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Biodegradation of Carboxymethyl Cellulose using *Aspergillus flavus*

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ABSTRACT

Background: Carboxymethyl cellulose (CMC) and their derivatives are frequently used in drilling fluids as viscosifiers and fluid-loss reducers. Cellulose is one of the most abundant organic macromolecules in the Ecosystem. Cellulolytic enzymes play an important role in natural biodegradation processes where plant lignocellulosic materials are efficiently degraded by cellulolytic fungi and bacteria. In industry, celluloytic enzymes have found novel applications in the production and processing of chemicals, foods and manufactured goods such as paper, rayon and cellophane. **Methods:** The efficiency of degradation was measured by treating *A. flavus* with various concentrations of carboxymethyl cellulose (0.2, 0.4, 0.6 and 0.8 g/100 mL). The endoglucanase enzyme activity of the strain was measured and the effect of different concentrations of zinc and iron on degradation of carboxymethyl cellulose was also studied. **Results:** The 0.8% concentration of CMC was found to be effectively degraded by *A. flavus*. The parameters such as carboxymethyl cellulose concentration, endoglucanase, zinc and iron showed that there is a statistically significant difference due to concentration of carboxymethyl cellulose, iron and zinc as well as treatment period. **Conclusion:** The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortage. Cellulose degrading fungus produced enzymes which are similarly related to xylanases. Both enzymes are synergistic over substrate, especially for microorganisms isolated from environments where wood and agro residues are biodegraded. Based on the above discussion, the strain *A. flavus* can be recommended for bioremediation programmes to clear cellulosic wastes.

Key words: Carboxymethyl cellulose, cellulolytic enzymes, *A. flavus*, endoglucanase

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INTRODUCTION

Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. The research on both methods has for decades occupied the attention of many investigators worldwide. Because each cellulose molecule is an unbranched polymer of 1000 to 1 million D-glucose units, linked together with beta-1,4 glycosidic bonds and cellulose from various sources are all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemicals. It is this difference that makes possible a persistent research on cellulose. The model chemical compound most commonly used in today's research is carboxymethyl cellulose (CMC) which has a generally amorphous structure and Avicel, with a highly crystalline structure[1].

Carboxymethyl cellulose (CMC) is an important industrial polymer with a wide range of applications in flocculation, drag reduction, detergents, textiles, paper, foods, drugs and oil well drilling operation. CMC is a derivative of cellulose and formed by its reaction with sodium hydroxide and chloroacetic acid. It has a number of sodium carboxymethyl groups (CH_2COONa), introduced into the cellulose molecule which promotes water solubility. 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. The various properties of CMC depend upon three factors: molecular weight of the polymer, average number of carboxyl content per anhydroglucose unit and the distribution of carboxyl substituents along the polymer chains[2,3,4].

The initial biodegradation of CMC probably involves the enzymatic hydrolysis of the glycosidic linkages by cellulases[5]. The enzyme system can be roughly subdivided into three enzyme groups β-D-glucosidases or cellobiases (EC 3.2.1.21) cellobiohydrolases or exoglucanases (EC 3.2.1.91) and 1,4-D-glucanohydrolases or endoglucanases (EC 3.2.1.4). β-glucosidases hydrolyse cellobiose and some soluble cello-oligosaccharides to give glucose. Exoglucanases release terminal cellobioses from the non-reducing end

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of the cellulose chain and are active on the crystalline parts whereas the endoglucanases hydrolyse amorphous cellulose randomly to give glucose, cellobiose and cellodextrins[6,7].

Cellulose degrading microorganisms play an important role in the biosphere by recycling cellulose and are common in fields such as forest soils, in manure and on decaying plant tissues. Among the cellulose utilizing species there are aerobic, anaerobic, mesophilic and thermophilic bacteria, filamentous fungi, basidiomycetes and actinomycetes[8,9]. A diverse group of fungi utilize cellulose for their carbon and energy sources. Following treatment of soil with cellulose, there is a significant increase in the number of fungi, particularly if the nitrogen supply is adequate. Strongly cellulose degrading fungi are represented by species of the genera *Aspergillus, Chaetomium, Curvularia, Fusarium, Memoniella, Phomo, Thielavia* and *Trichoderma.* These strains have been extensively studied in their ability to produce extracellular cellulose degrading enzymes namely endoglucanases, exoglucanases and cellobiase which act synergistically on the conversion of cellulose to glucose[10]. In the present study, the biodegradative ability of *Aspergillus flavus* on carboxymethyl cellulose was evaluated with the following objectives: (1) Isolation and identification of cellulolytic fungi (2) Estimation of cellulose and endoglucanase activity and (3) Effect of metals on cellulolytic fungi.

MATERIALS AND METHODS

Collection of sample: Samples rich in cellulose and cellulose degrading organisms were collected from the places rich in carpentry waste and also from Shola forest, Kodaikanal. They were collected in sterile containers and immediately brought to the laboratory for analysis.

Isolation of fungi: Serial dilution followed by pour plate technique was carried out to determine quantitatively the number of viable cells in the soil sample. The sample was serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) with sterile water blanks which serve as diluents of known volume. The diluted samples were plated onto the potato dextrose agar plates and the plates were incubated at 37°C for four days.

Isolation and identification of cellulolytic fungi: Fungi obtained were plated onto the cellulose agar to isolate the cellulolytic fungi. Sabouraud agar cover slips or slide culture of the unknown fungus was prepared and incubated at 37°C. Microscopic mount was prepared using lacto phenol cotton blue and observed under low and high power objectives of the microscope.

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. The fungus was inoculated into the basal minimum media containing the salts, ammonium hydrogen sulphate, potassium chloride and magnesium sulphate[11] supplemented with carboxymethyl cellulose of different concentrations like 0.2, 0.4, 0.6 and 0.8% .The flasks were incubated at 30±2°C up to 7 days.

Estimation of cellulose by dinitro salicylic acid method: Dinitro salicylic acid reagent was prepared by dissolving 1 g DNS, 0.2 g phenol, 0.05 g sodium sulphite in about 80 mL of distilled water and to this 5 mL of 5 N NaOH was added and the volume was made up to 100 mL with distilled water. One mililiter of the sample was mixed with 3 mL of DNS reagent in a test tube. A blank was prepared by adding 3 mL of DNSA with 1 mL of distilled water. Standards were prepared by taking 0.1, 0.2, 0.3, 0.4 up to 1 mg of the working standard and made up to 1 mL in each tube with water and 3 mL of DNS reagent was added. The tubes were covered with marble and placed in a boiling water bath for five minutes. They were then rapidly cooled and Optical Density (OD) was observed at 540 nm. The sample was centrifuged before use to allow the spores and mycelia to settle down. The glucose content in the sample was found out using the standard graph.

Estimation of endoglucanase activity: The 0.45 mL of 0.1% carboxymethyl cellulose was mixed with 0.05 mL of enzyme extract at the temperature of 55°C. The mixture was incubated at 55°C for 15 min and 0.5 mL of DNS reagent was then added immediately to the enzyme substrate mixture. This was placed in a boiling water bath for five minutes. One microlitter 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 the glucose (mg) released per minute per mg of protein.

Detection of cellulolytic activity: The cellulolytic activity of *Aspergillus flavus* was detected using Coomassie 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 *Aspergillus flavus.* Further, the treated filter paper was stained with coomassie brilliant blue R 250 for 10 min and destained using acetic acid-methanol mixture for ten 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 without enzyme.

Effect of metals on cellulolytic fungi: The effect of metals, iron and zinc on the degrading ability of the fungus 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 reducing sugar released was determined by dinitro salicylic acid method.

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

RESULTS

The fungus isolated from soil was tentatively identified as *Aspergillus flavus* (Plate 1) which was able to grow efficiently on cellulose agar (Plate 2). The concentrations of carboxymethyl cellulose added for *Aspergillus flavus* were 0.2, 0.4, 0.6 and 0.8%. The maximum level of glucose was released on the third day on the degradation of 0.8% cellulose. The minimum level of glucose was released on the fifth day for the degradation of 0.2% cellulose (Fig. 1).

The endoglucanase activity of the fungal strain *Aspergillus flavus* during the degradation of carboxymethyl cellulose is shown in Fig. 2. Results revealed that the fungus exerted higher level of endoglucanase activity on fourth day and the level decreased on fifth day. Figure 3 divulges the effect of iron on the degradation of carboxymethyl cellulose by *Aspergillus flavus*. At lower concentration, iron showed no inhibitory action on the fungus. The *Aspergillus flavus* showed a steady increase in the level of glucose upto third day and then decreased.

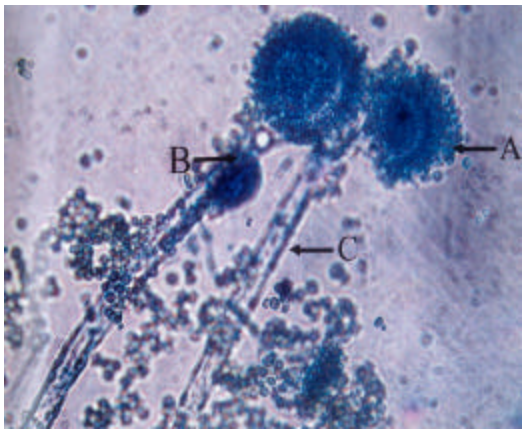

Plate 1: Microscopic view of *Aspergillus flavus*. A: Sporangia, B: Conidia, C: Sporangiophore

The effect of zinc on the degradation of carboxymethyl cellulose by *Aspergillus* flavus is exhibited in Fig. 4. Iron doesn't have any inhibitory action on the fungus. The maximum level of glucose released is 1.62 mg mL^{-1} on the degradation of optimum concentration of carboxymethyl cellulose in the presence of 1250 ppm of zinc (Fig. 4).

Both the metals exhibited inhibitory action at concentration 10,000-10,500 ppm. This was clearly visible by the presence of zone. The metal zinc had lesser effect on *Aspergillus flavus* than Iron (Plate 3).

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 smaller in filter paper coated with *Aspergillus flavus* (Plate 4, 5).

Table 1 shows the statistical analysis of variance for various parameters such as carboxymethyl cellulose

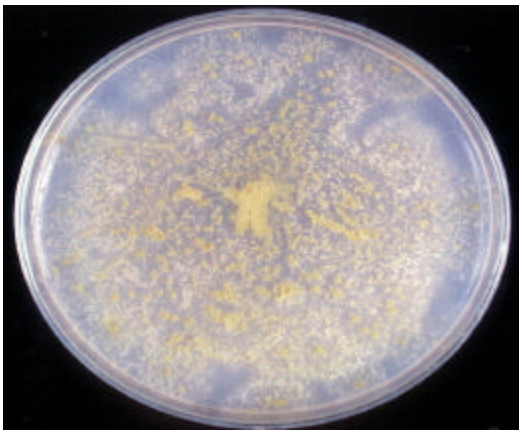

Plate 2: *Aspergillus flavus* in cellulose agar

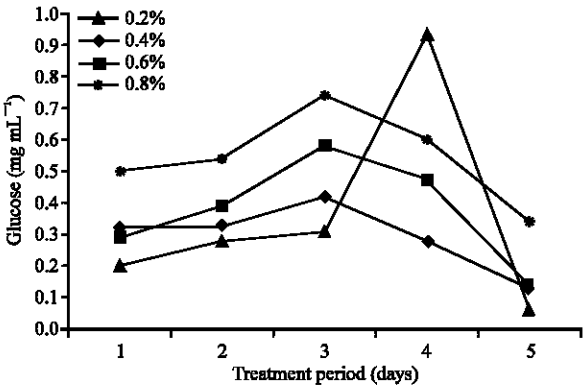

Fig. 1: Effect of *Aspergillus flavus* on the degradation of carboxymethyl cellulose

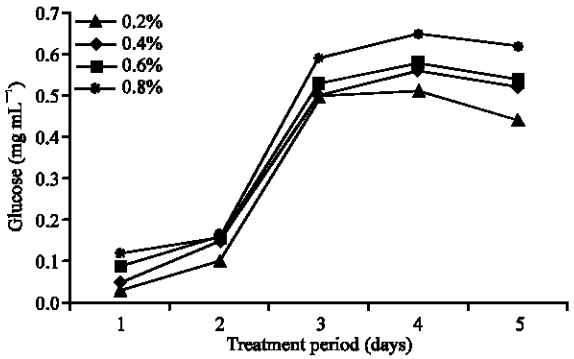

Fig. 2: Endoglucanase activity of *Aspergillus flavus* in the degradation of cellulose

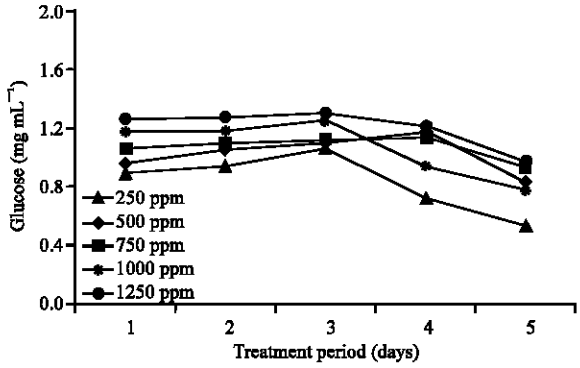

Fig. 3: Effect of iron on the degradation of carboxymethyl cellulose by *Aspergillus flavus*

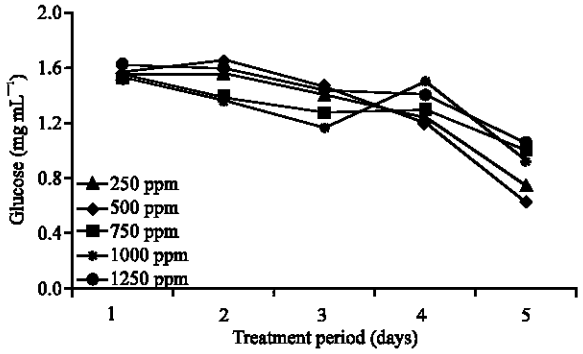

Fig. 4: Effect of zinc on the degradation of carboxymethyl cellulose by *Aspergillus flavus*

degradation by *Aspergillus flavus*, endoglucanase activity of the fungal strain and the effect of iron and zinc on the degradation of carboxymethyl cellulose. All these parameters showed that there is a statistically significant difference due to concentration of carboxymethyl cellulose, iron and zinc as well as treatment period.

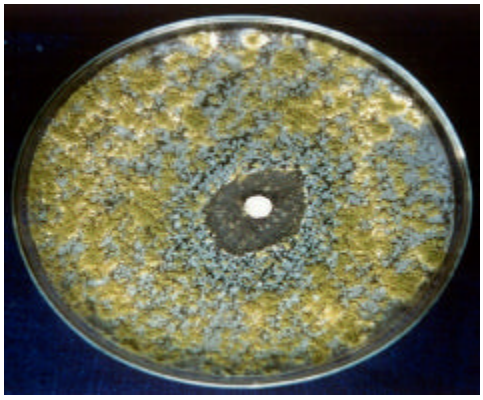

Plate 3: Inhibitory effect of zinc on the growth of *Aspergillus flavus* in potato dextrose agar

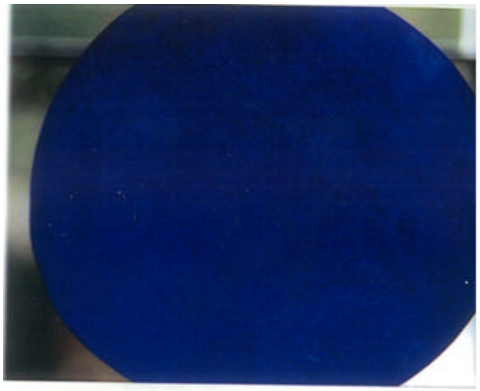

Plate 4: Control of Whatman filter paper No. 1 stained with Coomassie brilliant blue R-250

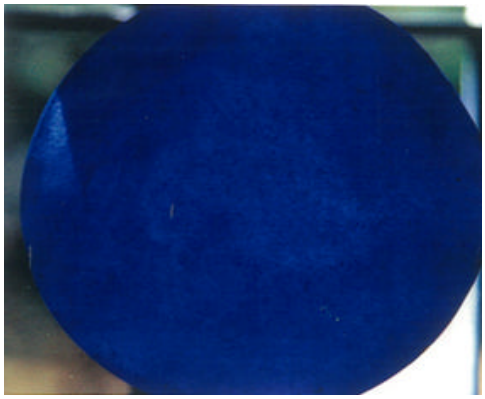

Plate 5: Activity of cellulose produced by *Aspergillus flavus* in Coomassie brilliant blue R-250 stained Whatman filter paper No. 1

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

Factor	Source of variation	df	MS	F value	p-value	Significance
Degradation of CMC employing *A. flavus*	CMC concentration	3	0.056565	2.34	0.12471012	Significant
	Treatment period	4	0.100812	4.17	0.0240158	Significant
Endoglucanase activity of *A. flavus*	CMC concentration	3	0.010960	20.647	4.97E-05	Significant
	Treatment period	4	0.233480	439.85	6.73E-13	Significant
Effect of Iron on the degradation of CMC by *A. flavus*	CMC concentration	4	0.090740	12.07854	0.000103	Significant
	Treatment period	4	0.094660	12.60033	8.04E-05	Significant
Effect of Zinc on the degradation of CMC by *A. flavus*	CMC concentration	4	0.014800	0.895253	0.4895002	Significant
	Treatment period	4	0.371100	22.4707	2.118E-06	Significant

DISCUSSION

Cellulose is the most abundant and renewable natural product in the biosphere with its estimated synthesis rate of 10 tonnes per year[10,12,13,14]. It is the major constituent of plant cell walls providing their rigidity[15]. It is a biopolymer consisting of insoluble, linear chains of β-(1-4)-linked glucose units linked with glucosidic bonds and the resulting biopolymers are assossiated by means of H-bonding. A matrix of lignin and hemicellulose encrusts and protects the cellulose of the plant cell wall[15].

The agricultural wastes are composed essentially of cellulosic or lignocellulosic matter. These are considered to be the cheapest source for the production of different utilizable products throughout the world[16]. Cellulose is commonly degraded by enzyme called cellulase. Complete enzymatic hydrolysis of cellulose requires synergistic action of 3 types of enzymes, namely cellobiohydrolases, endoglucanases or carboxymethylcellulase (CMCase) and B-glucosidases[17]. Wood and Garcia-Campyo[7] stated that there may be certain situations where cell-bound enzymes will be more efficient in the degradation of macromolecules. This includes the situation where the microorganisms are in an aquatic environment such as waste water treatment plants.

Endo-β-(1,4)-glucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), commonly referred to as endoglucanases, are characterised by their random hydrolysis of β-(1,4)-glucosidic linkages, although the degree of randomness may vary amongst the several different endoglucanases which are normally produced by a single organism[18]. Acting on soluble cellulose derivatives, their random cleavage causes rapid decrease in chain length and hence changes in viscosity relative to the release of reducing end groups. When acting on cellodextrins, the rate of hydrolysis increases with the degree of polymerisation within the limits of substrate solubility, with cellobiose and cellotriose being the major final products[18].

Fungi are found to be the major decomposers of cellulose and lignin[19]. The production of cellulase enzyme is a major factor in the hydrolysis of cellulosic materials[20]. *Aspergillus flavus* is capable of producing endoglucanase even from sawdust and corncob. It degraded carboxymethyl cellulose efficiently as it released high amount of reducing sugar (glucose). The *Aspergillus flavus* also possess the capacity to degrade the non-starch polysaccharide in the substrate to soluble sugar[21].

Endoglucanases randomly hydrolyze internal β-1,4-D-glycosidic bonds in cellulose[22]. As a result, the polymer rapidly decreases in length and the concentration of the reducing sugar increases slowly. Endoglucanase activities decreased sharply during the destruction of lignin-cellulose complex and the amount of carbohydrate in medium increased (30-40 mM)[23]. In our present study the *Aspergillus flavus* exerted higher level of endoglucanase activity and active in the degradation on fourth day and the level decreased on fifth day. So, fifth day endoglucanase activities decreased sharply during the destruction of carboxymethyl cellulose and the amount of carbohydrate in medium increased.

In our present study, the maximum level of glucose was released on the third day on the degradation of 0.8% cellulose. The minimum level of glucose was released on the fifth day on the degradation of 0.2% cellulose

Most of the cellulolytic microorganisms belong to eubacteria and fungi, even though some anaerobic protozoa and slime molds which can degrade cellulose have also been described. 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[9].

Many fungi and bacteria secrete a multicomponent enzyme system called cellulase that exhibits the ability to saccharify cellulose[24]. Bacterial cellulases are constitutively produced, whereas fungal cellulase is produced only in the presence of cellulose[25]. Filamentous fungi particularly *Aspergillus* and *Trichoderma* spp., are well known efficient producers of cellulases[26]. All four classes of enzymes have been identified in *Aspergillus*[27].

Heavy metals like cadmium, chromium, copper, mercury, zinc, nickel and lead are often present in a variety of industrial effluents and will inhibit biological activities. The presence of metals and metalloids (arsenic and selenium) will not allow otherwise degradable

organic matter also to get degraded[28]. In our present study, zinc and iron showed no inhibitory action on the fungus. There was a very high level of glucose till the third day and it decreased on the fifth day for iron. The maximum level of glucose released was 1.62 mg mL^{-1} on the degradation of carboxymethyl cellulose in the presence of 1250 ppm of zinc. The presence of the enzymes in the culture supernatant is proved by performing a simple experiment with Whatman filter paper No.1 stained with Coomassie brilliant blue R-250. Endo-β-1,4-glucanase has the role of randomly degrading-β-1,4-glucosidic bonds from the middle of the cellulose molecule[29]. This result in the formation of a clear zone in the enzyme treated Whatman filter paper No. 1 stained with Coomassie brilliant blue R-250. The parameters such as carboxymethyl cellulose, endoglucanase, zinc and iron showed a statistically significant difference due to concentration of carboxymethyl cellulose, iron and zinc as well as treatment period.

CONCLUSION

The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortage. Cellulose degrading fungus produced enzymes which are similarly related to xylanases. Both enzymes are synergistic over substrate, especially for microorganisms isolated from environments where wood and agro residues are biodegraded. Based on the above discussion, the strain *A. flavus* can be recommended for bioremediation programmes to clear cellulosic wastes.

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