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Highly efficient novel recombinant L-asparaginase with no glutaminase activity from a new halo-thermotolerant *Bacillus* strain

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Article Info

Article Type:
Original Article

Abstract

Introduction: The bacterial enzyme has gained more attention in therapeutic application because of the higher substrate specificity and longer half-life. L-asparaginase is an important enzyme with known antineoplastic effect against acute lymphoblastic leukemia (ALL).

Methods: Novel L-asparaginase genes were identified from a locally isolated halo-thermotolerant *Bacillus* strain and the recombinant enzymes were overexpressed in modified *E. coli* strains, Origami™ B and BL21. In addition, the biochemical properties of the purified enzymes were characterized, and the enzyme activity was evaluated at different temperatures, pH, and substrate concentrations.

Results: The concentration of pure soluble enzyme obtained from Origami strain was ~30 mg/L of bacterial culture, which indicates the significant improvement compared to L-asparaginase produced by *E. coli* BL21 strain. The catalytic activity assay on the identified L-asparaginases (*ansA1* and *ansA3* genes) from *Bacillus* sp. SL-1 demonstrated that only *ansA1* gene codes an active and stable homologue (ASPase A1) with high substrate affinity toward L-asparagine. The K_{cat} and K_m values for the purified ASPase A1 enzyme were 23.96^{s-1} and 10.66 μM, respectively. In addition, the recombinant ASPase A1 enzyme from *Bacillus* sp. SL-1 possessed higher specificity to L-asparagine than L-glutamine. The ASPase A1 enzyme was highly thermostable and resistant to the wide range of pH 4.5–10.

Conclusion: The biochemical properties of the novel ASPase A1 derived from *Bacillus* sp. SL-l indicated a great potential for the identified enzyme in pharmaceutical and industrial applications.
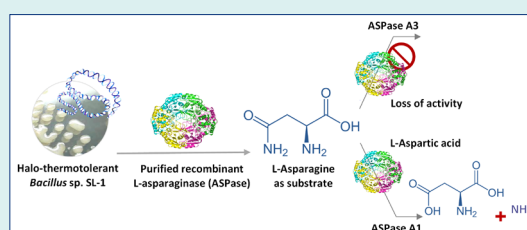
Introduction

L-asparaginase (EC 3.5.1.1; L-asparagine amidohydrolase) an important enzyme with known antineoplastic effect since 1967[1] catalyzes the conversion of L-asparagine amino acid (Asn) to L-aspartic acid and ammonia.[2] This therapeutic enzyme is applied primarily for the management of acute lymphoblastic leukemia (ALL). ALL is a case of leukemia affecting mainly children, contributing to approximately 80% of childhood and 20% of adult leukemias, respectively.[3,4] L-asparaginase is found in various organisms including animals, plant cells, yeast, fungi, and bacteria,[5] however, the bacterial enzyme has gained more attention in therapeutic application because of the higher substrate specificity and longer half-life.[1,2] Currently, *Erwinia chrysanthemi* and *Escherichia coli* are widely used in the pharmaceutical industry to produce L-asparaginase for the treatment of leukemia.[6] In the United States, 3 asparaginase formulations are widely used against ALL including native *E. coli* asparaginase (Elspar®), its pegylated form (Oncaspar®) and Erwinase from cultures of *E. chrysanthemi*.[3] In recent years, the PEGylation has widely been used in order to improve the blood-circulation half-life, to minimize the immunological clearances via reticuloendothelial system

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(RES) and to maximize the intratumoral penetration and biodistribution of the administered drugs.[7-9] The anti-leukemia activity of L-asparaginase is due to its depleting effect on the concentration of Asn in the extracellular fluid. Unlike normal cells, tumor cells, more specifically leukemia cells, have little or no asparagine synthetase enzyme and require exogenous asparagine to keep their rapid malignant growth.[10,11] Diminished level of L-asparagine and inability of leukemic cells to synthesize their own L-asparagine leads to the inhibition of protein synthesis, cell cycle arrest in the G1-phase, and ultimately apoptosis in susceptible leukemic cells.[12] In addition, L-asparaginase is enzymatically active against L-glutamine (Gln) but with a significantly lower affinity than that for L-asparagine.[10] This property leads to L-glutaminase activity associated side effects of L-asparaginase treatment. However, this activity contributes to the depletion of both Asn and Gln amino acids which have shown in some studies leads to the suppression of malignant cell growth in certain cancers.[3]

Despite significant advantages of L-asparaginase development, clinical application of this bacterial enzyme is limited by immune system response and antibody production against foreign L-asparaginase.[5,13] Anti-asparaginase antibodies are responsible for major toxicity and resistance to asparaginase therapy and also reduce the therapeutic efficacy of asparaginase in some cancer cases.[3] Rapid plasma clearance, shorter duration of activity, frequent injections to maintain the required therapeutic level, and development of immune responses, as well as the anaphylactic shock, are the main restrictions of current commercial L-asparaginases.[3,12] Low substrate specificity is another problem associated with the commercial L-asparaginases, which can cause liver dysfunction, pancreatitis, leucopenia, neurological seizures, and coagulation abnormalities leading to intracranial thrombosis or hemorrhage.[6]

It has been demonstrated that L-asparaginases derived from various microorganisms show different properties and do not share antigenic cross-reactivity. Therefore, when patients develop hypersensitivity to L-asparaginase from *E. coli*, enzyme from *E. chrysanthemi* can be replaced to avoid risk of allergic reactions.[5,14,15] This condition attracted scientist for discovering this activity in many other microorganisms. Hence there is a request for the novel and robust L-asparaginase from alternative GRAS (generally recognized as safe) microorganisms. Researchers are studying new microbial sources of L-asparaginase which have improved stability, lower glutaminase activity with high substrate affinity, and sufficient half-life under physiological conditions.[3,4] Halo-thermotolerant or halo-thermophile organisms, living under extreme physicochemical conditions, are a valuable source of stable and resistant enzymes that can potentially lead to advances in biocatalysis production.[16] Thermostable enzymes can be stored at room temperature

for long periods of time without significant loss of activities. Another advantage of these enzymes is their typical resistance to the variable concentration of sodium chloride.[17,18]

In the current study, a new halo-thermotolerant *Bacillus* sp. SL-1, isolated from Saline Lake of Iran[16] was investigated as the source of novel L-asparaginases.[19] Two ansA1 and ansA3 L-asparaginase genes from *Bacillus* sp. SL-1 were cloned and overexpressed in Origami (modified *E. coli* expression strain trxB2/gor2 double mutant) and *E. coli* BL21. The biochemical properties of the purified enzymes were characterized, and the enzyme activity was evaluated at different temperatures, pH, and substrate concentrations. The results of the present study revealed that only *ansA1* gene codes for a novel catalytically active L-asparaginase, with high catalytic activity, whereas *ansA3* gene was an inactive protein without any L-asparaginase activity.

Materials and Methods
Reagents
In the present study, all of the mentioned reagents were analytical grade. DNA extraction kit was obtained from Qiagen (Germany). Other kits such as Gel extraction, plasmid purification, and BM chemiluminescence western blotting kits were purchased from Roche Company (Germany). Micro BCA protein assay kit was obtained from Thermo Scientific (USA). DNA ladders, NdeI, XhoI, XbaI, SalI and T4 DNA ligase were acquired from Fermentas (Russia). Ni Sepharose 6 Fast flow medium was from GE Healthcare Life Sciences (Sweden). His-Tag monoclonal primary antibody and goat anti-mouse IgG-HRP conjugated secondary antibody were obtained from GE Healthcare and Santa Cruz companies, respectively. Tryptone and NaCl were from Scharlau (Barcelona, Spain). Finally, yeast extract, agar, and glycerol were provided from Applichem (Darmstadt, Germany). Clinically approved E. coli L-asparaginase (Leunase®) was purchased from Kyowa Hakko Kirin Company (Japan).

Cells and strains conditions
According to the authors previous study,[16] halo-thermotolerant *Bacillus* sp. SL-1 was isolated from the Aran-Bidgol salt lake located in the central region of Iran. Later on, this strain was donated into Iranian Biological Resource Center (IBRC-M 11052), recognized as an international culture collection, and is currently available for all scientists and investigators. In the current study, all of the utilized *E. coli* strains (i.e., Origami, DH5α, and BL21) were obtained from Novagen (Darmstadt, Germany).

Cloning and sub-cloning of L-asparaginase genes from *Bacillus* sp. SL-1
According to the manufacturer's instructions, QIAamp DNA kit (Qiagen, Germany) was utilized for Genomic

DNA extraction from *Bacillus* sp. SL-1. The principles, which were followed for primer design were based on the high similarity of 16S rDNA sequences derived from *Bacillus* sp. SL-1 (GenBank accession no. JQ996502) and *Bacillus licheniformis* (strain ATCC 14580). Polymerase chain reactions were carried out for amplification of the L-asparaginase genes (*ansA1* and *ansA3*) by utilizing the ansA1 forward (5′-TCTT*CATATG*AATAAAAAA GTAGCTCT-CATTACAACG-3′) and ansA1 reverse (5′- AACA*GTC-GAC*CTAATAGCA GAATTTGTCTTTTATGCCTT-3′) and ansA3 forward (5′- TATA*CATATG*AAAAAGTTAC TGCTGTTGACCACC-3′) and ansA3 reverse (5′- CAAG-*GTCGAC*TTATATGATGATATCGTCTGCAATC-3′) (Bioron, Germany, ordered via FAZA Biotech, Iran). It should be noticed that the italic scripts in the primers are the recognition sites for *Nde*I and *Sal*I endonucleases. Thereupon, *Nde*I and *Sal*I endonucleases cleaved the PCR products and those products were subsequently ligated into the pET28a+ expression vector at 16°C overnight. In a process known as bacterial transformation, the produced constructs were transferred into *E. coli* DH5α for plasmid amplification and extraction by utilizing plasmid purification kit. Subsequently, for examining the presence of *ansA1* and *ansA3* coding sequences, the purified plasmid constructs had to be tested. The aforementioned examination was conducted by PCR reactions utilizing the previously mentioned primers and universal primers for the pET vectors. Additionally, *Xba*I and *Sal*I endonucleases were utilized in order to sub-cloning the ansA1 and ansA3 genes into the pET22b+ expression vector. The sequencing of the generated constructs are required in order to confirm the sequence of the ansA1 and ansA3 genes, therefore the accuracy of the generated constructs was verified by Sanger sequencing[20] at Sequetech, USA.

Sequence analysis of L-asparaginase genes from Bacillus sp. SL-1

After analyzing the nucleotide sequences of L-asparaginase genes, they were also analyzed by BLASTn database search method provided by NCBI (http://www.ncbi.nlm.nih.gov). In addition, the translation tool provided by the ExPASy server (http://web.expasy.org/translate/) was utilized to translate the nucleotide sequence in order to obtain the amino acid sequences of *Bacillus* sp. SL-1 L-asparaginases. ClustalW and Jalview programs were also utilized for alignment. ProtParam (http://www.expasy.org/tools/protparam.html) was used for predicting the L-asparaginase related molecular mass and theoretical pI (isoelectric point) values.

Expression and extraction of recombinant L-asparaginase enzymes

For investigating the expression profile of the recombinant L-asparaginases, *E. coli* BL21 and Origami prokaryotic expression systems were utilized in aerobic condition in Luria-Bertani (LB) medium. The recombinant constructs

pET22-ansA1 and pET22-ansA3 were transformed into chemically competent *E. coli* cells (BL21 and Origami). The transformed cells were plated on LB ampicilin plates and incubated at 37°C overnight. A single well isolated colony was used to inoculate 10 mL LB+ampicilin medium followed by overnight incubation at 37°C. The starter cultures were diluted 1:100 in fresh LB medium supplemented with ampicillin in the scale of 100 μg/mL and incubated at 37°C with shaking at 180 rpm until optical density of 0.6. Then, the expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) in 0.4 mM and overnight incubation at 20°C with shaking at 140 rpm. Bacterial cells were harvested by centrifugation at 5000×g for 15 minutes. After discarding the supernatant, the pellet was resuspended in the lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 1% Triton X-100, 1.4 mM protease inhibitor (PMSF), 0.1% β-mercaptoethanol and 0.1 mg/mL lysozyme). For efficient disruption of the cells, three rounds of freezing and thawing steps were utilized using liquid nitrogen. Subsequently, the lysate was sonicated 5 times on ice at 60% amplitude for 30 seconds with 1 minute interval. Bacterial debris was removed by centrifugation at 13 000×g for 10 minutes at 4°C and then the soluble fraction was filtered through a 0.22 μm filter and subjected to purification step.

Purification of recombinant L-asparaginase, SDS-PAGE and western blot analysis

Purification of the recombinant L-asparaginase enzymes was performed by exploiting affinity chromatography in certain conditions (bath and gravity-flow conditions), as described previously.17 Briefly, the filtered soluble fraction was applied to Ni Sepharose resin pre-equilibration with lysis buffer and incubated at 4°C for one hour. After removal of the flow through, the affinity resin was washed (Tris 50 mM, NaCl 150 mM, β-mercaptoethanol 0.1%, and imidazole 20 mM) three times and the bound 6×His-tagged proteins were eluted by imidazole solution (imidazole 500 mM, sodium phosphate 20 mM, NaCl 500 mM, pH 7). To remove imidazole, the purified protein samples were dialyzed against the buffer containing 100 mM NaCl and 50 mM Tris-HCl adjusted at pH 8. The prepared purified recombinant protein concentration was determined by micro BCA protein assay method using bovine serum albumin (BSA) standard solutions and the obtained proteins named L-asparaginase A1 (ASPase A1) and L-asparaginase A3 (ASPase A3). Protein analysis at different steps of expression and purification was carried out on 12% polyacrylamide gel (SDS-PAGE) stained by Coomassie brilliant blue G-250. Immediately after SDS-PAGE, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane for western blotting. The membrane was blocked at 4°C overnight in 5% BSA dissolved in TBST (20 mM Tris–HCl, containing 150 mM NaCl and 0.05% Tween-20 at pH 7.5). Subsequently, the blocked membrane was incubated with

mouse anti-His monoclonal primary antibody (1:3,000) at room temperature for 1.5 hours. The membrane was washed three times with TBST for 5 min each at room temperature. Then, the membrane was incubated with goat anti-mouse IgG-HRP in 3% BSA at 1:8,000 dilution for one hour at room temperature and finally the protein bands were detected by chemiluminescence.

Enzyme activity assay of ASPase A1 and ASPase A3
The enzyme activities were determined by measuring the released ammonia during L-asparagine hydrolysis using Nessler's reagent.[21] In order to reach temperature equilibration the reaction mixture (2 mL), consisting of 50 mM Tris-HCl (pH 8.6) and 10 mM L-asparagine was incubated at 37°C for 5-6 minutes. After adding 100 μL diluted enzyme at zero time, the reaction proceeded at 37°C for precisely 10 minutes and paused by adding 100 μL of 1.5 M trichloroacetic acid (TCA) only to "Test" tubes. Prior to incubation, the blank tube was terminated by adding 100 μL of 1.5 M TCA. After centrifugation of the reaction mixture, 500 μL of clarified supernatant was mixed with 7.0 mL of ultrapure water and 1.0 mL of Nessler's reagent and incubated at room temperature for 10 minutes. All the measurements were done in triplicates by using spectrophotometer at 480 nm. Using the ammonium sulfate standard curve the micromoles of the released ammonia were determined. An amount of enzyme that under the specified conditions such as the temperature of 37°C and the pH of 8.6 is required to produce one micromole of the ammonia per minute is defined as one unit of the enzyme. According to the manufacturer's instructions, a micro BCA protein assay kit utilized in order to determine the protein concentration. Finally, the results of the recombinant ASPase A1 activity were compared with the clinically approved *E. coli* L-asparaginase (Leunase®) activity that named ASPase E in this study.

Biochemical characterization of recombinant ASPase A1 and substrate specificity assay
According to the spectroscopic method mentioned above, we used various concentrations of L-asparagine as a substrate in 50 mM Tris-HCl (pH 8.6) in order to determine the kinetic parameters K_m and K_{cat} of the purified recombinant ASPase A1. The data were fitted to the Michaelis–Menten equation by nonlinear regression using Prism software (version 6.01, GraphPad Software

Inc.). The Michaelis-Menten constant K_m indicates the concentration of the substrate when the reaction rate is one-half of the maximal velocity (V_{max}). The constant K_{cat} (catalytic rate) shows the number of substrate molecules turned over into the product by enzyme per second.[22] In order to determine the effect of pH on the activity and stability of ASPase A1, L-asparagine hydrolysis by the enzyme in 50 mM Tris-HCl (pH 8.6) was considered. At -20, 4, 25, 37 and 50°C and by using L-asparagine in 50 mM Tris-HCl and pH 8.6 during 120-hour incubation, the thermostability of ASPase A1 was investigated. In different intervals, samples were taken and immediately assayed for residual enzymatic activity by utilizing the L-asparagine hydrolysis. L-glutaminase activity of ASPase A1 was determined by 10 mM L-glutamine as a substrate in 50 mM Tris-HCl at 37°C and pH 8.6 using Nessler's method.

Results
Characterization of L-asparaginase sequences (ansA1 and ansA3) from Bacillus sp. SL-1
In this study, based on 97.0% similarity between the16S rDNA gene sequences of *Bacillus* sp. SL-1 and *B. licheniformis* (strain ATCC 14580),[16] the sequences for L-asparaginase enzyme from the *B. licheniformis* were used as a template for designing appropriate primers for identifying the corresponding genes from the genome of the *Bacillus* sp. SL-1. This approach led to the amplification of the coding sequences of ansA1 (969 bp) and ansA3 (990 bp) of *Bacillus* sp. SL-1. The DNA sequence analysis of the cloned ansA3$_{(SL-1)}$ showed the high degree of identity 99.90% to the same gene from *B. licheniformis* with just a silent T522A substitution (Tables 1 and 2). The ansA1$_{(SL-1)}$ gene (second homologous enzyme) was also identified from *Bacillus* sp. SL-1 with 99.07% similarity to ansA1 from *B. licheniformis* consists of 969 bp corresponding to 322 residues with five nucleotide substitutions (A177T, C184G, G229A, C462T, A946T) leading to tow silence and 3 residue changes in the protein sequence (None, Q62 to E, D77 to N, None, I316 to L), respectively (Tables 1 and 2). From the primary sequence alignment of ASPase A1 and A3, as shown in Fig. 1, it was clear that the identified L-asparaginases from *Bacillus* sp. SL-1 have conserved amino acids compared to the L-asparaginases from *B. licheniformis*, *Bacillus* sp. SL-1, *B. subtilis* 168, *E. chrysanthemi* and *E. coli*, represented in the dark blue columns. The primary structure of *Bacillus* sp. SL-1

Table 1. Comparison of the corresponding genes for L-asparaginase enzyme from the genome of *Bacillus* sp. SL-1 with *B. licheniformis* ATCC14580, *B. subtilis* 168, *E. coli* K-12, and *Erwinia chrysanthemi*

Genes	GenBank Accession numbers	Coding sequence alignment (Identity %)			
		B. licheniformis ATCC	*B. subtilis* 168	*E. coli* k-12	*Erwinia chrysanthemi*
ansA3	KX129701	99.90	72.04	44.37	42.66
ansA1	KX681674	99.48	46.35	41.99	45.20

Table 2. Pairwise alignment of nucleotide and amino acid sequences of L-asparaginase enzymes from *Bacillus* sp. SL-1 with *B. licheniformis* (ATCC14580) and analysis of their modifications

Enzymes	Protein sequence alignment		
	Nucleotide modification	*Amino acid modification*	*Identity (%)*
ASPase A3 (ansA3)	T522A	None	100.0
ASPase A1 (ansA1)	A177T	None	99.07
	C184G	Q62 to E	
	G229A	D77 to N	
	C462T	None	
	A946T	I316 to L	

ASPase A3 enzyme shows high sequence identity to *B. subtilis* 168 (79.0%) and *B. licheniformis* (ATCC14580) (100.0%) L-asparaginases. ASPase A1 sequence from *Bacillus* sp. SL-1 was more similar only to L-asparaginase from *B. licheniformis* (ATCC14580) (99.21%).

Characterization of the produced recombinant ASPase A1 and A3 from Bacillus sp. SL-1

In the present study, the L-asparaginase corresponding genes from *Bacillus* sp. SL-1 were successfully cloned and expressed in *E. coli* cells (BL21 and Origami) expression system under aerobic condition. Using Origami expression system under aerobic conditions, ASPase A1 and A3 were expressed significantly in the soluble fraction by

the appearance of a strong band with a molecular weight around ~37 kDa as shown in the SDS-PAGE analysis (Fig. 2) corresponding to the molecular weight of L-asparaginase monomers from *B. licheniformis* and *B. subtilis*. The result of the western-blot analysis was in agreement with that of the SDS-PAGE experiment. The production of enzymes in *E. coli* BL21 expression system led to precipitation and formation of insoluble aggregates as inclusion bodies in cytoplasmic space. After expression of 6×His-tagged ansA1$_{(SL-1)}$ and ansA3$_{(SL-1)}$ L-asparaginases, the clear soluble fractions were subjected to the Ni Sepharose resin for purification (Fig. 3). The concentration of soluble purified L-asparaginase in Origami expression system was ~30 mg per one-liter bacterial culture that showed improvement over the produced enzyme in *E. coli* BL21 strain under the same condition.

Activity assay of recombinant ASPase A1 and ASPase A3 from Bacillus sp. SL-1

The preliminary hydrolytic activity assay of recombinant ASPase A1 and A3 from *Bacillus* sp. SL-1 using L-asparagine as substrate was showed that the identified
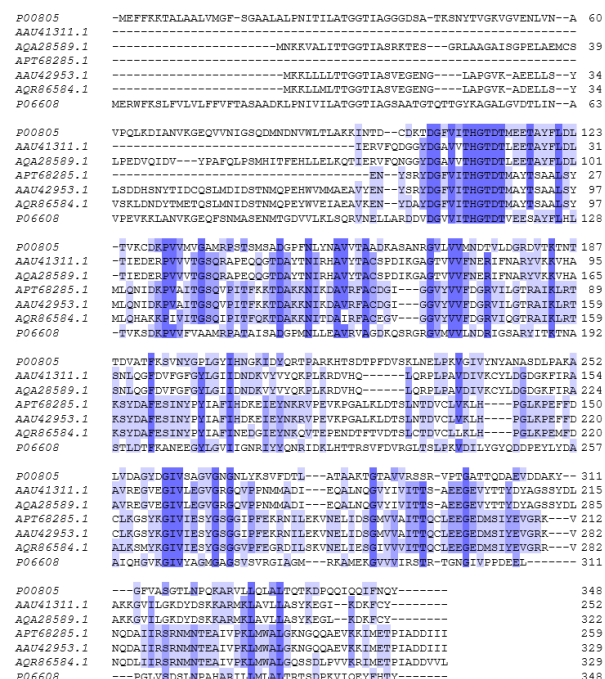

Fig. 1. Alignment of the amino acid sequence of ASPase A1 and A3 from *Bacillus* sp. SL-1 with identified L-asparaginase from closely related microorganisms. P00805, *E. coli* K12; AAU41311, ASPase A1 *B. licheniformis* ATCC; AQA28589, ASPase A1 *Bacillus* sp. SL-1; APT68285, ASPase A3 *Bacillus* sp. SL-1; AAU42953, ASPase A3 *B. licheniformis* ATCC; AQR86584, *B. subtilis* 168; P06608, *Erwinia chrysanthemi*. Highly conserved amino acid represented in the dark blue columns.
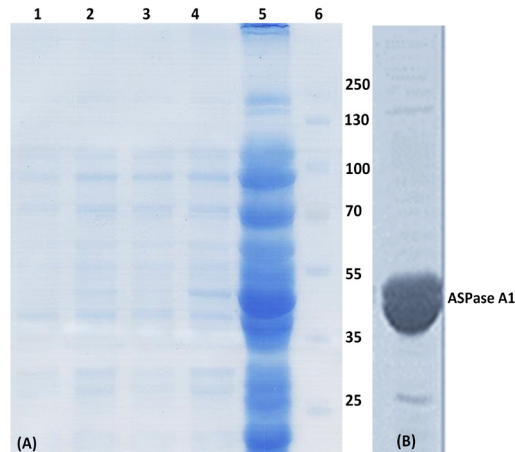

Fig. 2. The SDS-PAGE and western blot analysis of 6×His-tagged ASPase A1. (**A**) SDS-PAGE analysis of samples prepared from the soluble fraction of cell lysate prepared from Origami transformed with the plasmid harboring 6×His-tagged ASPase A1 gene. Lane 1, immediately after induction; lane 2, 1 h after induction; lane 3, 2 h after induction; lane 4, 3 h after induction; lane 5, 16 h after induction and lane 6, protein size markers. (**B**) Western blot analysis of 6×His-tagged ASPase A1 production in Origami expression system.
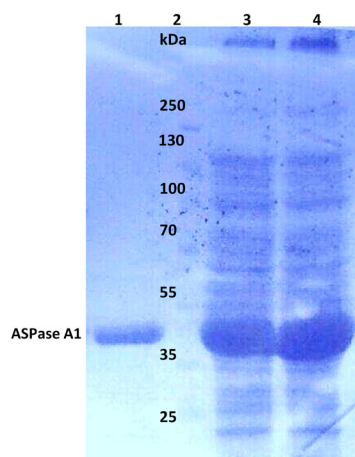
Fig. 3. The SDS-PAGE analysis of 6×His-tagged ASPase A1 prepared from the soluble fraction of the Origami lysate. Lane 1, purified ASPase A1 with the size of ~37 kDa; lane 2, protein size markers; lane 3, soluble fraction (total protein) of the Origami lysate for ASPase A1; lane 4, soluble fraction (total protein) of the Origami lysate for ASPase A3.

ASPase A3 is completely inactive regardless of the presence of 6×His-tags in either N- or C-terminal of the target protein. However, the second homologous enzyme ASPase A1 from *Bacillus* sp. SL-1 was produced in high purity and characterized with excellent enzymatic activity. To investigate the ASPase A3 inactivity and its relationship with polyhistidine-tag position, the 6×His-tag was moved from C- terminal to N-terminal of the L-asparaginase by sub-cloning of the ansA3 coding sequence into pET22b (+). The lack of activity for ASPase A3, in either case, shows that the presence of 6×His-tag was not the reason for the observed inactivity.

Biochemical characterization and kinetic parameters of recombinant ASPase A1 compared to ASPase E

The ASPase A1 expressed in Origami under aerobic condition was investigated for its kinetic and biochemical properties. As exhibited in Fig. 4, the K_m value of ASPase A1 was almost two times higher than the determined value for ASPase E, while the K_{cat} value of 7.727 s^{-1} for ASPase E was revealed to be 3 times lower than the K_{cat} of 23.96^{s-1} for ASPase A1 (Table 3). Purified ASPase A1 from *Bacillus* sp. SL-1 was stable in a wide range of pH values ranging from 4.5–10 over a period of 24 hours with maximum residual activity (100%). Temperature effect on enzyme
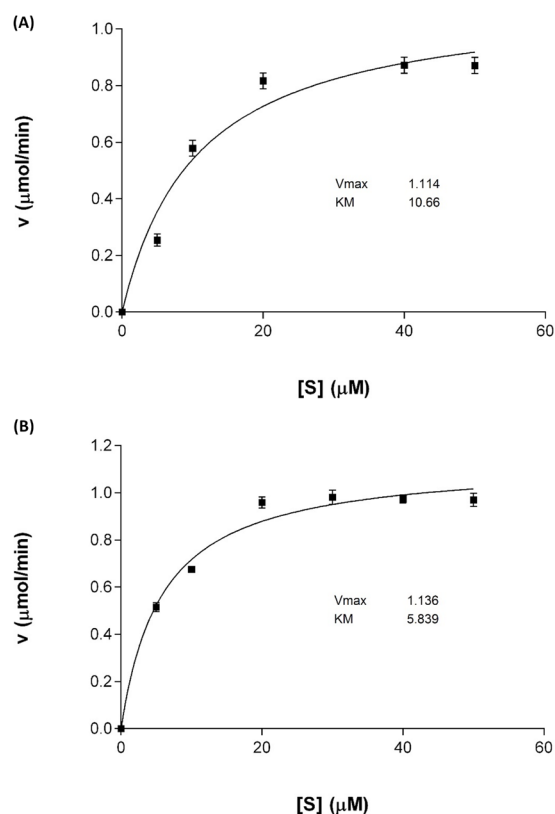

Fig. 4. Kinetic parameters analysis for ASPase A1 and ASPase E **(A)** Michaelis–Menten plot for ASPase A1 and **(B)** Michaelis–Menten plot for ASPase E.

stability and activity was studied between -20°C until 50°C at pH 8.6 during 24 until 120 hours. As shown in Fig. 5, the enzyme exhibited high stability at -20°C where its residual activity was about 100% after one-year storage at this temperature. In addition, the ASPase A1 retained about 40% of its residual activity after 72 hours incubation at 37 °C. The ansA1$_{(SL-1)}$ L-asparaginase showed maximum activity (~100%) after 24 hours incubation in room temperature (~ 25°C).

Discussion

To reach the main goal of the current work in developing the new recombinant efficient L-asparaginase with improved therapeutic properties, the focus of this study was the production of a soluble and functional enzyme with high-concentration and low L-glutaminase activity

Table 3. Kinetic properties of recombinant ASPase A1 from *Bacillus* sp. SL-1 in comparison with the L-asparaginase activity from other closely related organisms

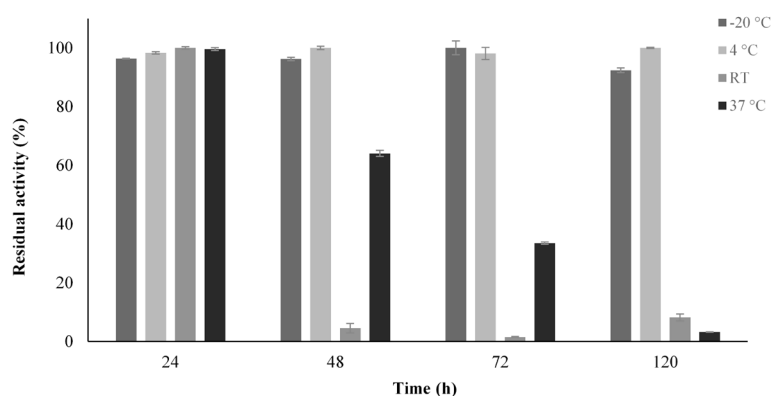| Source of L-Asparginase	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m	References
B. licheniformis SL-1	10.30	23.96	2.326	Present study
B. licheniformis MTCC 429	670	-	-	[4]
B. subtilis B11–06	430	-	-	[5]
E. coli k-12	5.788	7.727	1.339	Present study

Fig. 5. Thermostability assessment of the recombinant ASPase A1. The residual activity of the enzyme was determined after incubation for 24, 48, 72 and 120 hours at different temperatures.

within reasonable time and cost. Recently L-asparaginase from *B. licheniformis* with low-glutaminase activity has been considered as a key therapeutic agent in the treatment of ALL.[12] The glutaminase activity of L-asparaginase is probably related to the structural similarity between L-asparagine and L-glutamine amino acids although the latter has an extra methylene (-CH$_2$-) group.[23]

In accordance to our results, previous studies have shown that recombinant L-asparaginase from different bacterial and fungal sources overexpressed in various strains of *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) pLysS, *E. coli* JM109, *E. coli* BLR (DE3) can lead to different enzyme production profiles of soluble or insoluble forms.[4, 5, 24, 25] More recently, Saeed et al reported the overexpression of L-asparaginase from *Aspergillus terreus* in *E. coli* BL21 (DE3) pLysS that precipitated in the form of amorphous inclusion bodies upon induction with 2 mM IPTG for 18 h at 37°C.[26] In general *E. coli* is considered as a proper host for heterologous protein production in research and biotechnology industry.[27] However, the inability of *E. coli* to support the complex post-translational modifications is a serious drawback which can lead to misfolding, aggregation and lack of functionality of the heterologously expressed proteins in the cytoplasm of this expression system.[28] Solubilizing and refolding the proteins from inclusion bodies is considered as a common strategy to resolve this problem, because of the presence of a high concentration of target recombinant proteins in such aggregates.[29] However, the cost and time of the whole process must be considered if the goal is to produce a large-scale manufactured product. The soluble expression of recombinant proteins often leads to the production of properly folded, functional, and easy to purify products. Moreover, the refolding process of proteins from the aggregated inclusion bodies is not complete and can fail in many cases.[30] Therefore, in this study, the solubility is considered as a key factor for the production of recombinant L-asparaginase in heterologous expression systems. Based on previous experiences regarding the expression profiles for the other enzymes from *B. licheniformis* in different *E.*

coli strains, in the current study, the modified *E. coli* strain (Origami) was used to increase expression concentration and appropriate folding of L-asparaginase in soluble form. Moreover, it is noteworthy to mention that ansA1 from *Bacillus* sp. SL-1 compared to *B. licheniformis* ATCC 14580 have 3 residue variations (Table 3) which may be the reason for the observed dissimilar expression profile. It has been demonstrated that such limited alterations only change the structure of the enzyme very slightly, but may still be enough to make a different subunit interaction of the produced recombinant proteins leading to aggregation.[31] Due to native variations in Origami, which leads to its oxidative cytoplasmic environment, the soluble overexpression of L-asparaginases A1 and A3 have been facilitated leading to correct folding of the enzymes in this expression system.

According to the hydrolytic activity assay of recombinant ASPase A1 and A3, the identified ASPase A3 was completely inactive in the presence of 6×His-tags in either N- or C-terminal of the target protein. In contrast to our results, Sudhir et al have shown that the coding sequence ansA3 from another strain of *B. licheniformis* MTCC 429 produces an active and stable enzyme, while the enzyme from ansA1 is highly unstable.[4] The molecular basis of functional differences observed for the homologous enzymes is strongly related to their 3-dimensional structures.[32] The first possibility for enzymatic inactivity of ASPase A3 was the 6×Histidine residue-tag in C-terminal of protein that may interfere with protein folding, oligomerization and enzyme functionality.[3,33-35] However, this tag is present in ASPase A1 and hence rules out its adverse effect on the activity. The more possible reason for the observed activity difference can be attributed to the high sequence difference (44% similarity) between these two homologs due to the sequence divergence during the evolution may have rendered one of the proteins inactive while the other preserved the activity.[36]

Kinetic parameters of the purified enzyme were measured by assessing the hydrolysis of L-asparagine amino acid as a substrate at different concentrations

(Fig. 4). The ASPase A1 from *Bacillus* sp. SL-1 showed a Michaelis–Menten profile typical for such enzymes. The K_m value of ASPase A1 was approximately 43 and 67 times lower than the reported value for *B. subtilis* B11−06 and *B. licheniformis* MTCC 429, but the value was close to that of *B. licheniformis* RAM-8 L-asparaginase as shown in Table 3.[4,12] Interestingly, the K_{cat} value of ASPase A1 was comparatively higher than the calculated value for therapeutical ASPase E from *E.coli*, which unraveled the high potential of the ASPase A1 for being applied in the therapeutical and pharmaceutical fields.

The purified ASPase A1 from *Bacillus* sp. SL-1 possessed higher specificity for L-asparagine than L-glutamine amino acid similar to isolated L-asparaginase from *B. licheniformis* RAM-8 (soil isolate).[12] Purified ASPase A1 from *Bacillus* sp. SL-1 was stable in wide range of pH values ranging from 4.5–10 over period of 24 hours with maximum residual activity (100%) similar to the reported pH range 7.0-9.0 for L-asparaginase from *B. licheniformis* RAM-8.[12] L-asparaginase is one of the amidases that are commonly active and stable at neutral to alkaline pH, whereas, pH range 5.0-9.0 has also been reported to be optimum for amidase activity.[37] The thermostability of ansA1$_{(SL-1)}$ at -20 °C was similar to the stability of L-asparaginase from *B. licheniformis* RAM-8.[12] In another study, the L-asparaginase from *B. subtilis* B11−06 retains 80% and 75% of the initial activity after 10 h incubation at 20°C and 30°C, respectively. The ASPase A1 lost completely its hydrolytic activity after 24 hours incubation at 50°C, while the L-asparaginase reported from *B. licheniformis* RAM-8 retained 20% of its hydrolytic activity after 24 hours incubation at 60°C.[12] These biochemical and kinetic variations are attributed to the genetic nature of the microbial strain sources.[37] These results indicated that the thermostability profile of ASPase A1 from *Bacillus* sp. SL-1 was almost comparable with L-asparaginase from *B. licheniformis* RAM-8, *E. coli* K12 (ASPase E) and was higher than that reported for L-asparaginase from *B. subtilis* B11−06.[12]

Conclusion
In summary, the current study introduces a new and efficient recombinant L-asparaginase enzyme from locally newly isolated halo-thermotolerant *Bacillus* strain with high-level soluble overexpression in the modified *E. coli* strain (Origami). The cloning, expression and activity assay of recombinant ansA1 and ansA3 showed that only ansA1 gene produced an active and stable homologues enzyme with high substrate affinity toward L-asparagine. The concentration of soluble and functional recombinant L-asparaginase production was optimized in the modified *E. coli* expression system. The purified recombinant ASPase A1 was highly thermostable and resistant to the wide range of pH values. The L-glutaminase-free L-asparaginase activity and higher K_{cat} value for ASPase A1 from *Bacillus* sp. SL-1 in compared to other studied

Research Highlights

What is current knowledge?
√ L-asparaginase is an important anti-cancer enzyme against ALL.
√ Currently, *Erwinia chrysanthemi* and *Escherichia coli* are used in the pharmaceutical industry to production of L-asparaginase.

What is new here?
√ L-asparaginase enzymes from newly isolated Bacillus sp. SL-1 were investigated.
√ Cloned L-asparaginases were overexpressed in soluble form in Origami strain.
√ Only one of the two produced recombinant enzymes showed catalytic activity.
√ Novel active enzyme (A1) showed high K_{cat} value with no L-glutaminase activity.
√ Enzyme A1 was highly thermo-stable and resistant to wide range of pH values.

bacterial L-asparaginases reveals the potential of this enzyme for pharmaceutical and industrial application.

Ethical statement
None to be declared.

Competing interests
The authors declare that they have no conflict of interest.

Authors contribution
AS was the PhD student and conducted the experiments, gathered the data and drafted the manuscript. MHM was advisor in this study. SD and RM designed the experiments and finalized the manuscript and supervised the overall study.

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