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Molecular isolation, cloning and expressions of L–glutaminase encoded gene from the aquatic *Streptomyces* collected from Persian Gulf

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ABSTRACT

L-Glutaminase is a therapeutic enzyme found in various microbial source have been considered in the cancer therapy. Sampling was carried out from the shores of the Persian Gulf. After identifying and performing specific biochemical tests, marine *Streptomyces* was isolated and DNA extraction was performed. Through the PCR test, the strains of *Streptomyces* with the L-glutaminase enzyme gene were identified. The *L-gluta* gene was positively transmitted to the host bacterium *Escherichia coli* via a vector and cloned through the TA technique, and the Real Time PCR technique was used to measure the expression of genes in *E. coli* origami. The software clustalX and Mega5 were used to draw the phylogenetic tree. Out of 12 *Streptomyces* isolates, 58.3% of isolates were carried *L-gluta* gene. After cloning the L-glutaminase gene by colony selection (blue / white), the cloned strains were isolated. The real-time PCR test showed a successful expression of the *L-gluta* gene on the cloned strains. Phylogenetic results with the neighbor joining (NJ) method show that, *Streptomyces* species with bootstrap values 99% located in a clade which indicated their close relatedness. The results of this study showed that the Persian Gulf is one of the high potential sources with the production of secondary metabolites and useful antimicrobial products that can be used as a useful source of various biological products such as L-glutaminase.

1. Introduction

Actinobacteria make up a significant portion of the population of soil bacteria and coastal areas. These bacterial branches are very valuable to the pharmaceutical industry due to their unlimited ability to produce secondary compounds (Barka et al., 2016). These secondary compounds are highly diverse in terms of biological activity and chemical structure. *Streptomyces* spp are a major source of biologically active natural products. These Gram-positive bacteria that grows in several environments, and its shape look like filamentous fungi (Westhoff et al., 2020). The morphological differentiation of *Streptomyces* includes the development of a layer of hyphae that can differentiate into a chain of spores. The most notable property of *Streptomyces* is the ability to produce bioactive secondary metabolites, such as antivirals, antifungals, anti-tumorals, anti-hypertensives, immunosuppressant, and particularly antibiotics (Salwan et al., 2020).

One of the most important enzymes produced by these bacteria is L-glutaminase or -L-glutamine amidohydrolase (EC 3.5.1.2). Amino acid L-glutamine, as a nitrogen donor, is involved in the synthesis of purines and pyrimidines in living cells, and since cancer

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cells have no mechanism to produce this amino acid, they must receive it from the environment in the presence of the enzyme L-glutaminase (Sarkar et al., 2020). Glutamine is hydrolyzed and converted to L-glutamic acid and ammonia. By reducing the amount of amino acid L-glutamine, cancer cells die selectively, so that cancer cells die without damaging normal cells (Erickson et al., 2010). The enzyme is effective in Treatment of acute lymphocytic leukemia and AIDS. So, the L-glutaminase, produced by *Streptomyces*, also plays an important role in the food industry, especially in improving the taste of fortified foods such as soy sauce. L-Glutaminase is important in biological sensor to check the level of glutamine in cell culture (Curthoys et al., 1995). Advantages of producing this enzyme by microorganisms include short fermentation time, cheapness, simple screening methods, and rapid growth of microbes, some of which can be manipulated to optimize the reaction and increase product efficiency. Flexibility in selecting fermentation conditions and higher yields is one of the advantages of using L-glutaminase enzyme (Sabu et al., 2005). Due to the importance and widespread use of this enzyme, the aim of this study was cloning and sequencing of L-glutaminase gene isolated from seawater *Streptomyces* spp in the *Escherichia coli* Origami (DE3) strain for clinical, industrial and food use.

2. Materials and Methods

2.1. Sampling

In this cross-sectional study and in the spring and summer seasons of 2019, the shores of the Persian Gulf in Hormozgan province are divided into 5 stations (A, B, C, D, E) and in each station 6 water and 6 sediment samples from depths of 10, 20 and 30 cm were collected. Samples of rivers and sediments were also collected from the river estuary at these stations. At each station, the temperature, salinity and pH of the samples were measured with mercury thermometer, salinometer and pH meter, respectively. Water and sediment samples were collected in 50-ml sterile Falcon disposable tube in the ice bag and transferred to the laboratory and they were kept in the refrigerator until further tests.

2.2. Bacterial isolation

All samples were cultured within 24 hours. From each of the samples, 10^{-2} to 10^{-3} dilutions were prepared in the sterile state, and then 150 µl of each dilution were streaked on the Zobell marine agar (Merck, Germany) and incubated at 25 °C. Composition of Zobell marine agar used is as follows (in one liter of the medium, pH 7.6 ± 0.2): peptone, 5.0 g; yeast extract, 1.0 g; ferric citrate, 0.1 g; sodium chloride, 19.45 g; magnesium chloride, 8.8 g; sodium sulfate, 3.24 g; potassium chloride, 0.55 g; calcium chloride, 1.8 g; strontium chloride, 34.0 mg; sodium bicarbonate, 0.16 g; potassium bromide, 0.08 g; boric acid, 22.0 mg; sodium silicate, 4.0 mg; ammonium nitrate, 1.6 mg; sodium fluoride, 2.4 mg; disodium phosphate, 8.0 mg; agar, 1.5 g. Morphological features of colonies such as colony pigmentation were used for initial classification of the bacterial population.

2.3. Cloning of the L- Gluta gene in E. coli origami strain

Initially, the presence of the L-glutaminase gene in *Streptomyces* strains was investigated using PCR. For this aim, a single colony of all isolates were cultured in 50 ml of International *Streptomyces* Project Medium 2 (ISP2) for 18-24 h at 26˚C. Then the culture was centrifuged for 2 min at 3000 ×g and supernatant was discarded. Eventually the genomic DNA of lysed bacterial cells was precipitated with 0.6 volume of isopropanol and purified using 70% ethyl alcohol (Maleki et al, 2013). In order to reproduce a 110 region of the gene. To amplify the specific fragment of 234bp for 16S rDNA gene, a set of primers; F: 5′- CAA TGG ACA AAG CGC AAA C-3′ and R: 5′- GAA TTA AAG GCG TCA CCT G-3′ was used (Benga et al., 2014). PCR reaction was performed in an Eppendorf Gradient Mastercycler (Eppendorf, Germany) with a final volume of 25 µl per tube containing 1 µl of chromosomal DNA, 12.5 µl Taq DNA polymerase 2x Master Mix Red, 1 µl of each primer (10 pmol) and 9.5 µl ddH2O. The reaction mixture was achieved with the following PCR program: initial denaturation at 94°C for 4 min, 31 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1min and final extension at 72°C for 5 min. Amplified PCR products were evaluated using electrophoresis on 1.5% agarose gel in TBE buffer at 100 volts for 2 hours and

the DNA bands were visualized by DNA safe staining. The banding profile were analyzed by the NTSYS software (version.2.0) and cluster analysis was done to generate dendrogram by unweighted pair group method with arithmetic averages (UPGMA).

2.4. Cloning of the L-gluta gene in E. coli

The PCR amplicons of *L- gluta* gene was purified by the gel extraction kit (Roche) and ligated into the pTZ57R (MBI Fermentas) cloning vector. T4 DNA ligase (Fermentas) was used for the ligation. The resulting plasmids were transformed into competent *E. coli* origami^{TM} (DE3) by following the manufacturer's guidelines. The Mineral Salt Glutamine (Sigma Aldrich, Germany) comprising 100µg/µl ampicillin were used for selection of transformed colonies. The white colonies were selected and following an overnight cultivation, were subjected to plasmid extraction and PCR. In order to verify the fidelity of the cloned fragments, the selected recombinant plasmids were subjected to sequencing (MWG service) (Asadi et al, 2012).

2.5. Expression of the cloned gene

After 15h incubation (Late log phase), mRNA was extracted using RNeasy Midi Kit (Qiagen) instruction and cDNA was synthesis using a Smart PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The concentrations of RNA in all samples was quantified with a spectrophotometer at 260/280 nm. Real-time PCR was performed by using a 2× GreenStar Master Mix Kit (Bioneer, Korea) on a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). A typical PCR sample (25 µl) contained 10 µl of PCR Master Mix, 1 µl of cDNA, 0.5 µl of 0.8 µM solutions of both forward and reverse gene-specific primers and 13 µl of double distilled water. The Real time-PCR was performed according to the following program: the initial denaturation 95˚C for 1 minute, multiplication including 95˚C for 30 seconds, 59˚C for 40 seconds and 72 ˚C for 60 seconds in 35 cycles. Each sample was run in triplicate. A critical threshold cycle (CT) value was used to represent *l-gluta* transcripts quantitatively. The ΔCT for *l-gluta* transcripts was calculated against that for the β-actin gene. The *l-gluta* relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak et al, 2001).

Results

Plates based on morphological features and microscopic studies such as white with a dry, gypsum-like appearance and odor from their physiological activities, as well as gram staining and slide culture to study the characteristics of mycelium, 12 isolates of *Streptomyces* were isolated from the seawater samples. Gram staining showed the presence of gram-positive filamentous bacteria. (Figure 1). The biochemical properties of *Streptomyces* isolates are shown in Table 1. So, all strains were confirmed using universal 16s rRNA primer in PCR test.

The results of PCR reaction for the *L-gluta* gene showed that of 12 *Streptomyces* strains, 7 (58.3%) isolates were positive for this gene. All *L-gluta*-positive strains were sequenced (figure 2a and 2b).

After cloning the L-glutaminase gene by colony selection (blue/white), the cloned strains were isolated. In order to confirm the results of cloning, DNA were extracted and PCR sequencing was done. The presence of 234 bp indicates successful cloning. Finally, with the PCR sequence, the *L-gluta* gene clone in the *E. coli* was confirmed (figure 3a, 3b and 3c).

The real-time PCR test showed a successful expression of the *L-gluta* gene on the cloned strains with Tm= 86.79 (figure 4a). Phylogenetic results with the neighbor joining (NJ) method show that, *Streptomyces* spp with bootstrap values 99% located in a clade which indicated their close relatedness (figure 4b).

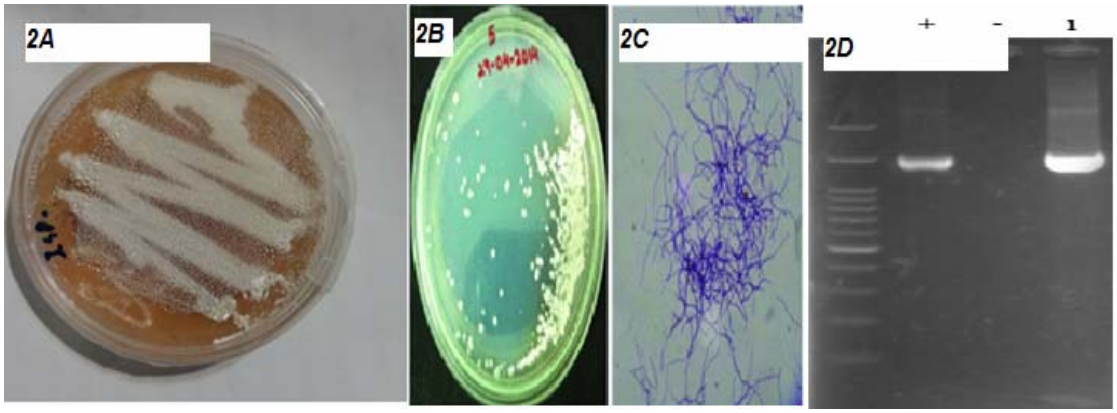

Figure 1. Morphological and microbiological characteristics of isolated *Streptomyces* in this study. 2A and 2B; Morphological and macroscopic examination, 2C; microscopic observation, 2D; 16s rRNA PCR test.

Table 1. The General characteristics of *Streptomyces* isolates

Biochemical properties	Results
Methyl red (MR)	-
Voges–Proskauer (VP)	-
Citrate	+
Casein hydrolysate	+
Nitrate reduction test	-
Oxidase	+
Catalase	+
6.5% NaCl	+
Color on the colony	Red, Peach, White
Color behind the colony	Red, Cream, yellow
Air Mycelium color	Gray, White with yellow pigments

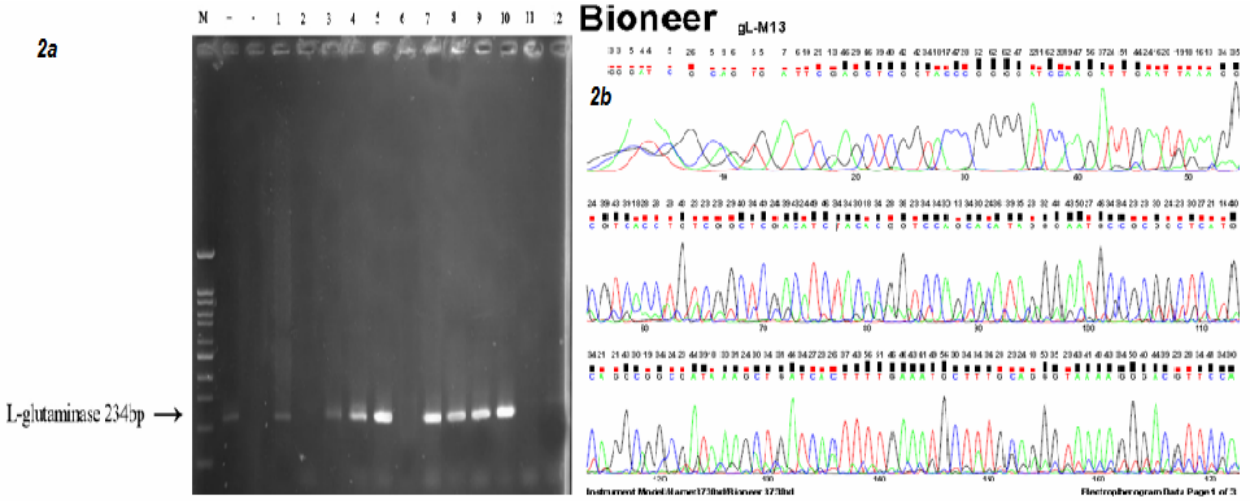

Figure 2. PCR results of *L-gluta* genes: 2a; C+: positive control, C -: negative control (distilled water), Wells 1 to 12: isolated *Streptomyces* strains (1 to 6 isolated from water and 7 to 12 collected from sediment), 2b; the result of sequencing and BLAST of the L-glutaminase gene.

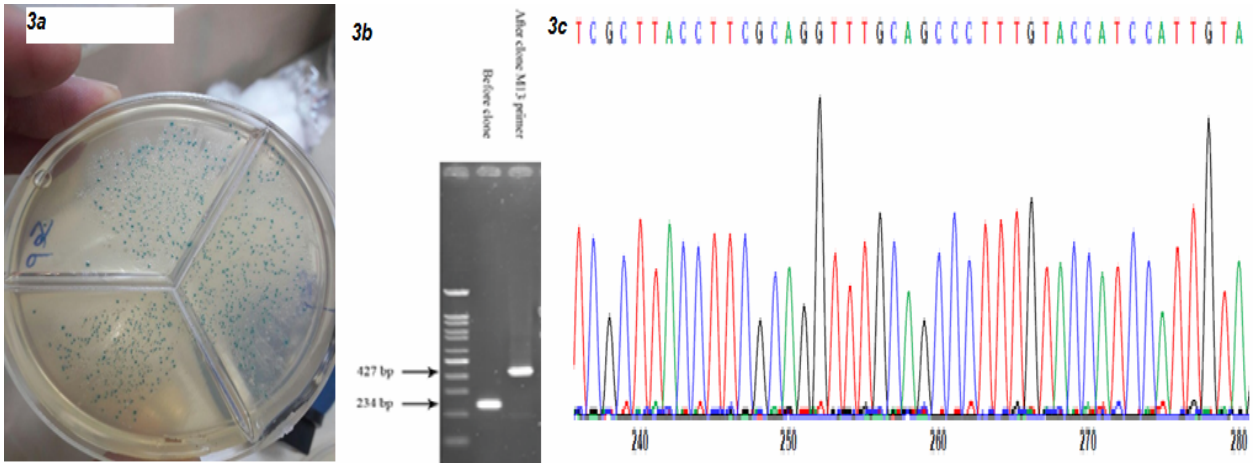

Figure 3. Results of clonal selection of L-glutaminase gene, (white and blue colonies).

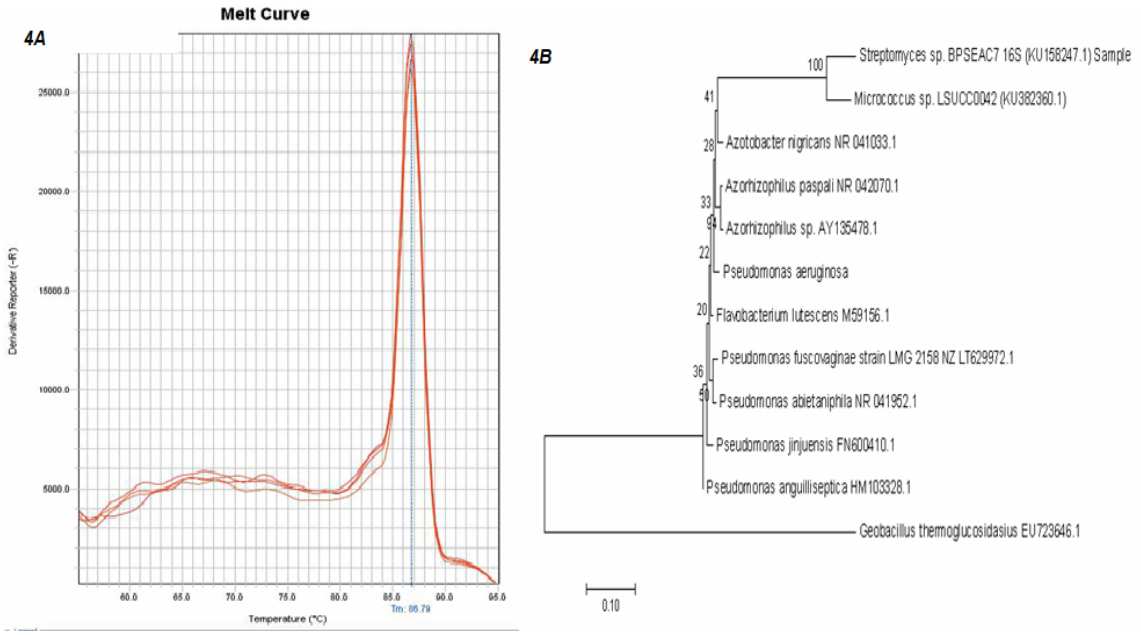

Figure 4. 4A; Real time PCR melting curve results, 4B; Phylogenetic tree drawn based on 16S r RNA gene sequence

4. Discussion

In general, enzymes are the most important products derived from microbial sources for humans, because of their cost-effectiveness, so, they are preferred to enzymes derived from plants and animals. One of the most important enzymes that has been considered in recent years is L-glutaminase, which has various uses in medicine, pharmaceuticals, food and industry (Chasanah et al., 2013). So far, many studies have been conducted to isolate L-glutamatase-producing microorganisms due to their anti-tumor properties. Most L-glutamitase microorganisms are obtained from the soil. However, there are also numerous reports of the isolation of L-glutamine-producing microorganisms from aquatic habitats (Wade et al., 1971; Spiers et al., 1976). L-glutaminase activity has been reported in a variety of bacteria, including Bacillus, Micrococcus, Pseudomonas, and Actinomycetes. But only

actinomycetes are commercially available in the production of L-glutaminase (Binod et al, 2017).

In 2010, Prakash et al. Used Mineral Salt Glutamine to produce L-glutaminase by bacteria (Prakash et al, 2010). They evaluated different environments for the production of the enzyme L-glutaminase. Their results showed that the highest amount of enzyme production was performed in Salt Glutamine mineral medium. For this reason, in the current study, this culture medium was used to produce L-glutaminase enzyme by productive bacteria. In agreement with our study, Balagurunathan et al. (2010) Isolated glutaminase-producing actinomycetes from seawater sediments. They were able to isolate 20 enzyme-producing strains (Balagurunathan et al., 2010). In study directed by Ranjbar et al (2015), 34 bacterial strains were isolated from different samples. Of the isolated strains, 11 bacteria produced the L-glutaminase enzyme (Ranjbar et al., 2015). In the present study, 12 strains of *Streptomyces* were isolated from sediment and seawater samples, of which 7 isolates had *L-gluta* gene as a result of PCR test. In 2009, Lyer and Singhal isolated Providencia from the seawater samples (Iyer and Singhal, 2009). The highest yield of enzyme was obtained at 25˚C (12.0 U / ml). Production of L-glutaminase in the 2-SFL strain of *Vibrio* and *Klebsiella oxytoca* strains isolated from aquatic and terrestrial habitats was 41U / ml and 44 U / ml, respectively. On the other hand, this amount of production was observed at 35° C and 33 °C, respectively, and cloning was performed in *E. coli* origami bacteria. Since in all studies, the environment used to produce the enzyme and the method of measuring the enzyme are similar, so the difference in the efficiency of the enzyme can be attributed to the study. Nazemi et al., 2014, in a study of molecular isolation and identification of anticancer L-asparaginase glutaminase enzyme produced by *Bacillus*, showed that these two enzymes play an important role in the treatment of acute lymphoblastic leukemia due to their Antineoplastic properties (Nazemi et al., 2019). In conflict with our study, Chasanah et al (2013) showed that identified bacteria based on 16S rRNA sequencing has revealed that the isolate was 96% similar to *Pseudomonas aeruginosa* strain CG-T8 (Chasanah et al., 2013). This discrepancy could be due to geographical and ecological differences and the materials used in the study.

Conclusion

The results of this study showed that the Persian Gulf is one of the high potential sources with the production of secondary metabolites and useful antimicrobial products that can be used as a useful source of various biological products such as L-glutaminase. Since L-glutaminase isolated from different bacteria has had different therapeutic effects, the search for microorganisms that produce this enzyme is one of the main ways to achieve an enzyme with ideal therapeutic properties. Examination of marine streptomycin due to its high potential in the production of L-glutaminase could pave the way for future studies, and these isolates could be considered as suitable candidates in the design and manufacture of anticancer drugs and acute lymphoblastic leukemia and AIDS and antimicrobial compounds and industries.

Potential conflicts of interest

All authors announce that there are no conflicts of interest.

Refereces

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