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Characterization of extracellular and substrate-bound cellulases from a mesophilic sugarcane bagasse-degrading microbial community

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ABSTRACT

Sugarcane bagasse (SCB) is a residual product of sugar processing, containing mainly lignocellulose. The biodegradation of cellulose is an economical approach to recycling SCB. To obtain an effective microbial community and study the SCB biodegradation process, a SCB-degrading mesophilic microbial community, EMSD13, growing at 50 °C was isolated. More than 77% of alkali pretreated SCB was degraded and over 83% of the cellulose it contained was utilized by the EMSD13 community within 6 days. To understand the biodegradation procedure of EMSD13, extracellular and SCB-absorbable cellulases were analyzed. The peak activity (42 mU/mL) of the extracellular cellulase in the supernatant occurred on the 3rd day of incubation. About 73.6 mg of substrate-bound proteins with a cellulase activity of 61.1 mU/mg were recovered from residual SCB by eluting with 3 M guanidine hydrochloride. Also, 15.9 mg of proteins with 28.6 mU/mg of cellulase activity were obtained from residual SCB by eluting with 6 M urea. SDS-PAGE analysis showed that cellulases in both elutes were similar, but most differed from those in the supernatant. This is the first report on substrate-bound cellulases from a microbial community.

1. Introduction

Sugarcane bagasse is the residue obtained after crushing of sugar cane during sugar production and contains mainly lignocellulose. The annual global production of dry cut sugarcane (sugar content: 55% dry basis) is about 328 Tg, and Asia (with 44%) is the primary production region [1]. About 180–280 kg of bagasse per ton of sugarcane could be produced after squeezing [2]. In China, sugarcane was planted in about 1368.2 kha in 2004, and the dry weight of sugarcane bagasse was over 9,000,000 t. The abundant sugarcane bagasse is a promising feedstock for industrial production of low-cost fuel ethanol [3,4] since it has high carbohydrate and low lignin content, and could also be used as low-cost sorbents for the removal of organic and heavy metal contaminants.

Many microorganisms are capable of degrading cellulosic materials, and the degradation has been well studied in the past decades [5]. Several cellulose-degrading microbial strains have been isolated [6], but they could not be widely used in industry for the disadvantage of feedback regulation and metabolite repression [7]. Thus, development of mixed cultures has been attempted to increase the production of enzymes and the digestion of cellulose due to the stability and high efficiency of mixed cultures [7,8]. However, not all effective strains in mixed cultures could be isolated and incubated [5] and this is a limitation to its use in the industry.

In nature, many cellulosic materials could be degraded rapidly by the microbial community, such as the rumen ecosystems, which was over ten times more rapid than the commercial digester [9]. For example, Haruta et al. [10] obtained a microbial community which was effective in degrading cellulosic materials such as paper, cotton and rice straw. These studies indicated an efficient way of biodegradation of cellulosic materials using a native microbial community. For now, little information is available on SCB biodegradation by a microbial community.

In the present study, an effective microbial community using SCB as the sole carbon source was obtained, and its cellulose

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degradation ability evaluated. To better understand the mechanism of the highly effective degradation achieved by this microbial community, extracellular and substrate-bound cellulases were characterized.

2. Materials and methods

2.1. Samples and chemicals

Organic samples including decayed straws, composts, cattle dung, and forest soils were collected in mid August from Jilin, Heilongjiang, Inner Mongolia, Hebei and Shandong provinces as well as Chongqing City of China. Samples were stored at 4 °C until use.

Carboxymethylcellulose (CMC), Avicels®, bovine serum albumin and chemicals used for SDS-PAGE were purchased from Sigma. Tryptone and yeast extract were obtained from OXOID. All other chemicals were of analytical grade and obtained locally.

2.2. Isolation of microbial community

PCS medium [10] was used for the isolation of microbial community and was prepared as follows: 5 g of tryptone, 5 g of filter paper (Whatman No. 4, cut into 3 cm × 1 cm), 5 g of NaCl, 2 g of $CaCO_3$, 1 g of yeast extract, and 1 L of dH_2O with pH 7.0. Aliquots of 150 mL PCS medium supplied with 1.3 g pretreated SCB by alkali (0.5% NaOH) [8] were autoclaved at 121 °C for 30 min in 150 mL flasks. A 0.5-g biological sample was added in each flask and incubated under static condition at 50 °C [10]. The culture inoculated directly with biological sample was identified as F_0. When the filter paper began to degrade, 5 mL (about 5×10^8 cells/mL) of each culture (F_0) was transferred into fresh medium with SCB, as described above, and the new culture was identified as F_1. This procedure was repeated until the degradation ability was stable, and the final culture was identified as Fn. Thereafter, the microbial community with high efficiency in degrading filter paper was stored in 25% (v/v) of glycerol at −20 and −70 °C. After 6 months, the residual biodegradation ability of all stored microbial communities (identified as Fa) was checked. The community able to degrade filter paper in less than 72 h and remain stable during storage was used for the biodegradation of SCB.

2.3. Growth conditions and biodegradation rate

To determine biodegradation, 5 mL of the seeding culture (about 5×10^8 cells/mL) was added to 150 mL of PCS medium in which the filter paper was replaced with alkali pretreated SCB. The cultures were incubated under static condition at 50 °C and then the residual SCB were filtered through preweighted filter paper (Whatman No. 1) [7]. The residue was washed three times with distilled water and dried to constant weight at 95 °C. The filtered cell suspension was used to determine the bacterial growth by measuring turbidimetrically at 600 nm and converted to dry cell weight using a calibration curve. All determinations were performed in three independent experiments.

2.4. Cellulose content of SCB

The α-cellulose content in residual SCB was determined at least thrice using a colorimetric method with the anthrone reagent [11,12].

2.5. Scanning electron microscopy (SEM)

A scanning electron microscope (Hitachi S-570 microscope) was used to observe the SCB residue as described in Beukes and Pletschke [13].

2.6. Protein and cellulase activity assay

Protein was quantified by the Lowry method [14] using bovine serum albumin as standard. Reactions for CMCase (carboxymethylcellulosase) and Avicelase assays were prepared using amorphous substrates CMC and crystalline substrates (Avicels®) as described in Beukes and Pletschke [13]. The absorbance was read at 520 nm and one unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of glucose equivalents per min under given conditions. Suitable controls were performed to assess the activities, and the reactions were performed in triplicate.

2.7. Extraction of extracellular and SCB-absorbable cellulases

The cultures containing SCB were incubated at 50 °C for 72 h and then centrifuged at low speed (1650 × *g*) at 4 °C for 10 min to separate the SCB residual. Then, the supernatant was centrifuged at 10,000 × *g* at 4 °C for 10 min to separate cells from the culture supernatant. Finely powdered $(NH_4)_2SO_4$ was added to the culture supernatant to 80% saturation. After 2 h on ice, the precipitate was collected by centrifugation, redissolved in 10 mM Tris–HCl buffer (pH 7.0) and dialyzed to remove $(NH_4)_2SO_4$. After dialysis nondissolved protein was removed by centrifugation at 10,000 × *g* for 10 min at 4 °C and the clear supernatant was concentrated by lyophilization.

The residual SCB was resuspended in 1 M NaCl at a solid–liquid ratio of 1–5 and then centrifuged at 5000 × *g* at 4 °C for 15 min. This washing step was repeated with distilled water, instead of 1 M NaCl. Then, the SCB residual was resuspended in two volumes of 3 M guanidine hydrochloride and incubated at 4 °C for 18 h with shaking at 150 rpm. The supernatant was recovered by centrifugation (5000 × *g* at 4 °C for 15 min), dialyzed against 15 mM sodium acetate buffer of pH 6.0 at 4 °C and concentrated by lyophilization. The pellet was resuspended in five volumes of 50 mM sodium acetate buffer of pH 6.0 and centrifuged at 5000 × *g* at 4 °C for 15 min. Then, the pellet was resuspended in 6 M urea at a solid–liquid ratio of 1–2 and incubated at 4 °C for 18 h with shaking at 150 rpm. The supernatant was recovered by centrifugation (5000 × *g* at 4 °C for 15 min), dialyzed against 15 mM sodium acetate buffer of pH 6.0 at 4 °C and concentrated by lyophilization [15].

2.8. SDS-PAGE analyses

The protein samples were analyzed by SDS-PAGE as described in Laemmli [16] using 12.5% separating gel. Protein bands were detected with a silver stain.

2.9. Activity staining of CMCase

For activity staining of CMCase after SDS-PAGE, the 12.5% separating gel containing 0.1% carboxymethylcellulose was prepared. After the protein samples (30 μg) were electrophorezed, the proteins in SDS gel were renatured by incubation overnight in three periodic rinses of 250 mL of 10 mM Tris–HCl (pH 7.0), 1% Triton-X 100. Finally, the gel was incubated in 10 mM Tris–HCl (pH 7.0) at 37 °C overnight. Then the gel was immersed in 0.5% Congo red for 15 min and destained with 1 M NaCl for 15 min. Active bands appeared as yellow halos on a red background [17].

3. Results and discussion

3.1. Isolation of microbial community

Among the samples used in this study, effective cellulose degrading microbial communities were found in compost, cattle dung and decomposed straw samples. When the samples were add into the medium, sample EMHN1, EMSD12–15, EMGX1, EMCBS1 and EMCBS2 had evident biodegradation effects, in which EMSD13, EMSD12, EMHN1 and EMCBS2 could degrade filter paper in 72 h at 50 °C, as shown in Table 1. Only sample EMSD13 from compost and sample EMCBS2 from Changbaishan maintained a steady biodegradation effect after continuous sub-culturing. Upon storing for 6 months to 1 year at −20 or −70 °C, EMSD13 retained a stable degradation effect on filter paper while EMCBS2 did not. Thus, EMSD13 was used for further study. The high degradation ability and stability, as reported in Haruta et al. [10], might be due to the coexistence of multiple cellulolytic and noncellulolytic species [18]. The stable microbial community obtained in the present

Table 1
Results of the construction of microbial community from different samples

No.	Resource	F_0[a]	F_1[b]	Fn[c]	Fa[d]
EMCQ1	Forest soil from Chongqing	–[e]	–	–	–
EMCQ2	Farmland soil from Chongqing	–	–	–	–
EMHN1	Cattle dung from Heilongjiang	++[f]	+[g]	–	–
EMSD13	Compost from Shandong	++	++	++	++
EMSD12	Cattle dung from Shandong	++	+	+	–
EMSD15	Decayed corn straws	+	–	–	–
EMSD14	Decayed wheat straws	+	–	–	–
EMCBS1	Soil from Jinlin Changbaishan	+	–	–	–
EMCBS2	Soil from Jinlin Changbaishan	++	++	++	+
EMCBS3	Soil from Jinlin Changbaishan	–	–	–	–
EMGX1	Soil from Guangxi forest	+	–	–	–
EMGX2	Soil from Guangxi farmland	–	–	–	–

[a] F_0 denotes fermented broth with sample.
[b] F_1 denotes new fermented broth transferred from F_0.
[c] Fn denotes the status after continuous transfer for 10 times.
[d] Fa denotes the status of seed activated after storing.
[e] – denotes that the filter paper could not be degraded.
[f] ++ denotes that the filter paper could be degraded in 72 h.
[g] + denotes that the filter paper could be degraded in 120 h.