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Molecular Cloning and Expression of a Xylanase Gene from Alkalophilic *Bacillus* sp.

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A 16 kilobase (kb) *Hin*dIII fragment of alkalophilic *Bacillus* sp. YC-335 containing a gene for xylanase synthesis was inserted at the *Hin*dIII site of pBR322 and cloned in *Escherichia coli* HB101. After subcloning of recombinant plasmid pYS52, the 1.5 kb fragment was found to code for xylanase activity, and the hybrid plasmid was named pYS55. The DNA insert of the plasmid was subjected to restriction enzyme mapping, which showed that pYS55 had single site for *Puv*II and *Sst*I in the 1.5 kb insert fragment. Southern hybridization analysis revealed that the cloned gene was hybridized with chromosomal DNA from alkalophilic *Bacillus* sp. YC-335. About 64% of the enzyme activity was observed in the extracellular and periplasmic space of *E. coli* HB101 carrying pYS55.

Xylan, a β-1,4-glycoside-linked polymer of D-xylose, is a major component of forest and agricultural materials such as hardwood, grain straw, corn cobs and grass (10, 17). After cellulose, it is the next most abundant renewable polysaccharide in nature, and a number of microorganisms which produce extracellular xylanolytic enzymes can solublize xylan readily (19).

The hydrolysis of xylan involves β-1,4-xylanase (1,4-β-D-xylan xylanohydrolase: EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase: EC 3.2.1.37) (2, 17). In general terms, the xylanase attacks the internal xylosidic linkage on the backbone and the β-xylosidase releases xylosyl residues by endwise attack of xylooligosaccharides (2). Xylanases are generally quite small proteins, with molecular weights ranging from 15,000 to 30,000 daltons, although higher-molecular-weight xylanases have occasionally been isolated (9).

In our laboratory, a xylanase gene (20) and a β-xylosidase gene (12) from alkali-tolerant *Bacillus* sp., which was isolated from soil (22), were cloned into *E. coli*. Our interest in xylanase was to obtain better enzyme activity and to manipulate the enzyme molecule for more desirable properties. To achieve this goal, alkalophilic *Bacillus* sp. YC-335 producing xylanase, β-xylosidase and other useful enzymes was isolated from soil (6, 7, 21), and the β-xylosidase gene was cloned into *E. coli* (23). This paper describes the cloning and expression of a xylanase gene from alkalophilic *Bacillus* sp. YC-335 in *E. coli* and analysis of some of its characteristics.

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Key words: Cloning, xylanase gene, alkalophilic *Bacillus* sp., cloning of a xylanase gene

MATERIALS AND METHODS

Bacterial Strain and Plasmids

Bacillus sp. YC-335, a potent xylan-hydrolyzing bacterium, was isolated from soil under alkaline condition (21). *E. coli* HB101 (*F*$^-$, *hsdS2* [*rB*$^-$ *mB*$^-$] *supE44 ara-14 galK2 lacY1 proA2 xyl-5 mtl-1* λ$^-$ *recA13*) (5) was used as the cloning host and the plasmid pBR322 (4) was used as the cloning vector.

Media

The alkalophilic *Bacillus* sp. YC-335 was cultured in the alkaline medium containing 10 g soluble starch, 5 g Bacto yeast extract, 5 g polypeptone, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 g Na_2CO_3 per liter (pH 10.3) (21). *E. coli* strains were grown on LB medium (10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl per liter, pH 7.0) or on LB medium supplemented with either ampicillin (50 μg/ml) or tetracycline (15 μg/ml) to select for transformants. An LB agar plate containing 0.5%

(w/v) xylan was used for screening transformants for the cloned xylanase gene.

Preparation of DNA

Chromosomal DNA of *Bacillus* sp. YC-335 was prepared according to the method of Rodriguez *et al.* (13). Plasmid pBR322 DNA was prepared according to Birnboim *et al.* (3) with modifications.

Cloning Procedure

The basic cloning procedure was conducted as described by Maniatis *et al.* (11). A partial digestion of *Bacillus* sp. YC-335 chromosomal DNA was prepared by *Hin*dIII. The vector plasmid pBR322, which had been linearized with *Hin*dIII and dephosphorylated with calf intestinal alkaline phosphatase, was ligated with partially digested *Bacillus* sp. YC-335 chromosomal DNA. Transformation of *E. coli* was performed using competent cells prepared by the calcium chloride heat shock procedure (1). The ampicillin resistant and tetracycline sensitive transformants were selected on an agar plate.

Screening of the Xylanase Positive Clones

The xylan plate for the screening of the recombinant colonies contained 0.5% xylan in LB medium supplemented with the appropriate antibiotics. Transformants were toothpicked onto the xylan plate and after 3 hours incubation at 37℃, 5 ml of 0.6% soft agar containing 3 mg D-cycloserine were overlayed onto the agar medium (16). The xylanase positive clone was selected by a clear zone in the agar medium after 18 hours incubation at 37℃.

Southern Hybridization

Chromosomal DNA from *Bacillus* sp. YC-335 and recombinant DNA pYS55 digested completed with *Hin*dIII was electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose paper. The 1.5 kb *Hin*dIII fragment of pYS55 was labeled with biotin-7-dATP and used as a hybridization probe. Hybridization was performed as described by Southern (15).

Enzyme Assay

Xylanase activity was measured on the basis of reducing sugar released from xylan by the enzyme, using the Somogyi method (14). The assay mixture consisted of 0.4 ml of 1% xylan and 100 μl of crude enzyme solution. The reaction was carried out for 20 min at 40℃, then mixed with 0.4 ml of alkaline copper reagent and heated in a boiling water water bath for 10 min. After cooling the mixture, Nelson's reagent was added. One unit of the enzyme activity was defined as the amount of enzyme releasing 1 μg of reducing sugar calculated as xylose per minute.

Distribution of Enzymes

Fractionation of extracellular, periplasmic and intracellular enzymes was performed by a modification of the method of Cornelis *et al.* (8). The culture broth was centrifuged at 8,000 g for 10 min and the supernatant fluid was used as the extracellular fraction. The cells were washed twice with an equal volume of culture broth containing 0.9% NaCl and resuspended in the same volume of 25% sucrose. The supernatant was shaken for 10 min at room temperature in the presence of 1 mM EDTA. The cells collected by centrifugation (8,000 g for 10 min) were quickly and vigorously resuspended in the same volume of ice-cold water. After 10 min shaking at 4℃, the suspension was centrifuged for 10 min at 10,000 g, and the supernatant fluid was removed. The cells were then resuspended in the same volume of 0.05 M phosphate buffer (pH 7.0) followed by sonication (20 kHz, 1 min). The extracellular enzyme activity was determined as the sum of the activities found in the culture broth supernatant, the two washes, and the EDTA treatment supernatant. The periplasmic enzyme activity was the activity found in the cold-water treatment supernatant. The cellular enzyme activity was in the sonicate supernatant.

RESULTS AND DISCUSSION

Cloning of a Xylanase Gene

*Hin*dIII generated chromosomal DNA from *Bacillus* sp. YC-335 was ligated with alkaline-phosphatase-treated pBR322, which had been linearized with *Hin*dIII. The hybrid plasmids constructed were introduced into competent cells of *E. coli* HB101. Among 5,000 transformants selected as Ap^r Tc^s strains, one clone was selected which produced a clear zone, indicating the presence of xylanase activity (Fig. 1A). The plasmid DNA isolated from this clone was retransformed into *E. coli* HB101, and all transformants were xylanase positive (Fig. 1B). This result indicated that the xylanase gene was plasmid-harbored and the recombinant plasmid DNA was stably maintained.

Subcloning of the Xylanase Gene

As shown in lane 6 in Fig. 2, the recombinant plasmid DNA contained a 16 kb insert into the *Hin*dIII site of pBR322. This chimeric plasmid was named pYS52. To determine the locus of the xylanase gene, pYS52 was partially digested with *Hin*dIII, and religated. The ligated DNA was introduced into competent cells of *E. coli* HB 101, and the transformants showing ampicillin-resistance and xylanase activity were isolated. The smallest plasmid contained in these transformants were examined. As a result, one subclone pYS55, which was constructed by insertion of the 1.5 kb *Hin*dIII fragment of pYS52 into the corresponding site of pBR322, was detected as shown in lane 7 in Fig. 2.

Physical Mapping of the Recombinant Plasmid

The DNA insert of the recombinant plasmid pYS55

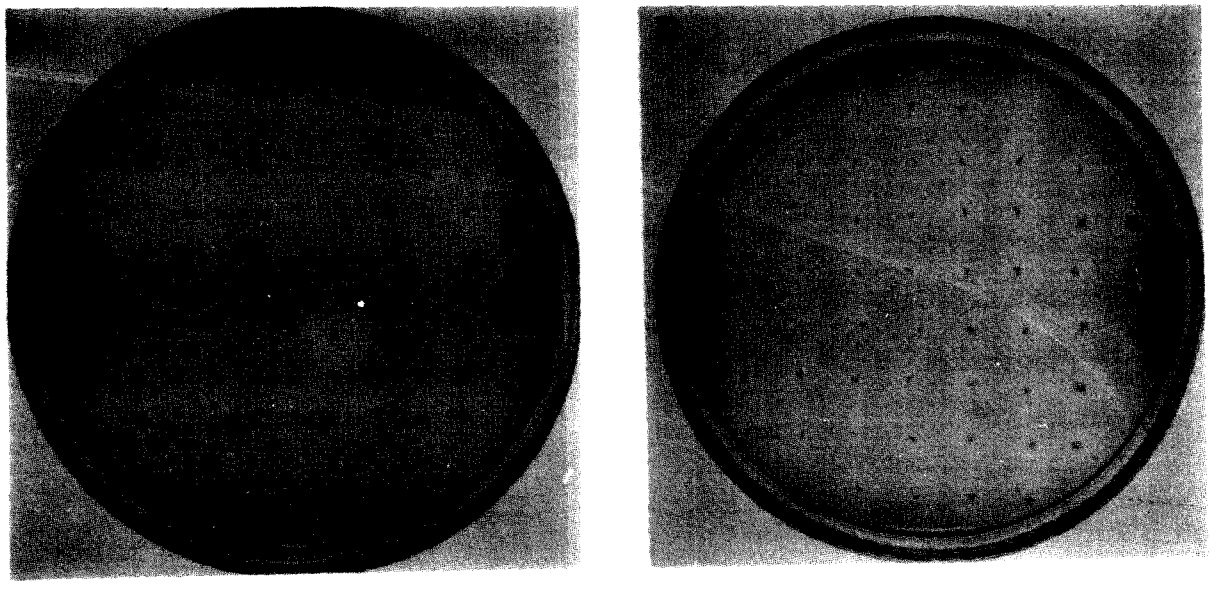

Fig. 1. Screening of *E. coli* HB101 containing recombinant xylanase gene onto LB agar plate complemented with 0.5% xylan.
Panal A; Xylanase positive clone, B; Retransformation of the recombinant plasmid pYS52.

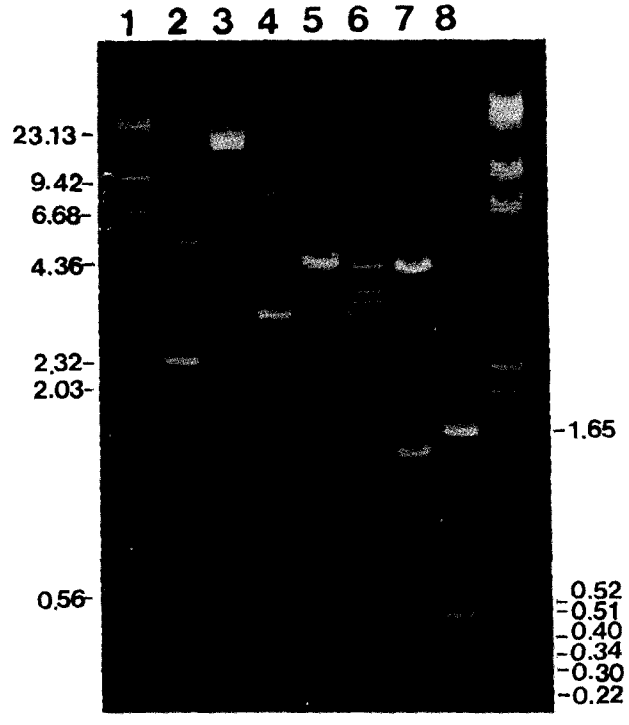

Fig. 2. Agarose gel electrophoresis of the recombinant plasmid carrying xylanase gene of *Bacillus* sp. YC-335.
A 0.9% agarose gel was used. Lane 1; λ-DNA digested with *Hin*dIII as a molecular weight marker (kb), lane 2; pBR322, lane 3, 4; undigested pYS52 and pYS55, respectively, lane 5-7; *Hin*dIII-digested pBR322, pYS52 and pYS 55, respectively, lane 8; pBR322 digested with *Hin*fI as a molecular weight marker (kb).

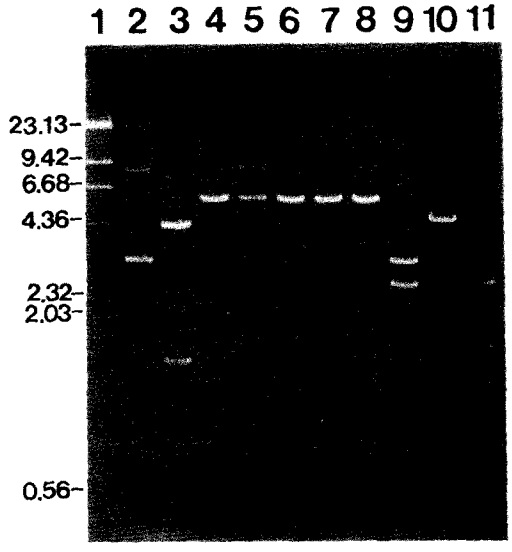

Fig. 3. Agarose gel electrophoresis of various restriction endonuclease digests of plasmid pYS55.
A 0.8% agarose gel was used. Lane 1; λ-DNA digested with *Hin*dIII as a molecular weigt marker (kb), lane 2; undigested pYS55, lane 3-11; pYS55 digested with *Hin*dIII, *Ava*I, *Bam*HI, *Cla*I, *Eco*RI, *Sst*I, *Pvu*II, *Sst*I+*Pst*I and *Pvu*II+*Bam*HI, consecutively. *Bcl*I, *Kpn*I, *Xba*I and *Xho*I sites were not existed.

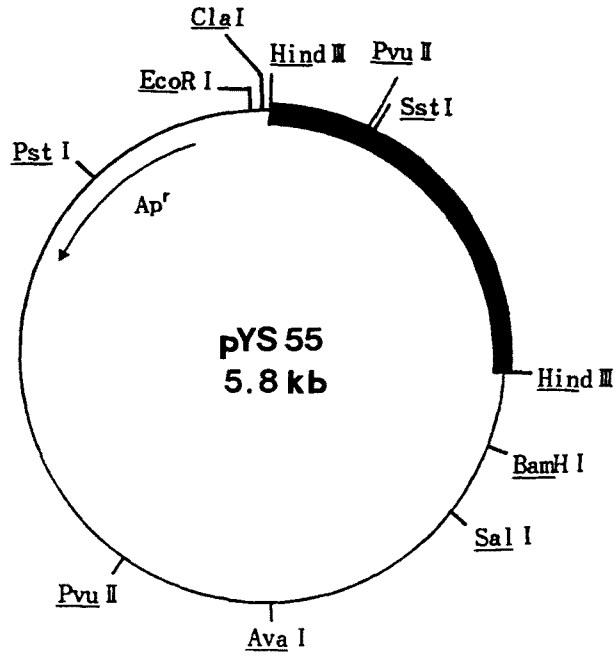

Fig. 4. Restriction endonuclease map of plasmid pYS55 carrying the xylanase gene.
The thick line represents DNA insert from *Bacillus* sp. YC-335 chromosomal DNA.

was subjected to restriction enzyme mapping. The isolated pYS55 was digested with various restriction enzymes and the resulting fragments were analyzed by agarose gel electrophoresis (Fig. 3). The physical map of pYS55, constructed from the above results, is shown in Fig. 5. The 1.5 kb DNA was revealed to be inserted at the *Hin*dIII site in pBR322. The recombinant plasmid pYS 55 had single sites for *Puv*II and *Sst*I in the 1.5 kb insert fragment. No cutting site for *Bcl*I, *Kpn*I, *Xba*I and *Xho*I was observed. This result was different from the those of Yang *et al.* (18), and Yu *et al.* (20).

Southern Hybridization

To conform the origin of the insert fragment of pYS55 to be the chromosomal DNA of *Bacillus* sp. YC-335, a hybridization experiment was performed as described in MATERIALS AND METHODS. The plasmid DNA and chromosomal DNA of *Bacillus* sp. YC-335 were digested with *Hin*dIII and hybridized with biotin-7 dATP labeled pYS55 plasmid DNA. Fig. 5 showed that the 1.5 kb band found in hybridization with *Hin*dIII-digested *Bacillus* sp. YC-335 DNA fragment corresponded to the size of the *Hin*dIII-digested fragments of the insert. This result supported the theory that the *Hin*dIII fragment of the chromosomal DNA of the donor was cloned in pYS55.

Distribution of Enzyme Activities

To analyze the effect of plasmid pYS55 which was

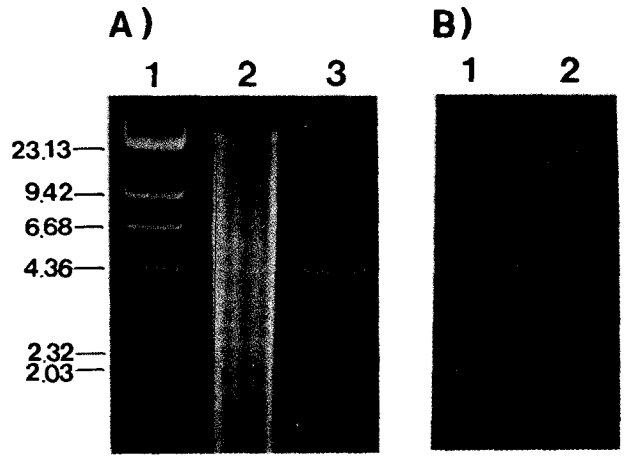

Fig. 5. Southern hybridization analysis of the *Bacillus* sp. YC-335 chromosomal DNA with the recombinant plasmid pYS55.
The chromosomal DNA from *Bacillus* sp. YC-335 and pYS 55 were digested with *Hin*dIII, fragments were separated on a 0.8% agarose gel (A). After being denatured and transferred to nitrocellulose filter, hybridization was carried out with biotin-labeled pYS55 (B). (A) lane 2 and (B) lane 2, *Bacillus* sp. YC-335 chromosomal DNA digested with *Hin*dIII; (A) lane 3 and (B) lane 1, *Hin*dIII-digested pYS55; (A) lane 1, λ-DNA digested with *Hin*dIII as a molecular weight marker. Fragments sizes (in Kilobases) are indicated at the left.

Table 1. Distribution of enzymes in *E. coli* HB101 carrying recombinant plasmid

Enzymes	Distribution of enzymes in fractions(%)			Total activity (U/ml)
	Extracellular	Periplasmic	Cellular	
Xylanase				
Bacillus sp. YC-335	80.0	20.0	0.0	12.26
E. coli HB 101	0.0	0.0	0.0	0.00
E. coli HB 101				
pYS52	31.4	30.0	38.6	69.56
pYS55	33.4	30.0	36.6	192.47
β-Galactosidase				
pYS55	30.8	0.0	69.2	
β-Lactamase				
pYS55	14.5	70.0	15.5	

introduced into *E. coli* HB101, the distributions of xylanase, β-galactosidase, β-lactamase activities were assayed. As shown in Table 1, *Bacillus* sp. YC-335, the donor strain of the xylanase gene, secreted 80% of xylanase into culture broth and no xylanase activity was detected in the cellular fraction. *E. coli* HB101 harboring pYS52 or pYS55 was cultured for 18 hr in LB broth at 37℃. As a reference, when *E. coli* HB101 was also cultured

under the same conditions as described above, xylanase activity was not detected. In *E. coli* HB101 harboring pYS55, about 70% of β-galactosidase, which is a typical cellular enzyme, and 70% of β-lactamase, which is a typical periplasmic enzyme, was observed on the cellular fraction and periplasmic space, respectively. The xylanase produced by *E. coli* HB101 harboring pYS55 was equally distributed in the extracellular, periplasmic and cellular fractions. 64% of the total xylanase was found in the extracellular and periplasmic space of *E. coli* HB 101 carrying pYS55, indicating that the gene code for a signal peptide which allowed the protein to traverse the cytoplasmic membrane. *E. coli* HB101 harboring pYS55 showed 16 times higher xylanase activity than *Bacillus* sp. YC-335.

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