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Molecular Detection of Cellulase -Encoding Gene in Thermophilic *Streptomyces* and Its Cloning in *Escherichia coli* Origami strain

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ABSTRACT

Background: *Streptomyces* is an aerobic filamentous Gram-positive bacterium frequently found in various environments worldwide. Cellulases are a group of glycosyl hydrolase enzymes that are frequently produced by bacteria. Thus, the aim of this study was to detect cellulase-encoding gene (*celA*) in soil-living *Streptomyces* strains and evaluate its cloning in *Escherichia coli* Origami strain.
Materials & Methods: Soil samples were collected from a depth of 5-10 cm in Tehran, Iran. After identification of *Streptomyces* isolates by morphological and biochemical tests, genomic DNA was extracted. Polymerase chain reaction (PCR) test was employed to identify *Streptomyces* strains harboring the cellulase gene. The *celA* gene was positively transmitted to the host bacterium *E. coli* via a vector and cloned through the TA technique. Real-time PCR was used to measure the overexpression of this gene. ClustalX and Mega5 software were used to draw the phylogenetic tree.
Findings: Out of 12 *Streptomyces* isolates, 25% were found to carry the *celA* gene. After cloning the *celA* gene, the cloned strains were chosen by colony selection (blue/white). The real-time PCR test showed the expression of the *celA* gene in the transformed strains. Phylogenetic analysis results using the neighbor-joining assay showed that *Streptomyces* spp. with 81% bootstrap were located in the same clade, indicating their close relatedness.
Conclusion: Soil is one of the high-potential sources of the production of secondary metabolites, which could be used as a valuable source of various biological products such as cellulase.

Keywords: *Streptomyces*, Cellulase, Cloning, TA cloning.

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Introduction

Cellulose $(C_6H_{10}O_5)_n$ as the most abundant carbon-based polymer is a polysaccharide consisting of an unbranched chain of β (1→4)-linked D-glucose units [1]. This organic polymer is an important structural constituent of the primary cell wall of green plants and many forms of algae and fungi [2]. Cellulase is a type of enzyme produced chiefly by various types of microorganisms. This enzyme catalyzes cellulolysis, which is the decomposition of cellulose and some associated polysaccharides [3]. Cellulases are a group of glycosyl hydrolase enzymes that hydrolyze cellulose β-1- and 4-glycosidic bonds; they are divided into three general categories: endoglucanases, exoglucanases, and glucosides [4]. Cellulase is used for commercial food processing in coffee [5]. Also, it is widely used in laundry detergents and in the textile industry [6]. They have also been used in the paper industry, fermentation of biomass into biofuels, and pharmaceutical applications [7]. Medically, this enzyme is used as a treatment for phytobezoars, a type of cellulose bezoar found in the human gastrointestinal tract. It has also been shown to be effective in degrading bacterial biofilms by disrupting β (1-4) glycosidic linkages in the structural matrix of exopolysaccharides of extracellular polymeric substances [8].

Streptomyces is a Gram-positive filamentous bacterium that grows in many niche and has the ability to produce a numerous types of hydrolytic and industrially important extracellular enzymes such as cellulases [9]. These organisms are of great importance due to their unique ability to produce secondary metabolites and biologically active substances including preservatives, enzymes, and antibiotics [10]. *Streptomyces* spp. are of special importance due to the high species diversity (more than 500 species) and the production of 75-80% of all antibiotics [11]. Soli *Streptomyces* strains appear to be a potential source of bioactive compounds as well as a rich source of secondary metabolites [12]. They have a special place in the objectives of research programs due to their ability to produce new metabolites as well as important drug molecules. In 2006, Hoenich demonstrated the use of cellulase in medicine [13]. Cellulase was used as a hemodialysis tube in the treatment of renal failure and wound care.

Objectives: The aim of this study was to detect cellulase-encoding gene in soil-living thermophilic *Streptomyces* strains and evaluate its cloning in *E. coli* Origami for used in wound dressing application.

Materials and methods

Sampling: In a period of eight months from April to November 2019, this cross-sectional study was performed in Tehran, Iran. In total, 60 soil samples were collected from a depths of 5 to 10 cm in pre-sterilized cellophane plastic bags and then sent to the laboratory. All samples were kept in the refrigerator until further used. During sampling, region-specific characteristics such as pH value and altitude were documented.

Bacterial isolation: In general, 5 g of collected soil sample was transferred into a sterile bottle, then 45 mL of distilled water was added and shaken for 35 min. Also, five sets of ten-fold serial dilutions were prepared from original supernatant, then 100 µL of the third concentration was used for inoculation on starch casein agar (SCA) (Merck, Germany). The plates were incubated for 10 days at 28 ˚C. Morphological characteristics of colonies such as colony pigmentation were used for initial identification of the bacterial population. Dry and powdery colonies were isolated from the agar surface.

Molecular confirmation of *Streptomyces* species: Finally, all suspected grown *Streptomyces* colonies were confirmed using 16SrRNA specific primers amplification.

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Thus, template DNA was extracted from the isolates using a DNA extraction kit (Iranian Genetic Center Storage Kit). PCR was performed by 16srRNA primers (F5'-AGAGTTTGATCCTGGCTCAG-3' and R:5'-AAGGAGGTGATCCAGCCGCA-3') in a Eppendorf master thermal cycler (Eppendorf, Germany) with a final volume of 25 µL per tube containing 0.09 µL of templet DNA, 11.5 µL of Taq DNA polymerase 2x Master Mix Red, 1 µL of each primer (10 pmol), and 10.6 µL of ddH_2O. The reaction mixture was attained with the next PCR program as follows: an initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 60 s, annealing at 53 °C for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min. PCR products were assessed using electrophoresis on 1.0% agarose gel in TBE buffer at 100 volts for 120 s, and DNA bands were imagined by DNA safe staining. The amplicon pattern was analyzed by NTSYS software (Version.2.0), and cluster analysis was performed to create dendrogram by unweighted pair group method with arithmetic averages (UPGMA) [12].

Detection of Cellulase-encoded gene: The *celA* gene amplification test was performed by specific primers, including F = 5'-AAGAGGACCTCGATATGATCTGGACACT-3' and R=5'-TCATCCCACATTCTAATGCCTGTAGGTA-3', in a master thermal cycler (Eppendorf, Germany) with 10 µL of PCR Master Mix 2x (Amplicon), 1 µL of each primers with a concentration of 10 pm/µL, 1 µL of template DNA (150 ng), and 12 µL of ddH_2O. PCR conditions were as follows: an initial denaturation at 95 °C for 7 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR amplicons were observed by electrophoresis on 1.5% agarose gel in TBE buffer stained by DNA safe staining.

***CelA* gene cloning in *E. coli* origami:** The *celA* gene PCR products were extracted, purified using a gel extraction kit (Roche), and ligated to the pTZ57R (MBI fermentas) cloning vector. Ligation was performed using T4 DNA ligase (fermentas). The plasmids were transferred into the competent *E. coli* origami™ (DE3) according to the manufacturer's procedures. The mineral salt glutamine (Sigma Aldrich, Germany) comprising 100 µg/µL ampicillin was used to select colonies. White colonies were chosen, overnight cultivated, and subjected to plasmid extraction and PCR. In order to confirm the accuracy of the cloned fragments, the selected recombinant plasmids were sent for Sanger sequencing (MWG service) [14].

Cloned *celA* gene expression by real-time PCR Extraction of mRNA was performed in a late log phase by RNeasy Midi Kit (Qiagen), and complementary DNA (cDNA) was synthesis using a Smart PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The RNA concentration was quantified with a spectrophotometer at 260/280 nm. Reaction was done using a 2× GreenStar Master Mix Kit (Bioneer, Korea) on a Corbett Rotor-Gene 6000 real-time rotary (Corbett Life Science, Australia). The quantitative real-time PCR (25 µL) reaction mixture contained 11.5 µL of PCR Master Mix, 1 µL of cDNA, 0.7 µL of 0.8 µM solutions of both forward and reverse gene-specific primers, and 11.1 µL of double distilled water. The real time-PCR was done based on the following program: an initial denaturation at 95 ˚C for 60 s, followed by 33 cycles of multiplication at 94 ˚C for 30 s, 57 ˚C for 40 s, and 72 ˚C for 60 s. All experiments were run in triplicate. A critical threshold cycle (CT) value was used to present *CelA* transcripts quantitatively. The ΔCT for *celA* transcripts was measured in comparison to the β-actin gene. The *celA* relative expression was assessed by the $2^{-\Delta\Delta CT}$ method [15].

Findings

Based on the morphological features and

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microscopic examination, such as whiteness, dryness, and gypsum-like appearance of colonies, odor due to physiological activities, and slide culture, 20% (n= 12 of 60) of the isolates were identifies as *Streptomyces* among the strains isolated from the soil samples (Figures 1A and 1B). All isolates were confirmed by 16s rRNA amplification test.

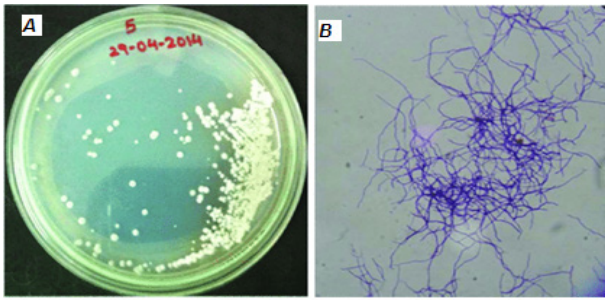

Figure 1) Morphological and microscopic characteristics of *Streptomyces* strains isolated in this study. 1A and 1B: white colonies and filamentous Gram-positive bacteria

The results of PCR reaction for the *celA* gene showed that out of 12 *Streptomyces* strains, 25% (n= 3 of 12) were positive for the presence of this gene. All *celA*-positive strains were sequenced by Sanger method (Figures 2A and 2B).

After TA cloning of the cellulase –encoding gene, the cloned strains were picked by colony selection (blue/white colonies). In order to confirm the results of cloning, transformed DNAs were extracted, and PCR sequencing was performed. The presence of a 234 bp fragment indicated a successful cloning. Lastly, the *celA* gene cloning in *E. coli* isolates was confirmed using PCR sequencing method (Figures 3A, 3B, and 3C). The quantitative real-time PCR test showed successful expression of the *celA* gene in cloned strains with Tm= 86.94 (Figure 4a). The results of phylogenetic relatedness using the neighbor joining (NJ) method showed that *Streptomyces* BPSEAC7 species with 81% bootstrap and *Streptomyces* MI02-7b were located in the same cluster, indicating their close relationship with each other (Figure 4b).

Discussion

Enzymes are proteinaceous substances that act as catalysts and accelerate biological reactions [16]. Cellulases are important textile, industrial, and medical enzymes that have attracted much attention in recent decades due to their diverse applications [6]. This enzyme is not actually a single enzyme, but a complex multi-enzyme system produced and secreted by specific microorganisms such as bacteria, yeasts, and fungi [8]. Of all the enzyme-producing strains, including, *Bacillus*, *Micrococcus*, *Pseudomonas*, and *Actinomycetes*, only *Streptomyces* strains (a genus from *Actinomycetes*) are commercially available [17].

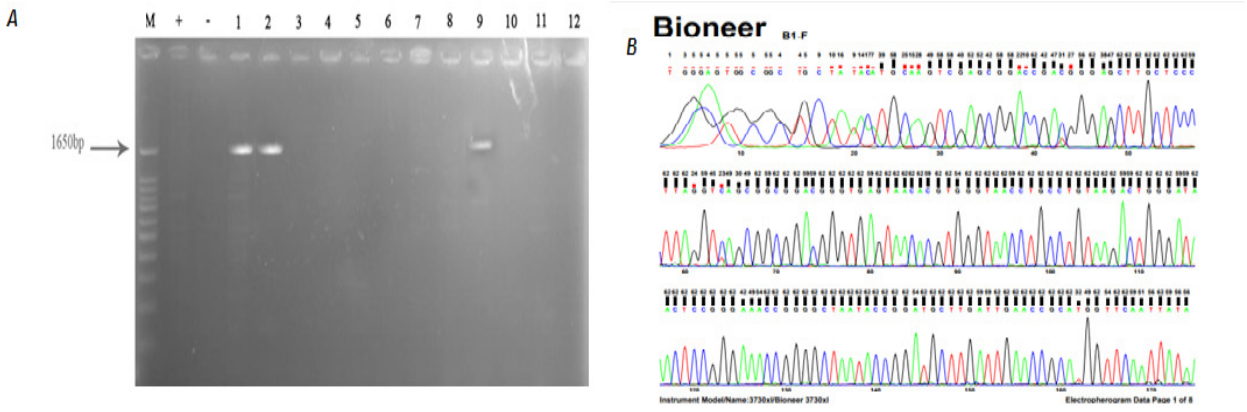

Figure 2) PCR results of *celA* genes: 2A: C+: positive control, C -: negative control (distilled water), and Wells 1 to 12: *Streptomyces* strains, 2b: the result of sequencing and BLAST of the amplified gene

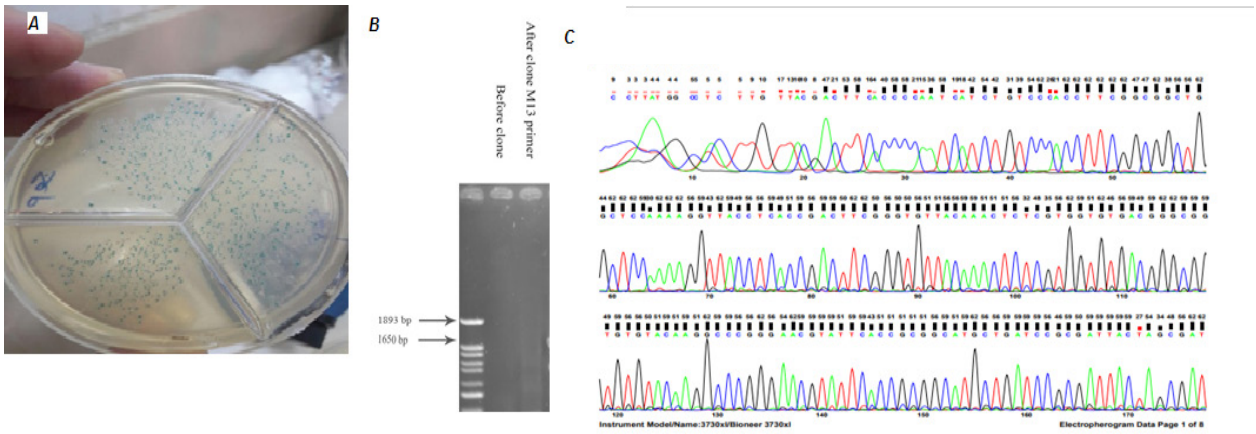

Figure 3) Results of A: clonal selection of *celA* gene (white and blue colonies), B: confirmation of cloning by PCR with M13 vector primers and its sequence, C: BLAST of PCR result

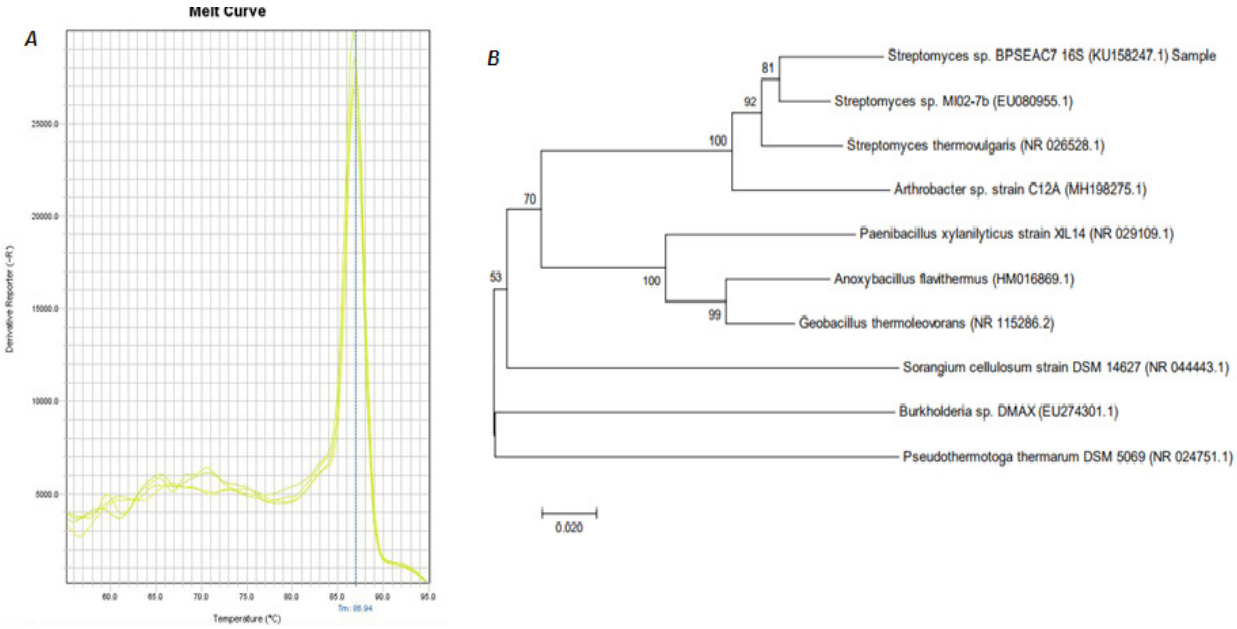

Figure 4) A: Real time PCR melting curve results, B: phylogenetic tree

Some previous studies have shown that the *celA* gene is found in soil *Streptomyces*; therefore, soil *Streptomyces* strains are a potential source for the production of this enzyme. In agreement with Behroozpour et al. (2019) [14] and Maleki et al. (2013) [18], *Streptomyces* strains in this study were isolated from the soil samples. In concordance with the present study, Maleki et al. (2013) [18] showed that soil of northwestern Iran could be used as a rich source to study novel *Streptomyces* strains with high potency of enzyme production. This could be due to the soil moisture content and pH. In the current study, of 12 *Streptomyces* isolates collected, three isolates carried the *celA* gene. In line with this study, Deepthi et al. (2012) [19] isolated the cellulase enzyme from soil *Streptomyces* isolates. Thakkar et al. (2014) [20] and Amore et al. (2012) [21] detected cellulase-encoding gene in soil-living *Streptomyces* isolates. The results of phylogenetic relatedness using the NJ method showed

that *Streptomyces* BPSEAC7 species with 81% bootstrap and *Streptomyces* MI02-7b were located in the same cluster, indicating their close relationship with each other. In agreement with the present study, Amore et al. (2012) [21] showed that the highest cellulolytic activity was identified by 16S rRNA sequencing and belonged to the genus *Streptomyces* and designated as *Streptomyces* sp. strain G12. Contrary to the present study data, in a study directed by Thakkar et al. (2014) [20], the purified PCR amplicon was sequenced, and the phylogenic relationship was determined by comparing the sequence data with the existing sequences available in the NCBI gene bank sequence database (Bethesda, MD, USA) and recognized as *B. amyloliquefaciens* denoted as MBAA3. Then the sequence was deposited in the gene bank under the accession number KF535140. This inconsistency could be due to geographical and ecological differences and the materials used in these study. Limitations of this work include soil type and cloning errors.

In a parallel study, Burlacu et al. (2016) showed that the best Xylanases activities were found in *B. amyloliquefaciens*, *Aspergillus brasiliensis*, *Penicillium digitatum*. and *A. niger* [22]. Vadala et al. (2021) showed that extracellular cellulase production by *B. subtilis* was confirmed firstly on the CMC agar, and then cellulase-encoding gene was detected by PCR test. This gene was then cloned into the pET21a expression vector. As a result, the researchers stated that soluble expression of active recombinant cellulase could be completed by subtle modification in the upstream procedure [23]. Wang et al. (2020) showed that the host strain *E. coli* BL21 (DE3) could express streptomycin-derived extracellular recombinant phospholipase D with high efficiency [24]. The differences observed between the results of the above and present studies could be attributed to the type of sample, the source of cellulase separation (thermophilic *Streptomyces* vs, other resources), fermentation conditions, difference in the type of expression vector, and difference in the cloning methodology. Cloning conditions also affect the product expression in the expression vector.

Sioud et al. (2009) showed that the *xylA* gene (encoding for glucose isomerase) from *Streptomyces* sp. SK was successfully expressed in *Streptomyces* sp. TN 58. Data showed that heterologous glucose isomerase could be expressed and folded effectively. Glucose isomerase activity of the constructed TN 58 recombinant isolate was about 18-fold higher than that of *Streptomyces* sp. SK [25]. These findings are certainly important due to the potential use of better strains in biotechnological methods to produce high-fructose syrup from starch.

Conclusion

It could be concluded that soil-living *Streptomyces* strains are one of the high-potential sources for the production of secondary metabolites, which could be used to produce various biological products such as cellulase. Since cellulase has different applications, the search for organisms producing this enzyme is one of the main ways to achieve enzymes with ideal properties.

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Ethical Permission: As the work is an industrial research we haven't received ethical permission yet.

Conflicts of interest: All authors declare that there are no conflicts of interest.

Authors Contribution: Conceptualization: SF, SS; Data curation: SF, SS; Formal analysis: SF, SS; Funding acquisition: SF, SS, KA; investigation: SF, SS; Methodology: SF, SS; Project administration: SF;Resources: SF, SS; Software: SF, SS; Supervision: SF; Writing of

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