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Original Research Article

Screening, Isolation and Identification of Cellulolytic *Chaetomium* species Collected from Deteriorating Cellulosic Samples

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Abstract: This research focuses on the screening, identification and evaluation of cellulolytic activity exhibited by different species of *Chaetomium,* a filamentous fungus known for its diverse enzymatic capabilities. Cellulose, a complex polysaccharide found in plant cell walls, is a promising feedstock for bioenergy and biorefinery applications. Different microorganisms, especially filamentous fungi, play an important role in the breakdown of cellulose using cellulase enzymes.The fungal cellulase ismore thermostable that makes them a better choice for industrial applications.During collection of deteriorated cellulosic samples,9 different genera of fungi were encountered. Among them the most dominant was *Chaetomium* with35 different species. These species were evaluated for their cellulolytic activities.The occurrence of several species of *Chaetomium* in the survey prompted further investigation of relatively less studied species.This evaluation was carried out based ondetermination of the cellulolytic activity in terms of loss in weight of filter paper followed by enzyme assays viz., determination of extracellular enzyme activities with respect to exoglucanase andendoglucanase activities of 10 selected species (which showed more than 25% loss in weight of filter paper) viz., *C. globosum, C. mollicellum, C. subspirale, C. olivaceum,C. convolutum, C. fibripilum, C. cochliodes, C. erraticum, C. Brasiliense* and *C. senegalensis*. *C. cochliodes* showed maximum cellulolytic activity in terms of loss in weight of filter paper where as *C. Subspirale* recoded maximum activities for both exoglucanase and endoglucanase enzymes. *C. olivaceum* exhibited least activities in both the cases. The findings contribute to the understanding of *Chaetomium* as a valuable cellulolytic resource for sustainable bioprocessing.

Keywords: *Chaetomium,* cellulolytic enzymes, cellulose, endoglucanase, exoglucanase, fungi

Introduction

Cellulose is the basic component of plant cell walls. It is the most abundant plant polysaccharide on earth (Horwath, 2007; Henriksson & Lennholm, 2009). About one third of the organic matter produced by green plants is cellulose (Srivastava *et. al*., 2021). Cellulosic biomass has gained significant attention as a renewable feedstock for bioenergy and bio-based products. Cellulolytic microorganisms, including filamentous fungi, play a crucial role in the enzymatic breakdown of cellulose (Saini & Sharma, 2021). The basic molecular structure of cellulose is very simple, it is linear polymer of glucose units linked end to end with β - 1, 4 linkages (Klemm *et. al*., 2005 and Zhu *et. al*., 2021). The polymeric chain of cellulose consists of over 10,000 D glucose units (Meena *et. al.,* 2014). The complexity in the structure of native cellulose also arises due to hemicellulose, lignin, and pectin along with cellulose as the cell wall components (Martínez-Sanz *et. al*., 2015). The biological degradation of cellulose is one of the largest mass hydrolytic reactions taking place in the nature

(Hawksworth, 1991 and Jayasekara & Ratnayake, 2019). Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity (Kadarmoidheen *et. al.,* 2012). It has been globally reported as being are capable of colonizing various substrates and are well-known for their ability to degrade cellulose and to produce a variety of bioactive metabolites.

The enzyme cellulase have shown their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture (Kuhad *et. al*., 2011). Cellulases are reportedly more stable at high temperatures than other plant cell wall degrading enzymes which makes them a better choice for industrial applications (Ejaz and Ghanemi, 2021). It is the third-largest industrial enzyme utilized around the world with a constant increase in demand (Jayasekara and Ratnayake, 2019). The commercial potential of using cellulases lies in its efficiency of converting lignocellulosic biomass into glucose through enzymatic hydrolysis which can be utilized to generate several value-added products such as ethanol (Patel *et. al*., 2019). *Chaetomium* is a genus of fungi belonging to the family Chaetomiaceae and order Sordariales (class Sordariomycetes in Ascomycota). These fungi are commonly found in various environments, including soil, decaying plant material, and indoor environments. They are known for their ability to decompose cellulose and other complex organic materials. *Chaetomium* species have been identified as potential cellulolytic organisms due to their ability to produce a diverse array of cellulolytic enzymes (Thapa *et. al.,* 2020). They are known to secrete a variety of cellulases with different specificities, including endoglucanases (endo-1-4-β-glucanase; EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). These enzymes work synergistically to break down cellulose into glucose units (Dadwal *et. al*., 2021). Endoglucanases cleave the internal glycosidic bonds of cellulose thereby releasing reducing and nonreducing chain ends, they are often called carboxymethyl cellulases (CMCase). The exoglucanases also known as cellobiohydrolases (CBH) act on the cellulose chain ends, releasing cellobiose. β-glucosidases then hydrolyse cellobiose into glucose, which can be further metabolized. (Andlar *et. al.,* 2018). Several authors reported that cellulolytic enzymes of *Chaetomium* are more efficient than other filamentous fungi (Al-Kharousi *et. al.,* 2015; Darwish & Abdel-Azeem, 2020). The present work describes the determination of cellulolytic activity of *Chaetomium*. Their cellulolytic activity has been determined by the loss in weight of filter paper as well as determination of total cellulase and endoglucanase enzyme activities.

Materials and methods

Mildewed and deteriorated samples of cellulosic materials viz., deteriorated paper and cotton samples were collected from libraries, warehouses, cotton fields and paper mills around Maharashtra and preserved in plastic bags at room temp. Small pieces of deteriorated portion of samples were cut (1g) and serial dilution method (10^{-6}) (Pramer & Schmidt, 1964) was used for isolation of cellulolytic fungi on selective media Czepek Dox Agar (CZA) (NaNO3. 3g, K2HPO4.1g, MgSO4.7H2O.0.5g, KCl. 0.5g, FeSO4.0.01g, cellolose.1%, agar. 15g, Distilled water.1000 ml. pH 5.5 before autoclaving). All the plates and flasks were incubated at room temperature i.e., 28°C. The culture plates and flasks were observed regularly for fungal growth. The visible fungal colonies were isolated by transferring them to new plates with a sterile nichrome loop. Streptopenicillin (antibiotic) was added in trace amount to

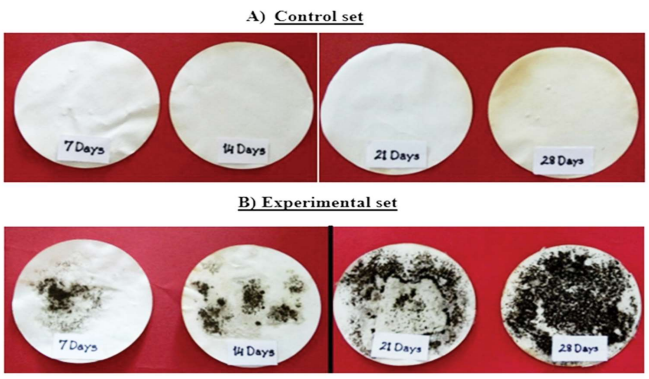

Fig. 1. (A). No cellulolytic activity in terms of loss in weight of filter paper is observed in control set (7-28 days). (B). Maximum cellulolytic activity in terms of loss in weight of filter paper shown by ***Chaetomium cochliodes*** (7-28 days).

suppress bacterial contamination at all stages during isolation and purification of the fungal cultures.

The isolated organisms were identified following the standard systems of fungal classification using available literature. (Ames, 1963; Gilman, 1976; Subramanyan, 1971 and Arx *et. al., .,* 1986). The identity of isolates was authenticated by Fungus Identification Service MACS, Pune and cultures were deposited in National Facility for Culture Collection of India (NFCCI) at Agharkar Research Institute Pune, India.

Determination of the cellulolytic activity in terms of loss in weight of filter paper

The experiment was based on the methods described by Fergus (1969). The experiments were conducted in Petri plates of 90 mm diameter (Fig. 1). Each Petri plate contained 1 filter paper disc of known weight and 10 ml of Czapek Dox Broth without sucrose ($NaNO_3$. 3g, K_2HPO_4.1g, $MgSO_4.7H_2O$.0.5g, KCl. 0.5g, $FeSO_4$.0.01g, Distilled water. 1000ml. pH 5.5 before autoclaving). A thin mat of surgical cotton was kept in each Petri plate below the filter paper for retention of moisture. A similar set at identical conditions was maintained as control. All plates were autoclaved at 15 lbs psi for 20 minutes. The plates were inoculated with 0.5 ml of spore suspension (10^{-6}). The plates were incubated at room temperature. All plates were observed at seven days interval up to 28 days. At the end of respective period of incubation, the filter paper discs were oven dried at 80°C, for 3 hours and allowed to cool down to ambient temperature in a desiccator and then weighed to the nearest mg on the electronic balance. The difference in weight of each filter paper was calculated by comparing it with the original dry weight and by the control set. The net loss in weight was attributed to the cellulose degradation.

The percentage loss in weight caused by isolate was calculated by using the formula (Ghewande, 1977).

$$\% \text{ loss in weight} = \frac{\text{Difference in weights}}{\text{Initial weight}} \times 100$$

Production of extracellular enzyme

The fungal isolates showing superior activities were selected from the previous experiment and further used for enzyme assay. A 100ml of Reese liquid medium (KH_2PO_4. 2.0g, KCl. 0.3g, Urea.0.3g, $NH_4(SO_4)$.1.4g, $MgSO_4.7H_2O$. 0.3g, Peptone. 0.05g, Yeast extract. 0.10g, and traces of $FeSo_4$, $MnSO_4$, $ZnSO_4$, D/W. 1000ml. pH adjusted to 5.2 before autoclaving) was poured in each 250ml conical flask. All flasks were autoclaved at 15 lbs psi for 20 minutes (Mandels *et. al.,* 1976). To this 0.5 g of cellulose (BDH, Chromatography grade) is added as the sole source of carbon was used to determine the extent of cellulase production by the isolates. The inoculum was obtained from 15 days old cultures of isolates grown on Czapek Dox Agar with filter paper strips. 10ml of sterile distilled water was added to the cultures and homogenized to form a suspension, 0.5ml of which was added to each flask. The flasks were incubated at room temperature for 7 days on a rotary shaker at 180 rpm. After the period of incubation, the broth was centrifuged at 5000 rpm for 15 minutes. The clear supernatant was further used as the enzyme source for determining cellulase activity.

Enzyme assay

The cellulolytic activity of the filtrate was determined by using the method described by Ghose (1987). Total Endoglucanase activity was assayed by measuring the amount of reducing sugar from carboxymethyl cellulose (CMC). The exoglucanase activity was analysed by measuring the amount of reducing sugar formed from cotton (substrate).

The production of cellulase enzymes using fungal organism is induced only in the presence of cellulosic substrate (Gosavi & Bagool, 2013). Several authors have suggested that cotton is the best substrate for exoglucanase activity whereas CMC is the best substrate to study endoglucanase activity of cellulase (Teeri, 1997; Enari & Markkanen, 1977; Soni & Soni, 2010 and Sulyman *et. al.,* 2020). For production of these enzymes using *Chaetomium* as organism same substrates are being used (Ravindran *et. al.,*2010).

The concentration of protein was determined by following the method of Lowry *et. al.,* (1951) using Bovin

Serum Albumin (BSA) as standard. The protein concentration was then estimated from a standard curve.

The enzyme activities of the extracts were determined by estimating the reducing sugars formed, using DNSA reagent (Mandels *et. al.,* 1976).

Exoglucanase activity

The dewaxed cotton was the substrate for exoglucanase activity of enzyme cellulase. The activity was determined using methods described by Erikson and Goysoyr (1977) and Wood and Bhat (1988). The assay mixture was made up of 50 mg of absorbent cotton, 0.5ml of 0.05M citrate buffer (pH4.8) and 1ml of enzyme extract. The reaction mixture was incubated at 50°C for 24 hours. A blank set as control was maintained under identical conditions. Total cellulose activity was analyzed by measuring the amount of reducing sugar formed from cotton used as a substrate.

Endoglucanase activity

The Carboxymethyl Cellulose (CMC) is used as the substrate for endoglucanase activity of enzyme cellulase. The activity was determined using methods described by Erikson and Goysoyr (1977) and Wood and Bhat (1988). The reducing sugars formed by the action of the enzyme on CMC were estimated. The assay mixture was made up of appropriately diluted 0.5ml of 1% CMC in 0.05M citrate buffer (pH 4.8). The reaction mixture was incubated at 50°C for 30 minutes. The reducing sugars produced were determined by using 3ml DNSA reagent; added to each tube. The test tubes were kept in boiling water bath for 10-15 minutes, and then cooled to room temperature. The content in each tube was adjusted to 20ml using distilled water. A blank set was maintained in triplicates under identical conditions. The optical densities were measured at 540 nm using colorimeter. The enzyme activity was expressed as mg of reducing sugars / mg of proteins.

The enzyme activity of exoglucanase and endoglucanase was defined in the international units (IU) and calculated as the amount of enzyme that released 1 µmol of glucose per minute under the assayed conditions (Patil & Talhande, 2023).

Results

A total of 53 isolates were obtained from the various samples collected. Nine different genera of fungi were encountered to association with cellulosic samples. The most dominant genus was *Chaetomium* which was represented by 35 species, followed by *Aspergillus* (6 species); *Penicillium* (3 species); *Trichoderma* (2 species); *Fusarium* (2 species); *Ascotricha* (2 species); *Thelavia* (1 species); *Curvularia* (1 species) and *Cladosporium* (1 species).

For the cellulolytic activity in terms of loss in weight of filter paper total 10 isolates showed more than 25% decrease in weight of filter paper. The highest activity was shown by *Chaetomium cochliodes* (43.93%) and the least by *Chaetomium olivaceum* (25.13%) at the end of 28 days of incubation (Table 1).Based on their activities in terms of loss in weight of filter paper 10 species were short listed for the determination of total cellulase and endoglucanase activities.The exoglucanase cellulase activity levels were lower than their corresponding endoglucanase for all isolates (Table 2 and Fig. 2).

The maximum endoglucanase and exoglucanase activities were exhibited by *Chaetomium subspirale* (13.96U.ml^{-1}.ml^{-1} glucose and 3.77U.ml^{-1}.ml^{-1} glucose respectively) and the least endoglucanase and exoglucanase activities were exhibited by *Chaetomium olivaceum* (2.22U.ml^{-1} .ml^{-1} glucose and

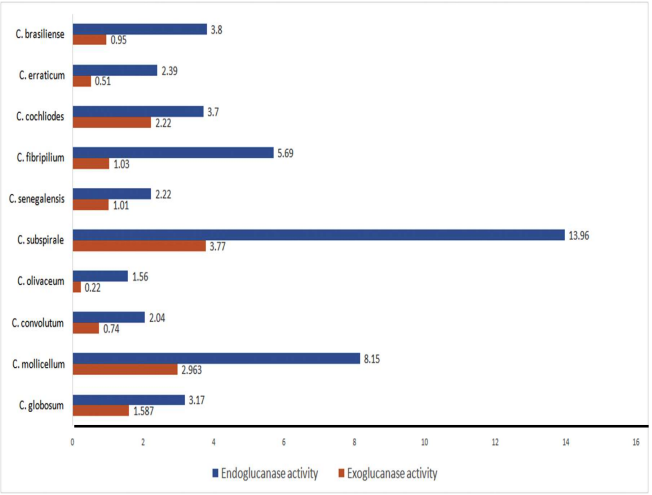

Fig. 2. Exoglucanase and Endoglucanase activities of selected *Chaetomium* species (U.ml-1 .ml-1 glucose)

Table 1. Cellulose degrading activity of isolated *Chaetomium* species isolates in terms of loss in weight of filter paper.

Organism	Period of incubation											
	7-Days			14-Days			21-Days			28-Days		
	Mean loss in weight (mg)	Mean Percent loss in weight	SE	Mean loss in weight (mg)	Mean Percent loss in weight	SE	Mean loss in weight (mg)	Mean Percent loss in weight	SE	Mean loss in weight (mg)	Mean Percent loss in weight	SE
C. globosum	73.33	12.83	0.27	95.33	16.46	0.52	150.67	25.99	0.26	155.33	26.64	0.25
C. mollicellum	102	17.57	0.14	131	22.92	0.17	160.33	27.34	0.33	171	29.7	0.9
C. mollicellum	83.67	14.46	0.29	92	15.98	0.02	103	18.07	0.5	105	18.81	0.91
C. subspirale	82.67	14.68	0.36	152.67	27	0.75	171.66	30.26	0.35	186.67	32.2	0.61
C. olivaceum	65	11.59	0.21	84	14.94	0.24	132	23.17	0.17	143.33	25.13	0.96
C. subspirale	121.38	21.08	0.51	159.67	28.39	0.38	175.34	31	0.4	188	33.12	0.9
C. cochliodes	105.2	18.66	0.32	175	30.17	0.6	220	39.19	0.42	258	43.93	0.52
C. cochliodes	56.67	10.11	0.49	78	13.93	0.52	101	18.05	0.24	124.33	22.2	0.2
C. fibripilium	134.33	23.78	0.29	161	28.79	0.4	188	32.57	0.16	205.67	36.05	0.54
C. globosum	74.2	13.26	0.64	90.17	16	0.58	109	19.24	0.2	120	21.07	0.07
C. cochliodes	87.66	15.62	0.31	124	21.92	0.09	141	24.75	0.38	148	25.9	0.66
C. cochliodes	65	11.59	0.21	83.67	14.46	0.3	90.33	16.23	0.39	103.67	19.4	0.31
C. erraticum	96.2	17	0.36	171	30.39	0.39	208.34	35.63	0.29	218	38.13	0.41
C. globosum	71.38	11.67	0.34	91.67	14.49	0.29	108.34	15.5	0.29	118.67	17.12	0.12
C. brasiliense	67.33	12.38	0.31	80	14.38	0.46	102	16.28	0.36	119.33	18.05	0.58
C. olivaceum	79.71	11	0.58	95.67	13.12	0.12	108.34	15.67	0.34	134.33	20.37	0.4
C. globosum	70.67	12.51	0.29	88	15.53	0.75	104.33	18.32	0.16	119.66	20.78	0.22
C. senegalensis	60.67	10.9	0.58	91	16.13	0.13	106.67	18.82	0.43	108.33	19.24	0.25
C. subspirale	61.33	10.67	0.6	96.33	16.75	0.25	116.67	20.29	1.19	132	22.96	0.58
C. brasiliense	88.33	15.27	0.37	113.67	19.4	0.31	122	21.33	0.34	144	25.89	0.49
C. mollicellum	57	11.65	0.68	78.67	14.9	0.49	92.67	17.16	0.6	103.33	19.1	0.49
C. globosum	53.33	9.87	0.13	79	14.62	0.62	103	19.07	0.41	126.67	23.45	1.08
C. convolutum	54.67	9.56	0.24	75.33	13.33	0.79	93.67	16.58	0.29	101.33	17.77	0.23
C. brasiliense	60.33	10.77	0.97	80.33	14.34	0.88	96.67	17.26	0.38	106.67	19.04	0.07
C. mollicellum	61.33	10.67	0.34	96.33	16.75	0.35	116.67	20.29	0.69	132	22.96	0.08
Control	10.0(+)	1.66(+)	0.08	10.0(+)	1.66(+)	0.08	10.0(+)	1.66(+)	0.08	10.0(+)	1.66(+)	0.08

*Mean values of three replicates are presented in this table

Mean initial weight = 550 mg '+' means gain in weight

0.22U.ml^{-1} .ml^{-1} glucose respectively). Followed by *Chaetomium subspirale*, *Chaetomium mollicellum* and *Chaetomium fibripilium* also showed good endoglucanase activity (8.15 U.ml^{-1}. ml^{-1} glucose and 5.69 U.ml^{-1} .ml^{-1} glucose) and *Chaetomium mollicellum* and *Chaetomium globosum* showed good exoglucanase activity (2.963 U.ml^{-1} .ml^{-1} glucose and 1.587U.ml^{-1} .ml^{-1} glucose).

Discussion

The interest in *Chaetomium* enzymes almost began when a method was devised for testing the effectiveness of mildew proofing agents on cotton fabrics in which the fungus, *Chaetomiumglobosum*, is used as the test organism. (Darwish and Abdel-Azeem, 2020). *Chaetomium*grows well and decomposes cellulose very rapidly, producing thermostable

Table 2. Exoglucanase and Endoglucanase activities of selected *Chaetomium* species.
Exoglucanase = mg of reducing sugars / mg of proteins in 24 hours
Endoglucanase = mg of reducing sugars / mg of proteins in 30 minutes

Organism	Exogucanse activity of Cellulase enzyme ($U.ml^{-1}$ $.ml^{-1}$ glucose)	SE	Endoglucanase activity of Cellulase enzyme ($U.ml^{-1}$ $.ml^{-1}$ glucose)	SE
C. globosum	1.587	0.05	3.17	0.04
C. mollicellum	2.963	0.03	8.15	0.13
C. convolutum	0.74	0.35	2.04	0.14
C. olivaceum	0.22	0.06	1.56	0.12
C. subspirale	3.77	0.18	13.96	0.96
C. senegalensis	1.01	0.1	2.22	0.1
C. fibripilium	1.03	0.02	5.69	0.04
C. cochliodes	2.22	0.15	3.7	0.12
C. erraticum	0.51	0.01	2.39	0.05
C. brasiliense	0.95	0.06	3.8	0.12

*Mean values of three replicates are presented in this table

cellulases (Sajith *et. al.,* 2016). True cellulolytic fungi, while growing on the cellulosic articles such as paper and cotton attack fibres and degrade the constituent cellulose to the final products, viz. CO_2 and water, which leads to a loss in the weight of the material. Therefore, loss in weight has been considered as a criterion for determining the cellulolytic activity of the test organisms. The loss in weight is said to correspond to the amount of cellulose degraded, which in turn is correlated with the cellulolytic activity of the respective organisms (Fergus, 1969; Park, 1976 and Paneerselvam and Saruvanamuthu, 1999). The enzyme activities were measured in terms of the production of reducing sugar end groups, which is taken to be indication of cleaves of cellulose molecules. *Chaetomium* species are known to produce cellulolytic enzymes. The endoglucanases, act on the amorphous regions of cellulose, cleaving the internal bonds and generating shorter cellulose chains. The exoglucanase hydrolyse the cellulose chains from their non-reducing ends, progressively releasing cellobiose units (Fan *et. al.,* 2012). Significant differences in activity were detected for two enzymes. Studies revealed that *Chaetomium* is a strong cellulose degrader. Cellulolytic enzymes play a significant factor in sustainability in various fields. Future research in this field is required to understandthe mechanisms, structure, function and substrate characteristics targeting efficient enzyme production.

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