5.1 Introduction

Enzymes are protein molecules consisting of amino acid chains, produced by all living organisms. The production of enzymes for use as drugs is an important facet of today's pharmaceutical industry. The last two decades have been characterized by the emergence of a large number of proteins as potential drug candidates to treat diseases. For pharmaceutical use these therapeutic proteins are extracted from sources like plants, microbes or human cells or are engineered in the laboratory (Adair and Ozanne, 2002). From a therapeutic perspective, proteins provide the distinct advantage of specific mechanisms of action and high potency (Pisal et al, 2010). Many enzymes have been used as drugs; one such enzyme, L-asparaginase had attracted much attention because of its use as an effective therapeutic agent against acute lymphoblastic leukemia (ALL). The main difference between other types of cancer that start in lymphocytes and ALL is that ALL mainly affects the bone marrow and the blood, and may spread to other places, while lymphomas chiefly affects the lymph nodes or other organs and then may spread to the bone marrow (American Cancer Society, 2013). Studies are in progress to find the most effective combination of chemotherapy drugs while limiting unwanted side effects.

5.1.1 Molecular Details of L-asparaginase

Studies at molecular level on L-asparaginase have been carried out in the past few years. Most of the bacterial L-asparaginases share similar tertiary and quaternary structures and also have common biochemical properties (Ramya et al, 2012). Molecular mass of *E.coli* L-asparaginase was 133-144 KDa (Kozak et al, 2002) and for different native asparaginases the molecular mass ranges between 140 and 150 kDa (Wikman et
al, 2005). The three-dimensional structure of *Pectobacterium carotovorum* was same as *E. coli* and *Erwinia* L-asparaginases. The structure of *Pectobacterium* (formally called *Erwinia carotovora*) has eight monomers. The eight monomers are arranged asymmetrically in two tetramers. An active site is located between two adjacent monomers. The whole molecule is considered as dimer of dimers (Yun et al, 2007).

![Molecular Active Site detail of L-asparaginase](image)

**Fig. 5.1 Molecular Active Site detail of L-asparaginase**

Each active site is formed by the distribution of amino acids in two adjacent monomers. The active site is comprised of the following amino acid residues: Thr, Tyr, Ser, Glu, Thr, Asp, Ala, Lys and only one active site residue Ser is present in the adjacent monomer (Asselin et al, 1999; Ramya et al, 2011)

Oncolytic enzymes are in great demand for use as therapeutic agents against many dreadful diseases. Cancer cells are more sensitive to oncolytic enzymes than the normal cells because enzymes dissolve the fibrous coating on cancer cells, allowing the immune system to work. The enzymes can also diminish the ability of cancer cells to attach to healthy organs or tissue. L-asparaginase is a typical oncolytic enzyme involved in the deamination of asparagine and glutamine. The enzyme L-asparaginase is found to
be a very promising agent in the treatment of acute lymphoblastic leukemia and other kinds of cancer. Normal tissue can synthesize L-asparagine but the cancer cells (especially Malignant and Carcinoma Cell) depend on the external source of L-asparagine for their growth and multiplication. Due to the presence of L-asparaginase activity the tumor cells are deprived of an important growth factor and fail to survive. Thus the enzyme L-asparaginase can be used as a chemotherapeutic agent for the treatment of ALL (mainly in children) as a potent antitumor or anti-leukemic drug. Various sources are found to be good producers of this enzyme which include bacteria, fungi, some of the plants and animal species. Apart from their role in the treatment of cancer, the enzyme L-asparaginase is also used in food industry, to reduce the formation of acrylamide, a carcinogen found in starchy food products.

5.1.2 Need of the Hour

In the food industry, it is desirable to have enzymes that are stable and active over a very wide range of temperature and pH, since baking and frying often involve very high temperatures such as 120°C. However asparaginases studied to date are not stable at such high temperatures.

In the treatment of ALL, asparaginase therapy is often accompanied by serious side effects. This is mainly caused as additionally L-asparaginases hydrolyze glutamine. Glutamine is the major transport form of amino nitrogen in the blood and also an amino group donor for many biosynthetic reactions. A prolonged decline of plasma glutamine levels, therefore, impairs a variety of biochemical functions, especially those of the liver.
Another important factor is that because mainly bacterial L-asparaginases are used in therapeutic treatment, a major side effect is the development of hypersensitivity ranging from mild allergic reactions to potentially fatal anaphylaxis. The underlying development of antibodies is also known to be liable for the “silent inactivation,” which leads to a rapid decline in L-ASNase activity due to the presence of neutralizing antibodies (Killander, 1976).

For many pharmaceutical proteins, enzymatic proteolysis becomes problematic in maintaining appropriate serum levels (Kotzia et al, 2007). The use of L-asparaginase in therapy leads to significant increase in serum trypsin and lysosomal proteases cathepsin B and asparagine endopeptidase which hydrolyze L-asparaginase and expose the epitopes that are implicated in immune response.

Thus, the application of L-asparaginases in food industry and as a therapeutic agent is mainly restricted by lack of thermal stability for the food industry and premature inactivation and rapid clearance i.e short half-life necessitating frequent injections to maintain therapeutic levels in the serum and several types of side effects ranging from mild allergies, thrombosis, pancreatitis, renal complications resulting from glutaminase activity to development of immune responses and anaphylactic shock, which may be life threatening conditions, in the treatment of ALL.

5.1.3 Improvement of L-asparaginase

Despite the broad range of L-asparaginases being isolated, no single enzyme is completely suitable for its use in pharmaceutical or food industry. However, they have provided a starting point for the improvement of L-asparaginases. Typically, the use of
protein engineering technology has been mainly directed towards its catalytic function and thermal stability. Mutagenesis has provided a discreet means for studying the role of different amino acids affecting the catalytic activity and the thermal stability of the enzyme. For enhancing the characteristics of the enzyme different parameters like thermostability, pH optima and substrate specificity can be studied using a number of approaches.

5.1.4 Enzyme Engineering

If a naturally-occurring enzyme with the desired trait is not available, to obtain one, the simplest method is to create the change and select and study the desired traits. In this process a microorganism expressing an enzyme with properties as close to the desired traits as possible is selected, and the enzyme is mutated for the desired characteristic.

Enzyme engineering is currently undergoing the most profound and unprecedented expansion in its history, promising applications of modified or improved enzymes with desired physical and catalytic properties (Chen, 2001). Enzymes can be engineered by two strategies:

- Rational redesign
- Directed evolution

Rational redesign

In rational redesign, precise changes in amino acid sequence are preconceived based on a detailed knowledge of protein structure, function and mechanism, and are
then introduced using site directed mutagenesis (Chen, 1999). This technology holds strong promise for optimizing the desired properties for commercial applications. It also greatly enhances our basic understanding of enzyme binding and catalytic mechanisms, thus increasing the success of future enzyme engineering efforts and laying the foundation for functional prediction of new protein sequences in databases. The power of rational redesign has been demonstrated by the generation of a faster superoxide dismutase, one of the fastest known enzymes in nature (Getzoff et al, 1992) and complete inversion of coenzyme specificities for both isocitrate and isopropyl malate dehydrogenases (Hurley et al, 1996). In these studies, individual amino acid substitution or secondary structure engineering, generated enzymes with desired properties. However, despite these spectacular examples, numerous attempts at redesigning enzymes have failed. These failures might result, to some extent, from an incomplete understanding of the underlying mechanisms required to enhance the desired enzyme properties (Chen, 2001). However, what probably accounts for many failed ‘rational’ engineering attempts is that a significant number were based on primary amino acid sequence homologies as the only criterion for amino acid replacements.

In many cases, these substitutions were made without regard to the structural properties of the protein. Such ‘homology-based engineering’ frequently leads to substituting rigidly conserved amino acids that do not affect the desired enzyme properties and render the enzyme inactive because of changes in protein conformation. This process overlooks key amino acid residues, particularly when comparing highly divergent enzymes. The conventional approach of rational redesign requires confirmation of the mutation by sequencing and then by purification of the mutant
enzymes following each round of mutagenesis in order that kinetic and functional properties can be determined. Such an approach is tedious and expensive, and might be impractical for multiple cycles of mutagenesis. Recently, an efficient strategy for identifying beneficial mutants using kinetics has been elaborated that would greatly facilitate the rational redesign of enzymes that require many cycles of mutagenesis to improve their properties.

**Directed Evolution**

Directed evolution does not require information about how enzyme structure relates to function (Kuchner and Arnold, 1997; Stemmer, 1994). This technique employs a random process in which error-prone PCR is used to create a library of mutagenized genes. High throughput screening of library is required to identify the mutants that possess improved properties. Mutants thus obtained might be subjected to further cycles of mutation and screening for enhancing the original beneficial mutation. Directed evolution has been improved significantly using *in vitro* recombination or DNA shuffling. These methods help in rapidly combining beneficial mutations obtained from random mutagenesis. Thus, these methods help in expanding the sequence diversity derived from small pools of homologous genes. In the last few years, directed evolution has been widely adopted by industry and has proven to be extremely valuable for improving enzymes, as well as ‘evolving’ new metabolic pathways. Although these techniques have been relatively successful in improving enzyme catalytic activity and physical properties, engineering substrate specificity appears to be more challenging. In the majority of cases, the enzymes had low catalytic activity and modest substrate
specificity (Zhang et al., 1997) and enzymes with new functions were rarely demonstrated.

Evolutionary analysis of enzyme families suggests that drastic changes in enzyme function might require considerable changes in polypeptide backbones. These changes cannot be achieved during the *in vitro* evolution process. In *in vitro* evolution process, enzymes are mainly improved by point mutation with a significant bias for transitions over transversions, thus accession to a wider spectrum of substitutions is limited. In contrast, natural mutations typically result from sexual or homologous recombination i.e. deletions, insertions, duplications or fusions. Such mutations alter the spacing between amino acid residues and polypeptide chain segments and can result in large changes in specificity and new catalytic activities. Hence, a challenging task is to mimic the natural evolutionary process by introducing these natural mechanisms into directed evolution. Another limitation of directed evolution is the prerequisite for a sensitive and efficient method for screening a large number of potential mutants. Thus, development of novel enzyme assays suitable for high-throughput screening is needed to extend the applicability of directed evolution to many more reactions of industrial interest (Chen, 2001). Rational redesign and directed evolution both have their distinct advantages and yet the technologies are complementary.

In summary, enzyme properties can now be improved by rational redesign or directed evolution. Choosing the most effective approach for a particular enzyme-engineering task depends on the level that the mechanistic base of the desired property is understood, and if an effective selection scheme is available. With the rapidly increasing number of 3D protein structures available in databases and the development of powerful
protein modeling tools, rational redesign will become more efficient and broadly applicable. Meanwhile, emergence of novel high-throughput screening processes and strategies for increasing sequence diversity will extend the application of directed evolution to many more industrial enzymes and increase the feasibility for creating new functions. Although either rational redesign or directed evolution can be very effective, a combination of both strategies will probably represent the most successful route to improving the properties and function of an enzyme (Altamirano et al, 2000).

**Present Study**

In the present work, recombinant L-asparaginase from *Bacillus licheniformis* has been studied for its catalytic properties and its half-life. The recombinant L-asparaginase has also been modified using Rational redesign to enhance its catalytic properties along with the half-life of the enzyme.
5.2 Materials and methods

5.2.1 Prediction of L-asparaginase structure

In the present study, protein structure of L-asparaginase from *Bacillus licheniformis* was predicted with the help of freely available online software “ITASSER”, (Zhang, 2008; Roy et al, 2010; Roy et al, 2012). I-TASSER server is an internet service for protein structure and function predictions. It allows academic users to automatically generate high-quality predictions of 3D structure and biological function of protein molecules from their amino acid sequences. When users submit an amino acid sequence, the server first tries to retrieve template proteins of similar folds (or super-secondary structures) from the PDB library by LOMETS, a locally installed meta-threading approach.

In the second step, the continuous fragments excised from the PDB templates are reassembled into full-length models by replica-exchange Monte Carlo simulations with the threading of unaligned regions (mainly loops) built by *ab initio* modeling. In cases where no appropriate template is identified by LOMETS, I-TASSER will build the whole structure by *ab initio* modeling. The low free-energy states are identified by SPICKER through clustering the simulation decoys.

In the third step, the fragment assembly simulation is performed again starting from the SPICKER cluster centroids, where the spatial restraints collected from both the LOMETS templates and the PDB structures by TM-align are used to guide the simulations. The purpose of the second iteration is to remove the steric clash as well as to refine the global topology of the cluster centroids. The decoys generated in the second simulations are then clustered and the lowest energy structures are selected. The final
full-atomic models are obtained by REMO which builds the atomic details from the selected I-TASSER decoys through the optimization of the hydrogen-bonding network. For predicting the biological function of the protein, the I-TASSER server matches the predicted 3D models to the proteins in 3 independent libraries which consist of proteins of known enzyme classification (EC) number, gene ontology (GO) vocabulary, and ligand-binding sites. The final results of function predictions are deduced from the consensus of top structural matches with the function scores calculated based on the confidence score of the I-TASSER structural models, the structural similarity between model and templates as evaluated by TM-score.

5.2.2 Visualization

The results of the computational structure predicted by ITASSER of L-asparaginase from *Bacillus licheniformis* and its characterization and validation were visualized by using software “PyMOL”.

“PyMOL” is an open source, user sponsored, molecular visualization system created by Warren Lyford Delano and commercialized by Delano Scientific LLC, which is a private Software Company dedicated to creating useful tools that become universally accessible to scientific and educational communities. It is well suited to producing high quality 3D images of small molecules and biological macromolecules such as proteins. PyMOL is one of free open source visualization tools available for use in structural biology (Delano et al, 2002). (http://pymol.sourceforge.net/).
**Comparision of L-asparaginase structure**

The structure of L-ASNase (rBlIAIII) thus obtained was used to superimpose with the E.coli L-ASNase (PDB code: 3ECA) and Erwinia chrysanthemi L-ASNase (PDB code: 1O7J). pyMOL was used for determination of the superposition and structure alignment. The corresponding superpositions were visualized with the help of pyMOL and saved as images.

**5.2.3 Plasmid DNA isolation**

Recombinant plasmid harboring ansA3 gene was isolated from previously transformed E.coli DH5α cells (Chapter 4). This plasmid DNA was used throughout as a template in PCR reactions for generation of mutants.

**5.2.4 Site Directed Mutagenesis**

**Primer Design**

Different sets of primers were designed manually, complimentary to L-asparaginase (ansA3) gene for site directed mutagenesis. They are as follows:

- **BLAFP**: GAA CTC TGG **GGA TCC** ATG AAA AAG TTA CTG CTG TTG
- **GNF2F**: GGA AGC GGC AAC ATT CCT TTT
- **EAF2F**: ATT GTG ATT GCC AGC TAC GGA
- **QHF2F**: ACG GGT TCC CAT GTT CCG ATT
- **DVF2F**: CAG AAT ATT GTT AAG CCC GTC
- **BLARP**: AGG TTC CAA **GAA TTC** TTA TAT GAT GAT ATC GTC TGC
- **GNF1R**: AAA AGG AAT GTT GCC GCT TCC
- **EAF1R**: TCC GTA GCT GGC AAT CTC AAT
5.2.5 Generation of mutated fragments

**PCR-1 Amplification of single mutant gene**

*G238N Mutation:* Site directed mutation of glycine (G) to asparagine (N) was inserted using primers BLAFP and GNF1R at position 238. Reverse primer contained bases encoding asparagine in place of original amino acid glycine. The other fragment was amplified to synthesize full length gene using primers GNF2F and BLARP. The annealing temperature for the corresponding primers used in PCR reaction was 56°C.

*E232A Mutation:* In this mutation, glutamic acid (E) was replaced with alanine (A) using primers BLAFP and EAF1R at position 232. Here reverse primer contained bases encoding alanine in place of the original amino acid, aspartic acid. The other fragment was amplified to synthesize full length gene using primers EAF2F and BLARP. The annealing temperature for the corresponding primers used in PCR reaction was 56°C.

In a similar manner, the other two mutations i.e Q112H and D103V were carried out by site directed mutagenesis replacing glutamine (Q) with histidine (H) at position 112 and aspartic acid (D) with valine (V) at position 103, respectively. The annealing temperatures for the primers of Q112H and D103V mutants were 52.5°C and 50°C, respectively.

Following are the conditions used in a thermo cycler for a PCR:
Initial denaturation 94°C for 3 min

Denaturation 94°C for 1 min

Annealing 56°C for 30sec

Extension 72°C for 1 min

Final extension 72°C for 5 min

The PCR reaction system for the mutations was as follows:

- PCR Master Mix 6.25 µl
- dH₂O 3.25 µl
- Forward primer 1.00 µl
- Reverse primer 1.00 µl
- Template 1.00 µl
- Total 12.5 µl

The amplified products obtained after PCR reactions were checked on 1% agarose gel stained with ethidium bromide and observed using an UV transilluminator.

*Elution of amplified product*

The amplified product obtained after PCR were eluted from the gel with the help of gel extraction kit (Merck, India). Eluted samples were checked loading 3 µl of the same on 1% agarose gel stained with ethidium bromide and observed on an UV transilluminator.
**PCR-2 Overlapping fragments**

The eluted products harbouring mutations i.e 238\(^{th}\), 232\(^{nd}\), 103\(^{rd}\) and 112\(^{th}\) amino acid positions were allowed to overlap to get full gene with a single mutation. Following were the conditions used in thermal cycler for 5 cycles:

- **Initial denaturation**: 90°C for 5 min
- **Denaturation**: 90°C for 1 min
- **Annealing**: 40°C for 1 min
- **Extension**: 60°C for 2 min
- **Final extension**: 72°C for 5 min

**PCR-3 Final amplification**

The full length gene for each mutation was amplified with the help of BLAFP and BLARP primers. Overlapped products were used as a template in each PCR. The reaction system consisted 1µl forward primers, 1 µl of reverse primer, 1 µl of template, 6.5 µl of Emerald Amp GT PCR Master Mix (TAKARA), 3 µl of Milli Q.

The conditions for 30 cycles of PCR were as follows:

- **Initial denaturation**: 94°C for 3 min
- **Denaturation**: 94°C for 1 min
- **Annealing**: 56°C for 30 sec
- **Extension**: 72°C for 1 min
Final extension 72°C for 5 min

5.2.6 Cloning of mutant genes

The purified mutated PCR fragments and pRSET A vector were mixed in appropriate volume of TE buffer and the DNA concentration was determined by absorbance at 260 nm. Total volume of ligation reaction system was 20 μl. The system consisted of the purified digested insert fragments and purified digested plasmid vector pRSET A (3:1 ratio), 10x Ligation Buffer 2 μl, T4 DNA ligase enzyme 1.5 μl were added and make upto 20 μl with MQ into a 0.5 ml tube, followed by incubation at 16°C overnight.

5.2.7 Transformation

Preparation of competent cells of E. coli DH5α

For preparation of competent cells, a well isolated, single colony of DH5α was picked from the Luria Bertani (LB) agar plate and inoculated into 5 ml of LB broth which was incubated at 37°C (in a shaker) overnight. 1 ml of the overnight grown culture was further inoculated into 100 ml of LB broth and the flask was incubated at 37°C in a shaker till the O.D A600 reached 0.600. The cultured flask was chilled on ice for 10-20 min and it was aseptically transferred into sterile centrifuge tubes and spun at 4,000 x g for 8 min at 4°C. The supernatant was discarded and to the cell pellet, approximately 15 ml of cold 0.1M CaCl₂ solution was added aseptically. The pellet was suspended using pre-chilled pipette tips by aspiration. The tubes were transferred to ice for 30 min followed by centrifugation at 4,000 x g for 8 min at 4 °C. The aqueous phase was discarded and pellet was resuspended gently in 0.6 ml of cold 0.1M CaCl₂ solution.
These competent cells of *E. coli* BL21 were used for transformation. (Sambrook and Russell, 2002)

**Transformation of recombinant plasmid in *E. coli* DH5α Strain**

In a microcentrifuge tube, 10 μl of ligated sample was mixed with 100 μl of the competent cells and kept on ice for 30 min. Heat shock treatment was applied to the sample tube for 2 min at 42°C, followed by incubation on ice for 5 min. To the sample tube 900 μl LB broth was added and incubated at 37°C for 1 h. The sample was centrifuged at 6,000 x g for 5 min, the supernatant was discarded (around 900 μl) and the pellet was resuspended in 100 μl of LB broth. Aliquots of the sample were plated on LA (Luria agar) plates containing the 100 μg/ml ampicillin. The transformants were selected by their ability to grow on ampicillin containing LB plates. (Sambrook and Russell, 2002)

**5.2.8 Plasmid DNA Isolation**

Alkali lysis method was followed to isolate plasmids. Transformants from ampicillin containing plates were picked up and inoculated into 10 ml Luria broth supplemented with ampicillin (100 μg/ml) and incubated under shaking condition at 37°C shaker. 1 ml of the cell suspension was transferred in microcentrifuge tube and centrifuged at 10,000 x g for 1 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 100 μl of solution-I (glucose 0.9%, 0.025 M Tris, 0.01 M EDTA). 200ul of solution II (0.2 N NaOH, 1% SDS) was added and tubes were invert mixed gently once. 150 μl of solution III (3 M potassium acetate, 11.5% glacial acetic acid) was then added and tubes were invert mixed once more. Cell debris and proteins were removed by centrifugation at
19,000 g at 4 °C. The supernatant was transferred to fresh tube and 0.8th volume of isopropanol was added. The tubes were incubated at room temperature for 2 h and centrifuged at 20,000 x g for 25 min at 4 °C. The pellet was washed with chilled 70% ethanol and air dried. Finally the plasmids were dissolved in 25 μl of MQ. 5 μl of each were checked on 1% agarose gel.

5.2.9 Clone confirmation by gene specific PCR

Confirmation of the plasmids harboring G238N, E232A, Q112H and D103V mutant L-asparaginase genes was carried out by gene specific PCR. The plasmids isolated from E.coli DH5α cells were used as a template in a PCR using gene specific primers. The PCR consisted of 1 μl template, forward (BLAFP) and reverse (BLARP) primers 1 μl each, PCR master mix (TAKARA) 6.5 μl and 3 μl nuclease free water.

PCR conditions used for 30 cycles of thermal cycling process were as follows:

- **Initial denaturation**: 94°C for 3 min
- **Denaturation**: 94°C for 1 min
- **Annealing**: 56°C for 30 sec
- **Extension**: 72°C for 1 min
- **Final extension**: 72°C for 5 min
Transformation of G238N, E232A, Q112H and D103V mutants in E.coli BL21 cells

Competent cell preparation and transformation of the confirmed plasmids was carried out using E.coli BL21 cells for the expression of the cloned genes. The methods used for competent cell preparation and transformation were similar to the methods used for E.coli DH5α cells which were discussed earlier in this chapter.

5.2.10 Clone confirmation of mutant genes through sequencing

The isolated plasmid of each mutation was sequenced to confirm the clones. The sequence obtained were used as query for Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/) in order to identify the mutant gene. Further each sequence were aligned to wild type L-asparaginase gene sequence of B. licheniformis to confirm the presence of inserted mutations in their respective clones i.e G238N, E232A, Q112H and D103V.

5.2.11 Expression of mutant genes

For the expression of each recombinant mutant L-asparaginase, in a conical flask (500 ml), 200 ml of fresh LB medium containing ampicillin (50 µg/ml) was inoculated with 1 ml of overnight grown culture of recombinant E.coli BL21 cells. These inoculated flasks were incubated overnight at 37°C in shaking condition. The protein expression was induced using 0.5 mM (Isopropyl-β-D-1-thiogalactopyranoside) IPTG by incubating flasks for 4 h after IPTG addition.

5.2.12 Cell lysis and purification

The cells thus grown were harvested with the help of centrifugation at 7,800 x g for 10 min. The pellet obtained was suspended in sodium phosphate buffer containing NaCl (0.3
M, pH 7.4). Cells were lysed with the help of ultra sonicator (Sonics vibra cell, USA) keeping its pulse 30sec on and 30sec off for 5 min. The cell debris was removed by centrifugation at 7,800 x g for 20 min at 4°C.

The cell free extract was loaded to a affinity chromatography column (Ni-NTA, Nucleopore, Genetix, India). The supernatant containing the crude recombinant mutant L-asparaginase was purified using a single step Ni-NTA affinity chromatography. The affinity column was equilibrated with equilibration buffer followed by the addition of crude sample. The column was then washed with 1 bed volume of equilibration buffer followed by a wash with 3 bed volumes of Wash buffer. Fractions were collected by the addition of Elution buffer. The recombinant protein i.e. His-tagged mutant L-asparaginase was eluted with an Imidazole step elution based on Ni-NTA affinity chromatography. Eluted fractions were confirmed for the presence of the mutant rL-asparaginase using Bradford’s method of protein estimation.

Buffers at pH 7.2 were used for the purification of G238N and D103V mutants whereas buffers at pH 7.4 were used for the purification of E232A and Q112H mutant.

To remove excess salts from the purified proteins, the protein solution was dialyzed at 4°C against the same equilibration buffer with continuous stirring and stored at -20°C.

5.2.13 SDS Polyacrylamide Gel Electrophoresis

SDS - PAGE was carried out in a vertical slab gel apparatus (Genei, India). To determine the subunit molecular weight, purified denatured proteins were separated according to their molecular weight on 10% SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) 1.5 mm gel (Laemmli, 1970). Denaturation of purified proteins was
done at 95°C for 5 minutes. The polyacrylamide gel was then stained overnight with the help of Coomassie brilliant blue R-250 dye or with the help of silver nitrate.

5.2.14 Enzyme Characterization

Effect of temperature on the Enzyme

The effect of temperature on the activity of the mutant enzyme was evaluated by incubating the enzyme with a standard reaction mixture containing 0.1 M Potassium Phosphate buffer (pH 8) and 40mM of L-Asparagine at temperatures 37°C, 45°C, 55°C, 60°C, 65°C, 70°C, 90°C and 100°C. The amount of product liberated was estimated by Nessler’s assay.

In-Vitro half life study

The stability of the enzyme i.e. half-life of the mutant enzyme was determined by incubating the enzyme with 0.1 M Potassium Phosphate buffer (pH 8) at 37°C. After different time intervals, 40mM of substrate L-Asparagine was added and further incubated for 15 minutes. The reaction was stopped by the addition of 2-2.5% TCA solution.

Kinetic parameters

The Michaelis-Menton constant (K_m) and the Maximum velocity (V_max) of the mutant enzymes were determined using L-Asparagine as substrate in the range of 10-250 mM.
5.3 Results and discussion

5.3.1 Structure

The best suited computational structures were predicted by ITASSER for recombinant ansA3 from *B. licheniformis* using amino acid sequence data of L-asparaginase (ansA3) of *B. licheniformis*. The predicted structure was then visualized with the help of “pyMOL” software.

![Visualization of rBliAIII protein with the help of pyMOL](image)

The predicted structure was scored according to the structural score known as C-score. C-score is a confidence score for estimating the quality of predicted models by I-TASSER. C-score is typically in the range of [-5,2], where a C-score of higher value signifies a model with a high confidence and vice-versa. TM-score and RMSD are known standards.
for measuring structural similarity between two structures which are usually used to measure the accuracy of structure modeling when the native structure is known. TM-score is a recently proposed scale for measuring the structural similarity between two structures (Zhang and Skolnick, 2004). A TM-score >0.5 indicates a model of correct topology and a TM-score <0.17 means a random similarity. These cutoff does not depend on the protein length.

The C-score of the present predicted structure was as followed:

<table>
<thead>
<tr>
<th>Name</th>
<th>C-score</th>
<th>Exp.TM-Score</th>
<th>Exp.RMSD</th>
<th>No.of decoys</th>
<th>Cluster density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model1</td>
<td>1.28</td>
<td>0.89±0.07</td>
<td>3.8±2.6</td>
<td>8512</td>
<td>0.7874</td>
</tr>
</tbody>
</table>

5.3.2 Structural comparison of *B. licheniformis* L-ASNase with *E. coli* and *Erwinia* L-ASNases

The structure generated with the help of ITASSER for *B. licheniformis* L-ASNase was further used to superimpose on *E. coli* and *Erwinia* L-ASNases. The structural data of later L-ASNases are readily available in protein data bank (PDB) (http://www.rcsb.org/pdb). Comparison of the structure of L-asparaginase from *B. licheniformis* was done by superposing the structure of L-asparaginase with *E. coli* (PDB code: 3ECA) and *Erwinia chrysanthemi* (PDB code: 1O7J) using pymOL.

Superposition of *B. licheniformis* L-asparaginase with both *E. coli* and *Erwinia* L-ASNases indicates that *B. licheniformis* L-ASNase is structurally identical with the overall structure of both *E.coli* L-ASNase (EcAII) and *Erwinia* L-ASNase (ErA).
Fig 5.3. 3D visualization of the superposition of *B. licheniformis* L-asparaginase (Green) with *E.coli* L-asparaginase (Pink).

Fig. 5.4 3D visualization of the superposition of *B. licheniformis* L-asparaginase (Green) with *Erwinia chrysanthemi* L-asparaginase (Blue).
However, there are significant differences in the loop region of rBliAIII when compared to EcAII and ErA. Being dimeric in nature, each monomer consisted of 329 amino acid residues. The computational structure of rBliAIII showed 9 α – helices and 9 β – strands present in two domains N – terminal (larger one) and C - terminal (smaller one) while the structural data of ErA, and EcAII showed the presence of 8 α – helices and 14 β – strands each (Aghaiypour et al, 2001). The active site loop residues of rBliAIII differed when compared to ErA and EcAII enzymes and it is a well established fact that flexibility and stability of the the active site loop plays a major role in enzyme functionality (Offman et al, 2011; Bansal et al, 2012).

### 5.3.3 Determination of active site by sequence homology

Binding site and active site of *B. licheniformis* L-ASNase were determined by aligning BliAIII protein sequence to EcoAII and ErA protein sequences with the help of online database, UNIPROTKB (www.uniprot.org). Alignment of the L-ASNase protein sequences showed that BliAIII has 22% amino acid sequence identical to the EcAII and 21% identical to ErA. Moreover, when the residues at the active site and binding site of EcAII and ErA were compared, it showed the presence of T (Thr) at the active site and S (Ser) at the binding site which showed presence of some conserved region at the active site and the binding site. Further, alignment of the protein sequences revealed the presence of same residues at active and binding sites i.e T and S, respectively. This further confirmed that presence of conserved regions at the active and binding site of BliAIII protein which are at 12th and 54th position in each case after signal peptide. The active site (Red) and binding site (yellow) are shown in Fig. 5.5.
5.3.4 Plasmid DNA isolation

Recombinant plasmid harboring ansA3 gene was isolated in order to be used as a template in a PCR reaction for the generation of mutants. Fig. 5.6 shows the presence of recombinant plasmid with insert (ansA3) at 4 kb.
5.3.5 Generation of mutated fragments

*PCR-1 Amplification of single mutant genes*

Using plasmid containing the insert as a template and the primers carrying the mutations for G238N, E232A, Q112H and D103V, amplification was carried out through PCR. For the mutations G238N and E232A, amino acid was to be replaced at 238 and 232 positions, in each case, the forward primer pair carrying the specific mutation should amplify a region of about ~700 bp whereas, the reverse primer pair should amplify a region of about ~300 bp for both mutations while, for the mutations Q112H and D103V, forward primer should amplify ~300 bp and reverse primer should amplify ~700 bp, thus, giving a total fragment of ~1 Kb on overlap.

As predicted, the first mutation of G238N showed 2 fragments (Fig. 5.7) without any non-specific amplifications likewise the same results were obtained in E232A (Fig. 5.8), Q112H (Fig.5.9) and D103V (Fig. 5.10) mutations. The forward primer pair gave an
amplicon of ~300 bp and the reverse primer pair gave an amplicon of ~700 bp in the case of G238N and E232A mutation and vice-versa in the case of Q112H and D103V mutations as determined by checking on 1% agarose gel and comparing with a standard 100 bp marker ladder.

Fig 5.7 Amplification of G238N fragments

(Lanes M: 100 bp Marker; Lane 1 – 300 bp fragment and Lane 2: 700 bp fragment)

Fig. 5.8 Amplification of E232A fragments

(Lanes 1: 700 bp fragment; Lane 2 – Standard 100 bp Marker and Lane 3: 300 bp fragment)
The mutations G238N, E232A, Q112H and D103V showed two fragments of desired size without any non-specific amplification. Thus, the amplification of mutated fragments was confirmed by agarose gel electrophoresis.
Elution of amplified products

The amplified products were eluted with the help of a gel extraction kit (Merck, India) and finally dissolved in 30 µl of Milli Q water. 3 µl of each eluted sample was checked on 1% agarose gel for its quality and quantity. The bands were found to have appropriate intensity which were further used for overlapping PCR to generate full length mutated genes.

PCR-2: Overlapping fragments

Overlapping PCR was carried out for annealing the fragments sharing similar portions present in both the primers used for mutations. These overlapped fragments were used further for the final amplification with the help of BALFP and BALRP primers.

PCR-3 Full length amplification of mutant gene

The overlapped product obtained after PCR-2 was used as a template for full length/overlap extension with the help of BALFP and BALRP primers. The final PCR yielded a band ~1 kb for each single mutation product i.e G238N, E232A, Q112H and D103V. This indicated the successful replacement of single amino acid into ansA3 gene. The full gene amplicons with mutation are shown in figures (5.11, 5.12 and 5.13). The products obtained were further proceeded for cloning experiments.
Fig. 5.11 Full gene amplification of G238N and E232A

(Lane M – Step up 1 kb Marker; Lane 1 – Full gene amplification of G238N; Lane 2 – Full gene amplification of E232A)

Fig. 5.12 Full gene amplification of Q112H

(Lane M – Step up 1 kb Marker; Lanes 1 & 2 – Full gene amplification of Q112H)
Fig. 5.13 Full gene amplification of D103V

(Lane M – Step up 1 Kb Marker; Lanes 1 to 4 – Full gene amplification of D103V)

Elution of the full length amplified product

~1 kb bands obtained after PCR-3 were eluted with the help of gel extraction kit (Merck, India). These eluted samples were finally dissolved in 30 µl of milli Q water. 3 µl of each sample was checked on 1% agarose gel to check its quantity and quality. These samples were further proceeded for restriction digestion.

Fig. 5.14 Eluted ~1 kb amplicons after PCR-3

(Lane M, Step up 1 kb marker; Lanes 1-4, eluted ~ 1 kb amplicons)
5.3.6 Restriction digestion of Mutant genes (insert) and vector

Insert and pRSETA vector were digested with the help of enzymes Bam HI and EcoRI at 37°C overnight and was checked on 1% agarose gel. After digestion, both insert and vector should show single band on agarose gel (Fig. 5.15). This confirmed the successful digestion of vector as well as insert. The restriction enzymes used created sticky ends and these digested samples were used for ligation into vector.

![Image](image.png)

Fig. 5.15 Vector and insert digested with restriction enzymes

(Lane M – Standard 1 kb Marker; Lane 1 – Digested pRSET A vector; Lane 2 – Digested Insert)

5.3.7 Cloning of mutant genes in *E. coli* DH5α cells

The purified insert and cloning vector (pRSET A) with sticky ends were ligated using T4 DNA ligase from NEB (New England Biolabs). Each ligated sample thus obtained was then transformed into competent *E. coli* DH5α cells by heat shock treatment. The transformed cells then were plated on Luria Agar plates containing ampicillin for selection of transformants. Presence of colonies on the plate showed successful transformation process. Further confirmation of the clone was done by plasmid isolation (as discussed in chapter 4). Plasmid isolation yielded desired size of bands (Fig. 5.16 and 5.17), when observed on 1% agarose gel on UV transilluminator, which further
confirmed the presence of inserted mutant gene. ~4 kb bands were obtained in each case as insert is ~1 kb and vector 2.9 kb. The bands obtained indicated the presence of desired size of plasmid. The isolated plasmid was further used to confirm the presence of gene through gene specific PCR.

(a) Lanes 1 & 2 – plasmid harboring G238N mutant gene, Lane M- step up 1 kb marker; (b) Lanes 3 & 4 – plasmid harboring E232A, Lane M- step up 1 kb marker

(Lane M – Standard 1 Kb Marker; Lanes 1 & 2- Q112H and Lanes 3 & 4 – D103V gene inserts in Plasmid isolated from transformed DH5α)
5.3.8 Confirmation of Clones through Gene Specific PCR

The plasmids showing desired size were used as a template for the amplification of the mutant genes for confirmation of ligation of the gene into pRSET A vector. Gene specific primers were used to amplify the gene from plasmid DNA of each clone harboring different mutant L-asparaginase genes. The PCR should show amplification of ~1 kb bands in each case. (Fig. 5.18 and 5.19) confirmed the presence of mutant genes in plasmid pRSET A which further confirmed the ligation of the gene in the vector in case of G238N, E232A, Q112H and D103V mutant genes.

![Gene Specific PCR Image]

Fig. 5.18 Gene Specific PCR of mutant gene using isolated plasmid as template

(Lane M – Step up 1 Kb Marker; Lane 1 – Gene Specific PCR amplicon of G238N; Lane 2 – Gene Specific PCR amplicon of E232A)

Following the confirmation of the presence of the insert, the presence of desired mutation in the insert was confirmed by sequencing the pRSET A vector harboring the insert.
Fig. 5.19 Gene Specific PCR of mutant gene using isolated plasmid as template

(Lane M – Standard 1 Kb Marker; Lane 1 – Gene Specific PCR amplicon of D103V; Lane 2 – Gene Specific PCR amplicon of Q112H)

5.3.9 Mutant clone confirmation through sequencing

The isolated plasmids were further sequenced to confirm the presence of L-asparaginase gene sequence and insertion of the mutations at the desired positions. The sequences obtained were checked for the nucleotide base change by aligning the sequence using a multiple sequence alignment program ClustalW2 (Fig. 5.20, 5.21, 5.22 and 5.23) Aligning the L-ASNase mutant gene sequences, i.e G238N, E232A, Q112H and D103V, with the wild type L-asparaginase gene sequence of *B. licheniformis* should reveal the mutation by nucleotide mismatch (Fig. 5.20, 5.21, 5.22 and 5.23).
Table 5.1 Codon replacement for respective mutations as observed in sequence alignment with wild type L-asparaginase gene sequence

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Codon</th>
<th>Replaced codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>G238N</td>
<td>GGC</td>
<td>AAC</td>
</tr>
<tr>
<td>E232A</td>
<td>GAG</td>
<td>GCC</td>
</tr>
<tr>
<td>Q112H</td>
<td>CAA</td>
<td>CAT</td>
</tr>
<tr>
<td>D103V</td>
<td>GAT</td>
<td>GTT</td>
</tr>
</tbody>
</table>

Fig. 5.20 Sequence alignment of G238N mutant with native ansA3 gene

The box highlights the nucleotide base substitution from GGC (Gly) to AAC (Asn).
Fig. 5.21 Sequence alignment of E232A mutant with native ansA3 gene

The box highlights the nucleotide base substitution from GAG (Glu) to GCC (Ala).

Fig. 5.22 Sequence alignment of Q112H mutant with native ansA3 gene.

The box highlights the nucleotide base substitution from CAA (Gln) to CAT (His).

Fig. 5.23 Sequence alignment of D103V mutant with native ansA3 gene.

The box highlights the nucleotide base substitution from GAT (Asp) to GTT (Val).
5.3.10 Expression of Mutant L-asparaginases

For the expression of the mutant genes, transformation of 5 µl plasmid harboring mutant gene was carried out into *E.coli* BL21. The transformants grown on LA plate containing ampicillin were picked and transferred to 10 ml LB broth and then subsequently into 200 ml LB broth and protein expression was induced adding 0.5 mM IPTG.

5.3.11 Purification of Mutant rL-asparaginases

The cell free extracts after sonication were used as crude enzyme and applied to affinity chromatography (Ni-NTA) column to purify the expressed recombinant proteins. The purified proteins were then checked on SDS-PAGE.

![SDS-PAGE separation of G238N mutant L-asparaginase](image)

*Fig. 5.24 SDS-PAGE separation of G238N mutant L-asparaginase*

(Lane M – Standard protein marker; Lane 1 – purified G238N mutant enzyme)

The homogeneity of the purified protein was evaluated by SDS-PAGE which showed a single denatured polypeptide of ~37 kDa. A single band was found at ~37 kDa when compared with the standard molecular weight markers.
Fig. 5.25 SDS-PAGE separation of E232A, Q112H and D103V mutant L-asparaginases (Lanes M – Standard protein marker; Lanes 1 – purified mutant enzyme (a) E232A, (b) Q112H and (c) D103V )

5.3.12 Activity of Mutant recombinant L-asparaginases

The purified recombinant mutant enzymes were incubated under assay conditions and the amount of ammonia liberated was estimated. After asparaginase assay, it was observed that only D103V mutant showed good asparaginase activity of 597.7 IU/mg whereas, G238N, E232A and Q112H mutants did not form the products as observed on
detection by Nessler’s assay indicating the loss of enzyme activity. The loss of enzyme activity can be attributed to the change in the surface charge.

The result of purified D103V mutant L-ASNase is summarized as followed in the table 5.2.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Activity (U)</th>
<th>Protein Concentration (mg)</th>
<th>Specific Activity IU/mg</th>
<th>Purification Fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>2229.17</td>
<td>114.4</td>
<td>194.85</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA Affinity Chromatography</td>
<td>1375.00</td>
<td>23.0</td>
<td>597.77</td>
<td>3.07</td>
<td>61.68</td>
</tr>
</tbody>
</table>

Table 5.2 Summary of characters of the purified D103V mutant L-ASNase

Comparing D103V mutant with the native recombinant enzyme (Table 4.1, chapter 4), it was found that the native recombinant enzyme had a specific activity of 407.65 IU/mg whereas the mutant had a specific activity of 597.7 IU/mg. The mutant, thus, exhibited higher activity.

Aspartate at position 133 in *Erwinina chrysanthemi* L-asparaginase (ErA) lies at the loop region required for the activity of the enzyme replacing Aspartate to Valine in ErA led to an increased flexibility due to non-participation in the hydrogen bonding network present locally (Kotzia and Labrou, 2009). On aligning the protein sequences (both rBliAIII and ErA) it was observed that position 133 in ErA was found to lie at amino acid 103 of the
rBliAIII protein sequence. 103 position of rBliAIII is present at the active site loop region and the replacement of Aspartate to Valine in rBliAIII increased the flexibility of the loop. This change in the property of D103V mutant enzyme may be attributed to restriction of the conformational freedom of the protein or to the energetic contribution of the newly formed H-bond and vanderwaals interactions. Similar kind of studies have been done which indicated that the flexibility of the loop region determines the catalytic activity of the enzyme (Randall et al, 2006).

However, the purified mutants G238N, E232A and Q112H L-asparaginases did not show asparaginase activity indicating the loss of enzyme activity. The charge on amino acid residues both in the interiors and the surface of the protein contribute to biological activity. The role of charged residues buried in substrate binding pockets in catalysis and substrate specificity has been well proven. However, the effect of altering charge on the protein surface even by replacement of a single residue has proven to provide structural determinants of enhanced stability in certain proteins (Sanchez-Ruiz and Makhatadze 2001; Gitlin et al, 2006; Strickler et al, 2006).

Mutations at well exposed positions in the native structure are less likely to be disruptive and significantly affect the structure and function of the protein and thus such mutations are advantageous. The effect of surface charge on the stability of the enzyme L-asparaginase II from Escherichia spp. was studied. It was concluded that the surface charge alteration contributed to the enhancement of stability without affecting the structure (Vidya et al, 2014).
5.3.13 Kinetic features of D103V L-asparaginase

*Effect of Temperature on D103V L-asparaginase Activity*

The reaction rate of the mutant D103V L-asparaginase was measured at different temperatures. Maximum activity was obtained at 37°C indicating the optimum temperature for enzyme activity. Although the L-asparaginase activity declined at higher temperatures, D103V L-asparaginase showed significant activity even at 90°C and 100°C.

![Fig. 5.26 Effect of temperature on D103V mutant enzyme activity](image)

Maximum activity at 37°C has been reported for many species such as *Pseudomonas aeruginosa* (El-Bessoumy et al, 2004), *Erwinia carotovora* (Kamble et al, 2006). However, activity of L-asparaginases above 90°C has been very rarely found, L-asparaginase from *Pyrococcus furiosus* being an example showing significant activity at 90°C (Bansal et al, 2010).
The enzyme activity at high temperatures after engineering has also been seen in *Escherichia coli* L-asparaginase after surface charge alteration. As in this case surface amino acid Aspartate, negatively charged was replaced with Valine, neutral, similar charge replacements in case of *Escherichia coli* concluded that optimization of surface charge contributed much to the thermal properties of the protein (Vidya et al, 2014).

**In-Vitro Half-life of D103V L-asparaginase**

In-vitro half-life of the mutant D103V L-asparaginase was determined by carrying out an experiment in which thermal stability of the enzyme was evaluated at physiological temperature i.e. 37°C. In this experiment the enzyme was pre-incubated at 37°C for different time periods. After pre-incubation, the enzyme was placed in the reaction mixture and the amount of ammonia liberated was measured. The stability of the enzyme deteriorated gradually with time.

The in-vitro half-life of the mutant D103V L-asparaginase was found to be upto 9 hours. On comparison with the native recombinant L-asparaginase, the in-vitro half-life was found to be ~3 hours. Thus the thermal stability of the mutant enzyme at 37°C increased more than 2.5 fold. This property of the enzyme makes it interesting as a therapeutic protein due to its long term stability.

Successful engineering for thermal stability of the current commercial L-asparaginases from *E. coli* (Li et al, 2007) and *E. carotovora* (Kotzia and Labrou, 2011) have already been studied. Increased thermal stability has often been attributed to changes in surface charge where a charged amino acid in neutral environment can destabilize the protein (Kotzia and Labrou, 2009).
Increase in thermal stability has also been observed due to chemical modification such as PEGylation of L-asparaginase (Kotzia et al, 2007). It is believed that PEGylation usually enhances the thermal stability of proteins by sterically blocking degradation pathways induced by hydrophobic interactions (Arakawa and Timasheff, 1985) and by causing non-specific steric hindrance of the intermolecular interactions that are involved in thermal instabilities (Hinds and Kim, 2002).

The non-specific steric hindrance caused by PEGylation also reduces the L-asparaginase activity (Kotzia et al, 2007). This reduction in enzyme activity conjugated with the immunogenic reactions caused in response to the chemical PEG (Wang et al, 2003) make genetic modification a better choice for engineering than chemical methods, as has been tried in the present study.

Better thermal properties such as higher thermal inactivation temperature or a higher in-vitro half-life can also have better applications in the food industry where it can play an
important role in better and faster degradation of acrylamide (Friedman, 2003; Ciesarová et al, 2006).

**Kinetic Parameters of D103V**

The steady state kinetic properties of the D103V mutant enzyme were studied at various substrate concentrations. The $K_m$ of the purified mutant L-asparaginase was calculated from the Lineweaver-Burk plot of $1/V$ versus $1/[S]$. The $K_m$ of the enzyme was 0.42 mM and $V_{max}$ of the enzyme was found to be 2778.9 µmol min$^{-1}$. The $K_m$ and $V_{max}$ of the native recombinant L-asparaginase was 0.6 mM and 36.6 µmol min$^{-1}$.

The mutant as compared to the native recombinant enzyme showed a lower $K_m$ and a higher $V_{max}$. Lower $K_m$ indicates a higher affinity towards its substrate, L-Asparagine which might relate to the degree of effectiveness in the treatment of ALL. However, the lowest $K_m$ for L-asparaginase has been observed in EcAII (Mashburn and Wriston, 1964).

![Substrate saturation curve of D103V mutant and native rL-asparaginase](image)

**Fig. 5.28 Substrate saturation curve of D103V mutant and native rL-asparaginase**
5.4 Conclusions

The predicted structure of rBliAIII showed overall structural homology with EcA and ErA with some dissimilarities at the loop regions of the enzyme, which makes it distinct from compared structures of EcA and ErA. Site-directed mutagenesis was carried out using the isolated plasmid harboring the ansA3 gene as the template. The mutations were carried out to enhance the characteristics of the native recombinant enzyme studied previously. Mutations were carried out to alter the surface charge on the native recombinant L-asparaginase enzyme from *B. licheniformis*. Mutations were incorporated in the ansA3 gene by nucleotide base substitution. The mutant D103V showed higher activity as compared to the native enzyme at 37°C whereas the mutants G238N, E232A and Q112H enzyme showed loss of activity. This loss in activity could be explained due to surface charge alterations. Thermal inactivation studies of the D103V mutant enzyme showed that though the activity decreased rapidly at high temperatures, the mutant

![Fig. 5.29 Lineweaver-Burk plot of D103V mutant L-asparaginase](image-url)
enzyme showed significant activity at temperatures up to 100°C. This suggested that replacement of aspartic acid (D) to valine (V) at amino acid position 103, is responsible for thermostability of the enzyme. In-vitro half-life of the mutant enzyme was determined at physiological temperature and it was about 9 hours whereas the wild type (native) enzyme had half life of about 3 hours, indicating 3 fold increase in half life. The results of this study provided a rare data on the basis of thermal stability of *Bacillus licheniformis* L-asparaginase. It also provides a basis for the new and improved forms of the enzyme for future therapeutic use. L-asparaginase being a therapeutic protein, should have higher stability in terms of shelf life and a higher half life in the body when delivered. Also this thermostable enzyme variant may prove to be a potential source for application in the food industry.
5.5 References


dihydropyrimidine derivative on some of its kinetic parameters. Indian J Biochem Biophys, 43: 391–394.


