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Original Article

Pectinase enzyme from *Streptomyces coelicoflavus* GIAL86 isolated from Meyghan Salt Lake, Arak, Iran

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Abstract: Aquatic saline ecosystems are suitable environments for isolation of microorganisms with high diversity and widely used biotechnology features. The pectinase enzyme is one of the most important commercial enzymes with high potential in food and pharmaceutical industries. Therefore, the discovery of microorganisms with new characteristics has always been a focus of research. As such, a pectinase producing actinomycete *Streptomyces coelicoflavus* GIAL86 was isolated from Meyghan Salt Lake of Arak located in Markazi Province of Iran. This strain was screened among 35 isolates of halotolerant actinomycetes with the highest production of pectinase enzymes. It was also found that production of pectate lyase, pectin esterase and polygalacturonase increased simultaneously with the logarithmic growth of the strain and its maximum production is at the time of stationary phase beginning. Also, some actinomycete strains with more pectinase activity were identified by molecular identification and their phylogenic relationships were investigated.

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Introduction

Pectinase enzyme is commonly referred to a group of enzymes that play a role in pectin biodegradation (Dai et al., 2018). Pectins are high molecular weight polysaccharides composing of α-1→4 linked D galacturonic acid residues with a few rhamnose residues in the main chain and arabinose, galactose and xylose on its side chain (Rangarajan et al., 2010). Pectinase enzymes are included three enzymes viz. poly galacturonase, pectine sterase, and pectate lyase based on their role on the pectin degradation (Khatri et al., 2015). Pectinase enzymes are extensively used in food, cosmetic and pharmaceutical industries (Jayani et al., 2005; Mohandas et al., 2018). As extraction of medicinal plants seeds by chemical solvents are harmful to human health, enzyme-assisted extraction is a green extraction technique. Pectinase enables the release of cell components with cell wall degradation (Kontiä, 2016).

Pectinases are produced by different organisms, including bacteria, fungi, plants and insects. Microbial pectinases are superior to other organisms, due to genetic manipulation ability. Although fungal pectinases have become more widely used in the industry, actinomycete pectinases have high industrial potential (Kumar and Suneetha, 2015). Actinomycetes belongs to a high GC class of gram-positive bacteria of prokaryote and produce different industrially useful extracellular enzymes like cellulase, xylanase and pectinase. As they are efficient degraders of plant debris, these enzymes are valuable for food and pharmaceutical industries. Also, actinomycetes have a high biotechnological significance in recent years due to the production of valuable metabolites produced by its various genera (Ballav et al., 2015).

However, many of the rare genera of actinomycetes have not been discovered for their biotechnological products. Studies on the extreme aquatic environments can be useful to produce novel enzymes form their microorganisms. The present investigation focuses on production and analysis of pectinase enzymes of halotolerant actinomycetes isolated from

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Meyghan saline lake and phylogenetic relationships of the strains.

Materials and Methods

Bacterial strains and culture medium: The Meyghan Lake, locating 15 km north east of Arak, Markazi Province of Iran. A total of 35 actinomycete strains were received from Iranian Biological Resource Center (IBRC), including those of Meyghan Lake. In order to revive and maintenance of actinomycetes, the salt free ISP2 medium was used (Salehghamari and Najafi, 2016). Then, they were cultured and incubated at 28°C for 5 days. Pure cultures of each strain was streaked on slants of ISP2 media and stored at 4°C for further study.

Screening of pectinolytic activities: The actinomycetes strains were assayed for their pectinolytic activity in agar plates containing pectin as a sole carbon source. Halos of hydrolysis were detected using I_2/KI (0.3% I_2 and 0.6% KI) (Soares et al., 1999; Aaisha and Barate, 2016) in a solid medium containing (g/l): pectin 10, yeast extract 1, and bacteriological agar 15. The plates were incubated at 28°C for 5 days. Clear zone formation due to pectin hydrolysis was evaluated around colonies. Several pectinolytic strains were isolated, and one strain was selected for a larger clear zone of hydrolysis for further investigation.

Enzyme production and medium condition: In order to measure the selected pectinase enzyme, 1 ml of 0.5 Mc farland suspension of the selected strain was inoculated into 100 ml of production medium containing (g/l): pectin 10, yeast extract 5 and peptone 5 (Saoudi et al., 2015) and incubated at 28°C for 72 hours and 180 rpm. The culture medium was centrifuged at 4000 g for 10 min to precipitate mycelia and the suspension was used for further enzyme study. Growth curve of the candidate was determined at the same production medium and condition by Bradford protein assay (Burgess and Deutscher, 2009).

Enzyme assays, pectin methyl esterase, pectate lyase and poly galactoronase: Pectate lyase activity was assayed by measuring the absorbance at 235 nm (Collmer et al., 1988). 0.05 ml from supernatant containing enzyme was added to a reaction mixture consisting of 0.001 ml of 0.01 M Tris–HCl buffer at pH 7, supplemented with 0.02 ml pectin 10% (w/v). One unit of enzyme activity was defined as the amount of enzyme required to produce unsaturated oligogalacturonides equivalent to 1 μmol/min under standard assay conditions. Pectin methyl esterase activity (U) was determined as the enzyme amount which releases 1 μmol of methanol from pectin per min at pH 7 and room temperature. 1 ml from supernatant was added to a reaction mixture consisting 0.5 ml of 3 mM potassium phosphate buffer at pH 7, supplemented with 2 ml pectin 10% (w/v) and 0.15 ml bromothymol blue 0.01% (w/v). The enzyme activity was assayed by measuring the absorbance at 620 nm (Hagerman and Austin, 1986). Poly galacturonase activity was determined by quantifying the amount of reducing groups expressed as galacturonic acid units, liberated during the incubation of a mixture of 0.5 mL of 10% pectin, 0.25 mL of 3 mM potassium phosphate buffer at pH 7, 1 ml dinitrosalicylic acid 3 mM and 0.5 mL of culture supernatant at 100°C for 15 min. The absorbance was measured at 540 nm (Miller, 1959). One unit of Poly galacturonase activity (U) was defined as the amount of enzyme that liberates 1 μM of galacturonic acid per min under standard assay conditions.

DNA extraction, *16S rDNA* amplification and phylogenetic analysis: Some of the actinomycete strains with the best pectinase activity were grown for 4 days at 28°C and 200 rpm in ISP-2 medium. Strains biomass were precipitated at 4000 rpm for 10 min and washed twice with 10% sucrose. About 200 mg of pellets were used for DNA extraction as described by Kieser et al. (2000). All of the *16S rDNA* genes were amplified using primers 9F (5´ AAGAGTTTGATCA TGGCTCAG 3´) and 1542R (5´ AGGAGGTGATCC AACCGCA 3´). The reaction mix (25 μl) contained 1 μl of genomic DNA, 25 μl of Taq Master Mix (2X), 0.5 mM of each primer and 5% DMSO. The reaction was started with an initial denaturation at 96°C for 300 second followed by 30 cycles of denaturation at 96°C for 30 second, annealing at 58°C for 30 second and extension at 72°C for 60 second, with a final extension

Figure 1. Some of the strains exhibited the large clear zone of pectin hydrolysis.

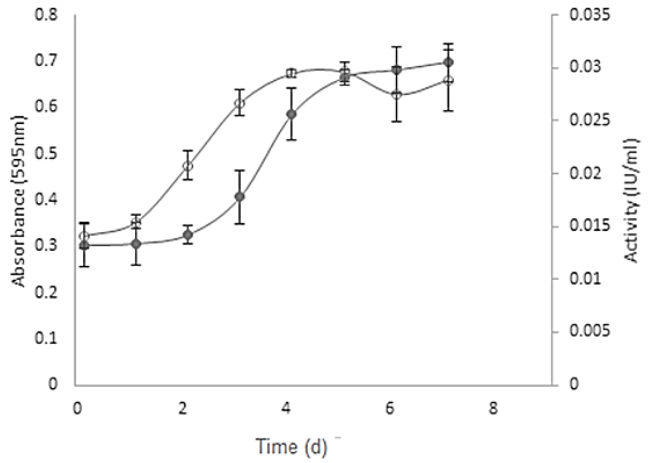

Figure 2. Pectin methyl esterase and growth curve of GIAL86 isolated strain. The empty circle is pectin methyl esterase and full circle is biomass absorbance.

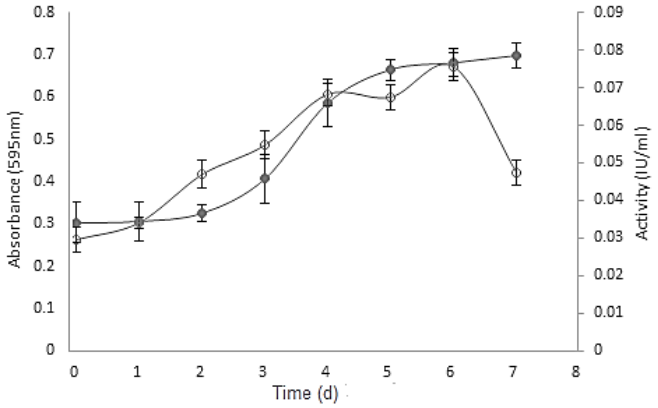

Figure 3. Pectate lyase activity and growth curve of GIAL86 isolated strain. The empty circle is pectate lyase and full circle is biomass absorbance.

at 72°C for 300 second. The PCR products were purified and analyzed by Macrogen Inc. (Seoul, Korea).

To identify phylogenetic neighbors and calculate pairwise *16S rDNA* sequence similarities, the eztaxon server (http://www.ezbiocloud.net/eztaxon) was used. To align the sequences, CLUSTALX software (version 2.0, Conway Institute, USA) was used. Phylogenetic tree was constructed by the neighbor-joining method using MEGA software (version 6.0, Biodesign Institute, USA). The bootstrap was calculated from 1,000 replicates.

Results

Screening of pectinase producing strains: In the current study, 35 actinomycetes isolates were screened for their pectinase activities. Fifteen isolates were shown the large clear zones of hydrolysis (Fig. 1) and they were selected for further assay to produce pectinase in liquid culture. To select the candidate, the fifteen isolates were cultured into liquid medium to produce pectinase and after five days they were assayed for pectin methyl esterase activity.

Pectinase enzyme activity and growth curve: Among pectinase producing strains, an actinomycete strain, called strain GIAL86 displayed the highest pectin methyl esterase activity (0.03 U/ml) after 5 days of incubation and therefore was selected for further assay of pectinase enzymes.

To find the best time of methyl esterase production during the strain GIAL86 life time, the production of this enzyme was monitored along with its growth curve for 8 days. As shown in Figure 2, the production of pectin methyl esterase enzyme increases simultaneously with the logarithmic phase, and its maximum activity is on the fifth day at the end of log phase.

Pectate lyase and polygalactoronase activity of strain GIAL86 were also assayed during 8 days. To determine production phase of these enzymes, enzyme activities were measured during growth curve of strain GIAL86 (Figs. 3, 4). The production of pectate lyase and polygalactoronase were increased simultaneously

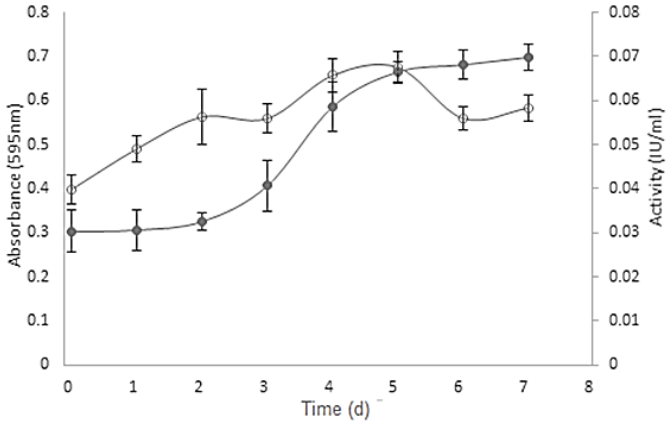

Figure 4. Poly galactoronase activity and growth curve of GIAL86 isolated strain. The empty circle is poly galactoronase and full circle is biomass absorbance.

with the logarithmic phase. Pectate lyase maximum activity was monitored on the seventh day and the maximum activity of polygalactoronase was measured on sixth day at the time of stationary phase beginning.

Bacterial identification and phylogenetic analysis: Genomic DNA of the 9 pectinase producing actinomycetes of Meyghan Lake were isolated and their *16S rDNA* genes were PCR amplified with specific forward and reverse primers. Based on the *16S rDNA* gene sequence, Meyghan pectinase producing actinomycetes shared high levels of sequence similarity with these genus, *Streptomyces* and *Nocardia*. They were more belong to genus *Streptomyces* (80%) and lesser to *Nocardia* (20%).

Isolate GIAL86 shared the high level of sequence similarity (100%) with the species *Streptomyces coelicoflavus* AB(184650). DNA sequence of GIAL86 strain was deposited in GenBank under accession number MH685384. The DNA sequence of other strains also were deposited in GenBank under accession numbers MK918501, MK418651, MK418652, MK418653, MK418654, MK418655, MK418656 and MK418657, respectively. Phylogenetic trees based on the *16S rDNA* sequences constructed with the NJ method (Fig. 5).

Discussions

Iran is among the countries where ecosystems, especially saline lakes, are found to be abundant, so that many arid and semi-arid regions of the country, including the central regions of Iran, have salty soil and water. One of these lakes, which is the origin of halophilic or halotolerant microorganisms with high biodiversity is Meyghan Lake. These microorganisms, and in particular actinomycetes, are highly capable of producing enzymes resistant to extreme environmental conditions such as temperature, pH and high salt concentrations, which are of great value in the field of biotechnology (Amoozegar et al., 2008).

In this study, the bacterial strains were subjected into agar plate and submerged fermentation to identify potent isolate with highest enzyme activity. Therefore, the production of pectinase in 35 actinomycetes of Maygan Lake was evaluated and 48% of them were pectinase producer. In 2009, Rohban et al. (2009) were able to screen 28 pectinase producing bacteria (12%) out of 231 halophilic strains isolated from Hoz_e Soltan Lake (Rohban et al., 2009). In 2013, in an area of India, 10 strains of *Streptomyces* isolated and all of them were a pectinase producer (Arijit et al., 2013). Oumer and Abate (2018) determined 95 isolates from Ethiopia with 35.5% of them having pectinase activity.

At the present study, quantitative assay of *S. coelicoflavus* GIAL86 pectinase was shown that all three enzymes were produced simultaneously with the logarithmic growth of the bacterium and eventually stabilized during stationary phase which this determined that production of pectinase is affected by fermentation time. Similar results were obtained with the other pectinase producing bacteria. Some researchers also reported that pectinase in *Bacillus* sp. is produced concomitantly with its growth curve (Roosdiana et al., 2013; Joshi et al., 2011). This coincidence also found between fungi producing pectinase. Xia et al. (2009) indicated that the trend of the growth curve of *Aspergillus niger* and the pectinase activity curve coincides in the exponential phase, which can be explained that the enzyme amount may change along with the growth of biomass.

Finally, due to the molecular identification of actinomycete producing pectinase, it was found that these bacteria are of the two genera, including *Streptomyces* and *Nocardia*. Different industries

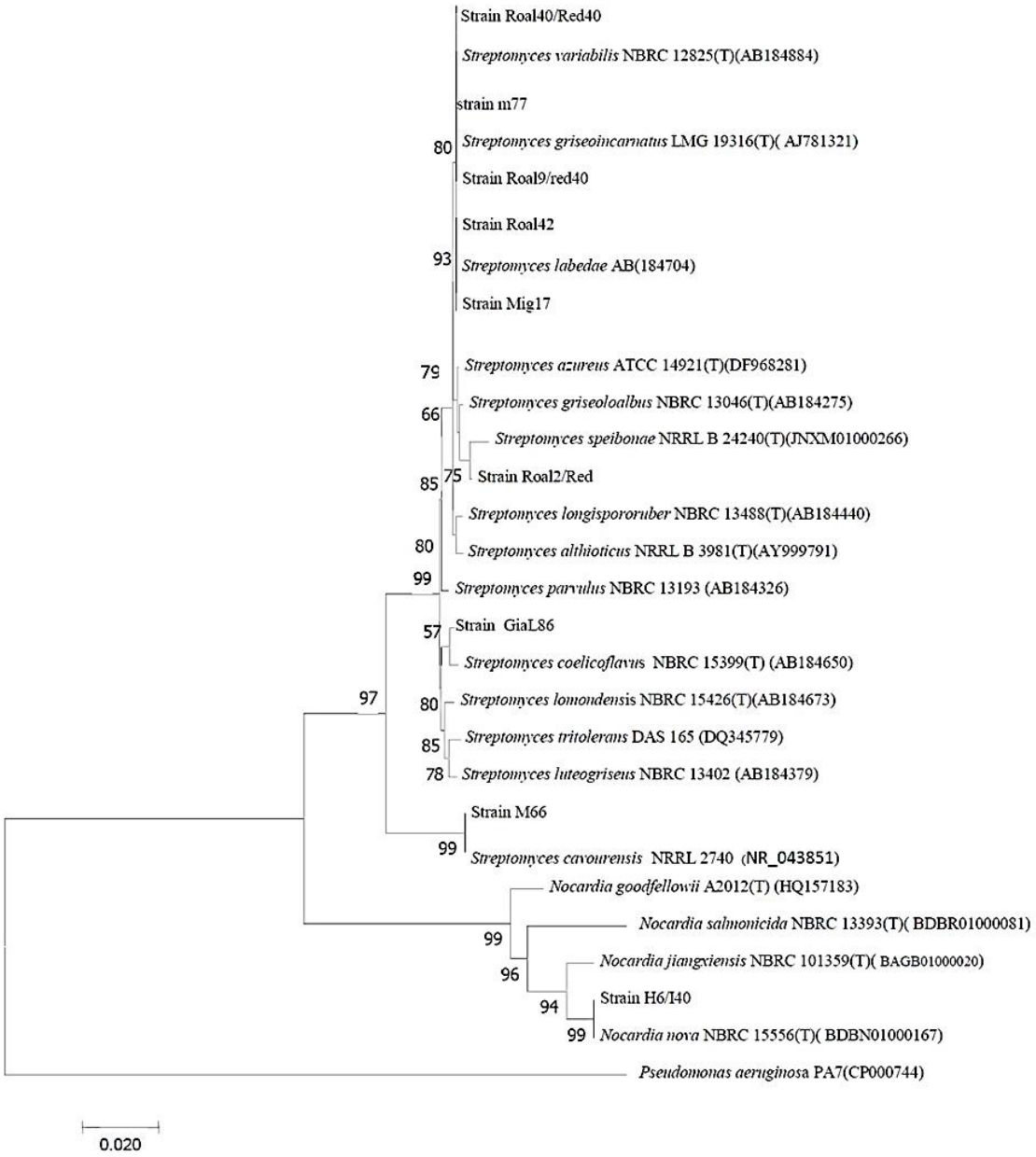

Figure 4. Phylogenetic tree, based on the Neighbor joining algorithm, of the 16S rDNA sequences, Bootstrap values are indicated at branch-points. Pseudomonas aeruginosa PA7(CP000744) was used as the outgroup. Bar = 2% sequence divergence.

require high-temperature and a wide range of pH, since pectinases produced in *streptomyces*, have good resistance to high temperatures and extreme pH, so these enzymes are good for use in the industry (Kumar and Suneetha, 2015). For better enzymatic production, it is necessary to screen novel species of *Streptomyces* from unique habitats.

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