

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

EFFECT OF LYS99GLN AND ASP97GLY MUTATION IN
***LACHANCEA THERMOTOLERANCE* ASPARAGINASE ACTIVE**
SITE ON SUBSTRATE AFFINITY

BERKCAN ŞENEL
A THESIS SUBMITTED FOR THE DEGREE OF
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THESIS SUPERVISOR
ASSOC. PROF. DR. BARIŞ BİNAY

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2023

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

LACHANCEA THERMOTOLERANCE
ASPARAGİNAZIN AKTİF BÖLGESİNDE
LYS99GLN VE ASP97GLY
MUTASYONUNUN SUBSTRAT AFİNİTESİ
ÜZERİNE ETKİSİ

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YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ANABİLİM DALI

TEZ DANIŞMANI
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2023



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SUMMARY

L-asparaginases (L-ASNase) are amidohydrolase enzymes which have a predominant L-asparaginase activity that catalyzes the conversion of L-asparagine to L-aspartic acid and ammonia. Also, some of them have secondary L-glutaminase activity. For this properties L-asparaginase is used to treat acute lymphoblastic leukemia (ALL). Elspar® and Erwinaz® L-asparaginases are widely used in the pharmaceutical industry to produce therapeutic products. But the hypersensitivity reactions are the main limitations of commercial L-asparaginases. This situation has forced scientists to search for promising L-asparaginases from new sources. Hence, the exploration of alternate sources of yeast L-ASNases holds promise in mitigating toxicity and improving efficacy; nevertheless, the investigation of these sources remains limited. This study examines the effects of site-directed mutagenesis on *Lt*ASNase enzyme structures from *Lachancea thermotolerance* (*Lt*). In the study focusing on the Lys99Gln and Asp97Gly regions of the enzyme, K_M and k_{cat} values were obtained and compared with the wild-type enzyme. SwissModel was used for the modelling study. When mutant and wild-type kinetic characteristics were examined, Lys99Gln *Lt*ASNase has a higher K_M and lower k_{cat} than wild-type. The Asp97Gly mutation has too low activity to estimate kinetic parameters. This study constitutes an important step for elucidating the *Lt*ASNase enzyme structure and interactions. In addition to the mutant enzyme kinetic studies mentioned above, storage activity studies were also carried out. As a result of the experiments, it was concluded that as the storage time increased, the relative activity of the enzyme decreased, and the most suitable storage temperature was -80°C.

Keywords: L-ASNase, Site-Directed Mutagenesis, Enzyme Kinetic, Homology Modelling.

ÖZET

L-asparaginazlar (L-ASNaz), L-asparajinin L-aspartik asit ve amonyağa dönüşümünü katalize eden baskın L-asparaginaz aktivitesine sahip amidohidrolaz enzimleridir. Ayrıca bazılarının ikincil L-glutaminaz aktivitesi vardır. Bu özellikleri nedeniyle L-asparaginaz, akut lenfoblastik lösemnin (ALL) tedavisinde kullanılır. Elspar® ve Erwinaz® L-asparaginazlar ilaç endüstrisinde terapötik ürünler üretmek için yaygın olarak kullanılmaktadır. Ancak aşırı duyarlılık reaksiyonları ticari L-asparaginazların ana sınırlamalarıdır. Bu durum bilim adamlarını yeni kaynaklardan gelecek vaat eden L-asparaginazları aramaya zorladı. Bu nedenle, maya L-ASNazlarının alternatif kaynaklarının araştırılması, toksisitenin azaltılması ve etkinliğin artırılması konusunda ümit vericidir; yine de bu kaynakların araştırılması sınırlı kalmaktadır. Bu çalışma, sahaya yönelik mutajenezin *Lachancea thermotolerance* (Lt) LtASNase enzim yapıları üzerindeki etkilerini incelemektedir. Enzimin Lys99Gln ve Asp97Gly bölgelerine odaklanan çalışmada K_M ve k_{cat} değerleri elde edilerek yabani tip enzimle karşılaştırıldı. Modelleme çalışması için SwissModel kullanılmıştır. Mutant ve yabani tipin kinetik özellikleri incelendiğinde Lys99Gln LtASNase'nin yabani tipe göre daha yüksek K_M ve daha düşük k_{cat} 'a sahip olduğu görüldü. Asp97Gly mutasyonu kinetik parametreleri tahmin etmek için çok düşük aktiviteye sahiptir. Bu çalışma LtASNase enziminin yapısını ve etkileşimlerini aydınlatmak için önemli bir adım oluşturmaktadır. Yukarıda bahsedilen mutant enzim kinetiği çalışmalarına ek olarak depolama aktivitesi çalışmaları da yapılmıştır. Yapılan deneyler sonucunda depolama süresi arttıkça enzimin bağıl aktivitesinin azaldığı, en uygun saklama sıcaklığının -80°C olduğu sonucuna varılmıştır.

Anahtar Kelimeler: L-ASNase, Bölgeye Yönelik Mutajenez, Enzim Kinetiği, Homoloji Modellemesi.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: Celsius degree
μl	: Microliter
μmol	: Micromolar
NaCl	: Sodium Chloride
NaH ₂ PO ₄ .H ₂ O	: Sodium Phosphate Monobasic Monohydrate
H ₂ SO ₄	: Sulfuric Acid
dH ₂ O	: Distillated water
ddH ₂ O	: Double distillated water
Asp	: Aspartic acid
g	: Gram
Gln	: Glutamine
Gly	: Glycine
h	: Hour
kDa	: Kilodalton
L	: Liter
LB	: Luria-Bertani
Lys	: Lysine
mL	: Milliliter
mM	: Millimolar
min	: Minute
MW	: Molecular Weight
nm	: Nanometer
rpm	: Rounds per minute
SDS-PAGE	: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Sec	: Second
SOC	: Super Optimal Broth with Catabolite Repression
WT	: Wild-type

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1. INTRODUCTION

1.1. Enzymes

Enzymes are mostly protein molecules that facilitate biochemical reactions as catalysts. They accelerate a reaction, thereby reducing the activation energy required for the reaction to occur. Enzymes are present in all organisms, including bacteria and humans. They are essential for survival because they allow cells to convert nutrients into energy and sustain metabolic processes [1]. Enzymes consist of a polypeptide chain of amino acids that fold into a distinct three-dimensional structure. This shape is crucial to the enzyme's ability to bind to specific substrate molecules. The enzyme forms an enzyme-substrate complex by binding to the substrate. This complex then undergoes a chemical reaction, producing new chemical compounds and releasing the enzyme molecule. This procedure continues until the completion of the reaction [2]. Figure 1.1 has also been given information about mechanism of enzyme action.

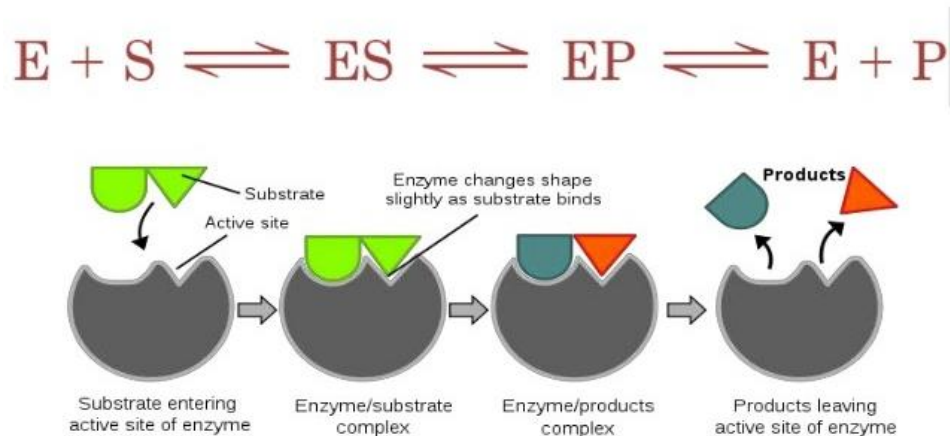


Figure 1.1: Mechanism of enzyme action [3].

Enzymatic reactions constitute a type of equilibrium process. Enzymes can regulate both forward and reverse reactions. The activation energy is the required energy for a chemical reaction to occur [4]. The Michaelis-Menten equation, which expresses the kinetics of enzyme reactions.

$$v_0 = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1.1)$$

The maximum rate (V_{max}) is the rate at which the enzyme reaches substrate saturation when the relationship between substrate concentration and reaction rate is taken into consideration. V_{max} is a theoretical value that will never be observed in practice. The K_M in the equation presented in Equation 1.1 represents the substrate affinity of the enzyme. At low K_M values, high enzyme activity and substrate affinity are possible [5].

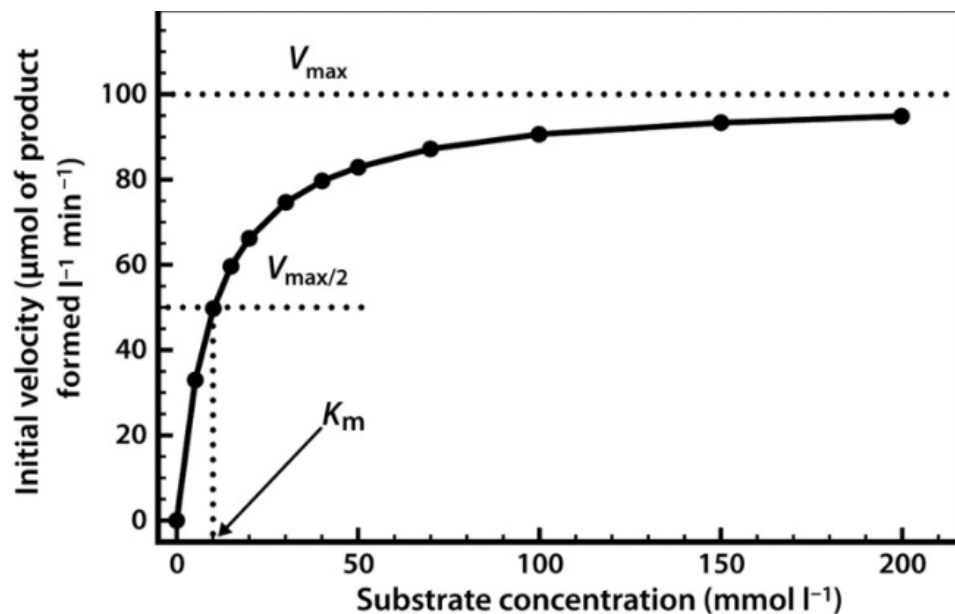


Figure 1.2: The correlation between the concentration of the substrate and the rate of the reaction [5].

Figure 1.2 demonstrates that K_M can also be regarded as the substrate concentration at which half of its maximum velocity is reached. pH and temperature are two parameters that influence enzyme kinetics. These values are representative of the questioned enzyme. Changing these values may result in functional alterations at the enzyme's active site. In addition to the effect of increasing temperature on reaction rate, the inactivation caused by denaturation above a certain temperature threshold must also be considered. Determining the optimal temperatures and measuring their thermal stability is, therefore, an additional crucial factor. k_{cat} is the quantity of substrate that an enzyme's active site converts into a product during an enzymatic

reaction. This is a guiding parameter for delineating the enzyme's efficiency and the functions of the metabolic network in which it resides [1], [5]. By studying enzyme kinetics, researchers can gain an understanding of the catalytic mechanism and control of enzyme activity, as well as the effects of drugs and enzyme activity modifiers [6].

The International Union of Biochemistry and Molecular Biology (IUBMB) has established a classification and naming system for enzymes. This classification system divides enzymes into six distinct classes, each with its own subclass. In addition, each subclass has its own set of naming rules. 1-oxidoreductases, 2-transferases, 3-hydrolases, 4-lyases, 5-isomerases, and 6-ligases are the six classes of enzymes. Within each class, sub-classes are designated by the type of chemical reaction they catalyze. Transferases are divided into kinases, phosphotransferases, and glycosyltransferases; hydrolases are divided into hydrolases, esterases, and peptidases; and lyases are divided into decarboxylases and lyases [7], [8]. They are used in a wide number of manufacturing processes, some of which include baking, brewing, the production of detergents and fermented goods, the processing of textiles and leather, and pharmaceutical manufacturing. Enzymes are also used to manufacture and enhance nearly 400 consumer and industrial products. In addition to technical applications, they are utilized in the food industry for processing raw materials and producing food products [9]-[10]. On the academic side, enzymes' potential for the production of biofuels, biopolymers, and enzyme engineering has been thoroughly investigated. In addition to their use in the production of drugs and materials, they are also employed in environmental applications [11]. L-Asparaginase is used to treat acute lymphoblastic leukemia (ALL), an aggressive form of blood cancer, by depleting levels of asparagine, a crucial amino acid for the development of cancer cells. It is also used in the food industry to prevent the formation of acrylamide, a potential carcinogen, in processed foods.

1.2. L -Asparaginases

L-asparaginase is a catalytic enzyme that enables the hydrolysis of L-asparaginase into L-aspartic acid and ammonia. This enzyme is also known as L-asparagine amidohydrolase, and Enzyme Code is 3.5.1.1. [12]. L-asparaginase is a tetramer protein composed of 4 identical monomers. The precise structure of the

tetramer can differ depending on the L-asparaginase enzyme source [13]. The enzyme L-asparaginase was discovered at the turn of the 20th century. In 1904, a German scientist named Karl Lang found the first evidence of asparagine-hydrolysis activity in cow tissue. He named the enzyme "asparaginase." Later on, in the year 1910, Furth and Friedmann confirmed that specific organs from horses and pigs possess enzymes that are able to break down the chemical asparagine. The discovery made by Furth and Friedmann increased our understanding of the asparaginase enzyme and served as a springboard for further research into the many L-asparaginase forms and characteristics [14]-[16]. The potential therapeutic application of L-asparaginase in the treatment of cancer was first discovered in 1953. This was accomplished by the observation that the formation of certain forms of lymphomas, which are a form of cancer that affects the immune system, in mice may be prevented by the administration of guinea pig serum. Broome then did more study and found that the enzyme L-asparaginase was the specific factor that caused this inhibition. This discovery has significant implications for comprehending and treating lymphomas with L-asparaginases [17], [18]. In addition, these enzymes have an extensive number of uses in a variety of different industries, including the medicine, the food industry, and also the biotechnology industry. In the field of biotechnology, L-asparaginases are utilized for the manufacture of L-aspartic acid, which is an essential intermediary in the synthesis of carbohydrates, amino acids, and antibiotics. In addition, these enzymes can be used to cut down on the amount of L-asparagine that is present in food products. This is important because high quantities of this amino acid have been linked to the production of acrylamide, which is thought to have carcinogenic properties [9]-[11]. As a cancer treatment method, L-asparaginases are widely applied in the field of medicine. In order to carry out their function, they degrade the amino acid L-asparagine, which functions as an essential nutrition for specific varieties of cancer cells, in particular 12 ALL cells. L-asparaginases are able to suppress the growth and proliferation of cancer cells by reducing the access that cancer cells have to the amino acid L-asparagine. This ultimately results in the death of the cancer cells. L-asparaginase is frequently combined with other chemotherapeutic agents to improve treatment outcomes [19]. Upon reviewing the existing literature, it becomes evident that numerous organisms have been employed as sources of enzymes in the aforementioned fields of L-asparaginase utilization study.

1.2.1 Sources of L-Asparaginase

L-ASNases have two distinct subgroups that can be synthesized within the same bacterial organism. While the amino acid sequences of both isozymes are known to be quite similar, it is notable that they display obvious distinctions in terms of their biochemical properties and structural characteristics. The isozymes, categorized as Type 1 and Type 2, are characterized based on their cellular localisation and their affinity for L-glutamine, and L-asparagine [20].

Type 1 L-ASNases are localized in the cytoplasm and exhibit constitutive enzymatic activity, indicating that their production is not influenced by external factors. These organisms exhibit deficiencies in several traits, including their limited ability to hinder the production of proteins and their reduced effectiveness in restraining the expansion of tumors, which can be attributed to their comparatively poor affinity for L-asparagine. In contrast, Type 2 L-ASNases are situated within the periplasmic region, which is the gap between the bacterial peptidoglycan and the cytoplasmic membrane. These enzymes exhibit anti-tumoral properties specifically targeting tumors that exhibit a strong affinity for L-asparagine and little action towards L-glutamine. The enzymes under consideration exhibit the property of being inducible, which implies that their synthesis can be augmented in response to alterations in the growth medium [21].

The utilization of Type II L-asparaginases derived from the gram-negative bacterial species *Escherichia coli* (*E. coli*), and *Erwinia* spp. is prevalent in the treatment of several types of blood malignancies, with a particular focus on acute lymphoblastic leukemia, owing to their strong affinity for L-asparagine. *E. coli*, a bacterium, with the capacity to biosynthesize both forms of L-asparaginases [22].

In recent research projects, investigations have been undertaken to ascertain an enzyme possessing enhanced attributes, economically viable production methods, and reduced negative impacts as compared to L-asparaginase obtained from *E. coli*. The investigation of alternate sources of L-asparaginase has garnered significant attention due to the potential for these sources to produce enzymes with unique characteristics and reduced adverse effects. The utilization of eukaryotic microorganisms, specifically fungi, as a prospective reservoir of L-asparaginase, has emerged as a notable field of investigation. Numerous fungal taxa, such as *Penicillium*, *Fusarium*, and *Aspergillus*

have been the subject of scientific inquiry on their capacity for L-asparaginase synthesis. At present, the food industry has utilized fungal recombinant L-asparaginase derived from *Aspergillus niger*, and *Aspergillus oryzae*. Additionally, promising outcomes have been documented with the utilization of endophytic fungus belonging to the genera *Talaromyces*, *Eupenicillium*, and *Colletotrichum*. Moreover, the presence of anti-neoplastic properties has been observed in fungi obtained from maritime habitats, particularly from endophytic seaweed. These fungi belong to many genera, including *Pithomyces*, *Cladosporium*, *Chaetomium*, *Nigrospora*, and *Curvularia*. Previous studies have demonstrated that the genus *Aspergillus* has the potential to be employed as a means of generating significant amounts of L-asparaginase. The findings of these investigations indicate that the utilization of eukaryotic microorganisms, particularly fungus, as a potential reservoir of L-asparaginase, has significant promise for further investigation and advancement in the field [16].

1.2.2 Biochemical Properties of L-Asparaginase

Asparaginases break the amide link, which results in the formation of aspartic acid and ammonia from the amino acid asparagine. During this reaction, the importance of the biochemical characteristics of enzymes is particularly crucial because of the reaction that is taking place. Hydrolysis is the reaction that takes place when anything hydrolyzes. The hydrolysis rate and the K_M in asparagine hydrolysis, for instance, are the primary reasons why asparaginases are an enzyme that is utilized in the treatment of leukemia. Another explanation is the glutamine reaction activity [23]-[25]. The amino acid sequences of asparaginases, as well as their substrate selectivity, biochemistry, and the organism in which they are generated, are used to categorize these enzymes into a few different classes. The structure of each asparaginase is very similar to that of the others. *ans A* and *ans B* are the genes that code for the eca I and eca II proteins that are produced by *E. coli*. It is essential that they share a homology in the sequence of their amino acids with one another. Asparaginases derived from bacteria are made up of 330 different amino acids. They are considered to be oligomers since a single chain is copied either two or four times along its substructure. In bacterial class II asparaginases, the regions Thr12, Tyr25,

Thr89, Asp90, and Lys162 were all conserved in their original state. Both class I, and II asparaginases are made up of identical four-membered subunits. Tetramers are generated when two dimers are linked together in a very close form [26]. The ASNases produced by *E. coli* have been measured to have molecular weights ranging from 134 to 142 kDa. Additionally, during the process of dimer formation, active site creation takes place at the interface between the two molecules. When looking at the structure as a whole, the ASNase II tetramer has four active sites (PDB: 3eca) . This is due to the fact that there are two active sites at each interface [27], [28]. Changes in pH and temperature may also occur, depending on the enzyme that is produced by the organism that serves as the source. It is critical to carry out research on the aforementioned conditions in order to activate enzymes. On the other hand, it can be demonstrated that 37 °C is the optimal operating temperature, and that the pH ranges from 8-10. In his investigation to evaluate the pH range of *E. coli*-derived ASNase utilized in the treatment of leukemia. Scientist identified a large range of pH values, such as 4.5 and 11.5 pH range of the enzyme. This broad range of pH values was observed in the enzyme [29]-[31]. L-ASNase II's periplasmic homo-tetramer structure has a high affinity for the substrate (asparagine), which is one of the advantages of this configuration. When compared to the other class, it has a K_M value that is significantly lower [32], [33].

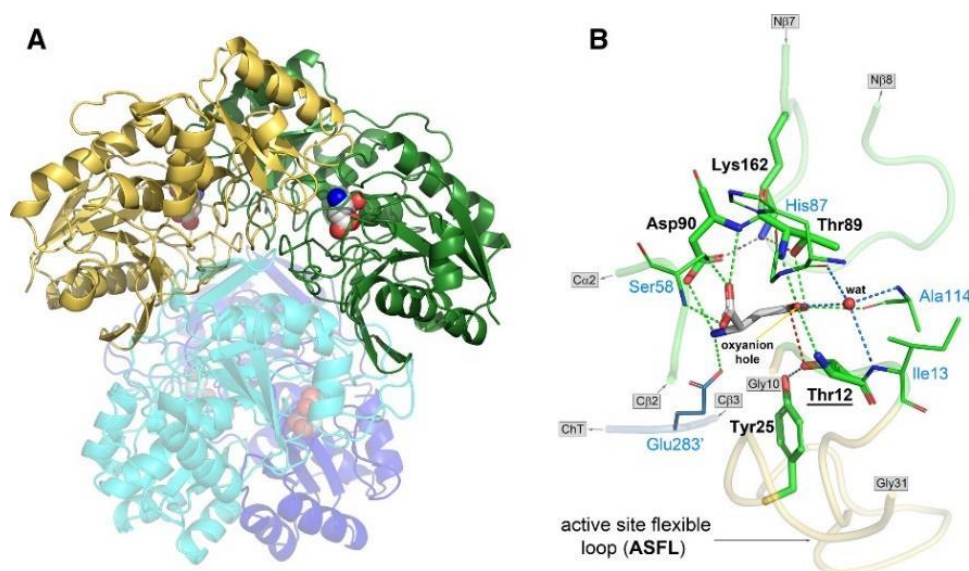


Figure 1.3: ASNase tetramer structure (A) and active side of ASNase I – ASNase II (B) [28].

One of the key objectives in the development of the asparaginase enzyme is to achieve improvements in toxicity. It is feasible to observe an immune-based allergy as well as a large antibody production when the substance in question is administered to living species. It is possible for asparaginase to develop a drug antigen, also known as a resistance. Research is being done to find ways to circumvent this barrier. In addition, it proposed that the study of silent antibodies is possible without having an effect on the activity of the asparaginase enzyme in toxicity improvements [33], [34]. Improvement the toxicity profile of the aforementioned L-asparaginase holds great significance, particularly in the context of cancer therapy.

1.2.3 The Role of L-Asparaginase in Cancer

Cancer is a medical condition characterized by the uncontrolled proliferation and reproduction of abnormal cells within the body. The neoplastic cells have an increased requirement for asparagine, which is essential for protein synthesis and cellular proliferation [33], [35]. The synthesis of asparagine by healthy human cells allows them to fulfil their own asparagine requirements, whereas cancer cells are incapable of producing sufficient amounts of asparagine endogenously. Consequently, the dependence of cancer cells on exogenous asparagine is a key factor that contributes to the efficacy of L-asparaginase as a therapeutic agent in the treatment of cancer. The enzyme L-asparaginase catalyzes the hydrolysis of the amino acid L-asparagine, leading to the production of L-aspartic acid and ammonia. This impedes the ability of cancer cells to obtain the essential nutrient L-asparagine, which is crucial for their proliferation and viability. The reason behind this phenomenon is because L-asparaginase effectively diminishes the intracellular levels of this vital amino acid within cancer cells, thereby impeding their ability to successfully go through the cell cycle [36]. The essential process of endogenous asparagine synthesis, which is assisted by the enzyme asparagine synthetase, plays a crucial role in maintaining cellular health. Nevertheless, among cancer cells, the insufficiency or scarcity of this particular enzyme leads to an incapacity to produce adequate quantities of asparagine. Consequently, these cells must rely on external sources to meet their metabolic requirements. The delivery of the L-asparaginase enzyme during chemotherapy leads to the reduction of asparagine levels in the bloodstream, resulting in a shortfall in the

G1 phase of the cell cycle, which is responsible for protein synthesis. The aforementioned insufficiency ultimately results in the initiation of programmed cell death, known as apoptosis, in cancerous cells, thereby impeding their ability to multiply [37].

The identification of the potential therapeutic application of L-asparaginase in the treatment of cancer was a notable advancement in the domain of oncology. L-asparaginases have been employed as a therapeutic modality for many cancers acute myelomonocytic leukemia, ALL, and Hodgkin's disease [38]. It is important to highlight that the L-asparaginases currently used in the treatment of leukemia are predominantly sourced from bacteria and demonstrate glutaminase activity. The use of these enzymes has been employed to deplete cancer cells of L-asparagine, resulting in their demise. Nevertheless, despite the positive outcomes observed in clinical settings for these enzymes, current research efforts are primarily directed towards the identification of L-asparaginases that possess a broader range of structural variations, while also demonstrating reduced levels of glutaminase activity, a lower K_M value, and a decreased molecular weight. Furthermore, ongoing research is focused on investigating the distinct responses of these enzymes to effector molecules [38], [40].

Table 1.1 presents a comprehensive overview of the commercial L-asparaginase enzymes that have been successfully introduced into the pharmaceutical market thus far [41]. In addition, there has been ongoing research into different drug delivery methods aimed at prolonging the presence of the L-asparaginase enzyme in the bloodstream. These methods include the utilization of polymers like polyethylene glycol to conjugate the enzyme, as well as the encapsulation of the enzyme in microcapsules or liposomes. The half-life of the enzyme in the bloodstream is a critical factor that significantly influences its anticancer efficacy. These strategies have the potential to enhance the effectiveness of the enzyme by prolonging its interaction with tumor cells, hence resulting in a more pronounced therapeutic impact [42].

Table 1.1: L-asparaginases available on the market for cancer treatment [41].

Bacterial Source	L-asparaginase	Manufacturer
<i>Erwinia chrysanthemi</i>	Erwinase [®]	Porton Biopharma, UK
	Erwinaze [®]	Jazz Pharmaceuticals, USA
<i>E. coli</i>	Oncaspar	Sigma-Tau Pharmaceuticals, Italy
	Elspar	Merck Sharp & Dohme, USA
	Crasnitin	Bayer, Germany
	Leunase	Kyowa Hakko, Japan
	Kidrolase	France Rhone-Poulenc, Canada & USA

There is a possibility that the wild-type of L-asparaginase or any other enzymes cannot be used in industry or in any of the other applications for which they are intended. Studies conducted today in the field of enzyme engineering strive to improve the target qualities of the enzyme that is desired.

1.3. Protein Engineering

Protein engineering is the process of creating proteins with desired properties. It is an interdisciplinary field that utilizes biochemistry, molecular biology, and bioinformatics knowledge. Engineered proteins have specific structures, functions, and activities [25]. Since the 1990s, the field has experienced rapid expansion and is now an integral part of biotechnology, drug design, and biomedicine. Protein engineering is also applied to improve the stability, activity, and specificity of already-existing proteins. This is achieved by introducing mutations into the protein-coding gene sequences [25], [43]. Scientists can gain a greater understanding of protein structure, function, and activity by systematically introducing mutations and testing their effects. Protein engineering has also been used to create proteins with novel functions, such as those that can bind to particular small molecules or other proteins. Recently, protein engineering has gained popularity as a technique for enhancing enzymatic activities, enhancing enzyme stabilities, and expanding product range.

There are primarily three different approaches to the manipulation of proteins. These three approaches are referred to as Partial Rational Design, Directed Evolution, and Rational Design respectively. [44], [45]. The three main protein engineering approaches are given in figure 1.4.

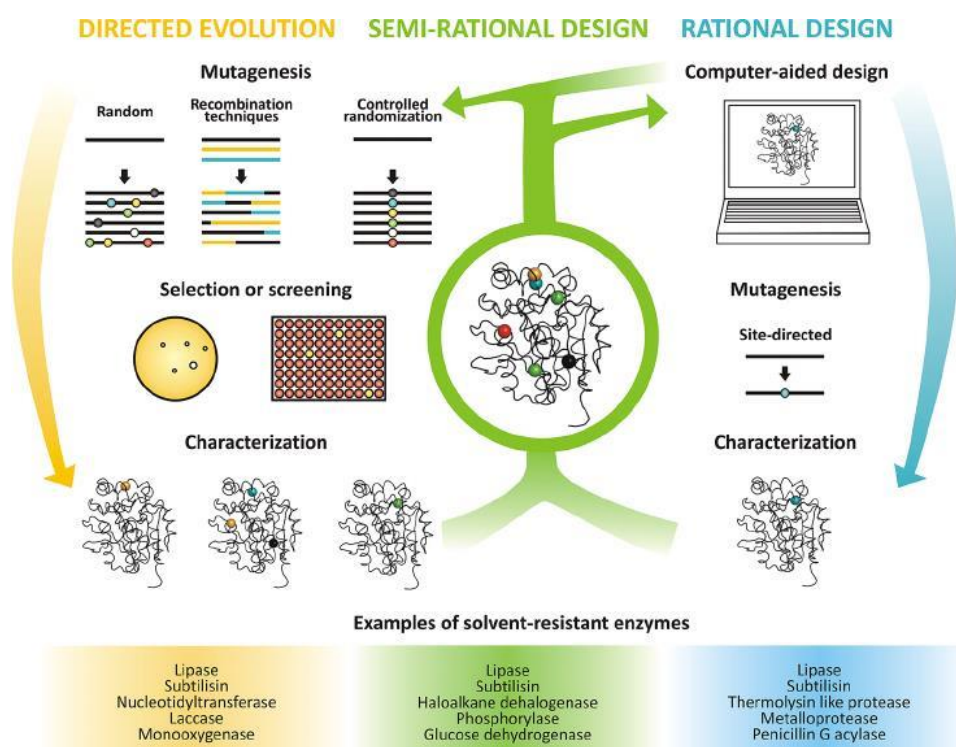


Figure 1.4: Schematic presentation of protein engineering approaches [46].

The most prevalent protein engineering technique is site-directed mutagenesis. In addition, a thorough understanding of the structure-function relationship of the enzyme is necessary. Locally directed mutation aims to replace an amino acid in a specific region with another desired amino acid, taking into consideration the protein's structure. In this technique, the first step involves the creation of a molecular model. Following this is the experimental design and analysis of the characteristic properties of the protein. This technique can be used to modify cofactor or substrate specificity, as well as to increase stability against oxidative stress and temperature. To change the enzyme's properties, the primary structure of the protein is modified. The structure and function of an enzyme are able to be altered with a single point mutation. Polymerase chain reaction with specific primers modifies the sequence of the gene [47], [48].

Based on Darwin's theory of evolution, which found widespread use in protein engineering at the end of the 19th century, directed evolution is a technique in which different protein variants are created without knowledge of their structure or function. This method involves the creation of an enzyme library and the experimental screening of the desired phenotype using screening techniques. Directed evolution techniques include gene manipulation, either by DNA shuffling or in vitro recombination, to improve or achieve the desired trait with traits that are not normally present. The mixed DNA technique relies on the homology of the protein sequence and the formation of hybrid sequences through the recombination of DNA fragments generated by brief PCR cycles. In order for sequences to recombine, a minimum of 70% homology between genes is required. Random mutation is another significant technique in directed evolution. It is common practice to use PCR and DNA polymerases that are prone to error. Typically, polymerases utilized in PCR reactions generate errors. This property is advantageous for random mutagenesis and can be augmented under less-than-ideal conditions [49], [50].

Using rational design, specific protein residues can be altered. Directed evolution, on the other hand, permits protein engineering by simulating the mechanism of natural selection and does not necessitate the protein's particular structure. Both approaches have advantages, but they also have drawbacks. As a result, the semi-rational method was created as a combination of rational design and directed evolution. This method requires knowledge of the protein's structure, though not as much as rational design. Around the region of interest, site-directed mutagenesis is applied. However, the primers utilized in this technique are degenerative primers whose amino acid compositions vary. Consequently, with directed evolutions, a library containing multiple mutations of the relevant protein is generated. [51], [52].

In particular, the Iterative Saturation Mutagenesis (ISM) method, one of the semi-rational design strategies, is very useful for developing the target enzyme with an effective and efficient screening technique for the relevant enzyme. The ISM focuses only on amino acid positions of the enzyme that are predicted to be functional. The mutant library obtained with ISM is smaller and gives better quality results for functional mutants. ISM is similar to rational design by using the structural information of the enzyme, and similar to directed evolution by creating a mutant library; it is a successful combination of both basic methods, resulting in better quality mutants with less work. For this, it uses the combined active site saturation test (CAST)

technique. The amino acid position of interest is chosen considering that it contributes to the property being attempted to be formed in the enzyme. The ISM/CAST technique is shown schematically in Figure 1.5.

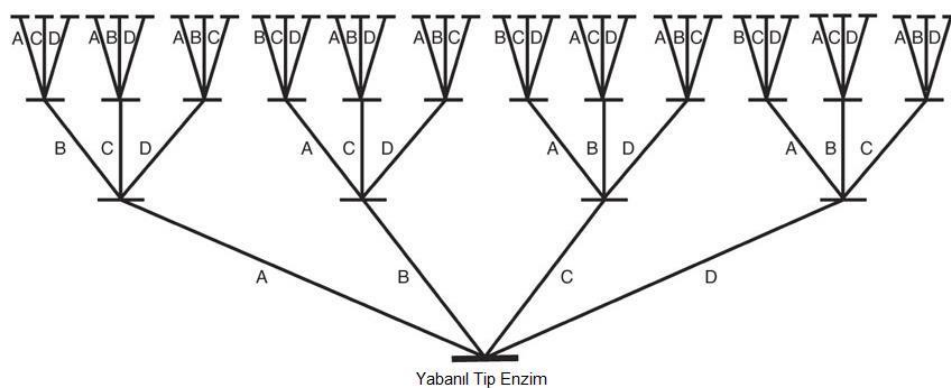


Figure 1.5: Schematic illustration of ISM/CAST technique [53].

Considering that the regions named A, B, C and D contain at least 2 amino acids; With each cycle of PCR, new mutants are obtained and mutants with the targeted trait can be obtained [53], [54].

The results of protein engineering experiments on L-asparaginases are presented in Figure 2.6. These results were collected during the process of conducting a literature review for this thesis.

Table 1.2: Sources of L-ASNase and mutation effects from literature review.

Source	Mutations	Results achieved	Reference
<i>E. coli</i> (Eca II)	N178P	Retention of 99% L-asparaginase activity at 50C (wild-type 71%).	Li et al., 2007 [55]
	N24G	AEP resistant-Retention of 45% L-asparaginase activity.	Patel et al., 2009 [56]
	Y176S	Increase of V_{max}/K_M for L-aspartic acid beta-hydroxamate	Mehta et al., 2014 [57]
	K288S/Y176F	10-fold less immunogenic	Vidya et al., 2014 [58]

Continued on next page

Table 1.2: Sources of L-ASNase and mutation effects from literature review.(Contd)

Source	Mutations	Results achieved	Reference
	K139A	Retention of 65% L-asparaginase activity at 65C (wild-type 40%)	Vidya et al., 2014 [58]
	Y176F	Increase of $V_{\max}/K_M$ for L-aspartic acid beta-hydroxamate	Verma et al., 2014 [59]
	D96A	Decreased activity against L-Glu and increased against L-Asp	Ln et al., 2011 [60]
	Y250L/R195S	Enhanced tetramer compactness and a decreased active site cavity	Offman et al., 2011 [61]
<i>Elwinia carotovora</i>	R206H	Enhanced resistance to trypsin degradation and higher thermostability	Kotzia et al., 2007 [62]
<i>Erwinia chrysanthemi</i>	D133V	Higher thermal stability	Kotzia and Labrou 2009 [63]
<i>Pectobacterium carotovorum</i>	N96A	Decreased glutaminase activity (30%) and increased asparaginase activity (40%)	Ln et al., 2011 [60]
<i>Pyrococcus furiosus</i>	K274E	Resistant to proteolytic digestion and no displayed glutaminase activity	Bansal et al., 2012 [64]
<i>Saccharomyces cerevisiae</i>	T64A, Y78A, T141A, K215A	99.9% loss of activity	Costa et al., 2016 [65]
<i>Bacillus subtilis 168</i>	S180N-D289TE260F-E292S	Compared with the wild-type enzyme, it exhibited an 8.1-fold increase in half-life at 65C and a 5.56C increase in melting temperature	Feng et al., 2017 [66]
	A26N-G29F	With 3.44-fold and 7.76-fold increases in specific activity and half-life at 65C in comparison with the wild-type	Feng et al., 2019 [67]
<i>Bacillus licheniformis</i>	D103V	Enhanced kinetic efficiency and thermal stability than wild type	Sudhir et al., 2016 [68]
<i>Wolinella succinogenes</i>	V23E/K24T	These mutations have been reported to develop resistance to trypsin with N-peptide that can bind to heparin, and its therapeutic efficiency remains high compared to reference enzymes.	Sannikova et al., 2016 [69]

1.4. Objective and Importance

An initial round of investigation into the structure of the *LtASNase* enzyme was carried out in advance of beginning of the laboratory experiments. In the framework of this study, mutation estimates were derived for the active site of the enzyme in order to investigate the feasible effects of enzyme mutation on the values of K_M and k_{cat} . Specifically, the structural models of *LtASNase* and *E. coli* *ASNase* were compared, and following that, the decision was made to apply the Lys99Gln and Asp97Gly mutations. When homology modeling of *LtASNase* was analyzed, it was hypothesized that the lysine amino acid at position 99 did not form a hydrogen bond with the substrate. Hopefully, after the Lys99Gln mutation is carried out, the enzyme will be able to bind with the substrate from this location. It was predicted that this mutation would result in a decrease in the enzyme's K_M value, which would be a positive outcome. Additionally, it was believed that the Asp residue at position 97, which is located relatively close to the substrate, forms hydrogen bonds with the substrate. With the Asp97Gly mutation that was created in this position, it is the intention to achieve an increase in the enzyme's k_{cat} value.

The primary constraints of commercial L-asparaginases are hypersensitivity responses. Therefore, the analysis of other sources of yeast L-ASNases has potential in reducing toxicity and enhancing effectiveness. In conjunction with these studies, it is believed that *LtASNases*, which have the potential to be utilized as a treatment for ALL, will contribute to the prevention of hypersensitivity side effects. In addition to this, its significance lies in the fact that it can be used as starting point for further academic endeavors.

2. MATERIALS AND METHOD

2.1. MATERIALS

2.1.1. Laboratory Equipments

Micropipette (Isolab-Axygen), vortex mixer (N-Biotek), autoclave (N-Biotek), ROTINA 380 benchtop centrifuge, magnetic stirrer/heater (IKA C-MAG HS 4), SIGMA 1-16K centrifuge, Allegra X-30 benchtop centrifuge, sterile cabinet (N-Biotek), PCR device - thermal cycler (Techne- 5PRIMEG/02), Gel Screening, Bluelight, Serva, -80°C deep freezer (ML Arctico), -20°C freezer (Vestel), refrigerator (Vestel), Milli-Q® IQ Water Purification System, shaker incubator (N-Biotek NB205-ML), Portable Meat pH meter Hanna Instruments, precision balance (Shimadzu–Radwag), oven (Nüve - EN 055), water bath (Stuart), UV-spectrophotometer (Epoch 2 Microplate), sonicator (BANDELIN SONOPULS HD 2070), SDS-PAGE (MINI-PROTEAN ® TETRA CELL BIORAD).

2.1.2. Enzymes, Kits, and Chemicals

For media preparation: LB Agar, LB Broth (LabM), Agar (Fluka), Yeast extract (LabM), Agarose (Biomax), Tryptone (LabM), Glucose (LabM), Lactose (Fisher BioReagents), Glycerol (Fisher BioReagents), Ampicillin (AppliChem), Autoinduction Studier Medium (Formedium), Glycine (Sigma) and Sodium chloride (Merck).

For SDS-PAGE visualizing technique: TEMED (Tetramethylethylenediamine) (Fisher), SDS-PAGE Gel Stain (Generon), Tris-Base (Formedium), Ethidium bromide, SDS-PAGE Running Buffer, SDS-PAGE Gels, Bis acrylamide (Fisher BioReagents), Acrylamide (Fisher BioReagents), Sodium dodecyl sulphate (Fisher BioReagents) and 2-mercaptoethanol (Alfa Aesar).

All used kit: Invitrogen PureLink (TM) Quick Plasmid Miniprep Kit (Cat. No. K210010) and Plasmid isolation kit (Invitrogen).

For PCR preparation mixture: Phusion High Fidelity DNA Polymerase (BioLabs) and dNTP (Thermo Sci.).

Other used chemical: Ammonium persulfate (Fisher BioReagents), *DpnI* (Thermo Sci.), Lysozyme (Serva), Sodium Bicarbonate, Sodium Phosphate (Fisher Scientific), Hydrochloric acid (Sigma-Aldrich), Imidazole (AppliChem), Sodium Formate (Sigma), Citrate (Riedel-de Haën) and Asparagine (Sigma), TCA (Merck).

2.1.3. Cells and Plasmid

The *ltasnase* gene was inserted into the *pET21a(+)* vector, which was then used as the plasmid. For the purpose of transformation, commercial *E. coli* BL21 (DE3) cells were utilized.

2.1.4. Mediums

Table 2.1: The media used.

	Yeast Extract	Sodium Chloride	Tryptone	Agar	ddH₂O
(LB) medium	1.5 g	1.5 g	3 g	-	300 mL
(LB) agar medium	1.5 g	1.5 g	3 g	4.5 g	300 mL

For the Luria-Bertani (LB) medium, the ingredients were weighed in the amounts specified in Table 2.1 and autoclaved at 121°C for 15 min.

For Luria- Bertani (LB) agar medium, the ingredients were weighed in the amounts specified in Table 2.1 and autoclaved at 121°C for 15 min. After the temperature had been lowered to 55°C, 1 µL/mL of ampicillin was added before being poured in petri dishes and kept in a sterile cabinet. For future use, the lids of the petri dishes were sealed, enveloped in parafilm, and stored at +4°C.

For Autoinduction Studier Medium pH was corrected to 7. The autoinduction Studier medium was put through 15 min. of sterilization in an autoclave at a temperature of 121°C.

2.1.5. Buffers and Solutions

•**50 mM pH 8.6 Tris-HCl Buffer:** Tris (6,057 g) was weighed and dissolved in ddH₂O before the pH was adjusted to 8.6 with HCl and the volume was brought to 1000 mL.

•**20 mM L-Asparagine Substrate:** Weighing and dissolving 2.6424 g of Asparagine in 100 mL Tris-HCl buffer.

•**1.5 M TCA Solution:** The TCA (4.901 g) was weighed and dissolved in 50 mL of deionized water.

•**Ampicillin Solution:** 1 g of ampicillin was dissolved in 10 mL of ddH₂O and filtered through a 0.45 µm sterile filter. The solution was then divided into 1 mL tubes and stored at -20°C.

•**Lysozyme:** 1 mL of Buffer A was used to dissolve 10 mg of lysozyme.

•**PMSF:** At a concentration of 0.5 mM, 0.174 g of PMSF was dissolved in 10 mL of isopropanol and stored at -20°C.

• **Running Buffer:** In 1000 mL of dH₂O, 3.03 g of Tris Base, 14.04 g of glycine, and 1 g of SDS were weighed and dissolved.

Utilizing HisTrap columns for protein purification allowed for the separation of bacterial lysate-derived proteins into their individual components. The following is a list of purification buffers:

• **NaPi:** In 800 mL of deionized water, 20 g of Sodium Phosphate Dibasic Heptahydrate and 3 g of Sodium Phosphate Monobasic Monohydrate were weighed and dissolved. Adjusted to a pH of 5.8 and diluted to a volume of 1L.

Table 2.2: Buffer A and B.

	NaPi (20 mM)	NaCl (500 mM)	imidazole
Buffer A	3.12 g	29.22 g	2.04 g (30 mM)
Buffer B	3.12 g	29.22 g	34.04 g (500 mM)

Buffer A mixture was dissolved in ddH₂O. The pH was adjusted to 7.4 and the volume was brought to 1000 mL.

Buffer B mixture was dissolved in ddH₂O. The pH was adjusted to 7.4 and the volume was brought to 1000 mL.

Table 2.3: Buffer A and B with imidazole concentration.

	Buffer A	Buffer B
Buffer A with 100 mM imidazole	42.50 mL	7.50 mL
Buffer A with 200 mM imidazole	32 mL	18 mL
Buffer A with 400 mM imidazole	10.50 mL	39.50 mL

2.2. Methods

2.2.1. Bioinformatic Analysis

In order to choose amino acid residues that interact with substrate, the crystal structure of *LtASNase* which can be found in the UniProt (UniProtKB – C5E3N2), was used as a guide. Primers for introducing these mutations into *LtASNase* have been created. The two potential residues, Asp97 and Lys99, which are near to the active site have been discovered. *E. coli* ASNase, which has a low K_M value and is used pharmaceutically, contains a glycine residue at position 97 and a glutamine residue at position 99. The decision to implement the Asp97Gly and Lys99Gln changes was made in light of these information. In order to do the comparative structure modelling, the SWISS-MODEL (SWISS-MODEL, 2022) server was utilized.

2.2.2. Primer Design

Both the forward and the reverse primers, which are displayed in Table 2.4, are performed convert; to Asp to Gly at mutation position 97 and Lys to Gln at mutation position 99 in *ltasnase* gene. For the purpose of primer design, the Sigma OligoEvaluator Primer and Probe Design tool (OligoEvaluator, 2022) was utilized.

Table 2.4: A list of the primer pairs that were utilized in the research.

WILD-TYPE	CGAACAGCTGTGTAATGTTGATAGTAAAGA AATTAACGAGAGCGTTC
PRIMERS	Sequence (5'-3')
ASP97GLY-F	5' GTGTAATGTTGGCAGTAAAGAAATTAAC '3
ASP97GLY-R	5' GTTAATTTCTTTACTGCCAACATTACAC' 3
LYS99GLN-F	5' GTAATGTTGATAGTCAGGAAATTAAC '3
LYS99GLN-R	5' GTTAATTTCTGACTATCAACATTAC '3

2.2.3. Performing Mutations by PCR, and Site-Directed Mutagenesis

Before establishing the PCR reaction, the plasmid containing the *ltasnase* gene was transferred to competent *E. coli* BL21 cells, amplified, and plasmid purification was performed again.

PCR was used with *pET21a(+)* vector to obtain mutations on the Asp97 (Asp97Gly) and Lys99 (Lys99Gln) regions of the *LtASNase* enzyme. The reaction was prepared using the materials shown in Table 2.5 and the reaction volume was adjusted to 50 μ L.

Table 2.5: The components and amounts of the reaction that were used for mutation.

Reactant	Amount (μ L)
HF Reaction Buffer	10
The plasmid containing the <i>ltasnase</i> gene	1
Forward Primers	1.25
Reverse Primers	1.25
10 mM dNTP mix	1
ddH ₂ O	35
Phusion High Fidelity DNA Polymerase	0.5
Total Volume	50

After the reaction mixture was prepared, it was placed in the PCR device and the program was started. The program was loaded to the device as indicated in Table 2.6. The PCR program was set up as a gradient and the binding step was applied at 55°C (Lys99Gln)/59°C (Asp97Gly)

Table 2.6: PCR protocol for site-directed mutagenesis of mutants.

Segment	Cycles	Temperature	Time
1	1	98 °C	30 sec
2	30	98 °C	10 sec
		55 °C/59 °C	20 sec
		72 °C	7 min 30 sec
		72 °C	7 min 30 sec
3	1	72 °C	7 min 30 sec

After the PCR protocol was completed, 5 µL of the PCR products were separated for control with agarose gel. 1 µL of *DpnI* was added to the remaining samples and incubated at 37°C for 1 hours. The remaining PCR products were used for transformation after 5 µL of the agarose gel was reserved from the samples that came out of the incubation.

2.2.4. Agarose Gel Electrophoresis

Before beginning the heating process, 0.50 g of agarose was first dissolved in an amount of 50 mL of 1X TBE solution. Once the gel had reached the desired temperature, 5 µL of Serva DNA Stain Clear-G was added to it. Then, this solution was poured into the agarose gel vessel. After gel freezing, 1X TBE buffer was added to the tank and samples were inserted. The first ten minutes were conducted at 80V, followed by 100V. The bands were exhibited using ultraviolet light.

2.2.5. Trasformation of Mutant *ltasnase* into *E. coli*

PCR products were transformed using *E. coli* BL21 cells, which have a high level of chemical competence. Initially, 2 µL of the PCR product were taken, and then 50 µL of competent *E. coli* BL21 cells were introduced. This created combination was allowed to incubate on ice for a total of 30 min. before being heated to 42°C for 50 sec. and then being allowed to incubate once more on ice for a total of 5 min. After removing the mixture from the ice and adding 250 µL of SOC medium, it was placed

in an incubator for one hour at 37°C and 230 rpm. After that, the cells that had been incubated were inoculated with the assistance of spreader beads onto LB-agar medium that included 1 µg/mL ampicillin, and the cells were then incubated at 37°C for approximately 16 hrs.

2.2.6. Plasmid Isolation

Plates of LB agar that had been treated with 1 µg/mL of ampicillin obtained after transformation were used to obtain individual colonies, which were then isolated. Then, these colonies were then inoculated into 5 mL of LB medium that included 1 µg/mL of ampicillin, and the combined solution was placed in an incubator at 37°C with 225 rpm for overnight. The pellet was obtained by precipitating of the cell culture after the incubation step was complete. Plasmid purification from the sample was performed according to the protocol suggested by the MN NucleoSpin Plasmid QuickPure kit (MN, 2022).

2.2.7. DNA Sequence Analysis

The obtained plasmid samples were sent to Eurofins for confirmation of the mutations by sequence analysis. In the reaction for the sequence analysis, the T7 promoter primer with the nucleotide sequence 5' TAATACGACTCACTATAGGG 3' was used. The analysis result was displayed in BioEdit and compared to the wild-type *LtASNase* using Clustal Omega (Clustal Omega, 2023).

2.2.8. Protein Expression

The *LtASNase* wild-type and mutant variants were introduced into *E. coli* BL21 for expression. One of the colonies obtained after transformation was chosen and incubated in 10 mL of LB broth containing 100 µg/mL ampicillin for 16 hrs at 37°C with 200 rpm. To induce the expression of the protein, the obtained culture was transferred to 400 mL auto-induction Studier medium (2 g yeast extract, 8 g tryptone, 5 g NaCl, 2.4 g Na₂HPO₄, 1.2 g KH₂PO₄, 0.2 g glucose, 2.76 mL of 87% glycerol and

0.84 g lactose) containing 1 µg/mL ampicillin (3x400 mL flasks) and incubated at 30°C for overnight at 180 rpm in a shaker. After cultivation, the cells were harvested by centrifuging them at 4000 rpm for 20 min, and then the purification procedure began.

2.2.9. *LtASNases* Purification Process

Following centrifugation, the pellet was resuspended in Buffer A, PMSF, and lysozyme the mixture was placed in an incubator on ice for a period of 30 min. The cells that had been resuspended were first disrupted with the use of sonication, and then they were cleared by centrifugation at 16000 rpm for 20 min. After passing through a filter with a pore size of 0.45 µm, the lysate was loaded onto a HisTrap column that had previously been pre-equilibrated with Buffer A. Following the rinsing of the column with 5 mL of Buffer 1, *LtASNase* was eluted using Buffer that had imidazole concentrations ranging from 100 to 200 and 400 mM. After determining whether fractions contained pure protein through the use of SDS-PAGE, the fractions were mixed and diluted to a volume of 50 mL with the addition of 50 mM Tris-HCl at a pH of 8.6. Concentrated proteins were passed through PD10 column to remove imidazole and other salts. Using nanodrop, the concentration of the purified protein was determined. SDS-PAGE was used to observe the expression efficiency.

2.2.10. SDS-PAGE Analysis of WT and Asp97Gly, Lys99Gln *LtASNases*

The samples were produced by combining 15 µL of the sample with 5 µL of the 4X sample loading buffer. 10 min. of heating at 95°C was followed by 10 min. of centrifugation at 13500 rpm. The SDS gel was added to the vessel, which was then filled with a running buffer. A protein standard of 5 µL was inserted into the first well, followed by 5 µL of each sample. Following that, the procedure was started with 80 V for ten minutes, and then it was continued with 100 V for an hr.

2.2.11. Activity Assay of WT and Asp97Gly, Lys99Gln *LtASNases*

For the *LtASNases* activity assay, the tubes were filled with 1.2 mL of a 20 mM asparagine substrate prepared with a 50 mM Tris-HCl buffer and 400 μ L of TCA were introduced to the blanks. Each enzyme sample, test tube, and blank tube were incubated at the specified temperature for five min. After preheating, 400 μ L of enzymes were introduced to the tubes, which were allowed to remain for 30 min. After 30 min at 37°C, the reaction was terminated by adding 400 μ L of TCA and centrifuging the tubes at 15000 rpm for 5 min. At the conclusion of the experiment, 118 μ L of each sample and 1647 μ L of dH₂O water were added to the tube. In three repetitions, 235 μ L of Nessler's reagent was added to each sample. The presence of ammonia is indicated by a yellow color; at higher concentrations, a brown precipitate may form. Using an ultraviolet-visible spectrophotometer at 480 nm, the yellow color was measured.

2.2.12. Kinetic Assay of WT and Asp97Gly, Lys99Gln *LtASNases*

Enzyme kinetic assay of WT and Asp97Gly, Lys99Gln experiments were performed at room temperature. All solutions were prepared in a 20 mM Tris-HCl buffer with a pH of 8.0. For substrate kinetics, asparagine was prepared at 10 different final concentrations in the range of 0.125-160 mM and 13 different final concentrations in the range of 0-50 mM ammonium standard. From the 50 mM master stock, 100 μ L of these ammonium standards were extracted and added to tubes. 1 mL of water was introduced. The addition of 145 μ L of Nessler was followed by a 20-min wait. It was then deposited upon the well-plate. This activity assay was conducted in accordance with the activity assay described in the article on *Streptomyces fradiae* published in the journal Nature, with the quantities modified to accommodate 1.5 mL of tube [70]. Following that, the methodology outlined in section 2.2.11 was carried out.

2.2.13. Storage Stability Assay

The wild-type and Lys99Gln *LtASNase* that had been prepared in tubes and stored at 4°C, 25°C, -80°C, and -20°C respectively were removed. Each temperature required the preparation of test tubes in addition to blank tubes. L-Asparagine at a concentration of 20 mM was produced by mixing 100 mL of Tris-HCl buffer. 50 mL of water was used in the preparation of 1.5 mM TCA. In each tube, 1 mL of L-asparagine, 100 µL of TCA, and 100 µL of enzymes were placed. The tubes were centrifuged for 5 min. at a speed of 10000 rpm. The 96-well plates were given an additional 150 µL of mutant supernatant in the second step of the process. After adding 50 µL of the Nessler reagent, the mixture was left to incubate at room temperature for 5 min. After centrifugation, 125 µL of deionized water and 25 µL of the WT and Lys99Gln *LtASNase* post-centrifuge sample were added. Ultraviolet-visible spectrophotometer was used to take a reading at 480 nm after 96 well plates had been incubated for 5 min. at room temperature.

3. RESULTS

3.1. PCR Analysis

The pET21a(+) vector containing the *ltasnase* gene was mutated using primers containing the mutant codon. Using agarose gel electrophoresis, the PCR products obtained were visualized. According to the obtained gel image, the PCR bands of the mutations of the *ltasnase* gene-containing pET21a(+) vector that were predicted to be approximately 5.770 kb in size were obtained at these values, as shown in Figure 3.1.

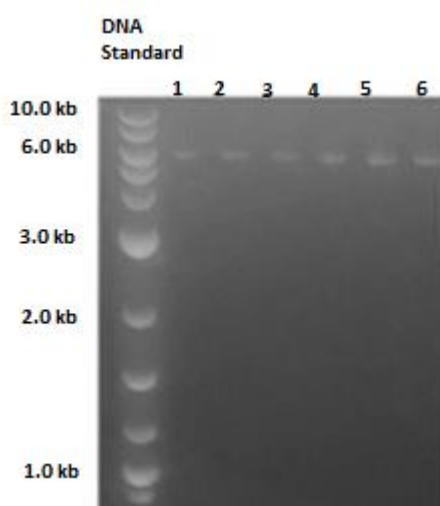


Figure 3.1: Visualization of agarose gel produced following PCR and treatment with *DpnI*. 1; control plasmid, 2. Asp97Gly recombinant PCR sample 3; example of Lys99Gln recombinant PCR. 4; control plasmid, 5 and 6; sample of recombinant PCR after *DpnI* incubation for Asp97Gly and Lys99Gln.

3.2. Expression of *LtASNase*

The effective transformation of PCR product into DH5 α competent *E. coli* cells was accomplished. Figure 3.2 displays bacterial colonies that were grown on selection plates that contained ampicillin at a concentration of 100 μ g/mL.

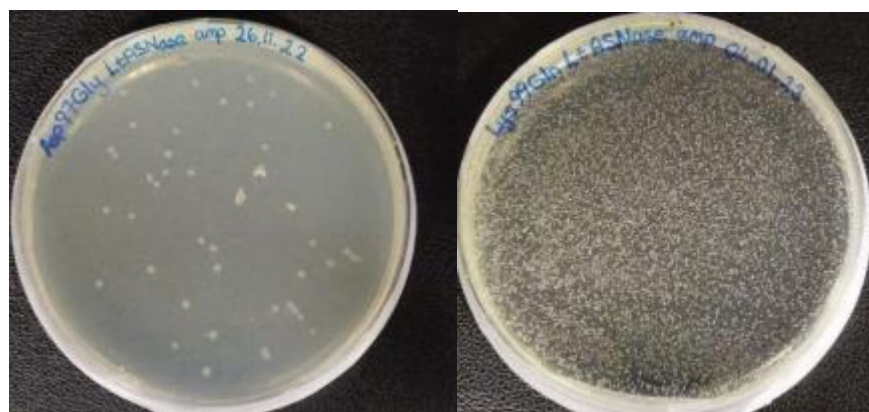


Figure 3.2: Colonies of recombinant organisms grown on plates following transformation (Asp97Gly on the left, Lys99Gln on the right, as viewed from the Reader's perspective).

3.3. DNA Sequence Analysis

After the results of the PCR were transformed, the plasmids that contained the *ltasnase* genes were purified according to the instructions in section 2.2.6. DNAs were sent to be sequenced. Using the BioEdit Sequence Alignment Editor program, the analysis outcomes were compared to *ltasnase* of the wild-type. Asp97Gly, and Lys99Gln mutations have been determined. Figure 3.3 illustrates the sequence analysis results of the mutations in the related region.

	300	310	320	330	340	350
Asp97Gly	TAGTGGCATTGTGAAATTGATTACGAACAGCTGTGTAATGTTG	GCAGTAAAGAAATTAA				
Lys99Gln	TAGTGGCATTGTGAAATTGATTACGAACAGCTGTGTAATGTTG	ATAGTCAAGAAATTAA				
Yabanil_tip	TAGTGGCATTGTGAAATTGATTACGAACAGCTGTGTAATGTTG	ATAGTCAAGAAATTAA				

Figure 3.3: The sequence analysis of wild-type and obtained mutant forms of *LtASNases*.

3.4. Purification of *LtASNases*

E. coli BL21 cells were transformed with designed vectors that included mutant *ltasnase* genes. Protein expression and purification were carried out in accordance with

the procedures outlined in Sections 2.2.8 and 2.2.9. At the end of the purification procedure, the collected fractions were loaded onto an SDS-PAGE gel, and the resulting image is depicted in figure 3.4. This image was acquired by loading the fractions.

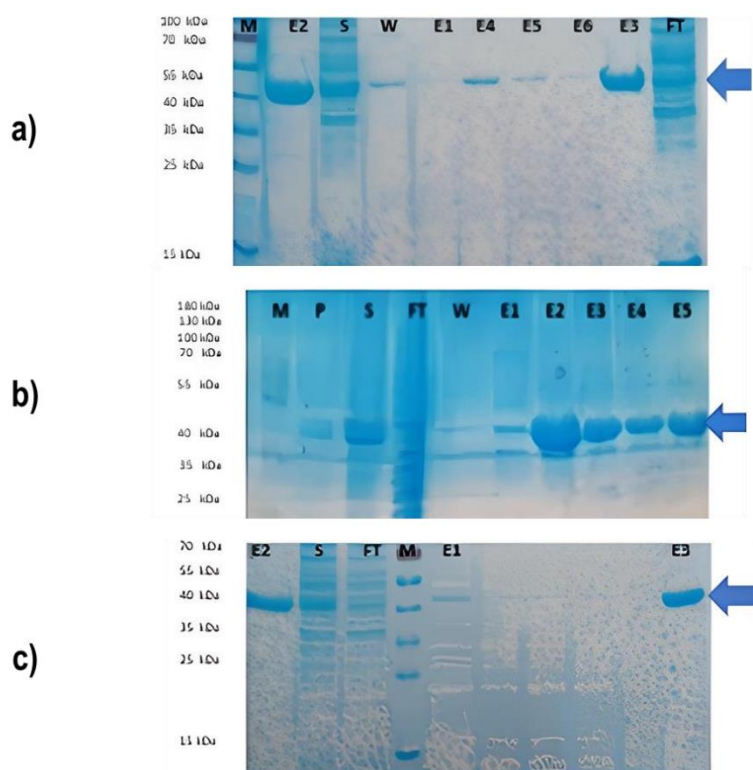


Figure 3.4: SDS-PAGE gel analysis of wild-type and mutant *LtASNases* (M: Marker, P: Pellet, S: Supernatant, FT: Flow-through, W: Wash fraction, E1-2-3-4-5: Elution fractions, a: Asp97Gly, b: Wild-type, c: Lys99Gln *LtASNase*).

The gel picture demonstrates that the size of *LtASNase* is ~40 kDa, and fractions that are pure enough for activity, and kinetic assay were obtained.

3.5. Specific Activity for *LtASNases*

During the experiment involving the specific activity, the pure fractions were pooled together and concentrated with the assistance of ultracentrifuge tubes. L - asparagine was utilized as a substrate for the purpose of determining the specific activities of mutant *LtASNase* enzymes that were purified in accordance with the

procedures described in section 2.9.2. Researchers measured the amount of ammonia that was produced as a by-product of the enzyme's breakdown of the substrate. This was done in order to determine the level of enzyme activity. In order to generate the calibration curve for enzyme activity, ammonium sulphate was first subjected to a series of dilutions. It can be seen in Figure 3.5 that the equation for the calibration curve is $y = 0,09x + 0,0319$. After the reaction, the values that were determined by measuring the samples at a wavelength of 480 nm were substituted into the equation, and the amount of ammonia that was released was determined to be in μmol .

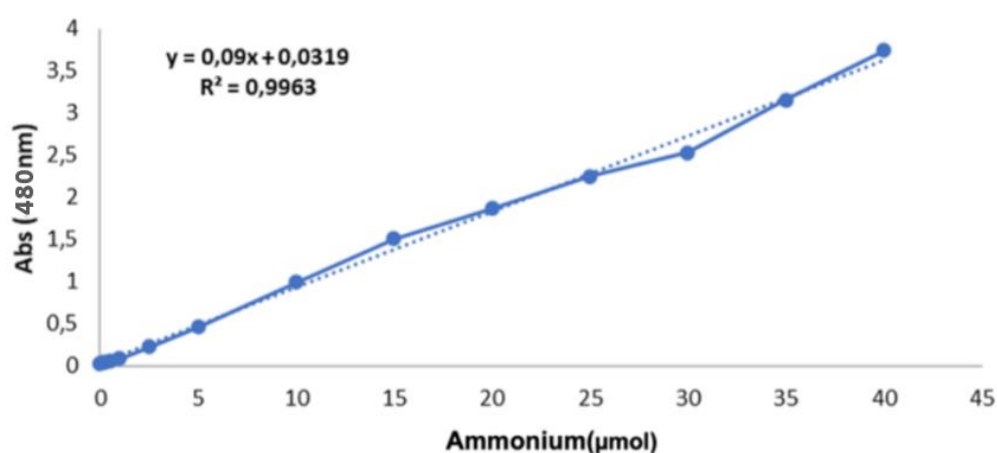


Figure 3.5: Ammonium calibration curve.

After incubating mutant *LtASNases* with the necessary enzyme quantities for the enzyme reaction in a buffer containing 50 mM Tris HCl, pH 8.6, 20 mM L -asparagine for 30 minutes, the amount of ammonia released was measured using the Nesslerization method described before. The results that were obtained about the wild-type and mutant *LtASNase* activities are summarized in Table 3.1. The specific activity of the enzyme was measured in units of U/mg.

Table 3.1: Comparison of wild-type and mutant *LtASNase* with regard to their purification yields and specific activities.

	Purification Step	Total protein (mg)	Total activity (U)	Specific activity	Purification Efficiency (%)	Purification Coefficient
Wild-type	Cell Lysate	54.40	7202.60	132.40	100	1
	Ni-NTA	0.70	219.70	313.80	1.30	2.40
Asp97Gly	Cell Lysate	34.50	-	-	100	-
	Ni-NTA	1.5	-	-	4.30	-
Lys99Gln	Cell Lysate	32.40	136.10	4.20	100	1
	Ni-NTA	0.90	12.20	13.60	2.80	3.20

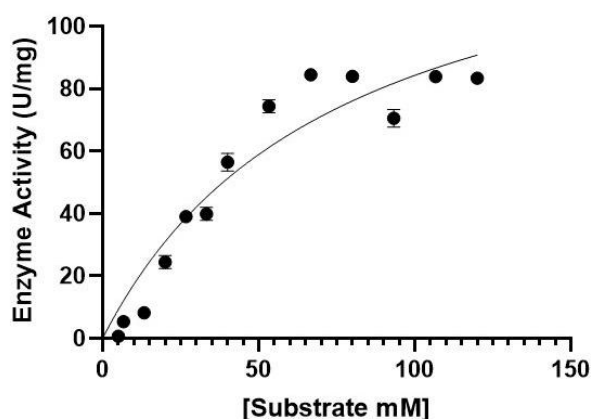
According to the data presented in Table 3.1, it can be observed that the Asp97Gly mutant exhibited a lost in enzymatic activity, in addition to a decrease in purifying efficiency.

3.6. Kinetic Parameters of WT and Lys99Gln *LtASNases*

Kinetic measurements were performed against asparagine in accordance with the procedures specified in section 2.2.12 at room temperature. Kinetic investigations were unable to be carried out because the Asp97Gly mutant did not display any detectable levels of activity. Michaelis-Menten graphs were made using the activity values that were determined at the optimal pH of each enzyme when it was 37 degrees Celsius. This was done in order to determine the kinetic parameters of the purified *LtASNase* enzymes. The program GraphPad Prism 9 (GraphPad Prism 9, 2023) was utilized in order to ascertain the values of the kinetic parameters $V_{\max}$ and K_M for the enzymes that had been purified. Table 3.2 provides a concise summary of the kinetic values. In addition, the Michaelis-Menten plots of *LtASNase* wild-type and mutant are displayed in Figure 3.6.

Table 3.2: Kinetic parameter of WT and mutant enzyme.

<i>LtASNase</i>	Asparagine		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($\text{s}^{-1}\text{mM}^{-1}$)
Lys99Gln	94.40	75.50	1.3
Wild-type	312.50	11.70	26.80

**Figure 3.6:** Michaelis-Menten plots of Lys99Gln *LtASNase*.

3.7. Storage Activity for WT and Lys99Gln *LtASNases*

The investigation of the storage activities of mutant *LtASNase* followed the methodology described in section 2.2.13. After putting 300 microliters of mutant *LtASNase* and wild-type *LtASNase* to tubes, the samples were kept at temperatures of +4, +25, -20, and -80°C, respectively. The activity test was carried out determined week. Following the completion of the activity test, calculations were done based on the absorbance values that were read in the spectrophotometer at 480 nm, and graphics were produced.

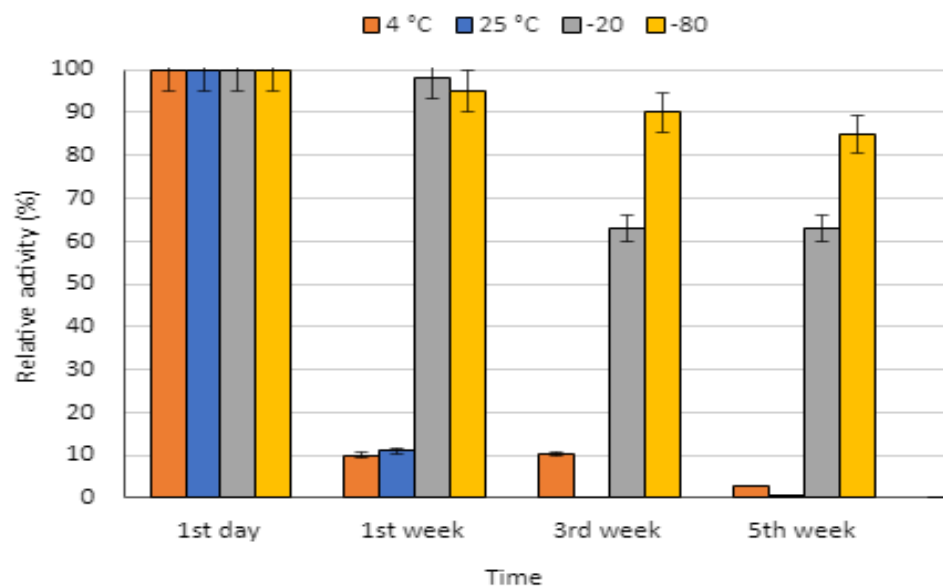


Figure 3.7: Storage stability of WT *LtASNase*.

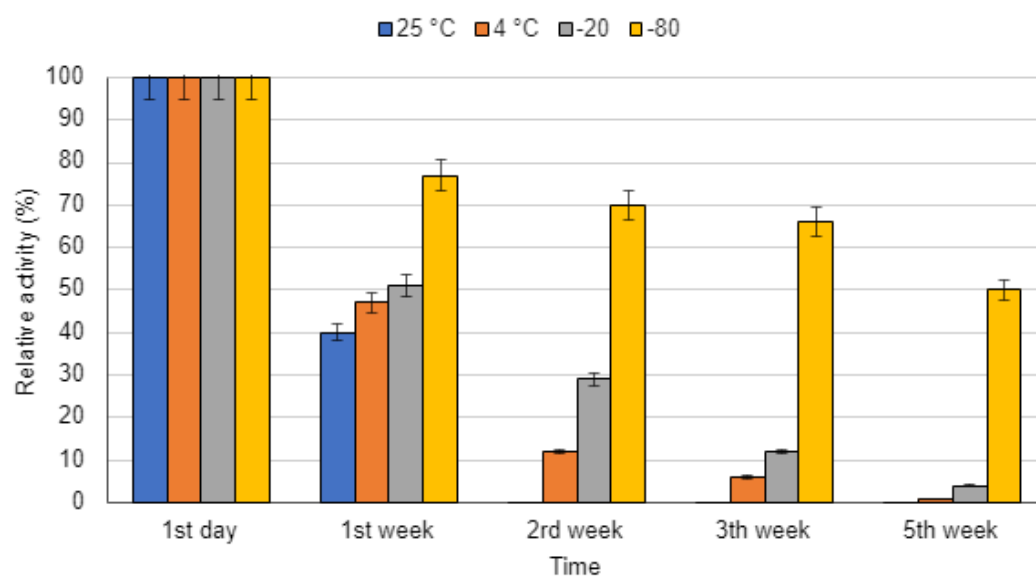


Figure 3.8: Storage stability of Lys99Gln mutant *LtASNase*.

4. DISCUSSION and CONCLUSION

This research focuses on examining the effect of point mutations on modelled structures of the enzyme *LtASNase* derived from *L. thermotolerance* yeast. The main purpose is to investigate how mutations near the enzyme-substrate interaction site affect the K_M and k_{cat} values. Another aim is to observe the effect of mutations on enzyme storage stability. For the aforementioned purposes, mutants of Asp97Gly and Lys99Gln were obtained by site directed mutagenesis.



Figure 4.1: Position of Asp97 (Red Circle) and substrate/product (Grey Arrow) at WT *LtASNase* active site.

In the literature study conducted at the beginning of the thesis study, it was observed that the 57th amino acid in the *E. coli* ASNase model was Gly. In the experiments performed, it was observed that the K_M and k_{cat} values were better than WT *LtASNase*. Based on these studies, the *LtASNase* Asp97Gly (*E. coli* number: 57) mutation was performed.

The substitution of Asp at position 97 with Gly had a sharp effect on the activity of the enzyme and caused the enzyme to lose its activity at an immeasurable level. Upon examination of the modelling studies, it is seen that Asp in the 97th position makes hydrogen bonds with the substrate/product. It can be thought that Asp has a critical importance in this region with the mutation. It can be understood that Asp keeps the substrate/product in the correct position with hydrogen bonding. Gly is the simplest and smallest amino acid among the 20 standard amino acids that make up proteins. It has a unique property due to its small size: it lacks a side chain, which means it has only a hydrogen atom as its side chain. This minimal side chain contributes to its flexibility and ability to fit into tight spaces within protein structures. [71],[72]. When examining Figure 4.1, it is seen that Asp in position 97 is close to the loop. Asp may be maintaining structural stability in this region. The replacement of Asp with Gly may have deteriorated the structural stability due to the flexible property of Gly.

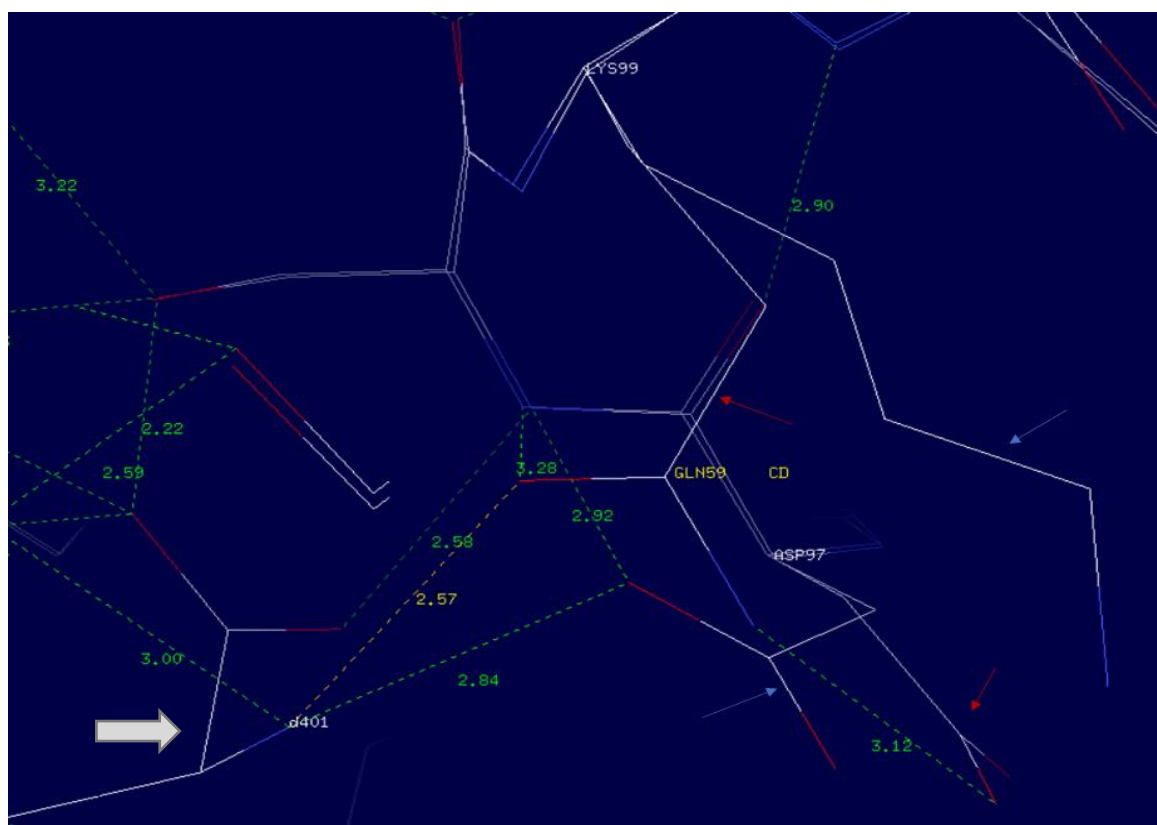


Figure 4.2: Visualization of WT and Lys99Gln *LtASNase* active site representation (Red arrow: WT *LtASNase* position; Blue arrow: Lys99Gln *LtASNase* position; Grey arrow: Substrate/Product).

In the literature study conducted at the beginning of the thesis study, it was observed that the 59th (PDB Number: 6pab) amino acid in the *E. coli* LtASNase model was Gln. In the experiments performed, it was observed that the K_M and k_{cat} values were better than WT LtASNase. Based on these studies, the LtASNase Lys99Gln mutation was performed.

As a result of the replacement of the amino acid Lysine at position 99 with Glutamine, a decrease in the specific activity of the Lys99Gln mutant was observed compared to the wild-type. Lysine contains an amino group in its side chain and this group has a suitable configuration to form hydrogen bonds. On the other hand, the glutamine side chain contains an amide group, which can also form hydrogen bonds [71],[7]. Considering the modeling study in Figure 4.2, it can be thought that Gln at position 99 in Lys99Gln mutation has a higher probability of hydrogen bonding than Asp at position 97. Furthermore, it can be hypothesized that the Lys99Gln mutation has altered the positioning of the Asp residue at position 97 within the active site. In this study, the K_M value of the Lys99Gln mutant increased approximately 6 times and the k_{cat} value decreased dramatically. Considering the test results, it can be deduced that the hydrogen bonding of Asp with the substrate yields more optimal results than the hydrogen bonding of Gln.

In the final phase of this investigation, enzyme storage activity experiments were conducted. Regardless of the temperature at which it is stored, the study revealed that the enzyme's activity decreases with increasing storage time. This could be due to the destruction of the enzyme's functional three-dimensional structure over time. In addition, as the enzyme's storage temperature decreased, its activity and functional structure were better preserved. In this instance, the optimal enzyme storage temperature is -80°C. Compared to WT LtASNase and mutant LtASNase, WT enzyme activity was more stable at -80°C. This may be due to the mutation disrupting the structural integrity of the enzyme.

There is more research on bacterial-derived ASNases than other source organisms, according to the literature. The yeast-derived asparaginases, which serve as alternatives to *E. coli* and commercial asparaginases, have received comparatively less attention in previous research endeavors. The existing literature on the structure and functions of yeast ASNase is limited, indicating a need for further research in this area. The identification and characterization of key residues within the active region

of *LtASNase*, a yeast-derived enzyme with high activity, will significantly advance the existing body of literature on yeast-derived ASNases.

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