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Cloning of an Endo-1,4-β-D-Glucanase Gene from *Clostridium josui* and Its Expression in *Escherichia coli*

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The gene for carboxymethyl cellulose-degrading enzyme (endoglucanase) from *Clostridium josui* (FERM P-9684) was cloned in *Escherichia coli* HB101 with pBR322. A 5.6-kilobase-pair *Hin*dIII fragment encoding an endoglucanase was hybridized with *C. josui* chromosomal DNA. The size of the cloned DNA fragment was reduced with *Pvu*II, and the resulting active fragment (2 kilobase pairs, with restriction sites of *Eco*RI and *Pst*I) was ligated into pUC118 at the *Sma*I sites (pUCJ1). The endoglucanase production by *E. coli* JM103(pUCJ1) in Luria-Bertani broth was enhanced up to approximately three times by maintaining the pH at 6.5 and using 80 mM NaCl.

Clostridium josui, a mesothermophilic strict anaerobe, has been reported as a potent bacterium which produces ethanol and other valuable compounds, including highly active cellulolytic enzymes from tough (crystalline) cellulosic materials such as Avicel, rice straw, and water hyacinths (11; J. Sukhumavasi, K. Ohmiya, M. Suwana-Adth, and S. Shimizu, J. Sci. Soc. Thail., in press). However, nothing is known about the properties of cellulases from *C. josui* because *C. josui* does not produce enough enzyme to be characterized. Moreover, the produced cellulase may form a complex like cellulosome (2), which is difficult to purify. In our present investigation, we cloned and expressed a *C. josui* endoglucanase gene in *Escherichia coli* by using pBR322 as the vector. The culture conditions for the effective production of the endoglucanase in *E. coli* were also examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. josui* FERM P-9684 produces endoglucanase in a synthetic medium under strictly anaerobic conditions. The host strains for transformation were *E. coli* HB101 [*proA2 lacY1 hasR hsdM ara-14 galK2 xyl-5 mtl-1 supE44* F *recA13 rpsL20* (Sm^r) Δ(*lac pro*) *thi strA supE endA sbcB hsdR* F′ *traD36 proAB lacI*^{q}ZΔM15] and JM103 [*endA hsdR supE sbcB thi-1 strA* Φ(*lac pro*) λ$^-$(F′ *traD36*) *lacI*^{q}ZΔM15]. The plasmids pBR322 (Ap^r Tc^r), pUC118 (Ap^r), and pUC119 (Ap^r) were used throughout this work. The methods for digestion by restriction enzymes, transformation of *E. coli* host cells, agarose gel electrophoresis, radiolabeling of DNA, Southern blotting (10), and autoradiography were essentially as described by Maniatis et al. (4).

Isolation of DNA. Chromosomal DNA was isolated from *C. josui* cells by the method of Saito and Miura (8). Plasmid DNAs were purified by the alkaline-sodium dodecyl sulfate method (3).

Cloning of the endoglucanase gene. Genomic DNA libraries of *C. josui* were constructed into the *Hin*dIII site on plasmid pBR322 after partial digestion of chromosomal DNA with restriction endonuclease *Hin*dIII. DNAs were digested with *Hin*dIII at 37°C for 2 h (pBR322 DNA) or for 1 h (*C. josui* DNA). Plasmid DNA (0.5 μg) and bacterial chromosomal DNA (2.5 μg) were mixed, ligated overnight with T4 DNA ligase at 16°C, and used to transform *E. coli* HB101 to ampicillin resistance. Transformants were plated onto Luria-Bertani (LB) agar medium (4) containing ampicillin (50 μg/ml) and grown at 37°C. The plates were overlaid with 1.0% agar containing 1.0% carboxymethyl cellulose (CMC). After being incubated for several hours, the overlaid plates were flooded with 5 ml of an aqueous solution of Congo red (1 mg/ml), incubated for 15 min more, and then washed several times with 5 ml of 1 M NaCl per wash (12).

Enzyme assay. Carboxymethyl cellulase (endoglucanase) activity was determined by evaluating the viscosity changes of a reaction mixture containing CMC (degree of substitution, 0.6; molecular weight, 180,000; Cellogen WS-C; Daiichi Seiyaku Kogyo, Kyoto) at 37°C and was monitored for 5 min by using a cone plate-type viscometer (Tokyo Keiki Co.). The reaction mixture contained CMC solution (1%) in 50 mM potassium phosphate buffer (5 ml; pH 6.8) with enzyme solution (1 ml). One unit of enzymic activity was defined previously (5) as the amount of enzyme required to reduce the viscosity of CMC by 1 cP in 1 min. The activity in 1 ml of enzyme solution was evaluated with the following equation: units = $[(1/\eta_s) - (1/\eta_o)] \times$ (1/reaction time [minutes]), where η_s is centipoise of the CMC solution after the enzyme reaction and η_o is centipoise of the CMC solution before the enzyme reaction.

The evaluation of the enzyme activity units based on the amounts of reducing sugar was unsuccessful, since negligible amounts of reducing sugar and large amounts of precipitate were detected in the enzyme reaction mixture by the method of Somogyi (9).

Cultivation of the transformant. *E. coli* JM103(pUCJ1) was cultivated in LB broth (1.5 liters) in a jar fermentor (Iwashiya K. Sawada Co., Tokyo) at 37°C. During cultivation, aeration was 1 volume of air per volume of medium per min (vvm), and the medium was agitated at 700 rpm. A range of NaCl concentrations was evaluated. The pH of the medium was controlled with a pH controller (Denki Kagaku Keiki Co., Tokyo).

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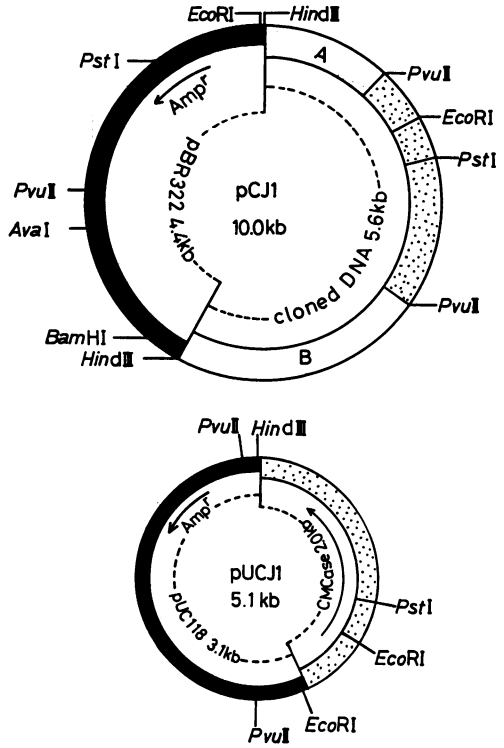

FIG. 1. Restriction maps of pCJ101 (top) and pUCJ1 (bottom). kb, Kilobase pairs; CMCase, carboxymethyl cellulase.

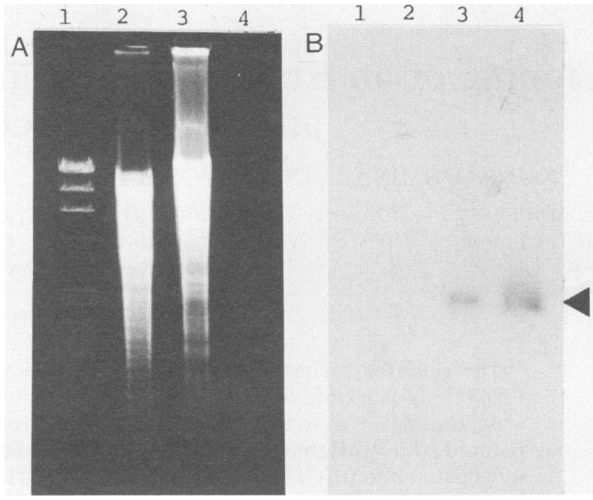

FIG. 2. Southern blot showing homology between cloned DNA and chromosomal DNA of *C. josui* digested with *Pvu*II. (A) Agarose gel electrophoretic pattern. Lane 1, *Hin*dIII fragment of λ DNA; lane 2, *Pvu*II fragment of *E. coli* DNA; lane 3, *Pvu*II fragment of *C. josui* chromosomal DNA; lane 4, *Pvu*II fragment of cloned DNA. (B) Hybridized pattern from panel A. The arrowhead indicates the position of the *Pvu*II fragment.

Enzyme preparation. For the extraction of whole intracellular substances, including enzyme from the transformants, cells were ground in a mortar with aluminum oxide powder and the intracellular substances were extracted with 50 mM phosphate buffer (pH 6.8). The extract was used as a crude intracellular enzyme solution. For the extraction of the enzyme located in periplasmic space, the transformant cells were suspended in Tris buffer (pH 8.0, 0.03 M) containing 20% sucrose solution for 10 min, harvested, and suspended in chilled Tris buffer (pH 8.0, 0.03 M). After being stirred for 10 min, the supernatant was used as a crude periplasmic enzyme solution.

RESULTS AND DISCUSSION

Cloning of *C. josui* cellulase gene. Two clones having endoglucanase activity were isolated by the initial screening of approximately 10,000 colonies described above. Both clones were found to carry the same recombinant plasmid DNA. The clone designated pCJ1 contained a 5.6-kilobase-pair (kbp) *Hin*dIII insert. A restriction map of pCJ1 is shown in Fig. 1. This map is completely different from those of *Ruminococcus flavefaciens* (1) and *Ruminococcus albus* (6), although both those strains are also strictly anaerobic bacteria.

To localize the essential endoglucanase gene sequence in the 5.6-kbp insert, pCJ1 was digested completely with *Pvu*II and the fragments were religated into pUC118 and pUC119 at the *Sma*I site. Of 800 recombinants, 6 clones having endoglucanase activity were isolated. Three of these contained the same plasmid, which contained a 2.0-kbp *Pvu*II fragment and was named pUCJ1 (Fig. 1). The transformant *E. coli* JM103(pUCJ1) expressed the endoglucanase activity in both the presence and the absence of isopropyl-thiogalactoside. The clone harboring pUCJ1R, which was constructed by inserting in reverse orientation the *Hin*dIII-*Kpn*I fragment from pUCJ1 into pUC119, gave high activity in the absence of isopropyl-thiogalactoside but low activity in its presence, indicating that the 2.0-kbp insert on pCJ1 or on pUCJ1 contains a promoter sequence for endoglucanase. To reconfirm that the cloned gene is the same as the gene from *C. josui*, we analyzed *C. josui* DNA by Southern hybridization (7, 10) with a ^{32}P-labeled 2-kbp *Pvu*II fragment, which was isolated from the insert on pCJ1 by complete digestion with *Pvu*II, as a probe. A single gene that hybridized with the probe was found in the genome of *C. josui* (Fig. 2B, lane 3), confirming that the *Pvu*II DNA fragment on pUCJ1 originated from the chromosomal DNA of *C. josui*.

E. coli JM103(pUCJ1) was aerobically cultivated overnight in LB broth in a shaking flask at 37°C. The endoglucanase activities in extracellular and intracellular fractions (including the periplasmic fractions) were 30 and 70%, respectively.

Production of endoglucanase from the transformant JM103(pUCJ1). The transformant JM103(pUCJ1) was cultivated in LB broth (working volume, 1.5 liters; pH 7.5; 170 mM NaCl) with an aeration rate of 1 vvm. The time course of growth (turbidity) and endoglucanase activity in the cells and in the supernatant were determined (Fig. 3). The growth rate of the transformant was slightly lower than that of the pUC118-containing host organism JM103(pUC118). The endoglucanase activity extracted from the transformant was only about 300 U/liter and reached a maximum after 5 to 7 h of cultivation, the end of the exponential growth phase. Further cultivation increased the cell concentration but not the endoglucanase activity. This sudden increase in growth and decrease in activity might depend on the stability of the cloned DNA in the host. In general, the cloned genes are foreign DNAs for the host DNA. Therefore, when the host microorganisms recognize the cloned genes as foreign contaminants, the host may become very active to get rid of the contaminants. When the host organisms succeed in getting

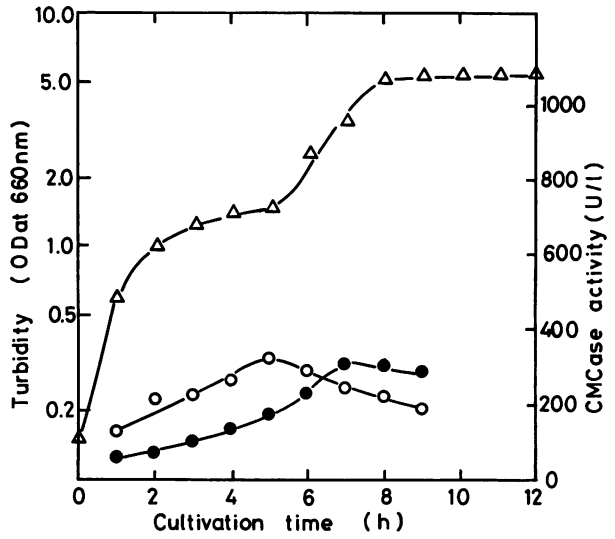

FIG. 3. Time courses of growth (△) and endoglucanase activities from cells (○) and culture supernatant (●) in the broth of the transformant JM103(pUCJ1) cultivated in LB broth at 37°C with the pH kept at 7.5 with 1 N HCl. The aeration rate was 1 vvm. OD, Optical density; CMCase, carboxymethyl cellulase. Enzymatic reaction was carried out at 60°C for 5 min.

rid of the cloned genes, the hosts cease consuming their own energy for synthesizing foreign proteins. In this case, they can use the energy for their own growth. That is why the growth rate of host organisms increased significantly after 6 to 8 h of cultivation (Fig. 3). Such a sudden increase in the growth rate of the host cells and such a decrease in the activity yield of the relevant enzymes were frequently observed during isolation of the cloned gene, as in the case of endoglucanases from both *C. josui* (this experiment) and *R. albus* (6).

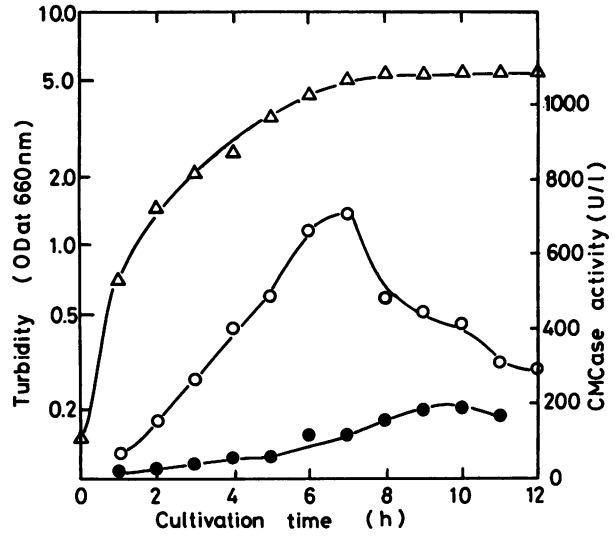

FIG. 4. Time courses of growth (△) and endoglucanase activities from cells (○) and culture supernatant (●) of the transformant JM103(pUCJ1) cultivated in LB broth and kept at pH 6.5 with 1 N HCl. The NaCl concentration in LB broth was adjusted to 80 mM, and the aeration rate was 1 vvm. OD, Optical density; CMCase, carboxymethyl cellulase. The enzymatic reaction was carried out at 60°C for 5 min.

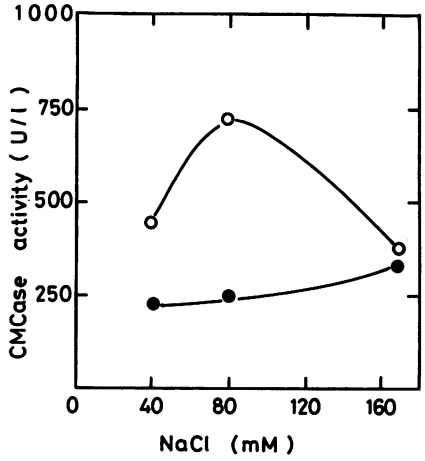

FIG. 5. Endoglucanase activity from cells (○) and culture supernatant (●) of *E. coli* JM103(pUCJ1) cultivated in LB broth at 37°C with a given concentration of NaCl at the controlled pH of 6.5 and at an aeration rate of 1 vvm. Enzymatic reaction was carried out at 60°C for 5 min. CMCase, Carboxymethyl cellulase.

For the efficient production of the endoglucanase from the transformant, the cultivation conditions were modified. Because the enzyme from *C. josui* kept its cellulolytic activity rather stably in the absence of reducing reagents such as mercaptoethanol and dithiothreitol, the cultivation of transformant for the accumulation of endoglucanase by limiting aeration during cultivation was not attempted. We have previously found (6) that the endoglucanase of *R. albus*, when grown in the neutral pH range, accumulated in the culture broth in an active state as a result of the regulation of the culture pH. Therefore, these cultivation methods were also employed for the transformant JM103(pUCJ1).

Aerated cultivation of the transformant in a jar fermentor was carried out in LB broth containing 170 mM NaCl at pHs of 5.5, 6.5, and 7.5, which were controlled by feeding 1.0 N HCl. The endoglucanase activity in the transformant cells harvested from the jar fermentor cultivation at each controlled pH was measured. The highest activity revealed at pH 6.5 was around 375 U/liter. The activity at pH 5.5 and pH 7.5 was almost one-third of that at pH 6.5. The activity (750 U/liter) of endoglucanase in the cells obtained from a cultivation time course experiment was the highest with 80 mM NaCl, while activity in the supernatant was depressed to around 200 U/liter. The typical time courses of cellulase production and cell growth of the transformant are shown in Fig. 4. The effects of NaCl concentration in LB broth on endoglucanase production at a controlled pH of 6.5 and an aeration rate of 1 vvm were studied (Fig. 5). The data plotted in Fig. 5 are the highest activities in each time course as shown in Fig. 3 and 4. The composition of LB medium was suitable for the growth of host organisms, but the remarkable increase in the pH of the culture (pH 8.0 to 8.5) rapidly inactivated the translated enzymes.

Fortunately, the endoglucanase from *C. josui* was produced at a high rate in the transformant *E. coli* under the adequate control of culture pH, as described above. Since this translation product was also a contaminant foreign to the host cells, this endoglucanase was rapidly inactivated at the late growth phase of the host *E. coli* (Fig. 4), even at the controlled pH of 6.5. Therefore, to get the highest activity, the transformant cells should be harvested after about 10 h of cultivation.

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