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L- Asparaginase from *Staphylococcus aureus*: Overexpression, Purification, Immobilization, Characterization, and Anticancer Activity

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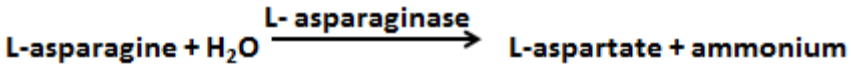
Abstract

L- asparaginases catalyze the conversion of L- asparagine to L- aspartate and ammonia. The current study focus on the cloning and expression of the L-asparaginase from *Staphylococcus aureus* into *Escherichia coli* strain BL21(DE3)pLysS. L-asparaginase enzyme was purified to homogeneity by glutathione sepharose 4B column chromatography. The enzyme was purified 117.6 times and showed a final specific activity of 1680.4 IU/mg protein with a yield of 67.7%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme showed that it was a single peptide chain with Mr 35 kDa. The enzyme was immobilized on Ca alginate beads. The immobilized enzyme retains most of its activity (78%) and reveals high stability at 4 °C. The enzymatic and structural properties of free recombinant and immobilized L- asparaginase were studied. The free enzyme showed maximum activity at pH 8.0 when incubated at 45 °C for 30 min. The immobilized enzyme displayed maximum activity at pH 8.5 after 30 minutes of incubation at 50 °C. The amino acid composition of the purified enzyme was documented. This approach offers the possibility of generating *Staphylococcus aureus* L- asparaginase with high efficiency that can be used to treat leukemia.

Keywords: Staphylococcus aureus - L Asparaginase – Overexpression- Purification- Immobilization, Optimization.

Introduction

L- asparaginase enzyme (EC 3.5.1.1) is found in all living organisms, from bacteria to humans, and plays a *vital role* in various activities. The enzymes hydrolyze L-asparagine to L-aspartate and ammonia. The substrate and product of this enzymatic reaction are crucial in a variety of metabolic activities in all species, from bacteria to humans. L-asparagine is the most common metabolite in plants for nitrogen storage and transport, which is used in protein production [1–2]. L-aspartate is a key precursor of ornithine in the urea cycle and transamination processes creating oxaloacetate in the gluconeogenic pathway leading to glucose in the human body [3–4].



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Initially, L- asparaginases produced interest because of their anticancer activities. L- asparaginase is utilized effectively to treat acute lymphocytic leukemia [5-6]. The antitumor activity of L-asparaginase is based on the dependence of specific tumor cells on the extracellular supply of L-asparagine. Unlike normal cells, malignant cells lack the enzyme L- asparaginase, resulting in slow L-asparagine synthesis. Thus, depletion of the circulating pool of L- asparagine by L- asparaginase leads to the destruction of tumor cells as protein synthesis cannot be completed [7].

Functional forms of L- asparaginase exist as tetramers of identical subunits with molecular weights ranging from 130 to 160 kDa [8-9]. Each of the four active sites is located between two neighboring monomers' N-terminal and C-terminal domains. Therefore, L-asparaginase tetramers can be considered dimers. Even in this case, the active enzyme is always a tetramer [10]. The L- asparaginase *Escherichia coli* and *Erwinia chrysanthemums* are currently being used clinically as effective drugs for the treatment of acute lymphocytic leukemia. They are also used in the treatment of Hodgkin's disease, acute myeloid leukemia, acute myeloid leukemia, chronic lymphocytic leukemia, lymphocytic leukemia, sarcoma, and Melan sarcoma [11-13]. Unfortunately, despite widespread use of L- asparaginase, patient response to treatment rarely progresses without signs of toxicity. The main side effects of L- asparaginase are liver dysfunction, pancreatitis, diabetes, leukopenia, neurological seizures, and clotting disorders that can lead to intracranial thrombosis or bleeding [14-15]. The anticancer activity of L-asparaginase is due to its high affinity for the substrate L- Asparagine (Km $=10^{-2}$ mM). Because tumor cells have low amounts of L-asparagine production, depletion of L-asparagine in the circulating pools starves them. L-glutamine can be hydrolyzed by some enzymes with L- asparaginase activity. When L-glutamine is the main ingredient, the enzymes are known as glutaminase type enzymes because they have superior substrate L- asparaginases [16].

Improvements in food technology have proved that fried and baked meals (especially starchy foods) contain high levels of acrylamide, a highly carcinogenic toxicant created by Maillard's reaction [17]. Before frying or baking, food items should be treated with L-asparaginase to reduce acrylamide generation and its harmful consequences [18]. L-asparaginases from a variety of origins (fungal, bacterial, animal, and plant) have been studied in this area. L- asparaginases from *Aspergillus* species are frequently utilized in the baking industry, however, they are thermolabile and only active over a small pH range. Since temperatures are rising, enzymes that are stable and active across a wide temperature and pH range are required [19].

Several studies have been carried out to improve L-asparaginase production culture conditions by changing growth parameters such as L-asparagine concentration, incubation temperature, fermentation time, medium pH, inoculum size, and carbon and nitrogen sources, all of which have a significant impact on L-asparaginase production. The properties of these enzymes have been demonstrated to vary depending on the enzyme source [20]. Thermophilic bacteria may survive in a wide temperature range (45-80°C). They are known for making thermostable enzymes with high stability and long shelf life [21-22].

The purpose of this study was to clone the gene responsible for L-asparaginase from a thermophile bacterial strain *Staphylococcus aureus*. Moreover, overexpression, purification, and immobilization of *Staphylococcus aureus* L-asparaginase on Ca alginate beads were achieved. The optimum conditions of both free and immobilized *Staphylococcus aureus* L-asparaginase were determined.

2. Material and Methods:

2.1. Molecular biology and analytical reagent grade chemicals were used in this study.

All experimental procedures were conducted in accordance with internationally accepted principles for laboratory animal use and care and were approved by the Ethics Committee in the Faculty of Science, Mansoura University, and Mansoura, Egypt. *Staphylococcus aureus* bacterial strain [23], *Escherichia coli* DH5 strain, BL21(DE3) strain, and pGEX-2T DNA plasmid were generously supplied by Dr. Picksley, S. M. Bradford University, UK (GST gene fusion plasmid IPTG inducible ApR).

2.2. Cloning of *Staphylococcus aureus* L-asparaginase

Staphylococcus aureus was cultured at 37 °C in a medium containing 1% peptone, 0.5% yeast extract, and 1% NaCl. After 24 hours, cells were pelleted by centrifugation and genomic DNA was isolated according to standard procedures [24]. GenBank BLASTN searches for *Staphylococcus aureus* strain ILRI_Eymole1/1 genomic sequence using request nucleotide sequence encoding L-asparaginase which was deposited in the Nucleotide Archive under the accession no LN626917.1

LN626917.1 generated a single open reading frame of 966 bp encoding the L-asparaginase gene. The oligonucleotide forward (5'TAT AGG A**GG ATC C**AT ATG AAA CAT3') and reverse (5'ATG TTA CG**G GAT CC**T TAT AAT TCA 3') primers were

synthesized with *Bam*H1 restriction site (underline) to amplify the entire L-asparaginase gene from the genomic DNA of *Staphylococcus aureus*. The PCR reaction was performed with a total volume of 50 µl containing 50 ng of each primer, 10 ng of *Staphylococcus aureus* genomic template DNA, 0.2 mM of each dNTP, 5 µl of 10x *Pfu* buffer, and 2 units of *Pfu* DNA polymerase (Stratagene, USA). The PCR procedure consisted of 60 seconds at 96 °C, 60 seconds at 46 °C, and 120 seconds at 72 °C. After 25 cycles, a final elongation was performed at 72 °C for 5 minutes. The obtained PCR product was digested with the *Bam*H1 restriction enzyme to facilitate the cloning process. The pGEX-2T DNA expression vector was linearized with *Bam*H1 restriction enzyme and treated with calf intestine alkaline phosphatase to eliminate the 5' phosphate ends of the plasmid. The linear pGEX-2T DNA was ligated with the *Staphylococcus aureus* L-asparaginase gene PCR product. The resulting expression construct was sequenced along both strands and used to transform BL21(DE3)pLysS *E. coli* competent cells.

2.3. Expression, and purification of *Staphylococcus aureus* L-asparaginase

E. coli cells containing the recombinant plasmid were grown at 37 °C in 1L of LB medium containing 100 µg/ml ampicillin. The overexpression of *Staphylococcus aureus* L-asparaginase protein was induced by the addition of 1 mM isopropylthio-β-galactoside (IPTG) when the absorbance at 600 nm was 0.6-0.8. Samples were taken each an hour for overexpression analysis. After 5 hours of induction, the bacterial culture was centrifuged at 10,000 xg (4 °C) for 20 minutes to collect cells, suspended in potassium phosphate buffer (20 mM, pH 8), and sonicated at 10,000 xg (4 °C). The cell lysate was centrifuged for 5 minutes to remove cell debris. The supernatant was collected and polyethyleneimine was added to a final concentration of 0.1% (w/v) to eliminate DNA contamination by centrifugation at 10,000 xg (4 °C) for 5 minutes. The supernatant was applied to a GST Sepharose column (5 ml, 3 cm x 2 cm, Pharmacia, USA) pre-equilibrated with potassium phosphate buffer (20 mM, pH 8). The undesired proteins were rinsed with a 30 ml equilibration buffer followed by a 30 ml potassium phosphate buffer (20 mM, pH 8). The bound *Staphylococcus aureus* L-asparaginase was eluted from the column with 0.2 M NaCl in potassium phosphate buffer (20 mM, pH 8.5).

2.4. Assay of *Staphylococcus aureus* L-asparaginase activity and protein

The *Staphylococcus aureus* L-asparaginase assay was performed at 45 °C using a Hitachi U2000 double beam UV-VIS spectrophotometer equipped with a cell holder with a thermostat (optical path length 10 mm). L-asparaginase activity was measured by determining the rate of ammonia formation utilizing glutamate dehydrogenase [25]. The final assay volume of 1 ml contained 70 mM Tris-HCl buffer, pH 8.0, 1 mM L-asparagine, 0.15 mM □-ketoglutaric acid, 0.15 mM NADH, 4 units of glutamate dehydrogenase, and a sample with L-asparaginase activity. Alternatively, the rate of ammonia production was also measured at 45 ° C using Nessler's reagent [26]. The unit of L-asparaginase activity is defined as the amount of enzyme that releases 1 mol of ammonia from L-asparagine at 45 °C / min. Protein concentration was determined at 25 °C using bovine serum albumin as standard [27].

1.5. 3D structural modeling, phylogenetic tree construction, and sequence analysis of *Staphylococcus aureus* L-asparaginase

The nucleotide sequence of *Staphylococcus aureus* L-asparaginase was analyzed and compared to previously deposited sequences in the database using the Basic Local Alignment Search Tool (BLASTn and BLASTp) provided by NCBI (https://www.ncbi.nlm.nih.gov/protein/ LN626917.1) and aligned using the ClustalO and DNA Star programs. The Phylogeny.fr Software (http://www.Phlyogeny.fr) was exploited to form the phylogenetic tree for *Staphylococcus aureus* L-asparaginase [28]. The Software from http://www.ebi.ac.uk/thornton-sev/ databases/sas/ was exploited to accomplish sequence annotation for *Staphylococcus aureus* L-asparaginase [29]. Following a template search against the Swiss-Model template library with BLAST and HHB lits, three-dimensional (3D) structure prediction and model construction was carried out. BLAST against the primary amino acid sequence present in the SMTL was utilized to catch the *Staphylococcus aureus* L-asparaginase target sequence. A total of 38 templates were revealed, and the template quality was predicted using target-template alignment features. For model construction, the highest-quality template was chosen. ProMod3 was then utilized to create models based on the target-template alignment. The template was utilized to copy coordinates that were conserved between the target and the template. Finally, the QMEAN scoring function [30] was utilized to evaluate the global and per-residue model quality.

2.6. *Staphylococcus aureus* L-asparaginase immobilization in Calcium Alginate-Gelatin Composites.

By combining gelatin (3%) with sodium alginate solution (5%) in water and then cross-linking with glutaraldehyde, gelatin-alginate combinations were formed. Sodium alginate (500 mg) and gelatin (300 mg) were typically combined in a conical flask with

distilled water (8 ml) and autoclaved for 15 minutes at 120 °C [31]. With steady stirring with a magnetic bead, the hot solution was allowed to cool to room temperature. After that, 3 mL of pure *Staphylococcus aureus* L-asparaginase was added, and the mixture was agitated for 15 minutes. The contents were agitated for another 15 minutes after adding glutaraldehyde (0.3 ml of a 25% solution in water). This slurry was then placed in a dropping funnel with a plastic tip and dropped into a cold $CaCl_2$ solution (4 °C) drop by drop. The beads were allowed to solidify in the $CaCl_2$ solution for 30 minutes. The beads were rinsed with distilled water and stored in the refrigerator after the supernatant was decanted.

2.7. Effect of pH and temperature on *Staphylococcus aureus* L-asparaginase activity

In the pH range of 6.0 to 10.0, the free and immobilized *Staphylococcus aureus* L-asparaginase activity was evaluated. 100 mM Tris–HCl (pH 6.0–10.0) was employed as a buffer. The reactions were carried out in a temperature-controlled water bath at their optimal pH values and throughout a temperature range of 20 to 80°C to investigate the effect of temperature on free and immobilized pure *Staphylococcus aureus* L-asparaginase activity.

2.8. Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli [32] on a slab gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Brilliant Blue R-250.

2.9. Cell culture and cytotoxicity test using Alamar Blue and MTT assay:

The THP-1 cell line was offered by ATTC® for this study. THP-1 cells were grown in RPMI 1640 medium, which included 10% heat-inactivated fetal bovine serum, 1% glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. On a 96-well plate, cells were seeded at a density of 10,000 cells per well, then treated with different amounts of purified *Staphylococcus aureus* L- Asparaginase and incubated for 48 hours at 37 °C in 5% CO_2. Untreated cells were seeded in the same circumstances as the treated cells, in a 20 mM potassium phosphate buffer (pH 8). Following incubation, each well-received 10 ml of alamarBlue reagent (10 percent alamarBlue, Invitrogen), and incubation was maintained at 37 °C for another 4 hours. The absorbance of the plates was measured at 570 nm for the plates and 600 nm for the reference using a micro-plate reader. The percentage of cell viability was expressed relative to the control cells after blank normalization [33]. Morphological changes in THP-1 cells were explored and documented using phase-contrast optical microscopy at a magnification of 40.

3. Results:

Staphylococcus aureus L-asparaginase gene identification and sequence analysis:

The genome of *Staphylococcus aureus* contained a unique L-asparaginase entire sequence was acquired and uploaded in the GenBank database (https://www.ncbi.nlm.nih.gov/protein/ LN626917.1). The L-asparaginase gene in *Staphylococcus aureus* has 966 base pairs and codes for a protein with 322 amino acids, allowing for sequence analysis (Figure 1). The NCBI Blast server's Blast P software was used to compare the protein sequence of *Staphylococcus aureus* L-asparaginase to L-asparaginase from other bacteria, with statistically significant high similarity scores (Table 1). The highest percentage of sequence identity (99.1%) was found in *Staphylococcus aureus* 966 bp (GenBank accession number Q2FYF4), while the lowest percentage of sequence identity (49.1%) was found in *Amphibacillus marinus* (GenBank accession number A0A1H8GDI3) (Table 1).

Figure 2A exhibitions the alignment of the deduced amino acid sequence of *Staphylococcus aureus* L-asparaginase with *Streptococcus pneumonia, Clostridioides, Deinococcus radiodurans, Escherichia coli* str. K-12 substr. MG1655 and *Escherichia coli* O157:H7 str. Sakai illustrative associate of the L-asparaginase family. The phylogenetic tree (Figure 2B) was built employing the neighborhood joining approach based on the L-asparaginase amino acid sequence of *Streptococcus pneumonia*, *Clostridioides, Deinococcus radiodurans*, *Escherichia coli* str. K-12 substr. MG1655, *Escherichia coli* O157:H7 str. Sakai common phylogenetic resemblances with *Staphylococcus aureus* L- asparaginase, but migrated to other clusters away from other bacterial species, such as *Streptococcus pneumonia, Clostridioides, Deinococcus radiodurans, Escherichia coli* str. K-12 substr. MG1655, *Escherichia coli* O157:H7 str. Sakai (Figure 2B), representing L-asparaginase deviation.

Table 1: *Staphylococcus aureus* L-asparaginase deduced amino acid homology with organisms

.

Entry	Organisms	Identity
Q2FYF4	*Staphylococcus aureus*	99.1%
Q4L6I3	*Staphylococcus haemolyticus*	72.6%
A0A2K4BWD7	*Staphylococcus auricularis*	72.9%
Q5HP67	*Staphylococcus epidermidis*	70.4%
Q49XT2	*Staphylococcus saprophyticus*	70.1%
K9B7S5	*Staphylococcus massiliensis*	70.1%
A0A178NU85	*Staphylococcus lentus*	59.5%
A0A328A7E9	*Macrococcus bohemicus*	61.5%
A0A4Q1BCE8	*Macrococcus sp. DPC7161*	60.5%
B9E6L8	*Macrococcus caseolyticus*	57.6%
A0A4V3BFC5	*Macrococcus lamae*	59.4%
A0A398BIN4	*Mesobacillus zeae*	55.5%
A0A0D6XRT9	*Staphylococcus microti*	69.1%
A0A3L7JXP6	*alsibacillus albus*	54.1%
A0A2N5G4E4	*Bacillus sp. V3-13*	53.9%
A0A4R1QJ80	*Thermolongibacillus altinsue*	53.8%
A0A0M2SY82	*Mesobacillus campisalis*	54.2%
Q9KCE7	*Alkalihalobacillus haloduran*	53.8%
A0A1H4CK39	*Thalassobacillus cyri*	53.6%
A0A0K9H072	*Peribacillus loiseleuriae*	51.6%
A0A4V1AN31	*Paenisporosarcina antarctica*	53.1%
A0A1H8GDI3	*Amphibacillus marinus*	49.2%

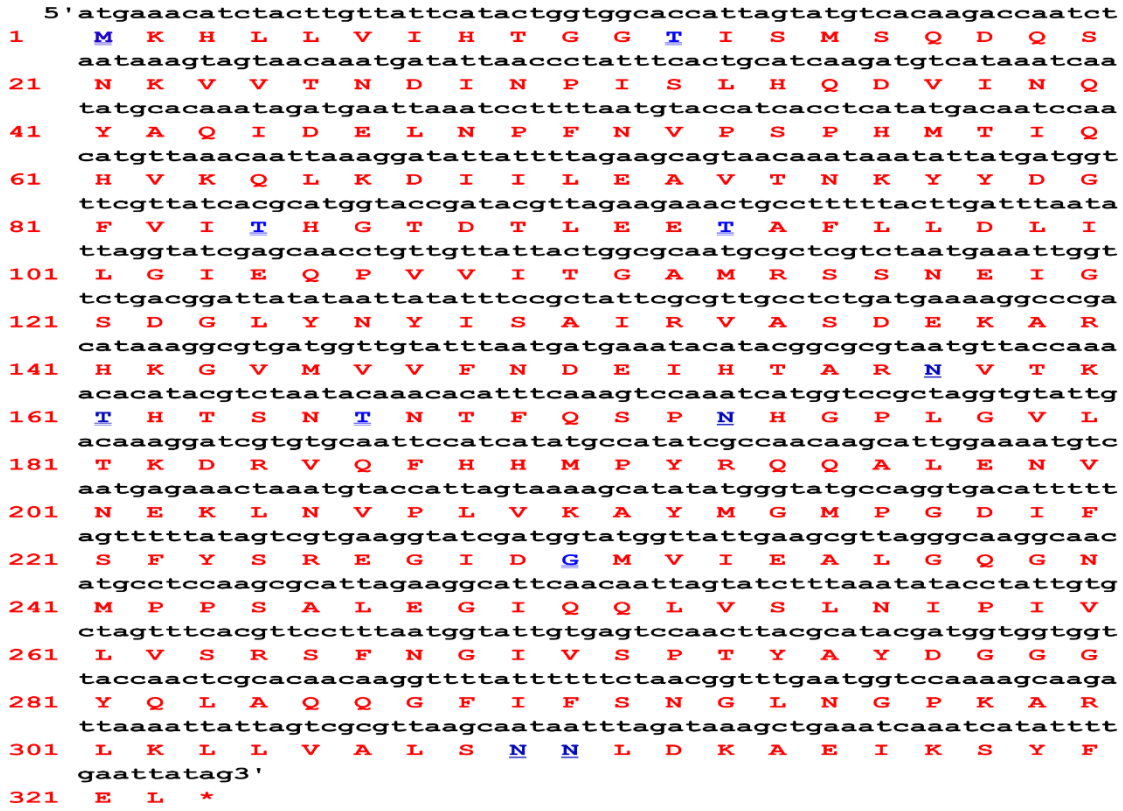

Figure 1: *Staphylococcus aureus* L-asparaginase nucleotide and deduced amino acid sequence. The L-asparaginase amino acid signature (residues Asparagine 157,173, 309, 310, Threonine 12, 84, 93, 161 and 166, and Glycine 230) is displayed in bold underlining. The start codon (atg, Methionine) is highlighted with a double underline, and the asterisk denotes the stop codon (tag).

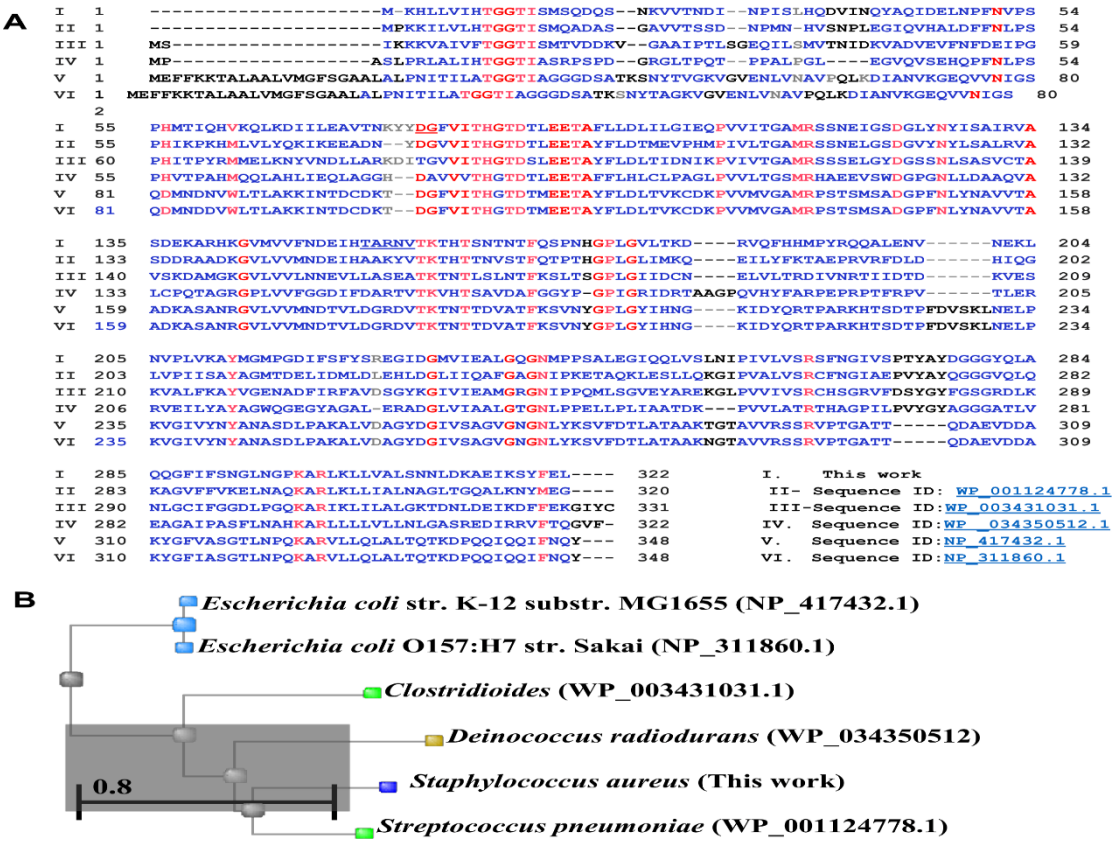

Figure 2: (A)-Pairwise alignment of L-asparaginase of *Staphylococcus aureus* (I) and L-asparaginases of *Streptococcus pneumonia* (II), *Clostridioides* (III), *Deinococcus radiodurans* (IV), *Escherichia coli* str. K-12 substr. MG1655 (V), *Escherichia coli* O157:H7 str. Sakai (VI). Red asterisks show the conserved segment near the N-and carboxy terminal end and the green asterisks show the conserved threonine residues representing the catalytic triad threonine 9, 12, 83, 92, 159 and 164 involved in catalysis. (B)- *Staphylococcus aureus* L-asparaginase and L-asparaginases of *Streptococcus pneumonia*, *Clostridioides*, *Deinococcus radiodurans*, *Escherichia coli* str. K-12 substr. MG1655, *Escherichia coli* O157:H7 str. Sakai phylogenetic relationship. Maximum probability tree based on GenBank-deposited full coding sequences.

Structures prediction for *Staphylococcus aureus* L- asparaginase

The primary structure of the *Staphylococcus aureus* L-asparaginase enzyme reveals that it has nonpolar, polar, hydrophobic, and aromatic amino acids as symbolized in Figure 3A. The sequence elucidation of *Staphylococcus aureus* L- asparaginases and secondary structural motif elements (Fig. 3B) exposed some interesting common characteristics. To begin, a signature for L-asparaginase was discovered, which included conserved invariant amino acid residues such as Asparagine 51, 126, 240, Threonine 9, 12, 84, 87,93, and Glycine 143, 175, 178, 230, 237, 239, all of which were involved in substrate (Asparagine) recognition, binding, and catalysis. The secondary structure of *Staphylococcus aureus* L-asparaginase (Fig. 3B) was predicted to have up to 16 helix structures (26.7%) and 13 strand structures (19.3%), as well as a substantial number of locations for advantageous coil and turn formation (54 %). *Staphylococcus aureus* L- asparaginase 3D was projected to be a homodimer with 16 helix and 13 strands (Fig. 3 C, D, and E), which was similar to h L-asparaginase3 in 3D structure. G_{80}FVITHGTDLEET$_{92}$ was found in *Streptococcus pneumonia*, *Clostridioides*, *Deinococcus radiodurans*, *Escherichia coli* str. K-12 substr. MG1655, *Escherichia coli* O157:H7 str. Sakai, and *Staphylococcus aureus* L-asparaginases. A_{154}RNVTKTHTSNTNT$_{168}$ was discovered to play a key role in the cleavage reaction and autoactivation of *Staphylococcus aureus* L-asparaginase in the presence of high threonine concentrations (Fig. 3A).

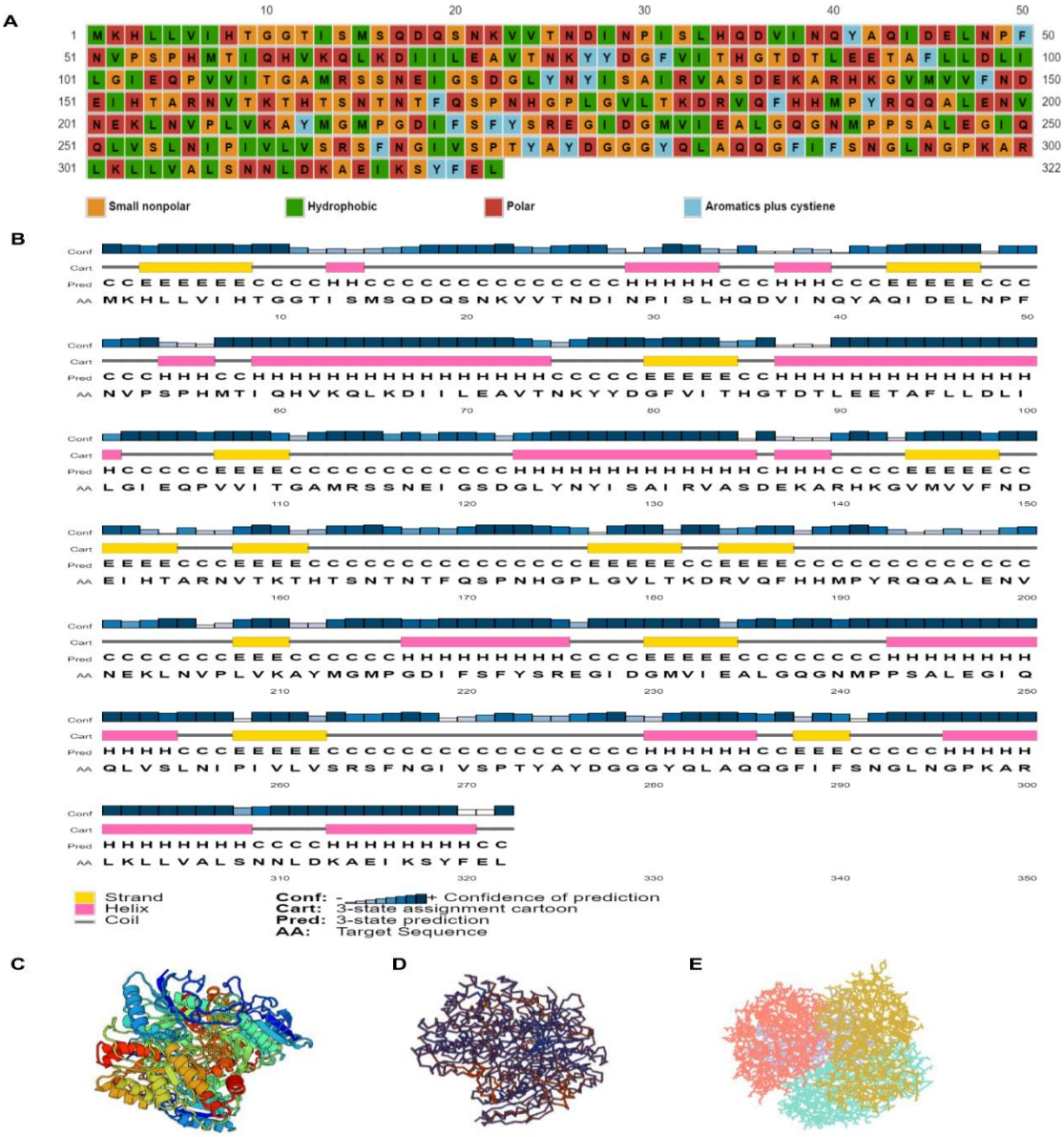

Figure 3: (A) Primary structure and position of nonpolar, polar, hydrophobic, and aromatic amino acid in *Staphylococcus aureus* L- asparaginase. (B) *Staphylococcus aureus* L- asparaginase secondary structure. Yellow boxes (- strand) and pink boxes (-helix) and gray boxes (-coil) represent protein secondary structural components. (C-E) A model of the expected 3D structure of *Staphylococcus aureus* L- asparaginase. predicted 3D structure - helix is blue, -strands are red, and coils are cyan in this cartoon representation of a tetramer structure.

Amino acids composition of Staphylococcus aureus L- asparaginase

The amino acid composition of *Staphylococcus aureus* L- asparaginase is recognized by the expasy database. The L- asparaginase enzyme has 322 amino acids with molecular Formula $C_{1587}H_{2504}N_{430}O_{479}S_{10}$ and a total number of atoms of 5010. The Theoretical molecular weight of this enzyme is 35.6 kDa with PI 5.93. The amino acids leucine and Isoleucine are repeated in *Staphylococcus aureus* L- asparaginase 31and 26 times with a percent of 9.3% and 8.1%, respectively. The amino acids arginine and threonine are found in *Staphylococcus aureus* L- asparaginase 24, and 18 times with percentages of 7.5% and 5.6%, respectively. The *Staphylococcus aureus* L- asparaginase does not have either cystine or tryptophane. The total number of negatively charged residues (Aspartic + Glutamine) is 31 and the total number of positively charged residues (Arginine + Lysine) is 24 (Table 2).

Table 2: Amino acids composition of *Staphylococcus aureus* L- asparaginase

Amino Acid		Symbol	Number	Percent
1.	**Alanine**	A	17	5.3%
2.	**Arginine**	R	9	2.8%
3.	**Asparagine**	N	24	7.5%
4.	**Aspartic**	D	16	5.0%
5.	**Cystine**	C	0	0.0%
6.	**Glutamine**	Q	18	5.6%
7.	**Glutamic**	E	15	4.7%
8.	**Glycine**	G	25	7.8%
9.	**Histidine**	H	12	3.7%
10.	**Isoleucine**	I	26	8.1%
11.	**Leucine**	L	30	9.3%
12.	**Lysine**	K	15	4.7%
13.	**Methionine**	M	10	3.1%
14.	**Phenylalanine**	F	12	3.7%
15.	**Proline**	P	15	4.7%
16.	**Serine**	S	22	6.8%
17.	**Threonine**	T	18	5.6%
18.	**Tryptophane**	W	0	0.0%
19.	**Tyrosine**	Y	12	3.7%
20.	**Valine**	V	26	8.1%

Time course and expression of *Staphylococcus aureus* L- asparaginase polypeptide

Using the required forward and reverse oligonucleotides primers, the L- asparaginase gene was amplified by PCR from *Staphylococcus aureus* chromosomal DNA, providing the predicted 1 kbp DNA product with nearby nucleotides (Fig. 4A), which included the 966 bp L- asparaginase gene with flanking DNA. Under the IPTG-inducible Tac promoter and the lacI repressor control, the PCR product was ligated into the *Bam* HI restriction site in the pGEX-2T DNA plasmid (Fig. 4B). The L-asparaginase gene was in-frame and appropriately located concerning the plasmid Tac promoter.

The appearance of putative induction of *Staphylococcus aureus* L- asparaginase polypeptides throughout time is depicted in Fig. 4C. At time 0 h, *E. coli* competent cells were transformed with the recombinant plasmid and the L- asparaginase protein overexpressed with 1 mM IPTG, samples obtained every 1 h. After 2 hours of IPTG induction, Staphylococcus aureus L- asparaginase overproduction was apparent (Fig. 4C, lane 5), and peak expression was reached after 5 hours (Fig. 4C, lane 8). The L- asparaginase polypeptide demonstrated the highest expression after 5 hours of IPTG induction. The coding sequence of *Staphylococcus aureus* L-asparaginase was cloned and produced in *E. coli* BL21 (DE3) pLysS under the control of the T7 promoter of the pGEX-2T DNA vector. The GST fusion recombinant L- asparaginase was bound to the glutathione sepharose 4B column matrix, and then eluted from the column using a buffer containing 10 mM reduced glutathione. Purified recombinant *Staphylococcus aureus* L- asparaginase was found to have a single band of 35 kDa on SDS-PAGE (Fig. 4D). The specific activity of the purified enzyme was 1680.4 U/mg protein, and the purified recombinant enzyme had a purification fold of 117.6, providing a total yield of 67.7 percent (Table 3).

Table 3: Purification of *Staphylococcus aureus* L- asparaginase

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	**60**	**452**	**6458**	**14.3**	**100**	**1.00**
Polyethyleneimine	**60**	**417**	**6084**	**14.6**	**94.2**	**1.02**
Glutathione Sepharose 4B	**10**	**2.6**	**4369**	**1680.4**	**67.7**	**117.6**

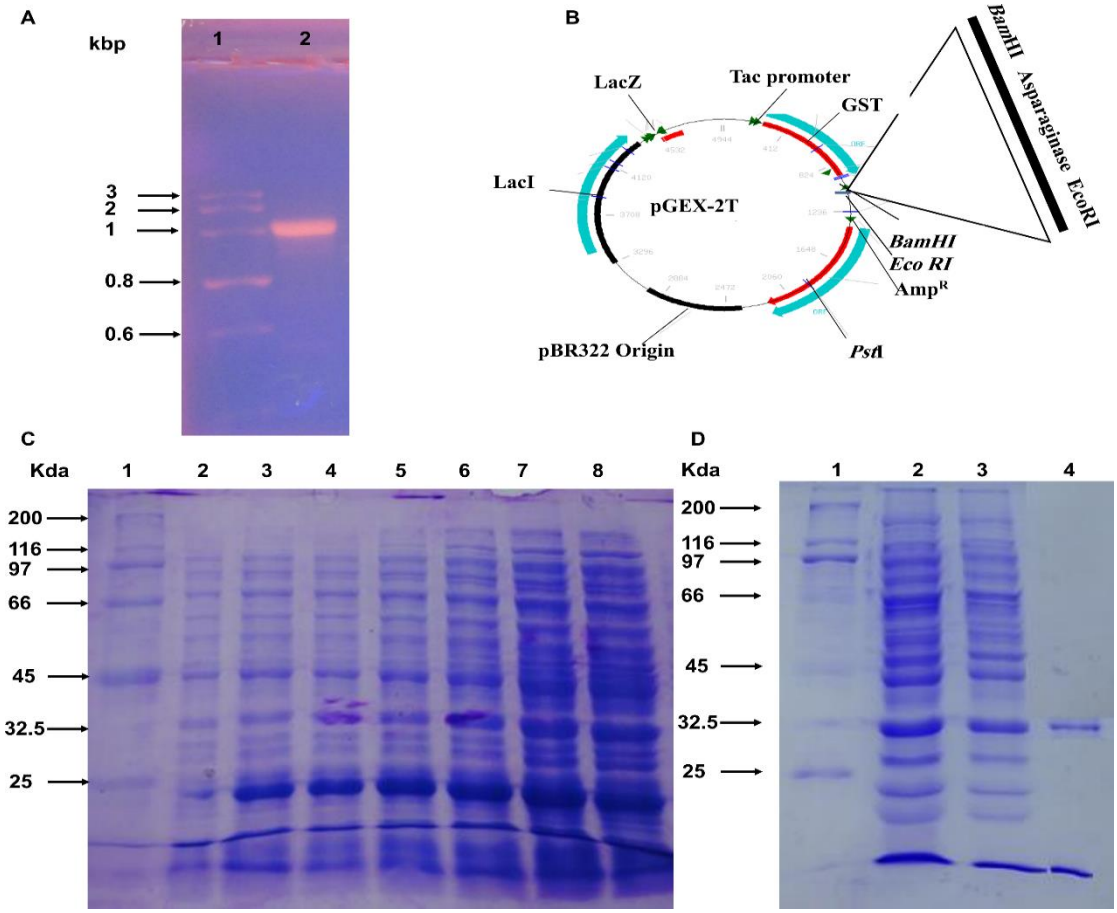

Figure 4: (A)-The PCR product of the 1kbP DNA fragment of the L- asparaginase gene of *Staphylococcus aureus*. The DNA fragment was analyzed on a 1.3% TAE agarose gel. Lane 1: DNA marker (Gel pilot Wide range ladder 100 -Qiagen). Lane 2: 1 kbP DNA fragment PCR product of L- asparaginase gene. (B) -Schematic diagram of the recombinant *Staphylococcus aureus* L- asparaginase overexpressions construct. The L- asparaginase gene was cloned downstream of the Tac promoter in the pGEX-2T DNA expression vector, which also contained the genes for lacI and lacZ repressors, pBR322 origin, and ampicillin resistance. (C) - Induction time course for overexpression of L- asparaginase protein. Early to the mid-log culture of *E. coli* BL21with L- asparaginase recombinant plasmid was induced at time 0 hours with IPTG at a final concentration of 1 mM and samples were taken and analyzed by 10% SDS-PAGE gel at times indicated (Lane 2-8), protein marker Lane 1 Sigma SD6H2 (M. Wt 25,000-200,000 KDa). (D)- The purification profile of the L- asparaginase protein on SDS-PAGE. Lane 1 protein marker, Lane 2 *E. coli* L- asparaginase crude extract, lane 3 Crude extract treated with PEI. Lane 4 Glutathione sepharose 4B column eluted L- asparaginase.

Immobilization of *Staphylococcus aureus* L-asparaginase

Staphylococcus aureus L- asparaginase in presence of glutaraldehyde is efficiently immobilized in calcium alginate gelatin composites. The immobilized enzyme had 78 percent of the activity of the native enzyme, showing that the immobilization approach is particularly appropriate for the *Staphylococcus aureus* L- asparaginase.

Effects of pH on *Staphylococcus aureus* L-asparaginase activity.

Figure 5A illustrates the effect of pH on the activities of free and immobilized *Staphylococcus aureus* L-asparaginase. The activities of free and immobilized *Staphylococcus aureus* L- asparaginase were measured throughout a pH range of 6 to 10. The perfect pH for the activity of free *Staphylococcus aureus* L- asparaginase was found to be 8.0 (Fig. 5A). Purified *Staphylococcus aureus* L- asparaginase is active in alkaline solutions. The immobilized enzyme's best pH was found to be pH 8.5. (Fig. 5A). The immobilized *Staphylococcus aureus* L- asparaginase has a higher optimum pH than the free enzyme.

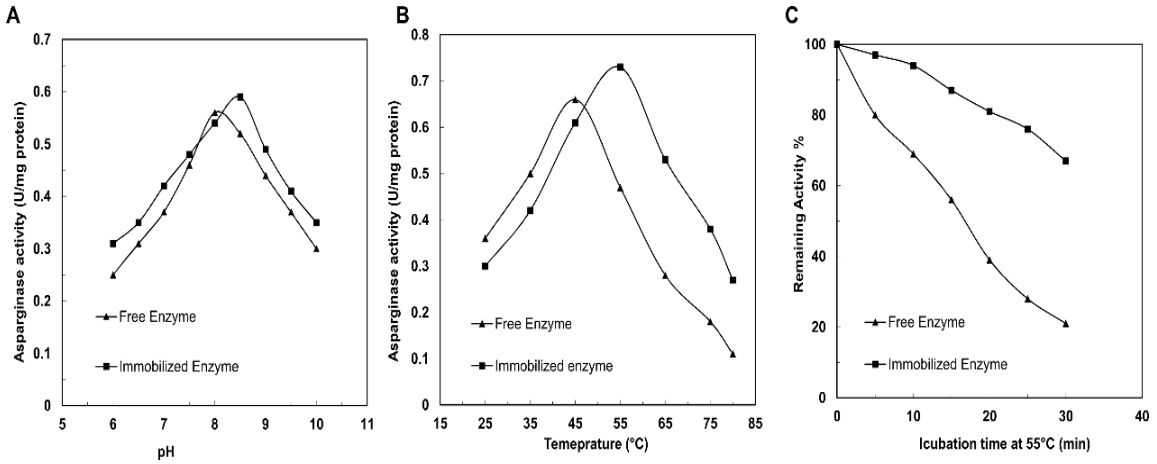

Fig. 5: (A)-Effect of pH on the activity of free and immobilized *Staphylococcus aureus* L- asparaginase. (B)- Effect of temperature on the activity of free and immobilized *Staphylococcus aureus* L- asparaginase in 100mM Tris–HCl buffer (pH 8.0 for free asparaginase II and pH 8.5 for immobilized enzyme). (C)- Thermal stability of free and immobilized *Staphylococcus aureus* L- asparaginase with incubation time at 60 ° C. Results are the mean ± SD of triplicate assays.

Effects of temperature on *Staphylococcus aureus* L-asparaginase activity.

After pre-incubating *Staphylococcus aureus*, L-asparaginase for 30 minutes at temperatures ranging from 20 to 80 °C, the optimum temperature for *Staphylococcus aureus* L- asparaginase activity was revealed. As indicated in Fig. 5B, the optimum temperature for free *Staphylococcus aureus* L-asparaginase activity was 45 °C and pH 8.0. The immobilized *Staphylococcus aureus* L- asparaginase optimum temperature is shifted to a higher temperature and recorded at 50 °C. The immobilized enzyme's optimal temperature is greater than the free *Staphylococcus aureus* L- asparaginase.

Heat stability of free and immobilized *Staphylococcus aureus* L- asparaginase

At 55 °C for the prescribed duration, the heat stability of the immobilized *Staphylococcus aureus* L-asparaginase was compared to that of the free enzyme (30 min). The thermal stability of the immobilized *Staphylococcus aureus* L- asparaginase was considerably improved, according to the data given in Figure 5C. For example, after heat treatment at 55 °C for 30 minutes, the free enzyme preserved 21 percent of its starting activity, whereas the immobilized enzyme retained 67 percent, indicating a 3-fold increase in heat stability at the defined conditions.

Substrate specificity of *Staphylococcus aureus* L-asparaginase

A notable advantage of using L-asparaginase to treat acute lymphocytic leukemia is the lack of glutaminase activity. A variety of reaction substrates were used to test the substrate specificity of *Staphylococcus aureus* L-asparaginase. At a concentration of 10 mM, the purified recombinant enzyme had the highest activity and selectivity for the reaction substrate L-Asparagine, with no activity for L-Glutamine, acrylamide, or urea.

Effect of metal ions, EDTA, and reducing agents on *Staphylococcus aureus* L- asparaginase

Sulfate and chloride metal ions, as well as reducing agents, were studied (Table 4). At a concentration of 1 mM, both NaCl and KCl increased *Staphylococcus aureus* L- asparaginase activity, whereas $ZnCl_2$, $CuCl_2$, $HgCl_2$, $MgCl_2$, and $CaCl_2$ inhibited it in the following order: $HgCl_2$> $CaCl_2$ >$ZnCl_2$ >$CuCl_2$> $MgCl_2$. In contrast, the majority of metal ions tested in sulfate forms inhibited *Staphylococcus aureus* L-asparaginase activity. At 1- and 5-mM concentrations, reducing agents like DTT and 2-mercaptoethanol decreased enzyme activity marginally (Table 4). The effect of the metal-chelating agent EDTA was also studied, and it was observed that EDTA reduced the activity of *Staphylococcus aureus* L- asparaginase by 62.4 percent and 47.9 percent, at concentrations of 1 mM and 5 mM respectively.

Cytotoxicity of recombinant *Staphylococcus aureus* L- asparaginase on cell lines

Using different doses of pure *Staphylococcus aureus* L- asparaginase, the effects of purified recombinant *Staphylococcus aureus* L- asparaginase on the human leukemia cell line THP-1 were examined. Significant morphological alterations were detected after 48 hours of therapy, according to our findings (Fig. 6 A and C). As a result of the formation of intracytoplasmic granules and apoptotic bodies, the number, size, and shrinkage of enzyme-treated cells were reduced. When

compared to untreated cells, cells treated with paclitaxel at a dose of 20 M as positive control showed similar morphological changes (Fig. 6 A-B). Cell viability and cell death appear to be hampered by these drastic changes in cell structure. The alamarBlue assay was used to see if recombinant *Staphylococcus aureus* L- asparaginase had any effect on THP-1 cell survival. The recombinant L- asparaginase reduced cell viability in a dose-dependent manner, with an IC_{50} of 0.75 IU (Fig. 6 D).

Table 4: The effect of reducing agents, EDTA, and certain metal ions (chloride and sulfate forms) on the activity of *Staphylococcus aureus* L-asparaginase.

Effector	**Residual Activity %**	
Control	**100 %**	
	1mM	**5mM**
NaCl	**109.5**	**87.9**
KCl	**107.3**	**85.4**
$HgCl_2$	**31.5**	**18.7**
$CaCl_2$	**80.4**	**71.1**
$CuCl_2$	**84.7**	**74.8**
$MgCl_2$	**94.8**	**84.3**
$ZnCl_2$	**82.7**	**76.6**
Na_2SO_4	**86.3**	**70.4**
$CuSO_4$	**63.9**	**55.7**
$MgSO_4$	**54.8**	**47.4**
$NiSO_4$	**72.5**	**64.7**
EDTA	**62.4**	**47.9**
DDT	**79.8**	**71.3**
2-C_2H_5SH	**98.4**	**93.7**

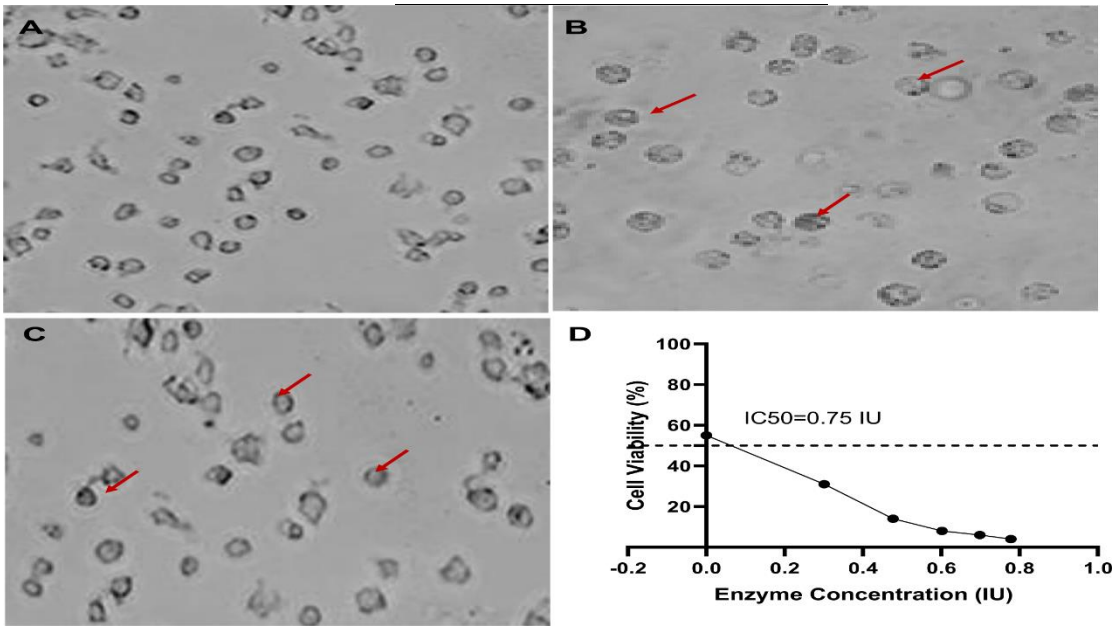

Figure 6: The shape of human leukemia THP-1 cells is altered by recombinant *Staphylococcus aureus* L-asparaginase. Purified recombinant L- asparaginase at a concentration of 1 IU was used to treat cells for 48 hours THP1 cells that had not been treated (A), paclitaxel-treated cells (B), and purified recombinant L- asparaginase - treated cells (C). The intracytoplasmic granules are indicated by red arrows. (D) THP-1, the cell line is killed by *Staphylococcus aureus* L- asparaginase. Different concentrations of *Staphylococcus aureus* L- asparaginase were utilized to treat the cell line for 48 hours. The percentage of cell viability was calculated using AlamarBlue and MTT tests. The IC_{50} of *Staphylococcus aureus* L- asparaginase for THP-1 was calculated.

Discussion:

The recombinant DNA technique was used in a different bacterial host to achieve overproduction of economically essential pharmaceutical enzymes like L-asparaginase. Several genetic elements found in diverse bacterial genera control this enzyme. In *Bacillus*, L-asparaginase is found in an operon with L-asparaginase B, which codes for L-asparaginase. L-asparaginase R inhibits the expression of the L-asparaginase AB operon, and the activity of L-asparaginase R is considered to be regulated by asparagine or aspartate. A non-pathogenic strain of *Staphylococcus aureus* was used to clone, over-express, and describe the L-asparaginase gene. The protein sequence of *Staphylococcus aureus* L-asparaginase was compared to L-asparaginase from *Streptococcus pneumonia*, *Clostridioides*, *Deinococcus radiodurans*, *Escherichia coli* str. K-12 substr. MG1655, *Escherichia coli* O157:H7 str. Sakai uses the Blast P program in the NCBI Blast server. *Staphylococcus aureus* L-asparaginase lacks the L-glutaminase active site signature present in most microbial L-asparaginases, including *E. coli* and *E. chrysanthemi*, according to sequence annotation by structure. These L-asparaginases show dual activity against both the reaction substrates, L-asparagine and L-glutamine, and account for 2–10% of their L-asparaginase activity [34]. This property of Staphylococcus aureus L- asparaginase is observed with significant significance due to the development of immunogenicity and cytotoxicity associated with the treatment of acute lymphoblastic leukemia patients [35]. L-asparagine and lysophospholipids are hydrolyzed by the 60-kDa lysophospholipase enzyme. This enzyme belongs to the bacterial type family and is related to E. coli type I and II L- asparaginase. This enzyme is identical to E. coli type I and II L-asparaginase. Human L-asparaginase is a lysosomal aspartylglucosaminidase and a plant type L-asparaginase that eliminates asparagine-bound carbohydrate groups [36 -37]. Third, human L-asparaginase is h asparaginase3, a plant type L-asparaginase that resembles E. coli type III L-asparaginase structurally [38- 39]. This conserved region, G_{228}IDGMVIEALGQG$_{240}$, is involved in h asparaginase3 auto-cleavage, self-activation, and catalytic activity in the presence of free amino acid glycine [40]. The catalytic triad of *Staphylococcus aureus* L-asparaginase has four threonine residues, thr 9, 12, 83, 92, 159, and 164, which are essential and responsible for the catalytic activity towards the L-asparagine substrate. Thr_{159}, which is not necessary for autocleavage but is required for catalysis because the hydroxyl group of Thr_{163} functions as an activator for the hydroxyl group of Thr_{159} [35], is the key and critical threonine residue in the sequence T_{159}KTHT$_{163}$. The third and fourth threonine residues in the catalytic triad of both h L-asparaginase3 and *Staphylococcus aureus* L-asparaginase are Thr_{219} (in humans) and Thr_{159} (in *Staphylococcus aureus*). The mobility of the glycine rich-region, which is a G_{227}IDG$_{230}$ loop at the N-terminal region of the L-asparaginase that alters the conformation between the cleavage and un-cleavage states, is influenced by this conserved threonine residue, as well as the neighboring glycine moiety (Gly_{227} or Gly_{230}). As a result, the catalytic mechanism for h asparaginase and Staphylococcus aureus L- asparaginase towards the L-asparagine substrate may be quite similar. The action begins with a nucleophilic attack by the Thr_{159} side chain on the carboxyl group of asparaginase, followed by the release of the amino group. The action also involves an amino group near the Asp_{229} side chain and the gln_{238} carbonyl atom. The oxyanion hole [41] has been proposed as a mechanism for stabilizing negatively charged tetrahedral intermediates. It is said to contain Thr_{159} and gln_{238} residues. Surprisingly, isolated recombinant *Staphylococcus aureus* L-asparaginase was revealed to have activity.

Pyrobaculum calidifontis thermostable L-asparaginase was shown to have an optimal temperature of at least 100 °C and a pH of 6.5 [42]. For pure thermostable L- asparaginase from *Bacillus amyloliquefaciens*, the ideal pH and temperature were 8.5 and 65 °C, respectively [43]. This observation is significant because glutaminase activity, which is often associated with *E. coli* and *E. chrysanthemi* L-asparaginase activity [34], causes cytotoxicity. Furthermore, the results of sequence explanation by structure, which revealed the absence of the L. glutaminase signature in *Staphylococcus aureus* L-asparaginase, corroborate these findings.

Hypertriglyceridemia, liver function, hepatic transaminase impairment, and bilirubin and alkaline phosphatase elevations have all been associated with L- asparaginase treatment in individuals with acute lymphoblastic leukemia [44]. In addition, 30–60% of patients receiving L- asparaginase as part of multiagent therapy had elevated hepatic transaminase, alkaline phosphatase, and bilirubin levels [45]. Although antileukemic and anticancer capabilities of L- asparaginase have been demonstrated [46], the effect of recombinant *Staphylococcus aureus* L-asparaginase on human leukemia and cancer cells has yet to be extensively investigated. According to our findings, the purified recombinant *Staphylococcus aureus* L- asparaginase is effective in killing human leukemia cells, THP-1, owing to the deamination of the non-essential amino acid L- asparagine to L-aspartic, reducing the asparagine pool. The characteristics of L- asparaginase are improved by producing L asparaginase from *Pseudomonas*

aeruginosa under solid-state fermentation [47]. This enzyme could be made inexpensively from untreated biomass residues. This enzyme's outstanding features, such as activity in the alkaline pH range at 37 °C, make it particularly useful in the chemotherapeutic treatment of leukemia. Although L- asparagine is a non-essential amino acid, it becomes angular to some leukemia and cancer cells for two reasons. First, L- asparagine is required to produce glycoproteins and other cellular proteins; second, these cells have low quantities of L-asparagine, the counteracting enzyme, leading to starvation and eventual cell death. Other investigators discovered that asparagine mRNA, protein, and activity levels in acute lymphoblastic leukemia patients vary significantly [48] and aren't necessarily associated with *in vitro* treatment resistance. L-asparaginase is a kind of asparaginase. As a result, there may be an additional mechanism of resistance to L- asparaginase in addition to asparagine regulation.

In conclusion, microbial L-asparaginase is a critical component of juvenile acute lymphoblastic leukemia, and identifying the L-asparaginase with the best clinical characteristics is difficult. Treatment-related toxicity necessitates proper management and the continual need for new enzyme sources and the innovation of existing products. This study established the overexpression, purification, and characterization of recombinant *Staphylococcus aureus* L-asparaginase with significant selectivity for L-asparagine without glutaminase activity. The recombinant enzyme caused cytotoxicity in human leukemia cell lines THP-1. As a result, recombinant *Staphylococcus aureus* L-asparaginase may be a promising alternative enzyme for the treatment of acute lymphoblastic leukemia, while more research is needed to assess its immunogenicity and toxicity. However, the potential for novel anti-leukemic medications discovered as a result of this research is expected to be significant.

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Author Contributions:
All authors are equal contributions.

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