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Molecular screening and cloning of the protease encoded gene from Streptomyces strains isolated from Persian Gulf

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ABSTRACT

Protease is an enzyme with various uses in medicine, industry and textile. One of the most important sources of protease production is bacteria such as *Streptomyces*. So, the aim of this study was cloning and sequencing of the protease gene in the *Streptomyces* spp isolated from Persian Gulf in *Escherichia coli* XL1blue. After collection of marine sediments from the Persian Gul, *Streptomyces* strains were identified using standard laboratory tests. All isolates were confirmed using 16S rRNA amplification test. Protease encoded gene were identified using specific primers in the PCR method. Protease gene was cloned in the *E. coli* host vector by TA cloning technique and finally the expression of the genes was measured using Real-time PCR method. ClustalX and Mega5 software were used to draw the phylogenetic tree. Twelve isolates of *Streptomyces* were isolated and 25% (n; 3/12) of them were positive for protease gene. After cloning of the gene, colony selection (blue / white colonies) were used for identification of success cloned strains. A relative expression of the protease gene was shown by real-time PCR test. Phylogenetic tree with the neighbor joining method show that, *Streptomyces* spp with bootstrap values 99% located in a clade which indicated their close relatedness. Protease enzyme production was performed by recombinant plasmid and TA cloning, and further studies could be helpful to optimize different conditions for this enzyme production. So, The Persian Gulf is a large pool for the protease producing *Streptomyces* for medical and industrial use.

1. Introduction

Streptomyces are aerobic gram-positive bacteria which belonged to the largest genus of Actinobacteria and frequently isolated from the various source such as; soil, water and marine sediments (de Lima et al., 2012). There are more than 500 species of *Streptomyces* spp. These bacteria have the ability to produce secondary metabolites such as enzymes and antibiotics. Due to their unique role in the production of secondary metabolites and biologically active substances, including enzymes, and enzyme inhibitors, this bacteria is one of the most important factors in biocontrol (Chater & Hopwood, 1993). Proteases include a very large group of enzymes that differ in properties such as substrate properties, active site, pH catalytic activity, and optimal temperature and stability. Proteases are divided into four groups based on proteolytic mechanism and active site including, Serine protease, aspartic protease, cysteine protease, and metalloprotease (Ellaiah et al., 2002). Protease enzyme has many applications in industry, including food industry, fermentation industry, detergents, Textile

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industry, poultry food supplement, oil industry, chemistry and pharmaceuticals. The main sources of proteases production are animals, plants and microorganisms. Production of this enzyme from plant and animal is limited due to climatic conditions and enough space issues. So, bacteria are the most important producers of this enzyme (Sawant & Nagendran, 2014). Separated protease enzymes have significant properties such as activity and stability in alkaline pH, activity in the presence of salt concentrations and organic solvents (Ward et al., 2011). So, alkaline protease to detergents considerably increase (35-40%) the cleaning effect (particularly in removing stains containing proteins, e.g., blood, cocoa, milk, eggs and sauces) and increase the consumption of surface-active substance, thereby improving the ecological situation (Varia et al., 2019). Recent studies have shown the pharmacological properties of alkaline protease in the pharmaceutical industry. Because this enzyme is also very important in the production of medical products. Applications of protease in medicine include (Bhatnagar et al., 2012), blood clots in ischemic stroke (Pu et al., 2019), wound debridement (Stone et al., 2020), antimicrobial therapy (Shao et al., 2019), anti-inflammatory activity (Sangeetha et al., 2019) and enzyme therapy (Balakireva et al., 2020). For example, protease is one of the enzymes in a pancreatic enzyme replacement therapy (PERT). It assists in the breakdown of proteins into amino acids and polypeptides (Kumar et al., 2008). The aim of this study was molecular isolation and expression Cloning of the Protease encoded gene from *Streptomyces* spp in the *Escherichia coli* Xl1 blue collected from Persian Gulf.

2. Materials and Methods

2.1. Sampling

In this analytical cross-sectional study, the shores of the Persian Gulf in the Hormozgan province are divided into 5 sections (A, B, C, D and E). In total, 6 water and 6 sediment samples from the 10 cm, 20 cm and 30 cm depth in the spring and summer time were. Water and sediment samples were collected from the river estuaries. Mercury thermometer (Zeal, French), salinometer (model: DMT-10) and pH meter (Metrohm, Switzerland) were used for determination of, the temperature, salinity and pH, respectively. Each sample was collected in the 50 ml sterile Falcon tube in the ice bag and transferred to the Pasargad microbiology laboratory located in Tehran, Iran. All samples were stored in 4˚C until further used.

2.2. Microbial isolation

In a sterile condition, serial dilution from 10^{-2} to 10^{-3} were prepared, and then 150 µl of all dilutions were cultured on the marine agar culture medium (Merck, Germany) and incubated at 25 °C. Composition of the marine agar culture media is as follows; peptone, 0.5 g; yeast extract, 0.1 g; iron phosphate, 0.1 g; and agar, 1.5 g. the acidity of the medium in 1 liter was 2.7 to 6.7. Macroscopic features such as colony morphology, color, pigment and odor, were used for early classification.

2.3. Nucleic acid amplification test

Finally, all *Streptomyces* colonies confirmed using the molecular identification test. Cellular DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Germany). The PCR reaction was performed using specific fragment of 16s rRNA including, F=5′ -AGA GTT TGA TCC TGG CTC AG-3′ and R=5′ -AAG GAG GTG ATC CAG CCC GCA-3′ . The PCR product was then sent to the Bioneer Company for the sequencing and the results were BLAST in the NCBI database (Srivastava et al., 2008).

2.4. Molecular identification of protease gene in Streptomyces isolates

The presence of the protease gene was evaluated using PCR. To amplify the specific fragment of protease gene, a set of primers including, F: 5′- GTA ACA GGA ACG AAT AAA GTA GGA ACT GGT AAA G -3′ and R: 5′- GTT TAC ACC AAC AGC ACA CAA ATG ATT GCT TAA C -3′ were used. PCR test was done in an Eppendorf Gradient Mastercycler (Eppendorf, Hamburg, Germany) with a total volume of 25 µl containing 0.9 µl of genomic DNA, 11.5 µl Taq DNA polymerase 2x Master Mix Red, 0.9 µl of each primer (10pmol) and 10.8 µl sterile distilled water. The temperature steps of the PCR reaction were performed as follows, initial denaturation at

95°C for 5 min, 35 cycles with denaturation at 95°C for 30 s, annealing at 56°C for 60 s and extension at 72°C for 1 min and final extension at 72°C for 6 min. Amplicons were analyzed using electrophoresis on 1.5% agarose gel in TBE buffer at 100 volts for 1 hours and the DNA fragments were visualized by DNA safe staining. DNA banding pattern analyzed by the NTSYS software (version.2.0) and cluster analysis was performed to draw a dendrogram by the unweighted pair group way with the arithmetic averages (UPGMA). The dendrogram was compared with Streptomyces ID: 1103098.

2.5. Cloning of the apr (alkaline protease) gene in E. coli xl1blue

The PCR products of *apr* gene was purified using the gel extraction kit (Cinna Clone Inc., Iran) and ligated into a linear vector called pTG19-T with the 3'-end of thymine base (MBI Fermentas as) cloning vector. Ligation was done using T4 DNA ligase (Fermentas). The resulting plasmids were transformed into the competent host *E. coli* XL1-Blue. The Inorganic Salt Glutamine (Sigma Aldrich, Germany) including 100 µg / µl ampicillin were used for selection of transformed colonies. The white / blue colonies were follow for 24h, and then recombinant plasmid was extracted. In order to confirm the fidelity of the cloned fragments, the selected recombinant plasmids were submitted to sequencing (MWG service) (Asadi et al., 2012).

2.6. Expression of apr gene by Real - time PCR method

In the Late log phase, messenger RNA was extracted by the RNeasy Midi Kit (Qiagen) and complementary DNA was synthesis using an AMV Reverse transcriptase enzyme (1U) in the Smart PCR cDNA synthesis kit (Clontech, USA). The concentrations of mRNA in all samples was quantified with a Nano-drop at OD; 260/280 nm. Real-time PCR was done using a 2× GreenStar Master Mix Kit (Bioneer, Korea) on a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett, Australia). A typical PCR sample contained 11.5 µl of PCR Master Mix, 1 µl of cDNA, 0.7 µl of both primers and 11.1 µl of ddH_2O. The Real time-PCR was done based on the following steps: the initial denaturation 95˚C for 1 min, extension at 95˚C for 30 s, 58˚C for 40 s and 72˚C for 60 s for 35 cycles. Each sample was run in triplicate and the β-actin housekeeping gene was used as an internal control of the test. A critical threshold (CT) value was used to presence of *apr* transcripts quantitatively. The ∆CT for *apr* transcripts was calculated in compare with the β-actin gene. The *apr* relative expression was measured by the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001).

2.6. Drawing a phylogenetic tree

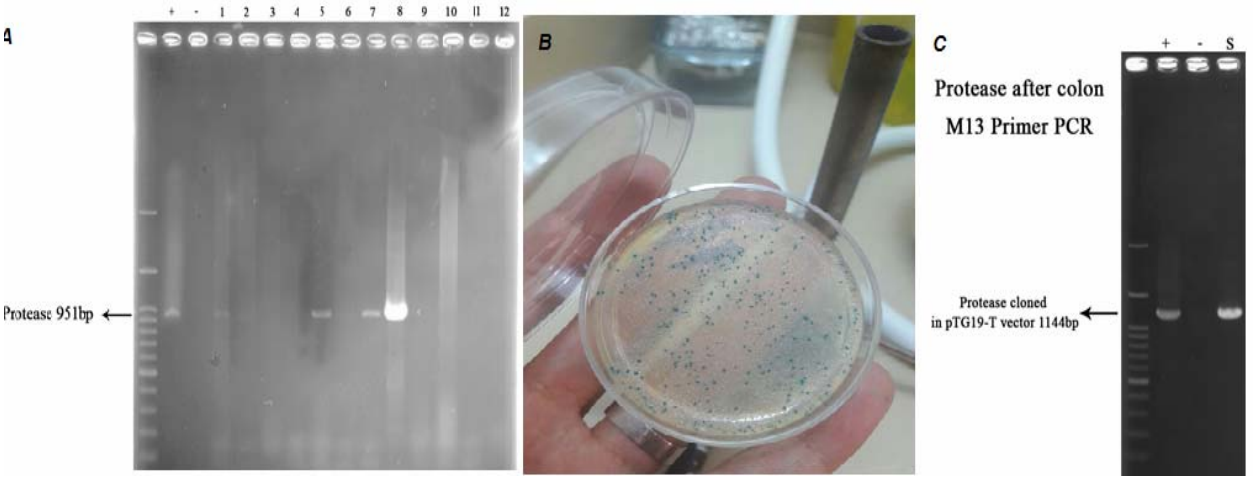
The results of sequencing were analyzed by the BioEdit software. The resulting sequences were compared with the strains registered in NCBI. Each sequence separately analyzed by software NBlast in GenBank BLAST search, and the sequences obtained were aligned by Clustal W software. Phylogenetic analysis After drawing sequences by Clustal W software, phylogenetic trees were drawn using 10.5 MEGA programs. Trees prepared using the MEGA 10.5 drawing annex program are located in Neighbor Joining (NJ).

3. Results

According to the microbiological and biochemical standard test, 12 *Streptomyces* spp were obtained, which 25% (n; 3) were positive for *apr* gene (figure 1A). After cloning the protease genes using colony selection (blue / white), transformed strains were isolated (Figure 1B). To confirm the results of DNA cloning, transformed DNA were extracted and PCR sequencing was performed. The existence of 1144 bp indicates successful cloning (figure 1C).

The real-time PCR test showed a successful expression of the *apr* gene on the transformed strains in the cycle 20 (figure 2a). Phylogenetic results showed that, *Streptomyces* spp with bootstrap values 99% located in a clade which showed their close relatedness (figure 4b).

4. Discussion



Microbial enzymes have various advantages over enzymes derived from plant and animal resources, including, diversity of catabolic activities, cheaper costs, abundant, continuous resources, and even greater quantity and relative stability.

Figure 1. (1A); PCR results of protease genes, (1B); Protease enzyme clone gene results and (1C); the presence of protease encoded in pTG19-T vector.

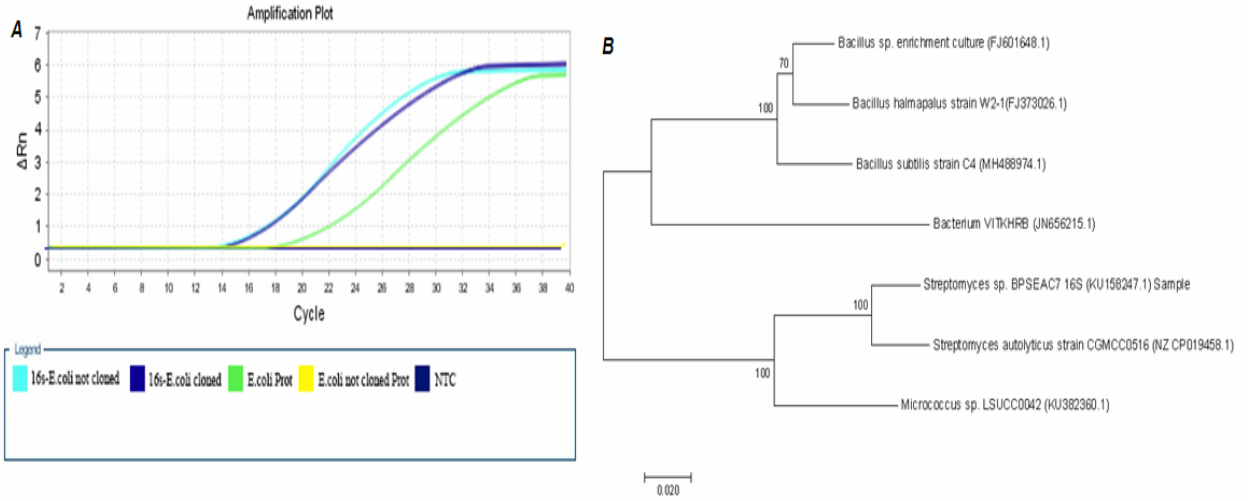


Figure 2. Real Time PCR Replication Curve Results and (2b) Phylogenetic tree drawn based on sequence of 16S r DNA gene

Marine bacteria produce different enzymes based on their habitat and ecological structure (Adrio and Demain, 2014). Because seawater is naturally saline and there are more chemicals similar to human blood plasma, therefore, marine microbial products, especially enzymes, have higher immunity and less cell toxicity and are generally more suitable for therapeutic application. Monadi et al. (2014) showed that antibiotic-produced by *actinomycetes* which isolated from the Persian Gulf (south of Iran) have 90% antimicrobial activity against at least one of the studied bacteria. The antibacterial effect was higher in gram-negative bacteria than in gram-positive. This is may be related to bacterial cell wall structure (Monadi et al., 2014). In a study directed by Raaiei Ardakani et al. (2010) halophilic bacteria that produce the

extracellular enzymes amylase, protease and lipase are separated from the water and sediments of the Persian Gulf. Based on the molecular analysis by 16S rRNA sequence, 9 isolates belonged to the *Pseudoalteromonas* spp. Most of the enzymatic activity was observed in the growth logarithmic phase (Raaiei Ardakani et al, 2010). In a study conducted by Lotfi et al (2015), 27 strains were produced the alkaline protease enzyme and the highest production of alkaline protease enzyme was observed in YPG Brats culture medium. Comparing the 18S rDNA sequence of the separated yeast with the sequences in the genomic database showed the maximum similarity with *Yarrowia lipolytica* yeast. The researchers concluded that due to the industrial use of these enzymes and the harmlessness of their microbial source, these strains could be considered as a potential source for the production of these enzymes for various uses (Lotfi et al., 2015). Mehrabi et al. (2019) studied the separation, extraction, and purification of the properties of a type of protease enzyme through the bacterium Bacillus SB1. Initially, enzyme production conditions were optimized. The enzyme filtration gel was then purified using chromatography. Using PAGE-SDS, the molecular weight of the protease was estimated at 34 kDa. This enzyme at pH = 8 has optimal activity and stability (Mehrabi et al., 2019). In a study directed by Dawoodi et al, (2013), various actinomycetes were screened for soil samples from Khuzestan, Chaharmahal and Bakhtiari and Isfahan provinces (Dawoodi et al., 2013). Among the actinomycetes that produce alkaline-generated soil screenings, most of the enzymatic activity belonged to the *Streptomyces diastaticus* strain. This study was the first report on the production of alkaline proteases by *S. diastaticus* in Iran. The tests performed in this study partially confirmed the previous international hypotheses in the production of alkaline protease enzymes by actinomycetes.

Conclusion

Due to the many applications of alkaline protease enzymes in various industries and due to the segregation of native enzymes from the enzymes that produce this enzyme, we can hope to produce this enzyme in the country and provide the ground for self-sufficiency and prevent its increasing import. In the present study, the bacterium that produces the protease gene was isolated from the seabed and the gene was purified.

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Contradiction

There is no contradiction between the authors of the article.

Refereces

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