Summary of Thesis

L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) catalyzes hydrolytic deamidation of L-glutamine into L-glutamic acid and ammonia. This enzyme plays a major role in the cellular nitrogen metabolism by supplying nitrogen required for biosynthesis of a variety of metabolic intermediates. This enzyme has important application in food and pharmaceutical industry. Although L-glutaminase is produced by almost all living cells for catabolism of glutamine, microbial L-glutaminase has received greater attention for its potential biotechnological applications and easiness in large scale production. In recent years, this enzyme has been commonly used as an excellent antitumor agent for glutamine-dependent tumor cells. It has been also used for treatment of acute lymphocytic leukemia and HIV (Roberts et al., 2001). Some microbial glutaminase showed low K_m for glutamine substrate, good activity in a physiological milieu, high inhibitory effect on growth of tumor cells and hence preferred as enzyme supplementation therapy for cancer. The discovery of new microorganism producing glutaminase with good therapeutic effect is highly desired (Moharam et al., 2010). Hence an attempt was made to produce glutaminase from bacterial source with desired therapeutic effect. The production, purification, characterization and antitumor property of L-glutaminase from *Bacillus cereus* MTCC 1305 were studied and reported in this thesis. The results of the study are summarized below:

- Among different bacterial strains screened, *Bacillus cereus* MTCC 1305 was selected as most potent bacteria for production of L-glutaminase.

- The parameters for estimation of the enzyme and, cultural conditions and media components for production of the enzyme were optimized by conventional method of varying one parameter at a time and using statistical tools like RSM and ANN

methodologies. ANN model was found to be the better predictor of the results in comparison to RSM with higher values of root mean square error, coefficient of determination and lower value of average absolute deviation.

- The activity of L-glutaminase was obtained as 628.035U/l at predicted optimum assay condition viz., pH of reaction mixture (7.5), reaction time (20minutes), incubation temperature (35˚C), substrate (L-glutamine) concentration (40mM) and enzyme volume (0.5ml). L-glutaminase activity was enhanced by 1.485 fold than the activity of L-glutaminase obtained under un-optimized assay conditions (423U/l).

- The production of L-glutaminase was enhanced up to 667.23U/l after conducting experiments at predicted optimum cultural conditions like, pH (7.5), fermentation time (40hrs), temperature (34˚C), inoculum size (2%), inoculum age (10hrs) and agitation speed (175rpm).

- The optimum media components were obtained as sucrose (2.5g/l), peptone (2.5g/l), L-glutamine (5.0g/l), Na_2HPO_4 (6.0g/l), NaCl (0.5g/l), $MgSO_4$ (0.5g/l) and the amount of L-glutaminase produced was estimated as 1630U/l, which was enhanced by 3.85 times than glutaminase produces in un-optimized media containing glucose (1.0g/l), yeast extract (5.0g/l), L-glutamine (3g/l), NaCl (0.5g/l), $Na_2HPO_4 \cdot 2H_2O$ (3g/l), KH_2PO_4 (2g/l), $MgSO_4 \cdot 7H_2O$ (0.5g/l), and $CaCl_2 \cdot 2H_2O$ (0.015g/l).

- Kinetic models were developed using logistic equation for cell growth, Luedeking Piret equation for L-glutaminase production and modified Luedeking Piret equation for sucrose utilization. Maximum production of L-glutaminase was

found at 40hrs fermentation period with maximum specific growth rate (μ_{max}= 0.4912hr^{-1}). L-glutaminase fermentation was non growth associated process with higher value of non-growth associated constant (β=0.1625U/g-cell/hr) in comparison to growth associated constant (α= 0.0048U/g-cell).

- The production of L-glutaminase in fermenter is necessary to produce sufficient data for evaluation of its large scale production efficiency. The frequently used scale-up method in aerobic fermentation process is generally based on the proper mixing of media and utilization of the oxygen mass transfer coefficient. The effect of different agitation rate (100, 200, 300, 400rpm) and aeration rate (1, 2, 2.5vvm) on the production of L-glutaminase in 5litre fermenter was studied. The optimum level of agitation speed and aeration rate was obtained as 300rpm and 2vvm respectively. Dissolved oxygen concentration was maintained >20% saturation over the entire fermentation process. Volumetric mass transfer coefficient (K_La) was determined as 30.67 hr^{-1}.

- For further increase in production of L-glutaminase, extractive fermentation with aqueous two phase system was studied. The maximum partition coefficient of L-glutaminase was obtained in PEG4000 (8.5%)/dextranT500 (9.5%) and hence selected as suitable system for partitioning of L-glutaminase. The overall volumetric productivity of L-glutaminase was enhanced by 1.35 times after extractive fermentation as compared with the homogeneous fermentation.

- The enzyme was purified to homogeneity using the purification techniques like ammonium sulphate precipitation, dialysis, DEAE cellulose chromatography, and electrophoresis. This enzyme exhibited homotetrameric nature with molecular

weight of each subunit and the native enzyme as 36KD and 144KD respectively. The purified enzyme showed maximum specificity for L-glutamine as substrate. The purified enzyme was characterized with optimum pH (7.5), temperature (35˚C), Michaelis constant (K_m=6.25mM), and maximum reaction velocity (V_{max}=100µmole/minute/mg-protein). This enzyme did not show any significant change in its activity in presence of EDTA indicating non metalloenzymatic poroperty. The inhibitory effect of thiol-binding agents and stimulatory effect of reducing agent on the activity of the purified enzyme indicates presence of sulfhydryl group in its catalytic site. The thermal deactivation of the purified enzyme was studied at temperature 35, 45, 50, and 60˚C. The higher value of energy of deactivation (E_d=152.1038KJ/K) and enthalpy ($\Delta H \approx 149 KJmol^{-1}$) indicates more energy is required for thermal denaturation of this enzyme. The positive value of free energy at all these temperatures showed that the process of deactivation of L-glutaminase was thermodynamically non spontaneous reaction. Negative value of entropy (ΔS) revealed that this enzyme protein was in more ordered state. The decrease in half-life ($t_{1/2}$) for deactivation of L-glutaminase with increase of temperature 35˚C to 60˚C showed higher thermal stability at temperature 35˚C.

- The peptide sequences of pure L-glutaminase from *Bacillus cereus* MTCC 1305 was obtained using MALDI-TOF/TOF analyzer and its 3D model structure was developed using X-ray crystallographic structure of L-glutaminase from *Bacillus subtilis* (PDB:1MKI_A) as template model. The model structure was refined using the bioinformatic tools like ANOLEA, QMEAN, GROMOS 96 and PROCHECK. Ramachandran plot showed distribution of most of the amino acid residue (86.1%)

in favorable region indicating good geometry of the predicted structure. Amino acid viz., Asp56, Trp65, Glu66, Phe120, Glu142, Glu156, Tyr182, Asp212, Tyr225 and Phe261 were found as conserved residues in active site of this predicted model structure. The active site of predicted 3D structure of L-glutaminase bound to substrate L-glutamine with high affinity.

- The purified enzyme showed good inhibitory effect against growth of Hep-G2 hepatocellular and HCT-116 colon carcinoma cell lines and hence can be used as enzyme supplementation therapy for cancer in future. .