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# Cloning of a β-Xylosidase Gene from Alkalophilic *Bacillus* sp. and its Expression in *Escherichia coli*

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**A gene coding for β-xylosidase in alkalophilic *Bacillus* sp. YC-335 isolated from soil was cloned into *Escherichia coli* HB101 using plasmid pBR322. The recombinant plasmid pYK40 was isolated, and the cloned *Hin*dIII fragment was 15 kilobases (kb). To reduce the size of the inserted DNA fragment of pYK40, the 15 kb *Hin*dIII fragment was subjected to a series of subclonings. A 6 kb subfragment was found to code for β-xylosidase activity, and the recombinant plasmid was named pYK44. Southern hybridization analysis revealed that the cloned gene hybridized with 3.5 kb, 1.5 kb, and 1.0 kb of *Hin*dIII cleaved chromosomal DNA from *Bacillus* sp. YC-335. β-xylosidase activity produced by recombinant *E. coli* was found to be 11 times higher than that produced by *Bacillus* sp. YC-335. Xylan was required to induce the production of β-xylosidase in *Bacillus* sp. YC-335.**

Plant cell wall is composed of three major polymeric constituents: cellulose, hemicellulose, and lignin. Among them, hemicellulose is noncellulosic polysaccharides including glucans, mannans, and xylans (14). After cellulose, xylan is the next most abundant renewable polysaccharide in nature and its main component is D-xylose, a five-carbon sugar which can be converted to fuels and chemicals (1, 9). In recent years there has been considerable interest in the utilization of plant material as a source of fuels and chemicals that can partially replace fossil fuels. In this viewpoint, the conversion of xylan to useful products is a good way to process the lignocellulose biomass and to produce the energy from renewable resource.

Complete breakdown of a xylan requires the action of several hydrolytic enzymes. The best known are endo-1,4-β-xylanase, which attacks the polysaccharide backbone, and β-xylosidase, which hydrolyses xylooligosaccharides to D-xylose (5).

Recently, we had been cloned the xylanase gene and β-xylosidase gene from alkali-tolerant *Bacillus* sp. into *E. coli* and described their expression (11, 12). To obtain the better xylanolytic enzyme producer, we isolated alkalophilic *Bacillus* sp. YC-335 from soil (10). This paper describes the cloning of β-xylosidase gene from *Bacillus* sp. YC-335 and the culture condition of enzyme production.

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Key words: Cloning, β-xylosidase gene, alkalophilic *Bacillus* sp.

## MATERIALS AND METHODS

### Bacterial Strains, Plasmids, and Media

The donor strain, *Bacillus* sp. YC-335, was isolated from soil under alkali condition (10). The cells were grown in alkaline medium (10g soluble starch, 5g Bacto yeast extract, 5g polypeptone, 1g $K_2HPO_4$, 0.2g $MgSO_4 \cdot 7H_2O$, 100 ml 10% $Na_2CO_3$ per liter, pH 10.3) (10) at 37℃ with agitation. The recipient strain, *E. coli* HB101 (*supE44, hsdS20* $r_B^-$ $m_B^-$ *recA13 ara-14 proA2 lacYl galk2 rpsL20 xyl-5 mtl-1*) (3) was grown in LB (10g Bacto tryptone, 5g Bacto yeast extract, 10g NaCl per liter, pH 7.0) at 37℃. The plasmid pBR322 (2) was used as cloning vector. For the selection of β-xylosidase positive clone, LB agar plate containing 0.1% (w/v) *p*-nitrophenyl β-D-xylopyranoside (Sigma Co.) was used. When required, antibiotics (Sigma Co.) were added to the medium at a final concentration of 50 μg/ml and 15 μg/ml for ampicillin(Ap) and tetracycline(Tc). respectively.

### Preparation of DNA

Chromosomal DNA of *Bacillus* sp. was prepared from

cells grown in alkaline medium at 37℃ according to Rodriguez (6). Plasmid DNA harbored in *E. coli* cells was prepared from cleared lysate by the miniscreen method (6).

**Cloning Procedure**

The basic procedure was carried out according to the method of Maniatis *et al.* (7). In construction of pYK44, chromosomal DNA of *Bacillus* sp. YC-335 was partially digested with *Hin*dIII and ligated to pBR322 linearized with *Hin*dIII followed by calf intestinal alkaline phosphatase treatment. Transformation of *E. coli* was done using $CaCl_2$-treated cells (13). The ampicillin-resistant, tetracycline sensitive transformants were selected on an agar plate and a β-xylosidase positive clone was selected as a yellow color producer on an agar plate containing 1 mg/ml *p*-nitrophenyl β-D-xylopyranoside.

**Southern Hybridization**

Chromosomal DNA from *Bacillus* sp. YC-335 and recombinant DNA pYK44 digested completely with *Hin*dIII was electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose paper. The 6 kb *Hin*dIII DNA fragment from pYK44 was labeled with biotin-7-dATP and used as a hybridization probe. Hybridization was performed as described by Southern (8).

**Enzyme Assay**

*Bacillus* sp. and *E. coli* cells were disintegrated by sonication at 20 MHz for 1 min in 50 mM phosphate buffer, pH 7.0 at 0℃. The supernatant obtained by centrifugation at 10,000×g for 10 min was used as crude enzyme solution. β-xylosidase activity was measured with 1 mg/ml *p*-nitrophenyl β-D-xylopyranoside in 50 mM phosphate buffer, pH 7.0 (4). The reaction mixture, composed of 100 μl substrate solution and 100 μl properly diluted crude enzyme, was incubated at 37℃ for 20 min. The reaction was stopped by the addition of 2 ml 0.4 M $Na_2CO_3$ and the absorbance of the nitrophenol released was measured at 405 nm. One unit of enzyme was defined as the amount of enzyme capable of releasing 1 μmol nitrophenol per min.

## RESULTS AND DISCUSSION

**Cloning of a β-Xylosidase Gene from *Bacillus* sp. YC-335**

*Hin*dIII generated chromosomal DNA from *Bacillus* sp. YC-335 were 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 4,000 transformants selected as $Ap^rTc^s$ strains, one clone was judged to be β-xylosidase-positive from the yellow color around the colony on the LB agar plate containing *p*-nitrophenyl β-D-xylopyranoside (Fig. 1A). The plasmid

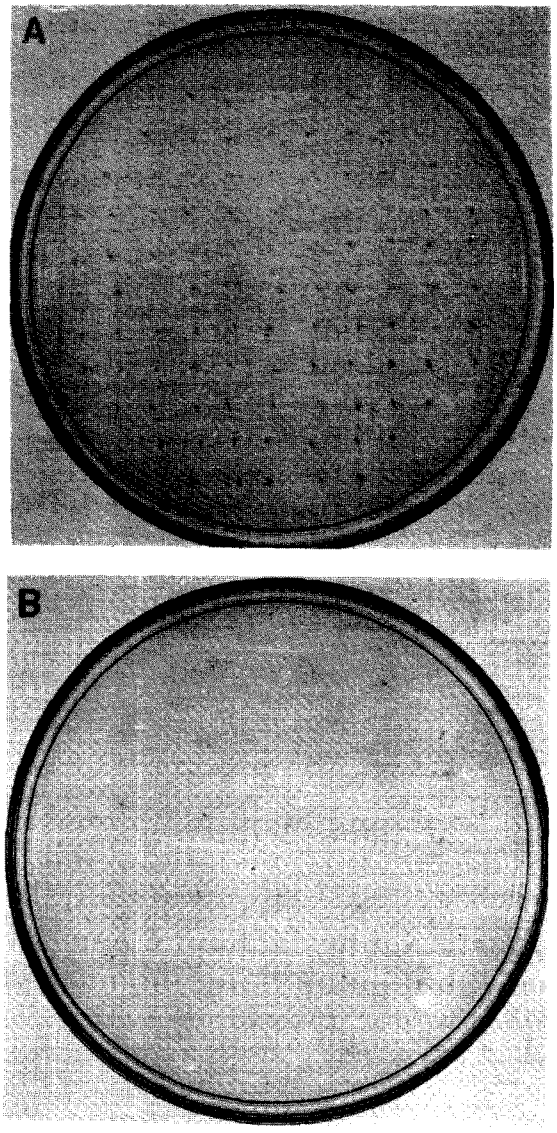


**Fig. 1. Screening of recombinant *E. coli* HB101 containing β-xylosidase gene onto LB agar plate complemented with 0.1% *p*-nitrophenyl β-D-xylopyranoside.**
Panal A: β-xylosidase positive clone, panal B: retransformation of the recombinant plasmid pYK40.

DNA isolated from this clone was retransformed into *E. coli* HB101, and all transformants were β-xylosidase positive (Fig. 1B). This result indicated that the β-xylosidase gene is plasmid-harbored and the recombinant plasmid DNA is stably maintained in recipient strain.

**Subcloning of β-Xylosidase Gene**

As shown in lane 6 in Fig. 2, the recombinant plasmid contained a 15 kb insert into the *Hin*dIII site of pBR322, and had 9 sites for *Hin*dIII in the insert fragment. This recombinant plasmid was named pYK40. To determine the locus of the β-xylosidase gene, pYK40 was partially

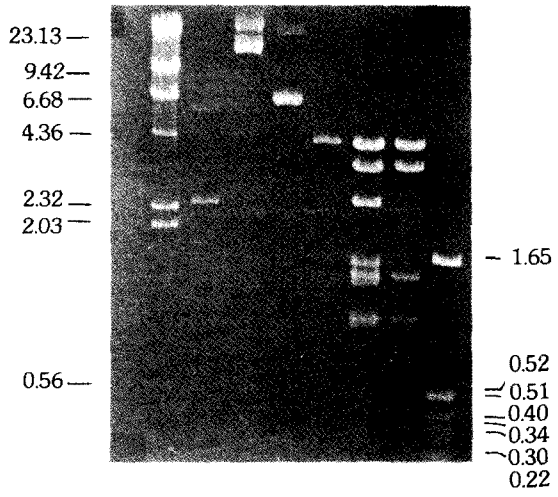


**Fig. 2. Agarose gel electrophoresis of recombinant plasmid containing β-xylosidase gene digested with various restriction endonucleases.**
Lane 1; λ DNA digested with *Hin*dIII, lane 2; pBR322, lane 3 and 4; undigested pYK40 and pYK44, respectively, lane 5-7; *Hin*dIII-digested pBR322, pYK40 and pYK44, respectively, lane 8; pBR322 digested with *Hin*fI (kb).

digested with *Hin*dIII, and religated. The ligated DNA was introduced into competent cells of *E. coli* HB101, and the transformants showing ampicillin-resistance and β-xylosidase activity were isolated. The smallest plasmid contained in these transformants were examined. As a result, plasmid named pYK44 contained a 6 kb insert and had 3 sites for *Hin*dIII in the insert fragment as shown in lane 7 in Fig. 2. Three fragments of DNA insert generated by *Hin*dIII digestion were ligated with pBR322 in combination, and transformed into *E. coli* HB101. Transformants showing β-xylosidase activity were not detected. These results indicate that 3 insert fragments (3.5 kb, 1.5 kb, 1.0 kb) were necessary for the expression of β-xylosidase gene.

**Southern Hybridization**

In order to confirm that the insert in the recombinant plasmid pYK44 was originated from *Bacillus* sp. YC-335, a hybridization experiment was performed as described in MATERIALS AND METHODS. Following digestion of the chromosomal DNA of *Bacillus* sp. YC-335 and pYK44, hybridization was performed using biotin-7-dATP labeled pYK44 probe. Fig. 3 shows that the three bands of 3.5 kb, 1.5 kb, 1.0 kb in size found in the hybridization with *Hin*dIII-digested *Bacillus* sp. YC-335 DNA correspond to the size of the *Hin*dIII-digested fragments of the insert. The 2.5 kb band may correspond to fragment of different size origination from a partial digestion.

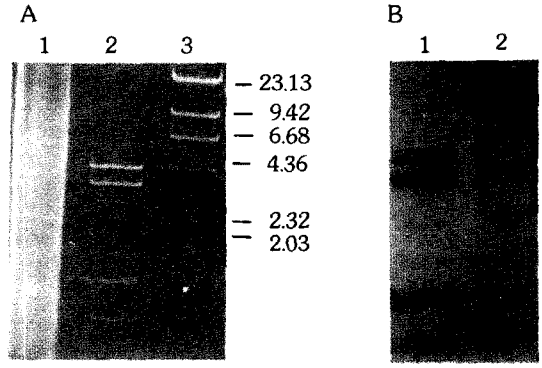


**Fig. 3. Southern hybridization analysis of the *Bacillus* sp. YC-335 chromosomal DNA with the recombinant plasmid pYK44.**
The chromosomal DNA from *Bacillus* sp. YC-335 and pYK 44 were digested with *Hin*dIII, fragments were separated on a 0.8% agarose gel (A). After being denatured and transferred to nitrocellulose filter, hybridization were carried out with biotin-labeled DNA of pYK44 (B). (A) lane 1 and (B) lane 2, *Bacillus* sp. YC-335 chromosomal DNA digested with *Hin*dIII; (A) lane 2 and (B) lane 1, *Hin*dIII-digested pYK44. (A) lane 3, *Hin*dIII digested λ DNA; Fragments sizes (in kilobases) are indicated at the right.

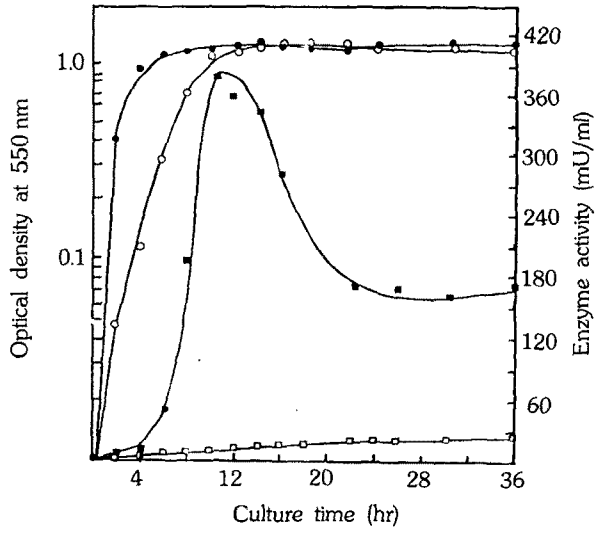


**Fig. 4. Growth and enzyme production of *Bacillus* sp. YC-335 and *E. coli* HB101 (pYK44).**
○, ●; Growth of *Bacillus* sp. YC-335 and *E. coli* HB101 (pYK44), respectively, □, ■; β-xylosidase activity of *Bacillus* sp. YC-335 and *E. coli* HB101 (pYK44), respectively.

**Cultural Conditions for Production of β-Xylosidase**

**Time courses of β-xylosidase production and cell growth:** β-xylosidase production and cell growth of *Bacillus* sp. YC-335 and *E. coli* HB101 harbouring recombinant plasmid pYK44 were examined (Fig. 4). The growth rate of *Bacillus* sp. YC-335 was somewhat

lower than that of *E. coli* HB101 harbouring plasmid pYK44. β-xylosidase activity of *Bacillus* sp. YC-335 was increased after the end of the exponential phase, but showed very low activity in comparison with that of *E. coli*. In *E. coli*, enzyme activity was remarkably increased to 390 mU/ml until 12 hrs and decreased to 170 mU/ml until 20 hrs.

**Effect of Carbon Sources**

The effect of various carbon sources on the production of β-xylosidase was examined (Table 1). The cells were cultivated for 18 hrs in the media containing 0.5% (w/v) carbon source and the enzyme activity in the cell extracts was measured. In *Bacillus* sp. YC-335, xylan was the most effective on β-xylosidase production. When other carbon sources were added to the medium, enzyme activity was very low. These results mean that β-xylosidase produced by *Bacillus* sp. YC-335 is inducible with xylan. In *E. coli*, fructose was the most effective on β-xylosidase production. When β-xylosidase activity was measured at various concentration of xylan and fructose, β-xylosidase was maximally produced in *Bacillus* sp. YC-335 at a concentration of 0.5% (w/v) xylan. Whereas, *E. coli* showed the maximum enzyme activity at a concentration of 0.3-0.5% (w/v) fructose (Fig. 5).

**Effect of Nitrogen Sources**

The effect of various nitrogen compounds was examined in the media with the addition of 0.5% (w/v) nitrogen sources (Table 2). *Bacillus* sp. YC-335 showed high enzyme activity when yeast extract and polypeptone were added simultaneously at a concentration of 1.0% (w/v), respectively (Fig. 6). In case of *E. coli*, polypeptone was effective on β-xylosidase production, and its optimum concentration was 0.1% (w/v).

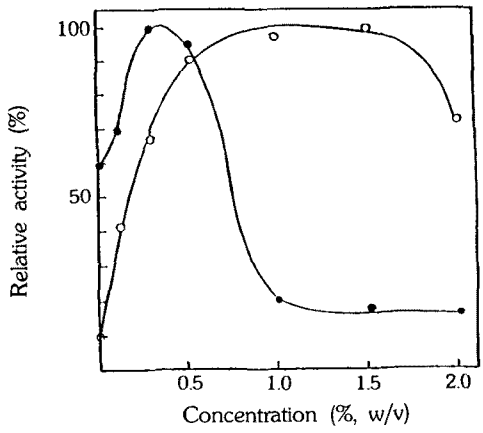


**Fig. 5. Effect of fructose and xylan concentration on the β-xylosidase production.**

○; Xylan concentration in *Bacillus* sp. YC-335, ●; fructose concentration in *E. coli* HB101 (pYK44).

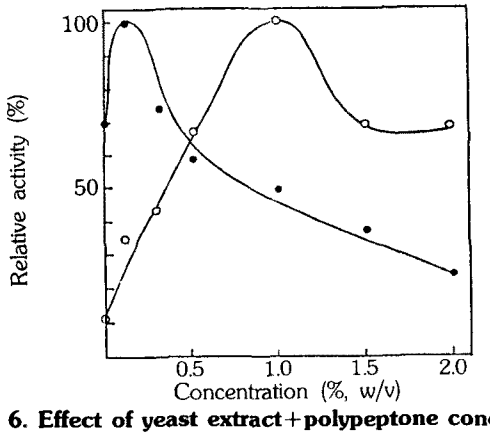


**Fig. 6. Effect of yeast extract+polypeptone concentration on the β-xylosidase production.**

○; Yeast extract+polypeptone concentration in *Bacillus* sp. YC-335, ●; polypeptone concentration in *E. coli* HB101 (pYK44).

**Table 1. Effect of carbon sources on the β-xylosidase production**

| Carbon sources (0.5%, w/v) | β-xylosidase activity (%) | |
|---|---|---|
| | *Bacillus* sp. YC-335 | *E. coli* HB101 (pYK44) |
| None | 3.2 | 78.0 |
| Fructose | 3.2 | 100.0 |
| Galactose | 0.0 | 47.6 |
| Glucose | 44.4 | 17.0 |
| Glycerol | 0.8 | 64.2 |
| Maltose | 16.1 | 87.1 |
| Raffinose | 1.6 | 79.2 |
| Sucrose | 3.2 | 71.3 |
| Starch | 50.0 | 58.0 |
| Xylan | 100.0 | 75.5 |
| Xylose | 33.1 | 43.4 |

**Table 2. Effect of nitrogen sources on the β-xylosidase production**

| Nitrogen sources (0.5%, w/v) | β-xylosidase activity (%) | |
|---|---|---|
| | *Bacillus* sp. YC-335 | *E. coli* HB101 (pYK44) |
| None | 42.8 | 20.1 |
| Casamino aicd | 86.0 | 10.1 |
| Corn steep liquor | 98.3 | 60.5 |
| Polypeptone | 28.0 | 100.0 |
| Skim milk | 0.0 | 10.9 |
| Soy bean meal | 28.4 | 11.6 |
| Urea | 28.8 | 27.9 |
| $(NH_4)_2SO_4$ | 14.4 | 31.8 |
| Yeast ext. | 42.8 | ND[a] |
| Yeast ext. +Polypeptone | 100.0 | ND |

[a]ND; Not determined.

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