T.C.

KIRSEHIR AHI EVRAN UNIVERSITY
INSTITUTE OF NATURAL SCIENCES
ADVANCED TECHNOLOGIES DEPARTMENT

Molecular Cloning, Heterologus Expression and Structural Modeling of L-asparaginase from *Pseudopedobacter saltans* in *E.coli*

MUNA SHAREEF ABED

M.Sc. THESIS

KIRŞEHİR / 2022

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MUNA SHAREEF ABED

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June, 2022 MUNA SHAREEF ABED

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LIST OF ABBREVIATIONS

Icons	Description
α	: Alpha
β	: Beta
g	: Gram
mg/mL	: Milligram per millilitre
U/ µL	: Unit per microliter
µM	: Micromole
µL	: microliter
mM	: milliMole
mL	: Milliliter

abbreviations	explanation
ºC	:Degree Celsius
dNTP	:Deoxynucleotide triphosphate
DNA	:Deoxyribonucleic acid
MgCl2	:Magnesium Chloride
PCR	:Polymerase Chain Reaction
rpm	:Revolution per minute
SDS	:Sodium Dodecyl Sulfate
PAGE	:Polyacrylamide gel electrophoresis
TBE	:Tris-Borate-Ethylene diamine tetra acetic acid
UV	:Ultraviolet
v/v	:Volume/Volume
w/v	:Weight/Volume
IMAC	:Immobilized Metal Affinity chromatography
PDB	:Protein Data Bank
OD	:Optical Density
NaCl	:Sodium Chloride
bp	:Base Pair
KDa	:Kilodalton
V_{max}	: Maximal Velocity of the Reaction
K_m	: The Michaelis Constant
DW	:Distal Water
H	:Hour

ÖZET

YÜKSEK LİSANS

*E.coli'*de *Pseudopedobacter saltans*'tan L-asparaginazın Moleküler Klonlama, Heterolog Ekspresyonu ve Yapısal Modellemesi

MUNA SHAREEF ABED

KIRŞEHİR AHİ EVRAN ÜNİVERSİTESİ
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L-asparaginaz (L-ASP) veya l-Asparagin aminohidrolaz (EC 3.5.1.1), L-Asn'yi aspartik asit ve amonyağa hidrolize edebilen hidrolazlar sınıfına ait bir enzimdir. Su kullanımı yoluyla amino asit asparajinin bölünmesini destekleyen ve peptit olmayan karbon-azot bağlarını parçalayan akut lenfoid lösemi tedavisinde kullanılır.

Bu güncel çalışmada, yeni L-asparaginaz geninin klonlanması, *E. coli*'de *Pseudopedobacter saltans* DM12145'ten yapılmıştır. Yeni bakteri *Pseudopedobacter Saltans'ın* DM12145'in, L-asparaginaz genine sahip olduğunun belirlenmesiyle ilgili tüm genetik bilgileri, BLAST web sitesi NCBI'den (//www.ncbi.nlm.nih.gov) elde edilmiştir.

Pseudopedobacter saltans L-asparaginaz'ın tam uzunluğu 1019 bp'dir ve moleküler ağırlığı, 37.8 kDa olarak değerlendirilmiştir. PCR, GenBank'ta belirtildiği gibi tam bir açık okuma çerçevesi ORF'si ile nükleotid dizileri [(ileri) 5'-ATCGGATCCATCACCATT-3' ve (geri) 3'-GCGAAGTTTAGTTAGATGATGATT-5'] ile ileri ve geri primerler kullanılarak kodlanmıştır.

Pseudopedobacter saltans DSM 12145'ten L-asparaginaz gen tip I'i kodlayan bu açık okuma çerçevesi (ORF), NC_015177.1 erişim numarasıyla *Pseudopedobacter saltans* DSM 12145 genomundaki 4301275-4302294 nükleotidden yayılmıştır. L-asparaginaz tip I geninin bu nükleotid dizisi, Genscript Co., ABD tarafından EZ Clone yöntemiyle sentezlendi. Protein

ID: (WP_013634621.1") ile 1019 bp'ye sahiptir. pET-28a (+) ekspresyon vektörü üzerine klonlandı. Yapı, pET-28a (+)/asp_pseudopedo olarak aday gösterildi.

Rekombinant L-asparaginaz'ın başlangıç seviyesi, *E. coli* BL21 (DE3) Rosetta suşunda 6 His etiketli bir füzyon proteini olarak ifade edildi. pET-28 His (+)/L-asparaginaz yapısını barındıran 0.0003 U/mL idi. Uygulanan optimize edilmiş strateji, LB büyüme ortamının ikame edilmesi üzerine rekombinant Rosetta suşunun hücre lizatında izlenen L-asparaginaz aktivitesini başarılı bir şekilde arttırdı. Transformasyon koşulu, ilgili antibiyotik kanamisin (34 g/mL) ile LB agar ortamı üzerinde kültürlendi ve gece boyunca 37 °C'de ve 200 rpm'de 0.5 ila 600 nm'lik bir optimal yoğunluk ulaşana kadar inkübe edildi.

Ekspresyon indüksiyonu, 1 mM IPTG (izopropil-D-tiogalaktopiranoz) kullanılarak kültür ortamının 200 rpm'lik bir çalkalama hızıyla 30°C'de 18 saat inkübe edilmesiyle gerçekleştirilmiştir. Ertesi gün, GeneJET plazmit mini hazırlık kiti (Thermo-Fisher "Co." ABD) kullanılarak üç kültürden plazmit mini hazırlık yapıldı. Tek bir koloniden kaynaklanan saf bir kültürden izole edilen her plazmit, eklenen L-asparaginaz tip I geninin amplifikasyonu için MluI ve XhoI ve PCR kullanılarak kısıtlama sindirimine tabi tutuldu.

İzole edilen DNA plazmitleri, pET-28a (+) vektörü üzerinde *Pseudopedobacter saltans*'tan L-asparaginaz tip I geninin varlığını kanıtlamak için PCR şablonları olarak kullanıldı. PCR reaksiyonu, pET-28a (+) vektörü T7 promotör /T7 terminatörün evrensel primer seti ile gerçekleştirildi. Evrensel primer seti T7 promotör /T7 terminatörünün dizisi aşağıdaki gibidir: T7 promotörü (5'-TAATACGACTCACTATAG-3'), T7 terminatörü (5'-TAATTGCTCAGGTGG-3'). Amplifiye edilmiş genomik PCR ürününün (1500 bp) beklenen boyutu, *Pseudopedobacter saltans* L-asparaginaz tip I genini (1019 bp) içerir. Bununla birlikte, evrensel primer seti T7 promotörü ve T7 terminatörü, ekin (*Pseudopedobacter saltans*'tan L-asparaginaz gen tip I) başlangıcından ve sonundan uzak olduğu sürece, PCR ürünündeki ekstra uzunluk vektöründen kaynaklanmakatadır. Bu nedenle, amplifiye edilmiş genomik ürünün, yaklaşık 1500 bp'lik L-asparaginaz I geninden biraz daha büyük bir boyuta sahip olması ve kalan ekstra nükleotidin vektörün kendisinden olması beklenir. Genomik dizilemeden sonra doğru nükleotid dizisi doğrulandı. Sonuç olarak, *Pseudopedobacter saltans*'tan elde edilen L-asparaginaz geninin rekombinant proteini, ardışık 6 histidinden (6xHis) oluşan bir kuyruk (etiket) ile N-terminal kısmında eksprese edildi. İmmobilize metal afinite kromatografisi (IMAC) ile saflaştırma yöntemi olarak yaygın olarak kullanılan basit bir etikettir.

Bu kuyruğun (etiket) kullanımı, eksprese edilen protein ile kromatografik kolon (verimi artıran) arasında doğrudan etkileşimden kaçınmanın yanı sıra, üretim maliyetinden tasarruf (tek aşamalı saflaştırma) sağladığı için protein üretim adımında avantajlar sağlar. çünkü sadece His-tag, kromatografik reçineyi oluşturan mikro kürelerde tutulan Ni^{2+} ve Co^{2+} metalik iyonları ile etkileşime girer.

Rekombinant L-asparaginazın saflaştırma işlemi için, 3 yıkama tamponu (300 mM NaCl, Gliserol %5 ve 25-, 50- ve 100-mM imidazol içeren 50-mM Tris) ve bir elüsyon tamponu (300 mM NaCl, Gliserol) %5 ve 50-mM Tris ve 250 mM imidazol) hazırlandı. Her ikisi de nikel iyonu (Ni+) ile konjuge edilmiş agarozdan oluşan HisTrapTM HP kolonu kullanılarak pH 8.0'da hazırlandı. IMAC Kromatografisi ile protein saflaştırmasından sonra, rekombinant protein, çözünürlüğünü değerlendirmek ve aynı zamanda rekombinant proteinin moleküler ağırlığını (M.W) belirlemek için Sodyum Dodesil Sülfat-Poliakrilamid Jel Elektroforezi (SDS-PAGE) ile analiz edildi. *Pseudopedobacter saltans* protein L-asparaginaz I enziminin beklenen molekül ağırlığı 37.8 kD'dir. SDS-PAGE analizi, protein bandının boyutunun 36.0 kDa'da tam olarak beklendiği gibi olduğunu ortaya çıkardı. Bu nedenle, asp_pseudopedo'nun rekombinant proteini, çözünür fraksiyonda eksprese edildi ve enzimatik aktivitesini korudu. L-asparaginaz, L-asparagine etki ettiğinde, amonyak açığa çıkar ve alkali koşullarda Fenol Red'in sarı renginin pembeye dönmesine neden olur.

Asp_pseudopedo enziminin aktivitesi üzerindeki pH varyasyonunun neden olduğu etkinin değerlendirilmesi, pH 3 ila 11 arasında değişen farklı tamponlar (Sitrat tamponu (0.2 mM)-fosfat (0.1 mM)- Tris tamponu (50 mM) ve Glisin tamponu) kullanılarak kolorimetrik Nesslerizasyon yöntemine dayalı olarak gerçekleştirilmiştir.

Test, 20 °C ila 90 °C arasında değişen bir sıcaklık yükselme rampası ile asp_pseudopedo'nun enzimatik aktivitesi için optimum sıcaklığı belirler. Varyasyonu 62.5 M ila 9.91 mM arasında değişen kademeli L-asparagin konsantrasyonları ile asp_pseudo enziminin kinetik mekanizması değerlendirildi. Elde edilen veriler, asp_pseudopedo enziminin optimal pH'ı 8,0 ve maksimum aktivite sıcaklığının 60 ^{o}C olduğunu, değişken kinetik Km değeri 3 mM ve Vmax'ın 168,2 mol/dak/mg olduğunu ortaya koyan kinetik parametreleri belirlemek için SigrafW programında kullanıldı.

Silico analizi, filogenetik *Pseudopedobacter saltans* L-asparaginaz geninin ağacını oluşturmak için Phylogeny.fr yazılımı [91]. Kullanılarak yapıldı. İkincil yapının tahmini, SAS online programına (yapıya göre açıklamalı dizi) göre yapıldı [92]. Daha sonra, verileri

elde etmek için proteinin sekansı SWISS-MODEL sunucusuna gönderilerek boyutsal yapı beklentisi yapılmış ve PDB görüntüleyici programı kullanılarak 3 boyutlu yapısal tahmin analiz edilmiştir. BLASTP benzerlik dizisi araştırması, diğer farklı L-asparaginazların ve Pseudopedobacter saltans L-asparaginaz genlerinin protein dizileri arasında %99.34-91.84 arasında değişen yüksek bir dizi özdeşliği sağladı.

Siliko analizinin sonucu, *Pseudopedobacter saltans*'tan elde edilen L-asparaginaz enziminin, L-asparajin için belirgin bir şekilde spesifik olan ve homodimer sitozolik proteinler olarak işlev gören *E. coli*'nin L-asparaginaz I'ine oldukça benzer olduğunu ortaya koymaktadır.

Siliko analizinin bulgularına göre, *Pseudopedobacter saltans*'tan elde edilen L-asparaginaz enzimi, *E. coli*'den elde edilen tip L-asparaginaz tip I genine oldukça benzerdir ve L-asparajin için belirgin bir şekilde spesifiktir ve homodimer sitozolik proteinler olarak işlev görür. Bu, sitozolde bulunan ve çözelti içinde bir homodimer olarak hareket eden L-Asparajin'e belirgin bir şekilde özgüdür.

Sonuçlarımız, bildirilen diğer çalışmalarla uyumludur, örneğin, *E. coli* L-asparaginaz tip I *Pseudopedobacter saltans* L-asparaginaz I, *Vibrio cholerae* L-asparaginase ile yaklaşık (%99) ve yaklaşık (%92) *Yersinia pseudotuberculosis* ve *Escherichia coli* L-asparaginaz enzimi tip I ile benzerlik gösterir..

Pseudopedobacter saltans L-asparaginaz, değişmez amino asit kalıntıları ile karakterize edilen, mikrobiyal L-asparaginaz için ortak olarak korunmuş benzersiz imzasıyla sınıflandırılır; Thr^{12}, Ala^{23}, Lys^{25}, Ser^{88}, Asp^{89}, Lys^{165}, Leu^{291}, katalize dahildir.

Ayrıca, *Pseudopedobacter saltans* L-glutaminaz I'e L-asparaginaz katalitik tortusu, amino asit tortuları; Thr^{12}, Ala^{23}, Ser^{88}, Glu^{286}, Leu^{291}. Son olarak, çözünür ve aktif istatistiklerde yeni bir bakteriyel *Pseudopedobacter saltans* L-asparaginaz enziminin klonlanması ve ekspresyonu başarıyla sağlandı.

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Anahtar Kelimeler: *Pseudopedobacter saltans*, L-asparaginaz, IPTG I sopropil β-D1-tiogalaktopiranosid, homodimer, Klonlama, ekspresyon.

ABSTRACT

M.Sc. THESIS

Molecular Cloning, Heterologus Expression, and Structural modeling of L-asparaginase from *Pseudopedobacter saltans* in *E.coli*

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L-asparaginase (L-ASP), or l-Asparagine aminohydrolase (EC 3.5.1.1), is an enzyme that belongs to the class of hydrolases that is capable of hydrolyzing L-Asn into aspartic acid and ammonia, which is used in the treatment of acute lymphoid leukemia, which promotes the cleavage of the amino acid asparagine through the use of water and cleaves non-peptide carbon-nitrogen bonds. In this current study, cloning of the novel L-asparaginase gene from *Pseudopedobacter saltans* DM12145 was performed in *E. coli* with accession number NC_015177.1. The full-length of *Pseudopedobacter saltans* L-asparaginase is 1019bp, a protein-encoding 339 amino acids; and the molecular weight was evaluated to be 37.8 kDa, with a theoretical (pI) of 6.13. It was cloned on the expression vector pET-28-His (+) by the EZ Clone method, synonymously called ligation independent cloning (LIC) with protein ID WP_013634621.1 by Genscript Co., USA. It was cloned onto the expression vector pET-28a (+). The construct was nominated as pET-28a (+)/asp_pseudopedo. The recombinant L-asparaginase I gene of *Pseudopedobacter saltans* was expressed in pET28-His and transformed into *E. coli* BL21 (DE3) as a 6 His-tag fusion protein. Induced by one mM of IPTG for 18 h at 30 ^{o}C, and purified by IMAC chromatography, then analyzed by SDS-PAGE to assess the solubility and molecular weight of the recombinant protein band, which was exactly as expected at 36.0 kDa. The findings of this study reveal that the L-asparaginase I enzyme maintained its enzymatic activity at a pH of 8.0, the temperature of 60 ^{o}C, with a variable kinetics K_m value equal to 3 mM and a V_{max} of 168.2 mol/min/mg. The Silico

analysis reveals that it is quite similar to L-asparaginases I of *E. coli*, which are distinctly specific for L-asparagine and act as homodimer cytosolic proteins. Finally, the cloning and expression of a novel-bacterial *Pseudopedobacter saltans* L-asparaginase enzyme in soluble and active stats were successfully achieved.

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Keywords: *Pseudopedobacter saltans*, L-asparaginase, IPTG Isopropyl β-D-1-thiogalactopyranoside, homodimer, Cloning, expression.

1. INTRODUCTION

L-asparaginase (L-ASP), or l-Asparagine aminohydrolase (EC 3.5.1.1), is an enzyme that belongs to the class of hydrolases that is capable of hydrolyzing L-Asn into aspartic acid and ammonia[1], which is used in the treatment of acute lymphoid leukemia, which promotes the cleavage of the amino acid asparagine through the use of water and cleaves non-peptide carbon-nitrogen bonds[2].

The enzymatic reaction of L-asparaginase takes place through hydrolysis, which occurs in two steps, with the formation of an intermediate compound. In the first step, a nucleophilic residue of LASP is activated by a strong base and attacks the amide carbon atom of L-Asn (one of the substrates), generating an intermediate product: the beta-acyl enzyme. The second step is an attack on the ester carbon performed by a nucleophilic activation through a water molecule [30]; [31].

It was reported that the first L-ASP obtained from a gram-negative bacterium. Two types of L-ASPs that differ in substrate affinity (L-Asn) and location were observed: type I, found in the cytosol, and type II, in the bacterial periplasm [46]. At present, type II L-asparaginase is most commonly used in the pharmaceutical industry because it has a greater affinity for L-Asn (K_m = 0.0115 mM for type II L-ASP versus K_m = 3.5 mM for type I L-ASP, with parameters dosed for both proteins at a pH of 7 and a temperature of 25 ^{o}C)) [47]; [48]. It is noteworthy that type II L-ASP is active when it forms a tetramer of identical 35.6 kDa subunits [49].

L-asparaginase is divided into two groups: L-asparaginase, which hydrolyze asparagine with high specificity, and L-asparaginase-glutaminases, which hydrolyze asparagine and glutamine with the same efficiency, but with less specificity. Asparagine hydrolysis results in aspartic acid and ammonia, while glutamine hydrolysis results in glutamic acid and ammonia [8]. The L-asparaginase from gram-negative bacteria, particularly *Escherichia coli*, is one of the most widely studied. This bacteria has an L-asparaginase that has anti-lymphoma activity, unlike enzymes present in yeast [22], and *Bacillus coagulants* [23]. L-asparaginase is divided into two types, EC-1 and EC-2, which catalyze different reactions. Although both have catalytic activity of Asparagine and produce aspartic acid and ammonia,

EC-1 has no apparent antitumor effect while EC-2 has a characteristic antitumor activity [24].

In the early ages of studies on the antitumor capacity of microbial L-asparaginase, identified that enzymes from different sources have great variation in their ability to inhibit tumor growth, with L-asparaginase from *E. coli* being the enzyme that presented better results. Then it was postulated that this high tumor inhibition capacity was due to three main factors [22]. The high affinity of the enzyme for L-asparagine translates into a low Michaelis-Menten (KM) constant, which allows the enzyme to maintain its activity even at the low concentrations of L-asparagine found physiologically in the blood; *E. coli* L-asparaginase remains highly active even after it is removed from the blood, possibly by sequestration by liver cells; The half-life of the enzyme is increased when lymphoma cells are implanted in guinea pigs.

Although L-asparaginase from gram-negative bacteria L-ASPs is widely used in the treatment of Acute Lymphoid Leukemia (ALL), its administration requires a crucial control for the maintenance of the patient's wellbeing, considering that its glutaminase activity is identified as the reason for the side effects. Allergic reactions (difficulty breathing, rash, fever, pain, redness, swelling in the injection area), symptoms of liver problems (darkness of urine, nausea, loss of appetite), pancreatitis (stomach pain), neurological seizure, and induction of anti-L-asparaginase antibodies are among the side effects [2].

It is considered that the new L-asparaginases to be used as medications must have pharmacological activity, causing minimal side effects. Thus, it is expected to have a high affinity for the substrate, a half-life time sufficient to avoid administration of several doses, low immunogenicity, and high stability. It was also postulated that low cross-glutaminolysis activity is important to avoid excessive side effects of the enzyme treatment [13].

Therefore, several research activities have been developed for the isolation of microbial strains that produce this important enzyme, such as *Pseudomonas fluorescens, Serratia marcescens, Escherichia coli, Erwinia carotovora, Proteus vulgaris, Saccharomyces cerevisiae, Karnatakensis Streptomyces, Streptomyces venezuelae*, and several genera of fungi such as *Aspergillus, Penicillium*, and *Fusarium* [17] were identified as producers.

1.1. THE AIM OF THE STUDY

In this present study, we aim to describe the cloning, expression, and purification of recombinant L-asparaginase from a novel bacterium, *Pseudopedobacter saltans*, into *E. coli* BL21 DE3. The aim was extended to analyze the sequence encoding of L-asparaginase in silico.

1.2. IMPORTANCE OF THE STUDY

Production of Novel recombinant L-asparaginase enzyme for therapeutic use in pediatric leukemia. Since this enzyme is currently used in the treatment of leukemia in children. This enzyme is so expensive. And many patients cannot afford it. In addition, an undesirable immune response to this enzyme as a drug may occur over time in the serum of patients treated with this drug. Therefore, the worldwide L-asparaginase enzyme market is in urgent need of searching for new L-asparaginases to cope with the pharmaceutical demands of such enzymes.

2. LITERATURE REVIEW

2.1. Structure of L-Asparaginase

L-asparaginase (L-ASP), or l-asparagine aminohydrolase (EC 3.5.1.1), is an enzyme that belongs to the class of hydrolases that is capable of hydrolyzing L-Asn into aspartic acid and ammonia [1], which is used in the treatment of acute lymphoid leukemia and promotes the cleavage of the amino acid asparagine through the use of water and cleaves non-peptide carbon-nitrogen bonds [2], as shown in Figure (2.1). This mechanism directly interferes with the energy production of the tumor cell, halting disease progression by lowering serum levels of the amino acid [3].

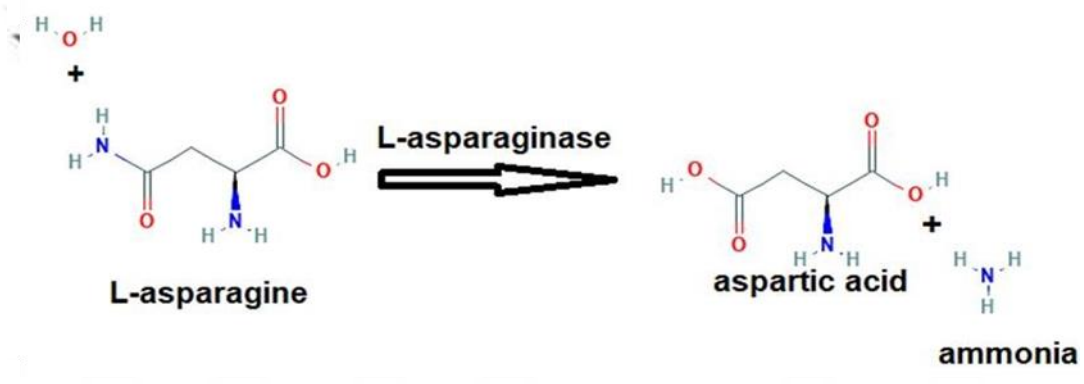

Figure 2. 1: Simplified mechanism of action of L-asparaginase [4].

The L-asparaginase enzyme was first observed in the early 20th century by Lang (1904), but its use as a tumor inhibitor was only reported in 1950 when Indian pig serum was injected into lymphosarcoma and mammary carcinoma, both of which showed gradual regression of size. In the mid-1960s, its presence was confirmed in bacterial and culture media. Currently, the main L-ASP formulations adopted in chemotherapy protocols for ALL, Hodgkin's Lymphoma, and other malignant neoplasms originate from gram-negative bacteria [5].

In general, microbial L-asparaginase comes in two isoforms: l-asparaginase I (L-ASP I), a cytoplasmic enzyme with a K_m of (3.5x10-3 M) for the substrate L-asparagine, and L-asparaginase II (L-ASP II), an extracellular enzyme whose K_m is 1.2x10-5 M [6], by presenting a lower K_m, L-ASP has a greater affinity for the substrate, so this isoform is considered the coadjuvant biopharmaceutical in chemotherapy protocols, usually combined with vincristine (Oncovin®) and glucocorticoids [7].

2.2. Characterization of L-asparaginase

L-asparaginase is an enzyme that hydrolyzes amino acids that have an amide group in their side chains. In bacteria, the main function of L-asparaginase is to maintain cell growth in an ammonia-deficient environment through the degradation of asparagine and glutamine as nitrogen sources [8].

L-asparaginase is divided into two groups: L-asparaginase, which hydrolyze asparagine with high specificity, and L-asparaginase-glutaminases, which hydrolyze asparagine and glutamine with the same efficiency, but are therefore less specific. Asparagine hydrolysis results in aspartic acid and ammonia, while glutamine hydrolysis results in glutamic acid and ammonia [8].

Although L-asparaginase from gram-negative bacteria (EcA) is widely used in the treatment of ALL, its administration requires a crucial control for the maintenance of the patient's wellbeing, considering that its glutaminase activity is identified as the reason for the side effects. Allergic reactions (difficulty breathing, rash, fever, pain, redness, swelling in the injection area), symptoms of liver problems (darkness of urine, nausea, loss of appetite), pancreatitis (stomach pain), neurological seizure, and induction of anti-L-asparaginase antibodies are among the side effects [2].

Although we are aware of these consequences caused by the glutaminase action of L-asparaginase, there is an unclear question about the real effects of this activity. It is not known how much the therapeutic action of the drug improves with the decrease in glutaminase action or if this enzyme activity is necessary for the desirable effects to exist [9]. One hypothesis suggests that the drug's therapeutic potential increases with the increase in its glutaminase activity and, at times, it is strictly necessary for there to be a fight against cancer cells. The decrease in glutaminase activity is responsible for a greater increase in toxicity than in the anticancer activity, contributing to a decrease in the therapeutic index. On the other hand, there is evidence that the therapeutic index of L-asparaginase increases due to the decrease in glutaminase activity [10].

The mechanism of action of L-asparaginase (Figure 2.3) is not fully elucidated. However, it is known that hydrolysis occurs in two steps, forming beta-acyl-enzyme as an intermediate compound. In the first step, the nucleophilic threonine attacks the carbonyl radical, forming

the intermediate and releasing an ammonia molecule. Then, a water molecule attacks the intermediate compound, forming aspartic acid [11].

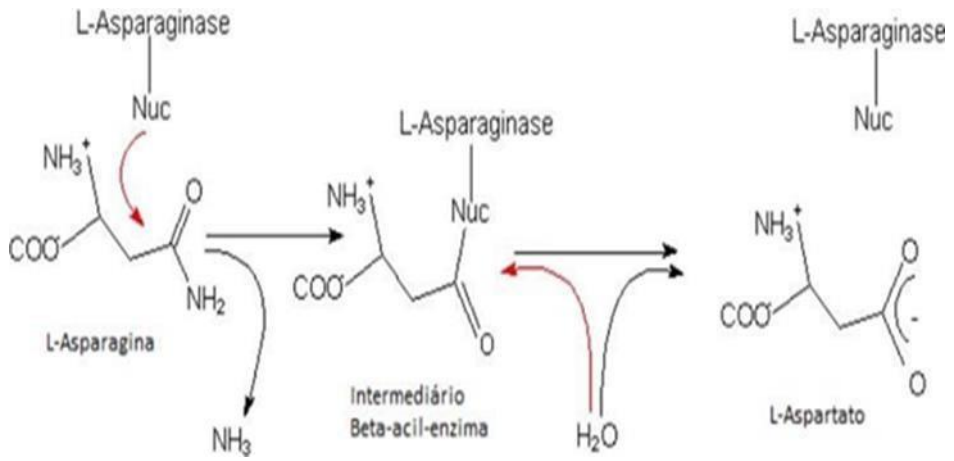

Figure 2. 2: L-asparaginase action mechanism [12].

2.3. Historical Background of L. asparaginase

Lang (1904) was the first to notice the enzymatic hydrolysis of L-asparagine to L-aspartate and ammonia. After a few years, Friedmann and Furth (1910) confirmed Lang's findings by detecting L-asparaginase hydrolysis in horse and pig organs, concluding that all animal tissues had the same level of L-asparaginase activity [13]. Clementi (1922) reported the presence of L-asparaginase in the serum of the guinea pig, but the anti-tumor properties of the enzyme were only recognized years later [14]. Mashburn and Wriston (1964) demonstrated that L-asparaginase from *E. coli* could inhibit tumors in rats, thus proving the potential for applying the enzyme and opening up horizons for its commercial production [15]. However, the great interest in L-asparaginase began when Broome (1961) discovered that the regression of lymphosarcoma transplants in rats treated with guinea pig serum as a result of the nutritional dependence of malignant cells on exogenous L-asparagine [16]. Since then, several research programs have been developed aiming at the isolation of microbial strains that produce this important enzyme, such as *Pseudomonas fluorescens, Serratia marcescens, Escherichia coli, Erwinia carotovora, Proteus vulgaris, Saccharomyces cerevisiae, Karnatakensis Streptomyces, Streptomyces venezuelae* and several genera of fungi such as *Penicillium, Aspergillus*, and *Fusarium* [17].

2.4. Asparagine

Asparagine is a non-essential amino acid and its biosynthesis is based mainly on two pathways: glutamine transamination (Figure 2.3) or direct addition of ammonia to aspartate (Figure 2.4). It has several functions in the body, including the restitution of a free amine group through its degradation for the synthesis of other amino acids or nucleic acids, as well as direct participation in protein synthesis [18].

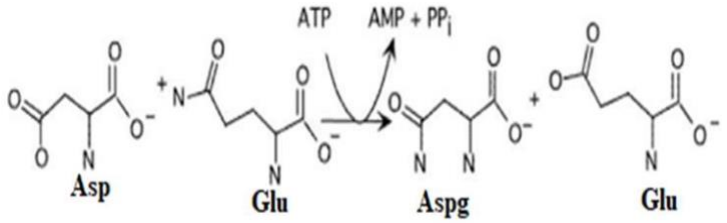

Figure 2. 3: Glutamine transamination reaction [19].

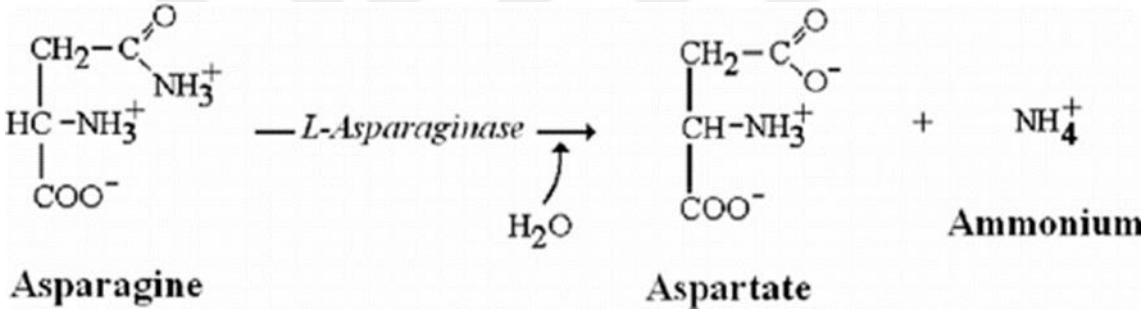

Figure 2. 4: Ammonia addition reaction to aspartate [19].

One of the chemical reactions that synthesize asparagine uses glutamine as a precursor, and this has a direct relationship with metabolic pathways, such as the citric acid cycle, in which its synthesis can occur from alpha-ketoglutarate through glutamate [18]. Thus, glutamine synthesis is induced by the inhibition of the alpha-ketoglutarate dehydrogenase complex, which can occur because of large concentrations of NADH, ATP, and succinyl-CoA, causing an accumulation of alpha-ketoglutarate that is converted into glutamate by glutamate dehydrogenase (Figure 2.5), [18].

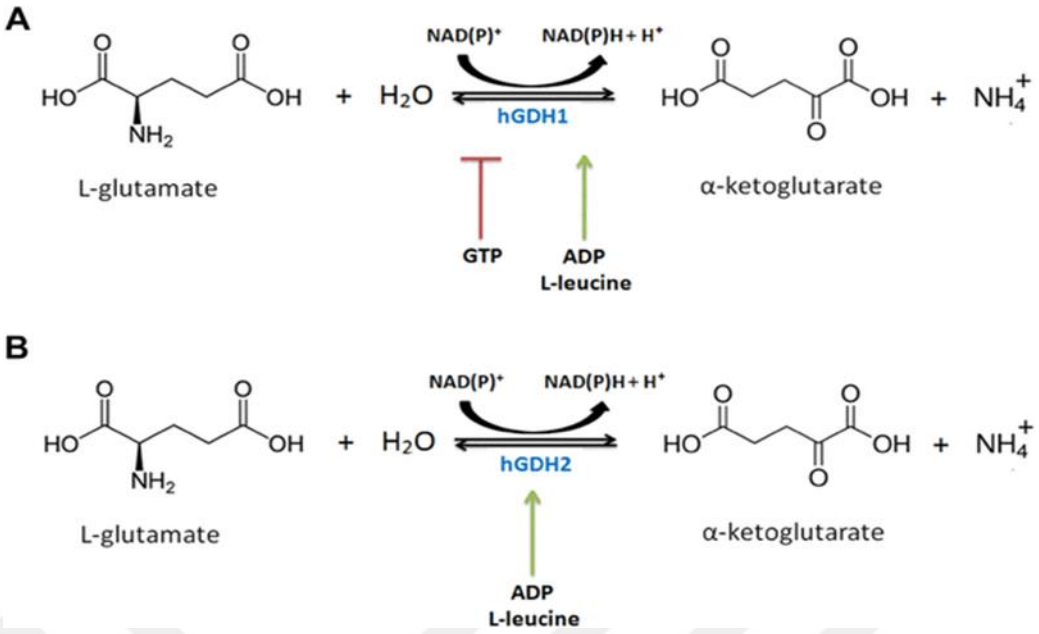

Figure 2. 5: Conversion reaction between alpha-ketoglutarate and glutamate [12].

2.5. Pseudopedobacter saltans

The gram-negative bacterium *Pseudopedobacter saltans* type strain (113T) is one of the many species of the genus *Pseudobacter* inside the *Sphingobacteriaceae* family. *Pseudopedobacter saltans* is heparinolytic, with short rods with rounded, slightly tapered round ends and a gliding, dancing motility, as shown in Figure 2.6. It does not form spores and is strictly aerobic (not pathogenic) [20].

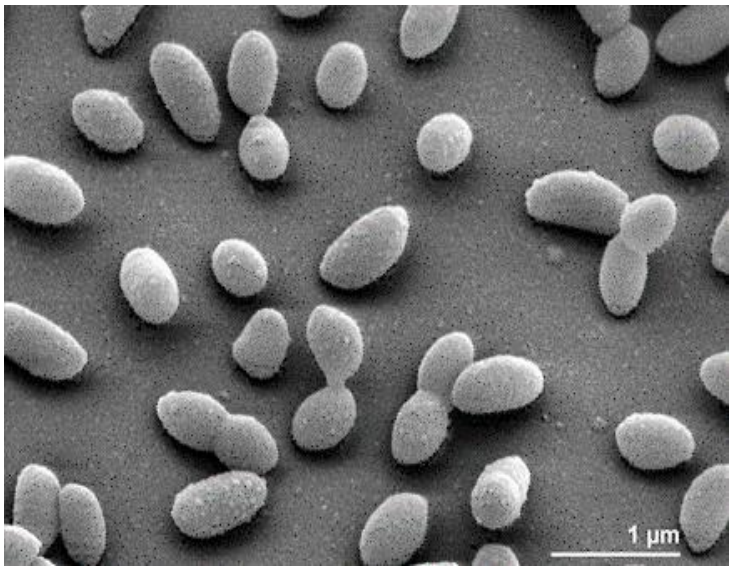

Figure 2. 6: Scanning electron micrograph of *Pseudopedobacter saltans* strain 113T [20].

Pseudopedobacter saltans can easily be recognized from other *Psedopedobacter* species by their capability to exploit glycerol and the disability to ingest D-cellobiose, and it does not reduce nitrate. The gram-negative organism *Pseudpedobacter saltans* strain 113T general genome properties include (4,635,236 bp) chromosome with a G + C content of (36.6%) percent of the (3,921) predicted genes. 3,854 of genes were protein-coding, 67 of which were RNAs, and (62) pseudogenes [20]. The gram-negative bacteria produce heparinase cell walls consisting of menaquinone-7 as the predominant menaquinone system and sphingolipids. The fatty acids in strain 113T are as follows: iso-C15: zero (31.4%), C16:17c (19.6%), iso-C17: zero 3-OH (12.7%), iso-C15: zero 2-OH (8.9%), iso-C16: zero (4.6%), anteiso-C15: zero (2.9%), iso-C15: zero 3-OH (2.8%), C15: zero (1.4%), C15: zero (1.4%), it's far taken into consideration as traditional acids of the genus [20].

2.6. Bacterial L-asparaginase

Bacterial L-asparaginases are characterized by having enzyme activity only when in a tetramer, even though they have an active site in all monomers [21]. The L-asparaginase from gram-negative bacteria, particularly *Escherichia coli*, is one of the most widely studied and property L-asparaginases (Figure 2.7). This bacterium has an L-asparaginase that has anti-lymphoma activity, unlike enzymes present in yeast [22], and *Bacillus coagulants* [23]. L-asparaginase is divided into two types, EC-1 and EC-2, which catalyze different reactions. Although both have catalytic activity of Asparagine and produce aspartic acid and ammonia, EC-1 has no apparent antitumor effect while EC-2 has a characteristic antitumor activity [24].

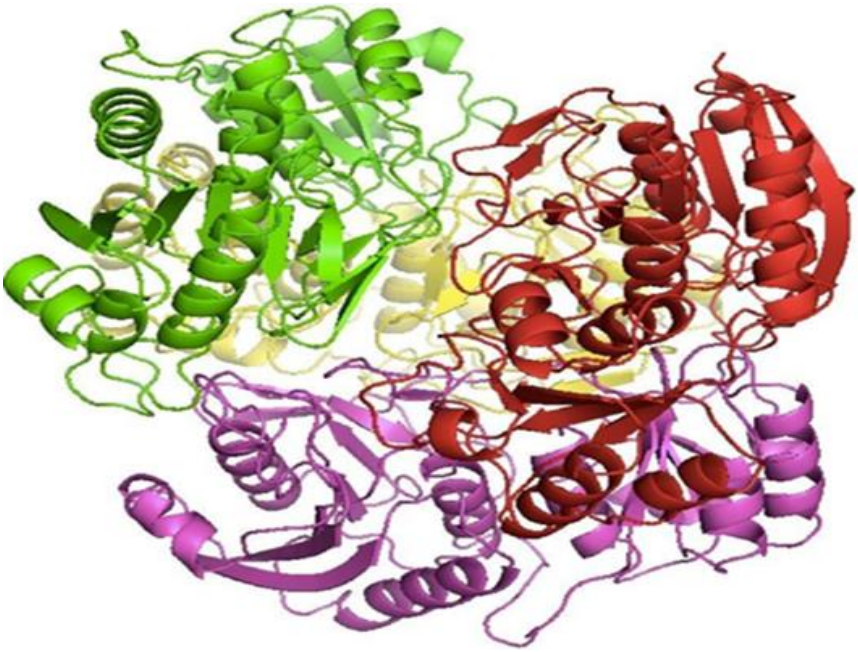

Figure 2. 7: Crystallized structure (PDB code 3eca) of L-asparaginase from *E. coli*, in which the 4 monomers and their organization for the formation of the homotetramer are observed [21]

2.7. General considerations on microbial production of L-asparaginase

The main microbial sources for the production of L-asparaginase are bacteria, filamentous fungi, and yeast. At the beginning of studies on the antitumor capacity of microbial Lasparaginase, Broome (1968) identified that enzymes from different sources have great variation in their ability to inhibit tumor growth, with L-asparaginase from *E. coli* being the enzyme that presented better results. Then, it was postulated that this high tumor inhibition capacity was due to three main factors [22]:

1) The high affinity of the enzyme for L-asparagine translates into a low Michaelis-Menten (K_m) constant, which allows the enzyme to maintain its activity even at the low concentrations of L-asparagine found physiologically in the blood.

2) *E. coli* L-asparaginase remains highly active even after it is removed from the blood, possibly by sequestration by liver cells.

3) The half-life of the enzyme is increased when lymphoma cells are implanted in guinea pigs.

Since then, several microorganisms have been evaluated to obtain an L-asparaginase with greater antitumor activity compared to that produced by *E. coli* and that was produced in an

economically viable way. It is considered that the new L-asparaginases to be used as medications must have pharmacological activity, causing minimal side effects. Thus, it is expected that they will have a high affinity for the substrate, a half-life time sufficient to avoid administration of several doses, low immunogenicity, and high stability. It was also postulated that low cross-glutaminolysis activity is important to avoid excessive side effects of the enzyme treatment [2].

2.8. Aspects of L-asparaginase production by bacteria

The enzymes produced by the enterobacteria *Escherichia coli* and *Erwinia chrysanthemi* are the only ones currently used in antileukemic therapies with L-asparaginase. In the first studies on L-asparaginase-producing microorganisms, the bacteria *Pseudomonas fluorescens, Mycobacterium phlei, Staphylococcus bacteria, Tetrahymena pyriformis*, and *Thermus aquaticus*, a thermophilic bacterium, were identified as producers [24]. Geckil et al. (2004) proposed that fermentative bacteria, in general, have the synthesis of L-asparaginase repressed by high concentrations of glucose due to the formation of acids, which reduce culture pH, and due to catabolic repression, which reduces intracellular cAMP levels [25]. It is believed that L-asparaginase is synthesized anaerobically in *E. coli* due to induction of the reducing protein of fumarate and nitrate and upregulation by the cAMP receptor protein [26]. Verma et al. (2007) cited those organic acids and the amino acids L-leucine and L-methionine as increasing L-asparaginase synthesis by *E. coli* [24]. The mechanism of regulation of L-asparaginase production by *Staphylococcus aureus* was studied by Rozalska and Mickucki (1992), in which carbon sources such as sucrose, maltose, galactose, lactose, mannitol, and mannose inhibited L-asparaginase production [27]. On the other hand, its production was stimulated when the medium was supplemented with exogenous cAMP [28]. Other more recent studies on the selection and optimization of periplasmic L-asparaginase production under submerged fermentation by bacteria are reported in Table 2.1 [29].

Table 2. 1: Non-recombinant L-ASP source bacteria, associated asparaginase activity values, Km, Kcat, optimal reaction pH, and temperature [29].

Source Organism	**Activity L-ASP**	**Km**	**pH**	**T(ºC)**
Yersinia pseudotuberculosis	62.7 U/mg	0.017mM	8	60
Bacillus aryabhattai	680.5 U/mg	0.257 mM	8.5	40
Pseudomonas fluorescens	168.4 U/mg	110mM	8	37
Bacillus licheniformis	697.1 U/mg	0.014mM	6a 10	50
Nocardiopsis alba	158.1 U/mg	0.127mM	8	40
Streptomyces noursei	0.803 U/mg	25 μM	7.5	50
Pectobacterium carotovorum	35.24 U/mg	657 μM	8 a 10	40
Streptomyces parvulus	146 U/mg	25 μM	7.5	50
Bacillus subtilus	23.8 U/mg	430 μM	7.5	40
Streptomyces ginsengisoli	3.32 U/mg	25 μM	7.5	30
Streptomyces thermoluteus	68.9 U/mg	1830 mM	8 a 9	63.6
Phytobacterium sp	20 U/mg	760 mM	7	25
Pyrococcus furiosus	550 U/mg	12000 mM	9	85
Bacillus licheniformis	597.8 U/mg	0.420 mM	8	37

2.9. L-ASP catalytic reaction and its use in the treatment of Acute Lymphoid Leukemia

The enzymatic reaction of L-asparaginase takes place through hydrolysis, which occurs in two steps, with the formation of an intermediate compound. In the first step, a nucleophilic residue of LASP is activated by a strong base and attacks the amide carbon atom of L-Asn (one of the substrates), generating an intermediate product: the beta-acyl enzyme. The

second step is an attack on the ester carbon performed by a nucleophilic activation through a water molecule [30]; [31], as shown in (Figure 2.8).

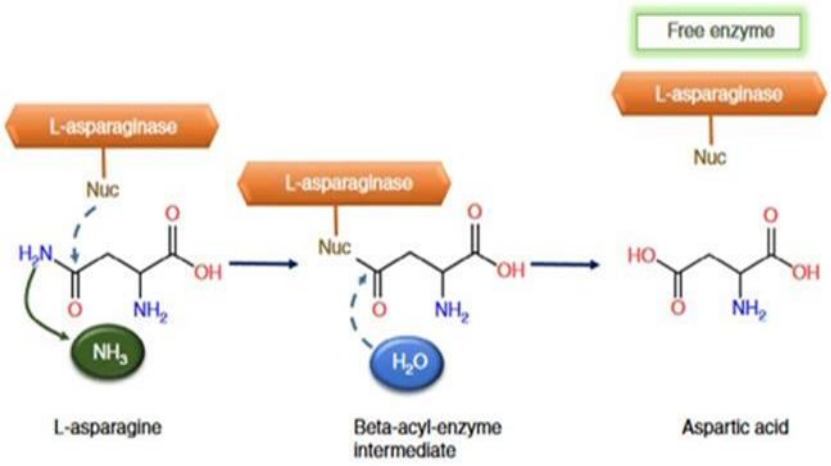

Figure 2. 8: Mechanism of the l-asp catalysis reaction in leukemia [32].

Many L-asparaginases also can catalyze another competitive reaction, acting on L-Glutamine (L-Gln) and forming L-Glutamate (L-Glu) and ammonia (NH3), with L-Gln being a competitive inhibitor of the hydrolysis of L-Asn (Figure 2.9) because of the similarity of their structural structures [33].

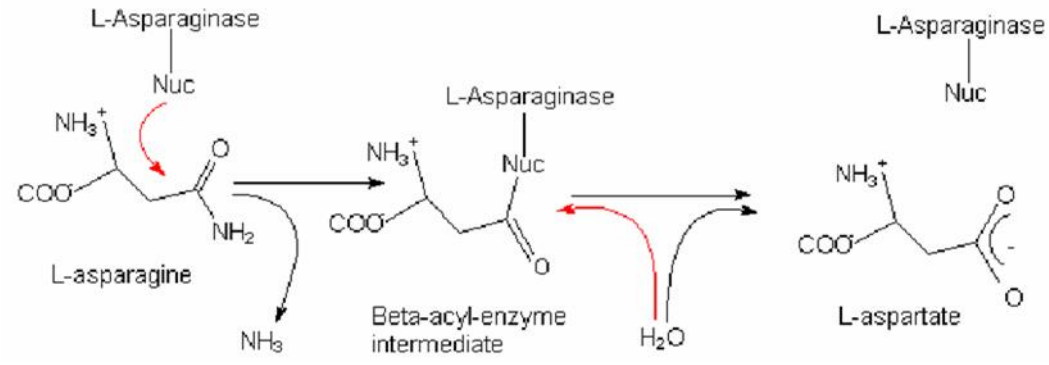

Figure 2. 9: Mechanism of the L-asparaginase / L-glutaminase catalysis reaction [34].

Other L-asparaginases from some microorganisms can hydrolyze 5% of L-Gln when compared to L-Asn hydrolysis, such as the L-ASP from *Serratia marcescens*, *Escherichia coli*, and *Erwinia chrysanthemi*, while other L-asparaginases from other microorganisms, such as *Pseudomonas* sp. [35]; [36], and that of *Acinetobacter glutase*, have the same level of L-asparaginase and glutase activity. Later, some L-asparaginases that do not show glutaminase activity detectable by the methodology used were described, such as the one derived from *Wolinella succinogenes* [37].

L-Gln is an amino acid abundant in plasma and necessary for the synthesis of amino acids and nucleotides. Many types of human cancers, such as pancreatic cancer, colon cancer, liver cancer, and leukemia, are dependent on L-Gln for their proliferation and survival [38]. It has been observed that L-Gln depletion in the medium induces cancer cell apoptosis with greater selectivity than L-Asn depletion [39].

The anticancer activity of L-asparaginase from *E. coli* was demonstrated by Marshburn and Wriston (1964), who observed the inhibition of tumors in rats. However, the greatest interest in its use as a biopharmaceutical began in 1965, when Broome identified a regression of lymphosarcomas in transplanted rats treated with guinea pig serum, reporting for the first time that this was due to nutritional dependence on L-Asn by malignant cells [22].

Thus, the success of L-asparaginase in treating some types of cancer, particularly lymphatic cancers, depends on the cells that make up these cancers require a lot of L-Asn to grow [40]. Treatment with L-asparaginase becomes effective when administered to patients with ALL cells that do not produce Asparagine synthetase (AS) due to their inability to synthesize the amino acid L-Asn [41]. When administered effectively, the enzyme leads to a decrease in the L-Asn substrate in the extracellular environment, selective apoptosis of LLA neoplastic cells [41], and a definitive cure in 80 to 90% of children and 30 to 60% of young people and adults, when applied in conjunction with other anticancer drugs [42]. When L-ASP was administered alone in the 1970s, it brought about a complete remission in 40 to 60% of pediatric cases [43].

2.10. Determining factors of the antileukemic activity of L-ASPs and formulations of commercial L-asparaginase

It was observed that the antileukemic activity of L-asparaginases depends on the hydrolysis rate and affinity of L-asparaginase with its substrate, and pharmacological factors of the serum clearance of the enzyme [41]. The development of L-asparaginase resistance in tumor cells [44], the presentation of antibodies against L-asparaginase by the host immune system, and the contribution of L-Asn to both biosynthesis and how much L-Asn is obtained from metabolism by nutrient intake [45]. There are three formulations of L-asparaginases used commercially under the brands. In the following paragraphs, such biopharmaceuticals produced on a large scale are provided in detail.

Tsuji (1957) reported the first L-ASP obtained from a gram-negative bacteria. Two types of L-ASPs that differ in substrate affinity (L-Asn) and location were observed: type I, found in the cytosol, and type II, in the bacterial periplasm [46].

At present, type II L-asparaginase is most commonly used in the pharmaceutical industry because it has a greater affinity for L-Asn (K_m = 0.0115 mM for type II L-ASP versus K_m = 3.5 mM for type I L-ASP, with parameters dosed for both proteins at a pH of 7 and a temperature of 25 ^oC) [47]; [48]. It is noteworthy that type II L-ASP is active when it forms a tetramer of identical 35.6 kDa subunits [49].

The other commercial L-ASP is obtained from *Erwinia chrysanthemi*, which is less immunogenic and less toxic, but less efficient in comparison to the L-ASP from *E. coli* [50][51].

Alternatively, there are PEGylated L-ASPs, obtained from *E. coli* or *E. chrysanthemi*, by the covalent modification by conjugation of mono-methoxy polyethylene glycol (PEG) units. This modification improves the biostability and bioavailability of the compound, influencing the pharmacokinetic and pharmacodynamic properties of the enzyme in patients (increased half-life, slower clearance, and reduced immune response) [52]. However, this results in the loss of part of the biological activity of the conjugate. As a result, PEGylated L-ASP can be given in smaller quantities and at shorter intervals [53].

L-ASP derived from any of the formulations presented undergoes continuous degradation in the body, either by hydrolysis or by enzyme inactivation. Two lysosomal proteases (L-asparaginase endopeptidase (AEP) and cathepsin B (CTSB)) are known to degrade and inactivate L-ASP by hydrolysis, contributing to therapy failure [54]. AEP and CTSB are highly expressed by Philadelphia leukemic cells (Ph) and by cells with iAMP21 (Intrachromosomal Amplification of Chromosome), very aggressive leukemic cells that are poorly responsive to treatment [54]; [55]. Thus, CTSB hydrolysis limits its use in the treatment of L-ASP inactivating tumors [54].

In the United States of America (USA), there are three formulations of L-ASPs, one of which has been discontinued and two of which are currently used against ALL. The non-pegylated native L-ASP obtained from *E. coli* (Elspar®, Merck & Co., Inc., West Point, PA, USA), whose production for human use was discontinued in 2012 due to adverse effects [56], the PEGylated L-ASP (Oncaspar®, Sigma-Tau Pharmaceuticals, Inc., Gaithersburg, USA); and

the *Erwinia chrysanthemi* (This last formulation is approved in the UK as a second-line treatment for patients with hypersensitivity (from 15 to 20%) to the two previous formulations. Native L-ASPs are offered under different brand names in Europe, except in the UK, and Asia [41].

Currently, all commercial formulations are available in lyophilized form (between 3,750 and 10,000 IU), except for Graspa®, which is presented in the form of erythrocytes with encapsulated L-ASP (between 78 and 146 IU/mL). The mechanism of action consists of erythrocytes (Graspa®) capable of "pumping" L-Asn from the blood plasma into its interior, through transporters coupled to sodium (SNAT 3/5), and the encapsulated L-ASP degrades the amino acid. In this way, L-ASP increases its half-life and activity and prevents enzyme inactivation by immune responses [57].

2.11. Other uses of L-ASP

L-ASP can also be useful in treating other types of illnesses. The production and purification of a chimeric enzyme, L-ASP-EP-T/B (L-ASP plus B cell epitopes and T helper cell epitopes against tetanus toxin), resulted in high titers of anti-L-ASP-EP-T/B in mice after immunization with 3 doses. This enzyme paves the way for the development of a new vaccine against atherosclerosis [58].

By detecting and stratifying different levels of L-asparagine (one of the substrates of L-ASP) present in leukemic cells, L-ASP can be used as a biosensor for leukemic cells [59]. CMTN (Triple Negative Breast Cancer) cells are considered the most aggressive within the group of breast cancers. These cells show a marked dependence on extracellular glutamine for growth, and it was found that the deprivation of this amino acid, through the use of the glutaminase activity of L-ASP derived from *E. chrysanthemi*, leads to apoptosis of these tumor cells, thus allowing the development of a new biological medicine against CMTN cells [60].

Extra-nodal Natural Killer Cells and Nasal-type T-cells (LE-NK/T) Lymphoma is a highly aggressive lymphoma that is refractory to conventional chemotherapy. *In vitro* studies have shown that L-ASP can reduce the activity of normal NK cells and induce apoptosis in tumor NK cells [61]. Takahashi et al. (2017) found that L-ASP, in combination with chloroquine, has high cytotoxicity against glioblastoma cells [62].

Another use of L-ASP in the food industry. L-asparaginase prevent the formation of acrylamide when foods are processed at high temperatures, especially when they are fried [63]. The prevention of acrylamide formation is important due to neurotoxicity, being classified as a potential carcinogenic to humans [64]. Zyzak et al. (2003) demonstrated that when L-ASP is used before the process of cooking food at high temperatures, the formation of the acrylamide level is reduced by more than 99% in the final product, caused by an 88% reduction in the concentration of L-Asn by the enzyme L-ASP in the raw material [65].

2.12. Adverse effects presented by patients treated with L-ASP

L-ASP therapy causes adverse effects, attributed to glutaminase activity [10]; [37], usually started from the 25th week onwards, which induces treatment remission [66]. However, Zhao et al. (2014) affirm the importance of glutaminase activity in ALL AS-positive cells [67].

There have been many adverse effects on patients treated with native L-ASP derived from PEGylated or non-PEGylated *E. coli*. On the contrary, the administration of L-ASP from *E. chrysanthemi* shows the same side effects, but with lesser intensity. However, treatment success is reduced by having lower asparaginase activity [68].

3. MATERIAL AND METHODS

3.1. MATERIAL

3.1.1. Equipment and Instruments

All the instruments and equipment that were used in the procedure of the present study are listed below in Table 3.1.

Table 3. 1: The equipment and instruments used in this study with companies and origin.

No.	Equipment and instruments	Company	Origin
1.	Balance	Kernp	Germany
2.	Incubator	Memmert	Germany
3.	Water bath	Memmert	Germany
4.	Oven	Memmert	Germany
5.	Sensitive balance	Sartorius	Germany
6.	Micro-spin Centrifuge	Biosan	Germany
7.	Micropipettes (10-1000µl)	Human	Germany
8.	Thermal-magnetic stirrer	Ika-Werk	Germany
9.	Vortex	Digsystem	Germany
10.	Nanodrop	ThermoScientific®	Germany
11.	Autoclave	Tomy-Seiko	Japan
12.	Microscope	Olympus	Japan
13.	Spectrophotometer U- VIS	Shimadzu	Japan
14.	Digital camera	Canon	Japan
15.	Centrifuge	Hettich	USA
16.	Gel electrophoresis system	Apparatus	USA
17.	Dialysis Bags	Sigma	USA
18.	Thermal cycler device	Applied Biosysytem	USA
19.	Shaker-Incubator	Artisan Scientific	USA
20.	Eppendorf bench centrifuge	Hettich	USA
21.	NanoDrop2000	Thermo Fisher	USA
22.	Microwave	Gosonic	China
23.	Freezer (-20)	Haier	China
24.	Deep freeze (-80)	Haier	China
25.	Water distiller	Jrad	China

26.	Ultra violet light transilluminator	Vilberlourmat	Italy
27.	Burner	Amal	Turkey
28.	Refrigerator	TEKA	Spain
29.	Sonicator	Soniprep	UK
30.	Laminar flow hood	Gallenkamp	England
31.	Nanodrop	Bioneer	South Korea
32.	Disposable Petri dishes	Bioneer	South Korea
33.	Ultrasonic processor UP50H	Hielscher Ultrasonics GMBH©	Germany
34.	HisTrap™ HP	SLS International	England

3.1.2. Chemical Materials

The chemicals that were used in the experiences in this study are shown below in Table (3.2).

Table 3. 2: The chemicals used in this study with companies and origin

NO	Chemicals	Origin	company
1.	$MgCl_2$	abm	Canada
2.	Nuclease free water	abm	Canada
3.	Ethidium bromide	abm	Canada
4.	Ethylene-diamine tetra acetic acid (EDTA)	abm	Canada
5.	Agarose	abm	Canada
6.	DNA loading dye	abm	Canada
7.	Primers	IDT	Canada
8.	Bisacrylamide	abm	Canada
9.	Acrylamide (BDH)	abm	Canada
10.	Sodium Dodecyl Sulfate	abm	Canada

11.	Methanol	abm	Canada
12.	Glacial acetic acid, Ammonium persulphate	abm	Canada
13.	TEMED	abm	Canada
14.	NaHCO3	abm	Canada
15.	Bromophenol blue	abm	Canada
16.	Absolute ethanol	abm	Canada
17.	NaOH	BDH	England
18.	Glycerol 20%	BDH	England
19.	Sodium chloride (NaCl)	BDH	England
20.	Isopropanol	BDH	England
21.	Sephacryl S-1000	ROMIL pure chemistry	UK
22.	Kanamycin	Sigma Aldrich	UK
23.	IPTG	Interon	Korea
24.	Protein marker	Interon	Korea
25.	Agar-Agar	Santacruz	Germany
26.	TBE buffer (10X)	Himedia	India
27.	Shrimp-Alkaline Phosphatase (SAP)	Thermo Fisher	USA
28.	DNA Ladder	Promega	USA
29.	Tris-Acetate-TAE	Promega	USA
30.	A 10X loading buffer	Promega	USA

31.	bovine serum albumin (BSA)	Promega	USA
32.	Coomassie-brilliant blueG250	Promega	USA
33.	Tris-base	Promega	USA
34.	rCutSmart®Buffer (10X)	Promega	USA
35.	Triton X100	Ameresco	USA
36.	PMSF	Ameresco	USA

3.1.3. Kits

The kits that were used in the experiences in this study are shown below in Table 3.3.

Table 3. 3: All the kits that have been used in this study.

No.	Kit	Company	Origin
1.	Gene JET PCR purification	Thermo Fisher Scientific GE	USA
2.	PCR master mix	Healthcare	USA

3.1.4. Culture Media

The cultural media that were used in experiences in this study are shown below in the Table 3.4.

Table 3. 4: The culture media that have been used in this study.

No.	Culture media	Company	Origin
1	Laura Bertani agar	Difco Laboratories	India
2	Laura Bertani broth	Difco Laboratories	India
3	Modified ezapelc's dox broth	interon	Korea

3.1.5. Vectors and Bacterial Strains

The pET-28a (+) vector was used as an expression vector. DH5-α, was employed to keep the recombinant vector, and the *E. coli* (BL21) Rosetta strain from Promega (USA) was used for expression hosts for recombinant proteins. All bacterial strains were maintained for long-term preservation in 50% (v/v) glycerol stocks and stored at -80 ^{o}C.

3.2. METHODS

3.2.1. Methods of Sterilization

The in-process sterilization method of choice in the laboratory as follows:

- The autoclave uses pressurized steam to heat the items to be sterilized. It is a very effective method for culture sterilization, which is efficiently achieved by intense heat in the presence of water. Compressed steam has a high latent heat; at 121 ^{o}C for fifteen minutes.

- Dry Heat: This requires higher temperatures for sterilization of the glassware effectively by dry heat. That would generally require a temperature of 180 ^{o}C for two h.

3.2.2. Preparation of Chemical Solutions

All the solutions that have been used in this study were prepared according to the instructions of the manufacturer's protocol as follows :

1) Normal Saline (NaCl) (0.85%); dissolving 0.85 g of NaCl in (100 ml) of D.W, then, the volume was completed up to (100 ml) by D.W, then sterilized by autoclaving.

2) Ethidium Bromide; dissolving 0.25 g of ethidium bromide dye in 50 ml of D.W, then, obtain a solution with final concentration (5 mg /ml), and kept at 4°C.

3) Sodium Hydroxide (NaOH)/(10 N); dissolving 40 g of NaOH in 60 ml of distilled water, and the volume was completed up to 100 ml by distilled water and then stored at room temperature.

4) Ethylene Diamine Tetraacetic Acid (EDTA)/(50mm); dissolving 1.86 g of Na^{2}EDTA in 10 ml of D.W, then, complete the volume up to 100 ml by D.W, then the pH was adjusted up to 8.0, and sterilized by autoclaving.

3.2.3. Preparation of Chemical Buffers

All the buffers that have been used in this study were provided by IDT (Canada) and were prepared according to the instructions of the manufacturer's protocol as follows:

- Tris Borate EDTA Buffer 1X (TBE 1X); dilution of 100 ml of stock TBE solution (10X) up to one liter by adding 900 ml of D.W, to obtain 1000 ml of TBE (1X).

3.2.4. Primer Preparation and Store

These primers were supplied by IDT/DNA Company in a lyophilized form, dissolving the lyophilized primers in nuclease-free water until a final concentration of 100 picomol/µl as a stock solution, and the working solution of these primers was prepared by adding 10 µl of stock solution to 90µl of nuclease-free water to obtain a working primer solution of 10 picomol/µl (stored at freezer -20 ^{o}C).

3.2.5. Protein Purification Buffers and Solutions

- Lysis buffer was prepared by using 25 mM Tris HCl and 2 mM EDTA of pH 7.6.
- Elution buffer was prepared by employing 50 mM Tris at pH 8 in 300 mM from NaCl, 5% from Glycerol, and 400 mM from Imidazole.
- Wash buffer was prepared by using 50 mM Tris at pH 8 in 300 mM from NaCl, 5% Glycerol, and 20 mM from Imidazole.
- Tris-HCl buffer (0.05M, pH=8.0): This solution was prepared by dissolving 6.057g of Tris-HCl in 900 ml distilled water, adjusting pH to 8, and then completing the volume to 1000 ml by distilled water.
- Bradford Solution: It was prepared by dissolving 0.1 g of Coomassie Brilliant Blue G250 in 50 ml of 95% ethanol, then 100 ml of phosphoric acid (85%) solution was added with cooling and stirring. Then, the volume completes to a liter of distilled water. The stain was filtered twice by Whatman filter paper and stored in a dark bottle at 4 ^{o}C until used.

3.2.6. Preparation of Culture Media

In this study, Laurie-Bertani (LB) broth and LB agar were used for growing the *E. coli* (BL21) DE3 Rosetta strain [69].

1) In 1 liter of DW, we dissolve 10 g of tryptone and NaCl and 5 g of yeast extract.

2) For LB Agar, we added agar 1.5% to the final concentration. To dissolve the agar and the other components, the mixture was heated to boil.

3) For sterilization, we used the autoclave at a specific condition of 15 psi, at 121-124°C for 15 min.

4) Finally, we incubated the culture media at 37 ºC for 24 h to check the sterilization.

3.2.7. Bacterial Competence By Chemical Method

I. The bacterial strain of *E. coli* BL21 (DE3), Rosetta strain, was subjected to chemical competence by stressing with saline solutions of $MgCl_2$ (0.1 M) and $CaCl_2$ (0.1 M).

II. Pre-inoculums of the strain, from isolated colonies, were prepared in Luria Bertani Broth Miller (LB) medium without antibiotics, in volumes of 5 mL, and incubated under agitation (200 rpm) at 37 °C, overnight.

III. 5 mL of the pre-inoculum colonies were added to 300 mL of antibiotic-free LB and incubated at 37 °C and 200 rpm for 3 h until they reached an optical density (OD) of 600.

IV. Once the OD was reached, the bacterial culture volume was centrifuged at 4 °C for 10 min at 5000 g, and reserving only the cell pellet (pellet), for cell resuspension with 40 mL of ice-cold $MgCl_2$.

V. This was followed by new centrifugation at 4 °C, 3000 g for 10 min, and subsequently, the supernatant was discarded.

VI. The pellet was resuspended again, this time with 2 mL of cold $CaCl_2$, leaving the bacteria to rest for 20 min on ice, followed by the addition of 1 mL of 50% glycerol.

VII. 100 µL aliquots were placed in the Eppendorf tubes containing the glycerol stock of the chemically competent *E. coli* cells and stored at -80 °C.

3.2.8. Cloning Of L-asparaginase I Encoding Gene From *Pseudopedobacter saltans*

PCR was amplified by using forward and reverse primers with nucleotide sequences (fw) 5 '-ATCGGATCCATCACCATT-3' and (rv) 3 'GCGAAGTTTAGTTAGATGATGATT-5' with a complete open reading frame ORF, as indicated in GenBank.
The ORF open reading frame encoding for the L-asparaginase gene type I from *Pseudopedobacter saltans* DSM 12145 has 1019 bp (protein ID: WP_013634621.1".), that was retrieved from GenBank.

This ORF was spanned from 4301275-4302294 nucleotides in the genome of *Pseudopedobacter saltans* DSM 12145 with the accession number NC_015177.1. This nucleotide sequence of the L-asparaginase type I gene was synthesized by Genscript Co., USA. Then, it was cloned onto the expression vector pET-28a (+) by the EZ Clone method, synonymously called ligation independent cloning (LIC). As shown in Figure 3.1, the vector map of pET-28a (+) was synthesized by GenScript Co. to carry the L-asparaginase gene from *Pseudopedobacter saltans* DSM 12145. The construct was nominated as pET-28a (+)/asp_pseudopedo.

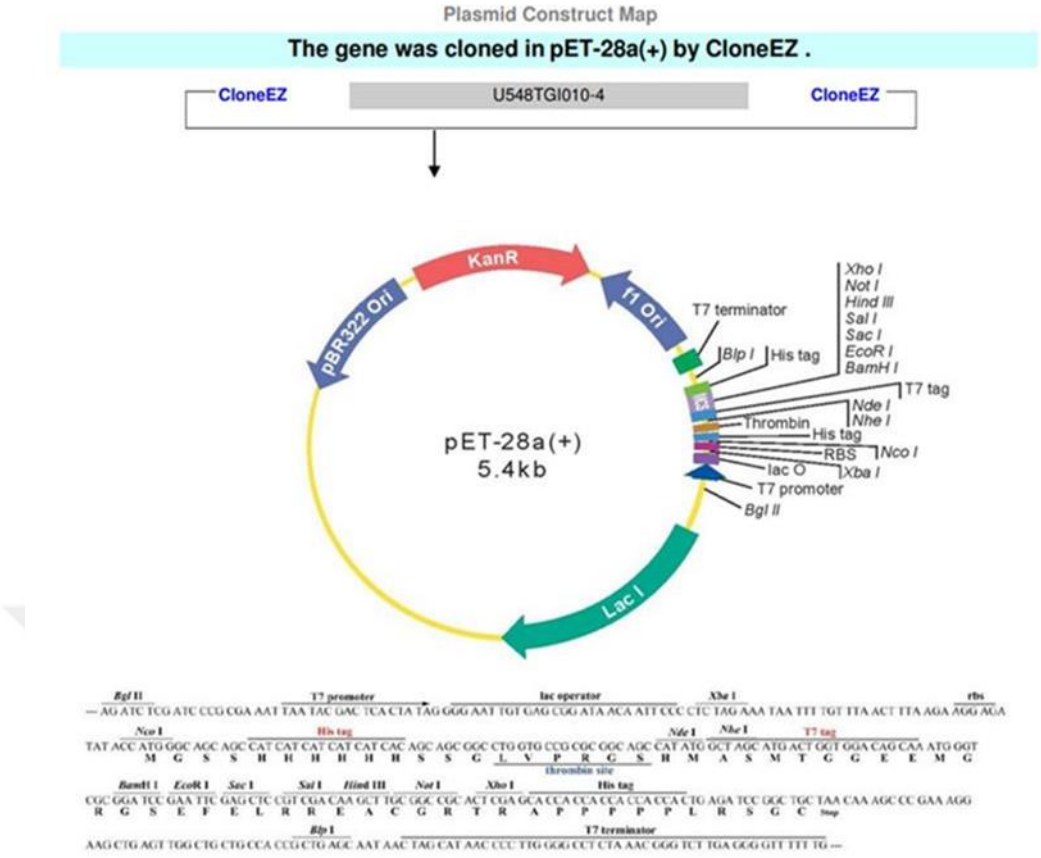

Figure 3. 1: The map of pET-28a (+) shows the method of insertion of the L-asparaginase type I gene of *Pseudopedobacter saltans* DSM 12145. U548TG1010-4 is the code given by Genscript Co. The full length of the plasmid vector is 5.4 kbp before insertion of the asparaginase type I gene.

3.2.9. Transformation Of *E. coli* DE3 Rosetta strain chemically competent cells

We added 2-3µL (50 ng) of the pET-28a (+) vector DNA to the chemically competent *E. coli* BL21(DE3) cells that were dispended in an Eppendorf tube (100 µL). In a parallel negative control experiment, *E. coli* cells were transformed with empty pET28a. Then, the mixture was kept for 40 minutes on ice, followed by the cells exposed to heat shock at 42 ºC for 45 sec in a water bath, returning to the ice for another 5 min to perform thermal shock. Then, 900 µL of LB medium was added to the Eppendorf tube for a period of regeneration under agitation for 3 h at 37 °C and at 200 rpm, which is enough time for the bacterial cells to be able to develop the antibiotic resistance gene. Therefore, the 1 mL mixture was plated onto an LB agar medium, containing its respective antibiotic kanamycin (34 µg/mL), and incubated overnight at 37 °C in a bacteriological incubator.

3.2.10. Heterologous Expression in Bacterial System

For the expression assay, 1% (v/v) of $MgCl_2$ (0.1 M) was added to 50 mL of LB medium, as well as (34 µg/mL) of kanamycin, and 1 mL of grown bacterial culture medium. This culture volume was incubated at 30 °C, 200 rpm until it reached OD from 0.5 at 600 nm. Expression induction was performed by using 1 mM of IPTG (isopropyl-β-D-thiogalactopyranose), incubating the culture medium at 30 °C for 18 h under 200 rpm of agitation.

3.2.11. Screening of the Transformants *E. coli* BL21 (DE3) Rosetta Cells Harbouring pET-28a (+)/asp_pseudopedo Construct

Three randomly selected single colonies from transformant cells, assumed to carry the pET-28a (+)/asp_pseudopedo construct, were transferred separately to 15 mL of LB/kanamycin in 50 mL falcon tubes. The inoculated broths were incubated for 16 h at 37 ºC and 200 rpm agitation speed. The next day, plasmid mini-prep was conducted from the three cultures by using the GeneJET plasmid mini-prep kit (Thermo Fisher "Co.," USA).

Each plasmid isolated from a pure culture originating from a single colony was subjected to restriction digestion using MluI and XhoI restriction digestion and PCR for amplification of the insert (L-asparaginase type I gene). The isolated plasmids were used as PCR templates to prove the presence of the L-asparaginase type I gene on the pET28a (+) vector, using the vector universal primer set T7 promoter and T7 terminator.

3.2.11.1. Screening Using Double Digestion with MluI and XhoI

The double digestion reaction was performed as follows: 1 µg of plasmid, 4 U of MluI, 4 U of XhoI, and 5 µL of (10X restriction digestion buffer) as shown in Table 3.5. The restriction digestion reaction was incubated overnight at 37°C. The next day, an aliquot of the restriction digestion reaction was applied to 1% agarose gel electrophoresis.

Table 3. 5: Recipe of restriction double digestion of pET-28a (+)/asp_pseudopedo construct with MluI and XhoI restriction enzymes.

Component	Volume (µL)
pET-28a (+)/asp_pseudopedo construct (30µg):	40.0
MluI (4 Units):	2.0
XhoI (4 Units):	2.0
rCutSmart®Buffer (10X):	5.0
BSA (100 ng):	1.0
Total volume:	50.0

3.2.11.2. Screening PCR of Transformed Colonies for Cloning Diagnosis By Using T7 Promoter and T7 Terminator Primer

The isolated DNA plasmids were used as PCR templates to prove the presence of the L-asparaginase type I gene from *Pseudopedobacter saltans* on the pET-28a (+) vector by PCR reaction was performed with the universal primer set of the pET-28a (+) vector T7 promoter/T7 terminator. The sequence of the universal primer set T7 promoter/T7 terminator was as follows: T7 promoter (5'-TAATACGACTCACTATAG-3'), T7 terminator (5'-TAGTTATTGCTCAGCGGTGG -3'). The recipe for the PCR reaction was outlined in Table 3.6. After PCR termination, aliquots of the PCR products were withdrawn to be checked on 1% agarose gel electrophoresis and visualized under UV-transilluminator.

Table 3. 6: PCR recipe and conditions for screening the colonies harboring the right pET-28a (+)/asp_pseudopedo construct using the universal T7 promoter/T7 terminator primer set.

<table>
<tr><th>PCR component</th><th>Volume (µL)</th><th>PCR conditions</th></tr>
<tr><td>Plasmid DNA from a white clone (50 ng):</td><td>5.0</td><td rowspan="8">Initial denaturation (1 cycle):
95 ℃, 5 min.
Amplification (16 cycles):
Each segment has:
➢ Denaturation: 98 ℃, 20 s
➢ Annealing: 69 ℃, 30 s
➢ Extension: 72 ℃, 30 s
Then amplification for 6 cycles
Each segment has:
➢ Denaturation: 94 ℃, 20 s
➢ Annealing: 58 ℃, 30 s
➢ Extension: 72 ℃, 30 s
Final extension (1 cycle):
72 ºC, 10 min</td></tr>
<tr><td>T7 promoter primer (0.5 µM):</td><td>2.0</td></tr>
<tr><td>T7 terminator primer (0.5 µM):</td><td>2.0</td></tr>
<tr><td>MyTaqTM Mix (2X) (Master mix):</td><td>25.0</td></tr>
<tr><td>Nuclease free water:</td><td>16.0</td></tr>
<tr><td>Total volume:</td><td>50.0</td></tr>
</table>

3.2.12. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed according to Sambrook's protocol [70]. Briefly, 1.0 g of agarose was added to 100 mL of TAE buffer (1X). The mixture was heated to completely melt the agarose mixture. Then, ethidium bromide (see 3.2.2. section) was added to the melted agarose at a final concentration of 0.5 µg/mL. The melted agarose was poured into the casting tray of the submarine gel electrophoresis unit (Cleaver Scientific Co., UK),

followed by comb insertion to create wells in the solidified gel. After gel solidification, the casting tray was immersed in the submarine tank. The TAE buffer (1X) was added until the gel was submerged under the surface of the buffer completely. Each DNA sample was mixed with 1X gel loading dye before loading onto the gel. The electrophoresis process was conducted at 5-8 voltage/cm between the anode and the cathode for 45 min. After the termination of electrophoresis, the agarose gel was visualized under ultra-violet (UV) light using a UV-transilluminator (Cleaver Scientific Co., UK).

3.2.13. Cells Harvest and Disruption

Cell lysis was carried out mechanically through sonication (amplitude 50%, cycle 1) using an ultrasonic processor UP50H from Hielscher Ultrasonics GMBH©. About 10 ml of lysis buffer (50 mM Tris and 300 mM NaCl containing 20 mM Imidazole (pH 7.6)), 20 µl Triton X100 and 100 µl PMSF (0.03 mM) were added to the pellet. *E. coli* was transformed with pET28a (+)/asp_pseudopedo, which was gently resuspended and rested in an ice bath throughout the procedure. The sample proceeded to the sonication step, with cycles of 30 seconds and 20 repetitions, until a homogeneous cell mixture was obtained. The cell lysate was centrifuged at 10,000 x g for 30 min at 4 °C to separate the cell debris and rescue the protein extract (soluble fraction), and a 100 µL aliquot of the lysate was centrifuged separately, then stored at -20 ºC.

3.2.14. Purification of L- asparaginase Protein by Affinity Chromatography for Immobilized Metals (IMAC)

For the purification process of recombinant L-asparaginase, 3 wash buffers (300 mM NaCl, Glycerol 5%, and 50 mM Tris containing 25-, 50-, and 100-mM imidazole) and an elution buffer (300 mM NaCl, Glycerol 5%, and 50-mM Tris and 250 mM imidazole) were prepared, both at pH 8.0. Approximately 2 mL of the HisTrapTM HP column, which is composed of agarose conjugated with nickel ion (Ni+), was washed with 5 mL of Milli Q water to remove the residual alcohol in which it is stored before being equilibrated with lysis buffer and incubated with approximately 10 mL of protein extract for 1 h in an ice bath with orbital shaking at 100 rpm. The volume was then placed on plastic support, and the chromatographic process began with approximately 35 mL of washing buffers in a crescent gradient of 25, 50, and 100 mM imidazole, followed by elution with 250 mM imidazole, where approximately 7 mL of purified fraction were rescued and stored at 4 °C until

application in polyacrylamide gel electrophoresis. The elution fraction in buffer containing 300 mM imidazole was dialyzed in 50 mM Tris buffer and 300 mM NaCl (pH 8.0) and concentrated into approximately 5 mL of fraction, with the aid of Vivaspin (GE Healthcare®) with a 10 kDa membrane, and stored at -80 °C.

3.2.15. Polyacrylamide Gel Electrophoresis (SDS-PAGE analysis)

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to Laemmli, 1970 [71]:

- 30% acrylamide-bisacrylamide solution was prepared by dissolving 29.2 g of acrylamide (BDH) in 70 ml of Distal Water with 0.8 g of bisacrylamide and completing the volume to 100 ml with DW. then filtered through a Millipore filter (0.45 µm) and stored in a sterilized opaque container for at least 30 days.
- Lower buffer (Tris –HCl buffer 1.5 M), pH =8.8; prepared by dissolving 17.15 g Tris-base in 80 ml, pH was adjusted to 8.8, then the volume was completed to 100 ml distal water and sterilized by filtration and stored at 4 °C.
- Upper buffer (Tris –HCl buffer 0.5 M), pH =6.8; prepared by dissolving 0.7 g Tris-base in 60 ml DW. pH was adjusted to 6.8, and the volume was completed to 100 ml via DW, is then filtrated and stored at 4°C.
- 10% sodium –dodecyl sulfate (SDS); prepared by dissolving 10 g SDS (BDH) in DW and mixed vigorously and completed the volume to 100 ml via distilled H_2O.
- 10% ammonium persulphate (APS); prepared by dissolving 10 g APS in distilled H_2O and mixed vigorously and completed the volume to 100 ml via DW.
- SAB; (1% SDS+10% β- mercapto ethanol + 10% glycerol + 0.05% bromophenol blue in 0.6 Tris, pH 6.8).
- 10X Running buffer electrode prepared by dissolving 6 g of Tris-base with 28,8 g Glycine and 2 g SDS by 200 ml of distilled water, then every 25 ml from running buffer complete to 250 ml of distal water for work with Electrode unit, stored at 4 °C.
- Staining solution prepared by dissolving; (Coomassie brilliant blue R-250,1.25 g; Absolut methanol, 227 ml; Glacial acetic acid,46 ml).

- Distaining solution prepared by mixing;(Methanol, 300 ml; Glacial acetic acid, 100 ml; Distilled water, 600 ml).
- Running gel; 10 ml of 12% resolving gel was firstly prepared using the following components: (3.3 ml distilled water; 4.0 ml Acrylamide/ bisacrylamide (30%); 2.5 ml Tris-HCl buffer (1.5 M, pH 8.8); 0.1 ml Sodium dodecyl sulphate (SDS 10%);0.1 ml Ammonium persulphnate (APS) 10%; 0.006 ml Tetramethylethylenediamine (TEMED).

Pooled purified plasmid pET-28a (+)/asp_pseudopedo (5 mg/ml) was mixed with 3X sample application buffer (SAB), then boiled for 3 min before loading into the wells. The dye was passed through the stacking gel at 15 mA, then the current was increased to 30 mA and 120 volts until it was ready to the bottom of the resolving gel. The gel was then removed and stained overnight using a Coomassie Brilliant Blue solution. Destaining was performed using a destaining solution.

3.2.16. Determination the Concentration of Recombinant Protein

According to the Bradford method, the concentration of the protein is determined by using BSA as a standard at a concentration of 0.5 µg/mL., using Coomassie-brilliant blue G250 and bovine serum albumin (BSA) as the standard protein [72]. Mention the Figure 3.2.

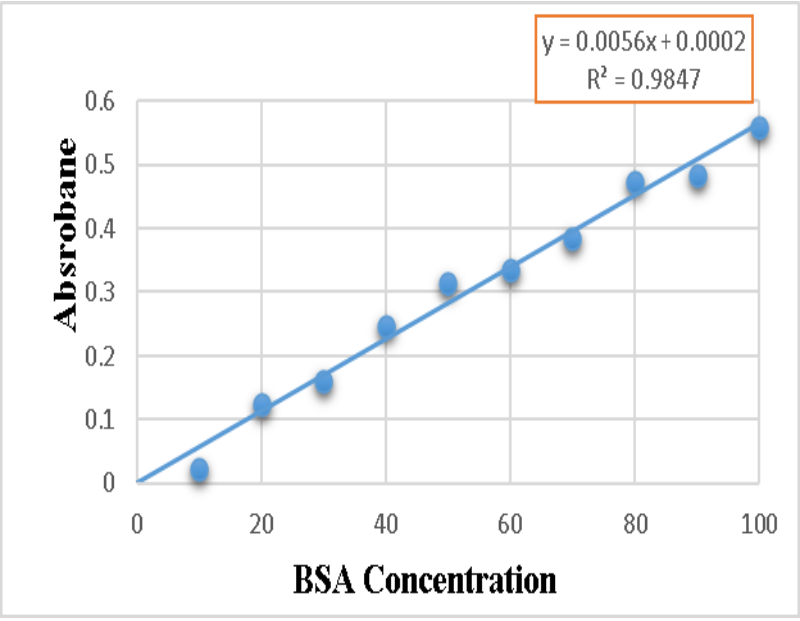

Figure 3. 2: Standard Curve of Bovine Serum Albumin Concentration, Absorbance at 595 nm.

3.2.17. Determination Of L-asparaginase Activity By Modified Ezapelc's Dox Broth

Modified ezapelc's dox broth method is used for enrichment of bacteria producing L-asparaginase enzyme. The media was supplemented with 0.01 M L-asparagine as substrate and phenol red as an indicator. When L-asparaginase acts on L-asparagine, ammonia is liberated, leading to the conversion of the yellow color of phenol red to pink in alkaline conditions. Ezapelc's dox media is composed of the following (in g/L): (di-potassium1; potassium dihydrogen phosphate 0.5; magnesium sulfate heptahydrate 0.5; sodium chloride 5; L-asparaginase 3; glucose 10), pH 6.8 at 25 ^{o}C, phenol red 0.0094, and sterilized by autoclaving at 121 ^{o}C for 20 min [36].

3.2.18. Effect of pH Variation on Enzyme Activity

The evaluation of the influence caused by pH variation on the activity of the asp_pseudopedo enzyme was carried out based on the colorimetric method of Nesslerization, using different buffers, which ranged from pH 3 to 11. Citrate buffer (0.2 mM) phosphate (0.1 mM) covered pH 3 to 6, Tris buffer (50 mM) pH 7 to 9, and Glycine buffer pH (10 and 11). The assay followed the enzymatic activity protocols [73].

3.2.19. Effect of Temperature Variation on Enzyme Activity

The assay determines the optimum temperature for the enzymatic activity of asp_pseudopedo, with a temperature elevation ramp ranging from 20 °C to 90 °C.

3.2.20. Enzyme Kinetics

To assess the kinetic mechanism of the asp_pseudopedo enzyme, with gradual concentrations of L-asparagine, whose variation ranged from 62.5 µM to 9.91 mM, were used for the construction of a curve with 10 points. Each point on the curve consisted of triplicates. The obtained data were used in the SigrafW program by Leone et al. (2005) to determine the kinetic parameters.

3.2.21. Silico Analysing the Sequence and Phylogenetic

The sequences of the nucleotides were gained for analysis and compared against the sequence database by using BLAST, obtained by the NCBI [93]. The sequence of

Pseudopedobacter saltans L-asparaginase amino acid was obtained by translating the nucleotide sequence using the translation tool on the ExPASy server [94].

Therefore, by using the Phylogeny.fr Software [91], the tree of the phylogenetic *Pseudopedobacter saltans* L-asparaginase gene was created [30]. The prediction of secondary structure was performed according to the SAS online program (sequence annotated by structure) (https://www.ebi.ac.uk/thornton-srv/databases/sas/).

Then, the dimensional structure expectation was done by submitting the sequence of the protein to the SWISS-MODEL server to obtain the data, and the 3D structural prediction was analyzed by using the PDB viewer program. The molecular mass and theoretical (pI) values of the *Pseudopedobacter saltans* L-asparaginase were expected [74] by using the ProtParam tool [95].

4. RESULTS

4.1. Sequence Of pET-28a (+)/asp_pseudopedo Construct

The full sequence of the pET-28a (+)/asp_pseudopedo construct is shown below in FASTA format program in Figure 4.1. Moreover, the vector construct was visualized by the SnapGene program as shown in Figures 4.2.

←aagaaataacgccggaacattagtgcaggcagcttccacagcaatggcatcctggtcatccagcggatagttaatgatcagcccac
tgacgcgttgcgcgagaagattgtgcaccgccgctttacaggcttcgacgccgcttcgttctaccatcgacaccaccacgctggcaccc
agttgatcggcgcgagatttaatcgccgcgacaatttgcgacggcgcgtgcagggccagactggaggtggcaacgccaatcagcaa
cgactgtttgcccgccagttgttgtgccacgcggttgggaatgtaattcagctccgccatcgccgcttccacttttcccgcgttttcgcag
aaacgtggctggcctggttcaccacgcgggaaacggtctgataagagacaccggcatactctgcgacatcgtataacgttactggtttc
acattcaccaccctgaattgactctcttccgggcgctatcatgccataccgcgaaaggttttgcgccattcgatggtgtccgggatctcga
cgctctcccttatgcgactcctgcattaggaagcagcccagtagtaggttgaggccgttgagcaccgccgccgcaaggaatggtgcat
gcaaggagatggcgcccaacagtcccccggccacggggcctgccaccatacccacgccgaaacaagcgctcatgagcccgaagt
ggcgagcccgatcttccccatcggtgatgtcggcgatataggcgccagcaaccgcacctgtggcgccggtgatgccggccacgatg
cgtccggcgtagaggatcgagatctcgatcccgcgaaattaatacgactcactataggggaattgtgagcggataacaatttcccctcta
gaaataatttgtttaactttaagaaggagatataccATGACAAAAATTTTTGTAATTTATACAGGTGG
AACGATTGGTATGATTACCGATCCAGAAACGGGTGCTTTAAAGCCGTTTACC
TTCGAACAGATAGAAGAAAATGTTCCCGAATTGAAACGGATGAATTGCAAAT
TAACTATTCATTCTTTTGATCCGATTATAGATTCTTCTAATATGACTCCTGAT
ATTTGGGCATATCTGGCTAAGTTAATAGAGGATAATTACGAAGATCATGATG
GATTCTTAATTCTGCATGGCTCCGATACGATGGCTTTTACCGCATCTGCATT
GAGTTTCATGTTAGAAGGATTAACCAAGCCGGTAATCTTCACAGGTTCGCAA
TTGCCAATATCTGAAGTAAGGACAGATGCAAAAGAAAATTTTATTACAGCTT
TAGAAATTGCATCGGCTAAGCTTGATGGCCGGGCTCGTGTGCCCGAAGTTTG
TATCTATTTCGAAAATAAGCTGTATAGAGGAAATCGTACATTCAAATATAATT
CAGCTAAGTTCGAGGCATTCCGATCTCCAAACTATCCGGTTCTGGTAGAGGC
AGGTGTGCATATTAAATATAATGATGCCGCTATTGGAAAATGTGAAAATTGT
ATATTGAAAGTACATTCTAACGTTAACAATAATATTGGTGTTTTGAAACTCTA
TCCGGGAATAAGCCCGGAAATTGTTGATGTTGTTTTAAATTCAGATGCTGAG
GCGATTATTATGGAAAGTTTTGGTACCGGGAACACAACAACAGCAAAGTGGT
TTTTGGATAAGTTAGAATCAGCAATAAAAAGAGGTAAGATTATTCTGGATAT
TTCCCAATGTAAAGTTGGGTCGGTTGAATTGGGTAGATATGAAACTAGCAGC
GAGCTGTTGCGGATGGGCGTCGTGAACGGTTACGACATGACTTTTGAGGCT
GCGGTTACCAAATTGATGTATTTGCTGGGAAGAAAATATCCAAAAGACAGAT
TGTTGAGAAGGTTGTCGACCTCAATTAGAGGTGAGCTTACTAAGCATctcgagca
ccaccaccaccaccactgagatccggctgctaacaaagcccgaaaggaagctgagttggctgctgccaccgctgagcaataactagc
ataaccccttggggcctctaaacgggtcttgaggggttttttgctgaaaggaggaactatatccggat

Figure 4. 1: The entire pET-28a (+)/asp_pseudopedo sequence is contained in a fasta file. The sequence in small letters is the pET-28a (+) vector. The sequence in bold capital letters is the L-asparaginase type I gene. The sequence highlighted in green is the start codon of the asparaginase type I gene. The sequence highlighted in yellow is 6: His tags. The sequence highlighted in violet is the stop codon. The sequences highlighted in faint blue and dark blue are the recognition sites for MluI and XhoI, respectively.

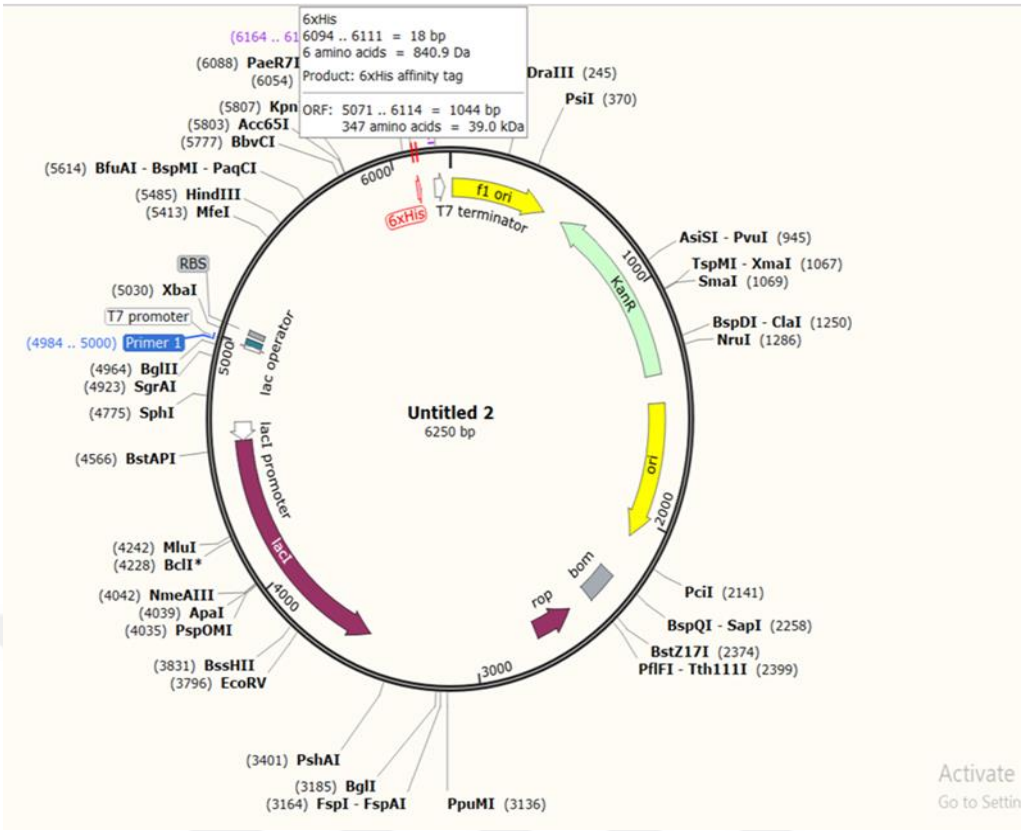

Figure 4. 2: The map of the recombinant construct pET-28a (+)/asp_pseudopedo, synthesized by GenScript Co., and generated by Snap Gene software, The full length of the recombinant plasmid after insertion of the asparaginase type I gene is 6,250 bp.

The full length (6250 bp) as demonstrated in Figure 4.2 and 4.3, the expected recombinant protein L-asparaginase type I should have a molecular weight of 1,058 bp and a protein level of 37.8 kDa in terms of nucleotide level and protein level, respectively. The length of the L-asparaginase type I gene is 1019 bp. The 6-His tag would introduce an extra 6 amino acids to the recombinant L-asparaginase type I protein. Moreover, the recognition sites of XhoI and MluI would introduce four extra amino acids. After omission of the stop codon, an extra three amino acids, thus, 13 extra amino acids would be introduced to the recombinant L-asparaginase type I protein, resulting in the expression of a recombinant L-asparaginase type I protein with 339 amino acids. The final recombinant protein would have a molecular weight of 352 amino acids. The expected theoretical molecular weight of the recombinant protein is 37.8 kDa.

4.2. Screening of the Transformants *E. coli* BL21 (DE3) Rosetta Cells Harboring pET28a (+)/asp_pseudopedo Construct

4.2.1. The Pattern of Double Restriction Digestion of pET-28a (+)/asp_pseudopedo Construct with MluI and XhoI

The double restriction digestion pattern of pET-28a (+)/asp_pseudopedo had displayed in Figure 4.3. The obtained pattern was two bands:

- The upper band represents the plasmid vector recognition site by MluI around (4,200 bp) before the insert of the L-asparaginase type I gene.
- The lower band represents the insert of the L-asparaginase type I gene from *Pseudopedobacter saltans*'s recognition site by XhoI around (1,999bp) and some parts of the vector itself.

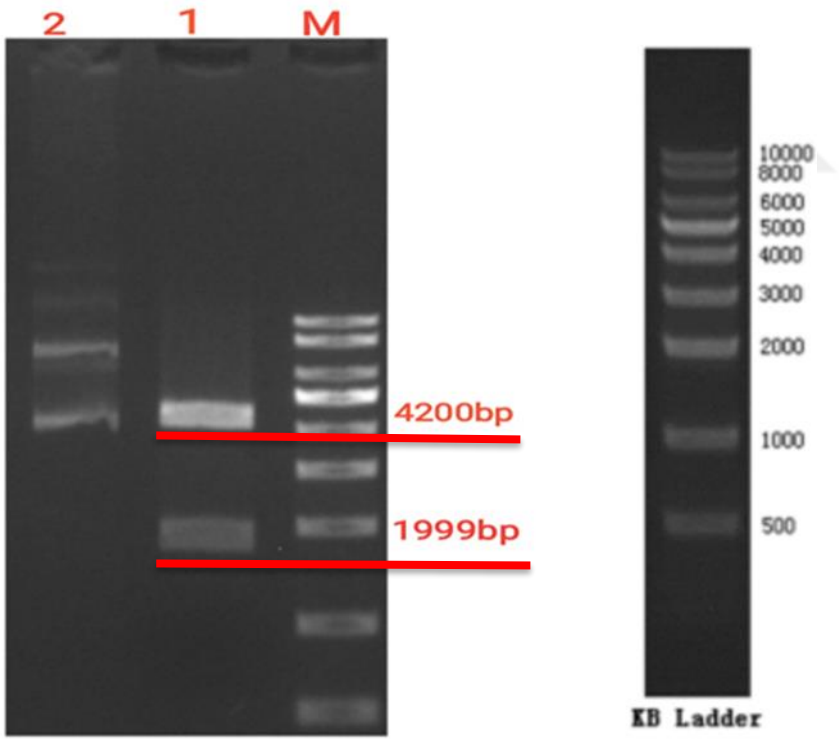

Figure 4. 3: Agarose gel electrophoresis (1%) shows a pattern of restriction digestion of pET-28a(+)/asp_pseudopedo with MluI and XhoI. The upper band is 4200 bp of the plasmid vector. The lower band: 1999 bp, including the inserted L-asparaginase type I gene. M is a 1 kbp DNA ladder.

4.2.2. The pattern of PCR Amplification of the Insert Using pET-28a (+)/asp_pseudopedo Construct as a Template and T7 Promoter and T7 Terminator Primer Set

The expected size of the amplified genomic PCR product (1,500 bp) includes the *Pseudopedobacter saltans* L-asparaginase type I gene (1,019 bp). However, the extra length in the PCR product came from the vector itself, as long as the universal primer set T7 promoter and T7 terminator were distal from the beginning and end of the insert (L-asparaginase gene type I from *Pseudopedobacter saltans*). Therefore, it is expected that the amplified genomic product has a size slightly larger than the L-asparaginase I gene of approximately 1,500 bp as shown in Figure 4.4. The remaining extra nucleotide was from the vector itself, after genomic sequencing, the correct nucleotide sequence was confirmed.

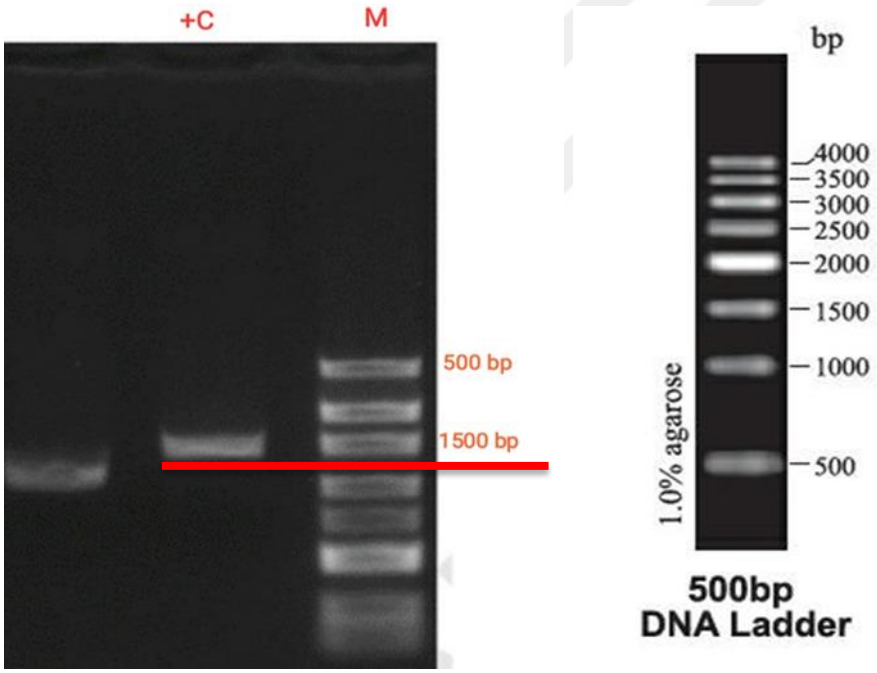

Figure 4. 4: This is a 1% agarose gel electrophoresis showing the PCR product resulting from using the universal primer vector set T7 promoter and T7 terminator and the pET-28a (+)/asp_pseudopedo construct as a template. 1% agarose gel demonstrating a positive colony for recombinant plasmid insertion pET-28a (+)/asp_pseudopedo; MM: molecular marker (500bp DNA ladder Promega®); and +C; pET-28a (+)/asp_pseudopedo]

4.2.3. SDS-PAGE Analysis of Heterologous Expression in Prokaryotic System and Purification of pET-28a (+)/asp_pseudopedo

The recombinant protein of the L-asparaginase I gene from the *Pseudopedobacter saltans* was expressed in the *E. coli* BL21 (DE3) Rosetta Strain; the host strain detects an able level of gene expression was achieved upon induction with one mM of IPTG for 18 h at 30 °C. The protein extract (soluble fraction) of the recombinant protein was purified with IMAC chromatography and then analyzed by SDS-PAGE to assess the solubility of the protein as well as to determine the molecular weight (MW) of the recombinant protein. The predictable MW of the protein L-asparaginase enzyme is 37.8 kDa. The SDS-PAGE analysis of purified L-asparaginase showed that a band with a corresponding molecular weight of about 36.0 kDa and the presence of L-asparaginase protein was confirmed by SDS-PAGE as shown in Figure 4.5.

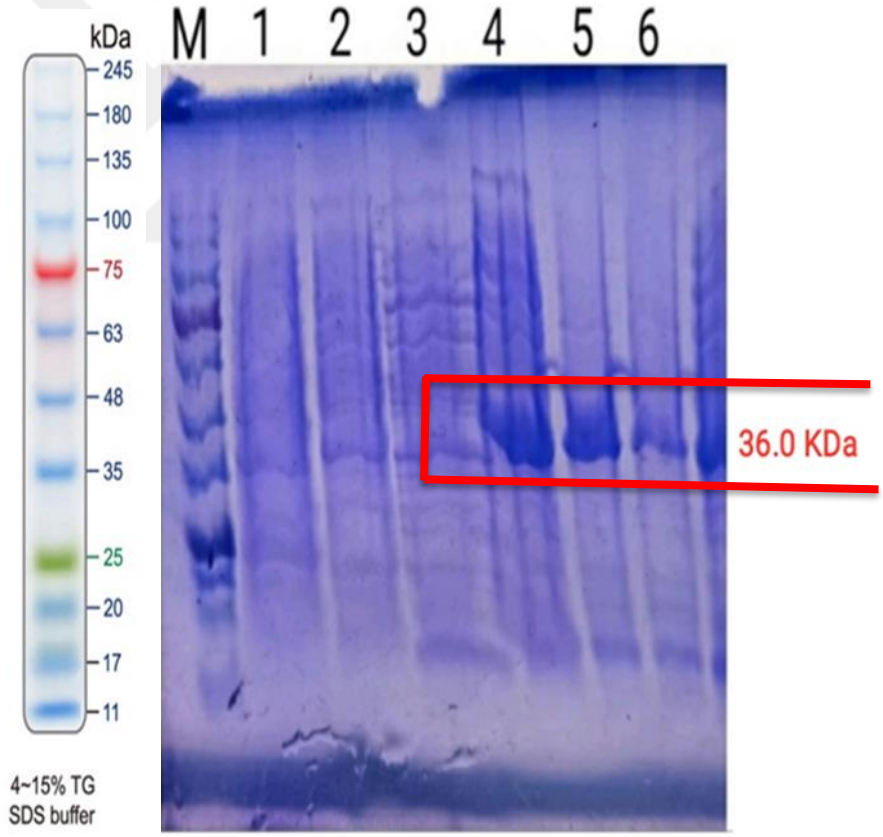

Figure 4. 5: SDS-PAGE (10%) showing M: protein ladder; the lanes (4; 5; 6) are the expression of recombinant L-ASP soluble fraction proteins of induced *E. coli* BL21 (DE3) Rosetta harbouring asp_pseudopedo, using 1 mM IPTG; and the lanes (1; 2; 3) represent the insoluble fraction proteins of uninduced E. coli BL21 (DE3) Rosetta asp_pseudopedo.

4.3. Determination of Recombinant Strain for L-asparaginase Production

The results, as shown in Figure 4.6, indicated that all of the colonies tested on modified ezapelc's dox media were able to produce L-asparaginase enzyme at varying levels, by the change in media color from yellow to pink, and that the most promising L-asparaginase producer was chosen from the colonies.

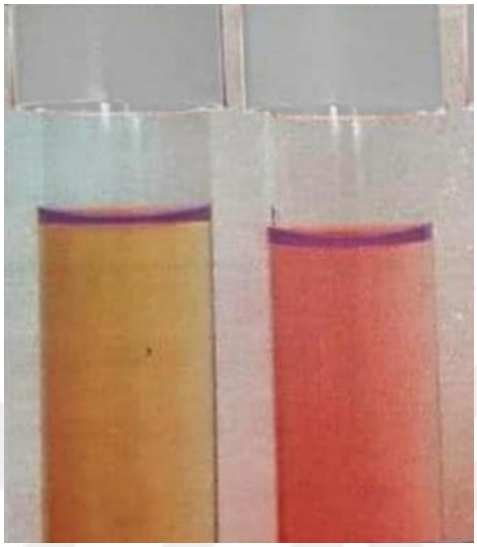

Figure 4. 6: The screening of L-asparaginase shows pink color modified ezapelc's dox media supplemented with phenol red; A/ yellow media devoid of L-asparaginase. Ammonium formation causes the pink color of phenol red in the basic medium.

4.4. Effect of pH and Temperature on Enzyme Activity

The range of the pH and the temperature at which a protein is exposed are important factors in its catalytic activity. The amino acid side chains, both from the active site and from other locations of the protein, which play an important role in maintaining the active pocket or its structure, can function as weak acids or bases for the conservation of a certain state of ionization [75]. Minimal temperature variations affect the entropy of a chemical system, such as in aqueous reaction systems when under atmospheric pressure (1 atm). Reactions involving enzymes are especially sensitive to such environmental disorders when considering characteristics such as binding energy between the atoms of the amino acids that compose them, so it would influence their catalytic efficiency [76]. In Figure 4.7, asp_pseudopedo showed maximum catalytic activity at pH 8.0, diverging from the results obtained by Vidya et al. (2011) [77], who established an optimal pH of 6.0, and Khushoo et al. (2004) around 7.0. Such disagreements can be explained by the influence of the buffers

used in the assays, and the method of measuring the enzymatic activity, the presence of modifications and tags in the proteins [78].

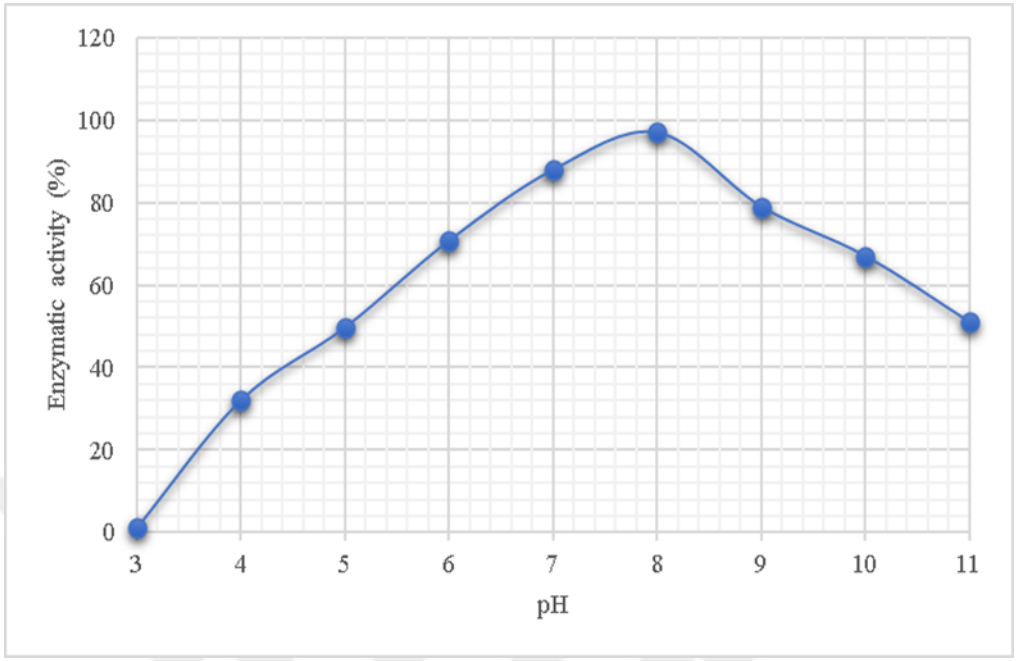

Figure 4. 7: asp_pseudopedo, enzymatic activity at different pH

Regarding the influence of temperature on the activity of asp_pseudopedo, an optimal temperature of 60 °C was obtained, as shown in Figure 4.8. This result diverges from Vidya et al. (2011), which reached an optimal temperature of 40 °C, possibly due to the factors already cited [77].

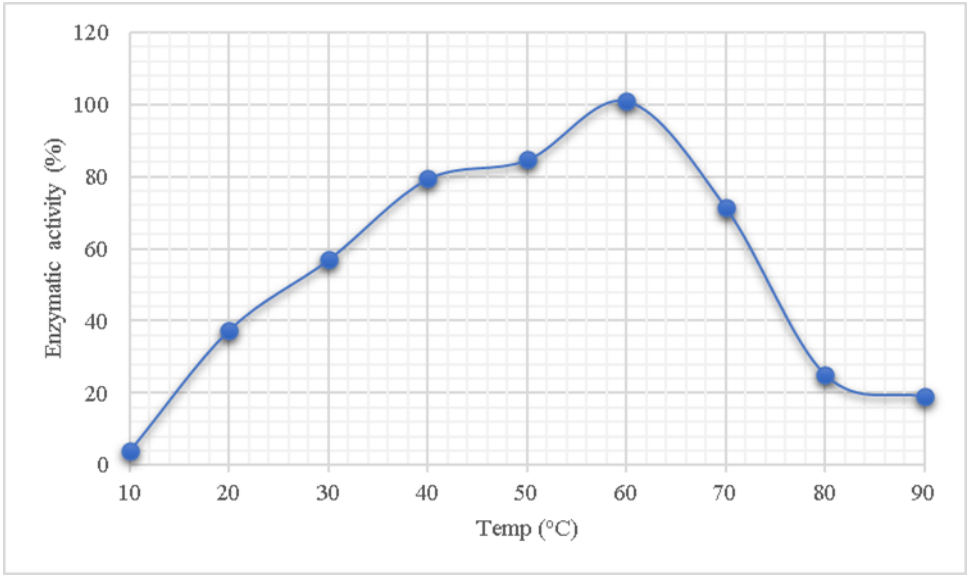

Figure 4. 8: asp_pseudopedo, enzymatic activity at different temperatures.

4.5. Enzyme Kinetics

The kinetic curve assay aims to evaluate the hydrolytic capacity of the enzyme against different substrate concentrations up to the complete saturation point of the catalytic sites, under optimum pH and fixed temperature. It is known that the K_m of a given substrate is its concentration in which the initial enzymatic reaction speed is equivalent to half of the maximum speed [75]. According to Segel (1979), this concept can also be applied to compare the catalytic efficiency between two enzymes, where the greater the K_m, the lower the affinity of the enzyme for the substrate [79]. As shown in Figure 4.9, asp_pseudopedo reaches the plateau at 8 mM L-asparagine, at which point it is indicated that all enzyme catalytic pockets are saturated by the substrate. Thus, the kinetic calculations were calculated using the Sigraf software and indicated a K_m value equal to 3 mM and a V_{max} of 168.2 µmol/min/mg, approaching that shown by Willis & Woolfolk (1974) [80].

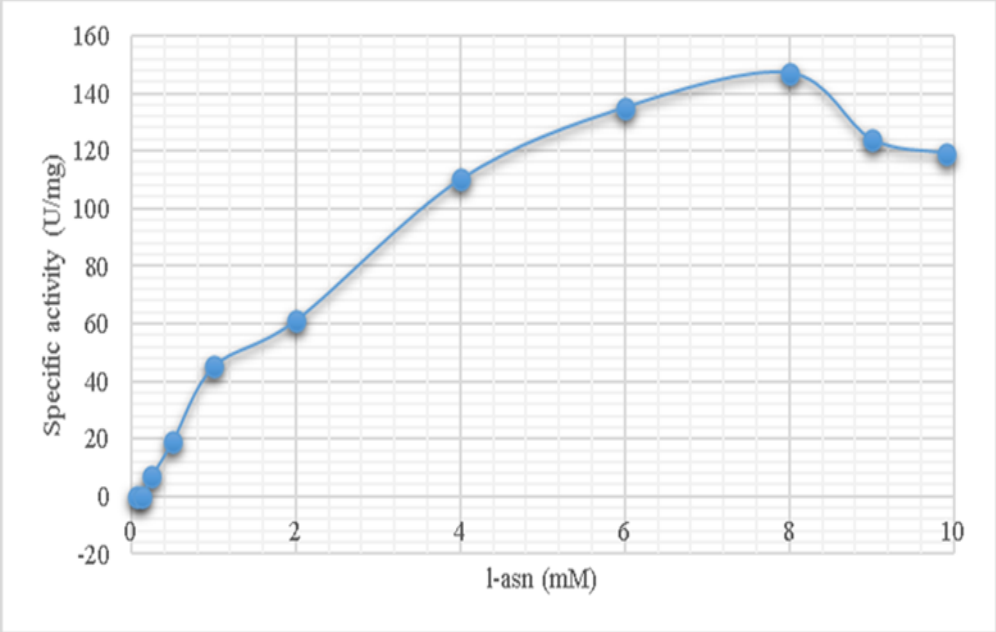

Figure 4. 9: Kinetic curve of asp_pseudopedo, enzyme.

4.6. In Silico Sequence and Phylogenetic Analysis

4.6.1. Phylogenetic Tree Analysis Of *Pseudopedobacter saltans*

Analysis of phylogenetic trees of *Pseudopedobacter saltans* by using the neighbor rejoining approach to obtain the genetic algorithms based on evolutionary distances was estimated from nucleotides and amino acid sequences of *Pseudopedobacter saltans* L-asparaginase I

The phylogenetic relationship of *Pseudopedobacter saltans* L. asparaginase I with other *Pseudopedobacter* species may be seen in the tree at the nucleotide and amino acid levels. Bacterial species shifted to separate clusters for the L-asparaginase gene at both nucleotide and amino acid levels (Fig 4.10), indicating that the organisms had diverged [74].

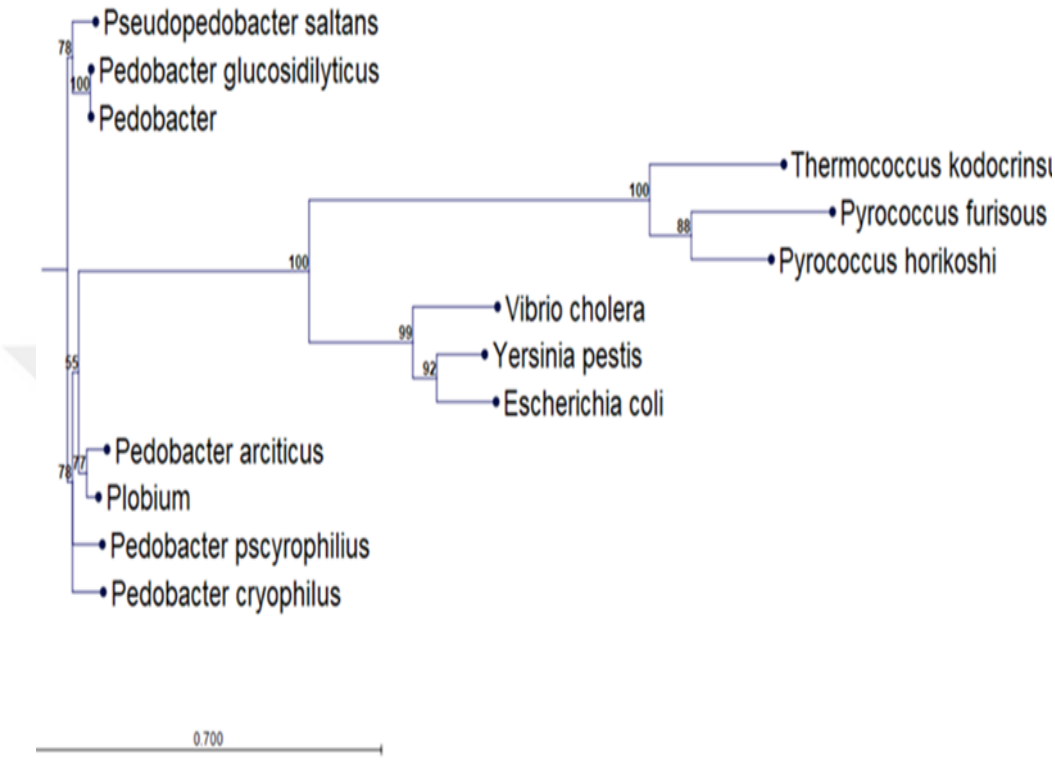

Figure 4. 10: The Phylogenetic Tree of *Pseudopedobacter saltans* by using the Phylogeny.fr Software (http://www.Phylogeny.fr).

4.6.2. Annotation of the *Pseudopedobacter saltans* Structure and 3D Structure Prediction

The secondary structure of the L-asparaginase type I gene was predicted by the SAS online program. The output of the SAS program revealed that the L-asparaginase type I from *Pseudopedobacter saltans* showed a similar sequence identity of 41.8% with the crystal structure of PDB (Protein Database Bank): 2OCD_A: *Vibrio cholerae* O1 biovar eltor str. N16961 L-asparaginase type I. The primary structure of *Pseudopedobacter saltans* L-asparaginase I and the protein module's secondary structure annotation expectations revealed some distinctive lineaments (for characteristic lineaments see Figure 4.11, 4.12, and 4.13); *Pseudopedobacter saltans* L-asparaginase I The unique signature of common conserved for microbial L. asparaginase is characterized by the invariant amino acid residues: Thr^{12}, Ala^{23},

Lys[25], Ser[88], Asp[89], Lys[165], Leu[291].The *Pseudopedobacter saltans* L-asparaginase I primary 2D structure involves a conserved catalytic residue to L. glutaminase I, the amino acid residues being Thr[12], Ala[23], Ser[88], Glu[286], Leu[291] . as shown in Figure 4.11 and Table 4.1.

Based on the amino acid composition, the predicted calculated pI for *Pseudopedobacter saltans* L-asparaginase was found to be 6.13 [74]. The result indicates that the *Pseudopedobacter saltans* L-asparaginase is quite similar to type I L-asparaginase from *E. coli*, which is distinctly specific for L-asparagine located in the cytosol and acts in solution as a homodimer. This homodimer is quite necessary for acting site formulation, and in turn, it is essential for catalysis.

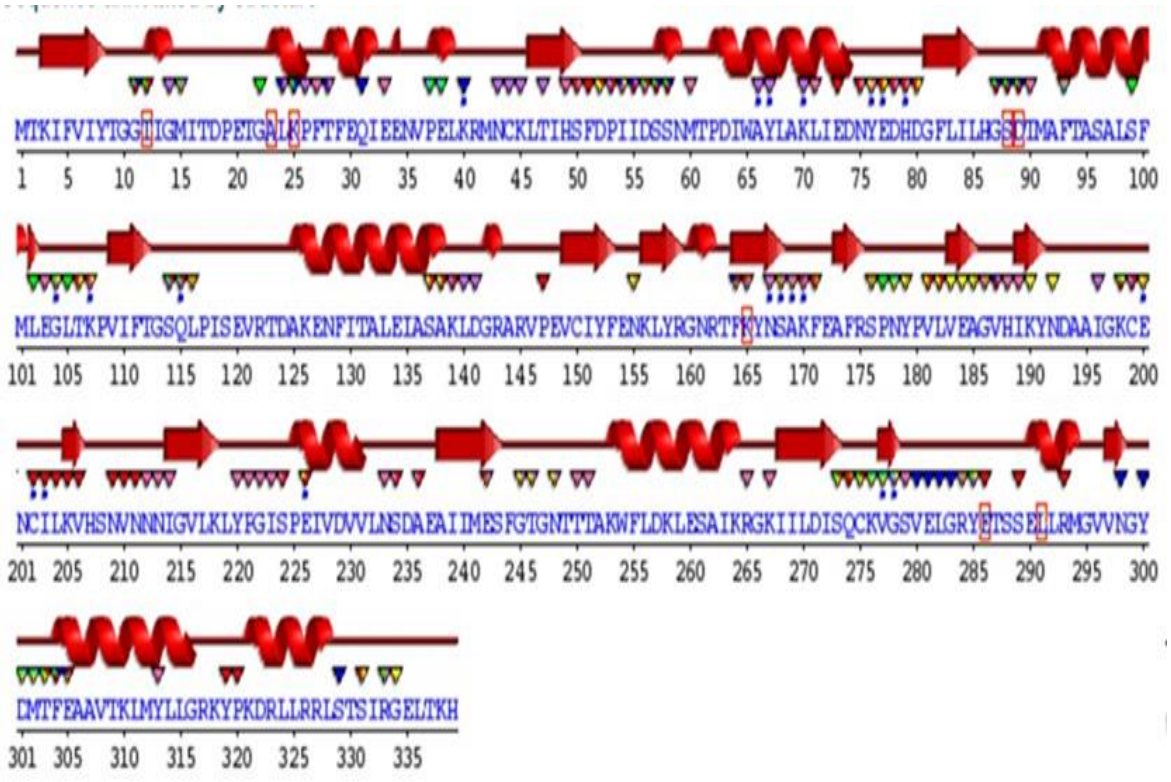

Figure 4. 11: Predicted Secondary structure of L-asparaginase type I from *Pseudopedobacter saltans* by SAS online program based on the template 2OCD_A: L-asparaginase type I from *Vibrio cholerae* O1 biovar eltor str. N16961. It consisted of 17 β-sheets and 15 α-helices. Amino acid residues highlighted in red rectangles refer to amino acid residues involved in the catalytic residues: Thre12, Ala23, Pro26, Ser88, Asp 89, Lys 165, Phe164, Glu286, and Leu291.

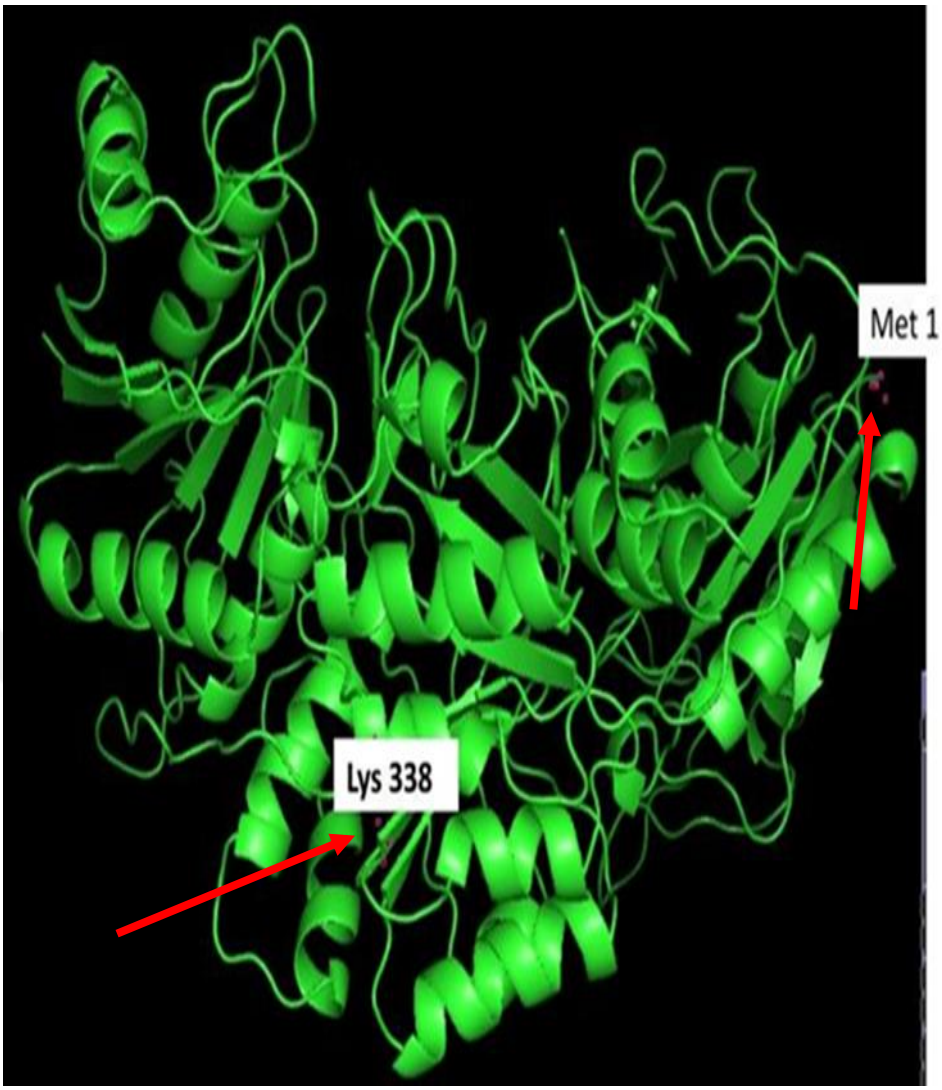

Figure 4. 12: Predicted three-dimensional structure of asparaginase from *Pseudopedobacter saltans* by SWISS-MODEl. The template used is the crystal structure of L-asparaginase from *Yersinia pestis* (3ntx.1. A) with a 44% sequence identity. The structure is homodimeric, which starts with Met 1 until it ends with Lys338.

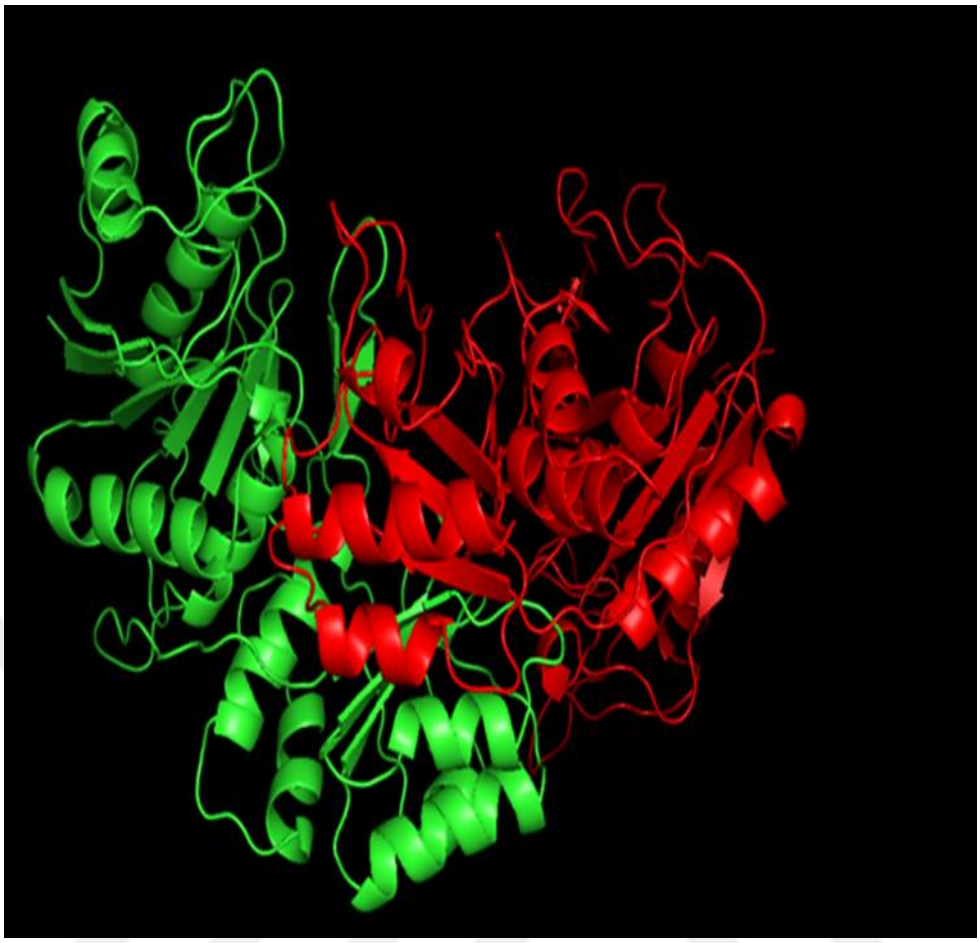

Figure 4. 13: Protein homodimer structure view of *Pseudopedobacter saltans* L-asparaginase I. the monomer in green color represents Methionine, and the monomer in red color represented the Lysin.

4.6.3. Translated Amino Acid Sequence of L-asparaginase from *Pseudopedobacter saltans* the Sequence is Retrieved From GenBank

MTKIFVIYTG GTIGMITDPE TGALKPFTFE QIEENVPELK RMNCKLTIHS FDPIIDSSNM TPDIWAYLAK LIEDNYEDHD GFLILHGSDT MAFTASALSF MLEGLTKPVI FTGSQLPISE VRTDAKENFI TALEIASAKL DGRARVPEVC IYFENKLYRG NRTFKYNSAK FEAFRSPNYP VLVEAGVHIK YNDAAIGKCE NCILKVHSNV NNNIGVLKLY PGISPEIVDV VLNSDAEAII MESFGTGNTT TAKWFLDKLE SAIKRGKIIL DISQCKVGSV ELGRYETSSE LLRMGVVNGY DMTFEAAVTK LMYLLGRKYP KDRLLRRLST SIRGELTKH

Table 4. 1: Preserved the *Pseudopedobacter saltans* L-asparaginase I of Amino Acid Residues That is Essential In Different Ligands and Metal Ions Binding

Annotation Features	Amino Acid Residues
Catalytic Residues; **Enzyme: 3.5.1.1 Asparaginase**	Thr12, Ala23, Lys25, Ser88, Asp89, Lys165.
Enzyme: 3.5.1.38 Glutamic-(asparagin-)ase.	Thr12, Ala23, Ser88, Glu286, Leu291
PDB SITE records:	
Site: AC1	Residues: Gly11, Thr12, Met15, Gly22, Lys25, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser99, Leu102, Gly104, Leu105, Ser114, Gln115, Pro177, Asn178, Lys198, Ser273, Cys275, Lys276, Val277, Tyr285, Asp301, Met302, Thr303, Phe304, Arg333.
Site: AC9	Residues: Gly11, Leu24, Lys25, Thr28, Gln31, Ile54, Ile55, Ser57, Ser58, Gly87, Ser88, Asp89, Phe164, Phe304, Glu305.
Site: AC5	Residues: Gly11, Thr12, Ser50, Asp56, Ser57, Ser58, Glu73, Asn75, Glu77, Asp78, His79, Asp80, Gly87, Ser88, Asp89, Thr106, Lys107, Ser114, Gln115, Phe164, Lys165, Lys170, Phe171, Leu204, Lys205, Val206, Asn209, Val210, Asn211, Glu242, Ser273, Gln274, Cys275, Thr303, Phe304, Glu305, Tyr319, Pro320, Ser331.
Site: BC8	Residues: Lys165, Ser168.
Site: BC5	Residues: Pro37, Glu38, Asn178, Lys276, Val277, Gly278, Asp301, Met302.
Site: BC9	Residues: Val187.
Site: CC1	Residues: Gly245, Thr246.
Site: AD8	Residues: Phe93, Asn248, Tyr285.
Site: AD4	Residues: Val187, Cys202, Ile203
Site: AC3	Residues: Asp52, Ile54, Tyr76, Glu77, Asp80, Gly104, Thr106, Lys107, Ser137, Ala138, Asn155, Asn178, Tyr179, Val181, Leu182, Val183, Glu184, Ala185, Lys190, Asn192, Lys198, Glu200, Glu226, Asn248, Ser273, Cys275, Lys276, Val277, Gly278, Tyr285, Asp301, Met302, Thr303, Ser331, Gly334.

Site: AC4	Residues: Gly14, Met15, Pro26, Asn43, Cys44, Lys45, Thr47, Pro53, Ile55, Ala66, Tyr67, Lys70, Gln115, Lys139, Leu140, Asp141, Lys165, Asn167, Ser168, Ala169, Lys170, Asn178, Ile196, Cys199, Glu242, Gly245, Thr246, Asn248, Ser273, Cys275, Lys276, Gly278, Arg284, Tyr285, Met302, Phe304.
Site: AC2	Residues: Leu24, Phe27, Thr28, Glu33, His49, Ser50, Ile54, Asp56, Ser58, Met60, Leu71, Asn75, Tyr76, Asp78, His79, Thr90, Glu103, Gly104, Lys107, Asn167, Ala169, Lys170, Asn178, Gly186, Val187, His188, Ile189, Asn212, Asn213, Ile214, Tyr220, Pro221, Gly222, Ile223, Ser224, Asn233, Ser234, Ala236, Thr251, Arg265, Lys267, Cys275, Lys276, Val277, Gly278, Ser279, Tyr285, Met302, Phe304, Tyr313, Arg333.
Site: AC6	Residues: Met15, Ser58, Gly104, Leu116, Asn155, Phe164, Lys165, Ser168, Ala169, Phe171, Ser176, Tyr179, Ala185, Gly186, Glu200, Glu242, Gly245, Thr246, Asn248, Ser273, Gln274, Cys275, Arg284, Tyr285, Thr303, Phe304, Glu305.
Site: AC7	Residues: Ser137, Ala138, Lys139, Pro147, Phe164, Val181, Leu182, Lys198, Cys199, Glu226, Glu242, Ser273, Gln274, Cys275, Glu286, Ser289, Arg293, Thr303, Phe304, Glu305.
Site: AE2	Residues: Gly245, Thr246, Asn248.
Site: AD3	Residues: Lys40, Tyr285.
Site: BC2	Residues: Gly11, Ser58.
Site: BC1	Residues: Asp56, Ser57, Gly87, Ser88, Asp89, Ser114.
Site: DC1	Residues: Phe93.
Site: BC3	Residues: Phe164, Glu242, Ser273, Gln274, Cys275, Thr303, Phe304, Glu305.
Site: BC4	Residues: Asn178, Lys276, Gly278, Asp301, Met302.
Site: CC7	Residues: Gly245, Thr246.
Site: CC3	Residues: Asn248.
Site: AD7	Residues: Pro37, Glu38.
Site: AF1	Residues: Phe93.
Site: AD5	Residues: Phe164, Glu242, Ser273, Gln274, Cys275, Thr303, Phe304, Glu305.
Site: AD2	Residues: Asn167, Ser168, Ala169, Lys170.
Site: AD6	Residues: Asn178, Lys276.

Site: AE7	Residues: Gly245, Thr246, Asn248
Site: AD1	Residues: Val277, Gly278, Val280, Glu281, Leu282, Gly283, Asn298, Tyr300, Ser329.
Site: CC2	Residues: Phe93.
Site: AC8	Residues: Phe51, Asp52, Pro53, Ile55, Asn178, Ser224, Glu226, Ser234, Ala236, Lys276, Gly278, Asp301, Met302.
Site: BC7	Residues: Asn248.
Site: AE3	Residues: Phe93.
Contact(s) to ligands:	
Ligand: ASP	Residues: Gly11, Thr12, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Gln115, Asn248, Tyr285
Ligand: ASN	Residues: Gly11, Thr12, Met15, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Gln115, Phe164, Glu242, Asn248, Ser273, Gln274, Cys275, Tyr285, Thr303, Phe304, Glu305.
-Ligand: CIT - Citric acid	Residues: Gly11, Thr12, Met15, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Asn248.
Ligand: EPE- 4-(2-Hydroxyethyl)-1-Piperazine ethane sulfonic acid. **- 4-(2-Hydroxyethyl)-1-Piperazine ethane sulfonic acid**	Residues: Glu73, Ile203, Leu204, Lys205, Val206.
Ligand: LEU-VAL-VAL-ASN	Residues: Phe5, Glu30, Ile48, His49, Ser50, Val181, Leu182, Val183, Glu184, Thr251, Ala252.
Ligand: FLC-Citrate anion	Residues: Gly11, Thr12, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Gln115, Glu286, Ser289, Arg293.
Contact(s) to metals:	
Metal: Chloride ion	Residues: Lys40, Ala66, Tyr67, Lys70, Tyr76, Glu77, His79, Gly104, Lys107, Gln115, Asn167, Ser168, Ala169, Lys170, Glu200, Cys202, Ile203, Glu226, Val277, Gly278.

5. DISCUSSION

In this study, all the genetic information of the novel bacteria *Pseudopedobacter saltans* DM12145 that was related to the determination of its possession of the L-asparaginase gene was obtained from the BLAST website, NCBI [93]. The L-asparaginase gene type I from the *Pseudopedobacter saltans* DSM 12145 with the accession number NC_015177.1. associated with protein ID WP_013634621.1". It was cloned on the expression vector pET-28a (+) by the EZ Clone method, synonymously called ligation independent cloning (LIC), by Genscript Co., USA. Setting the optimized conditions required to improve the production of the recombinant protein bioprocess output is a mandatory task. Therefore, the production of the total native L-asparaginase-producing- bacterium *Pseudopedobacter saltans* was maximized using the OVAT approach. Regarding the effect of temperature and PH on L-asparaginase production from *Pseudopedobacter saltans*, it imposed a dramatic decline in the level of L-asparaginase activity. Unlike the present finding regarding the effect of pH and temperature on L-asparaginase production, it was reported that PH and temperature promoted the L-asparaginase production technique by Abdel-Fattah et al., (1995) [81].

Considering the initial pH of the L-asparaginase production medium, pH 7.0 was the best initial pH of the L-asparaginase production medium for *Pseudopedobacter saltans*. Unlike the current discovery of the optimal initial pH and temperature of the L-asparaginase production medium, the optimum initial pH and temperature that induces the highest L-asparaginase production were found to vary greatly among different bacterial strains, recorded to be pH 6.0 and 32 ^{o}C by Gurunathan et al. (2012) [82], also around pH 8.5 and 45 ^{o}C by Abdel-Fattah et al. (1995) [81]. However, there are no uniform conditions that could be applied to maximize the L-asparaginase production from all L-asparaginase-producing bacteria. In other words, the optimal conditions required for L-asparaginase production are questionable according to the bacterial species and strains. The molecular mechanisms underlying the discrepancy in the optimized conditions among diverse L-asparaginase-producing bacterial members should be unveiled [83].

Recently, many microbial genome projects have introduced a hill of complete microbial genome sequences into the international repository nucleotide databases (e.g., GenBank, EMBL, and DDJA) with putative and annotated L-asparaginase from numerous bacteria. Consequently, the above-mentioned does address the indispensable need for the

heterologous expression of the putative bacterial L-asparaginase sequences to better understand the nature and mode of action of these enzymes.

In this study, the L-asparaginase from the *Pseudopedobecter saltans*, was cloned and expressed in *E. coli* BL21 (DE3) for the first time. A BLAST similarity sequence search conferred a high sequence identity that ranged from 99.34–91.84% among protein sequences of other different L-asparaginases and *Pseudopedobacter saltans* L-asparaginase genes. The amino acid residues are responsible for the first type of activity characterized by most Several microbial L-asparaginases such as those of *E. coli* and *Erwinia* have dual enzymatic activities toward l-Asn and l-Gln. Consequently, they can be classified into type I cytosolic enzymes with low glutaminase activity (2–10%) and type II periplasmic L-asparaginase with comparable L-asparaginase and l-glutaminase activities [84]; [68].

Heterologous expression in the microbial cell factory Because *E. coli* is easy to grow and the overall process is cost-effective, it is one of the well-established strategies for both small- and large-scale production of recombinant proteins. The formation of large, insoluble, misfolded versions of the recombinant proteins in the cytoplasm, namely inclusion bodies (IBs), is the main obstacle that hinders the consecution of the overall process, obtaining high levels of active soluble proteins. Therefore, to determine how to sequester the recombinant protein in an active state, we followed some techniques for obtaining high-level gene expression in *E. coli* according to Markrides (1996) evaluations, to collect high yields (> 50%) of soluble and active proteins [85].

The initial level of recombinant L-asparaginase expressed in the *E. coli* BL21 (DE3) Rosetta strain as a 6 His-tag fusion protein, harboring the pET-28 His (+)/L-asparaginase construct was 0.0003 U/mL. The applied optimized strategy successfully enhanced the traced L-asparaginase activity in the cell lysate of the recombinant Rosetta strain upon substituting the LB growth medium. Consequently, the recombinant protein of the L-asparaginase gene from *Pseudopedobacter saltans* was expressed in its N-terminal portion with a tail (tag) of 6 consecutive histidines (6xHis), a simple tag widely used as a purification tool by immobilized metal affinity chromatography (IMAC) developed by Porath (1988) [86]. The use of this tail confers advantages in the protein production step, as it provides production cost savings (single-step purification), in addition to avoiding direct interaction between the expressed protein and the chromatographic column (which increases yield), since only His tag interacts with Ni^{2+} and Co^{2+} metallic ions trapped in microspheres that make up the

chromatographic resin [87]. After protein purification by IMAC chromatography, the recombinant protein was analyzed by SDS-PAGE to assess the solubility as well as to determine the molecular weight (MW) of the recombinant protein. The expected MW of the protein L-asparaginase I enzyme of *Pseudopedobacter saltans* is 37.8 kDa, where the SDS analysis revealed that the size of the protein band was exactly as expected at 36.0 kDa. Therefore, the recombinant protein of asp_pseudopedo was expressed in the soluble fraction and maintained its enzymatic activity. Also, it had an optimal pH of 8.0 and a temperature with a maximum activity of 60 ^{o}C with a variable kinetics K_m value equal to 3 mM and a V_{max} of 168.2 mol/min/mg.

According to the finding of this study, the enzyme is quite similar to type I L-asparaginases which is distinctly specific for L-Asparagine that is located in the cytosol and acts in solution as a homodimer. Our result agrees with other reported studies for example *E. coli* L-asparaginase type I [31]; [88]. *Pseudopedobacter saltans* L-asparaginase I, share similarities with *Vibrio cholerae* L-asparaginase about 99% [89], also about 92% of both *Yersinia pseudotuberculosis* and *Escherichia coli* L-asparaginase enzyme type I [90]; [18].

The *Pseudopedobacter saltans* L-asparaginase is classified by its unique signature of common conserved for microbial L-asparaginase that is characterized by the invariant amino acid residues; Thr^{12}, Ala^{23}, Lys^{25}, Ser^{88}, Asp^{89}, Lys^{165}, Leu^{291}. that included in catalysis.

Also, the *Pseudopedobacter saltans* L-asparaginase catalytic residue to L-glutaminase I, the amino acid residues; Thr^{12}, Ala^{23}, Ser^{88}, Glu^{286}, Leu^{291}; as shown in Figure 4.11 and Table 4.1, the predicted calculated pI for *Pseudopedobacter saltans* L-asparaginase was found to be 6.13 [74].

6. CONCLUSION AND RECOMMENDATIONS

- Conclusively, this study successfully produced the L-asparaginase from the novel bacteria *Pseudopedobacter saltans* through cloning and heterologous expression of the L-asparaginase in *E. coli* for the first time. It is now ready to study its therapeutic properties against cancer in the future.
- The findings of this study revealed quite similarities to type L-asparaginases I of *E. coli*, which is distinctly specific for L-asparagine and located in the cytosol and acts in solution as a homodimer.
- Finally, a novel bacterial *Pseudopedobacter saltans* L-asparaginase enzyme was cloned and expressed in soluble and active stats.

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