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Extracellular Cellulolytic Enzymes of *Bacillus circulans* Are Present as Two Multiple-Protein Complexes

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ABSTRACT

The cellulase system of *Bacillus circulans* F-2 effectively hydrolyzed carboxymethyl cellulose (CMC), xylan, avicel, cellobiose, filter paper, cotton, and *p*-nitrophenyl-β-D-cellobioside, and the crude enzyme produced mainly glucose from digestion of avicel. Two major and one minor peaks of enzyme activities were eluted on DEAE ion-exchange chromatography, and designated cellulase complex I(C-I) and complex II(C-II) for the two major peaks, and cellulase-III for a minor peak. C-I and C-II were further purified on gel filtration column of a TSK-Gel SW G3000 xL. The molecular masses of C-I and C-II were estimated to be about 669 and 443 kDa, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the C-I and C-II complexes showed that the C-I complex was present as a multiple protein complex, consisting of at least five CMCases and two xylanases, and that the C-II complex was consisted of at least three CMCase and four xylanases. C-I showed high activities of cellohydrolase, CMCase, xylanase, and β-glucosidase, whereas C-II showed high activities of CMCase, xylanase, avicelase, and β-glucosidase. The outstanding property of the C-II was its high hydrolytic activity toward filter paper, a highly resistant substrate against enzymatic degradation. However, cellulase-III showed only strong avicelase activity. These results indicated that the cellulase system of the strain exists as multiple complex forms.

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Index Entries: Endo-β-glucanase; β-glucosidase; Avicelase; filter paper-hydrolase; enzyme complex; cellulo-xylanosome; *Bacillus circulans* F-2.

Abbreviations: CMC, carboxylmethyl cellulose; FP, filter paper; kDa, kilodalton; DNS, 3′,5′-dinitrosalicylic acid; pNPC, *p*-nitrophenyl-β-D-cellobioside; pNPX, *p*-nitrophenyl-β-D-xylopyranoside; pNPG, *p*-nitrophenyl-β-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; CBH, cellobiohydrolase; SDS, sodium dodecyl sulfate.

INTRODUCTION

Cellulase (EC 3.2.1.4) is produced by some fungal species (*1–4*) when grown on appropriate media and by certain bacteria (*5–7*). There have been many papers about the cellulolytic enzymes, its producing microorganisms, industrial usages, and multiplicity of cellulases (*8,9*). Of the cellulase producing microorganisms, *Trichoderma reesei* was known to be the best producer of cellulases (*10,11*). Some attempts on the breeding in combination with optimization of the culture conditions has been long done to increase the cellulase productivity and stability (*12*).

Microorganisms synthesise a number of different enzymes in order to utilize cellulose or hemicellulose since they exist as complex substrates. In some microorganisms many of these enzymes are found in a cell-associated complex called the cellulosome (*13*) or xylosome (*14*). Other microorganisms appear to secrete their cellulases into the culture fluid. Two enzyme complexes containing 18 endoglucanases have been found in the culture fluid of *R. flavefaciens* FD-1 (*15*), whereas *Clostridium* strain C7, *Cellulomonas fimi*, and *Trichoderma* sp. produce extracellular cellulase complexes (*16,17*). The cellulase complex is a multicomponent complex with an extraordinary capacity to degrade crystalline cellulose, producing glucose or cellobiose as the nearly exclusive product. Even though this complexity of the multicomponent cellulolytic system secreted by microorganisms is the subject of current investigation and speculation, relatively little is known of the nature of the enzymatic components that account for the marked superiority of the bacterial cellulase system. Some of the problems relate to the multienzyme complex of the cellulase of bacteria. Other problems associated in part with examining isolated components of the system arise from the anaerobic nature of some *Clostridium* sp.

There is thus still much to be learned of the cellulase system before successful application to biotechnological processes. The complete saccharification of cellulosic materials into glucose units became the most important and basic condition for the cellulose fermentation process. To produce glucose exclusively from cellulose, it needs strong β-glucosidase activity in reaction solution that is therefore important for cellulose fermentation.

B. circulans F-2 is a bacterium that produces several carbohydrate-related enzymes such as raw starch-digesting amylase (*18,19*), α-glucosidase (*20*), and a bifunctional amylase-pullulanase (*21,22*), and the bacterium has a very interesting synthetic system of those enzymes, which is sensitively regulated by glucose catabolite repression (*23*). Recently, in the course of study on carbohydrase system of this bacterium, it was found that *B. circulans* F-2 produces crystalline cellulose-degrading cellulase on the medium containing avicel as a carbon source. Here we describe the isolation and analysis of *B. circulans* F-2 β-glucanases that are present in the forms of cellulase complexes.

MATERIALS AND METHODS

Materials and Microorganisms

Cellulose powder, laminarin, carboxymethyl cellulose (with a grade of substitution, DS=0.65 and average mol wt=110,000) and Avicel SF were obtained from Tokyo Kasei Co. (Tokyo, Japan). Oat spelt xylan, *p*-nitrophenyl-β-D-cellobioside (pNPC), *p*-nitrophenyl-β-xylopyranoside (pNPX), *p*-nitrophenyl-β-D-glucopyranoside (pNPG), and cellobiose were purchased from Sigma Co. (St. Louis, MO). Filter paper (1×0.6 cm^2) was from Whatman Co. (Maidstone, UK). Other chemicals used were of the purest grade commercially available.

B. circulans F-2, which was isolated from potato starch granules by Taniguchi et al. (*18*) and which has been deposited in our laboratory, was used in this study. The culture was prepared with Avicel SF in Medium F-2 (*18*) supplemented with 2 mg of each $MnSO_4 \cdot H_2O$ and $ZnSO_4 \cdot 7H_2O$/L. Cultures were incubated with vigorous shaking at 45°C. Culture supernatants were used as crude enzyme preparations and could be stored at −20°C in the presence of 0.03% (wt/vol) sodium azide for several weeks without any appreciable loss of enzyme activity.

DEAE-Ion Exchange and Gel Filtration Chromatography

The concentrated enzyme solution was applied to a DEAE Toyopearl M650 column (1.6×64 cm) previously equilibrated with 0.05*M* Tris-HCl buffer (pH 8.0). After washing with the equilibration buffer, the adsorbed materials were eluted with a 600-mL linear gradient of 0.1*M* to 0.6*M* NaCl in the same buffer. Fractions (10 mL) were collected at a flowrate of 36 mL h^{-1}. The fractions from the two activity peaks, C-I and C-II, were concentrated by ultrafiltration through a PM30 membrane (millipore) and then each of C-I and C-II was applied to a HPLC TSK Gel Toyopearl HW-65S column (1.6×90 cm; Tohso Co., Tokyo, Japan) equilibrated with 50 m*M*

sodium chloride. Protein was eluted with the equilibrating buffer (flowrate 15 mL h^{-1}).

Enzyme Assays

CMCase or xylanase activity was determined as described previously (*5*) using the dinitrosalicylic acid (DNS) method (*24*). One unit of each activity corresponds to the release of 1 μmole equivalents of reducing groups/min. For detection of β-glucosidase, β-xylosidase, and pNPC-hydolase activities, the substrates of pNPG, pNPX, and pNPC were used according to the method of Desphande et al. (*25*): One unit of enzyme activity is defined as the amounts of enzyme that liberated 1 μmole of pNP in 1 min at 40°C. For hydrolysis of cellobiose, the glucose released was determined by the glucose-oxidase method (Glucose-oxidase detectiom kit AR-II, Wako Co., Tokyo, Japan). Avicelase or filter paper-hydrolyzing activity (FPase, F.P.U.) (*26,27*) was assayed as described previously (*5*). One unit of each activity corresponds to the release of 1 μmole of glucose equivalent/min (*5*). The procedure for measuring the activities of other glycan-hydrolyzing enzymes was essentially the same as described above.

Analytical Methods

Protein content was determined by the method of Lowry (*28*) with bovine serum albumin as the standard. Protein in the column eluants was routinely followed by the absorbance at 280 nm. The amount of total sugars was determined by the phenol-sulfuric acid method (*29*) with cellobiose as the standard. The effect of pH and temperature on enzyme activities was measured as described previously (*30*). The following Mr standards were used to calibrate the column: blue dextran, 2000 kDa, thyroglobulin, 669 kDa, apoferritin, 443 kDa, and catalase, 232 kDa (Sigma Co.). Polyacrylamide gel electrophoresis (PAGE) was performed in 7.5% (w/v) gels with or without SDS by the method of Laemmli (*31*). CMCase or xylanase activities in mildly denatured gels were detected as described (*14*). For determination of mol wt, the standards (Bio-Rad) used were myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin. Time course hydrolysis of CMC, cellobiose, xylan, avicel, filter paper, and cotton was carried out according to our previous method (*5*).

RESULTS AND DISCUSSION

Cellulase Production and Hydrolysis of Cellulosic Substrates by Crude Enzyme

The bacterium *B. circulans* F-2 is unique in that it produces cellulases when it is preferently cultivated on medium containing raw substrates of

avicel and xylan even though soluble CMC can induce *B. circulans* F-2 cellulase to some extent. The bacterium did not produce cellulase when it was cultivated on medium containing soluble oligosaccharides such as glucose, fructose, and cellobiose, resulting from possible catabolite repression of the enzyme biosynthesis (data now shown). As shown in Table 1, the enzyme system produced by *B. circulans* F-2 was capable of hydrolyzing various kinds of glycans. The important aspect of the enzyme system ws 41 U/mL of cellobiose-hydrolyzing activity as β-glucosidase activity. The culture broth hydrolyzed filter paper and cotton with higher extents than that of other *Bacillus* sp. (*32–35*). It was also found that the reducing sugars (40%) from xylan hydrolysis was greater than that released from other substrates such as filter paper, cotton, and Avicel (data not shown), indicating that the enzyme system may be able to degrade other insoluble materials such as hemicellulose and laminarin, because of its high xylanase activity. Avicel is less degraded and closer to the forms of cellulose expected in nature although less complex than that of cotton or filter paper. Nevertheless, Avicel offers itself as a useful substrate for examining cellulolysis, provided its limitations are recognized (*36*). Approximately 95% (0.4 U/mL) of the Avicelase activity was present in the fluid of cultures of the strain (late-log phase), with the remainder being cell-associated (data not shown). The amount of reducing sugar, soluble sugar, and glucose in hydrolysis of Avicel SF with the culture filtrate was further investigated: The enzymatically digested product of microcrystalline cellulose avicel was almost glucose (Fig. 1). Therefore, it appeared that cellulolytic enzyme system produced by *B. circulans* F-2 shows superior characteristics of solubilizing activity of crystalline cellulose powder to produce the almost soluble sugar that was exclusively glucose. This result indicates that the *B. circulans* F-2 cellulase system is useful for the direct saccharification of raw crystalline cellulose to the glucose production. Of well-known cellulase-producing microorganisms, the *Aspergillus* (*37,38*) and *Clostridium* (*7*) genus are known to distribute β-glucosidase activity and insoluble cellulose-degrading activity. However, the amounts of these enzymes produced are not enough for successful saccharification of cellulosic materials.

Behavior of Cellulase Components on DEAE Anion and Gel Chromatography

Preliminary experiments involving DEAE ion chromatography of culture supernatant fluids indicated that cellulase activities were associated only when high-mol wt protein fractions (data not shown). Two major peaks of enzyme activity were resolved by DEAE ion chromatography and designated complex I (C-I) and complex II (C-II). On the other hand, one minor peak of enzyme activity, named cellulase-III, was eluted between C-I and C-II (Fig. 2). C-I had CMCase, pNPC-hydrolase, β-glucosidase, and xylanase activity, whereas C-III showed CMCase, xylanase, Avicelase,

Table 1
Enzyme Activities of the Culture Broth, C-I Complex, C-II Complex, and Cellulase-III

Enzyme	CMCase[a]	Avicelase[a]	FPase[a]	Xylanase[a]	β-1,3-Glucanase[a]	β-Glucosidase[b]	PNPC-Hydrolase[b]	β-Xylosidase[b]
C_B[c]	102.5	0.4	0.3	142	1.4	41	0.07	2.9
C-I[d]	652.4	< 0.1	< 0.1	137	< 0.01	62	0.19	2.2
C-II[e]	852.1	0.7	0.5	252	3.2	88	N.D	6.2
Cel-III[f]	12.1	0.6	0.5	< 0.1	< 0.01	< 0.01	N.D.	N.D.[g]

[a]Reducing sugar, μmol min^{-1} mg^{-1} protein.
[b]pNP, μmol min^{-1} mg^{-1} protein.
[c]C_B, Culture broth.
[d]C-I, Complex-I.
[e]C-II, Complex-II.
[f]C-III, Cellulase-III.
[g]N.D, Not determined.

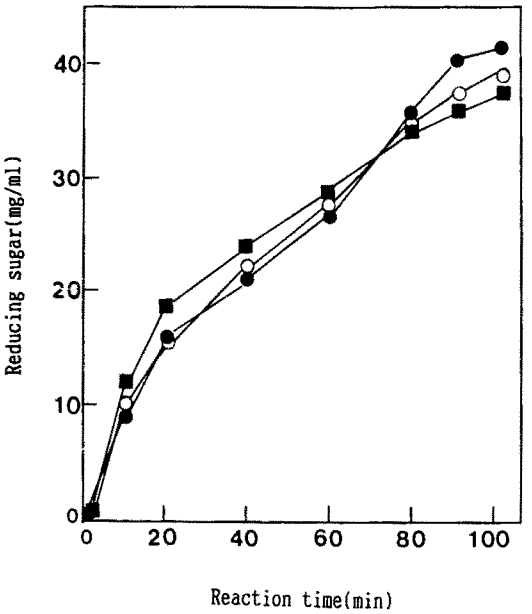

Fig. 1. Saccharification of microcrystalline cellulose (Avicel SF) with the cellulolytic enzyme system produced by *B. circulans* F-2. The reaction was carried out with the same condition as the method described here. At intervals, the amounts of reducing sugar and soluble sugar were determined by the method described in Material and Methods and the amount of glucose by the glucose oxidase method (Glucose-oxidase detection kit AR-II, Wako pure chemicals Co., Tokyo, Japan). ○, glucose; ●, soluble sugar; ■, reducing sugar.

and β-glucosidase activity. C-II, but not C-I, showed high activity against filter paper. On the contrary, minor cellulase-II showed only avicelase activity. When C-I was subsequently applied to a gel filtration column of HPLC and eluted, one peak having CMCase, pNPC-hydrolase and β-glucosidase activities were eluted in the 669,000-M_r region (Fig. 3). On the other hand, when C-II was subsequently applied to a same gel chromatography, seemingly four activities of CMCase, avicelase, β-glucosidase, and xylanase were observed in the 443,000-M_r region as one peak (Fig. 3). It was not possible to do a similar analysis on minor peak, as only Avicelase activity was detected. It is therefore likely that C-I and C-II exist as a multisubunit protein complex. Mild denaturation of the pooled C-I and C-II complexes were analyzed by treating them with SDS at room temperature before SDS-PAGE (Fig. 4). C-I complex revealed at least five CMCase bands and weak two xylanase bands from the dissociation of the 669,000-M_r multiple protein complex. Seemingly, the mildly denatured C-II showed at least three strong CMCase and four xylanase components from the dissociation of the 443,000-M_r multiple protein complexes (Fig. 4). The results

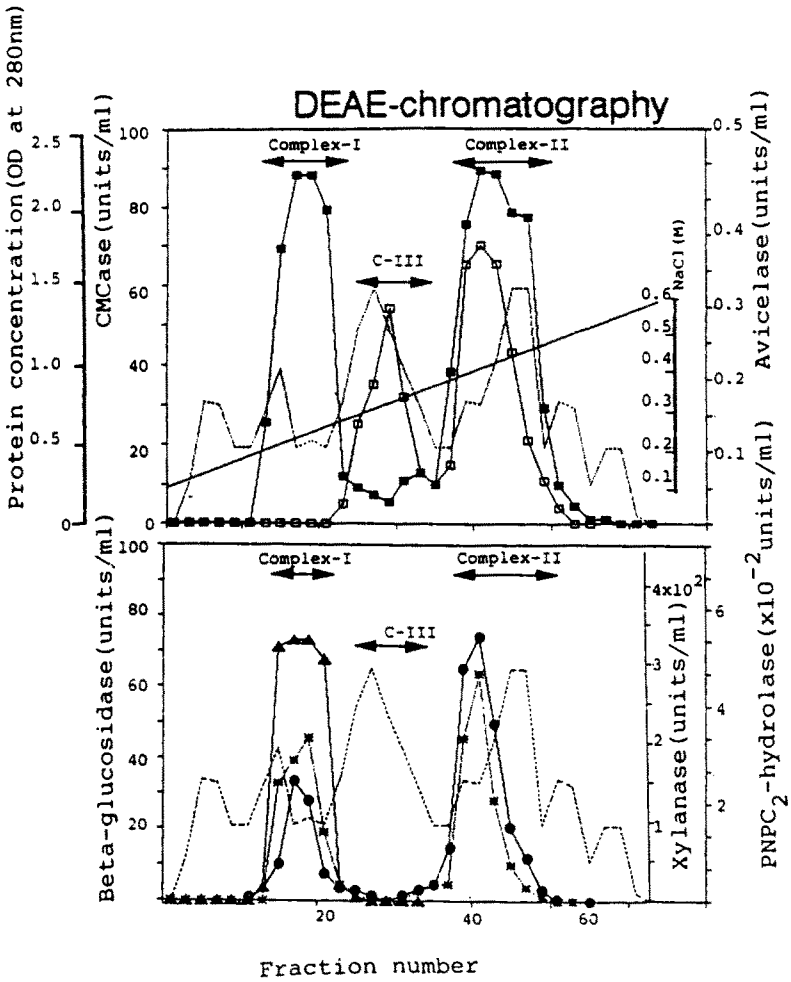

Fig. 2. DEAE-Toyopearl M650 column chromatography of extracellular culture filtrate from *B. circulans* F-2. Fractions of 5 mL were collected: ·····, protein (absorbance at 280 nm); □, Avicelase; ■, CMCase; ▲, $PNPC_2$-hydrolase; ●, β-glucosidase; *, xylanase; —, sodium chloride buffer gradients.

described above indicated that the cellulase activities resided in large protein complexes, with mol wts of approx 669,000 and 443,000, that apparently consisted of several proteins. Current ideas on the nature of the cellulase complex of microorganism have focused almost wholely on the cellulosome of high M_r values as they occur in isolated form or in association with large molecular complexes. The present work shows, at least with our strain, that an alternative approach to the problem allows different enzymes of cellulase and xylanase to be recognized in a cellulo-xylanosome complex. It would be of interest to see whether other strains of microbes respond likewise to the new approach. The finding reported

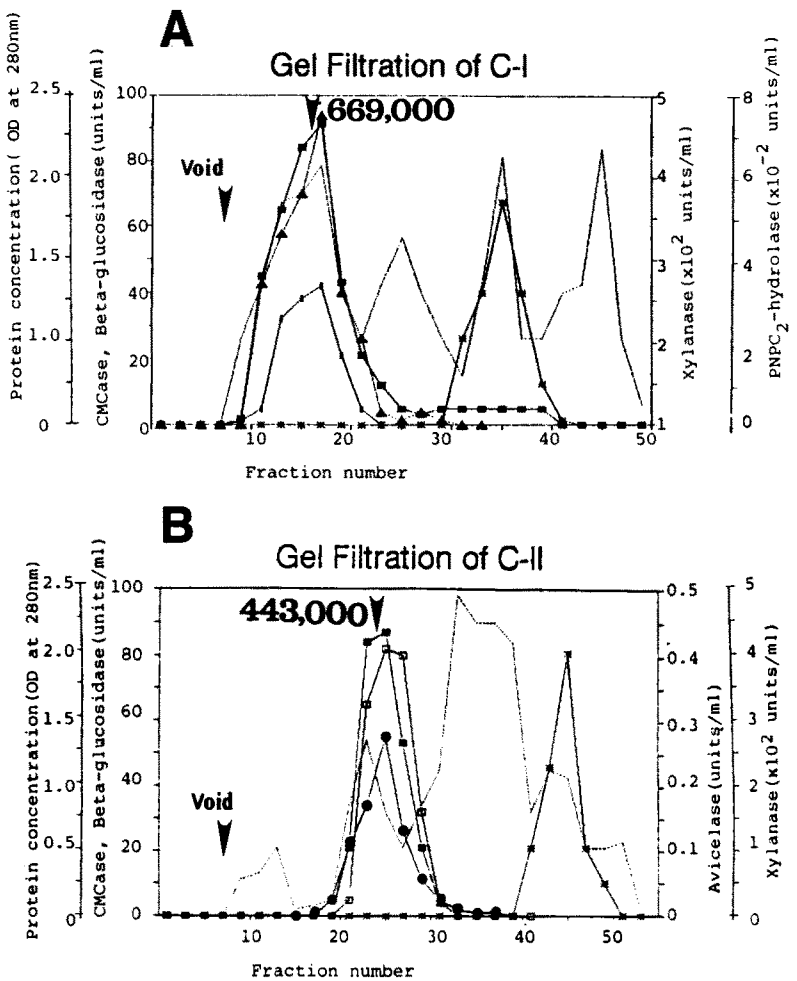

Fig. 3. Gel filtration chromatography on TSK Gel Toyopearl HW-65S column of pooled fractions from DEAE Toyopearl chromatography. The elution positions of thyroglubulin (669,000), apoferritin (443,000) and the void volume are designated by arrow heads. ·····, protein (absorbance at 280 nm); □, avicelase; ■, CMCase; ▲, $PNPC_2$-hydrolase; ●, β-glucosidase; *, xylanase.

above provides a new insight into the cellulase system of the present microbe and perhaps also of other cellulolytic microbes. The culture supernatant and the C-I and C-II complexes were tested for their ability to hydrolyze various carbohydrates and aryl-glucosides (Table 1). The concept of a cellulosome, a cell-associated multiprotein complex containing cellulases, some of which also have xylanase activity, and cellulose-binding factors has gained considerable acceptance for certain cellulolytic bacteria such as *C. thermocellum* (*39*). Recently, Cavedon et al. (*16*) and Lin et al. (*14*) have shown that *Clostridium* c7 and *Butyrivibrio fibrisolvens* secrete multiprotein cellulolytic complexes into the culture supernatant. *R. flavehaciens* secretes

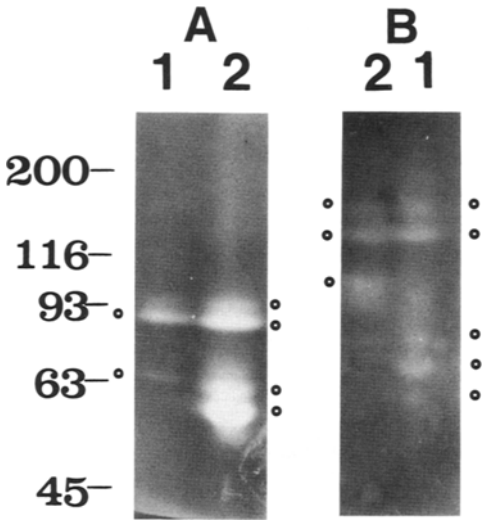

Fig. 4. SDS-PAGE zymograms of mildly denatured enzyme-active gel-filtration column fractions. Gels were stained for CMCase and xylanase activity. All samples contained 20 μg of protein. A. CMCase; B. xylanase. Lane 1, C-I complex; lane 2, C-II complex. The numbers indicate M_rs in thousands. Open circles correspond to enzyme activity bands.

two multiple protein complexes in which all the endoglucanases had xylanase activity (*15*). There are, therefore, precedences for the secretion by cellulolytic and xylanolytic bacteria of multiprotein complexes into the culture supernatant. However, as far as we are aware, this is the first demonstration of the secretion of two complexes composed predominantly of β-xylosidase, xylanase, Avicelase, FPase, and CMCases, which can therefore be called a cellulo-xylanosome.

Cellulolytic Activities and Properties of the Two Multiple Enzyme Complexes and Cellulase-III

The fractions (C-I and C-II complexes and cellulase-III) were tested for their ability to hydrolyze various carbohydrate and aryl-glucoside substrates (Table 1). As expected, C-I had predominantly CMCase and pNPC-hydrolase, and C-II predominantly CMCase, xylanase, β-1.3-glucanase and avicelase with some activity of β-glucosidase. Both complexes showed some activity on laminarin (β-1.3-glucan). CMCase activity in C-I was quite different from that in C-II, as the latter showed activity on pNPC. pNPC-hydrolase activity could be a result of the presence of a cellobiohydrolase (CBH), which together with the endoglucanases in C-I and C-II as indicated by CMCase activity, should be able to degrade crystalline cellulose.

However, Avicelase activity was not detected in C-I even though high CBH activity was found in C-I. These results indicated that the Avicelase is not related to the activity of CBH.

The optimum pHs of cellulolytic enzyme activities of two cellulase complexes were observed at pH 4.5 (C-I) and 5.0 (C-II), respectively. The enzyme activities of the C-I complex were found to be stable at pH 4.0–8.0, but enzyme activities of C-II complex were stable at pH 5.0–9.0. Interestingly, constituting enzyme activities of C-I and C-II complex exhibited the same pH spectra of pH 4.5 and 5.0, respectively. The range of pH optima of enzyme components in the C-I and C-II complex supports the proposal that they are multi-enzyme complexes. The optimum temperatures of enzyme activities in C-I and C-II complex were observed at about 50°C. The effect of temperature on the enzyme stability in the absence of a substrate was also examined. The enzyme activity was stable up to 65°C (data not shown).

Studies in progress in our laboratory are currently focusing on the cloning and analysis of the cellulase and xylanase genes from this strain. An understanding of their molecular nature should provide information regarding the ability of *B. circulans* F-2 to grow and degrade crystalline cellulose and xylan efficiently and may open the way for genetic information of cellulo-xylanosome-forming microbes.

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