

Persian Gulf is a Bioresource of Potent L-Asparaginase Producing Bacteria: Isolation & Molecular Differentiating

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ABSTRACT: L-asparaginase is a candidate enzyme for anti-neoplastic agent against acute lymphoblastic leukemia and also extensively use in the food industry for prevention of acrylamide formation. L-asparaginase is widely distributed among microorganisms. In this study, marine bacteria were isolated from Persian Gulf and screened for L-asparaginase activity. Production of L-asparaginase was carried out by using M9 medium. Among L-asparaginase producing strains, 12 potent strains were differentiated based on nucleotide sequences of *16S rDNA*. 12 potent strains included 2 strains of *Pseudomonas spp.*, 8 strains of *Bacillus spp.*, one strain of *Zobellella spp.* and one strain of *Oceanimonas spp.* identified and consequently the sequences published in the NCBI databases under the specific accession numbers. This is the first report on L-asparaginase activity of *Zobellella spp.* from this region. The highest (1.6 IU/ mL) and also the lowest (0.20 IU/ mL) productivity of L-asparaginase enzyme were recorded for *Pseudomonas sp. PG_01* and *Bacillus sp. PG_13* respectively. This study revealed marine bacteria are potential source of L-asparaginase enzyme. *Pseudomonas sp. PG_01* with high productivity can be used for production of L-asparaginase. Therefore, Persian Gulf can be considered as a potential bioresource for L-asparaginase producing strains which may be effectively used in the large scale production by related gene cloning in purpose of medical application and food industry in the future.

Key words: L-asparaginase, marine bacteria, *16S rDNA*, Persian Gulf

INTRODUCTION

Marine microorganisms are particularly attractive because they have high potency required for bioactive compounds, which is effective in the marine environment due to the diluting effect of seawater and they have not been as extensively exploited as their terrestrial counterparts (Lu *et al.*, 2009; Sereshti *et al.*, 2014; Reboleiro-Rivas *et al.*, 2013; Dorgham, 2013; Jahanpanah and Savari, 2013; Kwasniewska *et al.*, 2013; Rahimi *et al.*, 2012; Zhang *et al.*, 2012). These microorganisms can be considered as the potential sources of different enzyme with medical and industrial applications. L-asparaginases (L-asparagine amino hydrolase EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine to L-aspartate ammonia. L-asparaginase is also able to hydrolyze L-glutamine but with less effective (Ghasemi *et al.*, 2008, Georgia *et al.*, 2011). L-asparaginase (also commercially known as oncaspar, colaspase, crasnitin, kidrolase, erwinase and elspar) is

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an enzyme of high therapeutic value due its use in certain kinds of cancer therapies, mainly in acute lymphoblastic leukemia (ALL) (Geckil *et al.*, 2004). Its antineoplastic activity is resulted from depletion of the circulating pools of L-asparagine by asparaginase activity. Malignant cells because of slowly synthesize L-asparagine, are killed by lacking exogenous supply. However, normal cells are protected from asparagine-starvation due to their ability to produce this amino acid (Li *et al.*, 2007). The enzyme can also be used to reduce the formation of acrylamide in fried and oven-cooked foods especially in potato chips. Since, acrylamide formation in heated foods is mainly due to the reaction of free asparagine and reducing sugars, deamination of asparagine prevents acrylamide formation (Mahajan *et al.* 2012). L-asparaginase is widely distributed among microorganisms, plants and animals, but it has been proved that microorganisms are better source for producing the L-asparaginase due to large-scale production (Savitri *et al.*, 2002).

Microorganisms known to produce this enzyme including *Escherichia coli*, *Erwinia carotovora* (Warangkar & khobragade, 2008), *Vibrio* (Kafkewitz & Goodman, 1974), *Aerobacter*, *Bacillus*, *Serratia*, *Xanthomonas*, *Photobacterium* (Peterson & Ciegler, 1969), *Streptomyces* (Dejong, 1972), *Pseudomonas aeruginosa* (El-Bessoumy et al., 2004) and *Aspergillus tamari* (Sarquis et al., 2004). Although L-asparaginase is produced by various microorganisms, but L-asparaginase from *Erwinia chrysanthemi* and *Escherichia coli* are currently in clinical use. Unfortunately, therapeutic response by patients rarely occurs without some evidence of toxicity and the side effects are due to allergic responses. In addition, contamination with glutaminase is one of the causes of toxicity of these enzymes (Kumar et al., 2011).

Therefore, search of new enzymes with different serological properties, high therapeutic activity and free of glutaminase activity is important for biotechnology and clinical oncology. Hence, in the present study, we have tried to isolate & identify L-asparaginase producing bacteria from Persian Gulf, where it is still virgin area in view of microorganisms in order to find novel sources of the enzyme from new microorganisms.

MATERIALS & METHODS

Seawater and marine sediments samples were collected from different coastal locations in north of Persian Gulf (Hormozgan province), Iran (totally, 40 samples from the sediments and 20 samples from the seawaters). The samples were kept in sterile screw cap bottles and brought to the laboratory for isolation of marine bacterial.

10g of sediment samples were added to 95mL of sterile distilled water and these suspensions were considered as 10^{-1} dilution. 10mL of seawater sample was added to 90mL of sterile distilled water and these suspensions were considered as 10^{-1} dilution. Nutrient agar media were used for isolation of marine bacteria. Serial dilutions were done and streaked on the surface of nutrient agar medium. All plates were incubated at 35°C for 24 hours. The mixed bacterial colonies were purified by repeated streaking on the same medium. In order to get pure cultures of marine bacteria, individual colonies were picked up and sub-cultured on nutrient agar. Finally, the isolated colonies were sub-cultured on to nutrient agar slants and kept in 20% glycerol at -20°C as stock culture (Ramesh & Mathivanan, 2009). The L-asparaginase activity of purified bacterial isolates was screened by using modified M9 medium supplied with L-asparagine and phenol red indicator dye. The cultures were streaked on modified M9 agar plates and incubated at 35°C for 48 hours. Colonies with pink zones were considered as L-asparaginase producing active strains (Poorani et al., 2009; Gulati et al., 1997).

The active strains from L-asparaginase plate assay were selected and cultured on M9 broth, pH 7.0 and incubated at 35°C with shaking at 200 rpm for 48 hours. L-asparaginase activity was measured by the known method of Imada et al., (1973). The cultures were centrifuged at 10,000 rpm for 15 minutes and supernatant was considered as enzyme crude extract to determine the L-asparaginase activity. The reaction was started by adding 0.5mL of crude extract into 0.5mL 0.04M L-asparaginase and 0.5mL 0.05M Tris (hydroxyl methyl amino methane) buffer, pH 7.2 and incubated at 37°C for 15 minutes in a water bath shaker. The reaction was stopped by the addition of 0.5mL of 1.5M Trichloro acetic acid (TCA). The ammonia released in adding 0.2mL Nessler's reagent into tubes containing 0.1mL supernatant and 3.7mL distilled water and incubated at room temperature for 15 minutes, then the absorbance of the supernatant was measured using a UV-Spectrophotometer at 450nm. The liberated ammonia content was determined and one unit of asparaginase was defined as the amount of enzyme, which catalyzed the formation of 1µm ammonia per min at 37°C. Different concentrations of ammonium sulfate were used to draw standard curve (Poorani et al., 2009, Imada et al., 1973).

Twelve potent strains were identified based on cellular morphology, growth conditions, Gram staining, motility and biochemical tests such as oxidase, indole production, catalase, citrate utilization and urease.

Total DNA was extracted from the 12 potent strains by using PeqGold Bacterial DNA Kit (PeQLab, Germany) according to the manufacture's instructions. The *16S rDNA* was amplified using a set of universal primers 27F (5'AGAGTTTGATCCTGGCTCAG 3') and 1525R (5'AAGGAGGTGATCCAA 3'). A total of 50 µL of reaction mixture consisted of 1µL of each primer (10 pmol), 2µL of template DNA, 5µL of 10X PCR-buffer, 5µL of MgCl₂ (5mm), 1µL of dNTPs (10mm) and 1µL of *Taq* DNA polymerase (5U/µL). The PCR amplifications were carried out with an initial denaturation step at 95°C for 1 minute, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minute. Negative controls (no DNA added) were included in all sets of reactions. The 1.5 Kb amplified *16S rDNA* fragments were determined by 1% (w/v) agarose gel electrophoresis (Nimnoi et al., 2010). PCR products were sent to MacroGen Company (Holland) for sequencing using ABI-3730XL capillary sequencer. The sequence similarity searches were done using the BLAST program that is available from the National Center for Biotechnology Information (NCBI, 2013). The nucleotide sequences of the *16S rDNA*. 12 potent strains were aligned and phylogenetic tree was constructed using Neighbour-joining method in the

Molecular Evolution Genetics Analysis (MEGA) software version 4.0 (Tamura *et al.* 2007).

RESULTS & DISCUSSION

Nowadays, both academic and industrial interest in marine microorganisms is on the rise, because unique and biologically active metabolites have been reported from these organisms (Ramesh & Mathivanan, 2009). Microorganisms as the fastest means to search for food and medical applications may often use cell-free extracellular enzymes. Marine bacteria secrete different enzymes and compounds based on their habitat and their ecological functions. Marine bacteria enzymes have become the focal point of interest and several enzymes have drawn the attention of microbial prospectors (Chandrasekaran & Rajeev Kumar, 2010). This is the first report regarding to isolation the L-asparaginase producing bacteria from marine environment of the Persian Gulf.

Totally 60 different marine samples were collected from various locations of north coast of Persian Gulf, Iran. 35 and 146 bacterial strains were isolated from seawater and marine sediments respectively. All 181 isolated bacterial strains were investigated for their potency in production of the enzyme L-asparaginase. 57 strains were able to produce the enzyme and showed the L-asparaginase activity. 6 strains (nearly 10%) isolated from seawater samples and 51 strains (nearly 90%) isolated from marine sediments were able to produce the L-asparaginase enzyme. 57 active strains were selected from L-asparaginase plate assay in order to measure the enzyme activity. The number of isolated strains from sediment samples was higher than from seawater samples. In addition, most of the active strains were isolated from sediment samples. This may indicate that the marine sediments can provide a unique biological niche for growth and activities of microorganisms. As marine sediments usually contain a large global reservoir of organic carbon and essential nutrients that profoundly effect on global biogeochemical processes and influence carbon degradation and preservation (Toffin *et al.*, 2004), therefore it is supposed that enzyme activities also increase in marine sediments as compare to seawater niche. In our study, 12 potent strains were identified to be active in producing L-asparaginase among the strains. The maximum L-asparaginase activity was 1.6 IU/mL. Similar to this result, was reported by Ebrahiminezhad *et al.*, (2011). They isolated L-asparaginase producing bacteria with maximum activity of 1.64 IU/mL. But higher activity was reported by Shukla & Mandal (2013). They isolated 12 bacterial strains from soil. Five of them showed L-asparaginase activity. The strain A10 had highest activity of 2.1 U/mL. Different amounts of producing L-asparaginase have been reported in different studies. This difference may be due to microorganisms, culture media and method of assay of

L-asparaginase activity. More amount of enzyme with higher activity can be obtained by optimization the different growth parameters.

Because, the identification and classification in base of morphological, physiological and biochemical characteristics is cumbersome and time-consuming, therefore molecular biology method of *16S rDNA* sequence analysis used for classification of 12 strains with high potentiality for L-asparaginase. This method is an important tool for correct identification of microbial species (Poorani *et al.*, 2009). BLAST test using the NCBI (National Center for Biotechnology Information) website as the Gene Bank showed similarity of derived sequences in compare to the other sequences belonging to the recorded species of studied bacteria. Phylogenetic tree was constructed based on the 1500 bp for *16S rDNA*. The tree was generated using neighbor joining (NJ) a distance-based algorithm for phylogenetic analysis. All 12 haplotypes of the species clustered into two major clades with reasonably high bootstrap support of values (Fig. 1). The analysis of *16S rDNA* sequences demonstrated that the sequences of eight strains were most closely related to *Bacillus spp.*, which constructed the first clade and the other strains including *Pseudomonas spp.* (two strains), *Zobellella spp.* (one strain) and *Oceanimonas spp.* (one strain) were constructed the second clade, showed common ancestor with the first clade. Phylogenetic analysis based on *16S rRNA* gene sequencing also showed that the strains probably belong to two different phylum including Firmicutes and Proteobacteria. Eight strains (*PG_02*, *PG_03*, *PG_04*, *PG_05*, *PG_07*, *PG_08*, *PG_11* and *PG_12*) belong to the Bacilli subdivision of Firmicutes and Four strains (*PG_01*, *PG_06*, *PG_09* and *PG_10*) belong to the Gammaproteobacter subdivision of Proteobacteria but these four strains related to two different orders including Aeromonadales and Pseudomonadales. *Methanobacterium formicicum* (AY196659) was used as outgroup to root the tree (Fig. 1). The sequences were published in the NCBI databases under the specific accession numbers.

In our study, potent L-asparaginase producing bacteria were belonged to *Pseudomonas*, *Zobellella*, *Oceanimonas* and *Bacillus* genera. Audipudi *et al.*, (2013) have reported L-asparaginase producing bacteria from mangrove soils, East Coast of India. These bacteria were identified as *Pseudomonas* and *Bacillus spp.* Kamble *et al.* (2012) isolated L-asparaginase producing bacteria from water, saline and farm soils. They were belonged to the genera *Pseudomonas aeruginosa*, *E.coli*, *Serratia spp.*, *Bacillus*, *Aeromonas* and *Proteus* species. All these studies show that most L-asparaginase producing bacteria belong to genera *Bacillus* and *Pseudomonas* isolated from environmental samples. We also observed

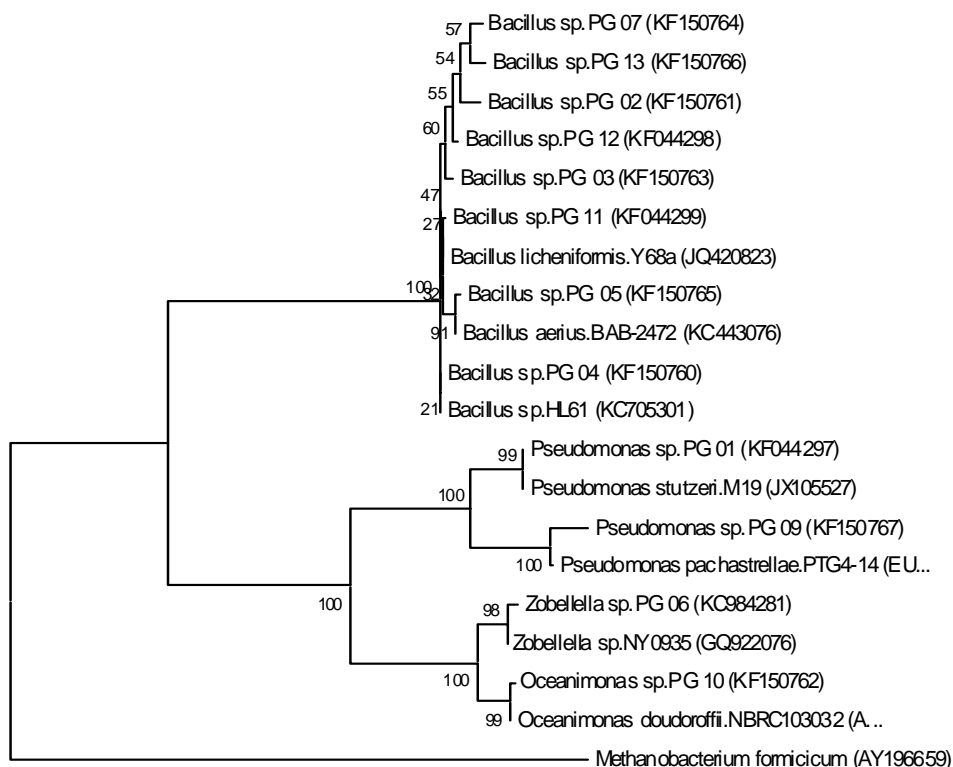


Fig.1. Neighbour-joining tree based on 16S rRNA gene sequences showing the positions of 12 potent producing L-asparaginase bacteria and related reference species. The bootstrap values indicate confidence limits of the phylogenies, based on percentages of 100000 replications. The bar represents 0.02 showed substitutions per nucleotide position. The sequence of *Methanobacterium formicicum* (AY196659) was used as outgroup

that the most isolated bacterial strains were belonged to genus *Bacillus*. Among identified strains 67% were *Bacillus spp.* (Fig.2). The maximum and minimum activity of L-asparaginase was found in *Pseudomonas sp. PG_01* (1.6 IU/mL) and *Bacillus sp. PG_13* (0.20 IU/mL), respectively (Table 1). *Pseudomonas* is well known for their high genetic and physiological diversity and ability to produce extracellular enzymes. Loperena *et al.*, (2012) analyzed four genres that strain belonged to genus *Pseudomonas* showed highest enzyme activities. Ebrahiminezhad *et al.*, (2011) isolated 32 bacteria from Maharloo salt lake in the south of Shiraz, Iran. The most bacterial isolates were *Bacillus*. From 32 isolated strains, 23 strains (71.9%) were *Bacillus spp.* Endospore formation universally found in the genus *Bacillus* and aerial distribution of these dormant spores probably explains the occurrence of *Bacillus* in most habitats examined. These findings agree with the results of our study that *Bacillus* genera are widespread in sediment habitats.

Pseudomonas sp. PG_01 was clustered in one clade with two sister groups. This strain was most closely related to *Pseudomonas stutzeri* strain M19 (JX105527) with 99% similarity. We did not see any genetic divergence between the *Pseudomonas sp.*

PG_01 isolated in our study in compare to *Pseudomonas stutzeri* strain M19 which isolated and reported by Batool & Faisal (2013) (:// www.ncbi.nlm.nih.gov/nuccore/451319234). Phylogenetic analysis showed that *Pseudomonas sp. PG_01* was grouped in one clade and showed the same phylogenetic structure with high (100%) bootstrap support which has common ancestor with *Zobellella* and *Oceanimonas* species (Fig.1). In this study we isolated seven haplotypes of the *Bacillus sp. PG_02* isolated in this study is closed to *Bacillus licheniformis* Y68a (JQ420823). Our study is the first report for *Zobellella spp.* with considerable L-asparaginase activity. The isolated strains, *Zobellella sp. PG_06* and *Oceanimonas sp. PG_10*, belong to Aeromonadales order of Gammaproteobacteria. *Zobellella sp. PG_06* was most closely compared to *Zobellella sp. NY0935* (GQ922076.1) with 99% similarity. This strain can be considered as a new and futuristic potential source of L-asparaginase for clinical and food industry applications.

CONCLUSION

The present study indicates that marine is a potential source of L-asparaginase producing bacteria and

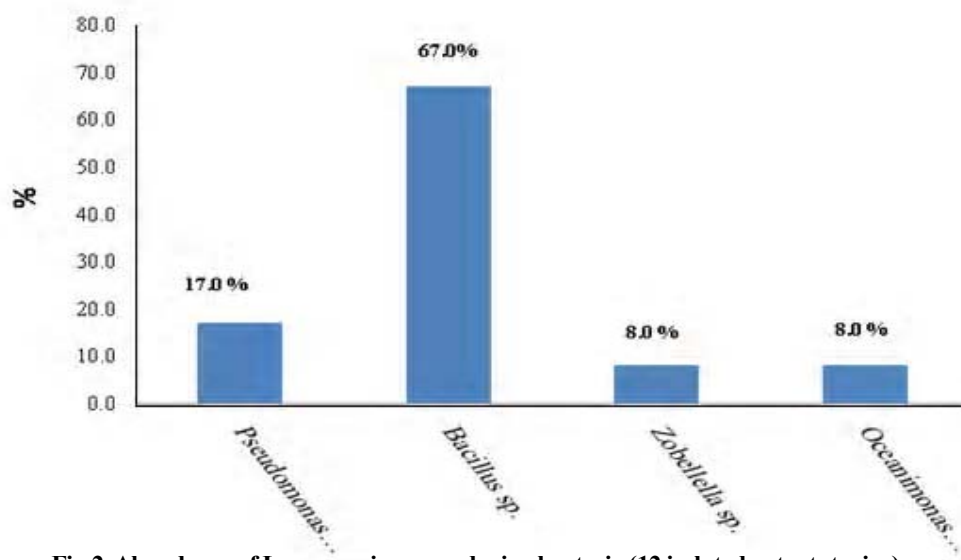


Fig.2. Abundance of L-asparaginase producing bacteria (12 isolated potent strains)

Table 1. The L-asparaginase activity of different strains of marine bacteria, the accession numbers published sequences at the NCBI and Nature of samples

Bacterial strain	Accession No.*	L-Asparaginase activity (IU/mL)	place of Samples
<i>Pseudomonas sp.PG_01</i>	KF044297	1.6	Sediment
<i>Bacillus sp.PG_02</i>	KF150761	0.73	Sediment
<i>Bacillus sp.PG_03</i>	KF150763	0.68	Sediment
<i>Bacillus sp.PG_04</i>	KF150760	0.67	Sediment
<i>Bacillus sp.PG_05</i>	KF150765	0.49	Sediment
<i>Zobellella sp.PG_06</i>	KC984281	0.41	Sediment
<i>Bacillus sp.PG_07</i>	KF150764	0.41	Sediment
<i>Pseudomonas sp.PG_09</i>	KF150767	0.27	Sediment
<i>Oceanimonas sp.PG_10</i>	KF150762	0.25	Sediment
<i>Bacillus sp.PG_11</i>	KF044299	0.21	Sediment
<i>Bacillus sp.PG_12</i>	KF044298	0.21	Sediment
<i>Bacillus sp.PG_13</i>	KF150766	0.20	Sediment

* These accession numbers has been registered in Genebank by this research work

sediments are excellent sources for isolation active strains. *Pseudomonas spp.*, *Basillus spp.*, *Zobellella spp.* and *Oceanimonas spp.* is potential sources of L-asparaginase. However, more detail investigation is required to characterize this bacterial enzyme, which may be effectively used in the large scale production for medical and food application in the future. Meanwhile, gene cloning of L-asparaginase enzyme with potent activity and high kinetic parameters as well as investigation on effects of *in-vitro* expressed enzyme against malignant cells has been considered for further studies.

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