus sojae* (Ito et al., 2013)*, Debaryomyces* sp. (Dura et al., 2002) was reported as dimer.

Protein can also be separated electrophoretically on the basis of their relative contents of acidic and basic residues. The isoelectric point (pI) for purified protein is defined as pH at which net charge of protein is zero and thus its electrophoretic mobility is zero. Each protein will move until it reaches a position in the gel at which the pH is equal to the pI of the protein. This method of separating proteins according to their isoelectric point is called isoelectric focusing.

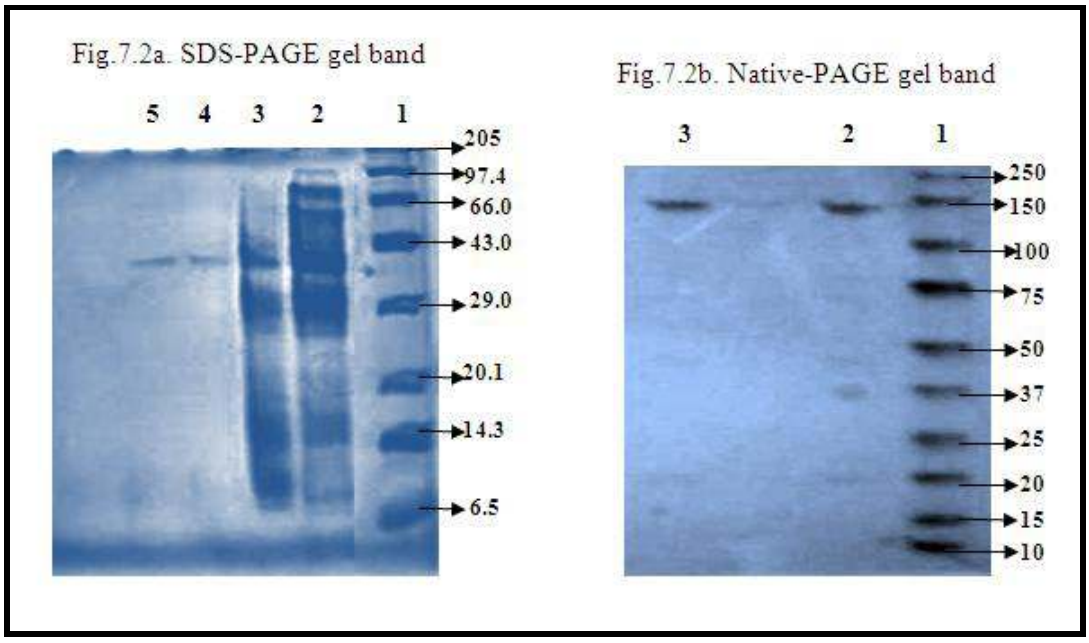

Fig.7.2. Assessment of homogeneity and molecular weight of L-glutaminase from *Bacillus cereus* MTCC 1305 after electrophoresis (a) SDS-PAGE gel band with electrophorized lanes 1, 2, 3, 4, and 5 for molecular weight marker (in Kilodalton), crude extract, $(NH_4)_2SO_4$ precipitated sample, purified fraction1, and purified fraction 2, respectively (b) Native-PAGE gel band with lanes 1, 2, and 3 for molecular weight marker (in Kilodalton), purified fraction 1, and purified fraction 2

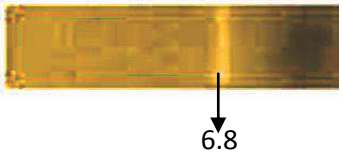

Fig.7.3. Estimation of pI for purified L-glutaminase from *Bacillus cereus* MTCC 1305 on gel band stained with coomassie brilliant blue R-250

A gradient of pH will be developed after loading poly-ampholytes having different pI values on the gel band. The isoelectric point (pI) of purified L-glutaminase from *Bacillus cereus* MTCC 1305 was found close to 6.8 (Fig7.3). L-glutaminase produced by *Clostridium welchii* (Kozlov et al., 1972), *Escherichia coli* (Prusiner et al., 1976), *Acinetobacter glutaminasificans* (Holcenberg et al., 1978), *Pseudomonas* (Holcenberg and Teller, 1976), *Cryptococcus albidus* (Yokotsuka and Sasaki, 1986), *Pseudomonas acidovorans* (Davidson et al., 1977), and *Bacillus pasteurii* (Marcus et al., 2002) showed different pI as 5.5, 5.4, 8.43, 5.8, 3.9, 4.1, and 4.9 respectively.

7.3.2. Effect of pH on activity of L-glutaminase

The relative activity of purified L-glutaminase was obtained as about 90% in pH range from 7.0 to 8.0 and about 30% at pH 8.5. The maximum activity of L-glutaminase was obtained at optimum pH-7.5 (Fig7.4). The optimum pH was reported as 6.0, 7.5, and 9.0 for glutaminase produced from *Cryptococcus albidus* (Iwasa et al., 1987), *Lactobacillus reuteri* KCTC3594 (Jeon et al., 2010), *Bacillus pasteurii* (Klein et al., 2002) respectively. Glutaminase produced from *Saccharomyces cerevisiae* was reported to exhibit two forms of GlsA and GlsB with optimum pH 7.5 and 8.1 respectively (Soberon and Gonzalez, 1987). Glutaminase from *Escherichia coli, Proteus morganii* and *Clostridium welchii* showed optimum activity at about pH 5 (Wade et al., 1971). Glutaminases with optimum pH above neutrality was found suitable for clinical purposes (Roberts et al., 1970, Wade et al., 1971). The optimum pH for crude extract of L-glutaminase was reported as 7.5 in Chapter 3 which was found to be similar to optimum pH obtained for purified L-glutaminase. This result

indicates that the presence of metal salts and other interfering substance in the fermented broth of L-glutaminase does not affect the optimum pH of L-glutaminase.

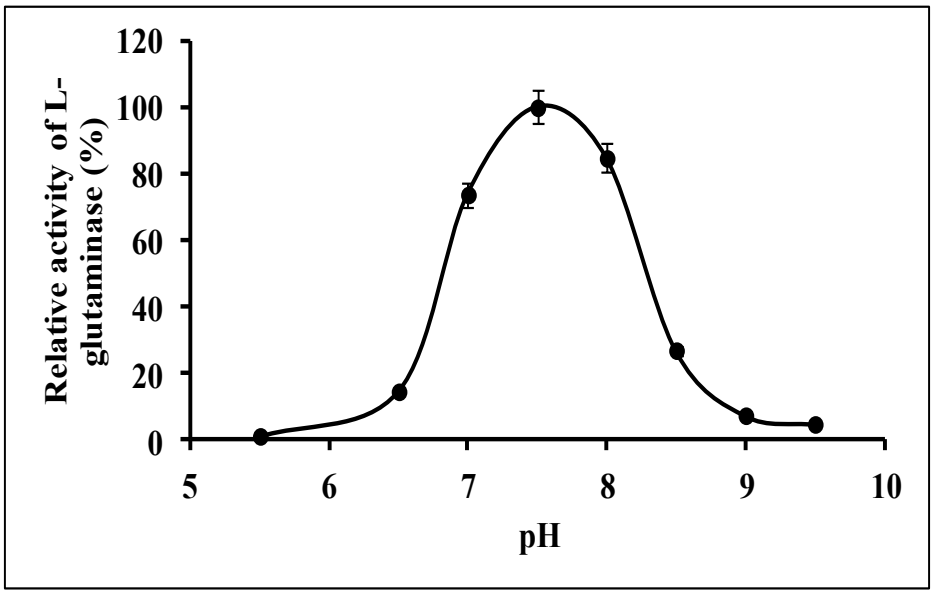

Fig.7.4. Effect of pH on activity of L-glutaminase produced from *Bacillus cereus* MTCC 1305

7.3.3. Effect of temperature on activity of L-glutaminase

Each enzyme shows reduction in catalytic activity beyond the optimal temperature range. This may be due to change in the secondary and tertiary levels of protein structure which occurred due to disturbance in the intra molecular attractions (hydrogen bonding and dipole-dipole attractions) between polar groups as well as the hydrophobic forces among non-polar groups within the protein structure. The effect of temperature on L-glutaminase activity was studied by incubating the reaction mixture at different temperature ranging from 20 to 70˚C. The maximum activity of the purified L-glutaminase was observed at 35˚C (Fig7.5a). The enzyme showed relative activity above 70% for temperature 30, 45˚C, while showed about 20% activity at

50˚C. The optimum temperature was reported as 37˚C, 70˚C, 40˚C, 50˚C, and 30˚C for purified L-glutaminase from *Bacillus pasteurii* (Klein et al., 2002), *Cryptococcus albidus* (Iwasa et al., 1987), *Debaryomyces* sp. (Dura et al., 2002), *Aspergillus sojae* (Ito et al., 2013), recombinant *Bacillus licheniformis* (Sinsuwan et al., 2012). The maximum activity of L-glutaminase in cell free fermented broth was reported at 35˚C in Chapter 3 which was found to be similar to the optimum temperature (35˚C) obtained for purified L-glutaminase. This result indicates that the level of optimum temperature for L-glutaminase does not get affected in presence of metal salts and other interfering substance in the fermented broth of L-glutaminase.

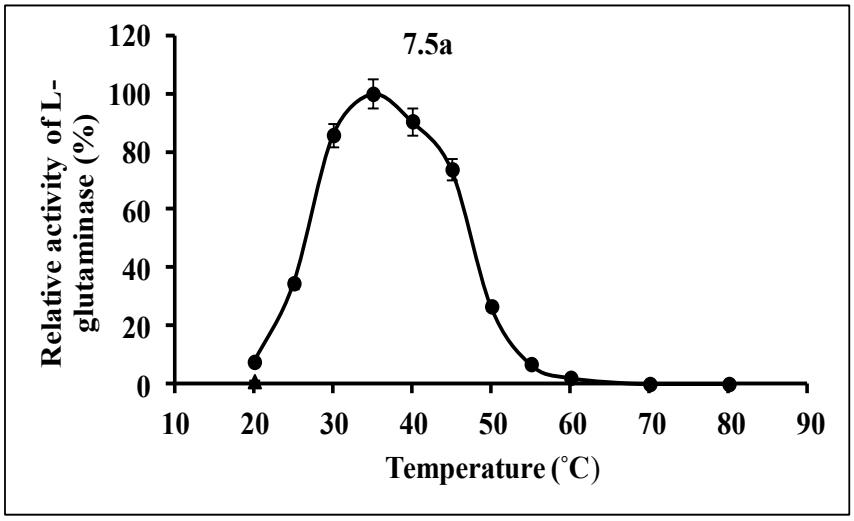

Fig.7.5a. Relative activity of purified L-glutaminase from *Bacillus cereus* MTCC 1305 at different temperature

The results of stability of purified L-glutaminase at different temperature (25-60˚C) are summarized in Fig7.5b. Purified L-glutaminase retained upto 95% relative stability at temperature 20, 25, 30, and 35˚C. The relative stability of purified L-glutaminase decreased at higher temperature and found to be upto 50% stable at 50˚C. Beyond this temperature, the stability of enzyme further decreased sharply.

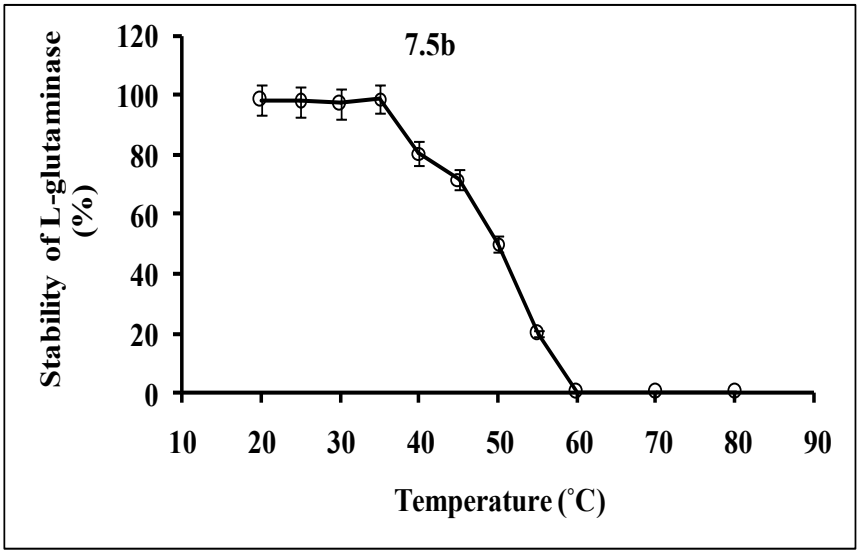

Fig.7.5b. Relative stability of purified L-glutaminase from *Bacillus cereus* MTCC 1305 at different temperature

7.3.4. Effect of different effectors on activity of purified L-glutaminase

The results of the studies of the effect of different metal ions on the catalytic activity of L-glutaminase are summarized in Table7.2. The activity of purified L-glutaminase was stimulated by 6-12% in the presence of monovalent cations (Na^+ and K^+) and phosphate ions at 5mM concentration (Table7.2). Some of the divalent metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Ca^{2+}, and Co^{2+} moderately inhibited the activity of the purified L-glutaminase, whereas addition of Hg^{2+}, Cd^{2+}, and $Cu^{2+,}$ showed strong inhibitory effect. The strong inhibition on the activity of the purified L-glutaminase in presence of Hg^{2+} and Cd^{2+} indicates the presence of essential sulfhydryl group in catalytic site of L-glutaminase. The addition of EDTA did not produce any significant change in the activity of this enzyme indicating its non-metalloenzymatic property.

The purified glutaminases from *Debaryomyces* sp. (Dura et al., 2002), *Pseudomonas* sp. (Abe et al., 1974), *Escherichia coli* (Hartman, 1968), and *Acinetobacter* (Holcenberg et al., 1978) were reported as non metalloenzymes. The purified L-glutaminase was strongly inhibited on treating with thiol-binding agents like p-chloro mercuric benzoic acid, iodoacetamide, which may indicate the presence of sulfhydryl groups in its catalytic site. The similar effect of thiol binding agent was reported for glutaminase from *Debaryomyces* sp. (Dura et al., 2002), *Lactobacillus reuteri* KCTC3594 (Jeon et al., 2010), *Pseudomonas acidovorans* (Davidson et al., 1977). Reducing agents such as β-mercaptoethanol, glutathione, L-ascorbic acid, cysteine and dithiothreitol showed stimulatory effect on the activity of purified L-glutaminase from *Bacillus cereus* MTCC 1305, which may indicate the presence of sulfhydryl group at the active site of the enzyme. Glutaminase from *Saccharomyces cerevisiae* also showed higher activity in presence of reducing agents (Penninckx and Jaspers, 1985).

Table7.2. Effect of metal ions, chelator, reducing agents, and thiol-binding agents on activity of purified L-glutaminase from *Bacillus cereus* MTCC 1305

	Enzyme activity (% of control)			
	5mM	**10mM**	**50mM**	**100mM**
Control	100	100	100	100
Metal ions				
Mg^{2+}	96.21	85.09	78.01	74.29
Mn^{2+}	81.34	79.02	77.98	76.23
Zn^{2+}	94.21	89.09	83.09	81.12
Pb^{2+}	80.29	79.09	73.76	69.27
Ca^{2+}	83.08	80.99	79.02	72.59
Co^{2+}	79.96	75.38	74.36	70.53
Cu^{2+}	12.52	1.50	0	0
Hg^{2+}	10.21	1.23	0	0
Cd^{2+}	6.23	1.93	0	0
Na^{+}	107.21	113.34	118.21	120.35
K^{+}	112.04	113.21	129.32	139.43
$(PO_4)^{3-}$	106.12	119.73	138.72	143.13
Chelating agent				
EDTA	101.15	103.45	105.18	109.49
Reducing agents				
β-Mercaptoehanol	113.24	119.43	123.39	138.22
Glutathione	101.23	106.16	112.52	129.32
Dithiothreitol	104.12	116.01	121.09	125.13
L-Ascorbic acid	109.23	113.12	123.12	128.23
Cysteine	105.12	119.32	123.13	128.15
Thiol-binding agents				
p-CMBA	1.02	0	0	0
Iodoacetamide	19.21	2.16	0	0

7.3.5. Substrate specificity tests for L-glutaminase

The substrate specificity test of glutaminase for different compounds is shown in Table7.3. L-glutaminase showed highest hydrolysis rate (100% relative activity) for L-glutamine substrate and this was considered as control. This enzyme showed lower specificity for D-glutamine and L-glutamic acid with relative activity as 48.5% and 13.5% respectively. This enzyme showed negligible activity for substrate like L-asparagine, D-asparagine and L-γ glutamyl para- nitroanilide. These results indicate that L-glutaminase produced from *Bacillus cereus* MTCC 1305 is very specific to L-glutamine substrate. L-glutaminase from *Pseudomonas acidovorans* (Davidson et al., 1977), *Lactobacillus reuteri* KCTC3594 (Jeon et al., 2010), *Bacillus pasteurii* (Klein et al., 2002), *Debaryomyces* sp. (Dura et al., 2002), and *Bacillus licheniformis* (Sinsuwan et al., 2012) also showed higher specificity for glutamine substrate.

Table7.3.Substrate specificity of purified L-glutaminase from *Bacillus cereus* MTCC 1305

Substrate	Concentration (mM)	Relative activity of L-Glutaminase (%)
L-Glutamine	40	100
D-Glutamine	40	48.40
L-Glutamic acid	40	13.56
L-Asparagine	40	0.00
D-Asparagine	40	0.00
L-γ Glutamyl para-nitroanilide	40	0.0

7.3.6. Estimation of Kinetic parameters

The substrate kinetics of L-glutaminase produced by *Bacillus cereus* MTCC 1305 was studied by using different concentrations of L-glutamine (1, 4, 8, 16, 20, 24, 40, 50mM). The maximum activity of L-glutaminase was observed at 40mM concentration of L-glutamine as shown in Fig7.6. The steady-state kinetics of enzyme-catalyzed reactions predicts a hyperbolic relationship between the steady-state velocity (V) and substrate concentration [S] which is expressed in term of following Michaelis-Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad \text{.....(7.11)}$$

Where, V_{max} is maximum velocity and K_m is Michaelis constant

Michaelis constant (K_m) is defined as the degree of affinity of enzyme towards the substrate. A rectangular hyperbolic plot through the origin was obtained for reaction velocity [V] against substrate concentration [S].

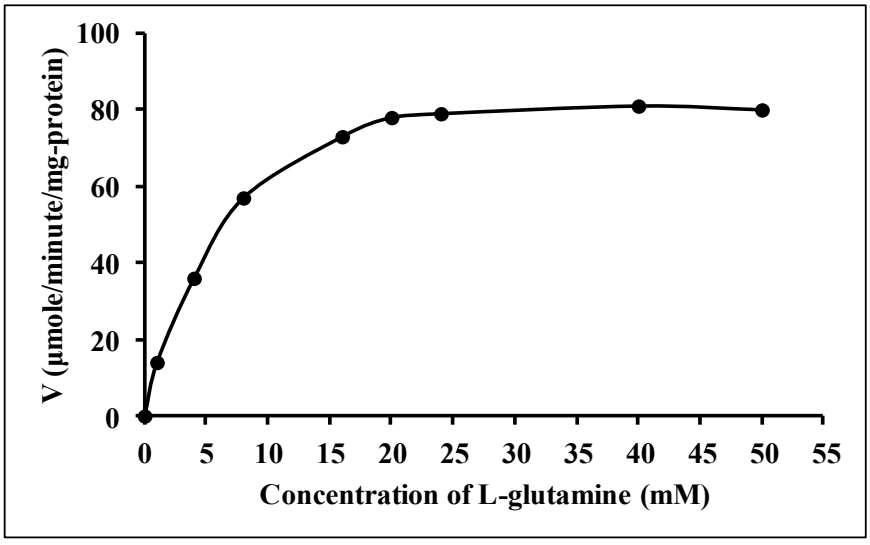

Fig.7.6. Effect of different concentration of L-glutamine substrate [S] on reaction velocity (V) of glutaminase kinetic process

This plot couldn't be used to determine accurate value of V_{max} and K_m, because the asymptotes cannot be approached closely enough (Eisenthal and Cornish-Bowden, 1974). Most biochemists have used Lineweaver Burk plot (1/V versus 1/[S]) to determine kinetic parameters by considering following equation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]} \quad \text{.....(7.12)}$$

The kinetic constant values (K_m and V_{max}) of purified L-glutaminase from *Bacillus cereus* MTCC 1305 were obtained as 6.25mM and 100µmole/minute/mg-protein respectively (Fig7.7) using Lineweaver Burk plot. The purified L-glutaminase from *Pseudomonas acidovorans* (Davidson et al., 1977), *Achromobacteraceae* (Roberts et al., 1972), *Acinetobacter* sp. (Holcenberg et al., 1978), *Cryptococcus welchii* (Kozolov et al., 1972), *Pseudomonas 7A* (Roberts, 1976), M*icrococcus luteus* (Moriguchi et al., 1994) and *Bacillus subtilis* RSP-GLU (Satish and Prakasham, 2010) was reported with different value of K_m as 2.2×10^{-5}M, $4.8\pm1.4\times10^{-6}$M, $5.8\pm1.5\times10^{-6}$M, 10^{-3}M, $4.62\pm0.4\times10^{-6}$M, 4.4×10^{-3}M and 2.21×10^{-3}M respectively towards L-glutamine substrate. Turnover number (K_{cat}) and catalytic efficiency for this enzyme was calculated as 1.3888sec^{-1} and $2.22\times10^{2}M^{-1}sec^{-1}$ respectively.

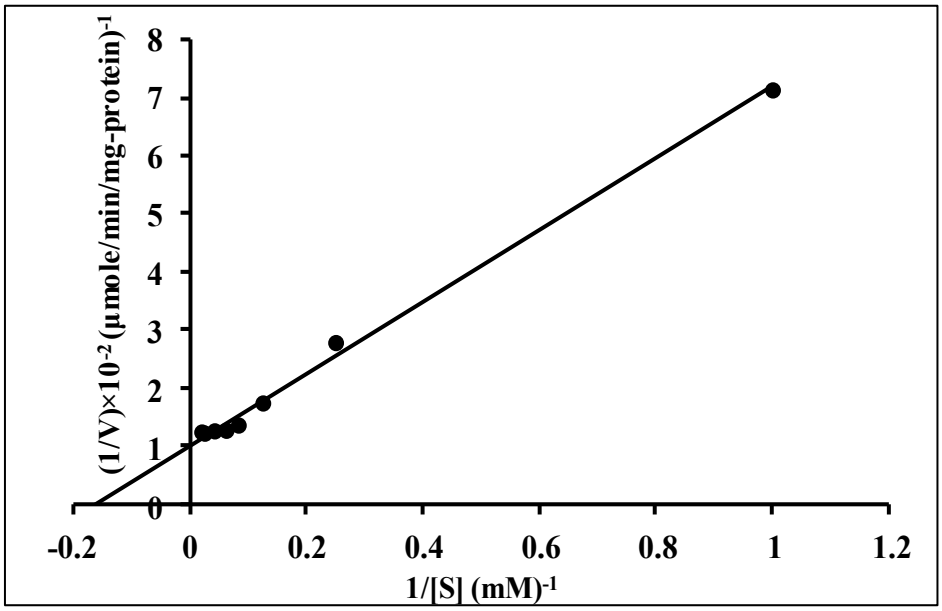

Fig.7.7. Lineweaver Burk Plot for reciprocal of reaction velocity (1/V) and reciprocal of concentration of L-glutamine substrate (1/[S])

7.3.7. Thermal deactivation studies of L-glutaminase

The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with a concomitant increase in the enthalpy of deactivation (Pal and Khanum, 2003). The unfolding of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of inactivation (Vieille and Zeikus, 1996). The behavior of deactivation kinetic varies with different enzymes. Some enzymes show exponential decay of activity with time and some follows different non-exponential kinetics. The thermal deactivation of L-glutaminase produced from *Bacillus cereus* MTCC 1305 was measured over the temperature range from 35˚C-60˚C. The deactivation rate constant (K_d) for L-glutaminase was obtained from slope of plot of natural logarithm of relative activity of L-glutaminase $Ln[A/A_0]$ with reaction time at different temperature of 35, 45, 50, and 60˚C as shown in Fig7.8. The temperature dependence of the natural logarithm of

deactivation rate constants Ln(k_d) of L-glutaminase followed the Arrhenius plot as shown in Fig7.9.The energy of activation (E_d) for thermal denaturation was determined as 152.1038KJ/K from the slope of plot between Ln(k_d) and reciprocal of absolute temperature (1/T). This high value of activation energy may indicate a very stable and compact L-glutaminase enzyme that is highly resistant to heat denaturation. The higher value of activation energy indicates more energy is required for thermal deactivation of enzyme (Klibanov, 1983).

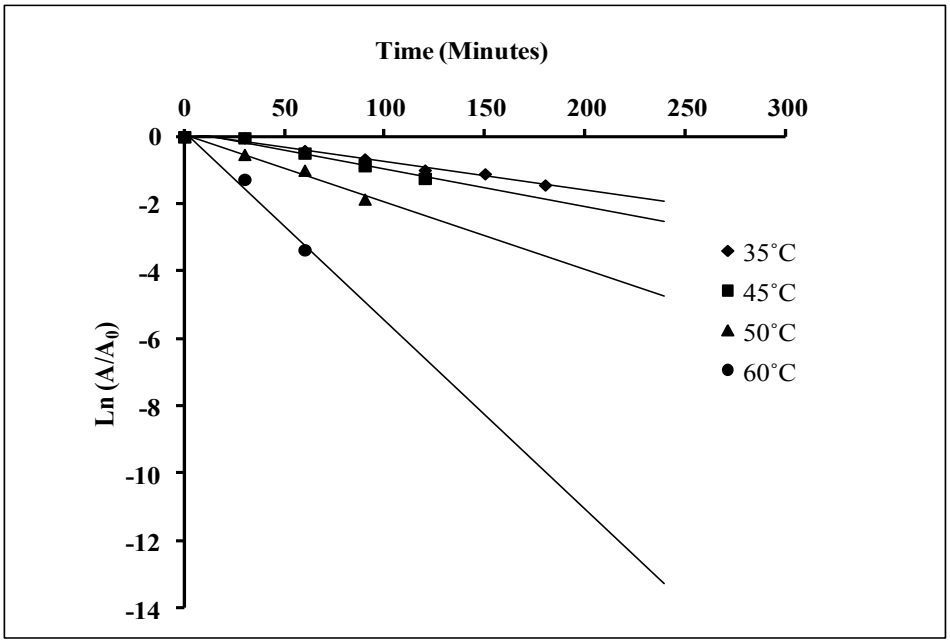

Fig.7.8. Effect of reaction time on relative activity of purified L-glutaminase Ln(E_d/E_0) obtained at different temperature

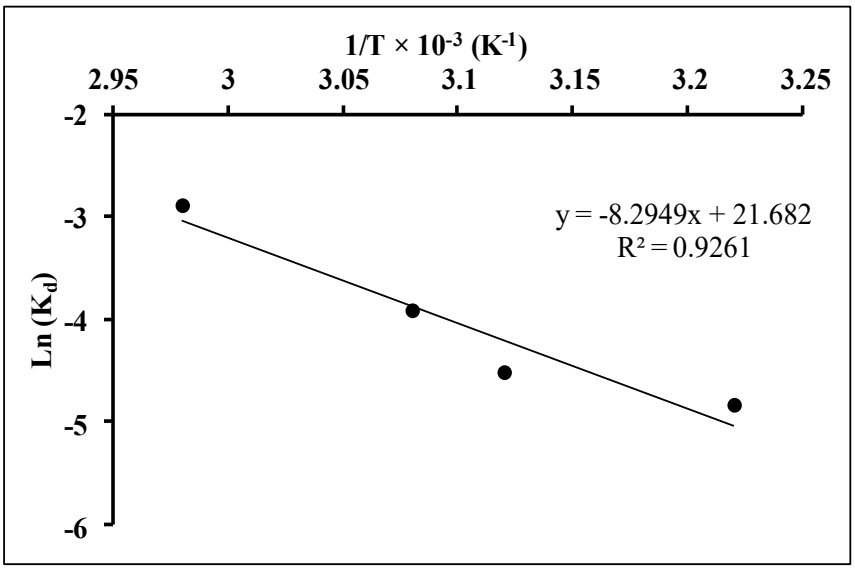

Fig.7.9. Arrhenius plot showing temperature dependence of the natural logarithm of deactivation rate constants Ln(K_d) of L-glutaminase from *Bacillus cereus* MTCC 1305

Table7.4. Deactivation kinetics parameters enthalpy (ΔH), free energy (ΔG), Entropy (ΔS) and $t_{1/2}$ (half life) of the purified L-glutaminase from *Bacillus cereus* MTCC 1305

Parameters	Temperature (˚C)			
	35	45	50	60
Free Energy (ΔG) $KJ.mol^{-1}$	169.671	170.324	170.651	171.302
Enthalpy (ΔH) $KJ.mol^{-1}$	149.525	149.442	149.401	149.317
Entropy (ΔS) $KJ.mol^{-1}K^{-1}$	-0.0650	-0.0653	-0.0654	-0.0656
Half life ($t_{1/2}$) Minutes	86.625	63	34.478	12.375

The thermodynamic parameters like half life time ($t_{1/2}$), enthalpy (ΔH), entropy (ΔS), and free energy (ΔG), for thermal denaturation of purified L-glutaminase from *Bacillus cereus* MTCC 1305 were determined at temperature 35, 45, 50, and 60˚C using equations 7.6, 7.8, 7.9, and 7.10 respectively and results are summarized in Table7.4. Enthalpy (ΔH) for thermal deactivation of L-glutaminase was determined as

149.525KJmol^{-1} at temperature 35˚C and its value was decreased with rise in temperature, revealing that lower energy was required to denature the enzyme at higher temperatures. Gibbs free energy (ΔG) for thermal unfolding was found to be increased with increase of temperature from 35˚C to 60˚C. The positive value of free energy at all these temperatures showed that the process of deactivation of L-glutaminase was thermodynamically non spontaneous reaction. Moreover, higher value of free energy of thermal denaturation at 60˚C (171.302KJmol^{-1}) indicates the resistance of L-glutaminase enzyme towards thermal unfolding at higher temperatures which indirectly indicates its stability.

The unfolding of enzyme structure was accompanied with an increase in disorder or entropy of deactivation. Negative value of entropy (ΔS) was obtained for thermal deactivation of the purified L-glutaminase at temperature 35, 45, 50, and 60˚C which revealed that this enzyme protein was in more ordered state (Table7.4). Half-life ($t_{1/2}$) for deactivation of L-glutaminase was determined as 86.625minutes at temperature 35˚C. With increase in temperature, the value of $t_{1/2}$ was decreased with increase of temperature and showed a value of 12.375 minutes at 60˚C indicating that the enzyme was unstable at higher temperature. The study of all these thermodynamic parameters revealed that purified L-glutaminase from *Bacillus cereus* MTCC 1305 showed high thermal stability at temperature 35˚C. The thermal stability of alkaline protease (Rao et al., 2009), xylanase (Cobos and Estrada, 2003; Pal and Khanum, 2010), glutaminase (Ziade et al., 2003), and glucoamylase (Bhatti et al., 2005) was also reported to be decreased with increase of temperature.

7.4. Conclusion

L-glutaminase from *Bacillus cereus* MTCC 1305 was purified with specific activity (7.44U/mg) and exhibited homotetrameric form with molecular weight of native enzyme as 144KD and its subunit as 36KD. The purified enzyme is specific to L-glutamine substrate and showed maximum activity at pH 7.5, temperature 35˚C. The purified L-glutaminase did not show any significant change on treating with EDTA indicates that this enzyme is not metalloenzyme. The stimulatory effect of reducing agents and inhibitory effect of thiol binding agents on the activity of purified L-glutaminase indicates the presence of sulfhydryl group in the catalytic site of L-glutaminase. The kinetic constant values K_m and V_{max} were determined as 6.25mM and 100µmole/minute/mg-protein respectively for the purified L-glutaminase. The higher value of energy of deactivation (E_d=152.1038KJ/K) and enthalpy ($\Delta H \approx 149 KJmol^{-1}$) indicates that more energy is required for thermal denaturation of this enzyme. The process of deactivation of L-glutaminase was found to be thermodynamically non spontaneous reaction with positive value of free energy. The negative value of entropy (ΔS) revealed that this enzyme protein was in more ordered state. The enzyme showed higher thermal stability at temperature 35˚C with high value of half-life ($t_{1/2}$) of deactivation.