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Original Research Article

Exploring bacillus subtilis isolated from soil as a promising alternative for L-asparaginase enzyme production

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ABSTRACT

In this study, eight bacterial isolates were obtained from the 35 soil samples of Golestan province in Iran. Samples were collected from different soils and cultured in an M9 medium. Enzyme-producing colonies were distinguished by the color change from yellow to pink. 16s rRNA sequencing identified L-asparaginase (L-ASNase)-producing bacterial species in soil samples. The presence of L-ASNase in *Bacillus subtilis* was shown by M9 culture and confirmed by spectrophotometry. *Bacillus subtilis* was identified by 16S rRNA gene sequencing. L-ASNase isolated from *Bacillus subtilis* showed effective antioxidant activity and also anti-bacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The present study demonstrated the presence of L-ASNase-producing *Bacillus subtilis* strain in soil samples with antioxidant and antibacterial activity.

***Keywords*:** L-asparaginase, soil bacteria, antioxidant activity

INTRODUCTION

Tumor cells have a heightened demand for non-essential amino acids, including asparagine, to support their development and progression [1,2]. L-asparaginase (L-ASNase) hydrolyzes, causing nutritional deficits and inhibiting protein synthesis by converting extracellular L-asparagine into

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L-aspartate and ammonia [3]. The administration of L-ASNase, which depletes circulating Asn, leads to nutrient deprivation and preferential induction of apoptosis in leukemic blasts [4-6]. L-ASNase plays a critical role in the therapeutic approach for patients diagnosed with ALL [7,8]. Therefore, it is imperative to identify and develop novel and robust L-ASNase derived from unexplored microorganisms that possess enhanced stability, reduced glutaminase activity, high substrate affinity, and low Km values. These optimized L-ASNase can serve as more effective therapeutic agents for treating ALL and other diseases that critically depend on extracellular Asn [9].

Microorganisms are the preferred choice for large-scale production of L-ASNase for clinical and industrial applications [10,11]. The systemic administration of bacterial-derived L-ASNase has proven to be an effective strategy for reducing the bioavailability of Asn and eliminating rapidly dividing ALL cells [4,12]. Since this enzyme has potential antimicrobial properties, there has been an increasing interest in developing methods to use it as an antimicrobial agent [13]. There have been several studies highlighting L-ASNase's antimicrobial properties and advocating for its potential application in infection control [14,15].

L-ASNase have been identified in numerous bacterial species, with the Bacillus genus being particularly abundant due to its ability to withstand harsh environmental conditions [16]. Bacillus spp. offer a diverse range of L-ASNase enzymes that can be utilized for therapeutic applications as well as in the food industry [17]. The *Bacillus subtilis* isolated from marine sponges represents a source for producing glutaminase-free L-ASNase. This L-ASNase has potential applications in developing anti-cancer drugs associated with reduced or no side effects[18]. *Bacillus subtilis* isolated from soil has demonstrated that it can be utilized for the industrial production of L-ASNase. By optimizing the growth conditions, L-ASNase production can be increased by up to 2-fold [19]. Hence, there is a pressing requirement for novel L-ASNases with improved immunological attributes, particularly those that exhibit reduced allergenicity, to minimize the occurrence of allergic reactions [20,21]. The current study used experimental approaches to evaluate the production of L-ASNase from the recovered soil isolate, *Bacillus subtilis*, in Golestan province, Iran. The study gives

evidence for introducing ASNase derived from *Bacillus Bacillus* as an additional source to those of the two FDA-approved ASNases to be used as anti-cancer agents.

MATERIALS AND METHODS

Sampling and Culture

Soil samples were collected from various locations in Gorgan, Golestan Province in Iran. The samples were taken from a depth of 10 cm and placed in plastic containers, which were stored at 4 °C. The soil samples were serially diluted by mixing with sterile normal saline. The tubes were centrifuged at 1000 rpm for 3 min, and then 25 microliters of supernatant from the 10^{-4} and 10^{-6} dilutions were cultured on 8 cm plates containing M9 Modified culture medium under sterile conditions. The cultures were spread evenly on the plates using an L-shaped Pasteur pipette. The plates were incubated at 37 °C for 48 h [22,23].

M9 Medium

The M9 medium used in this study consisted of the following components: 1 % glucose, 0.05 % K2HPO4, 0.02 % MgSO4.7H2O, 0.001 % FeSO4.7H2O, 0.001 % CaCl2.2H2O, 0.1 % L-asparagine, and 0.1 % trace elements (ZnCl2, MnCl2.4H2O, CoCl2.6H2O, NiCl2.6H2O, CuCl2.2H2O, and NaMoO4.2H2O). Additionally, it contained 1.5 % agar with phenol red (0.04-0.36 ml of a solution containing 2.5 %) to maintain a pH of approximately 7.

Colony selection

After the incubation period, colonies that produced the L-ASNase enzyme (indicated by a change in color from yellow to pink) were selected for further analysis. They were cultured on another plate containing M9 Modified culture medium to obtain pure colonies, where the only nitrogen source was replaced with asparagine. The selected colonies were then kept in slant environments and a refrigerator at 4 °C for future use [24].

Screening of L-asparaginase production

Screening for L-ASNase production involved analyzing soil samples for bacteria that produced a pink or reddish-pink color. Colonies grown on a modified M9 medium were evaluated for L-ASNase enzyme activity by measuring the amount of released ammonia using the Nesslerization method. Ammonia levels were determined by comparing them to ammonium sulfate solution, which served

as a standard. Nessler's reagent, which turns yellow to brown in ammonia, was used to detect even small amounts of ammonia. Nessler's reagent was used to determine enzyme activity as the L-ASNase enzyme produces ammonia by breaking down asparagine [25].

Identification of the bacterial isolates

The bacterial isolates that produced the enzyme were identified using standard biochemical methods of bacteriology. Tests including colony morphology, gram staining, catalase, citrate consumption, starch and casein hydrolysis, gelatinase, and motility were performed and recorded.

Molecular Identification of Enzyme-Producing Strains

Following enzyme extraction, conventional methods were employed to identify the producing strains. Subsequently, bacterial species containing L-ASNase enzymes were analyzed using molecular tests to determine their genus and species definitively. DNA extraction of selected bacteria was performed using the DN8115C kit from Sina Gene Company. Primer sequences designed for 16S rDNA were used for PCR amplification.

The resulting products were electrophoresed in a 1 % agarose gel, then photographed under UV rays using a uvidoc device to confirm band position. The size of each band was estimated using a gene ruler (Ladder) ranging from 250-10000 bp. The PCR products were sent to South Korea's Macrogen Company for sequencing. After determining the sequencing results, the sequences were BLAST on the NCBI site and recorded using Chromas 2.33 software.

Reductive Power (RP) Method

To measure antioxidant activity, the Reductive Power (RP) method [26] was utilized to determine the reductive power of bacterial enzymes. In this method, 100 microliters of cell culture supernatant containing enzyme were mixed with 100 ml of methanol, and dilutions ranging from 12.5 to 1000 (v/v) were prepared. Then, 1 ml of each dilution was poured into a test tube and mixed with 2.5 ml of phosphate buffer (6.6 pH) and 2.5 ml of potassium ferrocyanide before being thoroughly mixed again.

The mixture was placed in a bain-marie at 50 °C for 30 min. Afterward, 5 ml of trichloroacetic acid was added to each sample. The samples were then centrifuged

for 10 min at 1700 rpm. Next, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of iron III chloride in a test tube, and its absorbance was read at a wavelength of 700 nm. The percentage of reducing power of the existing L-ASNase enzymes was calculated using the following equation for absorbance readings:

$$RP (\%) = [(A_{Control} – A_{Sample})/A_{Control}] \times 100$$

Investigation of Anti-Bacterial Effect

The agar well diffusion method was used to evaluate the antibacterial effect of the enzymes extracted. In this way, after preparing the turbidity equivalent to 0.5 McFarland tube, the uniform culture of the test bacterial strains, including a gram-positive coccus (*Staphylococcus aureus*) and a gram-negative bacillus (*Escherichia coli*), was carried out on Mueller-Hinton agar culture medium plates. Then 6 mm diameter wells were dug in the culture media, and 100 µl of each enzyme supernatant sample was transferred into the wells. The plates were incubated for 24 h at 37 °C [27].

RESULTS

Screening of L-asparaginase-producing Bacteria

This study, 35 soil samples were collected from various environments such as industrial areas, gardens, forests, and fields to investigate the presence of *Bacillus subtilis* producing L-ASNase enzyme. The findings revealed that eight bacterial isolates could produce ammonia compounds in the environment by changing the color of the culture media color to pink due to pH changes as a result of asparagine production (Figure 1).

Among all the isolates, eight isolates produced an intense pink zone around the colonies. The highest positive isolates and the most intense pink zone were found in the soil around an industrial slaughterhouse. After purifying the primary colonies, enzyme activity was investigated for all isolates producing L-ASNase. The results showed that all isolates had enzyme activity, calculated and recorded based on the standard unit of one µmol of ammonia released in one min at 37 °C. The absorbance rate of the test and standard samples was measured using a spectrophotometer and calculated based on

the Beer-Lambert law. The results are presented in Table 2.

By plotting absorbance changes in different concentrations of standard samples, a standard absorbance chart (Figure 2) was obtained, and unknown sample concentrations were calculated using the line slope formula (Table 3).

Table 1. Primer sequence prepared for 16S rDNA synthesis

Primer name	Sequence
BAc08F	5'-AGAGTTTGATCCTGGCTCAG-3'
Uni 1390R	5'-GACGGGCGGTGTGTA CAA-3'

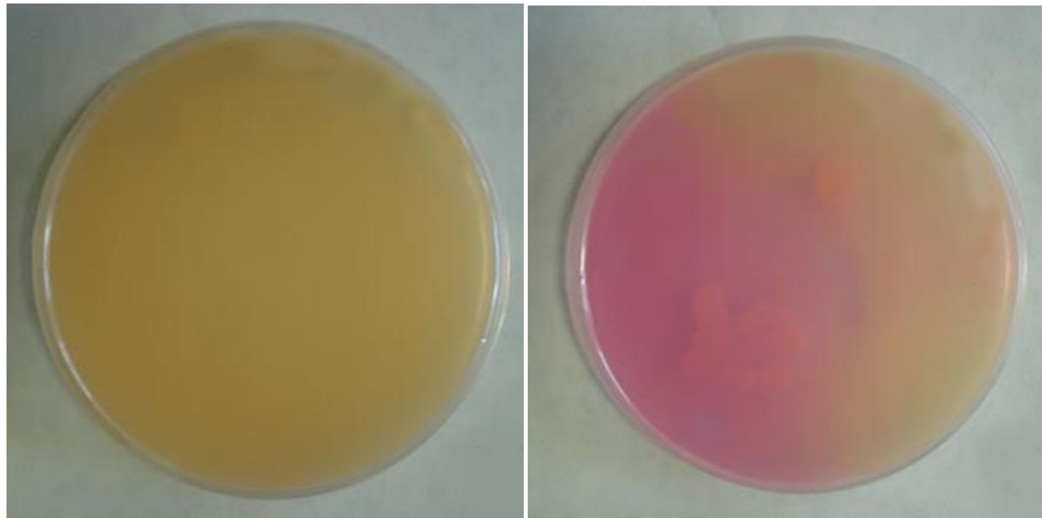

Figure 1. Detection of L-ASNase-producing isolates in the modified M9 medium

Table 2. Absorption of standard samples

Absorption rate (OD) 480 nm	Standard ascending concentrations Cs (µm/ml)
300	0.23
400	0.30
500	0.38
600	0.46
700	0.51
800	0.52

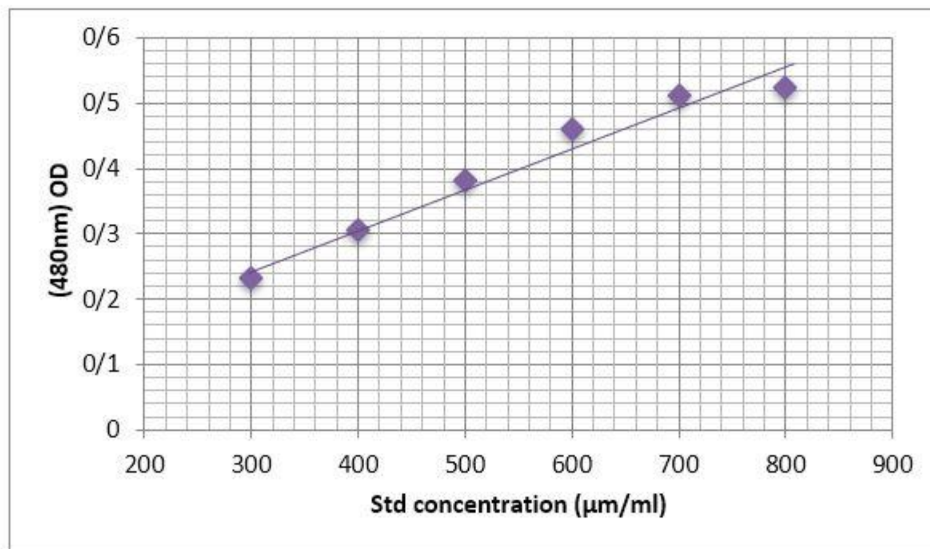

Figure 2. Standard absorption diagram of different investigated concentrations;

Slop = Δ OD/Δ C xSlop = 0.32/500 = 0.00064

Table 3. Absorption and unit of test samples (bacterial enzymes)

Sample number	Absorbance 480 nm	Calculated concentrations Cs (µm/ml)	Enzyme Units/ml
1	0.41	643.7	64.37
2	0.52	815.6	81.56
3	0.46	720.3	72.03
4	0.42	653.1	65.31
5	0.67	1043.7	104.37
6	0.45	709.4	70.94
7	0.44	684.4	68.44
8	0.39	614.1	61.41

Biochemical tests

Biochemical tests were conducted on enzyme-producing isolates, including colony morphology, gram staining, catalase, oxidase, indole production, citrate decomposition, Methyl Red (MR), Voges-Proskauer (VP), starch decomposition, gelatin hydrolysis, urea, motility, and Hydrogen Sulfide (H_2S) production. Results showed that out of 8 bacilli bacteria isolates tested, six were gram-positive and two were gram-negative. Table 4 displays the results of other biochemical tests based on the type of reaction for each bacterial isolate.

Evaluation of antioxidant effect of L-asparaginases

After identifying the producing strains of L-ASNases and purifying the enzyme by centrifugation, investigations related to its secondary metabolite were implemented in the antioxidant field. The antioxidant effect of the enzyme samples was calculated by measuring their reductive power on trivalent iron chloride based on an existing equation. IC50 values for each of the eight enzyme samples obtained

from productive soil bacteria were calculated using the absorption curve line drawn for different enzyme dilutions in the RP method. The reducing power of the enzyme samples was plotted in μg/mL compared to synthetic antioxidant BHA. For five isolates, the antibacterial effects were evaluated as favorable.

Evaluation of anti-bacterial effect of L-asparaginases

For five isolates, the antibacterial effects were evaluated as favorable, and the diameter of the halo of non-growth was visible. Some strains did not have an excellent antibacterial effect on gram-positive and gram-negative strains under investigation. The diameter of the no-growth halo has been measured (Table 4) (Figure 3).

Characterization and identification of Strain

Based on the data obtained from the observations, the top 4 bacterial strains in terms of L-ASNase enzyme production, which in the evaluations included enzyme activity, and antibacterial and antioxidant effects, had provided good quantitative and qualitative results for confirmation identification. Bacterial strains number 2, number 3, number 5, and number 6 were selected by molecular method, and after analyzing and determining the sequences made in this regard, sample number 3 is *Bacillus subtilis*.

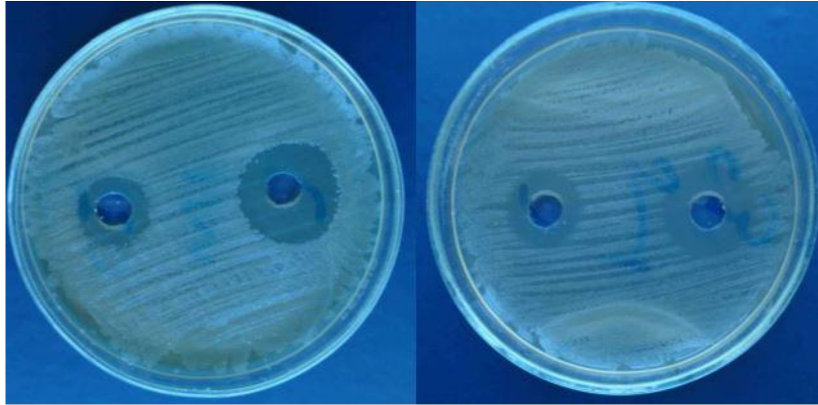

Figure 3. Antibacterial effect of L-asparaginases extracted from soil bacteria (Right: Escherichia coli; Left: Staphylococcus aureus).

Table 4. Antibacterial activity of L-ASNase in *Bacillus subtilis*

Test	***Bacillus subtilis***	**Positive Control**	**Negative Control**
Escherichia coli	9.0	18	0.0
Staphylococcus aureus	9.5	20.5	0.0

For bacteria, the diameter of the no-growth halo has been measured. All measured diameters are in millimeters. Tetracycline 5 % was used for positive control and broth medium without enzyme was used for negative control.

DISCUSSION

Despite the high compatibility of L-ASNase with the body's tissues and vital mechanisms compared to other chemotherapy drugs or more recent therapies such as oligonucleotide therapeutics, L-ASNase may still cause adverse side effects that can endanger the patient's life [28,29]. L-ASNase's derivation from bacteria has contributed to toxicity and hypersensitivity reactions which limited the use of L-ASNase in some populations of all patients. Consequently, it is crucial to identify alternative sources of L-ASNases.

The soil environment in the northern region of Iran, characterized by a diverse range of vegetation, represents a rich source of bacterial species that may harbor novel bioactive compounds with potential therapeutic applications [30,31]. In this investigation, out of eight bacterial isolates exhibiting positive L-ASNase activity, molecular analysis of the 16S rRNA gene confirmed the identity of the selected strain, designated as Strain 3, as *Bacillus subtilis*. The study focused on the soils of Gorgan County in Iran, where *Bacillus subtilis* were found through sampling. The antioxidant and anti-bacterial effects of L-ASNase enzymes produced by *Bacillus subtilis* isolated from with soil were evaluated and found to be positive.

Bacteria have adapted to various environmental conditions and produced different intracellular, extracellular, or both enzymes to catalyze chemical reactions [32-34]. *The Bacillus genus* represents a cost-effective source of enzymes owing to their ubiquitous distribution, ease of cultivation, safety in handling, and amenability to genetic manipulation [35]. *Bacillus subtilis*, a Gram-positive bacterium commonly found in the upper layers of soil, is an example of a microbial cell factory [36]. *Bacillus subtilis* has gained popularity as a microbial cell factory due to its superior fermentation characteristics, ability to produce high yields of desired products, and minimal generation of toxic by-products [36,37]. Therefore, *Bacillus subtilis* isolated from soil represents a promising source of anticancer L-ASNases, which can be produced through submerged fermentation [24,38].

This research aimed to identify readily available bacterial L-ASNase enzymes found in soil bacteria with the potential for anticancer applications [39]. As different

microorganisms produce L-ASNase enzymes with varying therapeutic properties and side effects, researchers have explored various natural sources to discover more desirable enzymes [40-42]. El-Fakharany et al. conducted tests on the antioxidant and anticancer activity of L-ASNase isolated from *Bacillus halotolerans*, revealing its cytotoxic effect as a promising candidate for further therapeutic applications as an antioxidant and antitumor drug [43]. Alrumman et al. have shown that *Bacillus licheniformis* isolated from the Red Sea is capable of producing glutaminase-free L-ASNase. The study found that the highest production of L-ASNase occurred at 72 h of fermentation time, at a pH of 6.5 and a temperature of 37 °C [9].

Researchers have reported isolating L-ASNase-producing *Bacillus subtilis* from different sources with desirable properties. Shukla and Mandal have shown that optimum production of L-ASNase (18.4 U/ml) was obtained by using *Bacillus subtilis* isolated from soil after 48 h of incubation time [44]. Pradhan *et al*. found that *Bacillus subtilis* strain hswx88 exhibited extracellular enzyme production capacity. The enzyme production was 1.7 and 14.5 times higher than the reference organisms Pectobacterium carotovorum and Bacillus sp., respectively. This isolate can utilize for large-scale production of thermophilic L-ASNase, which can be used in the pharmaceutical industry and acrylamide-free fried food products [45]. Jia *et al.* isolated *Bacillus subtilis* B11-06 from the soil, which produced a putative L-ASNase. The overexpressed gene encoding L-ASNase, and the recombinant enzyme demonstrated high thermostability and low affinity to L-glutamine, with optimal pH and temperature values of 7.5 and 40 °C, respectively [46]. Gholami et al. isolated *Bacillus subtilis* from the soil as a potential source for glutaminase-free L-ASNase production. Optimization studies found that the maximum production of L-ASNase occurred at a pH of 7.0 and a temperature of 37 °C, utilizing 1 % whey as the preferred carbon source and 0.2 % asparagine as the nitrogen source [19]. In a similar study, a soil isolate of *Bacillus subtilis* was identified as a producer of L-ASNase, with the glutaminase activity of the enzyme found to be 5.9 times lower than its L-ASNase activity. The enzyme was found to be stable in the pH range of 6.5 to 8.5 and up to a temperature of 55 °C. Furthermore, the purified L-ASNase demonstrated anticancer activity against

MCF-7 cell lines, with an IC50 value of 21 μg/mL [47].

Our study showed that the L-ASNase – producing *Bacillus subtilis* has an anti-bacterial and antioxidant effect. The results confirmed the potent antimicrobial activity of *Bacillus spp* against pathogenic microorganisms in previous studies [27]. Further research with a broader scope could be beneficial for assessing the applicability of this isolate for the production of L-ASNase for optimization of ALL treatments using this enzyme to avoid side effects such as toxicity and hypersensitivity reactions in patients. This approach would promote using cost-effective and readily available compounds in downstream processes while achieving long-term economic goals for the project.

There are some limitations to our study that need to be considered. The isolation, purification, activity, and kinetic parameters of L-ASNase must be investigated in future research in order to characterize L-ASNase activity comprehensively. The presence of the enzyme could be validated by SDS-PAGE and compared with a standard enzyme. Comparative analysis can be conducted between the produced and commercial L-ASNase to understand L-ASNase better. In light of these limitations, there is a need for further research to address these issues and to advance our understanding of the enzymes of *Bacillus subtilis*.

CONCLUSION

In conclusion, the present study successfully identified the production of L-ASNase enzyme from the soil bacteria *Bacillus subtilis*. The enzyme exhibited anti-oxidative activity and anti-bacterial against *E. coli* and *S. aureus*, indicating its promising therapeutic potential. Overall, the results of this study provide a targeted approach for the production of L-ASNase. Our study highlights the potential of the L-ASNase enzyme from *Bacillus subtilis* as an alternative candidate for developing novel anti-cancer drugs.

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