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Lactose Enhances Cellulase Production by the Filamentous Fungus *Acremonium cellulolyticus*

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Acremonium cellulolyticus **is a fungus that produces cellulase and has been exploited by enzyme industry. To promote cellulase production by *A. cellulolyticus* strain C-1, we evaluated the effects of the saccharides: Solka Floc (cellulose), soluble soybean polysaccharide (SSPS), pullulan, lactose, trehalose, sophorose, cellobiose, galactose, sorbose, lactobionic acid, and mixtures as carbon sources for cellulase production. Solka Floc with SSPS enhanced cellulase production. Lactose as the sole carbon source induced cellulase synthesis in this fungus, and the synergistic effects between lactose and Solka Floc was observed. Various enzyme activities and the protein composition of crude enzyme produced by cultures with or without addition of lactose were analyzed. The results showed that lactose addition greatly improves the production of various proteins with cellulase activity by *A. cellulolyticus*. To our knowledge, this is the first report on production of cellulases by lactose in the *A. cellulolyticus*.**

[**Key words:** bioethanol, lactose, cellulolytic enzymes, *Acremonium cellulolyticus*]

The impending depletion of fossil resources and fossil fuel combustion as the primary cause of global warming (1), have sharply increased global demand for bioethanol, which is the most promising renewable and carbon neutral alternative liquid fuel, since the Kyoto Protocol was established in February 2005. Thus, technology to produce bioethanol should be developed based on the cellulolytic biomass, which is an abundant, renewable and underutilized global carbon source. The cellulosic biomass must be hydrolyzed to fermentable sugars by cellulolytic enzymes or acids to produce bioethanol. Enzymatic hydrolysis is environment-friendly and can achieve high sugar yield for ethanol fermentation, and thus should be useful for industrial applications. However, the high cost of cellulases presents a key barrier to the economical enzymatic production of bioethanol from lignocellulose materials.

Cellulase is an enzyme complex found in some fungi and bacteria. Cellulases from *Trichoderma* and *Aspergillus* species have been investigated in detail over the past few decades. However, little is understood about cellulases from *Acremonium cellulolyticus*. This organism was isolated in 1982 from soil in northeastern Japan, and it secretes sufficient amounts of cellulolytic enzymes to completely convert cellulose materials to glucose (2). The involved enzymes have notably higher β-glucosidase activity than cellulases from *Trichoderma* species (2). Mutant strains have been engineered to produce cellulase and hemicellulase (Fukasawa, T. *et al.*, U.S. patent, 20040091469, 2004; Nojiri, C. *et al.*, Japanese patent, 2003-174892, 2003).

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Although most microbial cellulases are induced in the presence of cellulose, cellulose itself probably cannot directly trigger the induction because it is insoluble. It has been postulated that a basal level of cellulases production, which occurs in the absence of glucose, can degrade cellulose and generate smaller inducing molecules in *Trichoderma reesei* (3). Then soluble saccharides such as cellobiose, sophorose, lactose, trehalose, sorbose, galactose, and lactobonic acid might serve as inducers of cellulases synthesis in *T. reesei*, *Clostridium papyrosolvens*, *Acidothermus cellulolyticus* (4–12). Furthermore, the induction of cellulase activity has been investigated using various concentrations of inducers and cellulose culture media (8, 13). However, few studies have examined the effect of soluble saccharide on cellulase production in *A. cellulolyticus*.

To further understand the mechanism of cellulase synthesis, cellulase induction in *A. cellulolyticus* should be investigated. At the same time, an efficient inducer should be identified to include in culture media to enhance cellulase production. The present study investigates cellulase production in the filamentous fungus *A. cellulolyticus* strain C-1 using different carbon sources and mixtures of these sources. We analyzed various enzyme activities and the protein composition of crude enzyme. We also discuss the possible mechanism of synergism among carbon sources on cellulase production in *A. cellulolyticus* based on our results.

MATERIALS AND METHODS

Fungal strain *A. cellulolyticus* C-1 (FERM P-18508) obtained from Tsukishima Kikai Co. Ltd. (TSK; Tokyo) is a cellulase hyper-producing mutant (Yamanobe, T. *et al.*, Japanese patent,

2003-135052, 2003). The strain was inoculated on potato dextrose agar (PDA; Difco, Sparks, MD, USA) slants, incubated for 7 d at 30°C and then stored at 4°C.

Culture medium The media were based on those described by Ikeda *et al.* (14) and contained the following (in grams per liter): carbon source, 50 or 60; KH_2PO_4, 24; corn steep liquor (only in preculture), 10; Tween 80, 1; $(NH_4)_2SO_4$, 5; potassium tartrate hemihydrate, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; $ZnSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 6H_2O$, 0.01; $CuSO_4 \cdot 7H_2O$, 0.01; urea, 2 (in preculture) or 4 (in production culture). The pH was adjusted to 4.0 with H_2SO_4 and KOH before sterilization. Distilled water and all components of the production medium, except $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 6H_2O$, $CuSO_4 \cdot 7H_2O$ and urea were sterilized at 121°C for 20 min. We mixed $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 6H_2O$, $CuSO_4 \cdot 7H_2O$ in distilled water and separately sterilized the mixture. The appropriate amount of urea in distilled water was passed through a sterilized 0.22 μm microfilter (Millex GV; Carrigtwohill, Cork, Ireland).

D-Glucose (40 g/*l*) as a sole carbon source was added to the preculture. Carbon sources included in the production culture were Solka Floc (80% pure cellulose; CAS 9004-34-6; Fiber Sales and Development, Urbana, OH, USA) as a cellulose resource, soluble soybean polysaccharide (SSPS; Soyafibe S-DN; Fuji Oil, Osaka), pullulan, lactose, trehalose, cellobiose, sophorose, galactose, sorbose, lactobionic acid and mixtures of these. Other chemicals of analytical grade were purchased from either Wako Pure Chemical Industries (Osaka) or Nacalai Tesque (Kyoto).

Culture conditions Two colonies of *A. cellulolyticus* strain C-1 were inoculated into test tubes (ϕ16.5×165 mm) containing 5 ml of preculture medium and shaken (200 shakes per min; spm) in a reciprocal shaker (NR-10; Taitec, Saitama) at 30°C for 2 d. Thereafter, 0.5 ml of preculture was inoculated into 100-ml Erlenmeyer flasks containing 10 ml of production medium with various saccharides. The *A. cellulolyticus* strain C-1 was cultivated with Solka Floc at the same saccharide concentration as control in every experiment. The culture was rotated and 220 revolutions per min (rpm) in a rotary shaker (BR-42FL; Taitec) at 30°C for various periods. Samples were removed after various intervals and centrifuged to remove mycelia and undigested cellulose. The supernatants were analyzed after centrifugation as crude enzyme preparations.

Enzyme assay The filter-paper (FPase) activity (FPA) of cellulase was measured as described by Ghose (15) and recommended by the Commission of Biotechnology, IUPAC. The activity of CMCase was assayed based on that of Mandels *et al.* (16). Appropriately diluted supernatant and 0.5 ml of carboxymethylcellulose (CMC, 2% w/v) in citrate buffer (50 mM, pH 4.8) were mixed in equal volumes, and then the enzyme reaction proceeded at 50°C for 30 min. Avicelase activity was determined under similar conditions, except that the enzyme reaction proceeded for 2 h with 1.0 ml of acetate buffer (0.1 M, pH 4.8), 10 mg of avicel as a substrate and 1.0 ml of diluted supernatant. The reduced sugars released were analyzed using the DNS assay. One unit of enzyme activity was defined as the amount required to produce 1 μmol of reducing sugars in 1 min.

We measured β-glucosidase activity as follows. Diluted supernatant and 1.0 ml of 15 mM cellobiose in citrate buffer (0.05 M, pH 4.8) were mixed in equal volumes, and then the enzyme reaction that proceeded at 50°C for 30 min was terminated by heating at 100°C for 5 min. The glucose concentration in the supernatant was measured using a laboratory test kit (Glu-CII; Wako Pure Chemicals). The amount of enzyme at which reducing sugar is produced in an amount corresponding to 2 μmol of glucose or 1 μmol of cellobiose per min is defined herein as 1 unit (IU).

Protein concentration The soluble protein concentration was determined by the method of Lowry *et al.* (17).

Measurement of intracellular adenosine triphosphate (ATP) The ATP concentration reflects fungal growth (18) and was determined using a kit (CheckLite 250 Plus; Kikkoman, Tokyo) according to the manufacturer's instructions and the Lumitester (C-100; Kikkoman) based on the luciferin-luciferase reaction.

Sizing and analysis of protein from crude enzymes Proteins were sized and analyzed according to the instruction manual provided with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) in combination with Protein 230 assay software (Version B.02.05; Agilent Technologies) and the Protein 230 LabChip Kit (Agilent Technologies), which contains chips and reagents. Each chip contains an interconnected set of gel-filled microchannels that electrophoretically separate proteins by size.

RESULTS

Fungal growth and cellulase production with various saccharides The maximal ATP concentration in the cultures was observed after 1 d of cultivation in the presence of 1.0% (w/v) various saccharides and the maximal FPA of the supernatant was observed after 5 d. The ATP concentration was increased with all carbon sources although to various degrees (Table 1). These findings showed that *A. cellulolyticus* utilized all carbon sources for mycelial growth. Lactose was a good water-soluble carbon source for cellulase production by *A. cellulolyticus* followed by Solka Floc (cellulose) because a high ATP concentration indicated good fungal growth and a high FPA proved the powerful induction of extracellular cellulase activity (Table 1). *A. cellulolyticus* did not secrete any detectable cellulases in the presence of any other saccharides except cellobiose.

Effects of water-soluble saccharides on cellulase production Cellulose was the optimal carbon source for cellulase production among the sources tested (Table 1), but cellulose is insoluble and therefore cannot be taken up by *A. cellulolyticus* for cellulase synthesis. We therefore examined the effect of the water-soluble polysaccharides, SSPS and pullulan, on cellulase production. SSPS is produced from fibrous bean curd residue (Okara) and pullulan is produced by microorganisms. Figure 1 shows that improvement of total saccharides concentration from 5.0% (w/v) to 6.0% enhanced increasing of FPA. However, it is notable

TABLE 1. Maximum intracellular adenosine triphosphate (ATP) concentration in cultures after 1 d of incubation and maximal filter paper activity (FPA) of supernatant after 5 d with various saccharides of 1.0% at 30°C by *A. cellulolyticus*

Saccharides	ATP (mmol/*l*)	FPA (U/ml)
Solka Floc[a]	10.2±1.2	3.4±0.4
SSPS[b]	6.5±0.5	ND[c]
Pullulan	13.3±0.8	ND
Trehalose	9.2±0.6	ND
Lactose	8.5±0.4	1.2±0.2
Cellobiose	12.8±0.2	0.7±0.2
Sophorose	7.4±0.4	ND
Lactobionic acid	1.6±0.3	ND
Sorbose	1.9±0.2	ND
Galactose	6.4±0.2	ND

The values are mean (n=3)±standard deviation.
[a] Solka Floc contains 80% pure cellulose.
[b] SSPS, Soluble soybean polysaccharide (see Materials and Methods for details).
[c] ND, Below 0.37 U/ml.