



Molecular Characterization and Cellulolytic Capacity of *Chaetomium globosum* Isolates

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Authors' contributions

This work was carried out in collaboration among all authors. Author EGED designed the study, carried out practical experiments, wrote the protocol and wrote the first and final draft of the manuscript. Author YMS performed the statistical analysis and some practical experiments and managed the literature searches. Author YAG wrote the first and final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Chaetomium species are saprophytic fungi, have the ability to degrade cellulose with hydrolytic enzymes. Hydrolytic enzymes are a cluster of enzymes, which have the flexibility of breaking down complicated molecules into smaller molecules. The objective of this study was to clarify the genetic diversity among the isolated *Chaetomium* population by using PCR-based methods, along with the study of their cellulolytic activities. *Chaetomium* spp. were isolated from twenty-eight samples of *Cuminum cyminum* and *Pimpinella anisum*, and identified morphologically and molecularly by ITS1 and ITS4 primers. The sequencing indicated that all the isolates of *Chaetomium* had a 99% sequence identity with *Chaetomium globosum* sequences from GenBank. Molecular techniques with Internal Transcribed Spacer (ITS) region sequencing and specific genes random primers polymerase chain reaction (SGRP-PCR) showed the existence of high DNA polymorphism of *Chaetomium globosum*. All isolates were tested for their ability of cellulases production. Nine of the

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thirteen isolates of *Chaetomium globosum* could produce cellulases. There are variations between *C. globosum* isolates and the source of the isolates and their habitat do not correlate to their ability to secrete cellulases.

Keywords: Saprophyticfungi; *Chaetomium globosum*; cellulases; ITS region; SGRP-PCR.

1. INTRODUCTION

Chaetomium is a genus of filamentous and dematiaceous fungus. It is found in air, soil and debris of plants [1]. Furthermore, researchers have indicated that these are cosmopolitan and prevalent components of various ecosystems in a wide range of environmental and climatic zones [1]. *Chaetomium* species exhibited significant infections to human, and the number of infections is increasing [1]. The infections include onychomycosis [2], keratitis [3] and subcutaneous [4]. Some of these species are neurotropic and thermophilic [5]. The genus contains various species with the most common ones being *Chaetomium strumarium*, *Chaetomium funicola*, *Chaetomium atrobrunneum* and *Chaetomium globosum* [6].

Chaetomium species belong to the ascomycetes (Sordariales). *Chaetomium* species are characterized by their superficial ascomata (mostly ostiolate perithecia) surrounded by hairs of various types [6]. *Chaetomium* is distributed worldwide and exist on various substrates as saprotrophs. Several types of *Chaetomium* species are coprophilous, cellulolytic, toxigenic and even pathogenic for humans [6].

Deoxyribonucleic acid (DNA) barcoding of *Chaetomium* could be a fast and precise strategy for recognition utilizing short DNA groupings [7]. The Internal Transcribed Spacer (ITS) of nuclear "Ribosomal Deoxyribonucleic Acid (rDNA)" is one of the most thoroughly sequenced molecular markers [8]. The sequence comparison of the ITS region is used for scientific categorization and molecular phylogeny since its simple structure make it amplifiable even from very little amounts of DNA, due to the high copy number of "Ribosomal ribonucleic acid (rRNA)" genes. The ITS region encompasses a high level of variation even between closely related species [9].

Hydrolytic enzymes are a group of enzymes, with the flexibility of breaking down complicated molecules into smaller molecules, such as cellulases that are capable of breaking down cellulose into aldohexoses. Cellulase is a family of enzymes hydrolysing β -1,4- glycosidic bonds of

intact cellulose and other related cellooligosaccharide derivatives. Cooperation action of three principal types of the enzymes, viz., endoglucanase, exoglucanase, and β -glucosidase. Endoglucanases responsible for hydrolyzing the amorphous (internal) regions of the fibrils randomly by degrading β -glucosidic bonds; cellobiohydrolases are exoglucanases releasing cellobiose, from the ending of the chains, while β -glucosidases complete the hydrolyzing cellobiose and other cellobiohydrolases with a low degree of polymerization to glucose units [10]. Fungi and several species of bacteria have the capability to secrete cellulases, and it has been indicated that the ability of fungi is significantly higher to secrete cellulases[10] as compared to various species of microorganisms. The most well known cellulolytic fungal species are *Trichoderma* sp., *Humicola* sp., *Penicillium* sp., *Chaetomium* sp. and *Aspergillus* sp. [11].

The aim of this work was to identify *Chaetomium* species, which were isolated from contaminated two types of spices. The genetic diversity of the isolated *Chaetomium* species was compared using PCR-based methods, and matched with their cellulolytic activities.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out at Botany & Microbiology Department, Mycology lab, Egypt, from January, 2017 to April, 2018.

2.2 Isolation of *Chaetomium* Strains

Thirteen different isolates of *Chaetomium globosum* were recovered from twenty-eight seed samples of two spices (14 samples were isolated from *Cuminum cyminum* and the other 14 samples from *Pimpinella anisum*). The spice seed samples were gathered from different markets in Qena city of Egypt, by using dilution-plate method on modified Czapek's Dox agar medium (by adding 20 g/L of cellulose instead of glucose). Rose Bengal (0.1 mg/ml) and chloramphenicol (0.5 mg/L) were used as bacteriostatic agents [12] (Table 1).

2.3 Morphological Identification of *Chaetomium* Strains

The *Chaetomium* isolates were purified by culturing single spores on modified Czapek's Dox agar medium. A preliminary characterization was conducted using macro- and microscopic observations and measurements, including colony colour, the diameter of asci, ascospores, perithecium, perithecial hairs and their morphology. In this respect, the *Chaetomium* isolates were identified according to [13].

2.4 DNA Extraction

Chaetomium isolates were cultured in 250 ml flasks containing 50 ml Potato -Dextrose Broth (PDB) for 2 days by using a rotary shaker for 25°C at a speed of 120-150 rpm. The mycelium was separated by filtration and grounded to a fine powder in liquid nitrogen. Fifty mg of the powder was transferred to a 1.5 ml Eppendorf tube and mixed with 0.7 ml 2x CTAB buffer (cetyltrimethyl ammonium bromide) and vortexed for 2 minutes. Eppendorf tubes were incubated at 65° C for 60 to 80 minutes, and 0.7 ml of chloroform was added and mixed briefly. The samples were centrifuged at 15.000 rpm for 10 minutes, and the supernatant was transferred into a new tube and mixed with 0.6 ml isopropanol, chilled to 20° C and centrifuged at 15.000 rpm for 5 minutes. The pellet was washed twice by 1 ml of 70% ethanol, followed by centrifugation steps for 3 minutes at maximum speed. After drying under vacuum the sample was dissolved in 0.1 ml TE (10 mMTris, 1 mM EDTA, and pH 7.5) buffer [14]. The DNA quantity and quality were assayed by electrophoresis on a 1.4 % agarose gel stained with ethidium bromide and visualized by UV trans-illumination.

2.5 Polymerase Chain Reaction Amplification for ITS Region and Sequencing

Polymerase chain reaction (PCR) amplification was done on a thermal cycler (Molecular Mycology, Laboratory, South Valley University, Egypt), with a reaction volume of 25 µL. The reaction mix included the DNA sample (about 100 ng), dNTPs Mix (200 µmol/L), ITS1(CTTGGTCATTTAGAGGAAGTAA) and ITS4(TCCTCCGCTTATTGATATGC) primers (0.1 µmol/L each) [15]. Taq DNA polymerase (2.5 U), and 1X buffer. The amplification reaction was: two minutes pre-denaturation at 94° C, one

minute denaturation under the same condition, one minute annealing at 55° C, and two minutes extension at 72° C. The process was recycled for 30 times, and a final extension of 10 minutes at 72° C. The amplified products of full length ITS regions were separated by electrophoresis in 1.2% agarose gel in Tris EDTA-acetate (TAE) buffer (1X), stained with ethidium bromide at 0.5 µg/ml, run at 80 V for 3 hours. The DNA was visualized under UV light and photographed with gel documentation unit (Syngene Inc., Cambridge, UK). The sequencing was performed by the Macrogen Company (South Korea).

2.6 Sequence Analysis

Maximum parsimony phylogenetic analyses were conducted by using PAUP*4.0b10 (Swofford, 2000) on the ITS data set. Bayesian analyses [16] were performed by MrBayes 3.1 [17]. The best fit models were determined by using the AIC criterion as implemented in Mr Model test version 2.3 [18].

2.7 Genetic Diversity of *C. globosum* Isolates

A single pair of specific primers (SGRP-PCR), identified as good random primers [19] were used in this study. SGRP-PCR was used to study genetic diversity among collected isolates of *C. globosum*. Primers Opa1 (CGG TGC TTC ATC ACC TTA G), Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Tef1 (GCY CCY GGH CAY CGT GAY TTY AT). Bt2a, Opa1 and Tef1 were used as arbitrary primers. Amplifications were carried out as described by [20], PCR reactions were done in 25 µL reaction mixture by the following: 12.5 µL PCR Master Mix (Jena Bioscience), 0.5 µL MgCl₂, 0.04 µL Taq polymerase, 3 µL of each primer, 1 µL template DNA, and 8 µL PCR water. PCR conditions were: 1 step for denaturation at 94 °C for 2.30 min followed by 40 cycles of : 94 °C for 45 s; 36°C for 40 s; 72°C for 2 min; and an additional cycle at 72°C for 10 min. The PCR products were resolved by electrophoresis on 1.4% agarose gel in 0.5 X Tris-borate-EDTA buffer, at 125 V for 2 h. Gels were stained by ethidium bromide and photographed under UV light using UVP Bioluminescence Imaging System (UVP). Dendrograms based on the banding profiles were obtained by the unweighted-pair group method with arithmetic average based on CAP software.

Table 1. The list of *Chaetomium globosum* isolates and their CMase activity studied in this investigation

isolates numbers	Code in South Valley University Culturecollection	Source isolates	CMase activity [cm]	Gen Bank accession numbers
1	CgSVU1	<i>Cuminum cyminum</i>	9	MG885806
2	CgSVU2	<i>Cuminum cyminum</i>	-ve	MG885807
3	CgSVU3	<i>Pimpinella anisum</i>	4.5	MG885808
4	CgSVU4	<i>Pimpinella anisum</i>	4.45	MG885809
5	CgSVU5	<i>Pimpinella anisum</i>	1.9	MG885811
6	CgSVU6	<i>Pimpinella anisum</i>	-ve	MG885812
7	CgSVU7	<i>Cuminum cyminum</i>	7.25	MG885813
8	CgSVU8	<i>Cuminum cyminum</i>	9	MG885814
9	CgSVU9	<i>Pimpinella anisum</i>	1.75	MG885815
10	CgSVU10	<i>Cuminum cyminum</i>	-ve	MG885816
11	CgSVU11	<i>Cuminum cyminum</i>	7	MG885817
12	CgSVU12	<i>Cuminum cyminum</i>	1.6	MG885818
13	CgSVU13	<i>Cuminum cyminum</i>	-ve	MG885820

CMase: Cellulase production by using Carboxy methyl cellulose

2.8 Screening of *Chaetomium globosum* Isolates for Cellulolytic Activity

Liquid fermentations were carried out in 250 ml Erlenmeyer flasks containing a modified Czapek-Dox broth medium (50 ml). Each flask was autoclaved at 121° C for 20 minutes. After cooling, each flask was inoculated by 0.5 ml of the spore suspension (prepared in Tween-80 (0.2%, v/v), from 4 days and nights). Then the inoculated flasks were incubated at periods in an incubator (5 days). The content of the flasks was filtered through a muslin cloth. The filtrate was centrifuged at 10,000 rpm for 10 minutes and the supernatant was used for the determination of cellulase enzyme activity [21]. Cellulase production was assayed by using a minimal agar medium containing (g/L): yeast extract, 1; K₂HPO₄, 4; NaCl, 2; MgSO₄.7H₂O, 1; MnSO₄, 0.05; FeSO₄.7H₂O, 0.05; CaCl₂. 2H₂O, 2; NH₄Cl, 2; agar, 20 and distilled water at pH 6.0. The medium was supplemented by 1% (w/v) carboxymethyl cellulose (CMC) and point inoculated by 1 ml of the *Chaetomium* filtrate. After incubation, 3 ml aqueous solution of congo red (0.1% w/v) was added to the plates for 30 minutes. The plates were washed by 1 ml of NaCl solution (1M) and left to dry for 15 minutes [22]. The formation of clear zones around the colonies indicated cellulolytic activity by the isolates.

3. RESULTS

3.1 Sequence Characteristics

The ITS sequences compared with accessible sequences from the GenBank nucleotide

database, indicated that the DNA sequences from all *C. globosum* isolates studied had 99% similar sequence identity with *C. globosum* sequences from GenBank (Table, 1). A neighbour-Joining tree was employed to study genetic diversity among thirteen strains from *C. globosum*, branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed; tree length= 599; consistency index (CI) = 0.0.8848; homoplasy index (HI) = 0.0.1152; retention index (RI) = 0.6550; rescaled consistency index (RC) = 0.5795, as it is shown in Fig. 1. Comparison of ITS sequences with sequences accessible within the GenBank nucleotide database indicated that the deoxyribonucleic acid sequences from all thirteen isolates of *C. globosum* collected during this study had 99% sequence identity with *C. globosum* sequences from GenBank. The phylogenetic tree revealed that the strains could be divided into 6 different clades and one strain (root) was achieved from the Genbank. The phylogenetic tree was rooted by FJ878052, which was obtained from Genebank. No correlation was found between the habitat and isolates' source. For example, strains MG885815 and MG885816 were grouped together with 99% bootstrap value, although they were isolated from anise and cumin seeds, respectively.

3.2 SGRP-PCR Characterization of *C. globosum* Isolates

Thirteen isolates of *C. globosum* were used to study the genetic diversity of *C. globosum* population isolated from the spices samples. In this respect, specific genes random primers polymerase chain reaction (SGRP-PCR)

technique was employed using three different primers (Fig. 2).

The three primers produced 37 SGRP/ PCR loci (183 fragments). Across strains percentage of polymorphism were 100% (no missing bands were observed). Phylogenetic analysis was carried out to detect the evolutionary relationship across species using the Cap method. The phylogenetic tree was presented in (Fig. 3). The phylogenetic tree consists of two large clades, the first clade comprised of MG885817 and MG885820. The second clade was divided into two lineages, MG885818 occupied a single lineage on its own, and the rest of the strains were assembled in another lineage. These results confirmed that *C. globosum* is a complex group with different subspecies.

3.3 Cellulase Screening

The thirteen isolates of *C. globosum* were tested for their ability to produce cellulase enzymes. The cellulase production varied among the *C. globosum* isolates. Nine of the 13 isolates of *C. globosum* showed capability to produce cellulase as shown with the congo red dye. The halos ranged from 1.6 cm to 9 cm in diameter. Four of the isolates of *C. globosum* did not show any reaction with congo red dye as was shown in Table (1) and Fig. (4).

4. DISCUSSION

The ITS region sequencing helpful in the identification at the species level or close to species level, alignment of the ITS region between major groups of fungi and even at intervals order and families was found to be useful sometimes [23]. These results are in agreement with [19] who collected ten isolates from various substrates that constituted one clade in the phylogenetic tree, including *C. globosum* with 98% bootstrap value. The results presented here confirmed the variations between species level of *C. globosum*. Chandra, et al. [24] studied forty four isolates of *Chaetomium* (fifteen isolates *C. globosum*) and subjected them to PCR amplification of the genes; β -tubulin, actin, calmodulin, tef-1 and rpb2. The sequences were subjected to maximum parsimony phylogenetic analysis. The clustering obtained was compared by the ITS based clustering. It was found that ITS region indicated the best grouping of the *Chaetomium* species through phylogeny although the cluster of *C. globosum* had several sub clades. Aggarwal, et al. [25] showed that ITS sequence data could differentiate 18 different isolates of the *Chaetomium* spp. were collected from different climates. Aggarwal, et al. [26] designed specific marker for identification of *C. globosum* to distinguish it from other different species of *Chaetomium* by forming a unique region of genomic DNA.

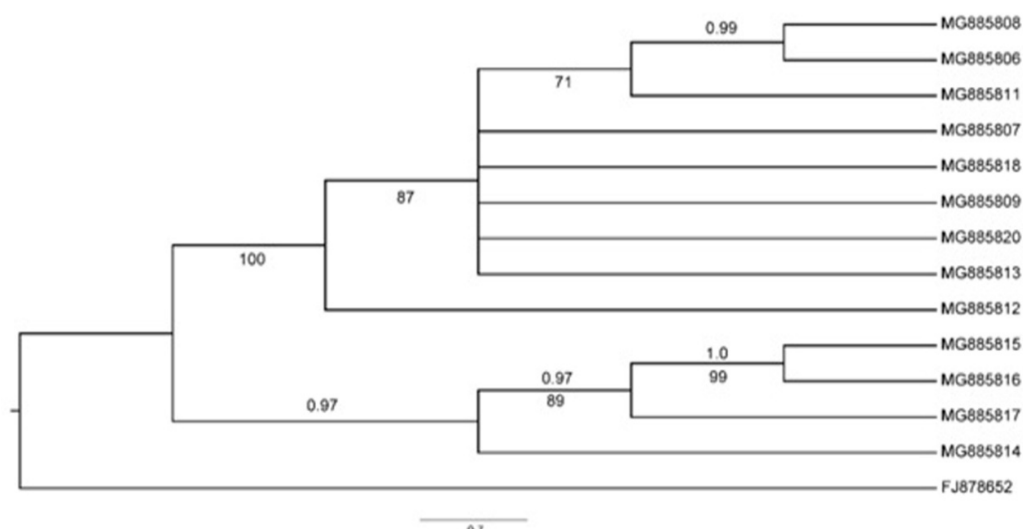


Fig. 1. Strict consensus cladogram of 9 Most Parsimonious (MP) trees found using the ITS sequence data showing the phylogenetic relationships among *Chaetomium globosum* isolates. Values above the branches indicate Bayesian posterior probabilities (≥ 0.95), and the maximum parsimony bootstrap support values ($\geq 70\%$) are given below the branches. The GenBank accession numbers of *C. globosum* were appeared besides the branches

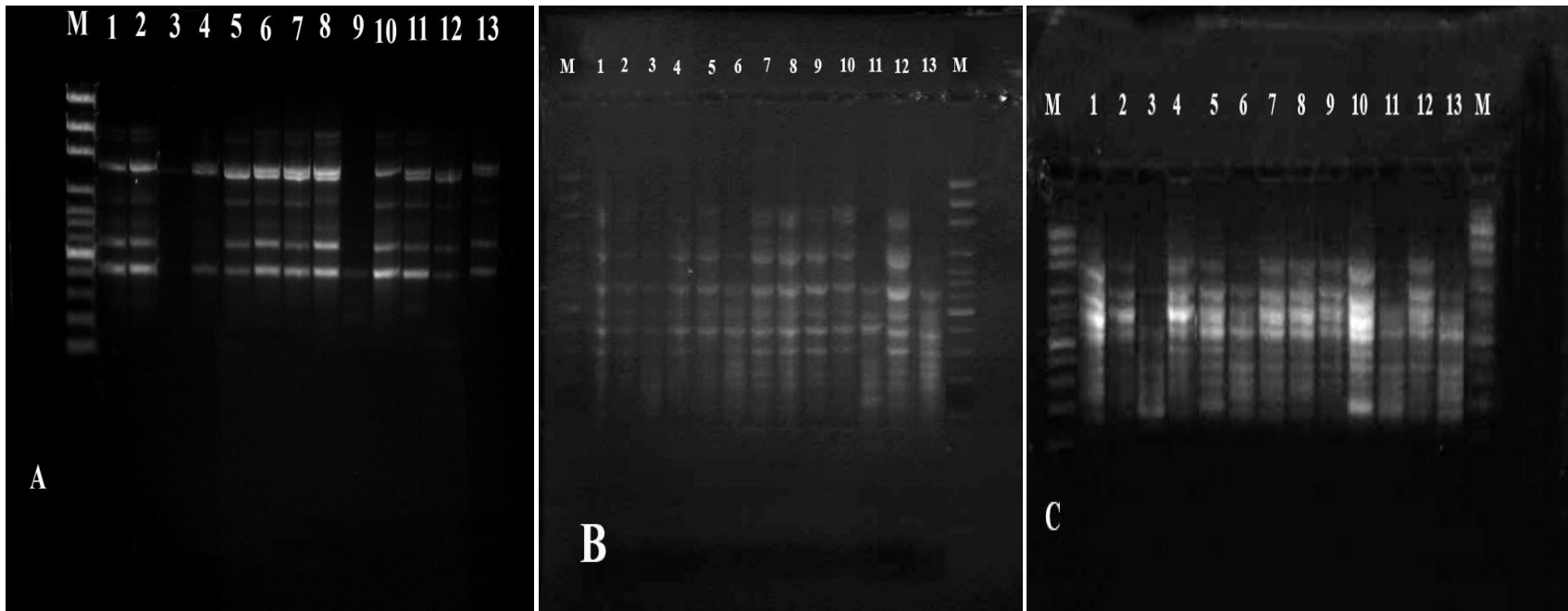


Fig. 2. DNA banding patterns from specific genes random primers polymerase chain reaction analysis of *Chaetomium globosum* isolates primed by Opa1 (A), Bt2a (B) and Tef1(C) primers. Lane Mis a 1-kb DNA ladder and lane 1-13 no. of isolates

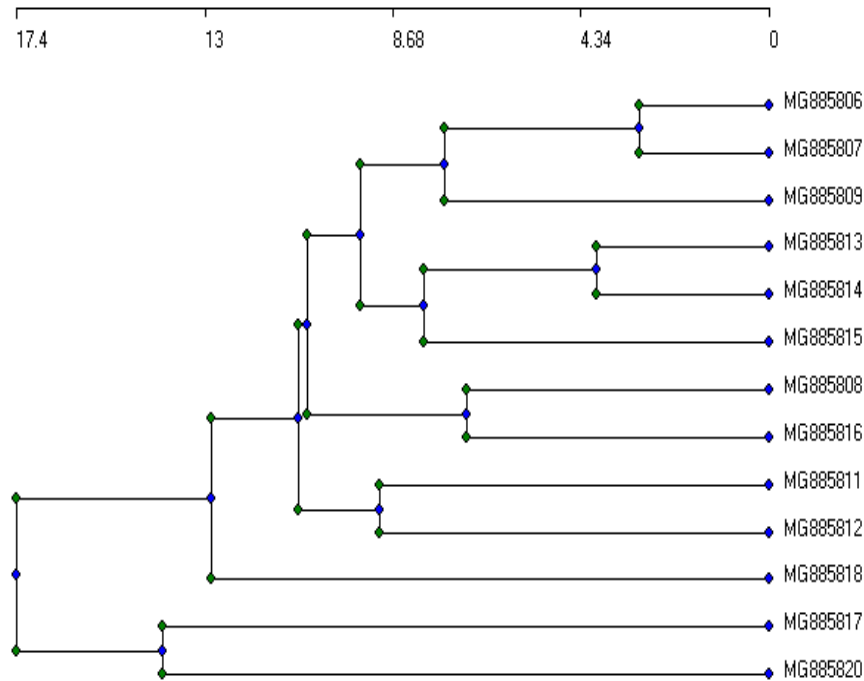


Fig. 3. Average linkage tree illustrating the relationship among the different isolates of *C. globosum* on the analysis of specific genes random primers polymerase chain reaction by using CAP software

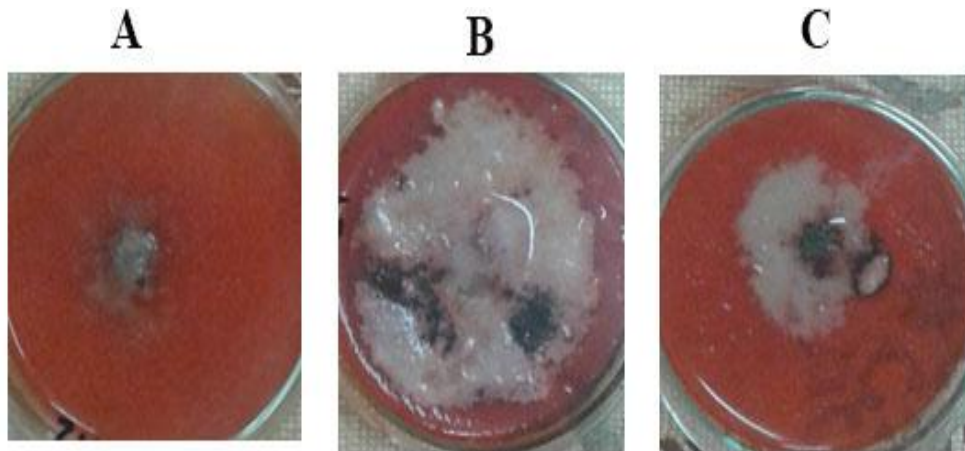


Fig. 4. *Chaetomium globosum* showed reaction with Congo red dye in B and C (= Positive isolates producing cellulase) and A= Negative isolate producing cellulase

The polymorphic fragmented patterns were found to occur by slight varieties in intensity. With the used primers, each isolate yielded up to 1 to 7 bands. Faint bands were only considered when consistently found in different experiments. It could be depicted that bands of higher intensities might be due to the amplification of repetitive sequences or the influence of neighbouring sequences on annealing to the target sequence

or might have been generated because of a lower degree of mismatch between primer and the target sequence [27].

The SGRP/ PCR findings confirmed the molecular identification by the ITS region. It is clear that there are significant differences under the species level of *C. globosum*. The results of the present phylogenetic analysis are similar to

the results of [19] on *C. globosum* isolates from various substrates.

Nine isolates of *C. globosum* produced cellulase with halo diameters ranging from 1.6 cm to 9 cm. Gupta, et al. [28] showed that CMC acts as an authentic indicator of cellulolytic ability. Isolated *Chaetomium* strains were primarily screened by employing an activity zone technique with acid-base indicator dye (congo red dye). Also, it was recognized that acid-base indicator dye could be a metachromatic dye, which is capable of reacting with the polysaccharide. The CMC is stained red to deep pink. Once the treatment of CMC with cellulase protein, the excess dye was washed gently by NaCl. Subsequently, the observation was created to notice the substrate utilization zone around the colony. On the other hand, unstained areas indicated the regions where the CMC have broken down to β -1. Suryadi, et al. [29] found that *Chaetomium globosum* has a high ability of cellulase production (by forming clear zone around the filtrate, 45 mm). Longoni, et al. [30] reported the functional role of two cellulases of *C. globosum* involved in preliminary degradation of plant cell wall polymers and also pointed to the functional role of other enzymes involved in cellulose degradation.

5. CONCLUSION

Identification of *C. globosum* with ITS1 and ITS4 primers is suggested to be used as a primary tool and need confirmation with more specific primers because there are several subspecies of *C. globosum* with the same morphological characters and similar to *C. globosum* species and these differences were confirmed by using SGRP/ PCR technique. Strains of *C. globosum* may be more helpful in the industrial production of cellulases.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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