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Isolation and Characterization of a Novel *Bacillus* sp. Strain that Produces L-asparaginase, an Antileukemic Drug

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ABSTRACT

L-asparaginase is an anti-neoplastic agent that has been particularly studied for its therapeutic aspects in the treatment of certain types of cancer, especially lymphoblastic leukemia. This study performed screening of soil microbial isolates that were potential producers of extracellular L-asparaginase. The isolates were mainly characterized using both biochemical and molecular approaches. Ninety-six of all L-asparaginase producer bacteria isolated from southern boundaries of the Caspian Sea, were found to belong to *Bacillus* sp. Among them, *Bacillus* sp. GH5 exhibited a distinctly high asparaginase activity and therefore underwent further analyses. This study specifically details the results of analyses performed on the mentioned isolate. After incubation at 80°C for 10 min, plate assay was performed on modified M9 medium supplemented with phenol red. *Bacillus* sp. GH5 showed a significant pink zone. L-asparaginase activity was assayed using direct Nesslerization method showing approx. 300 IU at 37°C. Molecular identification was carried out using the bacterial universal primer set of BAc08F and Uni1390R. Phylogenetic analysis of the 16S rRNA gene revealed that this isolate was a member of the genus *Bacillus*. Furthermore, effects of temperature and pH on both bacterial grow and enzyme activity was investigated and showed that maximum growth occurred at 37°C and pH 7. In addition, maximum asparaginase activity was observed at 37°C and pH 8.5. Accordingly, the results of this study suggest that *Bacillus* sp. GH5 is a promising candidate for treatment of leukemia considering its great enzyme activity and particular optimal conditions.

Key words: *Bacillus*, L-asparaginase, antileukemic, characterization

INTRODUCTION

L-asparaginases (L-asparagine aminohydrolases, EC 3.5.1.1) are a group of enzymes that catalyze the hydrolysis of asparagine to aspartic acid and ammonia (Hill *et al*., 1967). They can be produced by some microorganisms such as *E. coli* (Derst *et al*.,1994; Mercado and Arenas, 1999), *Erwinia cartovora* (Maladkar *et al*., 1993; Aghaiypour *et al*., 2001), *Thermus thermophilus* (Pritsa and Kyriakidis, 2001), *Enterobacter aerogenes* (Mukherjee *et al*., 2000), *Corynebacterium glutamicum* (Mesas *et al*., 1990), *Candida utilis* (Kil *et al*., 1995), *Staphylococcus aureus* (Muley *et al*., 1998) and *Pisum sativum* (Siechiechowcicz and Ireland, 1989).

Much attention has been focused on microbial asparaginases because of their applications as therapeutic agents in the treatment of certain types of human cancer (Gallogher *et al*., 1989). Over

the last few years, L-asparaginase isolated from *E. coli* and *Erwinia carotovora* have been being used in the treatment of acute lymphoblastic leukemia (Dhevagi and Poorani, 2006). They have also been used for the treatment of bovine lymphomosarcoma (Mosterson *et al*., 1988) and pancreatic carcinoma (Yunis *et al*., 1977).

A major disadvantage of using L-asparaginase as a drug is that it may cause hypersensitivity reactions. It can also be associated with a coagulopathy as a result of a decline in protein synthesis, including synthesis of coagulation and anticoagulant factors, leading to bleeding or thrombotic events such as stroke (Muller and Boos, 1998).

There is a growing body of research focused on decreasing of immune reactivity either by modifying the available L-asparaginases or by discovering new L-asparaginases that are serologically different but have similar therapeutic effects. The latter approach may require the screening of soil samples from various sources for isolation of potential microbes, which possess the ability to produce the desired enzyme. One of the bacteria that is well known for both being abundant in soils and being able to produce various extracellular enzymes is *Bacillus*.

Considering the above facts, an attempt was made for the first time on isolation, characterization and molecular identification of *Bacillus* species which were able to produce extracellular L-asparaginase from soils of south boundaries of the Caspian Sea.

MATERIALS AND METHODS

Materials: All materials and reagents were commercially purchased such as L-asparaginase (Sigma, USA) and Nessler's Reagent (Sigma, USA).

Isolation and characterization of *Bacillus*: In present study, three hundred soil samples were collected from a superficial layer of soil to the depth of 30 cm. The area of sampling covered all southern boundaries of the Caspian Sea (Fig. 1).

This particular geographical area was chosen considering the fact that by the time this study was planned, many local seafood retail markets used to discharge their wastewater directly into the nearby streams. As a result, during decades, the soil of these areas became protein-rich-an ideal environment for Asparaginase producing bacteria.

Fig. 1: The geographical area of sampling, The highlighted line along the southern boundaries of the Caspian Sea pointed by an arrow indicates the area of sampling, Soil samples were collected from the locations that were more likely to be protein-rich

A known quantity (1 g) of soil sample was diluted with sterile distilled water. In order to eliminate non-spore forming bacteria, dilutions were incubated at 80°C for 10 min and subsequently were cultured on nutrient agar medium and incubated at 28°C for two days.

Screening of soil isolates for L-asparaginase production: The isolates were screened for L-asparaginase production by sub-culturing on modified M9 medium (composition for 1 l: 5.0 g L-asparagine; 0.5 g $MgSO_4.7H_2O$; 6.0 g $Na_2HPO_4.2H_2O$; 3.0 g KH_2PO_4; 0.5 g NaCl; 0.014 g $CaCl_2.2H_2O$; 2.0% w/v glucose; and 20.0 g agar) supplemented with phenol red as a pH indicator (Prakasham *et al.*, 2007). The inoculated agar plates were incubated at 37°C overnight. L-asparaginase activity was identified by formation of pink zone around the colonies. The pinkish red colonies were picked from the plates and streaked on nutrient agar slants. The isolates were grown at 37°C and afterwards, were stored at 4°C. Two control plates were also prepared for each isolate using modified M9 media; one was prepared without adding dye and the other one without adding asparagine.

After isolation as pure cultures, the cultures were characterized using morphological (grams stained, observed under light microscope) and biochemical tests (carbon utilization, starch hydrolysis, liquefaction of gelatin, production of H_2S, degradation of urea, casein hydrolysis, catalase, citrate utilization, indole production).

The isolates that were able to exhibit a significant pink zone, were selected for further examinations.

Purification of L-asparaginase: The enzymes were partially purified by the following steps at 4°C. The modified M9 broth was centrifuged at 10,000 g for 10 min. The purification was carried out using crude enzyme extract (Distasio *et al.*, 1982). Finely powdered ammonium sulphate was added to the crude extract. The precipitate was collected by centrifugation at 9,000 g for 15 min, dissolved in 1 M Tris-HCl buffer and dialyzed against the same buffer. The dialyzed fraction was applied to a Sephadex G-100 column. The active fractions were pooled, dialyzed, concentrated and consequently applied to the column of CM Sephadex C-50. Finally, the active fractions at each purification steps were collected, dialyzed and concentrated.

Determination of L-asparaginase activity: L-asparaginase activity was assayed using direct Nesslerization method (Basha *et al.*, 2009). International unit IU of L-asparaginase was calculated from the amount of enzyme that liberates 1 micromole of ammonia in 1 min at 37°C. The isolates that proved to have a considerable L-asparaginase activity were chosen for molecular identification.

Molecular identification

Extraction of DNA: To determine the 16S rDNA sequences and identify the isolates, each genomic DNA was extracted with CinnaGen DNA Extraction Kit according to the manufacturer's directions.

PCR amplification and sequencing of 16S rDNA: PCR amplification and 16S rRNA sequencing were performed in order to confirm the identities of the isolates. The 16S rRNA genes were PCR-amplified from the genomic DNA using the bacterial universal primer set of BAc08F (5'-AGAGTTTGATCCTGGCTCAG-3') and Uni 1390R (5'-GACGGGCGGTGTGTACAA-3'), which were also used for sequencing. The PCR reaction mixture consisted of 5 μL of 10×PCR reaction

buffer, 1 μL of 10 mM dNTP mix, 1 μL of 10 pM primers, 2.5 μL of genomic DNA template and 2.5 U RED*Taq*™ DNA Polymerase (Sigma) prepared in a final 50 μL reaction volume. The thermal cycling program was included the following steps: 5min at 95°C for initial denaturation, 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 90 sec and a final extension at 72°C for 10 min. The amplified PCR products were analyzed by 0.8% (w/v) agarose gel electrophoresis.

Phylogenetic analysis: The 16S rDNA gene sequence of *Bacillus* sp. GH5 aligned with all the sequences available from the GenBank database by BLAST and sequences were retrieved from Genbank database and aligned using ClustalW 1.6 (Thompson *et al*., 1997). Phylogenetic analysis was performed by means of MEGA version 5.1 (Kumar *et al*., 2001) software.

Effect of temperature on growth and enzyme activity: To investigate the growth at different temperatures, *Bacillus* sp. GH5 was grown in modified M9 (pH 7) at three temperatures: 25, 37 and 50°C. The bacterial growth was developed in 250 mL Erlenmeyer flasks on an orbital shaker at 80 rpm and was quantified by reading the optical density at 600 nm. Growth was monitored hourly during an 8 hours period. In addition, asparaginase activity was measured after 24 h incubation in different temperatures in a range of 20 to 60°C.

Effect of pH on growth and enzyme activity: The pH of modified M9 medium was adjusted to 5, 6, 7, 8 and 9 with 0.1 N HCl and 1 N NaOH and then inoculated with fresh culture of the isolate. Flasks were incubated at 37°C on a shaker (80 rpm) for 24 h. For each assayed pH value, bacterial growth was evaluated as the optical density at 600 nm. Likewise, asparaginase activity was quantified at the same range of pH.

RESULTS

Basic identification of Bacterial isolates: After the heat shock, one hundred and eighteen isolates of survived bacteria showed positive result in rapid plate assay by exhibiting a significant pink zone on modified M9 media (Fig. 2). Therefore, they underwent further analysis. From those, ninety-six isolates proved to belong to the genus *Bacillus* by means of microscopic and biochemical examinations. In microscopic observations, *Bacillus* sp. GH5 was found to be gram-positive, rode shaped and spore forming. The results of biochemical testes and carbon utilization test on *Bacillus* sp. GH5 is shown in Table 1 and 2, respectively.

Assay of L-asparaginase: The partial purification of the L-asparaginase crude extract that was affected by the ammonium sulfate (80%) precipitation showed that most of the enzyme activity was preserved in the precipitate. The total protein decreased from 5,240 to 564 mg in the ammonium sulfate precipitation step. Further purification of the enzyme by Sephadex G-100 resulted in specific activity of 489 IU mg^{-1}, with approximately 34.2 folds purity and yield of 58%. The final purification of L-asparaginase was achieved by CM Sephadex C-50 column chromatography, which resulted in specific activity of 1835 IU mg^{-1}, approximately 128.3 folds purity and yield of 41% (Table 3).

Effect of temperature on growth and enzyme activity: *Bacillus* sp. GH5 was able to grow at 25, 37 and 50°C showing typical growth profiles with an exponential phase that began in the range of third to fourth hour (Fig. 3). Incubation at 25°C did not show a significant growth. The

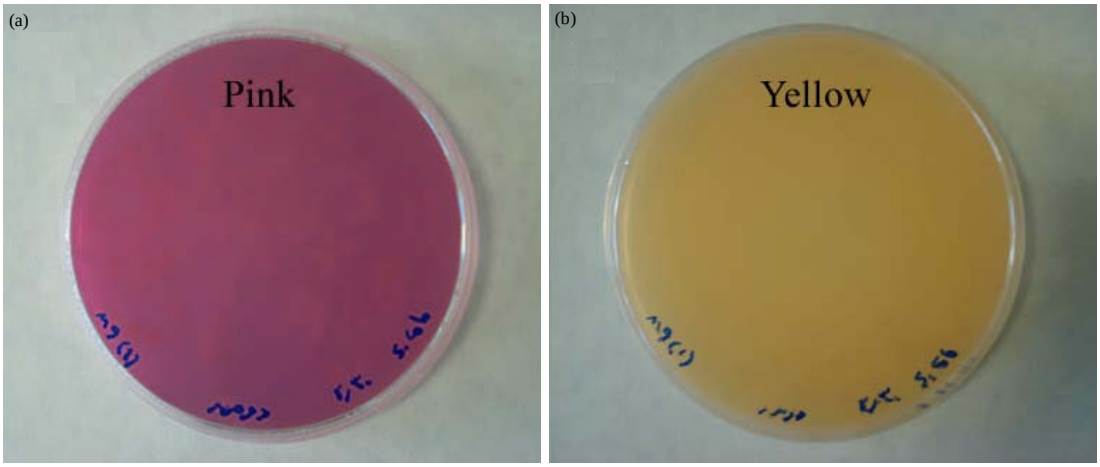

Fig. 2(a-b): Change of color in modified M9 media due to Asparaginase activity, Forming pink zones in M9 media indicates production of Asparaginase, Since the pink zones are a result of decreasing of pH, they readily develop and involve whole the plate. Therefore, single colonies were subcultured to make sure that the change of color was not affected by productions of other colonies, (a) Change of color in modified M9 media to pink due to Asparaginase activity and (b) The yellow color of the media indicates the lack of Asparaginase activity

Table 1: Biochemical properties of *Bacillus* sp. GH5

Parameter	Results
Starch hydrolysis	+
Production of H_2S	-
Liquefaction of gelatin	+
Casein hydrolysis	+
Degradation of urea	+
Citrate utilization	+
Indole production	-
Catalase	+

From eight biochemical tests performed on *Bacillus* sp. GH5, only two results were negative: starch hydrolysis and indole production

Table 2: Carbon utilization of *Bacillus* sp. GH5

Carbon source	Utilization
No carbon source (negative control)	-
Arabinose	+
Fructose	-
Galactose	+
Glucose (positive control)	+
Inositol	-
Mannitol	-
Raffinose	+
Sucrose	+
Xylose	-

Nine different carbon sources were used in carbon utilization test. Among them, the test results of five carbon sources (arabinose, galactose, glucose, raffinose and sucrose) were positive

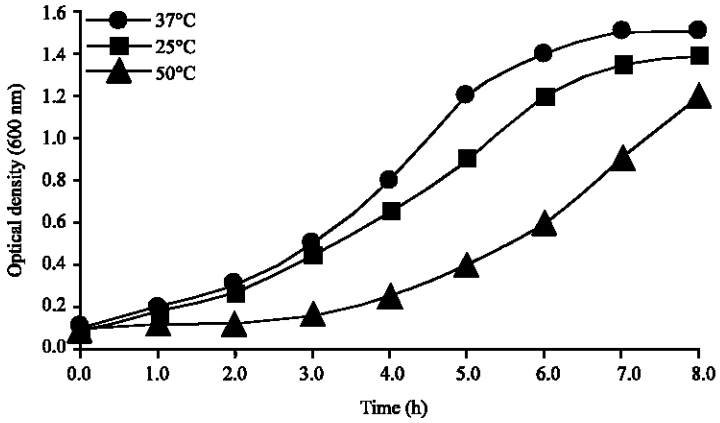

Fig. 3: Effect of temperature on cell growth, The growth was quantified by reading the optical density at 600 nm, *Bacillus* sp. GH5 was able to grow at 25, 37 and 50°C, The maximum growth was observed after 7 h incubation at 37°C, Incubation at 50°C did not show a significant growth in first 4 h

Table 3: Purification profile of L-asparaginase from *Bacillus* sp. GH5

Step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	75200	5240	14.3	1.0	100
$(NH_4)_2SO_2$ precipitation	68100	564	120.7	8.4	90
Gel filtration on Sephadex G100 column	43500	89	489	34.2	58
CM-sephadex C50 column	31200	17	1835	128.3	41

Decreasing of total protein from 5,240 to 564 mg occurred in the ammonium sulfate precipitation step, Further purification of the enzyme by Sephadex G 100 resulted in specific activity of 489 IU mg^{-1}, with approximately 34.2 folds purity and yield of 58%, The final purification of L-asparaginase was achieved by CM Sephadex C 50 column chromatography, which resulted in specific activity of 1835 IU mg^{-1}, approximately 128.3 folds purity and yield of 41%

maximum optical density was observed at 37°C. Therefore, this temperature was considered optimal for cell growth. The relation between the temperature and the asparaginase activity shown in Fig. 4 indicated that 37°C is not only the optimal temperature for growth but also for asparaginase activity of *Bacillus* sp. GH5.

Effect of pH on growth and enzyme activity: Figure 5 shows the kinetic growth profile at a range of pH 5.0 to 9.0. The maximum absorbance value was observed at pH 7.0 and the lowest absorbance was observed at pH 5.0. In addition, maximum asparaginase activity was observed at pH 8.5 (Fig. 6).

Phylogenetic analysis: The sequence of the 16S rDNA of *Bacillus* sp. GH5 was determined. Figure 7 shows the phylogenetic tree for *Bacillus* sp. GH5 that was drawn using MEGA software version 5.1. The GenBank accession number for the analyzed sequence is KC686402.

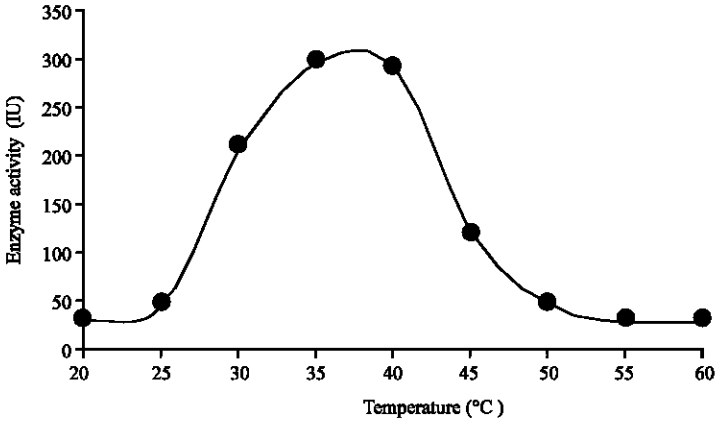

Fig. 4: Effect of temperature on L-asparaginase activity, Asparaginase activity of *Bacillus* sp. GH5 was maximum at 37°C while in temperatures below 25°C and over 50°C it was minimum

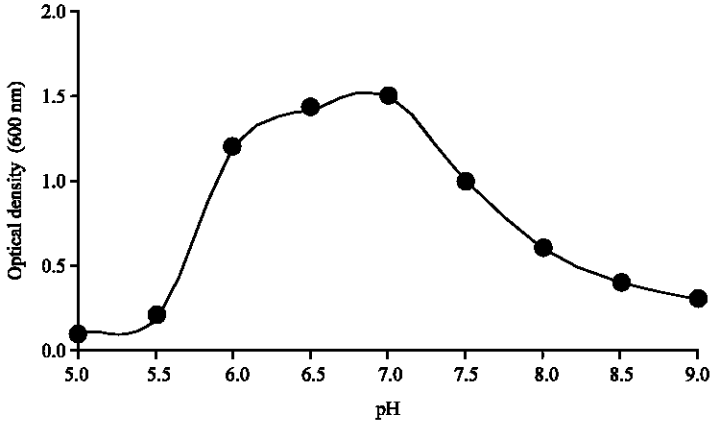

Fig. 5: Effect of pH on cell growth, The kinetic growth profile of *Bacillus* sp. GH5 at a range of pH 5.0 to 9.0 shows the maximum absorbance at pH 7.0 and the minimum absorbance at pH 5.0

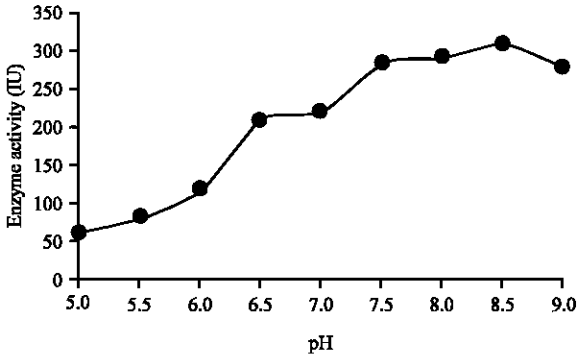

Fig. 6: Effect of pH on L-asparaginase activity, The maximum asparaginase activity of *Bacillus* sp. GH5 was observed at pH 8.5

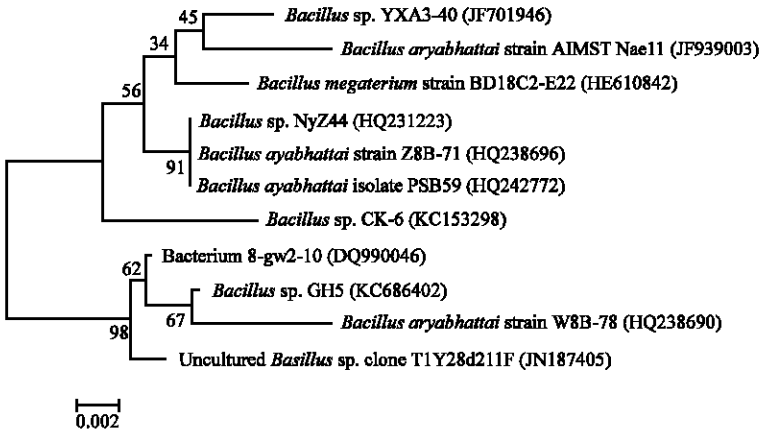

Fig. 7: Phylogenetic tree of *Bacillus* sp. GH5, Phylogenetic analysis of 16S rRNA gene for *Bacillus* sp. GH5 using MEGA 5.1 software, The significance of each branch is indicated by a bootstrap value, The accession number of each reference species is shown in parentheses

DISCUSSION

Bacillus sp. GH5 was preliminary screened for L-asparaginase activity by plate assay method. Screening of L-asparaginase activity using plate assay has been conducted on many bacterial strains such as *Bacillus circulans*? (Prakasham *et al*., 2010), *Streptomyces* sp. PDK7 (Dhevagi and Poorani, 2006) and *Streptomyces* sp. (Basha *et al*., 2009).

Enzyme purification was carried out by ammonium sulfate precipitation, Sephadex G-100 and CM Sephadex C-50 column chromatography. The final purification of L-asparaginase produced by *Bacillus* sp. GH5 showed 128.3 folds purity. L-asparaginase purity of 106 folds has been reported in a study on *Pseudomonas aeruginosa* 50071 (El-Bessoumy *et al*., 2004). In recent studies, L-asparaginase produced by *Streptomyces gulbargensis* (Amena *et al*., 2010) and *Escherichia coli* (Aljewari *et al*., 2010) has been purified up to 82.12 and 96 folds, respectively.

Although pH 7 was optimal for cell growth, maximum L-asparaginase activity was observed at pH 8.5. Similarly, maximum L-asparaginase activity of *Pseudomonas aeruginosa* 50071 (El-Bessoumy *et al*., 2004) and *Streptomyces* sp. PDK2 (Dhevagi and Poorani, 2006) has been obtained at pH 9 and 8, respectively.

According to the temperature profile, both maximum L-asparaginase activity and maximum growth accrued at 37°C. Similar results were obtained for L-asparaginases from *Bacillus* sp. DKMBT10 (Moorthy *et al*., 2010) and *Pseudomonas aeruginosa* 50071 (El-Bessoumy *et al*., 2004).

CONCLUSION

Since the known L-asparaginases have undesirable side effects, the need for discovering new bacterial sources of this enzyme still exists.

Newly isolated bacteria, *Bacillus* sp. GH5, proved that possess a considerable L-asparaginase activity. It also has an optimal temperature of 37°C for enzyme activity that is ideal for therapeutic applications. However, the enzyme activity is not the only parameter that is needed to be taken into consideration before suggesting an enzyme for medical purposes. One other very important

parameter is known to be the serological aspect of the enzyme. This study did not cover that area. Therefore, it can be suggested that a study should be done on serological properties of L-asparaginase produced by *Bacillus* sp. GH5 along with its efficiency in treatment of lymphoblastic leukemia.

Moreover, although the combination of morphological, biological and molecular tests revealed great information about taxonomic position of *Bacillus* sp. GH5, still more testes are needed to be performed before one can confidently announce the evolutionary relationships of the isolate and all its biochemical properties.

Finally, based on obtained data, a conclusion that can be drawn is that some soil bacteria may well be an unexploited source of potentially valuable products. Consequently, mass screening of common bacteria may be a practical approach for discovering such precious resources.

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