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Isolation and Identification of extremophiles producing anticancer L-glutaminase and L-Methioninas enzymes from Yale Gonbad hot spring in Qazvin, Iran

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ABSTRACT

L Glutaminase and L- methioninase, therapeutic enzyme obtained from microbial source have been considered in the treatment of cancer. A total 20 water samples were obtained from the Yale Gonbad hot spring in Qazvin province, Iran. Production capabilities L Glutaminase and L-methioninase were tested on m9 and mcd plates, respectively. Enzyme-producing isolates were identified by conventional test and then confirmed using molecular methods. The PCR amplicons were directed for sequencing and Phylogenetic tree were drawn using Mega version 6 software. Twelve colonies were obtained from all tested samples. Three extratermophilic isolates showed L-glutaminase activity in M9 medium but only 1 isolates have L-methioninase activity in MCD medium. The phylogenetic tree drawn from the 16 S rRNA gene sequence showed were 100% similar to *B. licheniformis* and strains of *S. epidermidis* and *L. sphaericus*. In this study, Bacillus licheniformis was identified as a dominant and a native strain for the production of L-glutaminase and L-methioninase.

1. Introduction

For many years' cancer treatment dependent to the antiproliferative agents like radiotherapy and low molecular weight chemicals (Shirazian et al., 2016). The previous acts by quickly killing proliferating cells. Though, the request of radiotherapy is limited to the treatment of solid tumors with no metastasis (Naidoo et al., 2019). So, there is a significant need for treatments with improved specificity in the tumor therapy (Courneya et al., 2007). Therapeutic digestive and metabolic enzymes can be used therapeutically for the treatment of numerous illnesses such as cancer, inflammation, cystic fibrosis, and digestive complaints (Deshpande et al., 2014). Many therapeutic enzymes like L-arginase, L-asparaginase, L-glutaminase, L-tyrosinase, L- methioninase, α- and β-glucosidase and β-galactosidase have been used in cancer therapy. Animals, plants, bacteria and fungi are the source of these enzymes (Reshma, 2019). Some tumors such as Acute Lymphoblastic Leukaemia (ALL) are

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auxotrophic for glutamine (Glut) because of their inadequate enzymatic pool. Then, glutamine-depleting enzymes can be valuable against these tumors. Furthermore, glutamine is obligatory for DNA synthesis. L-glutaminase (EC 3.5.1.2) is used in the treatment of cancer and AIDS (Dhevagi, 2006). L-Methionine-γ-lyase (EC 4.4.1.11; MGL), is a pyridoxal phosphate (PLP) dependent enzyme (Katikala et al., 2009). PLP decreases the energy for change of amino acids to a zwitterionic carbonion and considerably the apoenzyme catalyzes the cleavage of substrate bond yielding the product (Sharma et al., 2014).

Presently, de novo engineering of a human MGL has been followed for attaining systemic L-methionine depletion in tumor therapy (Liu et al., 2019). The medicinal use of therapeutic enzymes is imperfect because of the immunological responses in their long-term management; so new enzymes with novel immunological properties are obligatory (Safary et al., 2018). Thermophilic bacteria have revealed great potential in enzymes production with novel features in the detergent, dairy, baking, and leather businesses; though there are few documents about their enzymes as therapeutic agents (Turner et al., 2007). So, the aim of this study was isolation of extremophiles bacteria producing L-glutaminase and L-Methioninas from Yale Gonbad hot spring.

2. Materials and methods

2.1. Sampling

In this cross-sectional study, a total 20 water and sediments samples in a sterile condition were collected in a six months period of time from March till August, 2018. All samples were transferred to the laboratory under dark conditions. This condition prevents the reduction of bacterial load.

2.2. Isolation of thermophilic bacteria

For this aim, 10 cc of samples were centrifuged at 3000 ×g for 20 min. The supernatant was then discarded, and the sediment was streak-cultured on the see water plates (Merck, Germany) using a sterile cotton swab.

2.3. Detection of L-glutaminase producing isolates

L-glutaminase activity was assessed in the modified M9 medium. M9 medium (KCl 0.5g, MgSo4.7H2O 0.5g, NaCl 0.5g, agar 20.0g, KH2PO4 1.0g, L-Glutamine 0.5%, Phenol red 0.012g) supplemented with 0.5% L-glutamine as the only carbon and nitrogen source and phenol red as a pH indicator. The color change of the medium from yellow to pink is an indication of the extra cellular L-glutaminase production by the colony.

2.4. Detection of L- methioninase producing isolates

Screening of L-methioninase activity was performed by Gullati et al. (1997) using Modified Czapek Dox (mCD) plates. MCD medium (CaCl2 0.005 g/l, NaCl 0.5g, agar 20.0g/l, KH_2PO_4 2.0g/l, L- methionine 20 g/l, Glucose 2 g/l, bromothymol blue (BTB) 0.007%) contains 0.5% L- methionine as the sole carbon and nitrogen source and BTB as pH indicator.

2.5. Enzymes-producing bacteria identification

Standard microbiological and biochemical tests were performed to identify the strains as follows; gram stain, catalase, oxidase, MRVP, SIM, TSI, Simmon's Citrate and urease. All obtained colonies were confirmed using 16S rRNA PCR test. CinnaPure DNA extraction kit catalog number CAT NO: PR881613 was used for DNA extraction. The concentration and the quality of the extracted cellular DNA were assessed using a Nanodrop spectrophotometer (ND-1000; Thermo Scientific; Wilmington, DE, USA). The primer oligonucleotide sequences used in this work are recorded in Table 1. The

specimens were amplified in a Techne TC-512 thermo cycler (Eppendorf, Hamburg, Germany). PCR was performed for amplification of 16S rRNA gene in a volume of 1.0 µl of extracted cellular DNA was added to a final volume of 25 µl PCR reaction mixture counting 2.0 µl of 10× PCR buffer, 1.0 µl $MgCl_2$ (50 mM), 0.7 µl dNTPs (10 mM), 1.0 µl of each primer, 0.7 µl of Taq DNA polymerase (5 U/µl) (Amplicon Co., Denmark) and 17.6 µl distilled water. The reaction mixture was achieved with the following PCR process: initial denaturation at 94°C for 5 min, 33 cycles with denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1min and final extension at 72°C for 6 min. PCR amplicons were subjected on the 1.0% agarose gel, stained with Gel Red™ (Biotium, Landing Pkwy, Fremont, CA, USA) and photographed with ultraviolet illumination (Bio-Rad, Hercules, USA).

Table 1. Oligonucleotide primer sequences used in this project

Target genes	Oligonucleotide sequences (5'→3')	Product size (bp)
16 S rRNA	F5'-AGAGTTTGATCCTGGCTCAG-3' R5'- GGTTACCTTGTTACGCCTT-3'	605

2.6. DNA sequencing

The PCR amplicons were directed for sequencing (Bioneer, Seoul-Korea) in both directions with the equal set of primers used for the PCR by Sanger dideoxy chain termination method using an Applied Biosystems 3730/3730Xl DNA Analyser (API, California, USA). Similarity searches for the nucleotide sequences were done by BLAST algorithm software (http://www.ncbi.nlm.nih.gov/blast).

2.7. Phylogenetic tree

BioEdit Sequence Alignment Editor for Windows 95/98 / NT / XP software and Clustal W method were used to align the 16 SrRNA nucleotide sequences with other sequences in the gene bank. The phylogenetic tree was plotted using complete sequences of 16SrRNA gene with an approximate length of 1750 bp using Mega version 6 software and Neighbor Joining method with 1000-fold bootstrap test.

3. Results

Twelve colonies were obtained from all samples. These purified colonies were used for screening and identification of L-glutaminase and L-methioninase producing bacteria in specific media. Among the 12 strains grown in the previous stages, 3 extratermophilic isolates showed L-glutaminase activity in M9 medium. This activity is indicated by the pinking of the plates (Fig 1). Also in this study, only 1 extratermophilic isolates showed L-methioninase activity in MCD medium (Fig 2).

The PCR test was performed to confirm the prokaryoticity of the obtained isolates and then sequencing and BLAST data were performed online at https://www.ncbi.nlm.nih.gov/pubmed/ and genetic affinity determination was performed (Fig 3).

The phylogenetic tree drawn from the 16S rRNA gene sequence showed were 100% similar to *Bacillus licheniformis* and strains of *Staphylococcus epidermidis* and *Lysinibacillus sphaericus* (Fig 4).

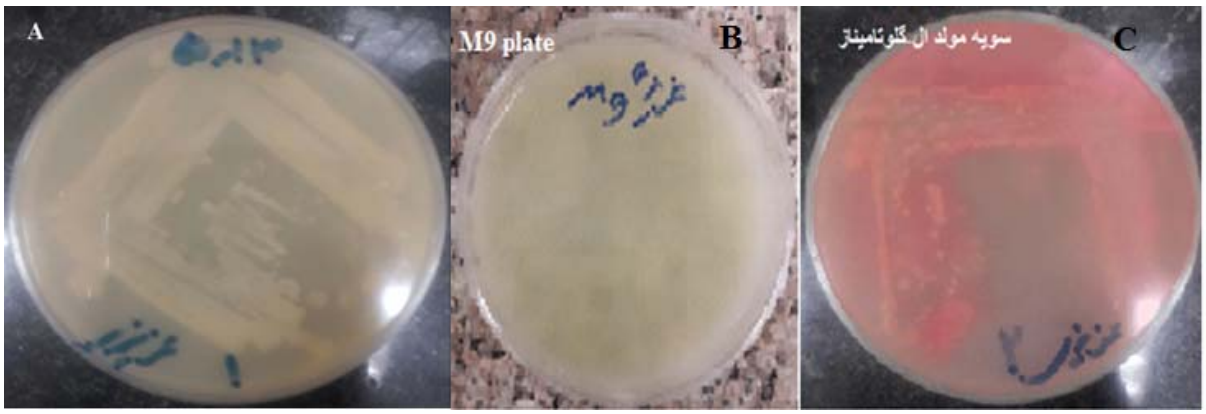

Figure 1. Primary Screening of Sample on (A) seawater Agar (B) M9 media (C) L-glutaminase-producing isolates.

Figure 2. Growth of colonies on MCD medium. Left to right, respectively, were L-methioninase-specific culture medium, negative control strain, 24-hour culture, and positive colonies incubated for 48 hours in the incubator.

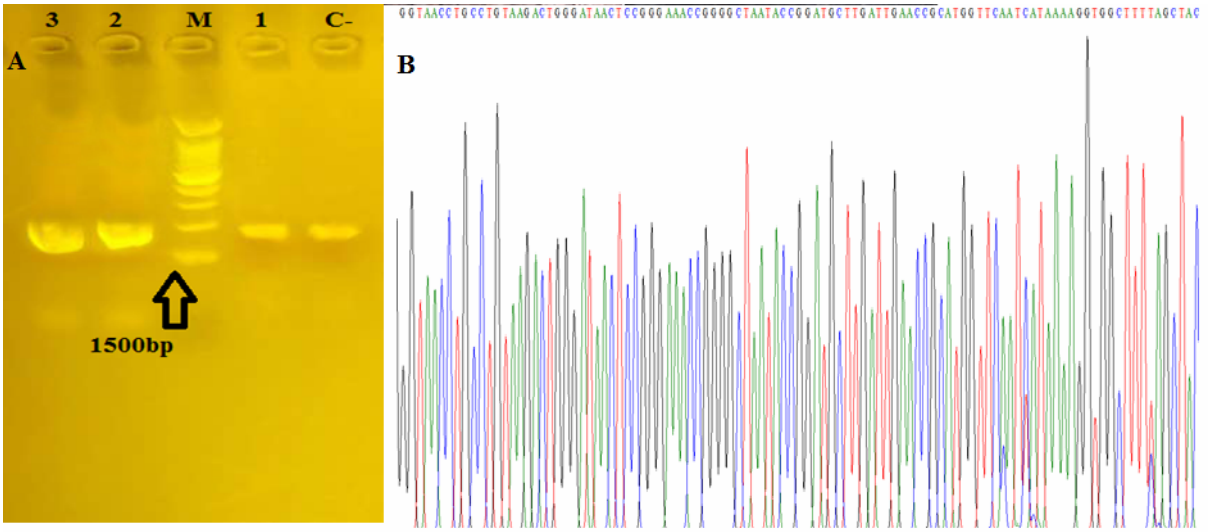

Figure 3. (A) M: Molecular mass standard (100 bp DNA Ladder, Sinaclone), wells of 1-3 of the strains studied and negative control (C-) is distilled water, (B) Sequencing results of one strain.

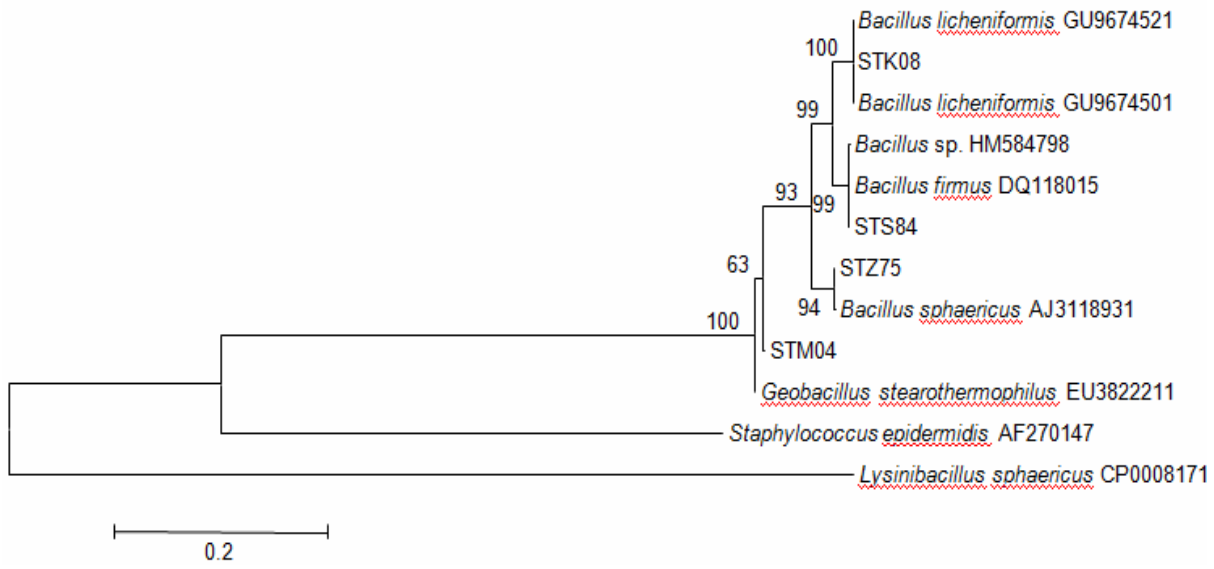

Figure 4. Phylogenetic tree with Bootstrap percentage and genetic strain determination

4. Discussion

Thermophilic bacteria are microorganisms whose optimum growth are above 45°C (Zeikus, 1979). Thermophilic microorganisms have abnormal proteins or specific physicochemical properties that maintain their activity in the degree of heat that destroys the proteins of other microbes (Wiegel et al., 1985). L-glutaminase is an amidohydrolase enzyme that plays an important role in the metabolism of nitrogen in living cells (Shirazian et al., 2019). Since it inhibits glutamine-dependent tumor cell elimination by glutamine, it is now known as a therapeutic enzyme. L-methioninase is also a pyrodexal-dependent enzyme and a multifunctional enzyme system that stimulates the γ- and α, β- elimination reactions of methionine and its derivatives (Liu et al., 2019). In the present study, 12 bacterial isolates were obtained from all samples. Out of 12 strains, 3 (25%) isolates exhibited L-glutaminase activity in M9 medium. In addition, only one isolate (8.3%) showed L-methioninase activity in MCD medium. Moreover, the results of BLAST 16 srRNA gene amplification indicated that all of these isolates belonged to *B. licheniformis*. Ranjbar *et al.* (Ranjbar et al., 2012) found that 11 strains of L-glutaminase-producing bacteria were isolated. The CH3-GLU strain showed the highest enzyme production (37±91.62 U/ml at 45°C after 96h). This isolate was identified as a strain of Bacillus subtilis. The results of this study showed that Bacillus subtilis CH3-GLU had high potential in the production of L-glutamine. These results are consistent with the Bioinformatics study in the present study at NCBI. L-glutaminase activity has been reported in various bacteria including *Bacillus* spp, *Providence* spp, *Micrococcus* spp, *Pseudomonas* spp and *Actinomycete* spp. In 2009, in Taiwan, a researcher, Adiguzel *et al.* (Adiguzel et al., 2009) isolated 1000 thermophilic bacteria from hot springs, the most important of which were Geobacillus spp and Brevibacillus sp. In 2010 in India, Balagurunathan *et al.* (Balagurunathan et al., 2010) isolated Actinomycete sp, producing glutamine from sea sediments. They were able to

isolate 20 enzymes-producing strains which showed a significant difference in the number of isolates producing enzyme compared to the present study (P-value = 0.07). This difference may be due to differences in the climate of the study area, sampling locations and time of study. Pseudomonas BTMS-51 is an L-glutaminase producing strain that was isolated from the soil habitat by Kumar *et al.* (Kumar et al., 2003) the highest production of L-glutaminase by this bacterium was 36.05 U / ml at 30°C. Sivakumar *et al.* (Sivakumar et al., 2006) indicated the production of L-glutaminase in *Streptomyces rimosus*, which was isolated from fish skin. This bacterium was able to produce the enzyme at 17.5°C and 17.51 U / ml. Prihanto *et al.* (Prihanto et al., 2018) in Indonesia indicated *Bacillus subtilis* UBTn7, a potent producer of L-methioninase isolated from *Rhizophora mucronata*. Hamed *et al.* (Hamed et al., 2016) in Egypt indicated the production of L-methioninase by *Chaetomium globosum* which it is an ascomycota fungi and it has belonged of dematiaceous. *C. globosum* was a producer of L-methioninase with specific activity of (2225 U/mg), so L-methioninase could be a good source for clinical use. In Egypt, El-Sayed (El-Sayed, 2009) showed that the combination of L-methioninase treatment, gene therapy, and chemotherapeutic drugs clearly explores the various therapeutic aspects of this enzyme.

Conclusion

In this study, *Bacillus licheniformis* was identified as a dominant and a native strain for the production of L-glutaminase and L-methioninase. These results are consistent with some of the studies cited above. However, due to some disagreements can be (1) geographical distance (2) location of sampling (hot springs, marine, salt environments, soil) (3) microbial isolation conditions (aerobic conditions) and anaerobic and microaerophilic (4) geographical and climatic conditions of the region (tropical, cold, temperate, etc.) and (5) distance from sampling and sea level study.

Potential conflicts of interest

The authors declare that there are no conflicts of interest.

Refereces

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