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Biodegradation of lignocellulosic waste by *Aspergillus terreus*

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Abstract

Biodegradation of lignocellulosic waste by *Aspergillus terreus* is reported for the first time. This isolate produced 250 CMCase (carboxymethyl cellulase or endoglucanase) $U.ml^{-1}$ and biodegraded hay and straw during 3 days and the biomass production on straw was $5g.L^{-1}$dry weight from 0.25 cm^2 inoculated mycellium. This strain secreted endocellulases and exocellulases in the culture medium, but some of the enzymes produced, remained cell membrane bound. Cell bound enzymes were released by various treatments. The highest amount of endoglucanase and exoglucanase was released when the cells were treated with sonication. *Aspergillus terreus* was added to two tanks containing sugar wastewater and pulp manufacturing waste, as a seed for COD removal. This fungus reduced the COD by 40–80 percent, also, ammonia was reduced from 14.5 mM to 5.6 mM in sugar beet wastewater. The effects of crude enzyme of this fungus for COD removal was studied.

Introduction

The biodegradation and bioconversion of lignocelluloses into useful products and biological alleviation of pollution from lignocellulosic wastes is an enormous environmental challenge. Lignocelluloses are generally composed of 30 to 56% cellulose, 10 to 27% or more hemicelluloses, 3 to 30% lignin, and 3.6 to 7.2% protein. Some crop residues like rice straw and bagasse also contain a large quantity of silica (Orth et al. 1994). Lignocelluloses gained attention during the 1980s as a possible source of single cell protein (SCP) to satisfy an increasing worldwide demand for new protein foods and enzyme production (Chahal 1994). The widespread application of enzymes in feeds to monogastric animals is a recent development. Amylases, glucoamylases, glucanases, cellulases, pentosanases, proteinases and xylanases are used in animal feed (Cowan 1996) and the market for industrial enzymes has been more than doubled since 1983. Environmental concerns and pressures for better use of regenerable resources, coupled with substantial advances in biotechnology have stimulated an explosive growth in the application of industrial enzymes. Delignification of lignocellulosic material by white rot fungi is of great interest and has been investigated to improve the digestibility of wood or straw for animal feed and to reduce costs for pulp and paper industries (Johansson & Nyman 1993; Vares et al. 1995). In the present paper we report the biodegradation of sugar beet and pulp paper wastewater by cell biomass and crude cellulase enzymes.

Materials and methods

Microorganisms and media

Aspergillus terreus was isolated from pulp wastewater and rotten wood. This microorganism was grown in the following medium ($g.L^{-1}$): wheat straw 10.0; peptone 0.5; KH_2PO_4, 2.0; $(NH_4)_2SO_4$ 1.4; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.005. pH was adjusted to 5.0. The medium received a 10.0% (v/v) inoculum of a pre-grown culture. 250 ml of this medium was incubated at 28 °C in a 1 litre flask on an orbital shaker. Cellulose or wheatstraw (10 $g.L^{-1}$), sucrose (5 mM) and glucose 5 mM was added to

the above medium to study their effects on enzyme production.

Enzyme assays

Exoglucanase (Fpase) activity in the culture supernatant fluid was assayed by the filter paper test. Filter paper is hydrolysed by the enzymes for 1 hour at 45 °C and pH 6. The reducing sugar produced is measured (as glucose) using dinitrosalicylate as described by Mandel and Weber (Mandel & Weber 1969). Cellobiase activity was assayed according to the method of Okada (Okada 1974). Protein concentration was measured according to the method of Bradford (Bradford 1976) by reference to a standard concentration curve for bovine serum albumin. Endoglucanase (CMCase) was determined by its activity against carboxymethyl cellulose (CMC, low viscosity). This was assayed by mixing 1ml of an appropriate enzyme dilution with 4 ml of CMC 10 $g.L^{-1}$ in 25 mM potassium phosphate (pH 6), and the mixture incubated at 50 °C. After 15 min the reaction was stopped by the addition of 2 ml of dinitrosalicylate. The resulting mixture was boiled for 10 min and reducing sugar content measured by absorbance at 640 nm (Yazdi et al. 1990; Emtiazi et al. 1999).

Determination of pH and optimal temperature and enzyme stability in presence of cations

To determine the optimal temperature for enzyme activities, assay was performed at 20–90 °C, pH 6.0. Assays for optimal pH were performed at 50 °C or 45 °C for exoglucanase (Fpase) in the pH range 2.5 to 9.0. For investigation of the effect of ions, the enzymes were incubated at various temperatures (30–100 °C) and various cations (500 $\mu g.ml^{-1}$) for 30 min.

Release of cell membrane bound enzymes

Experiments for the isolation of cell membrane bound enzymes were performed. In this procedure, *Aspergillus terreus* was grown in 100 ml of the cellulose medium. After 3 days, cells were removed by centrifugation at 10.000 rpm for 10 min, at 4 °C. Pellet was washed twice with 0.1 ml phosphate buffer and was used as the source for releasing the cell membrane bound enzymes. The enzymes were released by tween 80 or ultrasonication.

Study of COD and BOD removal from wastewater

In this study wastewater samples were collected by plant personnel in sterile tanks. About 20 L of wastewater from sugar beets industry and pulp industry were supplied in four 50 L sterile tanks. *Aspergillus terreus* was grown in hay straw and incubated at 32 °C and after 3 days it was filtrated and $5g.L^{-1}$ dryweight of it was added to two tanks as a seed for COD removal. After 5 hr aeration and 12 hr settlement the surface effluent was taken to measure COD, BOD and ammonia and compared with uninoculated control tanks. Also the effects of 1 ml crude concentrated CMCase (1800 CMC $U.ml^{-1}$) and Fpase (18 FP $U.ml^{-1}$) were studied.

Results

Aspergillus terreus was grown on cellulose broth and after 3 days incubation on shaker the activity of exoglucanase was 7 FP $U.ml^{-1}$ and the activity of endoglucanase was more than 250 CMC $U.ml^{-1}$ (Figure 1). The endoglucanase activities of this fungus was high compared to *Neurospora crassal* (Yazdi et al. 1988), *Trichoderma viride* (Beldman et al. 1984) and *Aspergillus foetidus* (Christov et al. 1999). As shown in Figure 1, exoglucanase (Fpase) is produced in 1 day, however the production of endoglucanase started on the second day. After 4 days incubation the production of these enzymes were reduced. Therefore the biomass and enzyme used for COD removal was obtained after 3 days. 1 ml of cell (7 $mg.ml^{-1}$ wetweight) biomass had 300 $U.ml^{-1}$ endoglucanase activity after cell sonication. Addition of T80 did not have any effect on enzyme production. The effect of 1–5 mM glucose and sucrose to induce the production of enzymes were studied. Glucose (5 mM) reduced the activity of endoglucanase and exoglucanase 15% and 33% respectively, however the addition of 1 mM glucose to cellulose broth did not have any effect on cellulase production. Sucrose (5 mM) reduced the activity of exoglucanase by 50% (Table 1). Thermal and pH stability of enzymes were studied. It was shown that both enzymes were stable at pH = 4–7 at 50 °C. The effect of pH on stability was tested by incubating the enzymes at 50 °C for 1 hour in different buffers of various pH and the residual activity was measured by the enzyme assay. Both enzymes were stable at 40–60 °C and pH = 4–7.

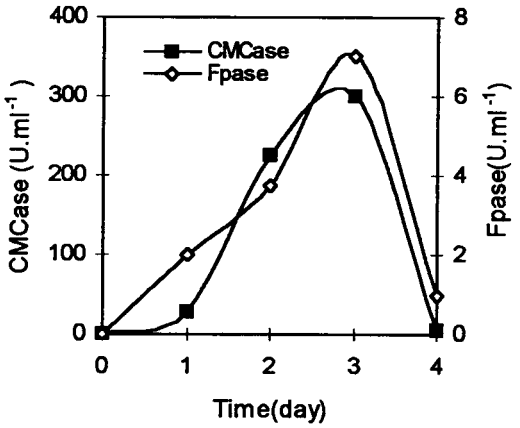

Figure 1. Production of exoglucanase (Fpase) and endoglucanase (CMCase) by *A. terreus*. In this experiment 100 ml of inoculated cellulose broth incubated at 30 °C.

Table 1. The effect of 5 mM glucose and sucrose on production of Exoglucanase (Fpase) and Endoglucanase (CMCase) in Cellulose broth. As it shows, these compounds did not have a positive effect on production of the enzymes

Substrate	Enzyme activity (%)	
	Endoglucanase	Exoglucanase
Cellulose (10 g.L^{-1})	100	100
Cellulose + Sucrose (5 mM)	100	50
Cellulose + Glucose (5 mM)	85	67

The effect of cations in enzyme activity

Figure 2 shows the activity of cellulase assayed in the presence of different metal ions. As shown in Figure 2, both enzymes were inhibited in the presence of Hg^{2+}. An increase of 40% of the endoglucanase activities is shown in presence of $CuSO_4$ and $FeSO_4$ and a reduction of 20–30% of the exoglucanase activities. Zn^{+2} and Pb^{+2} (500 μg.L^{-1}) did not have any effect on cellulase production by *Aspergillus terreus*.

The effect on COD removal by biomass and the enzymes of Aspergillus terreus

The effect of 5g.L^{-1} dryweight cell biomass of *Aspergillus terreus* was tested on COD removal of 20 L tank. The fungus reduced the COD of sugar wastewater and pulp industry by 40–80 percent. Also ammonia was reduced from 14.5 mM to 5.6 mM in sugar beet wastewater (Table 2).

The effect of 1 ml crude concentrated enzymes (1800 U.ml^{-1} CMCase and 18 U.ml^{-1} Fpase and 135 μg.mL^{-1} of protein) on to 30 ml of pulp waste was

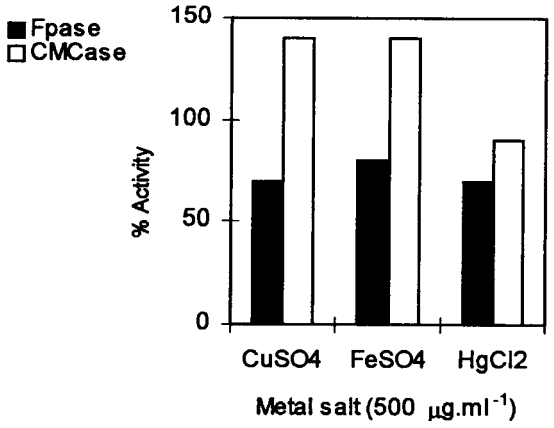

Figure 2. The effect of 500 μg ml^{-1} heavy metals ($HgCl_2$, $FeSO_4$ and $CuSO_4$) on stability of CMCase and Fpase.

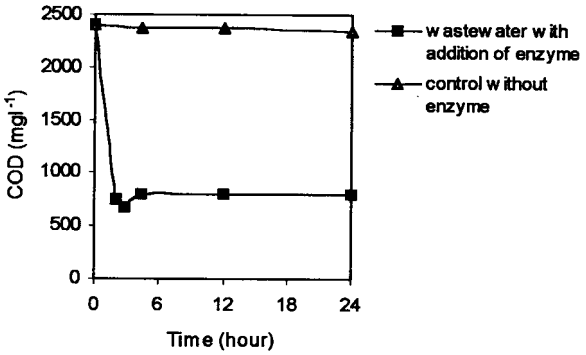

Figure 3. The effect of 1 ml crude concentrated enzyme (1800 U.ml^{-1}) CMCase and 18 U.ml^{-1} Fpase activities) on biodegradation of 30 ml pulp waste. As it showes COD removal of pulp waste was 70%. (The data are average of duplicated experiments).

investigated (Figure 3). It was shown that the crude enzyme could reduce the COD of pulp waste by 70% in 2 hours. Addition of 2 ml enzyme did not have any more effect on reduction of COD. The concentration of COD was stable after 2–24 hr, which showed that the crude enzymes could remove most of the COD in the first 2 hours treatments.

Discussion

One of the most important renewable resources and environmental contamination is cellulose. For this reason its conversion in wastewater is of considerable interest. Winter in 1980 (Winter & Cooney 1980) showed that a mixed culture enriched from sewage sludge and anaerobic digestor effluent was able to degrade cellulose to methane. He used a fed batch fermentation system to study the degradation of cel-

Table 2. Removal of COD from treated wastewater by *Aspergillus terreus*

	Sugar beet wastewater			Pulp industry wastewater		
	BOD5	COD	Ammonia (mM)	BOD5	COD	Ammonia (mM)
Wastewater	3020	6874	14.5	3000	6021	12
Effluent wastewater	400	1254	16.9	2800	3083	15
Effluent of treated wastewater with *Aspergillus terreus*	240	754.5	5.6	560	606.6	3.4

Data are the average of 3 experiments.

lulose with cultures enriched from sewage sludge. Here, natural media have been used for *Aspergillus terreus* to produce cellulase enzymes. Although high enzyme productions have been obtained and the media used were inexpensive, but using *Aspergillus terreus* to clear up cellulose in wastewater may cause another environmental contamination. The enzymes of this fungus are resistant to most cations, therefore the crude concentrated enzymes of this fungus not only have no negative effect on environment, but are also resistant to different environmental conditions. Also degradation of azo dyes in textiles wastewater by crude enzymes of this fungus was reported (Emtiazi et al. 2000; Habibi et al. 1998). In a study (Emtiazi, 1999) it was shown that the enzymes of this fungus could degrade fiber in pulp and sugar beet wastewater, pH (5–7), temperature (30–50 °C) and addition of inoculated enzymes (up to 3 ml) did not have any significant effects on biodegradation of cellulose in wastewater. It was also shown (Emtiazi, 1999) that *Aspergillus terreus* had peroxidase and laccase activities when grown on wheat straw and removed COD of textile dyes effluent. Decarboxylation of vanillic acid and oxallic acid by laccase and peroxidase is reported by Akamatsu et al. (1990) and Agemata et al (1991). Dezotti and co-workers (1995) showed that lignin peroxidase catalyzed 65% removal of COD from kraft effulent. Also Itavara and co-workers (1994) reported that a mixture of *Trichoderma reesei* culture filtrate, purified endoglucanase and β-glucosidase of *Aspergillus niger* degraded 70% of cellulose material to carbon dioxide. Also in a non-sterile system the dissolved organic carbon (DOC) released by the enzymes is subsequently utilized by the microbial community. Therefore in this study we have concluded that 70% COD removal of wastewater is due to the presence of mixed enzymes in the culture filtrated of *Aspergillus terreus* and microbial utilization.

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