



## Isolation and Characterization of Glutaminase-free L-asparaginase Produced by *Staphylococcus* sp. MGM1

Gholamhossein Ebrahimipour<sup>a</sup>, Mohammad Yaghoobi Avini<sup>a</sup>,  
Mahtab Ghorbanmovahed<sup>a\*</sup>

<sup>a</sup> Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.

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### Abstract

L-asparaginase is an important therapeutic enzyme used in combination with other drugs for therapy of Acute Lymphoblastic Leukemia (ALL). L-asparaginase catalyzes the conversion of asparagine to aspartic acid and ammonia. In recent years, this enzyme gained applications in many fields of science such as clinical research, pharmacological, and food industries. This study was aimed at isolation and identification of a strain with the ability to producing extracellular glutaminase free L-asparaginase from soil and determination of enzyme stability. The isolation was done on M9 medium. Biochemical tests and 16S rDNA sequence was used for strain identification. L-asparaginase was partially purified using ammonium sulfate precipitation, dialysis, and DEAE-anion exchange chromatography. The effect of pH and temperature on enzyme activity was investigated. The isolated bacteria were identified as *Staphylococcus* sp. The optimum pH and temperature for maximum L-asparaginase activity were found at 8 and 35 °C. The enzyme purification showed a single band around 115 kDa on SDS-Page. The optimal activity for the enzyme produced by MGM1 was similar to the physiological conditions of the human body, therefore, further studies on this enzyme would be of great value in finding a new efficient asparaginase enzyme.

**Keywords:** Isolation; L-asparaginase; Acute Lymphoblastic Leukemia (ALL); *Staphylococcus*; Glutaminase-free.

## 1. Introduction

L-asparaginase (L-asparagine amidohydrolase E.C. 3.5.1.1) is an important chemotherapeutic enzyme that hydrolysis L-asparagine to L-aspartate and ammonia. This enzyme has been used for the treatment of certain kinds of cancer like Acute Lymphoblastic Leukemia (ALL) and non-Hodgkin lymphoma [1, 2]. The antitumor action of asparaginase is due to the fact that ALL tumor cells have notability for de-novo synthesis of the nonessential amino acid asparagine because of absence of asparagine synthetase and therefore, require huge amount of asparagine to keep up their rapid malignant growth and mainly depend on the L-asparagine from the circulating plasma pools [3-5]. Supplementation of L-asparaginase results in the continuous depletion of L-asparagine [6]. The nutritional stress-induced by asparaginase due to the reduction of serum asparagine; inhibits DNA, RNA and protein biosynthesis in ALL and other asparagine dependent tumor cells, that causes subsequent apoptosis [5].

However, normal cells remain unaffected due to the presence of asparagine synthetase [1, 4]. Another application of L-asparaginase is found in the baking process, where the Millard reaction between sugar and asparagine at temperatures above 180 °C results in brown color and production of acrylamide which is a carcinogenic by-product [6]. L-asparaginase is an intracellular enzyme and produced by various microorganisms, plants, and animal tissue but

\* Corresponding author: [mghorbanmovahed@gmail.com](mailto:mghorbanmovahed@gmail.com)

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not human [7, 8]. Plants and animal-based enzyme sources produce in sufficient quantities. Microorganisms like bacteria, yeast, fungi, actinomycetes, and algae are very effective producers because they can be cultured easily and the extraction and the purification of L-asparaginase from them are also convenient, facilitating the large scale production [8, 9]. Among the common sources of L-asparaginase are microorganisms such as: *Escherichia coli* [10], *Erwinia carotovora* [11], *Enterobacter aerogenes* [12], *Corynebacterium glutamicum* [13], *Pseudomonas aeruginosa* [14], *Bacillus subtilis* [15], *Aspergillus tamari*, *Aspergillus niger*, *Aspergillus terreus* [16] *Staphylococcus aureus* and *Streptomyces* [17-19].

The enzyme from *Erwinia chrysanthemi* and *E. coli* are predominantly in use as effective drugs for various leukemia diseases [20, 21]. In 2001, the *Erwinia chrysanthemi* L-asparaginase was FDA-approved and joined the L-asparaginase treatment regimens [22]. Notably, these L-asparaginases harbor an L-glutaminase secondary activity, that is, a capacity to deaminate the amino acid L-glutamine (Gln). Whether this secondary activity has any clinical relevance is still being debated, but it is clear that many of the toxic side effects of L-asparaginase therapy can be ascribed to the L-glutaminase activity [23]. To reduce the L-glutaminase-related toxicity of L-asparaginase therapy, several groups focused on engineering EcA and ErA towards low L-glutaminase variants [24, 25]. Another approach is to search for enzymes that naturally have lower L-glutaminase activity. For successful clinical applications, it is necessary to identify glutaminase-free L-asparaginase, which is obtained from a novel producer organism. In the late 1970s, Distasio reported a "glutaminase-free" L-asparaginase variant from *Wolinella succinogenes* and Kumar Investigated L-asparaginase in *Pectobacterium carotovorum* MTCC 1428 with no glutaminase activity [26].

The present study aimed at the isolation of a bacterium from soil samples with low glutaminase activity and investigation of their quantitative production of L-asparaginase, which it might have even more efficient antineoplastic activity.

## 2. Materials and Methods

### 2.1. Sample Collection

Soil samples were collected in the sterilized bags from the depth of 10 cm from agriculture soils around Gharchak, Tehran, Iran. About 1 gram of soil was added into 10 ml sterile saline solution (0.9 % w/v). The suspension is serially diluted from 10<sup>-1</sup> to 10<sup>-8</sup> and 0.1 ml of it was spread onto an agar plate containing 0.1% L-asparagine as sole nitrogen source, 1% glucose, and 1.5% agar in tap water at pH 7 and incubated at 30 °C for 24 - 48 h. Morphologically different colonies were chosen and further purified by repetitive streaking method.

### 2.2. Screening Glutaminase Free L-asparaginase

L-asparaginase producing strains were screened by rapid plate assay method based on their capability to form a pink zone around colonies on agar plates of modified M9 medium. The medium contained: 1 g glucose, 0.02 g MgSO<sub>4</sub>.5H<sub>2</sub>O, 0.001 g CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.05 g K<sub>2</sub>HPO<sub>4</sub>, 0.001 g FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.1 g asparagine and 1.5 g agar per 100 ml of Deionized Water. Phenol red at 0.009 % was used as a pH indicator. In the presence of an enzyme, asparagine converted into aspartic acid and ammonia and the color of medium will be changed to pink. The amount of enzyme production is reflected in the diameter of the pink zone. L-glutaminase activity was investigated by replacing asparagine by 0.1 g of glutamine as a sole nitrogen source.

### 2.3. Identification of the Potent L-asparaginase Producing Strain

The best glutaminase free L-asparaginase producing strain was selected and identification was done based on 16S rDNA sequencing, phylogenetic analysis, and morphological and physiological tests. The sequence deposited in Genbank under accession No. KT361190. Multiple alignments were done with the clustering algorithm with type strains of nearest *Staphylococcus* species and *Salinicoccus roseus* as outgroup. A maximum likelihood (ML) phylogenetic tree was drafted with Mega-X software (ver 10.0.5) with the Tamura-Nei method and 100 bootstrap replications.

### 2.4. Enzyme Production Medium

The inoculum was prepared by transferring a loop full of strain MGM1 into 250 ml sterile medium followed by incubation at 30 °C and 100 rpm for 24 hours. The seeding medium contains: 1 % glucose, 1 % peptone, 0.1 % L-asparagine, 0.05 % K<sub>2</sub>HPO<sub>4</sub>; 0.02 % MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.001 % FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.001 % CaCl<sub>2</sub>.2H<sub>2</sub>O and 100 ml trace elements (ZnCl<sub>2</sub>, 70 mg; MnCl<sub>2</sub>.4H<sub>2</sub>O, 100 mg; CoCl<sub>2</sub>.6H<sub>2</sub>O, 200 mg; NiCl<sub>2</sub>.6H<sub>2</sub>O, 100 mg; CuCl<sub>2</sub>.2H<sub>2</sub>O, 20 mg; NaMoO<sub>4</sub>.2H<sub>2</sub>O, 50 mg; Na<sub>2</sub>SeO<sub>3</sub>.5H<sub>2</sub>O, 26 mg; NaVO<sub>3</sub>.H<sub>2</sub>O, 10 mg; and Na<sub>2</sub>WO<sub>4</sub>.2H<sub>2</sub>O, 30 mg in 1000 ml distilled water) in tap water at pH 7.0. This culture was used to inoculate 2.5 L of the enzyme-producing medium divided into two 5 L Erlenmeyer flasks. Flasks were incubated at 30 °C and 100 rpm for 48 hours. Afterward, the culture media were centrifuged at 10000 ×g for 6 minutes and the supernatant was stored as a crude enzyme.

## 2.5. Enzyme Assay

L-asparaginase and L-glutaminase assay were calorimetrically determined by measuring the amount of ammonia ion produced from L-asparagine or L-glutamine as substrate by nesslerization method. For assay of enzyme activity, the modified Mashburn and Wriston method was used [27], briefly, 0.5 ml of the crude enzyme was added to assay mixture containing 0.5 ml of 0.04 M L-asparagine and 0.5 ml of 0.1 M Tris-HCl buffer. This mixture was incubated for 2 h at 37 °C. Then the reaction was stopped by the addition of 0.2 ml of 0.9 M TCA and centrifuged at 13000 ×g for 4 min. One milliliter of supernatant was diluted by 3 ml of distilled water and treated with 0.2 ml of Nessler's reagent. The mixture was incubated at 37 °C for 15 min, then absorbance determined at 450 nm and compared with the standard curve which was prepared from ammonium sulfate solution in different concentrations. The one I.U. (International Unit) of L-asparaginase is equal to one mmol of ammonia released from L-asparagine per minute [28].

## 2.6. Enzyme Purification

Purification of the enzyme was performed by a protocol involving ammonium sulfate fractionation and column chromatography on diethylaminoethyl Sephadex (DEAE-Sephadex). Solid ammonium sulfate was added slowly to the culture supernatant with constant stirring to 40 % saturation point and held overnight at 4 °C. The precipitate was pelleted by centrifugation at 10,000 ×g for 10 min in 4 °C. Finally, the pellet was dissolved in 0.1 % Tris-HCl buffer (pH 8) and dialyzed against the same buffer. After dialysis, the tube was kept at 4 °C for 24 h. The equilibrated enzyme solution was loaded to a DEAE-Sephadex column (2×5 cm).

The column was equilibrated with 0.1 % Tris-HCl buffer (pH 8). After loading of proteins, the column washed with Tris-HCl buffer for removing of unbound proteins and eluted with one column size of 0.1, 0.2, 0.3, 0.4, 0.5 and 1 M of NaCl in the same buffer. Thirty fractions were collected and enzyme activity and protein concentration of each was evaluated after dialysis with Nesslerization and Bradford methods respectively [29].

## 2.7. Measurement of Molecular Mass

The molecular mass of a semi-purified enzyme was estimated by SDS-PAGE gel electrophoresis. The electrophoresis was performed on 10% of separating gel (pH 8.8) and 5% of stacking gel (pH 6.8). The protein sample was solubilized in sample buffer by incubation at 95 °C for 5 min. Then 0.05 ml of this solution was loaded in wells. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. Wide molecular weight markers (10-250 kDa) were used to compare the molecular weight of the purified enzyme.

## 2.8. Biochemical Characterization

### 2.8.1. Effect of Temperature and pH on Enzyme Activity

The effect of different temperatures i.e., 4 °C, 25 °C, 30 °C, 35 °C, and 40 °C on enzyme activity for three hours was evaluated with the above-mentioned method. For determination of optimum pH, different phosphate buffer with pH 4, 5, 6, 7, 8, 9 and 10 was used. Buffer systems were sodium dihydrogen phosphate-NaOH (pH 4-8) and disodium hydrogen phosphate-NaOH (pH 9-10).

### 2.8.2. Effect of Metal Ions and Inhibitors on Enzyme Activity

The effect of metal ions Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Fe<sup>3+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Hg<sup>2+</sup> and Zn<sup>2+</sup> at 5 mM concentration and glucose, urea, glutamine, and EDTA at 1 mM concentration were evaluated after 2 hours' incubation with the purified enzyme at 37°C and darkness. The enzyme activities were measured by the previously mentioned method and expressed relative to the observed activity of control [9].

## 3. Results

### 3.1. Isolation and Screening of L-asparaginase Producing Bacteria

Among 75 different strains isolated from agricultural soil samples; 3 strains showed asparaginase activity by changing the color of the modified M9 agar medium from yellow to pink. Between these strains, MGM1 showed the largest pink-colored zone around the colony and therefore was selected for further experiments.

### 3.2. Characterization and Identification of Strain MGM1

DNA extraction and PCR amplification results in a 1500 bp of 16S rDNA. The Ezbiocloud analysis demonstrated 99.13 % similarity to *Staphylococcus warneri*. Morphological and biochemical properties of the isolated strain were compared with this bacterium and *Staphylococcus pasteurii*.

The strain was a cocci-shaped, aerobic, non-oxidative, catalase-positive and, gram-positive bacterium. Summarized results of biochemical and morphological tests were shown in Table 1. Based on the physiochemical properties and the 16S rDNA sequence, this bacterium belongs to the *Staphylococcus* genus and designated as *Staphylococcus* sp. MGM1. The ML phylogenetic tree (Figure 2) placed MGM1 strain clearly in distinct branch among *Staphylococcus* genus.

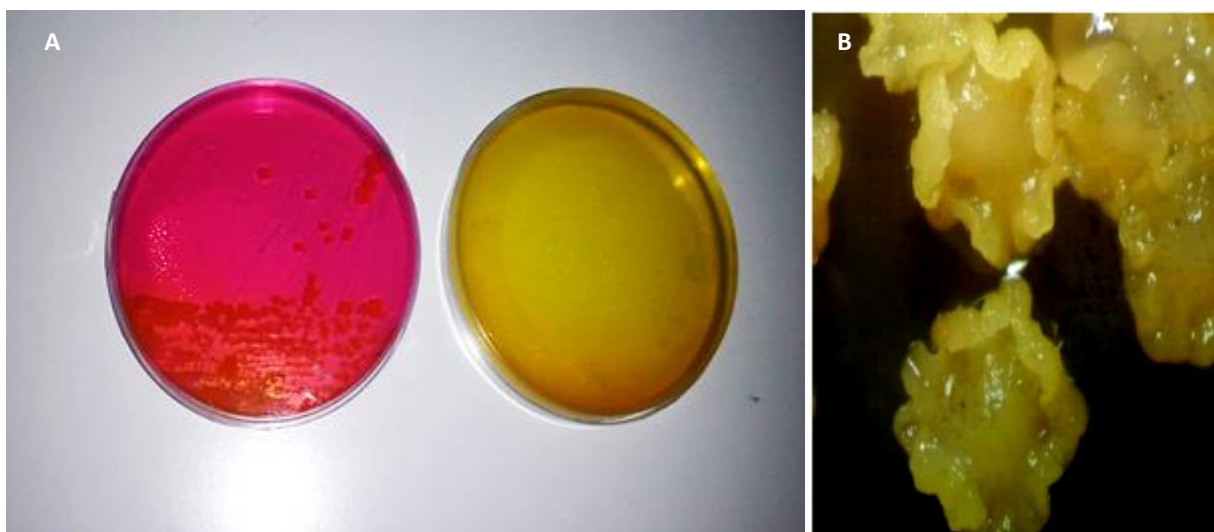


Figure 1. (A): right, M9 medium; left, the color of M9 medium turned to pink after cultured of MGM1; (B): MGM1 colony morphology under a stereomicroscope

Table 1. Some Morphological and Biochemical Characteristics of Strain MGM1

Characteristic	<i>Staphylococcus pasteurii</i>	<i>Staphylococcus warneri</i>	Strain MGM1
Shape	cocci	Cocci	cocci
Gram staining	positive	Positive	positive
Motility	-	-	-
Catalase	+	+	+
Oxidase	-	-	-
Anaerobic growth	-	-	+
Starch hydrolysis	ND	ND	+
Gelatin hydrolysis	-	-	+
Nitrate reduction to Nitrite	variable	week	+
<b>Acid production of:</b>			
Glucose	+	+	+
Glycerol	+	+	-
Mannitol	+	+	-
sucrose	+	+	+
Maltose	+	+	+
Lactose	+	+	-
Hemolysis	week	week	γ
Urease	+	+	+
Growth in 10-15% NaCl	+	+	+
Growth at 42°C temperature	+	+	+

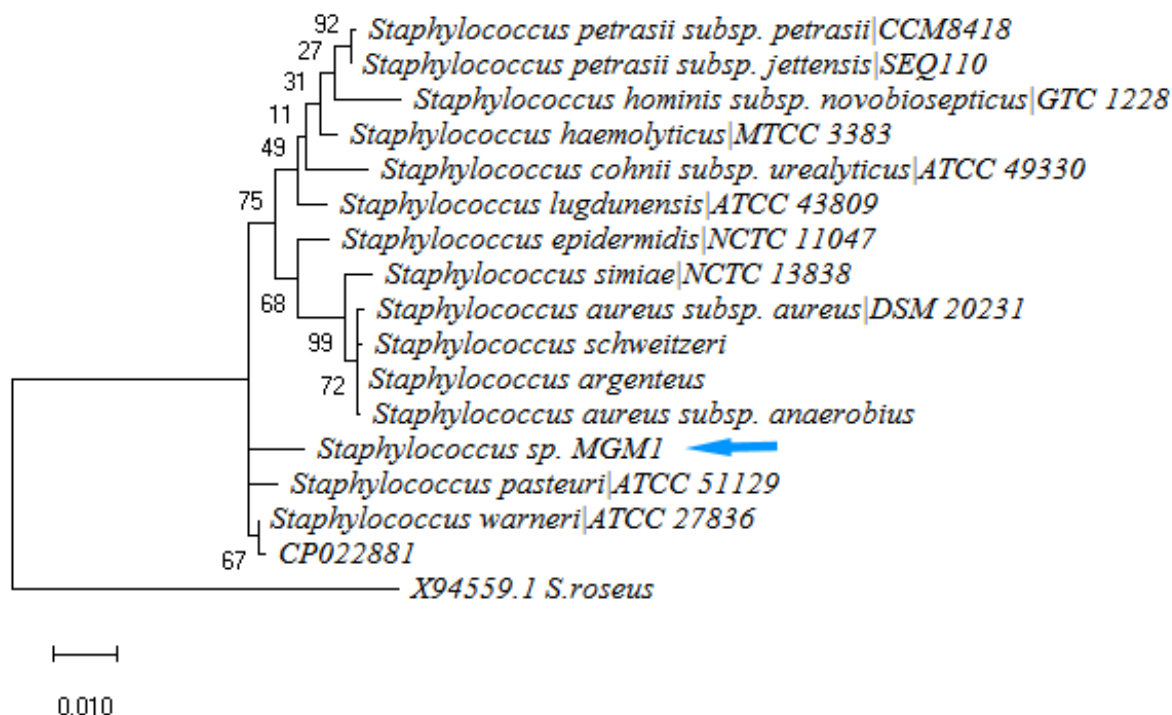


Figure 2. The maximum likelihood phylogenetic relationship of *Staphylococcus* sp. MGM1 as inferred by Tamura-Nei method with 100 bootstrap

### 3.3. Partial Purification of L-asparaginase and Molecular Mass Determination

The L-asparaginase was purified from the crude enzyme solution through salting out and anion exchange chromatography to a single band on SDS-PAGE. The steps of purification have been presented in Table 2 and Figure 3. The highest concentration of enzyme in centrifuged media was 4.93 IU/ml with 0.126 IU/mg specific activity. Ammonium sulfate precipitation recovered 19.47 % of the enzyme, whereas specific activity raised to 0.965 IU/mg. Final purification carried out by DEAE-Sephadex column showed the highest enzyme activity in fraction 3 with 1.63 IU/mg specific activity, which corresponds to 12.93 purification fold. This fraction was used for the SDS-PAGE analysis and showed a single band on gel coincident with a molecular weight of 115 kDa (Figure 4). The electrophoresis of other purification steps reveals the marked improvement in the purification of L-asparaginase using a DEAE-Sephadex column.

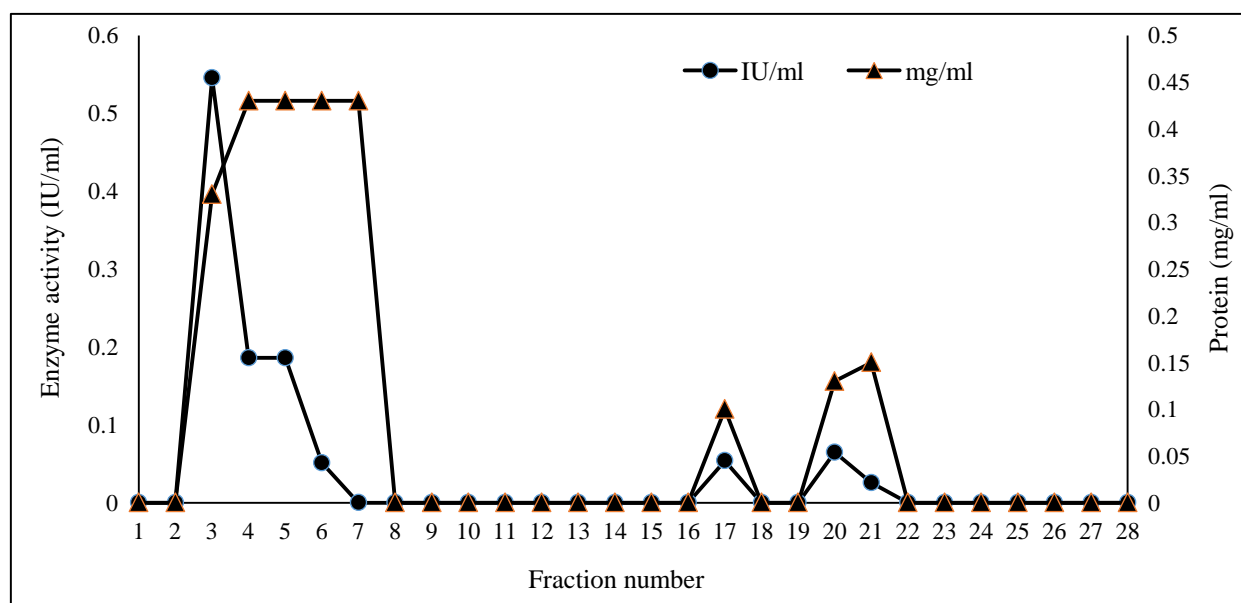
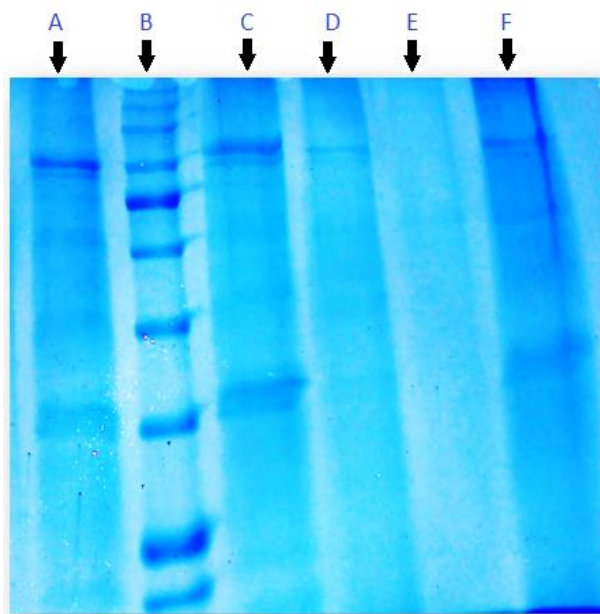


Figure 3. Elution profile of MGM1 L-asparaginase on DEAE-Sephadex A25; (●) total activity in each fraction; (▲) total protein in each fraction

**Table 2. Purification profile of L-asparaginase from *Staphylococcus* MGMI**

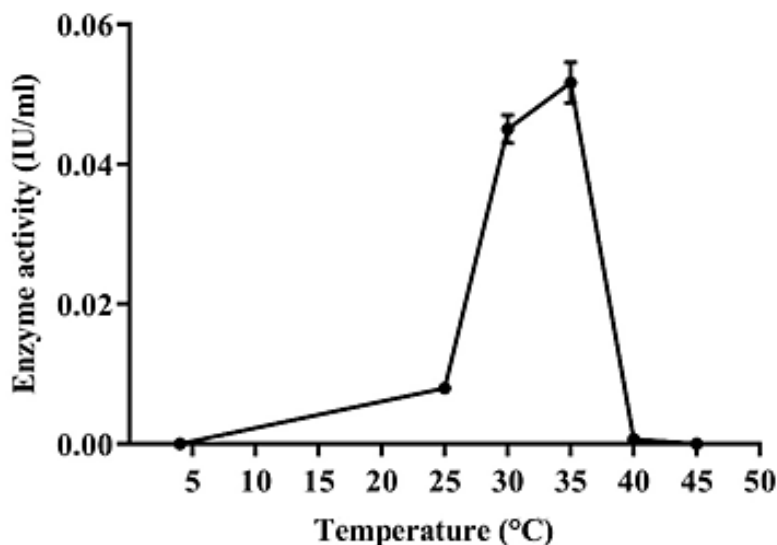
Step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Crude extract	4.93	39	0.126	-	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	0.96	1.38	0.695	5.79	19.47
DEAE-Sephadex	0.54	0.33	1.63	12.93	10.95



**Figure 4. SDS-PAGE of the purified enzyme and the molecular marker. A: supernatant of production culture; B: molecular weight marker; C: ammonium sulfate precipitate after dialysis; and D: fraction 3 of DEAE Sephadex anion exchange column.**

**3.4. Effect of Temperature and pH on Enzyme Activity**

The effect of temperature on L-asparaginase activity was measured at 4 °C, 25 °C, 30 °C, 35 °C, 40 °C and 45 °C in pH 7 by using L-asparagine as substrate. The thermal activity of the crude enzyme was obviously optimal at 35 °C and the enzyme retained greater than 50 % of the maximum activity from 30 °C to 37 °C (Figure 5). At 37 °C, it exhibited 60 % of the maximal activity. The effect of different pH on the enzyme activity was shown in Figure 5. The highest activity observed at 35 °C and pH 8.0 and more than 50 % of the maximum activity retained between pH 6.5 and 8.5 (Figure 6).



**Figure 5. The effect of temperature on enzyme activity and its stability at 4, 25, 30, 35, 40 and 45°C for 2 hours**

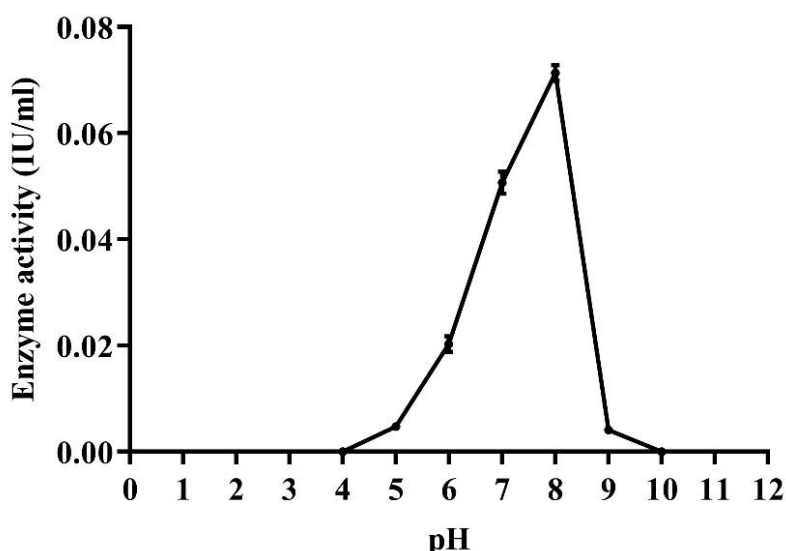


Figure 6. The effect of pH on enzyme activity at 35 °C and its stability for 2 hours in the range of 4 – 10

### 3.5. Effect of Metal Ions and Inhibitors on Enzyme Activity

Figure 7 demonstrates the effect of various metal ions and some of human blood serum components on L-asparaginase activity of *Staphylococcus* sp. MGM1. Glutamine and Mg<sup>2+</sup> had the least effect on the activity of L-asparaginase while K<sup>+</sup>, Fe<sup>3+</sup>, glucose, and Na<sup>+</sup> enhance its activity by 254, 236, 200, and 136 % respectively. However, urea and EDTA inhibit 45 % of the enzyme activity but Ca<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Hg<sup>2+</sup>, and Zn<sup>2+</sup> are potent inhibitors of the enzyme and reduce its activity by almost 95 %.

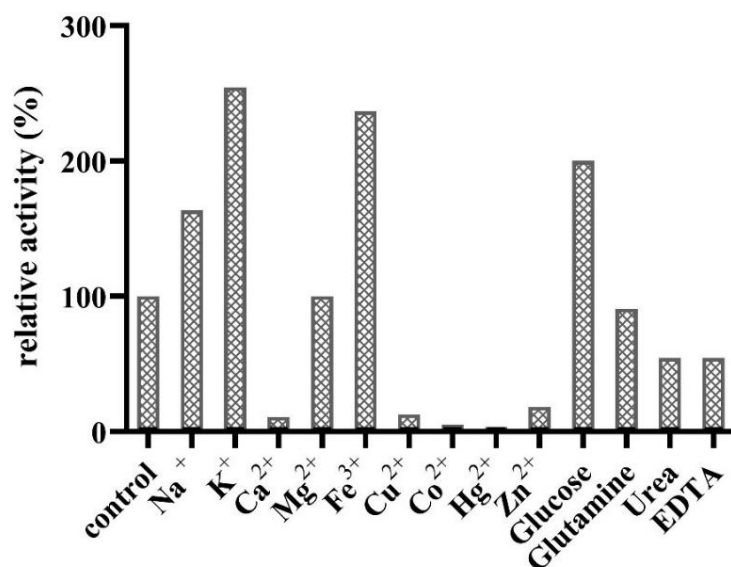


Figure 7. The effect of metal ions at 5 mM and some blood serum components at 1mM concentration

## 4. Discussion and Conclusion

Recent application of L-asparaginase in the treatment of certain kinds of leukemia such as acute lymphoblastic leukemia made it active research field in cancer therapy. However, like some other drugs, the longtime use of L-asparaginase may cause a side effect because of the glutaminase activity of the enzyme. In this study, we isolated a glutaminase free L-asparaginase producing bacterium from farm soil. According to 16S rDNA sequencing and morphological properties, the isolated bacterium was found to belong to the *Staphylococcus* genus. However, its physiochemical characteristics are considerably different from the nearest hit (*S. warneri*, 99.13 % homology) therefore; more experiments such as DNA-DNA hybridization should be done for exact identification of the bacterium. L-asparaginase from gram-positive bacteria other than *Actinobacteria* has been poorly studied and exploration of these species as new sources of L-asparaginases may be worthwhile. Sobis and Mikucki report an L-asparaginase from a *Staphylococcus* with optimum pH between 8.6 and 8.9 and optimum temperature around 30 °C [30]. *Staphylococcus* sp. – 6A [31] was found to produce 6 IU/ml L-asparaginase with the highest activity at 39 °C and pH 7.5 with unknown mass. In 2014, Han et al. [32] isolated NaCl-tolerant *Staphylococcus* sp. OJ82 from the

traditional Korean fermented food with the ability to producing 2.3 IU/ml L-asparaginase which was active in temperatures 37-50 °C and pH 8-10.

The enzyme was a dimer with 67 kDa molecular mass and subsequently cloned in *E. coli* and purified by affinity chromatography to 86.9 IU/mg specific activity and a 98.75-fold purification. The isolated *Staphylococcus* of this study produces 4.93 IU/ml enzyme with the best activity at the temperature of 35 °C and the pH 8. These results are consistent with other staphylococcal asparaginases reported elsewhere. The optimal pH and temperature of enzymes from *staphylococci* were near the physiological pH (7.4) and temperature (35-37 °C), thus these L-asparaginases might be suitable for therapeutic applications. Partial purification of L-asparaginase carried out by ammonium sulfate precipitation and DEAE anion-exchange chromatography result in an enzyme with 115 kDa molecular weight and 12.93-fold increase in the specific activity. L- asparaginases from other gram positive sources have a very broad range of molecular mass from 45 kDa in *Bacillus* sp. [33] to 140 kDa in *Streptomyces noursei* [19].

The monovalent cations exhibit a considerable increase in activity of L-asparaginase. The enzymes from *Enterobacter cloacae* and *Pectobacterium carotovorum* also showed the same effect [28, 34]. However, no change in activity was observed in only other study of metal ions effect on staphylococcal asparaginases [32]. The divalent cations except magnesium almost totally inhibit L-asparaginase in this study. Han et al. also have reported inhibition of SoAsn by  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  but increased activity by  $\text{Co}^{2+}$  (263.9 %), ferrous ion (203.7 %) and  $\text{Cu}^{2+}$  (109.3 %).  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Hg}^{2+}$  have been reported as inhibitors of *E. cloacae* enzyme [34]. Also reported the inhibitory effect of  $\text{Hg}^{2+}$ . L-asparaginases from *Vibrio* species [35, 36] and fungal sources such as *Fusarium culmorum* [37] and *Aspergillus nidulans* [38] were effected by  $\text{Cu}^{2+}$  from complete inhibition to 84% decrease in activity. Ferric ion markedly enhance activity of MGM1 asparaginase and inhibition of its half activity by EDTA suggest that  $\text{Fe}^{3+}$  is the cofactor of the enzyme. Among blood serum components glucose increase activity by 200 % while glutamine did not show any effect and urea inhibit 45 % of activity [9]. Have reported that 58 % of activity of *S. brolllosae* asparaginase decreased by 1 mM urea.

The glutaminase side activity of enzyme is the cause of several problems associated by glutamine depletion such as reducing the synthesis of important proteins like albumin, fibrinogen, insulin, and protein C. The enzyme reported here was very specific for L-asparagine, with no glutaminase activity in contrast to the *E. Coli* L-asparaginase and SoAsn from *Staphylococcus* sp. OJ82, which is associated with 19 % and 58.2 % relative glutaminase activity, respectively [32]. The present study clearly supports usefulness of simple soil screening techniques. The isolated L-asparaginase is highly active in physiological pH and temperature and easily purified in two steps. In addition, the enzyme is greatly enhanced in the presence of usual ions and other ingredients of human blood serum that makes it a potential candidate for enzyme therapy of leukemia.

Among the drugs used to treat leukemia, Asparaginase is one of the best drugs that can be used in the treatment of various types of leukemia like ALL. Nowadays, among the different enzyme-producing sources, only 2 of them "the enzyme extracted by *Erwinia* and *E. coli*" are used. However, it is ongoing to find new sources of producer Enzymes with more distinct and consistent features with the body. According to the obtained results in this study, like the stability of the asparaginase enzyme isolated from MGM1 sp in body physiological conditions such as temperature 35C, pH8, and various ions and compounds, as well as the absence of glutaminase activity, can be hoped that the producing enzyme, is an enzyme with good therapeutic properties and low side effects requiring clinical trials and Application to laboratory models and cancer cell lines. On the other hand, considering the results of this study and the enzymatic activity of the bacterium compared to similar studies, it can be said that the enzyme L-asparaginase produced by MGM1, is an isoenzyme with different characteristic properties, as well as it seems to be a new strain of *Staphylococcus* that needs further studies for its accurate identification. It is important in this respect that accurate identification of the source of the enzyme producer contributes greatly to the research and analysis of the enzyme produced by the bacterium.

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## 7. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



## 8. Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Shahid Beheshti University affiliated to Iran's Ministry of Science, Research & Technology and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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