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Original Article

Production of Cellulase by Soil Isolated *Streptomyces* sp.

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ABSTRACT

Cellulases have been considered effective biocatalysts because of their diversity of applications. A total of 165 cellulase-producing *Streptomyces* isolates were recovered from Egyptian soil samples via cultivation on carboxymethyl cellulose agar medium. The results of screening by the Congo red method showed that the tested isolates revealed different levels of cellulase activity. The isolates showed a high level of cellulase production (clear zones > 25 mm, n= 15) were submitted for quantitative evaluation of their cellulase productivities by using dinitrosalicylic acid assay. Cellulase production of these selected isolates ranged from 146.9 to 650.5 U/L. The highest cellulase-producing isolate (S11-6) was identified at the molecular level using 16S rRNA gene sequencing. The sequence homology analysis proved its proximity and relatedness to *Streptomyces coelicoflavus* strain NBRC 15399. Production improvement of cellulase was carried out by genetic manipulation using a dose of 4 Kilogray (KGy) of gamma radiation. M1 mutant showed 1.31 fold increments in cellulase production as compared to the wild-type strain of *S. coelicoflavus.*

Keywords: *cellulose; Streptomyces coelicoflavus; dinitrosalicylic essay; genetic manipulation; gamma radiation*.

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1. INTRODUCTION

Cellulase is one of the most important industrial enzymes. They are used in the textile industry for bio-polishing and stone washing **[1]**; in beverage for enhancing extraction and filtration **[2]**; in the food industry for the improvement of juice and oil production **[3]**; in the pulp and paper industries for better drainage and fiber improvement **[4]**, and they are even used for pharmaceutical applications **[5]**.

The increasing concerns about fossil fuel depletion, greenhouse gas emissions, and air pollution caused by incomplete fossil fuel burning resulted in a growing emphasis on the synthesis of bioethanol from lingo-cellulosic biomasses **[6]**. Cellulases have sparked renewed attention in recent years, owing to their role in converting cellulosic biomass into industrially viable products or fuels. Agricultural by-products, industrial waste, municipal rubbish, and other sources account for the majority of cellulosic biomass **[7]**. The elimination of these wastes is crucial for a variety of industries. Sugars, bioethanol, bio-methane, bio-hydrogen, and other chemicals could be manufactured from

them **[8]**. Several microbes, most often bacteria and fungus, synthesize this enzyme **[9]**. *Streptomyces* sp. is one of the best-known enzyme producers **[10]**.

The aim of the present study involved the isolation of high-cellulase-producing streptomycetal isolate(s) from Egyptian soil samples and improving its cellulase production through genetic manipulation.

2. MATERIALS and METHODS

2.1. Isolation of Cellulase Producing Bacteria

A total of 43 soil samples were collected from different Egyptian governorates. The samples were stored at 4 °C in sterile containers until use **[11]**. Ten-fold serial dilutions were prepared from each sample. Then 0.1 mL from each dilution was plated on carboxymethyl cellulose (CMC) agar plate (1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 0.3 g NH_4NO_3, 10.0 g CMC, 30.0 g agar per liter, pH 7) and incubated at 28 and 37 °C for 96 h **[12]**. Morphologically different colonies were isolated, purified, and stored on CMC agar plates at 4 °C.

2.2. Ability of the Collected *Streptomyces* Isolates for Cellulase Production by Congo Red Method

The selected isolates were cultured on CMC agar plates that were incubated at 28 °C for 96 h. After incubation, the plates were flooded with 0.1% Congo red for 20 min then washed with 1M NaCl for 30 min and the clearance zones (indicated CMC hydrolysis) formed around each colony were measured **[13]**. According to the size of formed clear zones, the isolates were divided into five groups; very strong (> 25 mm), strong (15-25 mm), moderate (10-15 mm), weak (5-10 mm), and very weak (0-5 mm) **[14]**.

2.3. Conditions of Bacterial Growth and Enzyme Production

Five-day-old cultures of the very strong producers were used to prepare a spore suspension in 10 mL sterile distilled water. Afterward, 0.5 ml of this suspension was used to inoculate 50 mL CMC liquid medium (1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 0.3 g NH_4NO_3, 10.0 g CMC per liter, pH 7) in 250 mL Erlenmeyer flasks and incubated in a shaking incubator (C25, New Brunswick Scientific, USA) at 200 rpm and 28 °C for 96 h. At the end of the incubation period, a known volume of the broth was centrifuged at 6000g for 20 min. The cell pellets were washed using sterile saline and dried in a hot oven at 60 °C to a constant weight. The supernatant was used for the determination of cellulase activity as described below **[5, 12]**.

2.4. Enzyme Assay

The crude enzyme (0.4 mL) was added to 1.6 ml of 0.5% CMC in 50 mM sodium phosphate buffer (pH 7). The reaction mixture was incubated for 30 min at 50 °C in a shaking water bath (GFL, Germany). Then the reaction was ended by adding 3 mL 3, 5-dinitrosalicylic acid (DNS) reagent. Then the mixture was boiled for 5 min to develop the color. To stabilize the color, 1 ml of 40% potassium sodium tartrate and then cooled. The optical densities (OD) were measured at 540 nm against a blank that contained the entire reagent without the crude enzyme. Results were determined in terms of cellulase activity in which the amount of cellulase that liberates 1 µmol glucose per minute under the above assay conditions was defined as one unit (U) of cellulase activity **[15, 16]**.

2.5. Molecular Identification of the Selected Isolate by 16S rRNA Analysis

The isolate with the highest cellulase activity was selected to be identified by 16S rRNA gene sequence analysis. DNA extraction, PCR amplification of the 16S rRNA gene, and

sequencing took place at Sigma Scientific Co. (Cairo, Egypt). The two primers used for PCR amplification of the 16S rRNA gene are StrepF: 5-ACGTGTGCAGCCCAAGACA-3 and StrepR: 5-ACAAGCCCTGGAAACGGGGT-3. To determine the degree of DNA similarity and sequence alignment, the BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed. The TREE VIEW program was used to align the sequences and create a neighbor-joining phylogenetic tree **[17]**.

2.6. Improvement of Cellulase Production by Gamma Irradiation

This was performed by exposure of the selected *Streptomyces* isolate to Gamma radiation as described by Khaliq *et al* **[18]** with a few modifications; a five-day-old culture of the chosen isolate was used to prepare a bacterial suspension of 1 X 10^8 CFU/mL. To find the best dose for killing 99.99 percent of the bacteria, the resulting suspension was irradiated with various doses of gamma radiation (1, 2, 3, 4, and 5 Kilo Gray (KGy)). At the time of the experiment, the source of gamma radiation was ^{60}Co from an Indian Gamma cell that was generating a dose rate of 1.43 KGy/h. The process was carried out at the National Center for Radiation Research and Technology, Atomic Energy Authority, Nasr City, Cairo, Egypt. The irradiated cell suspension was then diluted appropriately, plated on CMC plates, and cultured for 7 days at 28 °C. Colonies were randomly chosen, purified, and kept on CMC slants.

Using the Congo red staining method, the cellulase productivity of the chosen colonies was compared to that of the wild-type strain. After that, the enzyme productivity of the resultant colonies with larger clearance zones was quantitatively evaluated using DNS assay.

3. RESULTS

3.1. Isolation of Cellulolytic Bacteria from Different Soil and Compost Samples

A total of 165 cellulolytic bacterial isolates (they cultured on CMC agar medium which is selective for cellulase producers) were recovered from 43 soil samples from different Egyptian governorates at 28 °C. They demonstrated the typical growth of *Streptomyces* species (colorful chalky appearance with hard textures).

3.2. Ability of the Collected *Streptomyces* Isolates for Cellulase Production

The Congo red technique was used to assess the recovered *Streptomyces* isolates for their capacities to generate cellulase **(Fig. 1)**. *Streptomyces* isolates produced cellulase at different levels and they were classified as described by Jaradat *et al* **[14]**. Among the 165 isolates; 6 (4%), 6 (4%), 39 (23%), 99 (60%), and 15 (9%) isolates were classified as very weak, weak, moderate, strong, and very strong producers, respectively **(Fig. 2)**.

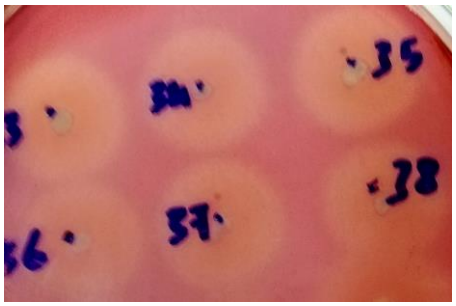

Fig. 1. Different CMC agar plates showing clear zones results from cellulase production by different *Streptomyces* isolates using Congo red technique.

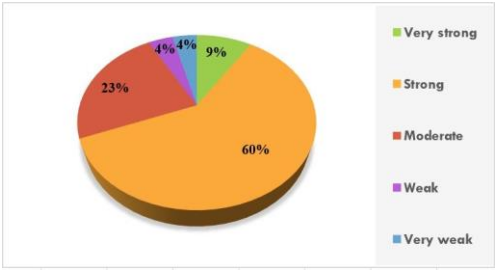

Fig. 2. Levels of cellulase production of the selected *Streptomyces* isolates (n=167) by using Congo Red Method; 9% evaluated as very strong , 60% as strong, 23% as moderate, 4% as weak and 4% as very weak producers.

3.3. Quantitative Determination of Cellulase Production by the Selected *Streptomyces* Isolates

The DNS assay was used for quantitative evaluation of the enzymatic activity of strong-cellulase-producing *Streptomyces* isolates (n= 15). According to the obtained results, the cellulase productivity by the tested isolates was ranged from 146.9 to 650.5 U/L **(Table 1)**. The isolate coded S11-6 showed the maximum cellulase productivity.

Table 1. Quantitative determination of cellulase activity and specific productivity of the selected *Streptomyces* producers of highest productivity using DNS assay

Isolate	Cellulase activity (U*/L)	Dry cell weight (g/L)	Specific productivity (U/g)
S11-6	650.48 ± 3.41	1.97	330.19
S33-1	626.58 ± 42.74	2.93	213.85
S07-1	590.72 ± 18.45	2.6	227.20
S24-1	577.48 ± 54.47	0.97	595.34
S08-3	496.4 ± 26.23	1.47	337.69
S13-3	492.52 ± 54.67	1.43	344.42
S18-1	478.95 ± 81.81	2.3	208.24
S41-1	461.83 ± 25.59	0.93	496.60
S26	445.04 ± 41.3	2.03	219.23
S02-3	444.07 ± 25.94	0.97	457.80
S25	423.39 ± 21.25	2.33	181.71
S15-1	368.15 ± 27.34	2.4	153.40
S08-1	261.88 ± 77.87	3.97	65.58
S03-1	208.58 ± 58.74	2.77	75.30
S30-7	146.88 ± 36.83	3.1	47.38

Table 2. Evaluation of the cellulase productivity of the obtained mutants compared to the wild type strain using Congo Red method

Isolate	No. of obtained mutants	No of mutants (Percentage)		
		Lower	Equal	Higher
S. coelicoflavus	45	32 (71%)	8 (18%)	5 (11%)

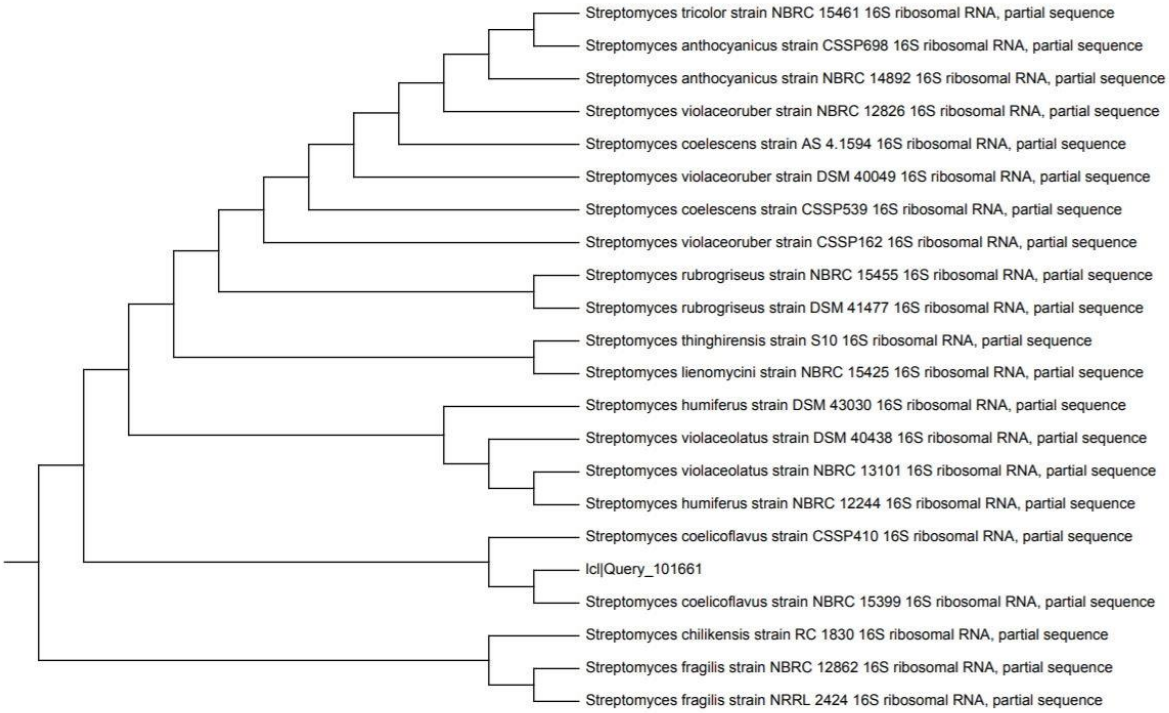

Fig. 3. Neighbor-joining tree showing the phylogenetic position of Streptomyces 11-6 and related species based on partial 16S rRNA gene sequence

3.4. Identification of the Selected *Streptomyces* Isolate Coded S11-6

The selected isolate (S11-6) was identified by 16S rRNA gene sequencing and deposited in the NCBI GenBank database under the accession number NR041175.1. It was identified as *Streptomyces coelicoflavus* strain NBRC 15399 with 99.9% homology of the 16S rRNA nucleotide sequence. The phylogenetic tree **(Fig. 3)** revealed that *S. coelicoflavus* is the closest isolate in similarity to the above-mentioned strain.

3.5. Improvement of The Enzyme Production of *S. coelicoflavus* by Gamma Irradiation

This was accomplished by exposing *S. coelicoflavus* to various gamma radiation dosages. According to the obtained results, the optimum dose of gamma radiation for the mutation that resulted in a 99.99 percent kill was 4 KGy. The grown colonies were selected for cellulase production testing.

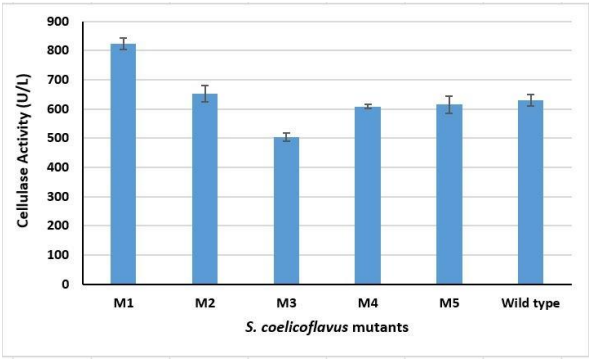

Fig. 4. Evaluation of cellulase activity of the selected mutants (M1-M5) using DNS assay as compared to that of *S. coelicoflavus* wild type.

The cellulase productivity of the obtained mutants was tested by Congo red method. The results revealed that the tested mutants had

different degrees of cellulase production **(Table 2)**. As compared to the wild-type strain, mutants with higher enzymatic productivity (n= 5) were chosen for the quantitative determination of their cellulase productivity by using DNS Assay. According to the obtained results **(Fig. 4)**, only the M1 mutant showed an increase in the cellulase productivity (823.1 U/L), while M2, M4, and M5 approximately had the same cellulase production level as compared to the wild type strain. On the other hand, M3 showed a significant decrease in cellulase production as compared to the wild-type strain.

4. DISCUSSION

Cellulase is one of the most significant industrial carbohydrates due to its various applications in several industrial sections. In this context, the selection of microorganisms and process techniques are of essential importance for a thriving industrial production of cellulases **[19]**. The species *Streptomyces* have been implicated over the years as a producer of numerous metabolites and biomolecules, including hydrolytic enzymes **[20]**. Different strains of *Streptomyces* sp. have been examined and proven to be good producers of cellulase **[12, 16, 21]**. *Streptomyces* sp. can be isolated from soil, compost, and water, among other sources. *Streptomyces* is the most prevalent soil microbe as it approximately accounts for 95% of the soil microbial isolates **[22]**.

In this study, 43 soil samples were collected from different Egyptian governorates. A total of 165 isolates were recovered and demonstrated the typical growth of *Streptomyces* sp. (colorful chalky appearance with hard textures). All of them could produce cellulase as they grow on CMC agar medium, a selective media for cellulase-producing microorganisms. The productivity of enzyme-producing microbial strains is closely linked to enzyme production costs. As a result, finding microorganisms with high enzyme productivities was critical **[23]**.

Congo red method was conducted to confirm the ability of the recovered isolates to produce cellulase. Congo red interacts strongly with polysaccharides containing contiguous β-(1-4)-bound-glucopyranosyl units as CMC. When cellulase-producing isolates grow on CMC agar plates, they hydrolyze CMC around their growth into smaller fragments than cellohexaose to which Congo red doesn't bind so clear zones formed **[24]**. Although overall strain growth rates on CMC agar medium are similar, a large variation in cellulase activity has been determined in the tested *Streptomyces* isolates, ranging from very strong cellulase producers to very weak producers. The very strong producers (n= 15) were subjected to a more quantitative test for characterizing their cellulase productivities using DNS assay. The use of DNS assay to detect cellulase activity is very common. The enzymatic hydrolysis of CMC, which liberates reducing sugars, is the basis for this approach. In an alkaline medium, 3, 5-dinitrosalicylic acid (yellow color) oxidizes the aldehyde functional group of these reducing sugars, which itself is reduced to 3-amino-5-nitrosalicylic acid (orange-red). The absorbance of the orange-red color of 3-amino-5-nitrosalicylic acid is measured colorimetrically at 540 nm **[25]**.

The S11-6 isolate that showed the maximum cellulase production was selected for further studies. It was identified by using 16S rRNA gene sequencing. The most prevalent method for detecting bacteria or building bacterial phylogenetic relationships is 16S rRNA gene sequence analysis **[26]**. This could be due to a variety of factors; first, the existence of this gene in practically all bacteria, frequently as a multigene family, or operons. Second, the 16S rRNA gene's function has remained constant over time, implying that random sequence changes are a more accurate measure of evolution. Finally,

the 16S rRNA gene (1,500 bp) is large enough to be used in informatics **[27]**. The selected isolate (S11-6) with the highest cellulase productivity was identified *Streptomyces coelicoflavus* with close genetic proximity to strain NBRC 1539.

Regarding strain improvement, random mutagenesis and fermentation screening have been reported as promising ways to boost the metabolic activity of microorganisms **[28]**. As a result, inducing mutations in the chosen isolate, *S. coelicoflavus*, may be beneficial in obtaining mutants with increased cellulase productivity. There are various mutagens either chemical, such as N-methyl-N-nitro-N-nitroso guanidine, or physical, such as UV or Gamma irradiation. The limitation of chemical mutagens' use is due to its ability to induce G/C-to-A/T transitions mutations resulting in amino acid substitutions that affect protein structure and function as well as truncation changes that prevent protein activity. Moreover, UV light appears to be a weak mutagen **[18]**. Accordingly, gamma irradiation was selected for mutation induction.

The use of gamma radiation mutation induction was documented previously by several investigators. Treatment of *Aspergillus niger* by using gamma irradiation causes an increase in lipase and glucose oxidase productivity by 3.11 and 3.66 fold as compared to the wild-type strain **[28, 29]**. Furthermore, Lv *et al* **[30]** documented 7.6 fold increase in adriamycin production by *Streptomyces viridochromogenes* using gamma radiation. Moreover, 1.44 fold increase in paromomycin production by Streptomyces rimosus was reported by Ibrahim *et al* **[31]**.

In the present study, the chosen isolate *S. coelicoflavus* was treated by different doses of Gamma radiation. The dose of 4 KGy was successful to kill 99.99 percent of microbial cells, after which the grown mutants were selected and their cellulase activity was evaluated. M1 mutant could increase the cellulase activity by 1.31 fold when compared to the wild-type strain.

Conclusion

The soil-isolated *S. coelicoflavus* can be considered a promising candidate for cellulase production and scaling-up. Improvement of cellulase production was achieved by mutagenesis using gamma radiation. A mutant coded M1 showed about 1.31 fold increase in cellulase production. The developed *S. coelicoflavus*-M1 mutant could be exploited as a possible industrial strain for cellulase production in future studies.

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Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data analyzed during this study are all included in the main manuscript.

Competing interests

No competing interests were declared by the authors.

Funding statement

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