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Overexpression, biochemical characterization, and anticancer activates of L-asparaginase from *Bacillus subtilis*

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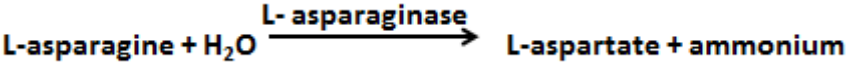
***Abstract*---**L-asparaginases convert L-asparagine into L-aspartate and ammonia. The L-asparaginase from *Bacillus subtilis* was cloned and expressed in the *E. coli* strain BL21(DE3)pLysS in the current study. Using glutathione sepharose 4B column chromatography, the L-asparaginase enzyme was uniformly purified 173.34 times, with a final specific activity of 1769.13 IU/mg protein and a yield of 56.14%. The isolated enzyme was identified as a 36 kDa polypeptide chain by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The immobilized enzyme was placed on top of the Ca alginate beads. The immobilized enzyme is quite stable and retains the majority of its activity at 4 °C (74 percent). The enzymatic and structural characteristics of free and immobilized recombinant L-asparaginase were studied. The activity of the free enzyme peaked after 30 min of incubation at pH 8.0 and 45 °C. After 30 minutes at 50 °C, the immobilized enzyme showed its peak activity at a pH of 8.5. The refined enzyme's amino acid makeup was identified. An enzyme that heals leukemia, *Bacillus subtilis* L-asparaginase, can be successfully mass-produced using this technique.

Keywords*---Bacillus subtilis*, L asparaginase, overexpression, purification, immobilization, characterization.

Introduction

The widely present enzyme L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) catalyzes the breakdown of L-asparagine into L-aspartic acid and ammonia. L-asparaginase is thought to have a considerable impact on how asparagine is metabolized in cells. Many microorganisms have been used as sources for this enzyme as *E. coli, Erwinia cartovora, Enterobacter aerogenes, Corynebacterium*, and *Burkholderia pseudomallei* [1-4]



There are two types of l-asparaginases: l-asparaginase I and l-asparaginase II [5]. The two enzymes differ from one another in terms of their location within the cell, solubility in ammonium sulfate, sensitivity to thermal inactivation, expression

requirements, and most significantly, their affinity for the substrate L-asparagine. Compared to the type I enzyme, which is produced in the cytoplasm, the type II enzyme, which is found in the periplasm, has a high affinity for L-asparagine. The high affinity of the type II enzyme considerably reduced mouse lymphomas in contrast to the low affinity of the type I enzyme [6]. Type II enzymes from *Escherichia coli* [7] and *Erwinia chrysanthemi* [8] *Pseudomonas aeruginosa* [9] have been thoroughly investigated for their anticancer potential. They have been successfully used for a long time to treat acute lymphoblastic leukemia [10]. Numerous studies have looked at the use of small deaminases to increase the level of the flavorful amino acid glutamate in processed foods [11]. The use of microbial glutamyl transpeptidase and glutaminase-asparaginase in food preparation has also been documented [12-13]. However, L-asparaginase has only been studied as a leukemia treatment. *Bacillus subtilis* is frequently used in the food industry. The existence and expression of *B. subtilis* two L-asparaginase genes, ansA and ansZ, have been confirmed [14- 15]. The amino acid sequence of the ansA gene product is similar to that of *E. coli* asparaginase I (EcAI), whereas that of the ansZ gene product is similar to that of *E. coli* L-asparaginase II (EcAII). Because they are utilized to treat leukemia, bacterial L-asparaginases are significant therapeutic enzymes. L-asparaginase, a periplasmic enzyme from *E. coli* with high affinity, is very helpful in the treatment of different malignancies [16]. Many lymphoblastic leukemia patients' malignant cells need an external source of L-asparagine to survive, although healthy cells can make L-asparagine on their own [17]. L-asparaginase is most usually used to treat Hodgkin's disease, acute lymphoblastic leukemia, acute myelomonocytic leukemia, and melanosarcoma [18] Asparaginase's main limitations for therapeutic use range from mild allergic responses to potentially fatal anaphylactic shocks [19]. Patients who experience allergic reactions to the *E. coli* enzyme can be treated with the *Erwinia* enzyme, but the opposite is also conceivable [20]. Research has focused on using microbial asparaginases therapeutically to treat particular types of human cancer [21-22].

The goal of this investigation was to clone the L-asparaginase gene from the thermophilic *Bacillus subtilis*. L-asparaginase from *Bacillus subtilis* overexpressed, purified, and immobilized on Ca alginate beads. Optimum conditions for both free and immobilized *Bacillus subtilis* L-asparaginase were determined.

Material and Methods

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

Dr. Picksley, S. M. Bradford University, UK, kindly provided *E.coli* DH5 bacterial strain, BL21(DE3) strain, and pGEX-2T DNA plasmid (GST gene fusion plasmid IPTG inducible Ap^R) and the *Bacillus subtilis* strain [23].

Cloning of *Bacillus subtilis* L-asparaginase

At 37 °C, *Bacillus subtilis* was grown in a medium that contained 1% peptone, 0.5 % yeast extract, and 1% NaCl. Cells were centrifuged into pellets after 24 hours, and genomic DNA was extracted according to Sambrook, [24]. Request nucleotide

sequence encoding L-asparaginase, which was deposited in the Nucleotide Archive under accession number M63264.1, is used in GenBank BLASTN searches for *Bacillus subtilis* strain ILRI Eymole1/1 genomic sequence. A single 990 bp open reading frame encoding the L-asparaginase gene was produced by M63264.1. The whole L-asparaginase gene from the genomic DNA of *Bacillus subtilis* was amplified using the oligonucleotide forward (5'ACG AGG AGG ATC CAT ATG CGA TCA3') and reverse (5'GTA CCA CGG GAT CCT TAT GGC TCA 3') primers, which were generated with the *Bam*H1 restriction site (underlined). 0.2 mM of each dNTP, 50 ng of each primer, 10 ng of *Bacillus subtilis* genomic template DNA, 5 l of 10x Pfu buffer, and 2 units of Pfu DNA polymerase were added to a total volume of 50 l for the PCR experiment (Stratagene, USA). 96 °C for 60 seconds, 46 °C for 60 seconds, and 72 °C for 120 seconds made up the PCR method. A final elongation was carried out at 72 °C for 5 minutes following 25 cycles. The resulting PCR product was digested with the *Bam*H1 restriction enzyme to speed up the cloning procedure. The 5' phosphate ends of the pGEX-2T DNA expression vector were removed by linearizing it using the *Bam*H1 restriction enzyme and calf intestinal alkaline phosphatase. The L-asparaginase gene PCR result from Bacillus subtilis was ligated with the linear pGEX-2T DNA. After being sequenced along both strands, the produced expression construct was used to convert BL21(DE3)pLysS E. coli competent cells.

Expression, and purification of *Bacillus subtilis* L-asparaginase

The recombinant plasmid-carrying *E. coli* cells were grown at 37 °C in 1L of LB medium containing 100 g/ml ampicillin. When the absorbance at 600 nm was between 0.6 and 0.8, 1 mM isopropylthio-galactoside (IPTG) was added to trigger the overexpression of the *Bacillus subtilis* L-asparaginase protein. Each hour, samples were collected for overexpression analysis. The bacterial culture was incubated for 5 hours before being centrifuged at 10,000 x g (4 °C) for 20 minutes to remove the cells. The cells were then suspended in potassium phosphate buffer (20 mM, pH 8), and 10,000 × g (4 °C) sonicated. To get rid of cell fragments, the cell lysate was centrifuged for five minutes. To remove DNA contamination, the supernatant was collected and polyethyleneimine was added to a final concentration of 0.1 percent (w/v) before being centrifuged at 10,000 x g (4 °C) for 5 minutes. The supernatant was added to a GST Sepharose column that had been pre-equilibrated with potassium phosphate buffer (5 ml, 3 cm x 2 cm, Pharmacia, USA) (20 mM, pH 8). After rinsing the unwanted proteins with a 30 ml potassium phosphate buffer, the equilibration buffer (20 mM, pH 8). With 0.2 M NaCl in potassium phosphate buffer, the bound Bacillus subtilis L-asparaginase was removed from the column (20 mM, pH 8.5).

Assay of *Bacillus subtilis* L-asparaginase activity and protein

Using a Hitachi U2000 double beam UV-VIS spectrophotometer fitted with a cell holder with a thermostat, the Bacillus subtilis L-asparaginase test was carried out at 45 °C (optical path length 10 mm). Utilizing glutamate dehydrogenase, the rate of ammonia production was used to determine L-asparaginase activity [25]. A sample having L-asparaginase activity, 70 mM Tris-HCl buffer, pH 8.0, 1 mM L-asparagine, 0.15 mM -ketoglutaric acid, 0.15 mM NADH, and 4 units of glutamate dehydrogenase were all included in the final test volume of 1 ml. Nessler's reagent

was also used to determine the rate of ammonia generation at 45 ° C (Sheng *et al.*, 1993). The amount of enzyme that releases 1 mol of ammonia from L-asparagine at 45 °C/min is considered one unit of L-asparaginase activity. Using bovine serum albumin as the reference material, protein content was assessed at 25 °C [26].

Bacillus subtilis L-asparaginase structural 3D modeling, phylogenetic tree construction, and sequence analysis

Using the Basic Local Alignment Search Tool (BLASTn and BLASTp) offered by NCBI (https://www.ncbi.nlm.nih.gov/protein/LN626917.1), the nucleotide sequence of Bacillus subtilis L-asparaginase was examined, compared to previously deposited sequences in the database and aligned using the ClustalO and DNA Star programs. The phylogenetic tree for *Bacillus subtilis* L-asparaginase was created using the Phylogeny.fr Software (http://www.Phlyogeny.fr) [27]. Software from http://www.ebi.ac.uk/thornton-sev/ databases/sas/ was used to complete the sequence annotation for the L-asparaginase from *Bacillus subtilis* [28]. After performing a template search using BLAST and HHB lits against the Swiss-Model template library, three-dimensional (3D) structure prediction and model construction were completed. To find the Bacillus subtilis L-asparaginase target sequence, BLAST was used against the SMTL's major amino acid sequence. We revealed a total of 38 templates and used target-template alignment attributes to forecast the template quality. The best template was selected for model development. Then, models based on the target-template alignment were made with ProMod3. The template was utilized to copy coordinates that were conserved between the target and the template. Finally, the QMEAN scoring function [29] was utilized to evaluate the global and per-residue model quality.

Immobilization of Bacillus subtilis L-asparaginase in Calcium Alginate-Gelatin Composites

Gelatin-alginate combinations were created by mixing gelatin (3%) with sodium alginate solution (5%) in water, followed by glutaraldehyde cross-linking. Typically, gelatin (300 mg) and sodium alginate (500 mg) was mixed with distilled water (8 ml) in a conical flask and autoclaved for 15 minutes at 120 °C [30]. The hot solution was allowed to cool to ambient temperature while being stirred continuously with a magnetic bead. After that, the mixture was stirred for 15 minutes while 3 mL of pure *Bacillus subtilis* L-asparaginase was added. Glutaraldehyde was added, and the mixture was stirred for a further fifteen minutes (0.3 ml of a 25 percent solution in water). Then, this slurry was put in a dropping funnel with a plastic tip and slowly poured into a 4 °C $CaCl_2$ solution. 30 minutes were given for the beads to set up in the $CaCl_2$ solution. After the supernatant was decanted, the beads were washed with distilled water and kept in the fridge.

Bacillus subtilis L-asparaginase activity and pH and temperature effects

The activity of free and immobilized *Bacillus subtilis* L-asparaginase was assessed in the pH range of 6.0 to 10.0. 100 mM Tris-HCl (pH 6.0–10.0) was used as a buffer. To examine the impact of temperature on free and immobilized pure *Bacillus subtilis*

L-asparaginase activity, the reactions were conducted in a temperature-controlled water bath at their optimal pH values and over a temperature range of 20 to 80°C.

Electrophoresis

According to Laemmli's method [31], SDS polyacrylamide gel electrophoresis was carried out on a slab gel containing 12.5 percent (w/v) polyacrylamide (running gel) and 2.5 percent (w/v) stacking gel. The Coomassie Brilliant Blue R-250 stain was used to highlight the protein bands.

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 *Bacillus subtilis* 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 Alamar Blue reagent (10 percent Alamar Blue, Invitrogen), and incubation were 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 reference using a microplate reader. The percentage of cell viability was expressed relative to the control cells after blank normalization [32]. Morphological changes in THP-1 cells were explored and documented using phase-contrast optical microscopy at a magnification of 40.

Results

***Bacillus subtilis* L-asparaginase gene identification and sequence analysis**

A unique L-asparaginase complete sequence from the *Bacillus subtilis* genome was retrieved and added to the GenBank database (https://www.ncbi.nlm.nih.gov/protein/LN626917.1). Sequence analysis is possible since the *Bacillus subtilis* L-asparaginase gene codes for a protein with 329 amino acids and contains 990 base pairs (Figure 1). The protein sequence of *Bacillus subtilis* L-asparaginase was compared to L-asparaginase from other bacteria using the NCBI Blast server's Blast P software, yielding statistically significant high identities scores (Table 1). *Bacillus spizizenii* and *Bacillus tequilensis* 990 bp had the highest percentage of sequence identity (99.7 percent each; GenBank accession numbers TR: E0U231 and TR: A0A291B5A4, respectively), while *Danio rerio* (GenBank accession number NP 001096091.1) had the lowest percentage of sequence identity (31.9%). (Table 1).

```
1     5'atg aaa aaa tta ttg atg ttg aca act ggg gga acg att gct tca gtt gaa ggg gaa aat    60
1      M   K   K   L   L   M   L   T   T   G   G   T   I   A   S   V   E   G   E   N     20
61     ggg ctg gct ccc gga gtc aag gct gat gaa tta tta agt tac gta tca aaa ctt gat aac    120
21     G   L   A   P   G   V   K   A   D   E   L   L   S   Y   V   S   K   L   D   N     40
121   gat tac aca atg gaa act cag tcg ctt atg aat ata gac agc acc aat atg cag cct gaa     180
41     D   Y   T   M   E   T   Q   S   L   M   N   I   D   S   T   N   M   Q   P   E     60
181   tac tgg gtg gaa ata gca gaa gcc gtt aag gaa aat tat gat gcc tat gac ggg ttt gtt     240
61     Y   W   V   E   I   A   E   A   V   K   E   N   Y   D   A   Y   D   G   F   V     80
241   att act cac ggt aca gat aca atg gcc tat aca tct gcc gca cta tcg tat atg ctg cag     300
81     I   T   H   G   T   D   T   M   A   Y   T   S   A   A   L   S   Y   M   L   Q    100
301   cat gcc aaa aag ccg att gtc atc acc ggc tcg cag att ccg atc acg ttc caa aaa acc     360
101    H   A   K   K   P   I   V   I   T   G   S   Q   I   P   I   T   F   Q   K   T    120
361   gat gcc aaa aaa aat att aca gat gcc att cga ttt gcc tgt gaa ggc gtg ggc ggc gtt     420
121    D   A   K   K   N   I   T   D   A   I   R   F   A   C   E   G   V   G   G   V    140
421   tat gtt gtg ttt gac ggc aga gtc att cag gga acg cgt gcg atc aaa tta aga acg aaa     480
141    Y   V   V   F   D   G   R   V   I   Q   G   T   R   A   I   K   L   R   T   K    160
481   agc tac gac gca ttt gaa agc atc aat tac cca tat atc gct ttt atc aat gaa gac ggg     540
161    S   Y   D   A   F   E   S   I   N   Y   P   Y   I   A   F   I   N   E   D   G    180
541   atc gaa tac aac aaa caa gta acg gaa cct gag aac gac acc ttc aca gtt gac act tca     600
181    I   E   Y   N   K   Q   V   T   E   P   E   N   D   T   F   T   V   D   T   S    200
601   cta tgc aca gat gta tgt ctg ctg aag ctg cat cca ggc tta aag ccc gaa atg ttt gat     660
201    L   C   T   D   V   C   L   L   K   L   H   P   G   L   K   P   E   M   F   D    220
661   gcc ctg aaa agc atg tac aaa gga att gtc att gag agt tat ggc agc gga gga gtg ccg     720
221    A   L   K   S   M   Y   K   G   I   V   I   E   S   Y   G   S   G   G   V   P    240
721   ttt gaa ggc aga gac att ttg tca aaa gtg aat gaa ctg atc gaa agc ggc att gtc gtc     780
241    F   E   G   R   D   I   L   S   K   V   N   E   L   I   E   S   G   I   V   V    260
781   gtc att acg act caa tgt ctt gaa gaa ggc gaa gac atg agc att tac gag gtt ggc cgc     840
261    V   I   T   T   Q   C   L   E   E   G   E   D   M   S   I   Y   E   V   G   R    280
841   aga gtc aac caa gac tta att atc cga tca aga aat atg aac aca gaa gca att gtg cca     900
281    R   V   N   Q   D   L   I   I   R   S   R   N   M   N   T   E   A   I   V   P    300
901   aaa ttg atg tgg gcg cta ggt cag tct tcg gat ctt cct gtc gtc aag aga att atg gaa     960
301    K   L   M   W   A   L   G   Q   S   S   D   L   P   V   V   K   R   I   M   E
961    acg ccg ata gct gat gac gtt gtc ctg taa3'
321    T   P   I   A   D   D   V   V   L   *
```

Figure 1: *Bacillus subtilis* L-asparaginase nucleotide and deduced amino acid sequence. The L-asparaginase amino acid signature (Active site residues Threonine 12, 85, Serine 54, and Asparagine, 86) is displayed in bold underlining. The start codon (atg, Methionine) is highlighted with a double underline, and the asterisk denotes the stop codon (tag).

Table 1: *Bacillus subtilis* L-asparaginase deduced amino acid homology with organisms.

Accession	Source	Identities %
SP: ASPG1	*Bacillus subtilis (strain 168)*	100.0
TR: E0U231	*Bacillus spizizenii*	99.7
TR: A0A291B5A4	*Bacillus tequilensis*	99.7
TR: A0A8G0S6N6	*Bacillus inaquosorum*	99.1
TR: A0A6H0WJ12	*Bacillus tequilensis*	99.1
TR: A0A1Q9FYR6	*Bacillus licheniformis*	99.1
TR: A0A6H2JS86	*Bacillus mojavensis*	98.5
TR: A0A410WFE2	*Bacillus vallismortis*	98.2
TR: A0A024DCD4	*Lactobacillus crispatus*	97.6
TR: A0A150F8V1	*Bacillus nakamurai*	93.0
TR: A0A1Y0X9N3	*Bacillus amyloliquefaciens*	92.7
XP_645400.1	*Dictyostelium discoideum*	36.40%
WP_011072398.1	*Shewanella oneidensis*	33.97%
NP_310501.1	*Escherichia coli O157:H7*	32.32%
NP_001122863.1	*Caenorhabditis elegans*	33.58%
NP_001262440.1	*Drosophila melanogaster*	32.00%
NP_001096091.1	*Danio rerio*	31.49%
WP_011278186.1	*Sulfolobus acidocaldarius*	34.21%

In Figure 2A, the deduced amino acid sequence of the *Bacillus subtilis* L-asparaginase is shown in alignment with those of *Priestia filamentosa, Bacillus licheniformis, Bacillus sp., Aneurinibacillus sp., and Desmospora active*, which are representative members of the L-asparaginase family. Using the neighborhood joining method, the phylogenetic tree (Figure 2B) was constructed using the L-asparaginase amino acid sequences of *Priestia filamentosa, Bacillus licheniformis, Bacillus sp., Aneurinibacillus sp., and Desmospora*. These bacteria shared phylogenetic similarities with *Bacillus subtilis* L-asparaginase but moved to different clusters away from others.

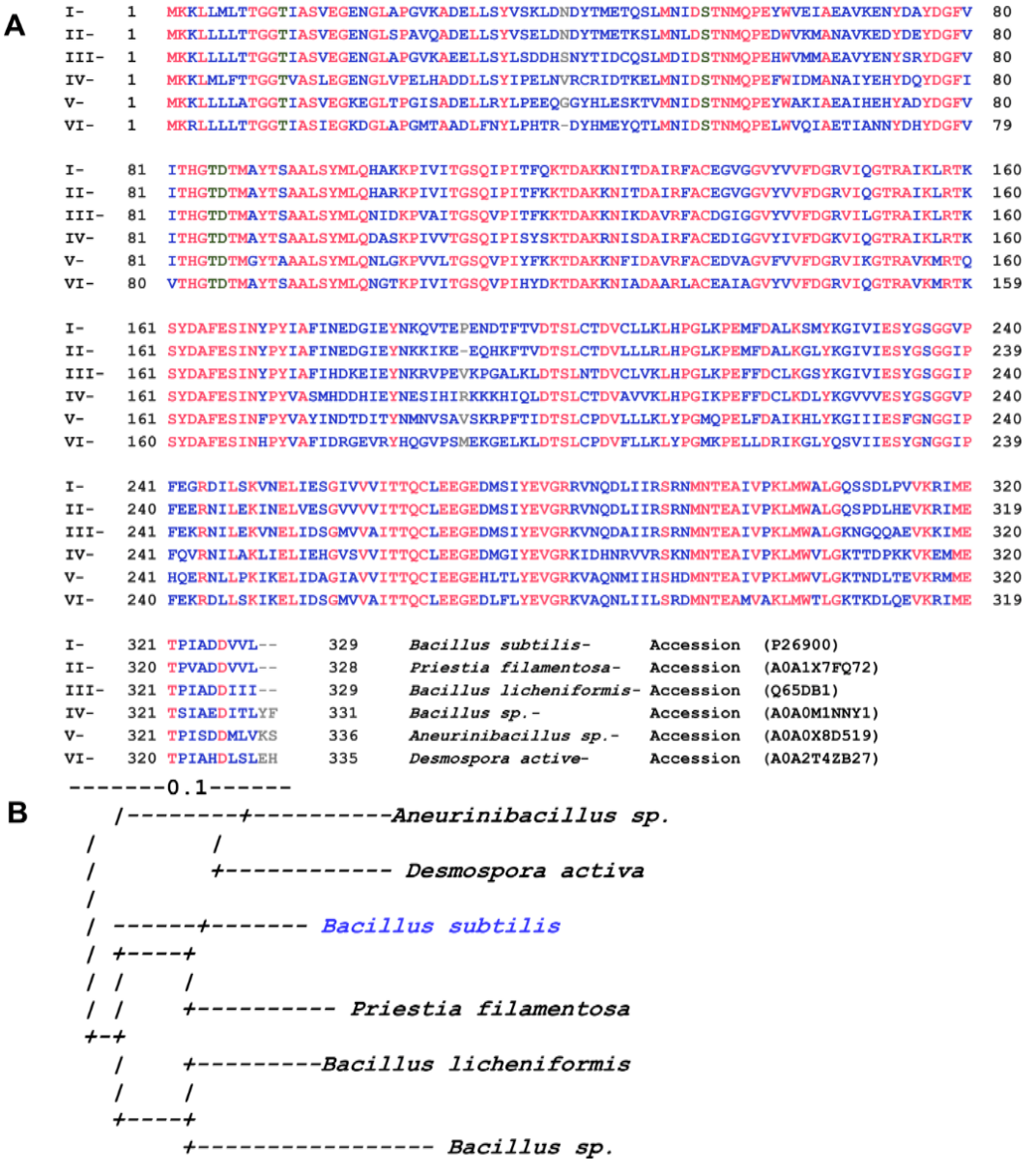


Figure 2: (A)-Pairwise alignment of L-asparaginase of *Bacillus subtilis* (I) and L-asparaginases of *Priestia filamentosa* (II), *Bacillus licheniformis* (III), *Bacillus sp.* (IV), *Aneurinibacillus sp.* (V), *Desmospora active* (VI). Red asterisks show the conserved segment near the N-and carboxy-terminal end and the green asterisks show the conserved threonine residues representing the catalytic triad threonine 12, 85, serine 54 and asparagine 86 involved in L-asparaginase catalysis. (B)- *Bacillus subtilis* L-asparaginase and L-asparaginases of *Priestia filamentosa, Bacillus licheniformis, Bacillus sp., Aneurinibacillus sp., Desmospora active*. phylogenetic relationship. Maximum probability tree based on GenBank-deposited full coding sequences.

Structures prediction for *Bacillus subtilis* L- asparaginase

The primary structure of the *Bacillus subtilis* L- asparaginase enzyme reveals that it has nonpolar, polar, hydrophobic, and aromatic amino acids as symbolized in Figure 3A. The sequence elucidation of *Bacillus subtilis* 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 86, Threonine 12, 85, and Serine 54, all of which were involved in substrate (Asparagine) recognition, binding, and catalysis. The Domaine from 3-187 is responsible for asparaginase-InterPro annotation. The active site Threonine 12 is Automatic Annotation and O-isoaspartyl threonine intermediate1 Automatic Annotation. Also, residues Threonine 85- Asparagine 86 is substrate binding1 automatic annotation. The secondary structure of *Bacillus subtilis* L-asparaginase (Fig. 3B) was predicted to have up to 13 Alpha helix structures (28.27%) and 14 Extended strand structures (14.29%), as well as a substantial number of locations for advantageous 22 Random coil and turn formation (57.45 %). *Bacillus subtilis* L-asparaginase 3D was predictable to be a homodimer (Fig. 3 C), which was similar to h L- asparaginase3 in 3D structure with 13 helix and 14 strands, (Fig. 3D and E). T_{82}HGTDTMAYTSAALSYMLQ$_{100}$ was found in *Priestia filamentosa, Bacillus licheniformis, Bacillus sp., Aneurinibacillus sp.,* and *Desmospora active*. L-asparaginases. S_{161}YDAFESINYPY$_{172}$ was revealed to play a key role in the cleavage reaction and autoactivation of *Bacillus subtilis* L-asparaginase in the presence of high threonine concentrations (Fig. 3A).

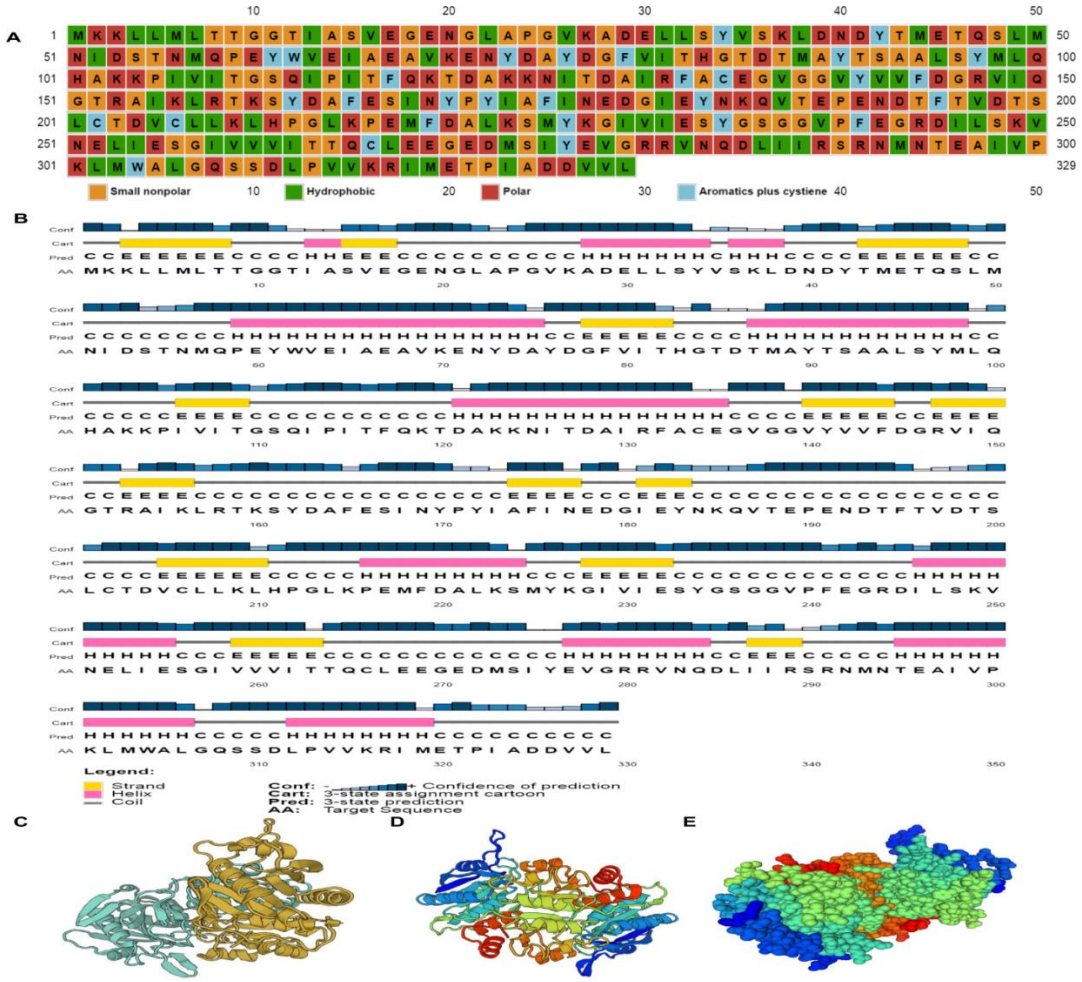


Figure 3: (A) Primary structure and position of nonpolar, polar, hydrophobic, and aromatic amino acid in *Bacillus subtilis* L- asparaginase. (B) *Bacillus subtilis* L-asparaginase secondary structure. Yellow boxes (- strand) and pink boxes (-helix) and gray boxes (-coil) represent protein secondary structural components. (C-E) A model of the expected 3D structure of *Bacillus subtilis* 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 Bacillus subtilis L- asparaginase

The expasy database recognizes the amino acid profile of *Bacillus subtilis* L-asparaginase. The chemical formula of the L-asparaginase enzyme, which has 329 amino acids and a total of 5125 atoms, is $C_{1618}H_{2571}N_{411}O_{508}S_{17}$. This enzyme has a theoretical molecular weight of 36454.68 Da with PI 4.65. *Bacillus subtilis* L-asparaginase repeats the amino acids leucine and isoleucine 25 and 28 times, with percentages of 7.6 percent and 8.5 percent, respectively. *Bacillus subtilis* L-asparaginase contains the amino acids arginine and threonine 10, and 25 times, respectively, with percentages of 3 and 7.6. There are four cysteine and two

tryptophane in the *Bacillus subtilis* L- asparaginase. There is a total of 30 positively charged residues (Arginine + Lysine) and 47 negatively charged residues (Aspartic + Glutamine) (Table 2).

Table 2: Amino acids composition of *Bacillus subtilis* L- asparaginase

Amino Acid		Symbol	Number	Percent
1.	Alanine	A	20	6.1%
2.	Arginine	R	10	3%
3.	Asparagine	N	14	4.3%
4.	Aspartic	D	20	6.7%
5.	Cystine	C	4	1.2%
6.	Glutamine	Q	10	3%
7.	Glutamic	E	25	7.6%
8.	Glycine	G	24	7.3%
9.	Histidine	H	3	0.9%
10.	Isoleucine	I	28	8.5%
11.	Leucine	L	25	7.6%
12.	Lysine	K	20	6.1%
13.	Methionine	M	13	4%
14.	Phenylalanine	F	9	2.7%
15.	Proline	P	12	3.6%
16.	Serine	S	20	6.1%
17.	Threonine	T	25	7.6%
18.	Tryptophane	W	2	0.6%
19.	Tyrosine	Y	15	4.6%
20.	Valine	V	28	8.5%

Time course and expression of *Bacillus subtilis* L- asparaginase polypeptide

The L- asparaginase gene was amplified by PCR from *Bacillus subtilis* chromosomal DNA using the essential forward and reverse oligonucleotide primers, yielding the predicted 1 kbp DNA fragment with adjacent nucleotides (Fig. 4A), which included the 990 bp L- asparaginase gene with surrounding DNA. The PCR product was ligated into the *Bam* HI restriction site of the pGEX-2T DNA plasmid along with the IPTG-inducible Tac promoter and the lacI repressor control (Fig. 4B). The plasmid Tac promoter was correctly positioned and the L-asparaginase gene was in-frame. Fig. 4C shows the progression of putative *Bacillus subtilis* L-asparaginase polypeptide induction throughout time. A recombinant plasmid was used to transform *E. coli* competent cells at a time of 0 hours, and 1 mM IPTG was used to overexpress the L-asparaginase protein, with samples being taken every hour. L-asparaginase overproduction in *Bacillus subtilis* was visible after 2 hours of IPTG induction (Fig. 4C, lane 5), and peak expression was attained after 5 hours (Fig. 4C, lane 8). The L-asparaginase polypeptide showed the greatest expression 5 hours after IPTG induction. Under the direction of the T7 promoter of the pGEX-2T DNA vector, the *Bacillus subtilis* L-asparaginase coding sequence was cloned and generated in *E. coli* BL21 (DE3) pLysS.

The glutathione sepharose 4B column matrix was used to bind the GST fusion recombinant L-asparaginase, and a solution containing 10 mM reduced glutathione was used to elute the enzyme from the column. A single 36 kDa band was discovered on SDS-PAGE for purified recombinant *Bacillus subtilis* L- asparaginase (Fig. 4D). With a total yield of 56.14 percent and a specific activity of 1769.13 U/mg proteins in the purified recombinant enzyme, purification was successful (Table 3).

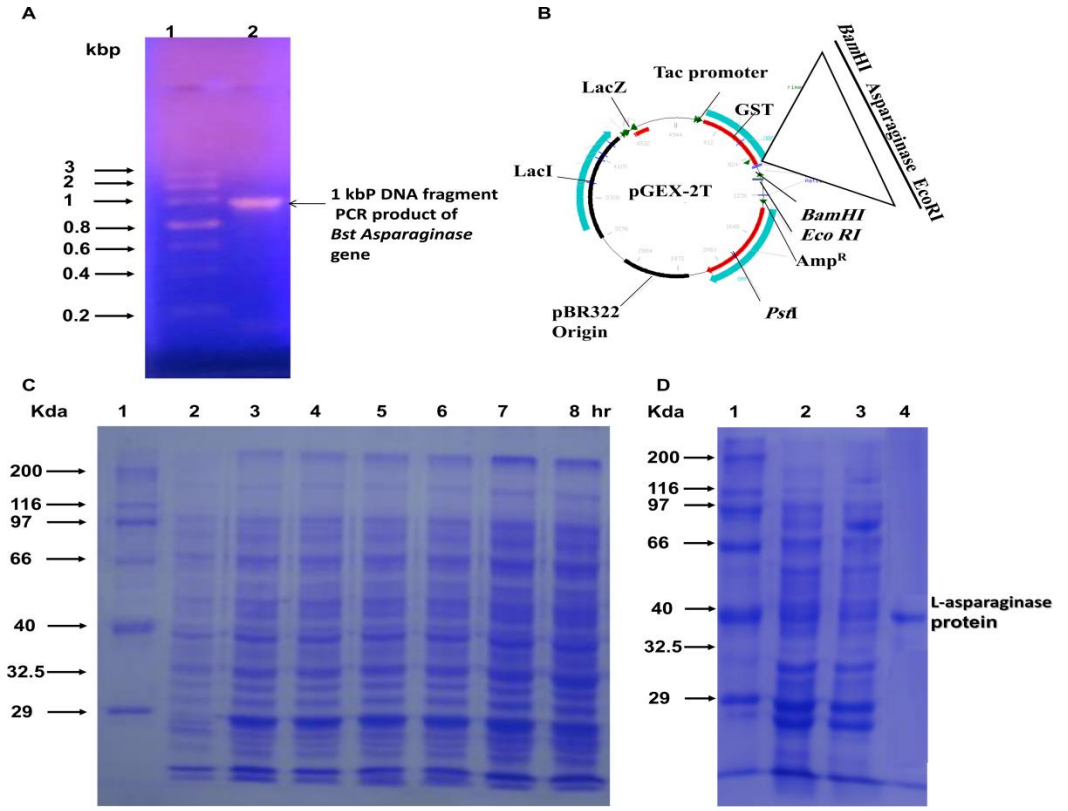


Figure 4: (A)-The PCR-produced L- asparaginase gene of *Bacillus subtilis*, a 1 kbP DNA fragment. On a 1.3 percent TAE agarose gel, the DNA fragment was examined. Lane 1: DNA standard (Gel pilot Wide range ladder 100 -Qiagen). Lane 2: PCR product of the L-asparaginase gene, a 1 kbP DNA fragment. (B) - Diagrammatic representation of the construct for the overexpression of Bacillus subtilis L-asparaginase. The ampicillin resistance gene, the lacI and lacZ repressors, the pBR322 origin, and the L- asparaginase gene were all included in the pGEX-2T DNA expression vector along with the Tac promoter. (C) - L-asparaginase protein overexpression induction time cycle. Samples were extracted and analyzed by 10 percent SDS-PAGE gel at the timings stated (Lane 2-8), protein marker Lane 1 Sigma SD6H2, and IPTG at a final concentration of 1 mM at time 0 hours of an early to mid-log culture of *E. coli* BL21 with L- asparaginase recombinant plasmid (M. Wt 25,000-200,000 KDa). (D) The L-asparaginase protein purification profile on SDS-PAGE. Protein marker in lane 1, crude E. coli L-asparaginase extract in lane

2, and crude extract that has been treated with PEI in lane 3. L-asparaginase was eluted from the glutathione sepharose 4B column in lane 4.

Immobilization of *Bacillus subtilis* L- asparaginase

Calcium alginate gelatin composites effectively immobilize *Bacillus subtilis* L-asparaginase in the presence of glutaraldehyde. It can be seen that the immobilization method is particularly suitable for the *Bacillus subtilis* L-asparaginase because the immobilized enzyme has 74% of the activity of the native enzyme.

Table 3: *Bacillus subtilis* L-asparaginase purification

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	50	718	7248	10.09	100	1.00
Polyethyleneimine	50	687	7139	10.39	98.62	1.03
Glutathione Sepharose 4B	6	2.3	4069	1769.13	56.14	173.34

Effects of pH on *Bacillus subtilis* L- asparaginase activity

The impact of pH on the functions of free and immobilized *Bacillus subtilis* L-asparaginase is shown in Figure 5A. In a pH range of 6 to 10, the activities of free and immobilized *Bacillus subtilis* L-asparaginase were assessed. It was revealed that pH 8.0 is ideal for the action of free *Bacillus subtilis* L-asparaginase (Fig. 5A). L- asparaginase from purified *Bacillus subtilis* is active in alkaline solutions. The ideal pH for the immobilized enzyme was discovered to be pH 8.5. (Fig. 5A). The optimal pH of immobilized L-asparaginase from *Bacillus subtilis* is greater than that of free enzyme.

Effects of temperature on *Bacillus subtilis* L- Asparaginase activity

Bacillus subtilis L- asparaginase was pre-incubated for 30 minutes at temperatures between 20 and 80 °C, and the ideal temperature for *Bacillus subtilis* L-asparaginase activity was revealed. As shown in Fig. 5B, pH 8.0 and 45 °C were the ideal conditions for free *Bacillus subtilis* L- asparaginase activity. The optimal temperature for the immobilized *Bacillus subtilis* L- asparaginase is now recorded at 50 °C. The optimum temperature of the immobilized *Bacillus subtilis* L-asparaginase is higher than that of the free enzyme.

Heat stability of free and immobilized *Bacillus subtilis* L- asparaginase

The heat stability of free and immobilized *Bacillus subtilis* L-asparaginase was compared at 55 °C for the specified amount of time (30 min). According to the information in Figure 5C, the thermal stability of the immobilized *Bacillus subtilis* L- asparaginase was significantly improved. For instance, after undergoing a 30-

minute heat treatment at 55 °C, the free enzyme still held 21% of its initial activity, whereas the immobilized enzyme retained 67%, showing a 3-fold increase in heat stability at the specified conditions.

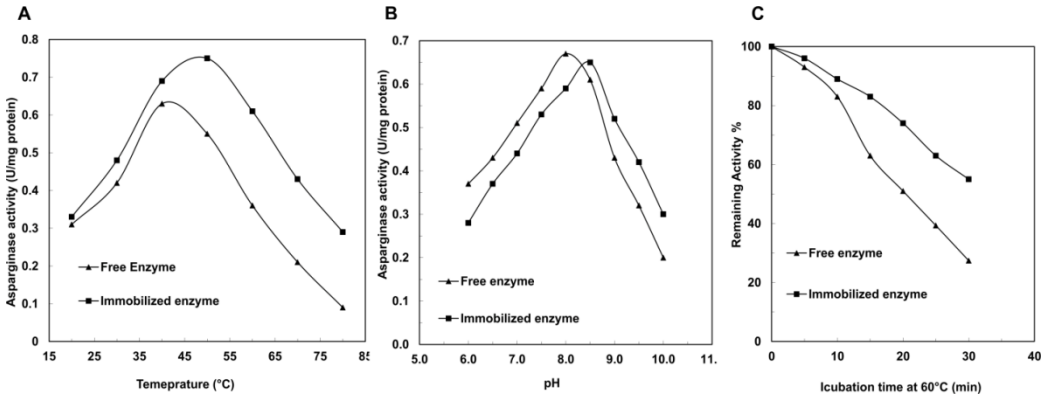


Figure 5: (A)-Effect of pH on the activity of free and immobilized *Bacillus subtilis* L-asparaginase. (B)- Effect of temperature on the activity of free and immobilized *Bacillus subtilis* L- asparaginase in 100mM Tris–HCl buffer (pH 8.0 for free asparaginase II and pH 8.5 for immobilized enzyme). (C)-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

Substrate specificity of *Bacillus subtilis* L-asparaginase

The lack of glutaminase activity in L-asparaginase makes it a major benefit in the treatment of acute lymphocytic leukemia. The Bacillus subtilis L-substrate asparaginase's specificity was examined using a range of reaction substrates. The pure recombinant enzyme had no activity for L-Glutamine, acrylamide, or urea at a concentration of 10 mM, but it did have the maximum activity and selectivity for the reaction substrate L-Asparagine.

Effect of metal ions, EDTA, and reducing agents on *Bacillus subtilis* L-asparaginase

Reducing agents and metal ions such as sulfate and chloride were examined (Table 4). At a concentration of 1 mM, $ZnCl_2$, $CuCl_2$, $HgCl_2$, $MgCl_2$, and $CaCl_2$ inhibited *Bacillus subtilis* L- asparaginase activity in the following order: $HgCl_2$> $CaCl_2$ >$ZnCl_2$ >$CuCl_2$> $MgCl_2$. The majority of the metal ions tested, on the other hand, reduced the activity of *Bacillus subtilis* L- asparaginase. Reducing chemicals like DTT and 2-mercaptoethanol somewhat lowered enzyme activity at 1- and 5-mM doses (Table 4). The impact of the metal-chelating agent EDTA was also investigated, and it was shown that at doses of 1 mM and 5 mM, respectively, EDTA reduced the activity of *Bacillus subtilis* L-asparaginase by 68.8% and 41.4 percent.

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

Effector	Residual Activity %	
Control	100 %	
	1mM	5mM
NaCl	103.5	89.9
KCl	104.9	88.6
$HgCl_2$	35.6	23.9
$CaCl_2$	74.7	63.1
$CuCl_2$	81.3	69.4
$MgCl_2$	91.2	82.5
$ZnCl_2$	74.9	57.8
Na_2SO_4	77.6	59.4
$CuSO_4$	70.2	53.1
$MgSO_4$	49.2	41.7
$NiSO_4$	55.2	43.5
EDTA	68.8	41.4
DDT	72.6	65.7
2-C_2H_5SH	97.4	90.6

Cytotoxicity of recombinant *Bacillus subtilis* L- asparaginase on cell lines

Purified recombinant *Bacillus subtilis* L- asparaginase was tested for effects on the human leukemia cell line THP-1 using varying concentrations of pure *Bacillus subtilis* L- asparaginase. The results showed that after 48 hours of therapy, significant morphological changes were found (Fig. 6 A and C). The quantity, size, and shrinkage of the enzyme-treated cells were decreased, and intracytoplasmic granules and apoptotic bodies were formed. The morphological alterations in cells treated with paclitaxel at a concentration of 20 M as a positive control compared to untreated cells (Fig. 6 A-B). These pronounced structural changes in cells appear to be detrimental to cell viability and cell death. Recombinant *Bacillus subtilis* L-asparaginase was tested to see if it had any impact on THP-1 cell survival using the Alamar Blue assay. With an IC_{50} of 1.2 IU, recombinant L-asparaginase decreased cell viability in a dose-dependent manner (Fig. 6 D).

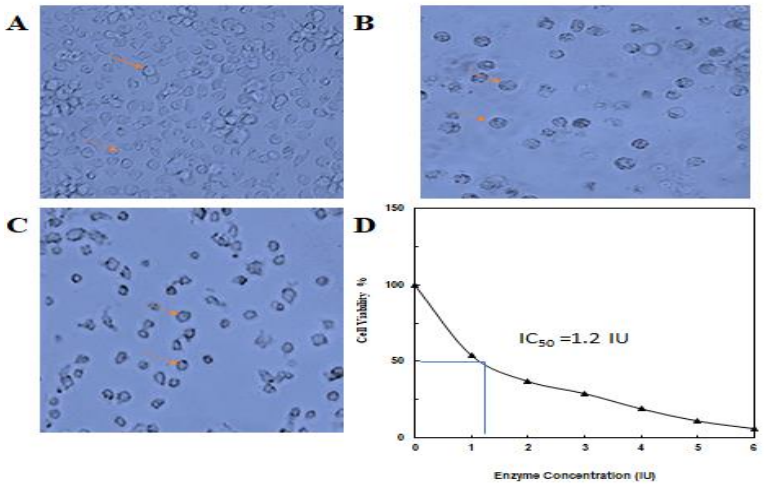


Figure 6: The shape of human leukemia THP-1 cells is altered by recombinant *Bacillus subtilis* L- asparaginase. Purified recombinant L- asparaginase at a concentration of 1 IU was used to treat cells for 48 hours THP1 cells that had not been treated (A), paclitaxel-treated cells (B), and purified recombinant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

Discussion

To induce overproduction of commercially fundamental pharmaceutical enzymes like L-asparaginase, the recombinant DNA technology was applied in a bacterial host. This enzyme is administered by several genetic components that are present in many bacterial genera. L-asparaginase is present in an operon with L-asparaginase B, which codes for L-asparaginase, in the bacterium *Bacillus*. The L-asparaginase AB operon is inhibited by L-asparaginase R, and asparagine or aspartate are thought to control L-asparaginase R's activity. The L-asparaginase gene has been cloned, overexpressed, and described in a non-pathogenic strain of *Bacillus subtilis*. The protein sequences of the L-asparaginases from *Streptococcus pneumonia*, *Clostridioides, Deinococcus radiodurans*, and *Escherichia coli* str. K-12 substr were compared to those of the L-asparaginases from *Bacillus subtilis*. *Escherichia coli* O157:H7 strain MG1655. Sakai utilizes the NCBI Blast server's Blast P software. Sequence annotation by structure reveals that the L-glutaminase active site signature, seen in the majority of microbial L-asparaginases, such as those from *E. coli and E. chrysanthemi*, is absent from the *Bacillus subtilis* L-asparaginase. These L-asparaginases exhibit dual activity against both L-asparagine and L-glutamine, the reaction substrates, and makeup 2–10% of their L-asparaginase activity [33]. Acute lymphoblastic leukemia patients' treatment has resulted in the development of immunogenicity and cytotoxicity, which has a substantial bearing on this *Bacillus subtilis* L- asparaginase property [34]. The 60-

kDa lysophospholipase enzyme hydrolyzes lysophospholipids and L-asparagine. This enzyme is related to the L- asparaginase found in *E. coli* types I and II and is a member of the bacterial type family. This enzyme is the same as the L-asparaginases found in *E. coli* types I and II. Human L-asparaginase is a plant-type L-asparaginase that breaks down asparagine-bound carbohydrate groups in lysosomes [35- 36]. Third, human L-asparaginase is h asparaginase3, a structurally similar plant type L-asparaginase to *E. coli* type III L-Asparaginase [37-38]. In the presence of the free amino acid serine, the conserved sequence G_{228}IDGMVIEALGQG$_{240}$ plays a role in the auto-cleavage, self-activation, and catalytic activity of h asparaginase3 [39]. Two threonine residues 12 and 85 make up the catalytic triad of *Bacillus subtilis* L-asparaginase. These residues are necessary for the enzyme's catalytic activity toward the L-asparagine substrate. The important and crucial threonine residue in the sequence T_{82}HGTDTM$_{88}$ is Thr85, which is not required for autocleavage but is vital for catalysis because the hydroxyl group of Thr85 acts as an activator for the hydroxyl group of Thr87 [41]. Thr219 (in humans) and Thr85 (in *Bacillus subtilis* L-asparaginase) are the third and fourth threonine residues in the catalytic triad of both h L-asparaginase3 and these enzymes (in *Bacillus subtilis*). This conserved threonine residue as well as the nearby glycine moiety influence the mobility of the glycine rich-region, which is a T_{82}HGTDTM$_{88}$ loop at the N-terminal region of the L- asparaginase that modifies the conformation between the cleavage and un-cleavage states (Gly_{84}). Because of this, there may be some similarities in the catalytic mechanism of h asparaginase and *Bacillus subtilis* L- asparaginase towards the L- asparagine substrate. The action begins with a nucleophilic attack by the Thr_{85} 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_{86} side chain and the Gly_{84} carbonyl atom. The oxyanion hole [40] has been proposed as a mechanism for stabilizing negatively charged tetrahedral intermediates and it is said to contain Thr_{12} and Thr_{86} residues. *Pyrobaculum calidifontis* thermostable L- asparaginase was shown to have an optimal temperature of at least 100 °C and a pH of 6.5 [42]. The optimal pH and temperature for pure thermostable L- asparaginase from *Bacillus amyloliquefaciens* were 8.5 and 65 °C, respectively [43]. This finding is important because glutaminase activity, which is frequently linked to L-asparaginase activity in *E. coli* and *E. chrysanthemi* [33], induces cytotoxicity. These findings are further supported by the sequence explanation by structure results, which showed that the L. glutaminase signature is not present in *Bacillus subtilis* L-asparaginase. L-Asparaginase therapy in people with acute lymphoblastic leukemia has been linked to hypertriglyceridemia, liver function, hepatic transaminase impairment, bilirubin, and alkaline phosphatase increases, among other side effects [44]. Additionally, hepatic transaminase, alkaline phosphatase, and bilirubin levels were raised in 30–60% of patients receiving L- asparaginase as a component of multiagent therapy [45]. Although L- asparaginase has been shown to have antileukemic and anticancer properties [46], further research is still needed to determine how recombinant *Bacillus subtilis* L- asparaginase affects human leukemia and cancer cells. Our research indicates that the deamination of the non-essential amino acid L- asparagine to L- aspartic, which lowers the asparagine pool, is the mechanism by which the pure recombinant *Bacillus subtilis* L- asparaginase kills human leukemia cells, THP-1. By creating L-asparaginase from *Pseudomonas aeruginosa* using solid-state fermentation, the properties of the enzyme are improved [47]. By

using untreated biomass leftovers, this enzyme might be produced at a low cost. Due to this enzyme's exceptional qualities, including its ability to function at 37 °C and an alkaline pH range, leukemia is one of its primary uses. Even though L-asparagine is a non-essential amino acid, it can cause two different types of cancer and leukemia cells to become angular. First of all, L- asparagine is necessary for the synthesis of glycoproteins and other cellular proteins. Secondly, because these cells have low levels of L- asparagine, the enzyme that balances it, they get starved and eventually die. Other researchers found that asparagine mRNA, protein, and activity levels in acute lymphoblastic leukemia patients differ greatly and aren't always linked to in vitro treatment resistance [48]. Asparaginase is a type of enzyme. As a result, in addition to asparagine control, there may be another mechanism of resistance to L- asparaginase.

Conclusion

Finding the L-Asparaginase with the optimum therapeutic properties is challenging since microbial L-asparaginase is a crucial component of juvenile acute lymphoblastic leukemia. The correct management of treatment-related toxicity calls for the ongoing development of new enzyme sources and improvements to current products. In this study, recombinant *Bacillus subtilis* L-asparaginase was overexpressed, purified, and characterized with notable selectivity for L-asparagine without glutaminase activity. Human leukemia cell lines THP-1 experienced cytotoxicity due to the recombinant enzyme. Therefore, further studies are required to determine the immunogenicity and toxicity of recombinant Bacillus subtilis L-asparaginase before it can be considered a promising alternative enzyme for the treatment of acute lymphoblastic leukemia. However, it is anticipated that this research will have a substantial impact on the development of new anti-leukemic drugs.

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References

1. Siechiechowcicz, K. and Ireland, R. (1989) Isolation and properties of an asparaginase from leaves of Pisum sativum. Phytochem. 28, 2275.
2. Mercado, L. and Arenas, G. (1999) Escherichia coli L-asparaginas induced phosphorylation of endogenous polypeptide in human immune cells. Sangre (Brac), 44, 438-442.
3. Youssef, M.M and Al-Omair, M. A. (2008). Cloning, Purification, Characterization, and Immobilization of L-asparaginase II from E. coli W3110. Asian Journal of Biochemistry 3(6):337-350. DOI: 10.3923/ajb.2008.337.350.
4. Darwesh, D. B; Al-Awthan, Y. S; Elfaki, I; Habib, S. A; Alnour, T. M;

Darwish, A. B. and Youssef, M. M. (2022). Anticancer Activity of Extremely Effective Recombinant L-Asparaginase from *Burkholderia pseudomallei.* J. Microbiol. Biotechnol. 32(5): 551-563. https://doi.org/10.4014/jmb.2112.12050.

5. Cedar, H., and Schwartz, J. H. (1967). Localization of the two-L-asparaginases in anaerobically grown Escherichia coli. J Biol Chem.242(16):3753-5.
6. Schwarts J.H., Reeves J.T., Broome J.D. (1966). Two L-asparaginase from E. coli and their action against tumors. Proc. Natl. Acad. Sci. USA, 56: 1516-1519.
7. Harms E., Wehner A., Jennings M.P., Pugh K.J., Beacham I.R., Roehm, K.H. (1991). Construction of expression systems for Escherichia coli asparaginase II and two-step purification of the recombinant enzyme from periplasmic extracts. Protein Exp. Purif., 2:144-150.
8. Aghaiypour K., Wlodawer A., Lubkowski J. (2001). Structural basis for the activity and substrate specificity of Erwinia chrysanthemi L-asparaginase. Biochemistry. 40: 5655- 5664.
9. Geckil H., Gencer S., Ates B., Ozer U., Uckun M., Yilmaz I. (2006). Effect of Vitreoscilla hemoglobin on production of a chemotherapeutic enzyme, L-asparaginase, by Pseudomonas aerugionosa. Biotechnol. J., 2: 203-208.
10. Clavell L.A., Gelber R.D., Cohen H.J., Hitchcock-Bryan S., Cassady J.R., Tarbell N.J., Blattner S.R., Tantravahi R., Leavitt P., Sallan S.E. (1986). Four agent induction and intensive asparaginase therapy for the treatment of childhood acute lymphoblastic leukemia. New Engl. J. Med., 315: 657-663.
11. Nandakumar R., Yoshimune K., Wakayama M., Moriguchi M. (2003). Microbial glutaminase: biochemistry, molecular approaches, and applications in the food industry. J. Mol. Catal. B, 23: 87-100.
12. Kijima K., Suzuki H. (2003). Improving the umami taste of soy sauce by the addition of bacterial γ-glutaminase as a glutaminase to the fermentation mixture. Enzyme Microb. Technol., 32: 431-438.
13. Wakayama M., Yamagata T., Kamemura T., Boontim N., Yano S., Yoshimune K., Moriguchi M. (2005). Characterization of salt tolerant glutaminase from Stenotrophomonas maltophilia NYW-81 and its application in Japanese soy sauce fermentation. J. Ind. Microbiol. Biotechnol., 32: 383-390.
14. Sun D., Setlow P. (1993). Cloning and nucleotide sequence of the Bacillus subtilis ans^{R} gene, which encodes a repressor of the ans operon coding for L-asparaginase and L-aspartase. J. Bacteriol., 175: 2501-2506.
15. Fisher S.H., Wray L.V. Jr. (2002). Bacillus subtilis 168 contains two differentially regulated genes encoding L-asparaginase. J. Bacteriol., 184: 2148-2154.
16. Cammack, K. A; Marlborough, D. I., and Miller, D. S. (1972). Physical properties and subunit structure of L-Asparaginase isolated from Erwinia carotovora. Biochem J.: 126(2), 361-379.
17. Broome, J. D. (1981). L-Asparaginase: discovery and development as a tumor-inhibitory agent. Cancer Treat Rep. 65 Suppl 4:111-4.
18. Sindhu, R; Manonmani, H.K. (2018) Expression and characterization of recombinant l-asparaginase from Pseudomonas fluorescens. Protein Expr

Purif 143:83–91. https://doi.org/10.1016/j. pep.2017.09.009.
19. Benchamin, D; Sreejai, R; Sujitha, S; Albert, C. (2019). Anti-proliferative activity of l-asparaginase enzyme from fungi on breast cancer. J Pharmacogn Phytochem 8(1):407–410.
20. Baskar, G; Aiswarya, R; Renganathan, S. (2019) Applications of asparaginase in food processing. In: Parameswaran B (ed) Green bio-processes, energy, environment, and sustain.nability. Springer, Berlin. https://doi.org/10.1007/978-981-13-3263-0.
21. Ahmad, N; Pandit, N; Maheshwari, S. (2012) l-asparaginase gene a therapeutic approach towards drugs for the cancer cell. Int J Biosci 2(4):1–11.
22. Benchamin, D; Sreejai, R; Sujitha, S; Albert, C. (2019) Anti-proliferative activity of l-asparaginase enzyme from fungi on breast cancer. J Pharmacogn Phytochem 8(1):407–410.
23. Youssef, M. M., Al-Omair, M. A. and Picksley, S. M. (2014). Genetic characterization of the Escherichia coli RecN protein as a member of the SMC family of proteins. Arabian Journal of Chemistry 7, 327–334.
24. Sambrook, H. Molecular cloning: a laboratory manual. (1989) Second ed., Cold spring harbor laboratory press, New York.
25. Balcao, V.M., Mateo, C., Fernandez-Lafuente, R., Malcata, F.X., Guisan, J.M., (2001). Structural and functional stabilization of l-asparaginase via multisubunit Immobilization onto highly activated supports. Biotechnol. Prog. 17, 537–542.
26. Bradford, M.A., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
27. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M. (2008). Phylogeny. fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36:465–469.
28. Milburn, D., Laskowski, R.A. Thornton, J.M. (1998). Sequences annotated by structure: a tool to facilitate the use of structural information in sequence analysis, Protein Eng. 11 (10) 855–859.
29. Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer F.T., de Beer, T.A.P., Rempfer, C., Bordoli. L. (2018). SWISS-MODEL: homology modeling of protein structures and complexes, Nucleic Acids Res. 46 (W1) W296–W303.
30. Youssef, M. M. (2015). Overexpression, Purification, Immobilization, and Characterization of Thermophilic Lipase from *Burkholderia pseudomallei*. American Journal of Microbiology and Biotechnology.2 (6): 82-91.
31. Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
32. Tabandeh, M.R. and Aminlari,M. (2009). Synthesis, physicochemical and immunological properties of oxidized inulin–L-Asparaginase bioconjugate, J. Biotechnol. 141 (3–4) 189–195.
33. Narta, U.K., Kanwar, S.S., Azmi, W. (2007). Pharmacological and clinical evaluation of L- Asparaginase in the treatment of leukemia, Crit. Rev. Oncol. Hematol. 61 (3) 208–221.

34. Nomme, J., Su, Y., Lavie, A. (2014). Elucidation of the specific function of the conserved threonine triad responsible for human L-Asparaginase autocleavage and substrate hydrolysis, J. Mol. Biol. 426 (13) 2471–2485.
35. Oinonen, C., Tikkanen, R., Rouvinen, J., Peltonen, L. (1995). Three-dimensional structure of human lysosomal aspartylglucosaminidase, Nat. Struct. Biol. 2 (12) 1102.
36. Youssef, M. M., Al-Omair, M. A. (2015). Identifying the putative interacting partners of the heat shock protein, HtpG through phage display library screening. Asian Journal of Biochemistry 10: 1-16.
37. Bush, L.A., Herr, J. C., Wolkowicz, M., N.E. Shore, A., C Flickinger, J. (2002) A novel LAsparaginase- like protein is a sperm autoantigen in rats, Mol. Reprod. Dev. 62 (2) 233–247.
38. Evtimova, V., Zeillinger, R. Kaul, S., Weidle, U.H. (2004). Identification of CRASH, a gene deregulated in gynecological tumors, Int. J. Oncol. 24 (1) 33–41.
39. Zuo, S., Zhang, T., Jiang, B., Mu, W. (2015). Reduction of acrylamide level through blanching with treatment by an extremely thermostable L-Asparaginase during French fries processing, Extremophiles 19 (4) 841–851.

40. Tikkanen, R., Riikonen, A., Oinonen, C., Rouvinen, R., Peltonen, L. (1996). Functional analyses of active site residues of human lysosomal aspartylglucosaminidase: Implications for catalytic mechanism and autocatalytic activation. The EMBO Journal 15(12):2954-60.
41. Nomme, J., Su, Y., Lavie, A. (2014). Elucidation of the specific function of the conserved threonine triad responsible for human L-Asparaginase autocleavage and substrate hydrolysis, J. Mol. Biol. 426 (13) 2471–2485
42. Chohan,S.M. Rashid,N Sajed,. M. Imanaka, T. (2019). Pcal_0970: an extremely thermostable L-Asparaginase from Pyrobaculum calidifontis with no detectable glutaminase activity, Folia Microbiol. (Praha) 64 (3) 313–320.
43. Yim, S. Kim, M. (2019). Purification and characterization of thermostable L-Asparaginase from Bacillus amyloliquefaciens MKSE in Korean soybean paste, LWT-Food Science and Technology 109 415–421.
44. Raetz, E. A. Salzer, W.L. (2010) Tolerability and efficacy of L-Asparaginase therapy in pediatric patients with acute lymphoblastic leukemia, J. Pediatr. Hematol. Oncol. 32 (7) 554–563.
45. Parsons, S. K. Skapek, S. X. Neufeld, E. J. Kuhlman, C. Young, M. Donnelly, L. M. Brunzell, J. D. Otvos, J. D. Sallan, S. E. Rifai, N. (1997). L-Asparaginase-associated lipid abnormalities in children with acute lymphoblastic leukemia, Blood 89 (6) 1886–1895
46. Swain, A., L., Jakolski, M., Housset, D., Rao, J.K., Woldawer, A. (1993). Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. Proc. Natl. Acad. Sci. 90: 1474-1478.
47. Roberts, J., Holcenberg, J.S., Dolowy, W.C. (1972). Isolation, crystallization, and properties of Achromobacteraceae glutaminase-asparaginase with antitumor activity. *Journal of Biological Chemistry*, 247 (1), pp. 84-90.
48. Fine, B.M., Kaspers, G.J.L. Ho, M., Loonen, A.H. Boxer, L.M. (2005). A genome-wide view of the in vitro response to L-Asparaginase in acute lymphoblastic leukemia, Cancer Res. 65 (1) 291–299.