

Metagenomic discovery and functional validation of L-asparaginases with anti-leukemic effect from the Caspian Sea

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Abstract

Background

L-asparaginase has been used for the treatment of acute lymphoblastic leukemia (ALL) for more than 30 years. However, efforts continued to find new enzymes with more desirable properties due to the immunogenicity, short half-life, rapid clearance and L-glutaminase side activity of the existing commercial enzymes. Screening for novel L-asparaginases in prokaryotes as a promising resource has been mainly hampered by the cultivation/expression bottleneck.

Results

By screening 27000 publicly available prokaryotic genomes, we recovered ca. 6300 type I and ca. 5200 type II putative L-asparaginase in 36 and 42 bacterial phyla respectively highlighting the vast potential of prokaryotes for L-asparaginase activity. Caspian water with similar salt composition to the human serum was targeted for *in-silico* screening of L-asparaginase. We screened ca. three million predicted Open Reading Frames of the assembled Caspian Sea metagenomes. *In-silico* screening resulted in 87 putative L-asparaginase genes from the Caspian Sea datasets. The L-asparagine hydrolysis was experimentally confirmed by cloning three selected genes (1092, 1218 and 1011 bp) in *E. coli*. Catalytic parameters of the purified enzymes including *K_m*, *V_{max}* and catalytic efficiency were determined to be among the most desirable reported values of microbial L-asparaginases. Two of the recombinant enzymes represented remarkable anti-proliferative activity (IC₅₀ less than 1 IU/ml) against leukemia cell line Jurkat while no cytotoxic effect on human erythrocytes or human umbilical vein endothelial cells was detected.

Conclusions

Similar salinity and ionic concentration of the Caspian water samples to the human serum highlights the secretory L-asparaginases recovered from these metagenomes as potential treatment agents.

Background

L-asparaginase (EC 3.5.1.1) is an amidohydrolase catalyzing the breakdown of L-asparagine into aspartic acid and ammonia. For over 30 years, L-asparaginase has been widely used as antineoplastic agent for treatment of acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma [1–3]. Its mode of function is that it selectively kills leukemic cells by depleting patient's serum of L-asparagine, an amino acid that tumor cells essentially depend on for the malignant growth [3]. The L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* have been extensively used for medical purposes, however, arising complications such as hypersensitivity, antigenicity, short half-life, temporary blood clearance and non-desirable L-glutaminase-dependent neurotoxicity necessitate continuous explorations seeking more suitable substitutes [4–6].

Various strategies have been adopted with the common aim of achieving L-asparaginases of optimal characteristics for therapeutic purposes (Fig. 1). While some researchers focus on sequence engineering to enhance enzyme's affinity or extinguish immunogenic epitopes [7, 8], others aim to protect the enzyme from immune system by conjugating it to a variety of organic or inorganic compounds or using carriers for drug delivery [9–11]. Since L-asparaginase gene is distributed in all domains of life, various sources including bacteria, fungi, plants and animals have been screened in hope of finding enzymes with superior features [12–14]. Prokaryotic L-asparaginase specifically the secretory type is involved in recycling the organically bound nitrogen through the ammonification process, thus playing a notable role in nitrogen biogeochemical cycling [1]. The high metabolic diversity of prokaryotes renders them a promising resource for L-asparaginase screening campaigns. However, all screening efforts so far have only targeted cultivated prokaryotes [15, 16]. One major limitation of this approach is due to cultivation bottleneck; majority of prokaryotes (up to 99%) are still evading the bound of culture [17], consequently; their novel genetic contents remain inaccessible. Additionally, prokaryotes represent selective expression for different types of L-asparaginases under certain environmental condition [18, 19], thus further limiting the culture/expression-dependent screening approaches. For example, model organism *E. coli*'s type I L-asparaginases is a cytoplasmic low-affinity protein continuously expressed and required for growth, whereas its type II enzyme is periplasmic with high-affinity toward L-asparagine, expressed under anaerobic and starvation conditions acting as a scavenger to absorb nitrogen from the environment [18].

One way to bypass the limitations caused by the culture/expression-dependent screening approaches is to explore prokaryotes' genomic potential through direct screening of sequenced environmental DNA i.e. metagenomics [20–22]. In this study, we leverage the availability of metagenomic datasets derived from the brackish samples of the Caspian Sea for *in-silico* screening of putatively novel L-asparaginase genes and further verify their activity via cloning and expression analysis. As the salinity and osmolality of brackish Caspian water are akin to blood serum [15], it is expected that secretory proteins screened from this ecosystem would represent optimal activity in physiologic condition. Additionally, culture-independent *in-silico* screening of metagenomes provide an untapped reservoir of unexplored genes already available in nature [23]. In this study, the assembled metagenomes of the Caspian Sea depth profile were screened for putative L-asparaginase genes. The enzymatic activity of three selected genes were verified by cloning and expression of the synthesized gene in *E. coli* host. The enzymes were purified to homogeneity. Kinetic and stability parameters and their anticancer activity were then studied. This is the first report of *in-silico* screening and *in-vitro* activity verification of L-asparaginases with potential as a chemotherapeutic agent.

Material And Methods

Chemicals and cell lines. All chemicals were of analytical grade purity. L-asparagine, L-glutamine, Dithiothreitol (DTT) and isopropyl- β -D-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (USA). All other chemicals were obtained from Merck (Germany) unless stated differently. The Jurkat and human umbilical vein endothelial cells (HUVEC) were procured from the Iranian Biological Resource Center (IBRC C10155) and Pasteur Institute of Iran (CRL-1730), respectively.

In-silico L-asparaginase screening. Metagenomics datasets derived from three different depths of the Caspian Sea (15, 40 and 150 meter) [24] were used for screening putative L-asparaginase enzymes. The sequenced reads of these datasets were quality checked using `bbduk.sh` (<https://sourceforge.net/projects/bbmap/>) and assembled using Megahit [25]. Open reading frames (ORFs) of the assembled contigs ≥ 1 kb were predicted using PRODIGAL [26]. Identified ORFs were annotated against TIGRFAM comprising curated multiple sequence alignments, Hidden Markov Models (HMMs) for 4488 protein families using `hmmsearch` [27]. ORFs annotated as putative L-asparaginase were affiliated to either of TIGR00519 or TIGR00520 HMMs. Putative L-asparaginase ORFs were further evaluated using NCBI's Conserved Domain Database (CDD) [28] and cross checked with both hallmark L-asparaginase genes of *E.coli* using BLAST. Sequences affiliated to the TIGR00520 were selected for further analysis for the purpose of cloning and expression. Subcellular localization of sequences was predicted using 5 different online servers: Phobius, mPLoc [29–31], SecretomeP 2.0 Server [32], SignalP 4.1 Server [33], TatP 1.0 Server (Twin-arginine signal peptide) [34], if the server enquired cell wall type of the organism (Gram category) as an input, the sequence was examined twice with both possible assumptions. structure and function of selected sequences were predicted using Phyre² web-based service [35]. Predicted secretory proteins were marked to be more desirable for further analysis. Sequences affiliated to fastidious bacteria were preferably selected for cloning. Additionally, species spotted in all three depths of 15, 40 and 150 m were assumed to be more desirable as are adapted to sustain wide range of environmental conditions.

Phylogenetic analysis. Reviewed L-asparaginase sequences were collected from the UniProt (n = 138). All open reading frames annotated as putative L-asparaginase (TIGR00519 and TIGR00520) in the Annotree database were also collected (n = 12553) together with the 87 verified L-asparaginase genes identified from the Caspian Sea metagenomes. The retrieved sequences were aligned using Kalign [36] and the maximum likelihood phylogeny was reconstructed using FastTree2 (WAG + CAT model, gamma approximation, 100 bootstrap replicates) [37].

Gene cloning. Three of the candidate genes annotated as TIGR00520 were codon optimized for expression in *E. coli*. These genes were synthesized and cloned into the pET21a (+) vector between *Nde*I and *Xho*I restriction sites in fusion with a histidine tag at C terminus (General Biosystems, USA). Following transformation into competent *E. coli* BL21 (DE3) cells, bacterial pools were plated on Luria-Bertani (LB) medium supplemented with ampicillin (100 μ g/ml). Cloning accuracy was further confirmed by plasmid extraction (Yekta Tajhiz Azma Kit, Iran) and sequencing (Bioneer, Korea).

Protein expression and purification. To select the optimal expression condition, different concentrations of IPTG (0.1, 0.2, 0.3, 0.5, 1 mM), induction duration (3, 5 and 7 h), culture media (Luria Bertani (LB), Terrific Broth (TB) and Yeast Extract Tryptone (2xYT)) as well as the induction temperature (20, 30 and 37 °C) were tested. At last, pursuant to overnight pre-culture of bacteria in 10 ml LB medium (100 μ g/ml ampicillin, 37 °C, 150 rpm), pre-culture was inoculated (1% v/v) into 250 ml of fresh LB medium and left to grow at 37 °C, 150 rpm. Reaching the optical density of 0.5–0.6 at 600 nm, protein expression was induced by addition of 0.2 mM IPTG for CASpI and 0.3 mM for CASpII and III besides temperature

decrement to 30 °C. Over a period of 3 h, cells were spun down (9000 g, 10 min), concentrated in 5 ml of ice-cold lysis buffer (50 mM potassium phosphate buffer, pH 7.8, NaCl 150 mM) and lysed by sonication (recurrent periods of 10 s pulsing and 5 s resting for 10 minutes, SYCLON Ultra Sonic Cell Sonicator SKL950-IIDN). Lysate was centrifuged (9000 g, 30 min, 4 °C) and supernatant was loaded on Ni-NTA agarose column pre-equilibrated with lysis buffer. Elution of bound proteins was accomplished by the buffer containing 100 mM of imidazole (pH 6.8). Purified protein solution was dialyzed against phosphate buffer (pH 7.2, 4 °C) overnight to remove imidazole. All protein purification steps were carried out at 4 °C.

SDS-PAGE (12% w/v acrylamide) was used to confirm the purity of the enzyme. Gels were stained by Coomassie Brilliant Blue R-250 (Bio-Rad, USA). Protein concentration was estimated by BCA method using bovine serum albumin as standard [38].

Enzyme activity assessment and biochemical characterization. 1 µg/ml of purified enzymes was incubated with 4 mM of L-asparagine for 2 min in 0.5 ml of 50 mM phosphate buffer (pH 7.2) and activity was determined by real time measurement of ammonia production via colorimetric assay (Nesslerization) [39]. Absorbance of Nessler reagent complexed with ammonia was determined by Perkin Elmer lambda 25 UV/VIS spectrophotometer at 410 nm. Reaction rate was examined at different substrate concentrations (1.25 to 30 mM L-asparagine) using a standard curve of known concentrations of ammonium chloride. The data were fitted to generate a Michaelis–Menten equation, and apparent kinetic values were calculated. One unit (U) of enzyme's catalytic activity was defined as the amount of enzyme which converts one micromole of L-asparagine to ammonia per min at the condition of assay. The specific activity was expressed as U/mg protein. All assays were performed in triplicate, the means and standard deviations were presented.

Effect of pH and NaCl concentration on enzyme activity. With the intention of finding optimal activity conditions, enzyme activities were studied in various NaCl concentrations (50, 100, 150, 200 mM in 50 mM potassium phosphate buffer, pH 7.2). Optimal pH for enzyme activity was studied by measuring activity at the pH range of 5–8 in a mixed buffer of 20 mM Tris-HCl (pKa 8.06), 20 mM Bis-Tris (pKa 6.46), and 20 mM sodium acetate (pKa 4.76) possessing buffering range of 3.5–9.2 [40].

Anti-proliferation studies. Jurkat cells, clone E6-1, acute T cell leukemia cell line, were used for examining the anti-leukemic effect of our purified enzymes. The cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) and 1% Pen/Strep (100 U/ml penicillin and 100 µg/ml streptomycin) and incubated under humidified 5% CO₂ atmosphere at 37 °C. 100 µl of culture medium containing 5×10^5 cells/ml was added to 96 well plates. Purified recombinant enzymes were concentrated to various concentrations using Amicon Ultra-15 centrifugal filter. The concentrated solutions were sterilized using hydrophilic 0.22 µm Millipore filters. 100 µl of sterile enzyme solutions (10, 100 and 1000 nM) was added to each well and incubated for 24 h at 37 °C. All three concentrations of each of CASpI, CASpII and CASpIII enzymes, were evaluated in quadruplicate. 100 µl enzyme-free PBS solution was used to serve as negative control. As described previously [41], MTT

(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide) assay was performed after 24 h in order to quantify the amount of cell death in enzyme-treated groups in comparison with the untreated control group.

Enzyme Cytotoxicity assessment on non-leukemic cells. In order to assess any possible cytotoxic effect of our purified L-asparaginases on non-leukemic cells, HUVEC (human umbilical vein endothelial cells) were exposed to purified L-asparaginases (10, 100 and 1000 nM). MTT assay was performed after 24 h of enzyme addition, and the results were compared against negative controls, as described above.

In vitro hemolysis test was also performed according to available protocols with minor modifications [42, 43]. The test was done based on standard phlebotomy procedures. Briefly, 10 ml of human blood was collected in K2-EDTA-coated Vacutainer tubes and centrifuged at 500 g for 5 min. Hematocrit was washed twice with 150 mM NaCl solution and resuspended in PBS, pH 7.2. 100 µl of sterile enzyme solution with different concentrations (10, 100 and 1000 nM) were mixed with 100 µl of blood in V-bottom 96-well plates and incubated at 37 °C for one hour in orbital shaker. Serial dilutions of Triton X-100 (0 to 20%) in PBS were used as controls. Plates were centrifuged at 500 g for 5 min to pellet intact erythrocytes. Then supernatants were transferred to new 96 well plate and absorbance was read at 541 nm. Hemolysis percentage was normalized against controls.

Results And Discussion

Taxonomic distribution of prokaryotic L-asparaginase. Prokaryotic L-asparaginase is ecologically involved in transformation of organic nitrogen to release ammonia via the ammonification process [18]. Nitrogen rich compounds, peptides and amino acids are among available nitrogen sources in aquatic habitats where type II secretory L-asparaginase can be utilized for scavenging nitrogen by a range of prokaryotes [18, 19]. The secretory L-asparaginase act as a public good; providing surrounding cells by nitrogen source. To explore the phylogenomic distribution of L-asparaginase containing bacteria we perform an extensive screening of 27,000 publicly available bacterial genomes/metagenome assembled genomes (MAGs) via Annotree [44]. Our results show a vast distribution of L-asparaginase genes in bacteria where 54 out of 112 bacterial phyla contain the genetic potential for L-asparaginase activity. Many of these bacterial phyla have no representative in culture and consequently remain inaccessible to culture/expression-dependent screening approaches (Fig. 2).

A total of 6329 bacterial genomes containing genes annotated as TIGR00519 (L-asparaginase, type I) were recovered. These genomes are affiliated to 36 different bacterial phyla. 5294 bacterial genomes affiliated to 42 different phyla contain genes annotated as TIGR00520 (L-asparaginase, type II) (Fig. 2). Representatives of eight bacterial phyla contain both type I and type II L-asparaginase genes in their genomes (*Proteobacteria* 817, *Bacteroidota* 249, *Firmicutes* 175, *Firmicutes_A* 8, *Myxococcota* 5, *Firmicutes_C* 5, *Riflibacteria* 4, and *Firmicutes_B* 2). Phyla *Proteobacteria* and *Firmicutes* contain more representatives harboring the secretory L-asparaginase compared to the cytoplasmic one, whereas phylum *Bacteroidota* represent the opposite with higher frequency of representatives containing

cytoplasmic L-asparaginase (**Supplementary Fig. S1**). These screened genomes/MAGs originate from different habitats ranging from aquatic, soil, to host associated microbes showing the vast distribution of this enzyme among bacterial representatives.

In-silico screening of L-asparaginase from the Caspian Sea metagenomes. The expansive medical use of L-asparaginase necessitate continuous screening for novel L-asparaginases of superior features in order to resolve emerging complications against the existing L-asparaginases used for therapeutic purposes. Screening novel environments by tapping into the vast metabolic reservoir of yet to be cultured prokaryotic majority can serve as a highly promising resource (Figs. 1 and 2). Current therapeutic L-asparaginase enzymes originating from *E. coli* and *Erwinia chrysanthemi* respectively lose 40 and 80 percent of their activity in blood salinity (0.9%) [15, 45]. Here we target the brackish microbiome of the Caspian Sea for in-silico metagenomics exploration of novel putative L-asparaginase. The brackish salinity of the Caspian Sea represents a highly similar salinity and main ionic concentration to the human serum (130–145 and 132.44 mM Sodium in the human serum and the Caspian Sea water respectively and 3.5–5.3 and 3.04 mM potassium in the human serum and the Caspian Sea water respectively [24, 46] (**Supplementary table S1**). Therefore, we hypothesize that secretory enzymes in the brackish microbiome of the Caspian Sea could potentially represent higher stability in physiologic conditions of the human serum thus, promising desirable therapeutic applications.

In total 703171, 1169997 and 1214607 predicted open reading frames (ORF) were screened for L-asparaginase activity respectively from 15, 40, and 150 m depth profile metagenomic datasets of the Caspian Sea. A total of 175, 296, and 284 putative L-asparaginase genes were annotated as TIGR00519 or TIGR00520 HMMs from 15, 40, and 150 m depth metagenomes respectively. The putative annotation of these genes was further evaluated by inspecting the conserved regions and protein secondary structure (as explained in the methods section). Amongst them 87 genes were verified for L-asparaginase activity after *in-silico* evaluations (18, 30, and 39 respectively from 15, 40, and 150 m metagenomes). Recovered L-asparaginase genes had the length in the range of 245 to 489 amino acids (median 336 amino acids) with the highest sequence identity to representatives of Bacterial taxa *Verrucomicrobiota*, *Gemmatimonadota*, *Alphaproteobacteria*, *Acidobacteriota*, *Chloroflexota*, *Bacteroidota*, *Gammaproteobacteria*, *Patescibacteria*, *Actinobacteriota*, *Cyanobacteria*, *Firmicutes_C*, and 'Candidatus Rokubacteria' (n = 84), a single gene showing 75.7% identity to an archaeal L-asparaginase and two genes with 86.2 and 87% identity to single cell eukaryotes affiliated to *Bathycoccus* and *Micromonas* respectively (**Supplementary Table S2**).

To assess the activity and other characteristics of recovered enzymes we have selected three candidates to synthesis the enzyme coding sequence to experimentally verify their activity, *K_m*, survival percentage at human serum salinity and cellular analysis. Sequences of secretory enzymes (**Supplementary Table S3**) related to fastidious bacterial taxa were preferably selected for cloning and enzyme activity verification. Additionally, bacteria spotted in all three depths of 15, 40 and 150 meter were assumed to be more desirable as are expected to be adapted to sustain wide range of environmental conditions. Three genes were selected CAspl from 40 m metagenomics dataset and affiliated to phylum *Acidobacteriota*

(79.9% sequence identity), CAspII also from 40 m metagenomic dataset and affiliated to phylum *Gemmatimonadota* (51.8% sequence identity), and CAspIII from 15 m metagenomics dataset affiliated to phylum 'Candidatus Rokubacteria' (50.7% sequence identity) based on their best BLAST hit against NCBI NR database (**Supplementary Table S2**). Phylogenetic reconstruction of L-asparaginase genes annotated from the Caspian Sea metagenomes together with the secretory L-asparaginase genes recovered from screening bacterial genomes through Annotree is shown in Fig. 3. Highlighting the enzyme phylogeny based on genome taxonomy bring forward the possibility of horizontal transfer of type II L-asparaginase gene between different taxa.

In-silico characterizations and cloning of putative L-asparaginase. To estimate the probability of possible cross-reaction between antibodies against *E. coli* and *Erwinia chrysanthemi* L-asparaginases and selected genes from the Caspian Sea metagenomes, pairwise nucleotide sequence alignment was performed. The maximum similarity was between CAspI and *Erwinia* (49.2% similarity, 34.3% identity) which is lower than the similarity between *E. coli* type II and *Erwinia* L-asparaginase (62.8% similarity, 42.3% identity). *Erwinia* L-asparaginase is used as the second line of chemotherapy in cases that show intense immune reaction against *E. coli* L-asparaginase. As antibodies against *E. coli* L-asparaginase do not cross-react with *Erwinia* considering their sequence similarity of 62.8%, we estimated that our genes should be safe against *E. coli* and *Erwinia* antibodies based on their sequence similarity (Table 1).

Table 1
pairwise nucleotide alignment results of the selected L-asparaginases from the enzymes from *E. coli* and *E. chrysanthemi*.

Pairwise sequence alignment (%)	<i>E.coli</i> type I	<i>E. coli</i> type II	<i>E. chrysanthemi</i>
CAspI	S: 36.5 I: 21.9	S: 47.0 I: 31.7	S: 49.2 I: 34.3
CAspII	S: 32.5 I: 19.7	S: 46.5 I: 31.1	S: 47.4 I: 34.0
CAspIII	S: 35.6 I: 22.5	S: 48.3 I: 32.6	S: 49.9 I: 32.3
<i>E. chrysanthemi</i>	S: 35.6 I: 19.0	S: 62.8 I: 47.3	
<i>E. coli</i> type II	S: 36.6 I: 23.6		

Three L-asparaginases screened from the Caspian Sea metagenomes named CAspI, CAspII and CAspIII (1092, 1218 and 1011 bp respectively) were codon optimized for expression in *E. coli*, synthesized and

cloned in pET21a (+) vector in fusion with hexa-histidine tag at C-terminus (**Supplementary Table S4**).

Protein expression and purification. Subsequent to plasmid transformation and accuracy confirmation by re-sequencing, enzyme expression and activity was examined. Successfully all three recombinant enzymes showed L-asparaginase activity while the specific activity of the crude enzyme from untransformed *E. coli* was negligible; indicating that our computation metagenome-wide screening method is a promising approach for functional annotation of predicted open reading frames. After culture condition optimization, maximum specific activity of crude extract was achieved by induction of mid-log phase transformed cells for three hours at 30 °C using LB as culture medium. Optimum IPTG concentration was found 0.3 mM for CAspl and 0.2 mM for CAspll and CAsplll (**Supplementary Fig. 2**).

Recombinant CAspl, CAspll and CAsplll were purified to homogeneity by nickel-agarose affinity chromatography. SDS-PAGE single band matching the enzymes estimated molecular mass indicates the accuracy of purification (**Supplementary Fig. 3**). Molecular mass of the monomeric enzymes using their amino acid sequences were predicted to be 38.2, 43.3 and 35.6 kDa respectively which was consistent with protein migration on SDS-PAGE gel. The molecular weight of L-asparaginase protein varies according to the enzyme source i.e. for bacterial L-asparaginases, the molecular weight according to SDS-PAGE analysis is usually in the range of 35 to 40 kDa [47–49]. However, there are reported cases of very low molecular weight of 11.2 kDa for *Streptobacillus* sp. KK2S4 [50] and the high molecular weight (near 97.4 kDa) of purified L-asparaginase from *Streptomyces tendae* [51]. The specific activities of the purified enzymes were 700, 240 and 100 U/mg respectively for CAspl, CAspll and CAsplll while the specific activity of the commercial L-asparaginases from *E. coli* and *E. chrysanthemi* are between 280–400 U/mg and 650–700 U/mg respectively [4]. Phetsri et al., reported maximum specific activity of 113 U/mg for *Streptococcus thermophiles* among four species of lactic acid bacteria tested [52]. Specific activities of 833 and 155 U/mg are also stated for the L-asparaginases purified from *Thermococcus Kodakarensis* and *Acinetobacter soli*, respectively [53, 54].

Kinetic parameters of recombinant L-asparaginases. Kinetic parameters of the enzymes were calculated according to the classical Michaelis–Menten equation using L-asparagine as substrate (Fig. 4 and Table 2). K_m values of 10, 0.35 and 0.15 mM were achieved for CAspl, CAspll and CAsplll respectively. Enzyme affinity toward its substrate is reflected by the value of the K_m ; The lower the K_m value, the better binding ability of the enzyme. While the K_m value of all three enzymes are higher than *E. coli* type II and *E. chrysanthemi* L-asparaginases [4], our data suggests that CAsplll K_m is among the lowest reported L-asparaginase K_m values to date. The K_m values of CAsplll is lower than the L-asparaginases produced by *Streptomyces fradiae* NEAE-82, *Halomonas elongata* and *Enterobacter cloacae* [15, 55, 56]. This gene was recovered from a 1161 bp long contig assembled from the 15 m depth metagenomes of the Caspian Sea showing the highest protein sequence identity (50.7%) to the reconstructed metagenome assembled genome 'Candidatus Rokubacteria' bacterium AR30 (The BioSample accession number SAMN08911936). This MAG was reconstructed from meadow soil samples at 30-40cm depth, Angelo Coast Range Reserve, CA, USA (The BioSample accession number SAMN08902845). Representatives of this candidate phylum so far have evaded the bound of culture and remained inaccessible to the culture/expression-screening

campaigns [57–59]. Representatives of this *Candidatus* phylum are shown to be involved in biogeochemical cycling of elements in the soil [57–59] and harbor a vast potential for secondary metabolites biosynthesis [60]. While the CAspIII is only distantly related to the L-asparaginase gene of the ‘*Candidatus* Rokubacteria’ bacterium AR30, it most probably belongs to the rare and fastidious microbiome of the Caspian Sea; however, the taxonomic affiliation cannot be assured only based on the BLAST identity. Additionally, the representatives of the ‘*Candidatus* Rokubacteria’ are ubiquitous in a diverse range of terrestrial ecosystems and subsurface habitats with no reported marine representative [59]. CAspII and CAspIII also revealed catalytic efficiencies of approximately 10-fold higher than CAspI suggesting that they could metabolize asparagine more efficiently [61].

Table 2
Biochemical characteristics of the recombinant L-asparaginases.

L-asparaginase	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)	V_{max} ($\mu mol\ min^{-1}$)
CAspI	446 ± 20	10 ± 1	44.6	0.35 ± 0.02
CAspII	174 ± 8	0.35 ± 0.02	497.14	0.12 ± 0.01
CAspIII	59.3 ± 4	0.15 ± 0.02	395.4	0.05 ± 0.004

Effect of pH and NaCl concentration on enzyme activity. The activity of the purified enzymes was studied in the pH range of 5–8 (Fig. 5a). All recombinant enzymes displayed maximal L-asparaginase activity at pH 7.5 that is favorable feature of these enzymes where maintaining optimal enzymatic activity at the physiological pH is one of the prerequisites for antitumor activity [62]. A sharp decrease in activity was observed at more acidic or basic pH in the case of CAspII. CAspI activity was increased gradually up to pH 7 and retained its maximum activity up to pH 8. CAspIII showed maximum activity at pH 6.5–7.5. The amidases enzymes such as L-asparaginases are mostly active and stable at neutral and alkaline pH ranges of 5–9 [63]. L-asparaginase, purified from alkaliphilic *Streptomyces fradiae* NEAE-82, exhibited maximum activity at pH 8.5 [55]. The optimal L-asparaginase activity from *Halomonas elongata* was reported to be at pH 6–9 [15]. Maximum activity at pH 8 was obtained for purified L-asparaginase from *Pyrococcus furiosus* [64].

Effect of NaCl concentration on the enzyme activity is shown in Fig. 5b. As expected, it can be observed that 140 mM NaCl (equal to physiologic 0.9% saline solution) had no adverse effect on enzymatic activity of the recombinant L-asparaginases; furthermore, it is consistent with blood Na⁺ concentration that ranges between 130–145 mM [46]. It should be noted that both commercial L-sparaginases and most reported enzymes show decreased activity when subjected to physiological salinity [45]. This reiterate the adaptive advantage of secretory enzymes recovered from the brackish waters of the Caspian Sea to retain their activity in the salinity of human serum due to similar ionic concentration. L-asparaginase isolated from the halophilic *H. elongata* also retained its maximum activity at physiologic salinity [15].

Anti-leukemic assessment. L-asparaginases have been isolated from various sources but all do not have cytotoxic effects on cancerous cells. The cytotoxicity of recombinant L-asparaginase was examined on

human lymphoblastic leukemia cell line, Jurkat, by MTT assay (Fig. 7a). After 24 h of incubation, CAspIII and II proved to be highly effective against the leukemic cell line with IC₅₀ of 120 and 33 nM which are equal to 0.6 and 0.06 IU/ml of the enzymes respectively. However, the commercial L-asparaginase from *E. coli* has IC₅₀ of 1.0 IU/ml and that of *Erwinia* has been reported to have IC₅₀ of 7.5 to 10.0 IU/ml [65]. These results clearly indicate that the purified recombinant CAspII and CAspIII can be considered as effective chemotherapeutic agents in killing human leukemic cell line, Jurkat, primarily due to depletion of the asparagine pool. Although asparagine is a nonessential amino acid, it is vital for some leukemia and cancer cells for two reasons. Firstly, asparagine is required for the synthesis of glycoproteins and other cellular proteins, and secondly, these cells have low expression level of L-asparagine synthetase for *de novo* synthesis of asparagine [64]. The IC₅₀ value for CAspI was higher than 1000 nM (13 IU/mL), the highest concentration used in the dose-response curve. As seen in Fig. 6a, no tested concentration of CAspI could reduce cell viability to less than 60%, therefore the IC₅₀ value is approximated (calculated based on the non-linear regression model).

Enzyme cytotoxicity. As a chemotherapeutic agent, asparaginase is routinely administered intravenously and thus it would come into contact with both leukemic and non-cancerous or non-blood cell types. As such, any probable anti-proliferative or cytotoxic effect of enzyme on both target and other cell types should be carefully assessed. Thus, we inspected the cytotoxicity of the purified enzymes with non-leukemic, non-myeloid cell line (HUVEC), along with the dominant cells of the blood, erythrocytes, to monitor any possible side effects of the enzymes on other cell types. No detectable adverse effect was observed for HUVEC cells (Fig. 6b **and c**). Additionally, no sign of erythrocyte hydrolysis was observed for any of the in vitro hemolysis test experiments conducted with the CAspI, II and III enzymes (data not shown).

Conclusion

L-asparaginase has been widely used as antineoplastic agent to deplete patient's serum from L-asparagin essentially needed by auxotrophic cancer cells. Its therapeutic potential for acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma has fueled the search for novel L-asparaginases to combat the complications associated with the existing therapeutic L-asparaginases. Harnessing the vast potential of prokaryotes via culture independent methods is a promising approach for finding novel enzymes. Prokaryotes express L-asparaginase as a common good to scavenge for nitrogen sources consequently screening the oligotrophic waters of the Caspian Sea mimicking the salinity and ionic concentration of blood serum can potentially lead to the finding of enzymes that can attenuate the complications with hypersensitivity, antigenicity, short half-life, and temporary blood clearance. Recognizing that the cancer has the properties of ecological systems have been suggested to be the way forward in devising sustainable treatments [66]. Leveraging the knowledge of ecology and tapping into the genetic potential of ecosystems similar to cancer ecosystem can facilitate the biodiscovery of treatment agents.

Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable.

Availability of data and materials. The putative L-asparaginase gene sequences together with the *in vitro* examined codon-optimized genes are provided in the supplementary Tables 2 and 3.

Competing interests. Authors declare no competing of interest.

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Figures

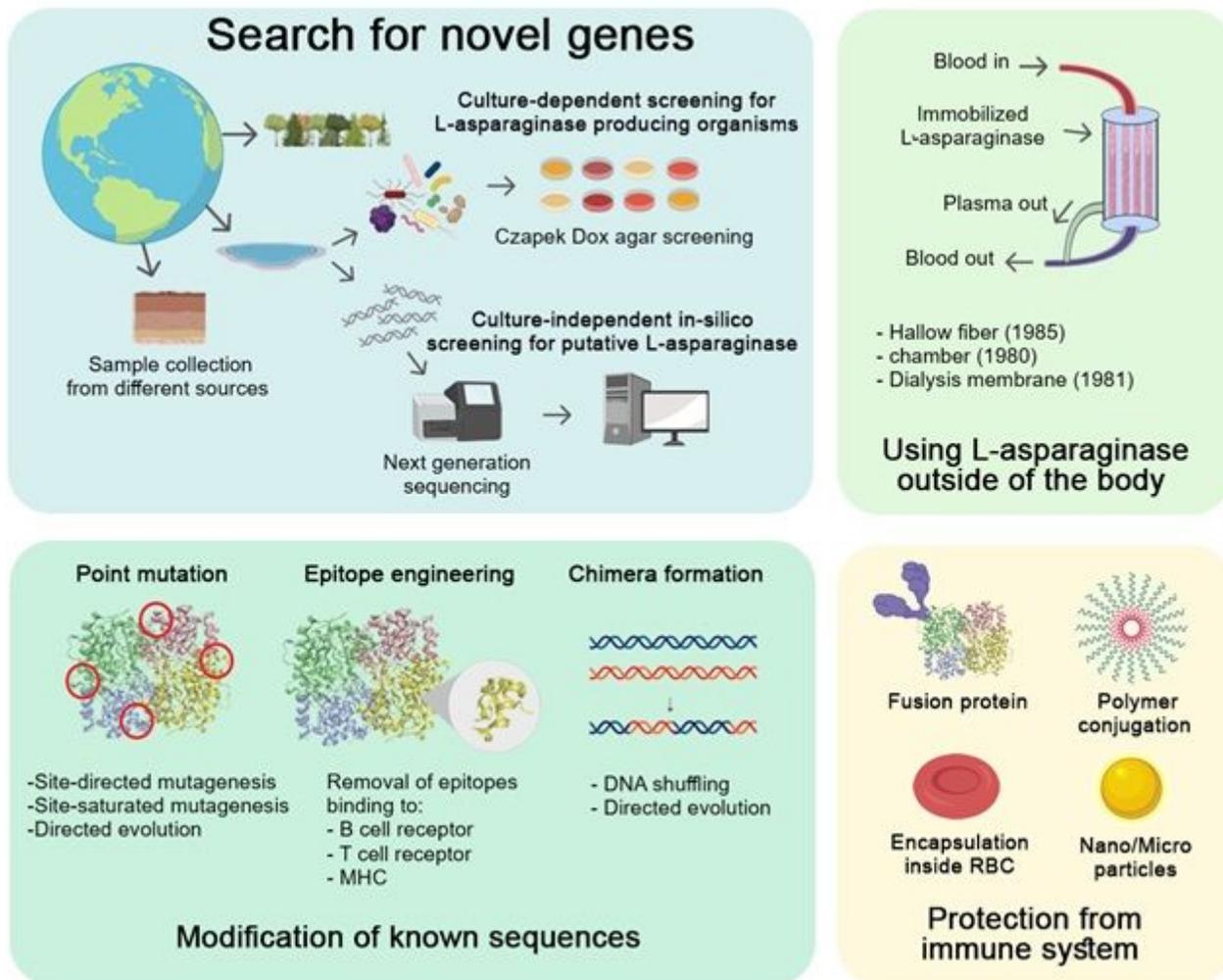


Figure 1

Schematic overview of approaches used to tackle the immunogenicity and low activity of the L-asparaginase enzyme in respect to its use for acute lymphoblastic leukemia treatment.

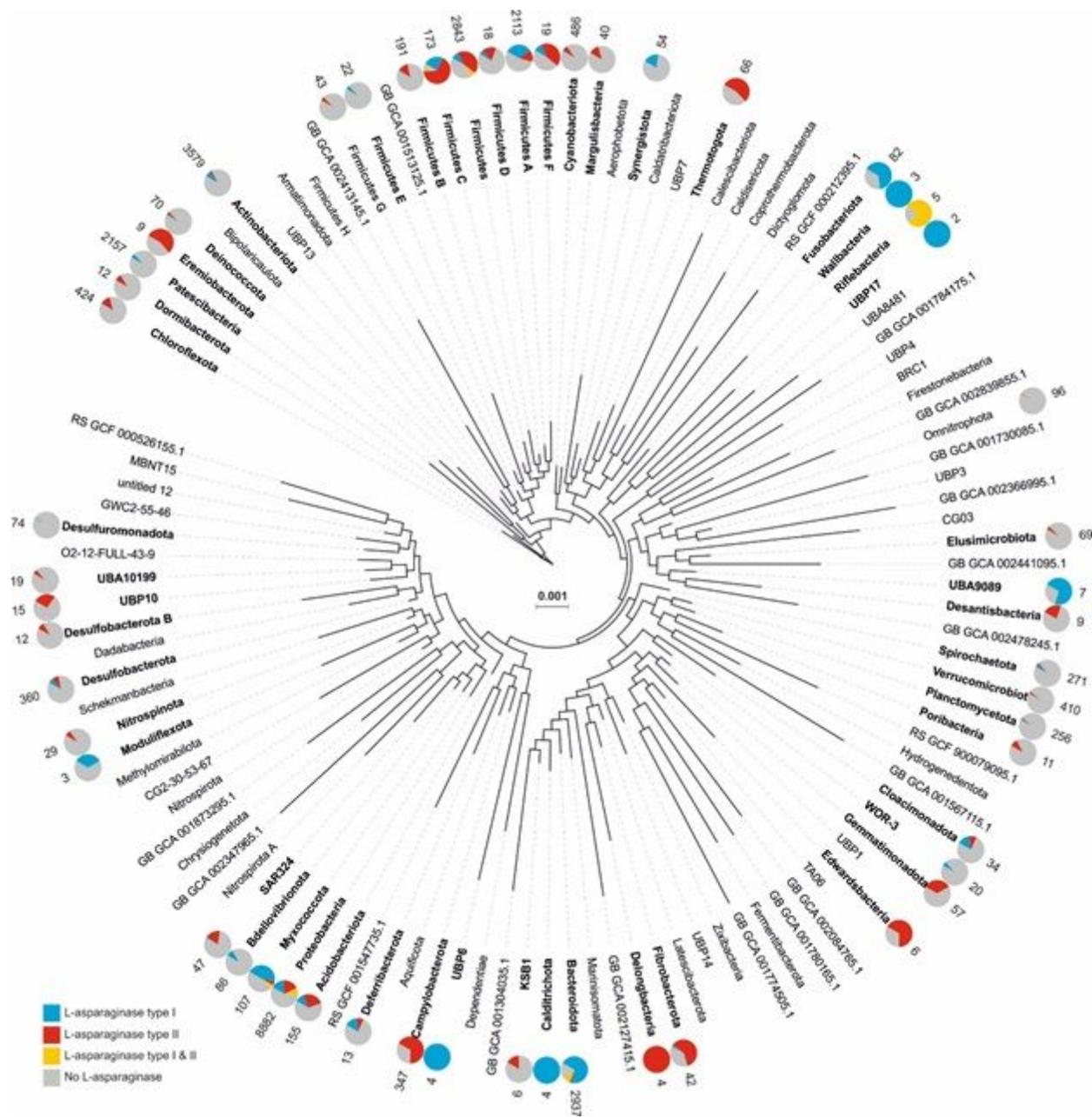


Figure 2

Phylogenetic distribution of L-asparaginase containing representatives across bacterial phyla. Phyla with representatives containing L-asparaginase genes are shown in bold. The pie charts show the distribution of genomes containing genes annotated as secretory or periplasmic L-asparaginase or those containing both L-asparaginase types simultaneously. The number next to the pie chart represents the number of genomes present in the phyla.

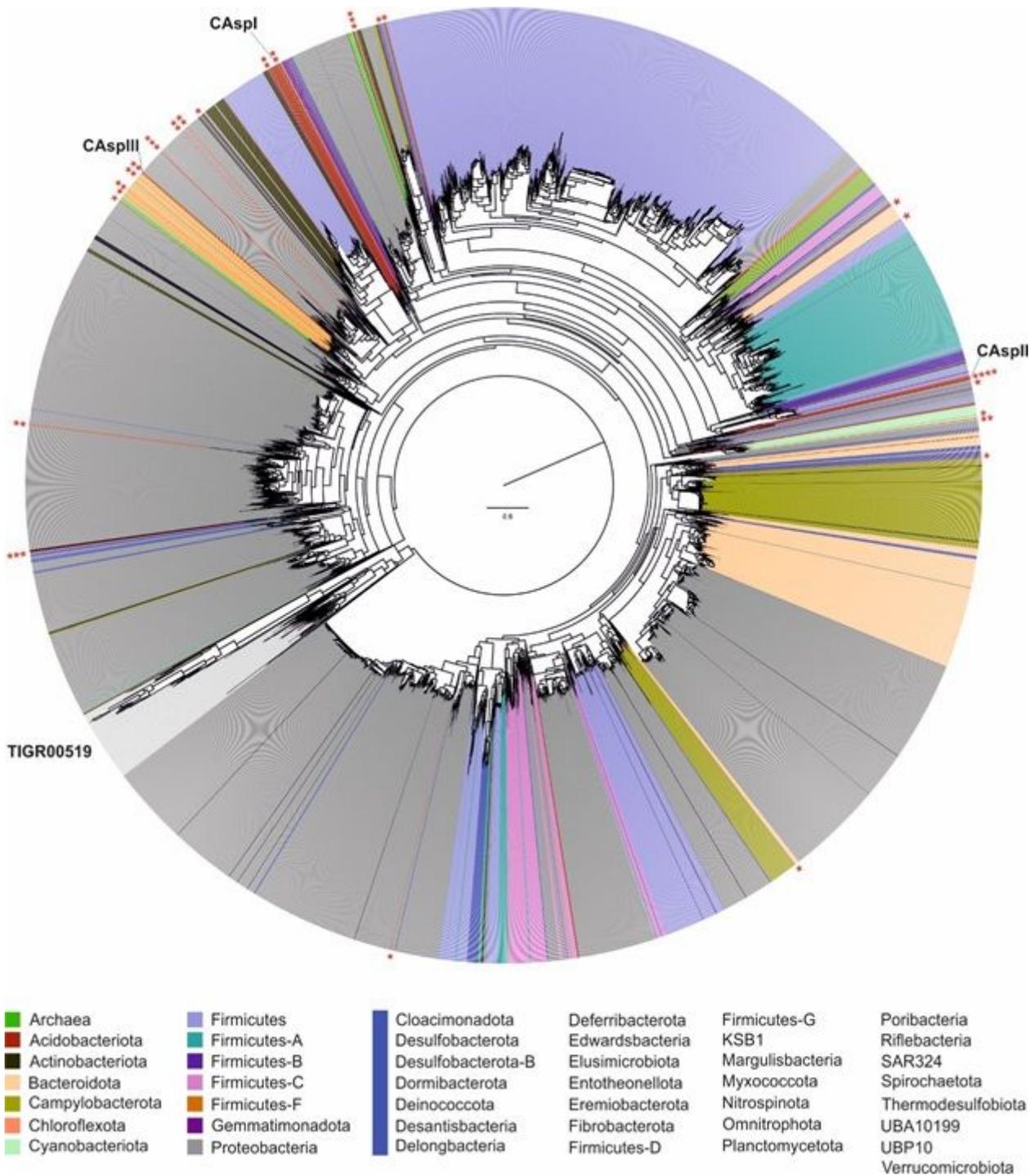


Figure 3

Phylogeny of the secretory (TIGR00520) L-asparaginase protein sequences recovered from Caspian Sea metagenomes together with L-asparaginase genes recovered from bacterial genomes annotated in the Annotree database together with the reference L-asparaginase protein sequences recovered from UniProt. The phylogenetic position of the Caspian Sea recovered L-asparaginases are shown with asterisk. The periplasmic L-asparaginase (TIGR00519) sequences were used to root the tree.

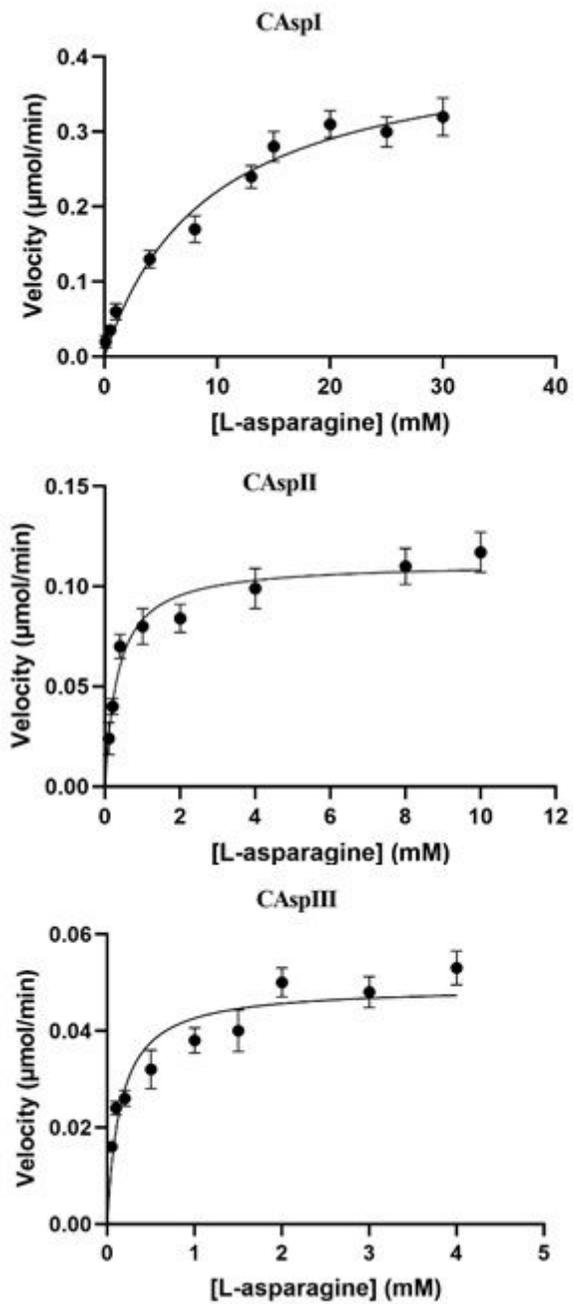


Figure 4

Michaelis–Menten kinetics of recombinant L-asparaginases towards L-asparagine as substrate.

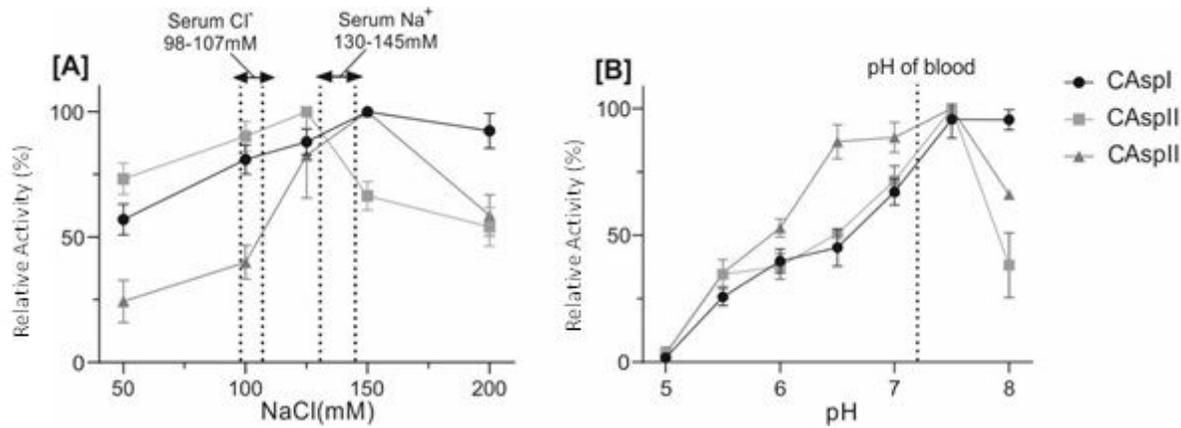


Figure 5

Optimum condition for enzyme activity. Effect of pH [A] and NaCl [B] concentration on enzyme activity. Dashed lines show the blood Serum conditions.

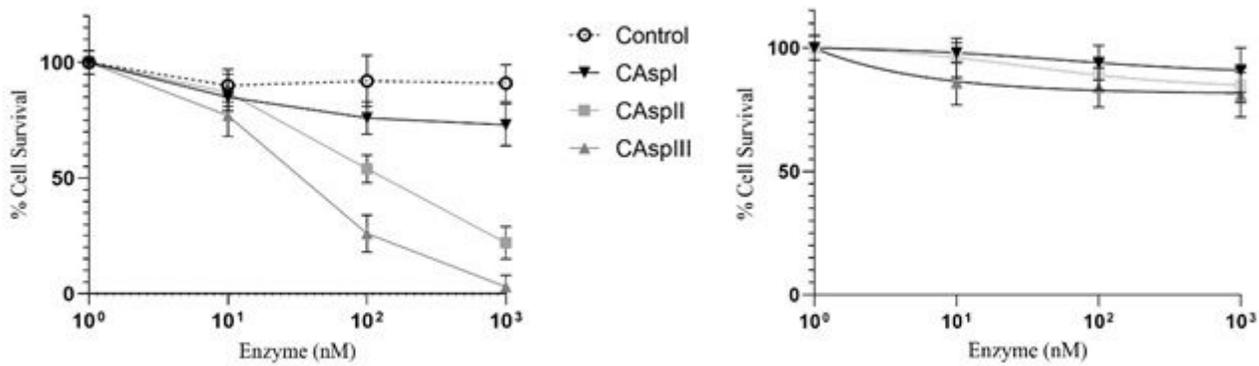


Figure 6

Anti-proliferative and cytotoxic activity assessment. Effect of purified L-asparaginases on Jurkat [A] and HUVEC [B] cell lines, [C] Enzymes effect on HUVEC cell line morphology. The cells were incubated for 24 h with purified enzyme (right), and cell morphology was compared with untreated cells (left).

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