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ORIGINAL ARTICLE

L-Asparaginase Production by Moderate Halophilic Bacteria Isolated from Maharloo Salt Lake

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Abstract L-Asparaginase is an anti-neoplastic drug used in lymphoblastic leukemia chemotherapy. Nowadays, this enzyme derived from bacterial sources, mostly L-asparaginase II from *Escherichia coli* and in lesser amount L-asparaginase of *Erwinia* sp. has medical utilization. The long-term usage of these agents leads to allergic reactions and new asparaginase with new immunological characteristics is required. Halophilic bacteria might contain L-asparaginase with novel immunological properties that can be used in hypersensitive patients. In this experiment, we have screened moderate Halophilic bacteria for L-asparaginase production ability and showed that Halophilic bacteria produce intra- and extracellular L-asparaginase. *Bacillus* sp. BCCS 034 was found to produce the highest L-asparaginase (1.64 IU/ml supernatant) extracellularly.

Keywords L-Asparaginase · Halophile · Bacteria · 16S rRNA

Introduction

L-Asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) is an anti-neoplastic agent, used in the therapy of certain lymphomas and leukemias in both experimental animals and humans and has been used in combination with other agents in the treatment of acute lymphoblastic leukemia chemotherapy [1, 2]. L-Asparaginase is an amidohydrolase that catalyzes the hydrolysis of the amino acid asparagine to aspartic acid and ammonia. Neoplastic cells cannot synthesize L-asparagine due the absence of L-asparagine synthetase and should obtain it from circulating sources. For this reason, the commonest therapeutic practice is to inject the enzyme intravenously in order to decrease the blood concentration of L-asparagine, selectively affecting the neoplastic cells [1]. Since the observation that L-asparaginase from *Escherichia coli* has an antitumor activity similar to that of guinea pig serum, there has been considerable interest in asparaginase from various sources specially microorganisms [3]. Although other microorganisms such as *Aerobacter*, *Bacillus*, *Erwinia*, *Pseudomonas*, *Serratia*, *Xanthomonas*, *Photobacterium* [1], *Streptomyces* [4], *Proteus* [5], *Vibrio* [6] and *Aspergillus* [3] have a potential for asparaginase production, just the purified enzyme from *E. coli* and *Erwinia* sp. [4] has been used as anti-tumor and anti-leukemia agent.

The medical utilization of asparaginase from these two mentioned sources was limited because of the immunological responses. However, L-asparaginase from bacterial origin can cause hypersensitivity in the long-term usage, leading to allergic reactions and anaphylaxis. So, we need new asparaginase with new immunological characteristics. For these reasons, considerable efforts have been devoted to the selection of microorganisms via sophisticated screening techniques and process methodology for the production of L-asparaginase with new antigenic properties.

Hypersaline lakes, with salinity ranges at or near saturation are extreme environments; yet, they often maintain remarkably high microbial cell densities and are biologically very productive ecosystems [5]. Microorganisms inhabiting these environments are expected to have proteins with different features than proteins of non-saline environment organisms. They have halophilic enzymes

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with modified structure that creates tolerance of high salt concentration and low water activity. Therefore, Halophilic bacteria may contain L-asparaginase with novel immunological properties that can be used in hypersensitive patients.

Moderately halophilic bacteria are a group of halophilic microorganisms able to grow optimally in the media containing a wide range of NaCl concentrations (3–15% NaCl). They constitute a heterogeneous group of microorganisms including species belonging to different genera, such as *Halomonas*, *Salinivibrio*, *Chromohalobacter*, and have been studied with regard to their ecology, physiology, biochemistry and more recently, their genetics [7]. In this study, we describe the screening for extra- and intracellular L-asparaginase producing moderately halophilic bacteria, isolated from Maharloo hypersaline lake located in the south of Shiraz, Iran.

Materials and Methods

Isolation of Moderate Halophilic Bacteria

Sediments and water samples were collected from different parts of Maharloo salt lake in the south of Shiraz, Iran. Suspensions were prepared by mixing of each sample with sterile distilled water. After sedimentation, the supernatants were strictly cultured and serially diluted. 100 µl of the 10^{-3} and 10^{-4} dilution tubes were spread on to saline nutrient agar containing 7% (w/v) NaCl and incubated at 37°C for 24 h, to obtain single colonies. Some of the colonies growing on the plates were purified by streaking on saline nutrient agar plates.

Identification of Bacterial Isolates

Different morphological, cultural and physiological characteristics of the bacterial isolates were studied for identification purpose and compared with standard description of Bergey's Manual of Determinative Bacteriology [8].

Analysis of 16S rRNA Gene Sequence

The purified bacterial isolates were cultured in saline nutrient broth (7% NaCl). After centrifugation at 4500×*g*, 10 min, at 4°C, and twice washing with distilled water, the pellets were selected for DNA extraction and PCR amplification. Bacterial DNA was extracted by heat extraction method. The 16S rRNA gene was amplified by PCR, using the universal prokaryotic primers 5′-ACGGGCGGTGTG-TAC-3′ and 5′-CAGCCGCGGTAATAC-3′, which amplify a ~800-bp region of the 16S rRNA gene. PCR was performed in a final volume of 50 µl containing PCR amplification buffer (1×), *Taq* DNA polymerase (2.5 U), dNTPs (4 mM), primers (0.4 µM) and template DNA (4 ng). Amplification conditions were initial denaturation at 94°C for 5 min, 10 cycles at; 94°C for 30 s, 50°C for 30 s and 72°C for 2 min. 20 cycles at; 92°C for 30 s, 50°C for 30 s, and 72°C for 2.5 min with a final extension of 72°C for 5 min. *Taq* polymerase was added to the reaction after initial denaturation. The lower denaturation temperature (92°C) during the 20 cycle step was used to avoid loss of enzyme activity [9] The samples were electrophoresed in a 1% (w/v) agarose gel, using TBE buffer containing ethidium bromide (1 µg/ml). A single ~800 bp DNA fragment was cut and extracted from the gel, using a Core Bio Gel Extraction Kit. The sequence was determined by the CinnaGen Company. The sequence similarity searches were done using the BLAST program that is available from the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and GeneDoc software, version 2.6.002.

Enzyme Solution Preparation

The isolated colonies were transferred to 250 ml Erlenmeyer flasks with 50 ml broth medium containing in 1 l; KH_2PO_4 (0.75 g), NaCl (0.5 g), L-asparagine (10 g), and glucose (1 g) [10] and incubated in a shaker incubator (150 rpm, 37°C) for 48 h.

After incubation, the cells were removed by centrifugation at 4000 × g for 5 min. The supernatant was used to assay extracellular L-asparaginase activity. For determination of the intracellular L-asparaginase, bacterial pellets were freeze-dried and 0.1 g of dry cell weight was resuspended in 80 µl sonication buffer (50 mm Tris and 10 mM EDTA, pH 7.5). The suspension was transferred to a 1.5 ml thin wall micro tube. Then, the tube was placed in water and ice mixture and treated with sonicator. Six treatments of one min each at an amplitude setting for Gram-negative bacteria and 10 for Gram-positive bacteria were performed. The disrupted cells were then brought down to the bottom of the tube by centrifugation at 13000×*g* for 20 min. The supernatant was used as enzyme solution for intracellular L-asparaginase activity assay [11].

L-Asparaginase Assay

L-Asparaginase activity was measured by Nessler's reaction. The assay procedure is based on direct Nesslerization of ammonia. Enzyme solution (30 µl) was added to Tris–HCl (pH 8.5, 50 mM) in a final volume of 1.5 ml. The reaction was started with addition of 0.5 ml L-asparagine solution (10 mM, in 50 mM Tris–HCl, pH 8.5) and incubation in 37°C water bath for 20 min. The reaction was terminated with addition of 0.5 ml trichloroacetic acid 15%

(w/v) and the volume was adjusted to 4.5 ml with distilled water. Nessler's reagent (0.5 ml, 45.5 g HgI_2 and 35.0 g KI in 1 liter distilled water containing 112 g of KOH) was added and the tubes were incubated at room temperature for 15 min. After vortexing, the absorbance was measured at 500 nm, using visible spectrophotometer [12, 13]. A standard curve was drawn with various concentrations of ammonia.

Results

Isolated Bacterial Strains

Totally, 32 bacterial isolates were obtained from sediment and water samples, of which 23 isolates (71.9%) were identified as *Bacillus* species. They were *Bacillus subtilis*, *Bacillus endophyticus*, *Bacillus thuringiensis*, *Bacillus pumilus*, *Bacillus simplex*, *Bacillus amyloliquefaciens*, *Bacillus vallismortis*, and *Bacillus aquimaris*. Other Gram-positive strains were one strain of *Paenibacillus* sp. and two strains of *Staphylococcus epidermidis*. Just three Gram-negative bacterial strains were obtained, two strains of *Aeromonas veroni* and one strain of *Chryseobacterium taeanese*. Also, two archaeal strains, *Halobacterium* sp., were isolated.

L-Asparaginase Activity

Among 32 isolated bacterial strains, 11 (34.4%) showed L-asparaginase activity, from which nine strains (81.8%) had both intra- and extracellular asparaginase and two strains (18.2%) just had intracellular asparaginase activity. *Bacillus* sp. BCCS 034 was found to produce the most L-asparaginase, 1.64 IU/ml supernatant (Table 1).

16S rRNA Gene Sequence Analysis

The PCR amplification of 16S rRNA gene revealed efficient amplification; a single band of amplified DNA product of ~800-bp was recorded. The DNA sequences were published in the NCBI databases under the specific accession numbers (Table 1). The result of PCR blasted with other sequenced bacteria in NCBI showed similarity to the 16S small subunit rRNA of other bacteria. Edited sequences were used as queries in BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to determine the nearest identifiable match present in the complete GenBank nucleotide database. A bioinformatic tool, GeneDoc software, version 2.6.002, was used for more 16S rRNA gene sequence investigation of the most L-asparaginase producing strain *Bacillus* sp. BCCS 034. A total of 802 nucleotides of the partial sequence of *Bacillus* sp. BCCS 034 were 99% similar to the 16S ribosomal rRNA genes in other recorded strains of *Bacillus* sp. in National Centre for Biotechnology Information (NCBI) (Fig. 1).

Discussion

The largest saline body of water, hypersaline environments, are generally defined as those containing salt concentrations in excess of sea water (3.5% total dissolved salts). A great diversity of microbial life is observed in brine from marine salinity up to about 3–3.5 mol/l NaCl, at which point only halophiles can grow. Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic

Table 1 The intra and extracellular L-asparaginase activity of various strains of moderate halophilic bacteria, the accession numbers and the length base pair of DNA published sequences at the NCBI

Bacterial strain	Accession number	Length (bp)	IU/ml cell extract	IU/ml supernatant
Bacillus sp. BCCS 036	FJ619744	802	1.10	1.16
Bacillus sp. BCCS 034	FJ607328	802	1.07	1.64
Bacillus subtilis BCCS 027	FJ517538	832	1.23	1.07
Bacillus subtilis BCCS 028	FJ517539	784	1.28	1.01
Bacillus sp. BCCS 029	FJ517540	683	1.14	1.16
Halobacterium sp. BCCS 030	FJ529204	821	1.21	1.30
Bacillus endophyticus BCCS 031	FJ426405	702	1.30	1.39
Bacillus sp. BCCS 040	FJ645730	787	0.17	1.50
Aeromonas sp. BCCS 037	FJ619745	802	1.03	0.00
Bacillus thuringiensis	FJ624483	804	0.2	0.00
Chryseobacterium taeanese BCCS 016	FJ422574	800	0.20	0.30

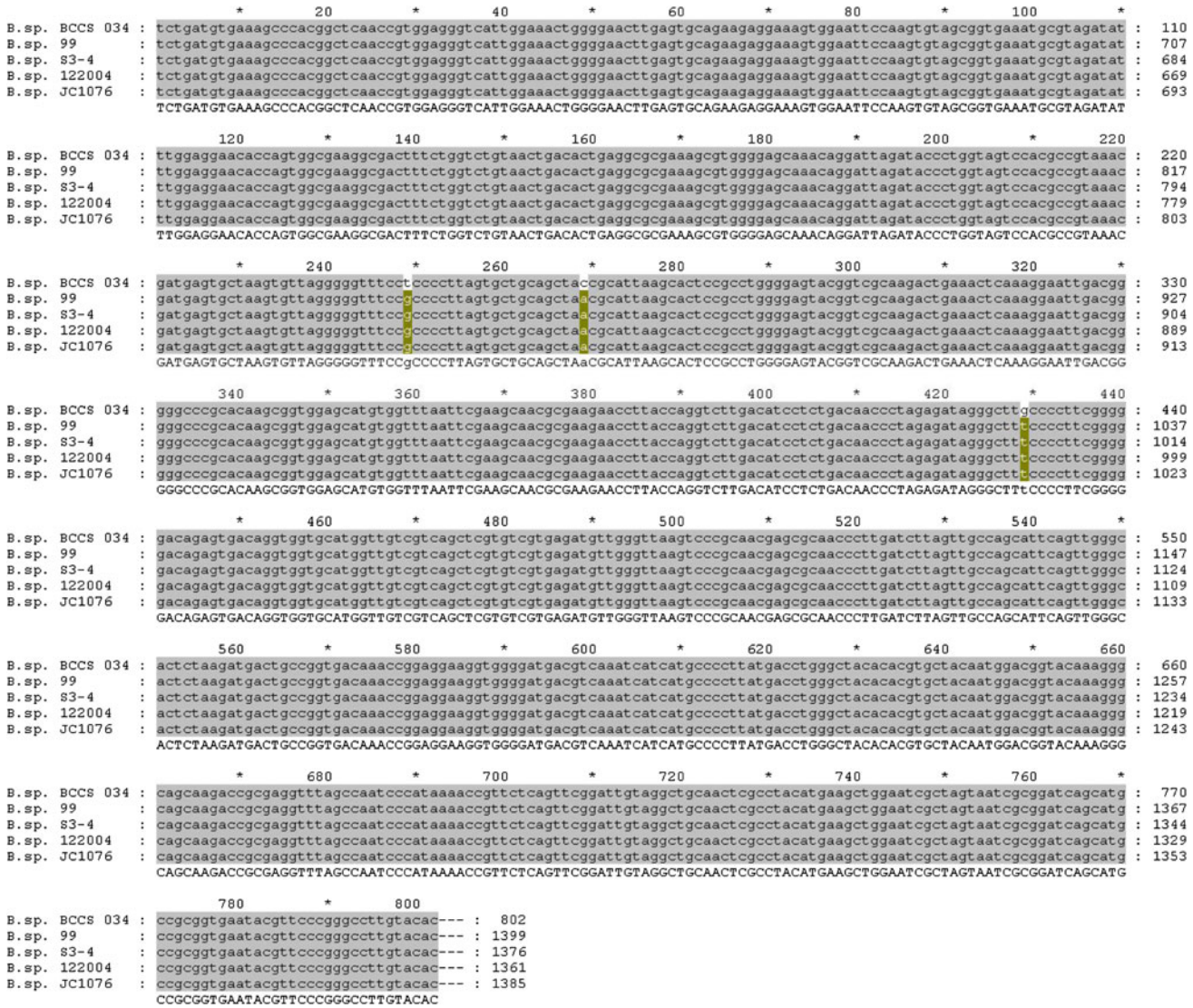

Fig. 1 16S rRNA gene sequence investigation of *Bacillus* sp. BCCS 034 by bioinformatic tool, GeneDoc software, version 2.6.002

and heterotrophic eukaryotes; and photosynthetic, lithotrophic, and heterotrophic bacteria. So far, many moderately halophilic, heterotrophic Gram-negative and Gram-positive bacteria were founded and isolated from hypersaline environments. They belong to genus; *Arhodomonas*, *Dichotomicrobium*, *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Alteromonas*, *Acinetobacter*, *Spirochaeta*, *Halomonas*, *Chromohalobacter*, *Marinococcus*, *Salinococcus*, *Nesterenkonia*, *Tetragenococcus*, *Halobacillus* and *Bacillus*. *Bacillus* strains were isolated from hypersaline environments around the world, for example; *B. diposauri*, from the nasal cavity of a desert iguana, *B. haloalkaliphilus*, from Wadi Natrun, and *B. halodenitrificans*, from a solar saltern in southern France [14]. Endospore formation universally found in the genus and aerial distribution of these dormant spores probably explains the occurrence of *Bacillus* in most habitats examined.

In this experiment as other studies, the most bacterial isolates were *Bacillus*. From 32 bacterial strains isolated, 23 strains (71.9%) were *Bacillus* spp. Also, *Paenibacillus* that has been isolated is originally included within *Bacillus* genera. Rohban and his associates isolated 231 moderate halophilic and 49 extremely halophilic bacteria from a hypersaline Howz Soltan Lake in the middle of Iran, among which there were 172 isolates (61.5%) Gram-positive rods, 52 isolates (18.5%) Gram-positive cocci and 56 isolates (20%) Gram-negative rods [15]. Olajuigbe and Ajele have isolated 25 bacterial isolates from soil, of which nine isolates (36%) were identified as *Bacillus*. They were *Bacillus brevis*, *B. licheniformis*, *B. subtilis*, *B. macerans*,

B. mycoides, *B. coagulans*, *B. polymyxa*, *B. cereus*, and *B. megaterium*. In another study, Waksman identified 29 isolates as *B. megaterium* and 24 isolates as *B. subtilis* out of 306 soil samples [16]. These findings agree with the results of our study that *Bacillus* genera are widespread in hypersaline and soil habitats.

Two kinds of L-asparaginase have been known to be produced by microorganisms; however, only one of these possesses antitumor activity. This activity has been known to vary with strains or culture condition of microorganisms. For example, *Mycobacterium tuberculosis* produces two kinds of L-asparaginase in the cell, but only one of them is effective against animal tumors. *E. coli* L-asparaginase I and II were produced simultaneously in the cells, but only the latter showed antitumor activity [3]. This different activity is due to differences in a number of properties, such as pH activity profile, sensitivity to thermal inactivation, clearance rate (half life in serum) and most significantly their affinity for L-asparagine [17, 18]. Genus *Bacillus* produces two L-asparaginases too. During *B. subtilis* genome sequencing project, the *ansZ* gene (previously called yccC) was identified to encode a functional L-asparaginase. The deduced amino acid sequence of *ansZ* shows 59% identity to the L-asparaginase from *Erwinia chrysanthemi* and 53% identity to L-asparaginase II from *Escherichia coli*. In addition, *B. subtilis* has another gene (*ansA* gene) that encodes L-asparaginase [17]. More investigations should be done to find out if these L-asparaginases are active against tumors. Gram-positive bacteria secrete enzymes to the medium and eliminates the cost of enzyme extraction.

Conclusion

Hypersaline environments, with salinity at or near saturation, are active environments which inhabited by a vast variety of microbial communities. These environments contain novel microbial strains with novel properties of biotechnological interest. The results of this work indicate that the bacterium, *Bacillus* sp. BCCS 034 displays a potential for asparaginase production that warrants further investigation.

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