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Biodegradation of Carboxymethyl Cellulose Employing *Cheatomium globosum* MTCC 2193

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ABSTRACT

Background: Cellulose, a major component of wood and cotton is the most abundant renewable organic molecule in the world, making it a huge resource of renewable energy. Carboxymethyl Cellulose (CMC) or cellulose gum is a cellulose derivative with carboxymethyl groups. As it is completely water soluble in nearly all of its applications, it eventually find its way into waste streams and ultimately into water surface thereby causing enormous environmental pollution with serious health consequences. Biological digestion of lignocellulosic materials can convert waste streams into valuable products like ethanol, acetic acid, lactic acid and antibiotics; it also reduces the waste disposal problems associated with landfills and burning forests. **Methods:** The present study investigates the biodegradation of various concentrations of carboxymethyl cellulose (0.25, 0.50, 0.75, 1.00 and 1.25%) in carpentry wastes using the fungal strain, *Cheatomium globosum*. The effect of the fungus on cellulose was measured by estimating several parameters like cellulolytic activity of endoglucanase, cellulolytic activity and effect of metals such as zinc and iron on cellulose degradation in addition to the determination of minimal inhibitory concentration. **Results:** *C. globosum* was found to be very effective in degrading 0.75% of carboxymethyl cellulose which was considered to be the optimum concentration. All the analyzed parameters showed that there is a statistically significant difference due to concentration of carboxymethyl cellulose, iron and zinc as well as treatment period. **Conclusion:** The results from this research support the general conclusion that introduction of cellulose degrading fungi is a beneficial microbiological tool to aid recovery of energy from degraded ecosystems. With a better understanding of the capabilities of the isolated fungus, industrial applications could be developed and the study of cellulolytic enzymes at the molecular level in future could reveal some features that contribute to their activity.

Key words: Carboxymethyl cellulose, *Cheatomium globosum,* biodegradation, endoglucanase

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INTRODUCTION

Cellulose is the most abundant renewable organic molecule in the world, making it a huge resource of renewable energy. Cellulose is produced by a variety of plants and bacteria, plus a few animals and it is the most abundant renewable organic molecule in the world, making it a huge resource of renewable energy[1]. Cellulose almost never occurs alone in nature but is usually associated with other plant substances like lignin and hemicellulose. This association may affect its natural degradation. Cellulosic materials have played an important role in everyday life as constituent of wood, paper, cloth, rayon film, plastic rope and fillers. The Carboxymethyl Cellulose (CMC), a derivative of cellulose is produced by chemical modification of cellulose, the most abundant polymer in nature and a major component of wood and cotton. CMC has been routinely used for many years now as a food additive (INS 466) in products such as ice creams and pre-cooked meals. Because of the water-solubility of the compound, it eventually finds its way into waste streams and ultimately into surface water. Therefore, knowledge about the biodegradability of CMC is desirable in order to assess the impact on the environment[2].

The biological decomposition of cellulose is the most important process in nature. It constitutes the major necessary steps in maintaining the balance between the synthetic and degradative phenomenon in carbon cycle. These are responsible for returning an estimated 85 billion tons of carbon as CO_2 in the atmosphere each year[3]. Annually, great amounts of cellulose wastes, which could be measured in many billions of tons, are produced worldwide as residues from agricultural activities and industrial food processing. They constitute a renewable resource from which many useful biological and

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chemical products can be derived. Accumulation of this biomass in large quantities every year results not only in deterioration of the environment but also in loss of potentially valuable materials that can be processed to yield energy, food and chemicals[4].

Biodegradation of redundant cellulose wastes from agriculture and food processing by continuous enzymatic activities of immobilized bacterial and fungal cells as improved biotechnological tools and, also, research concerning cellulose wastes biocomposting to produce natural organic fertilizers and, cellulose bioconversion into useful products, such as: 'single-cell protein' (SCP) or 'protein rich feed' (PRF) are reported[5].

Microorganisms with cellulase systems inhabit many different niches but thrive mainly in areas of higher concentrations of cellulose, such as soils, plants and in the guts of plant-eating animals and insects[6]. One of the most important features of cellulose as a substrate for microorganisms is its insolubility. Bacterial and fungal degradation of cellulose and other insoluble polymers occurs exocellularly, either in association with the outer cell envelope layer or extracellularly. This suggests that the assembly of enzyme systems, which may be extremely complex, also occurs exocellularly. Most cellulose is degraded aerobically but 5-10% is degraded anaerobically. Thus, vast quantities of cellulose are degraded by cellulose-fermenting microorganisms in anaerobic environments. Cellulolytic microorganisms can establish synergistic relationships with non-cellulolytic species in cellulosic wastes. The interactions between both populations lead to complete degradation of cellulose, releasing carbon dioxide and water under aerobic conditions and carbon dioxide, methane and water under anaerobic conditions[7].

Cellulase, a group of hydrolytic enzymes which hydrolyze the β-glycosidic bonds of native cellulose and related cellooligosaccharides, is the key enzyme of potential use for industrial saccharification of cellulosic materials into simple sugars. Cellulase production by different cellulolytic microfungi using various waste cellulosic materials is being vigorously studied for cost reduction strategies[8,9]. Many of the early studies of bacterial cellulases employed the fungal system as a model. Among the cellulolytic mircofungi, the genera *Trichoderma* and *Aspergillus* are notable cellulase producers. The cellulase systems of fungi (e.g., *Trichoderma reesei*) comprise three main activities, (1) Endoglucanases, which randomly hydrolyze β-1, 4 bonds within cellulose molecules, thereby producing reducing and non reducing ends, (2) Exoglucanases, which cleave cellobiose units from the non reducing ends of cellulose polymers and (3) β-glucosidases, which hydrolyze cellobiose and low-molecular-weight cellodextrins, thereby yielding glucose. These enzymatic components act synergistically in the hydrolysis of crystalline cellulose. Synergism has been explained by the proposal that endoglucanases attack amorphous regions of cellulose fibers, forming sites for exoglucanases which can then hydrolyze cellobiose units from more crystalline regions of the fibers[10].

Products of cellulose hydrolysis are available as carbon and energy sources for cellulolytic microorganisms or other microbes living in the environment where cellulose is being degraded. In fact, this release of sugars from cellulose is the main basis of microbial interactions occurring in such environments[11].

MATERIALS AND METHODS

The reference strain *Chaetomium globosum* MTCC 2193 was obtained from the Institute of microbial technology, Chandigarh.

Effect of fungi on cellulose: The efficiency of cellulolytic fungus to degrade cellulose was tested by providing fungus with different concentrations of carboxymethyl cellulose in the basal minimum medium[12]. The fungus was inoculated into the basal minimum media containing the salts, ammonium hydrogen sulphate, potassium chloride, magnesium sulphate supplemented with carboxymethyl cellulose of different concentrations like 0.25, 0.50, 0.75, 1.0 and 1.25% for *C. globosum*. The flasks were incubated at 30±2°C up to seven days.

Colorimetric assay of cellulase activity: Cellulase activity was determined by the DNS (3,5-dinitrosalicylic acid) method[13] through the determination of the amount of sugars reduction. The cellulase activity was determined by a standard graph with glucose in the concentration range of 50 to 1000 μg mL^{-1}.

Estimation of endoglucanase activity: Endoglucanase activity in culture supernatant was determined according to the method described[12]. The 0.45 mL of 0.1% carboxymethyl cellulose was mixed with 0.05 mL of enzyme extract and the mixture was incubated at 55°C for 15 min. After incubation, the reducing sugars released were assayed by adding 1 mL of DNS[13]. This was placed in a boiling water bath for five minutes. One milliliter of potassium sodium tartarate solution was added to the warm tubes and the solution was made up to 5 mL with distilled water. The tubes were rapidly cooled and the OD was observed at 540 nm and a blank was prepared without enzyme extract. The enzyme activity was expressed as glucose (mg) released per minute per mg of protein.

Detection of cellulolytic activity: The cellulolytic activity of *C. globosum* was detected using coomassie

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brilliant blue R 250 staining on Whatman No. 1 filter paper. The filter paper was pretreated with 75% ethanol and air dried followed by coating them uniformly with 1 mL of culture supernatant of *C. globosum*. Further, the treated filter paper was stained with coomassie brilliant blue R 250 for 10 min and destained using acetic acid-methanol mixture for 10 min. The enzyme active sites were visualized using this technique. The experiment was carried out for the control also using filter paper stained with the dye but without enzyme.

Effect of metals on cellulolytic fungus: The effect of metals, iron and zinc on the degrading ability of *C. globosum* was studied. The fungus was inoculated in the basal minimal media supplemented with different concentrations of iron and zinc like 250, 500, 750, 1000 and 1250 ppm. The flasks were maintained for five days at 30±2°C. The amount of released reducing sugar was determined by the DNS method.

Minimal inhibitory concentration: The minimal inhibitory concentration of the metals was determined using metal coated discs placed in plates swabbed with the fungus. Diameter of the inhibition zone was measured using a graph sheet.

Statistical analysis: Data presented are the averages of three replicates. The statistical analysis for standard deviation was carried out using instat+v3.33 and SPSS 10.0 software packages.

RESULTS

Plate 1 shows the microscopic view of *Cheatomium globosum* and Plate 2 shows the growth of *Cheatomium globosum* in cellulose agar.

Figure 1 reveals the amount of glucose released when CMC was degraded by *C. globosum*. The concentrations of CMC added for *C. globosum* were 0.25, 0.50, 0.75, 1.00 and 1.25%. The maximum level of glucose was released on the third day on the degradation of 1.25% cellulose and the minimum level of glucose was released on the first day on the degradation of 1.0% cellulose.

The endoglucanase activity of the fungal strain *C. globosum* during the degradation of CMC was estimated and the results revealed that the fungus exerted higher level of endoglucanase activity and active in the degradation on fifth day. There is a steady increase observed in the enzyme production up to fifth day and no fall in the enzyme level was observed till fifth day (Fig. 2).

Figure 3 and 4 divulge the effect of zinc and iron on the CMC degradation by *C. globosum*, respectively. Both the metals exhibited inhibitory action at the concentration 10,000 to 10,500 ppm. The fungus showed a very high level of glucose release till the fourth day and

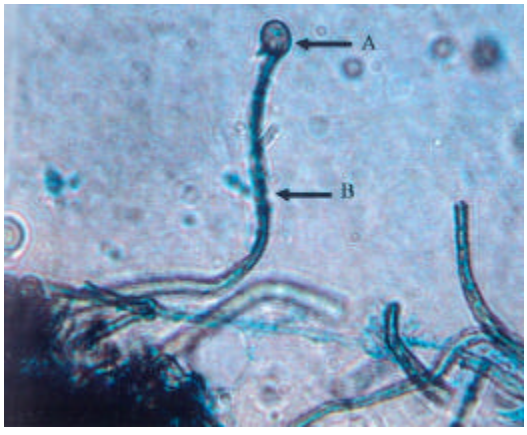

Plate 1: Microscopic view of *Cheatomium globosum*. A: Sporangium, B: Sporangiophore

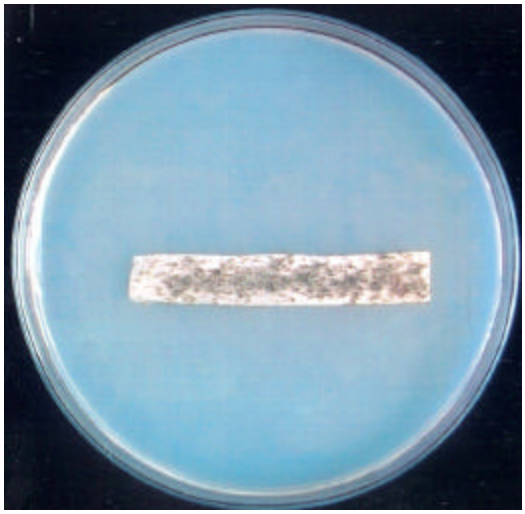

Plate 2: *Cheatomium globosum* in cellulose agar

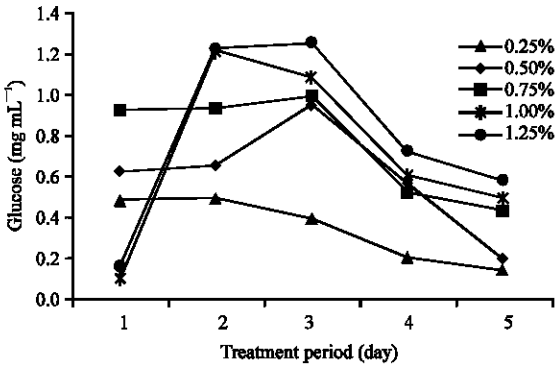

Fig. 1: Effect of *Chaetomium globosum* on the degradation of carboxymethyl cellulose

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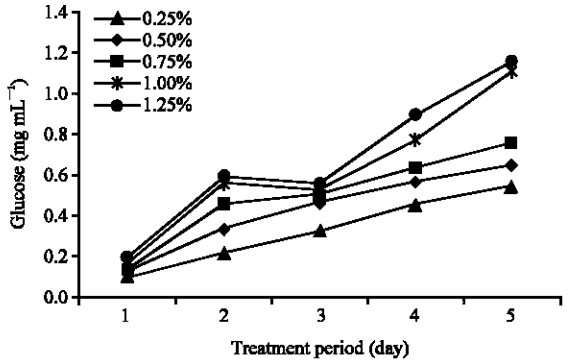

Fig. 2: Effect of endoglucanase activity of *Chaetomium globosum* on the degradation of carboxymethyl cellulose

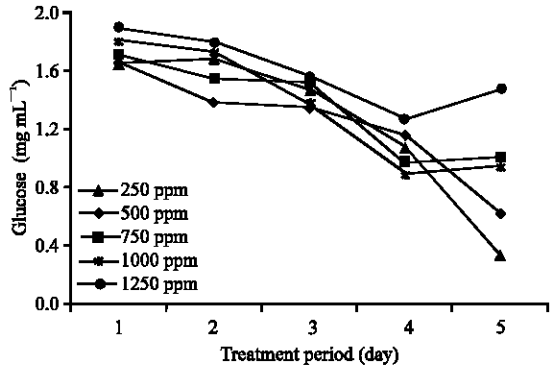

Fig. 3: Effect of Zinc on the degradation of carboxymethyl cellulose by *Chaetomium globosum*

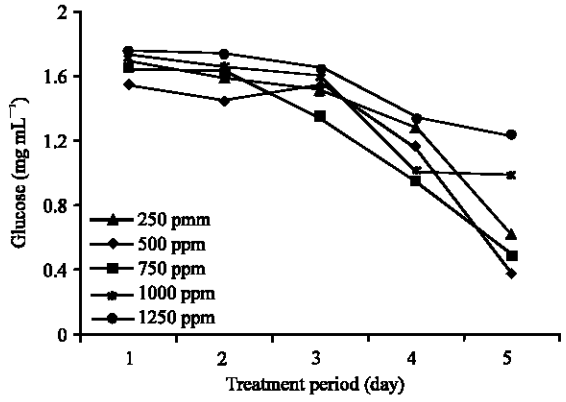

Fig. 4: Effect of iron on the degradation of carboxymethyl cellulose by *Chaetomium globosum*

decreased on the fifth day for both zinc and iron. Both the metals exhibited inhibitory action at This was clearly visible by the presence of zone. The metal iron effectively inhibited *C. globosum* than zinc (Plate 3).

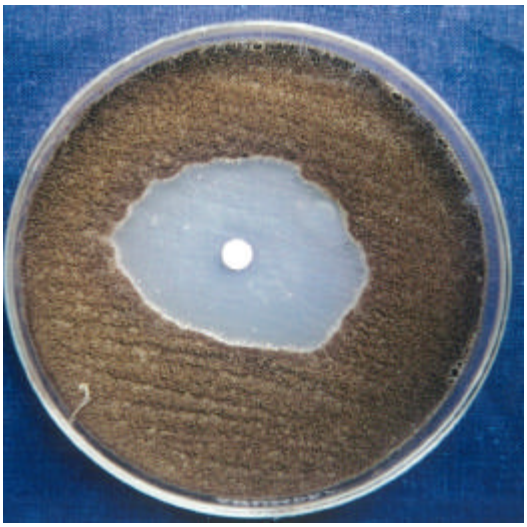

Plate 3: Inhibitory effect of iron on the growth of *Cheatomium globosum* in potato dextrose agar

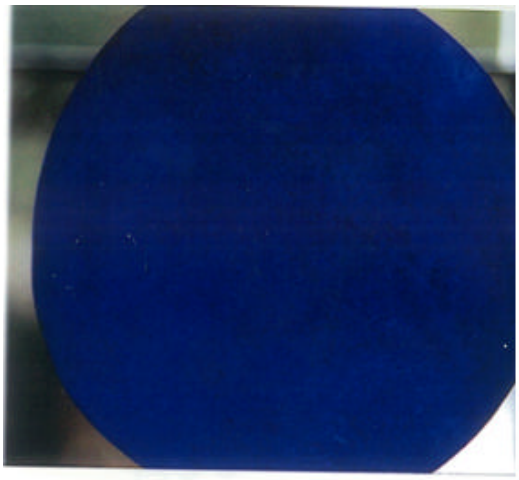

Plate 4: Control of Whatman filter paper No. 1 stained with Coomassie brilliant blue R-250

Cellulolytic activity seen on the Whatman No. 1 filter paper coated with culture supernatant, when stained with Coomassie brilliant blue R-250 showed clear white zone after destaining and the zone was clear and large in filter paper coated with *C. globosum* (Plate 4, 5).

Table 1 shows the statistical analysis of variance for various parameters such as carboxymethyl cellulose degradation by *C. globosum*, endoglucanase activity of the fungus strain and the effect of iron and zinc on the degradation of carboxymethyl cellulose. All these

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Table 1: Two way analysis of variance for the factors with the variables, treatment period and CMC concentration for *C. globosum*

Factor	Source of variation	df	MS	F-value	p- value	Significance
Degradation of CMC employing *C. globosum*	CMC concentration	4	0.1629460	2.73000	0.06582647	Significant
	Treatment period	4	0.0345076	5.79000	0.00445033	Significant
Endoglucanase activity of *C. globosum*	CMC concentration	4	0.1025660	10.93000	0.00018197	Significant
	Treatment period	4	0.3424360	36.49000	7.4428E-08	Significant
Effect of iron on the degradation of CMC by *C. globosum*	CMC concentration	4	0.0925000	3.981399	0.019867	Significant
	Treatment period	4	0.7647000	32.92602	1.54E-07	Significant
Effect of zinc on the degradation of CMC by *C. globosum*	CMC concentration	4	0.1106000	3.066045	0.047152	Significant
	Treatment period	4	0.6873000	19.04869	6.26E-06	Significant

Plate 5: Activity of cellulose produced by *Cheatomium globosum* in Coomassie brilliant blue R-250 stained Whatman filter paper No. 1

parameters showed that there is a statistically significant difference due to concentration of carboxymethyl cellulose, iron and zinc as well as treatment period.

DISCUSSION

Cellulolytic microorganisms, especially fungi, have attracted a great deal of interest as potential biomass degraders for large-scale applications due to their ability to produce vast amounts of extracellular cellulolytic enzymes. Previous reports indicated that fungal cellulases were produced only in the presence of cellulose as a growth substrate[14].

Since glucose is an end product of cellulolysis and inhibits extracellular cellulolytic enzymes, our study also suggests that the enzymes from *C. globosum* are inducible and regulated by catabolite repression. The enzymatic degradation of waste cellulose by fungal enzymes has been suggested as feasible alternative for the conversion of the cellulose into fermentable sugars and fuel ethanol[15].

The maximum level of glucose released on the third day of the degradation process was found to be 1.25% cellulose. For the complete hydrolysis of cellulose to glucose the cellulase systems must contain the enzymes such as endoglucanase, exoglucanase and cellobiase. Only the synergy of these enzymes makes possible the cellulose hydrolysis to glucose[16]. Therefore the results suggest that any one of these three enzymes could be present in *C. globosum*. Direct physical contact between enzyme and surface of cellulose molecules is a preliminary requirement to hydrolysis. Since the cellulose is an insoluble and structurally complex substrate, this contact can be achieved only by diffusion of the enzymes into the complex structural matrix of the cellulose[17]. The crystallinity degree of cellulose is one of the most important structural parameters which affect the rate of enzymatic degradation by hydrolysis. Products of cellulose hydrolysis are available as carbon and energy sources for cellulolytic microorganisms or other microbes living in the environment where cellulose is being degraded. This release of sugars from cellulose is the main basis of microbial interactions occurring in such environments. As it is evident from the results obtained in the present study, the fungus tested was able to decompose cellulose.

The main function of the enzyme endoglucanase is to cleave the β-1,4-linkage in amorphous region of cellulose to yield long chain oligosaccharides which is finally converted into glucose by the action of the enzyme cellobiase[18]. No decline in the level of endoglucanase enzyme production was observed during the period of study. Low endoglucanase activity at the beginning may be due to lower secretion of the enzyme during the initial stages of degradation because the organism would not prefer to use cellulose as a sole carbon source due to the availability of other enriched simple nutrients in growth media. Maximum enzyme production stage of the organism largely depends upon the type of microbial strains and their genetic makeup and on cultural and environmental conditions employed during growth of the organism[19].

When the metals like zinc and iron are present at lower concentrations, they showed no inhibitory action. As the concentration increases, they showed inhibitory action. This is seen clearly by the formation of the clear zone surrounding the disc. This shows that the presence of high amount of iron and zinc in the soil may inhibit cellulolytic fungi from degrading cellulose, which in turn leads to the accumulation of cellulose. The heavy metals like zinc, nickel, iron and chromium often present in a variety of industrial effluents will inhibit the biological activities. The presence of metals will not

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allow otherwise degradable organic matter also to get degraded. Amongst the organic matter solvents, chlorinated organics and alcohols are toxic to biological processes. Also phenols, pesticides and surfactants are inhibitory to biological activities in treatment plants[20].

Present study helps to identify the fungal species based on their cellulolytic capabilities for further characterization of the cellulolytic systems involved. Purification, cloning and characterization of novel cellulolytic enzymes from these species would enable development of technologies for cost-efficient cellulose degradation and ethanol production. To improve the conversion of cellulosic biomass to chemicals and fuels, many hyper cellulolytic strains have been used nowadays either as pure cultures or as mixed cultures with fermenting organisms[20]. Despite all the progress of the past three decades, many questions regarding cellulose biodegradation by fungi are still open. Thus, more effort will be necessary to elucidate above all the regulation of cellulolysis both on molecular and enzymatic levels.

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