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Subcloning and Enhanced Expression of the β-Xylosidase Gene Cloned from Alkalophilic *Bacillus* sp. K-17

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호알칼리성 *Bacillus* sp. K-17 의 β-Xylosidase 유전자의 Subcloning 및 발현증진

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To reduce the size of 5.0kb *Hin*dIII fragment containing β-xylosidase gene, the 5.0kb insert of pAX278 which was previously cloned was reduced by various deletions and thus 1.4kb *Eco*RI-*Xba*I fragment was subcloned into pUC19, and the recombinant plasmid was named pAK208. The β-xylosidase activity of *E. coli* harboring pAK208 was higher about 1.3times than that of pAX278. For the improvement of β-xylosidase activity, we cloned and expressed the β-xylosidase gene in *E. coli* using vector pKK223-3 containing a potent *tac*-promoter, and enzyme activity of the transformant harboring pKHR212 was increased about 3.3 and 1.8 times than that of *E. coli* (pAX278) and *Bacillus* sp. K-17, respectively. To obtain better expression of β-xylosidase gene, the whole 5.0kb *Hin*dIII fragment was recloned into pC194, and the *Bacillus* sp. K-17 transformant harboring the recombinant plasmid pCX174 showed higher activity than that of the *E. coli* (pAX278) and *Bacillus* sp. K-17, respectively. The characteristics of enzyme purified from transformants were consistent with those from alkalophilic *Bacillus* sp. K-17.

Xylan is one of the main components in plant biomass and account for nearly 30% of total sugar in some plant. Microbial degradation of xylan to xylose is thus important for the use of agricultural wastes. Xylan can be degraded to xylose by the sequential reaction of xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1, 4-β-D-xylohydrolase; EC 3.2.1.37) (1,2). β-Xylosidase degrades not only xylobiose but also xylooligosaccharides. Alkalophilic *Bacillus* sp. K-17, which isolated previously, produced two types of xylanase and a β-xylosidase. Although β-xylosidase from *Bacillus subtilis* and *Bacillus pumilus* was reported to be located in intracellular compartment, our strain secreted β-xylosidase into the medium (3). We have started to clone the genes for xylan degradation to elucidate gene structure and to develope the strain producing greater amount of β-xylosidase (3). In this paper, first, we performed the subcloning to reduce the 5.0kb insert size of pAX278 which was previously cloned. Second, for the purpose of increasing β-xylosidase activity, we recloned the subcloned fragment into pKK223-3 containing *tac*-promoter and expressed in *E. coli*. Third, the entire 5.0kb *Hin*dIII fragment of pAX278 cloned and expressed in alkalophilic *Bacillus* sp. K-17, which is the gene

Key words; β-Xylosidase gene, alkalophilic *Bacillus* sp. K-17, *tac*-promoter
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Table 1. Strains and plasmids used in this experiment.

Strains and plasmids	Relevant properties
Strains	
Bacillus sp. K17	Hyper-xylanase and β-xylosidase producing strain isolated in our lab.
E. coli JM109	*Δ(lac pro), thi, strA, supE, endA, sbcB, hadR⁻, F' traD36, proAB, lacI*^{q}, *Z ΔM15*, r⁻, m⁺
Plasmids	
pUC19	Am^r, *lacZ*
pC194	Cm^r
pKK223-3	Am^r, *tac*-promoter
pAX278	Am^r, Tc^s, xynB⁺
pAK208	pUC19 containing *Eco*RI-*Xba*I fragment of pAX278
pKHR212	pKK223-3 containing *Eco*RI-*Pst*I fragment of pAK208
pCX174	pC194 containing *Hin*dIII fragment of pAX278

source strain, using vector pC194.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used were show in Table 1. *E. coli* JM109 was used as the host for transformation done in this experiment. Alkalophilic *Bacillus* sp. K-17, which isolated previously, was used as a gene source strain (2). Plasmid pUC19 was used as a vector for subcloning of β-xylosidase gene, and plasmid pKK223-3 and pC194 were used as vectors to increase enzyme activity.

Media and growth conditions

E. coli was grown routinely at 37°C in LB medium (10g Bacto tryptone, 5g yeast extract, and 10g NaCl per l*l* of deionized water, pH 7.4). MacConkey agar was used as a selective medium for selection of *E. coli* transformants. Alkalophilic *Bacillus* sp. K-17 was grown at 45°C with shaking in alkaline medium containing 10g xylose, 5g polypeptone and 2g yeast extract. The pH of the medium was adjusted to 10.3 by adding 10% Na_2CO_3 which was separately sterilized.

Isolation of plasmids

For plasmid isolation, the method of Birnboim and Doly was employed (4).

Transformation of *E. coli* and *Bacillus* sp. K-17

Transformation of *E. coli* with plasmid was carried out by calcium chloride treatment previously described (5, 6). Transformation of *Bacillus* sp. K-17 was done as previously described (7, 8).

β-Xylosidase assay and localization

The activity of β-xylosidase was measured with *p*NPX (*p*-nitrophenyl-β-D-xylopyranoside) in 50 mM phosphate buffer (pH 7.0) (3). The reaction mixture, composed of 1 m*l* substrate solution (1mg/m*l*) and 1 m*l* properly diluted enzyme, was incubated at 40°C for 10 min. The reaction was stopped by addition of 2 m*l* of 0.5 M Na_2CO_3 and the optical density of *p*-nitrophenol released was measured at 410 nm. Fractionation of β-xylosidase were performed by the method of Cornelis *et al.* (9).

Subcloning of β-xylosidase gene

The subcloning was carried out according to the method described previously (6, 10). The 5.0kb insert DNA of pAX278 containing β-xylosidase gene was digested with one or two restriction enzyme, and the resulting fragments were analyzed by agarose gel electrophoresis (11). The recovered fragments were ligated with pUC19 by T4 DNA ligase at 15°C for 16 hours. The ligated DNA was transformed *E. coli* JM109 and transformants were selected on LB-MacConkey agar plates containing proper antibiotic (ampicillin; 50 μg/m*l*, chloramphenicol; 12 μg/ml). Ampicillin-resistant and white-color forming colonies were toothpicked onto LB plates containing antibiotics and 1 mM *p*NPX. After incubating at 37°C, yellow-color forming colonies were selected as the β-xylosidase producing strain.

Enzyme purification

Purification of β-xylosidase was done as published previously (1, 3).

Results and Discussion

Subcloning of β-xylosidase gene

A hybrid plasmid pAX278, consisting of the

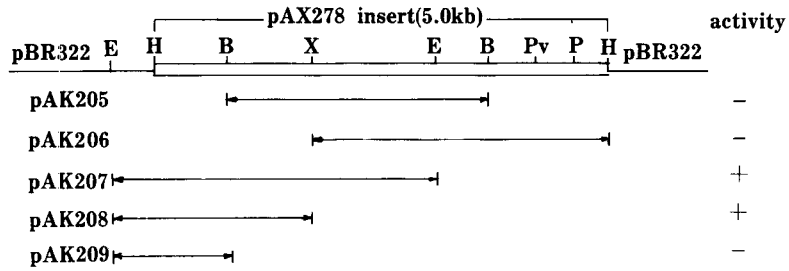

Fig.1. Strategy for subcloning of β-xylosidase gene (pAX278) in pUC19.
E; *Eco*RI, H;*Hin*dIII, B; *Bgl*II, X; *Xba*I, Pv; *Pvu*II, P; *Pst*I

5.0kb *Hin*dIII chromosomal fragment of alkalophilic *Bacillus* sp. K-17 and pBR322, contained the β-xylosidase gene. Fig. 1 shows a restriction map of the 5.0kb insert in plasmid pAX278. The pAX278 plasmid was digested with one or two restriction enzyme, and the restricted fragments were ligated with pUC19 digested with the same enzyme. The ligated DNA was used to transform the competent cells of *E. coli* JM-109. The white-color forming cells were assayed for β-xylosidase activity. As shown in Fig.1, several deletion derivatives were constructed by ligating the purified fragment of pAX278 and pUC19, and *E. coli* transformants harboring them were selected on LB medium containing ampicillin and 1mM *p*NPX. The derivative plasmid pAK205 was constructed by ligating the *Bgl*II fragment of pAX278 to the same site of pUC19, but no β-xylosidase activity was observed in the transformant. When the *Xba*I-*Hin*dIII fragment was subcloned (pAK206) into pUC19, the enzyme activity of *E. coli* carrying pAK206 was also not detected. For derivative pAK207, *Eco*RI fragment of pAX278 was ligated into pUC19, and now the *E. coli* transformant harboring the pAK207 produced active β-xylosidase. The *E. coli* transformant carrying the recombinant plasmid pAK208 containing *Eco*RI-*Xba*I fragment produced also the same value of β-xylosidase with that of the *E. coli* (pAK207). From the above results we could know that the *Hin*dIII-*Xba*I region was necessary to express β-xylosidase gene. Finally to confirm the more precise location of β-xylosidase gene, when the *Bgl*II-*Xba*I region was deleted, the enzyme activity was not detected. It is clear from the various deletions that the 1.4kb *Hin*dIII-*Xba*I region contained complete β-xylosidase gene indicating the locus of β-xylosidase gene within the whole 5.0kb *Hin*dIII fragment. Fig. 2 shows the electrophoresis pattern of plasmid pAK208.

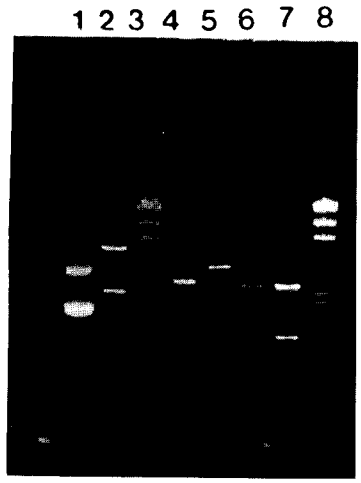

Fig. 2. Agarose gel (1%) electrophoresis of the pAK208 containing *Eco*RI-*Xba*I subfragment.
Lane1; pUC19, Lane2; pAX278 (*Eco*RI, *Xba*I), Lane3; λ DNA (*Hin*dIII), Lane4; pAK208, Lane5; pAK208 (*Bgl*II), Lane6; pAK208 (*Eco*RI, *Xba*I), Lane7; pAK208 (*Eco*RI, *Xba*I), Lane8; λ DNA (*Hin*dIII)

Cloning into *E. coli* expression vector, pKK223-3

The β-xylosidase activity of *E. coli* transformant having pAX278, which was constructed firstly, was lower than that of the gene donor strain, alkalophilic *Bacillus* sp. K-17 (3) as in the case of *Bacillus pumilus* (12) and *Bacillus subtilis* (13) published previously. Thus we attempted to clone the *Eco*RI-*Xba*I fragment to the expression vector pKK223-3 containing *tac*-promoter, to increase the expression efficiency of β-xylosidase gene. The *Eco*RI-*Pst*I fragment of pAK208 was ligated under the downstream in pKK223-3. This recombinant plasmid was named pKHR212. *p*NPX and IPTG (isopropyl-

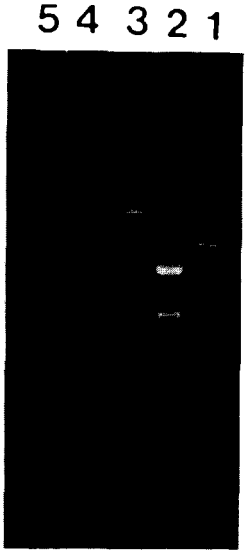

Fig. 3. Agarose gel (1%) electrophoresis of the pKHR212.
Lane1; pKK223-3 (*Eco*RI), Lane2; pAK208 (*Eco*RI,*Pst*I), Lane3; λ DNA(*Hin*dIII), Lane4; pKHR212, Lane5; pKHR212 (*Eco*RI, *Pst*I)

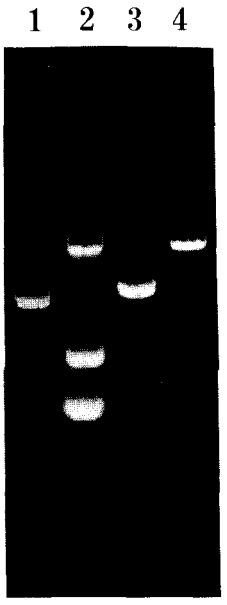

Fig. 4. Agarose gel (0.8%) electrophoresis of pC194, pCX174 and pAX278, transformed to *Bacillus* sp. K-17.
Lane1; pCX174, Lane2; pC194, Lane3; pAX278, Lane4; λ DNA (*Hin*dIII)

beta-D-thiogalactopyranoside) were added to selective medium for the purpose of selecting β -xylosidase producing transformants. Fig. 3 give the electrophoresis pattern of pKHR212. As shown in lane 6 and 7, *Eco*RI-*Pst*I fragment of pAK208 was revealed to be inserted at the same site in pKK223-3. The β -xylosidase activity of *E. coli* harboring pKHR212 was increased about 3.3 and 1.8 times than that of *E. coli*(pAX278) and *Bacillus* sp. K-17, respectively. It was concluded from the result that the transcription of β -xylosidase gene was under control of a *tac*-promoter on pKK223-3, and thus the enhanced β-xylosidase production was caused by the promotion of β -xylosidase expression.

Cloning and expression in alkalophilic *Bacillus* sp. K-17

To obtain better expression of β -xylosidase gene, the whole 5.0kb *Hin*dIII fragment of pAX278 was cloned and expressed in alkalophilic *Bacillus* sp. K-17 using plasmid pC194. Panbangred *et al.* (12) reported the molecular cloning of β -xylosidase gene from *Bacillus pumilus,*but no β -xylosidase gene was cloned and expressed in gene source strain. Because vector plasmid pC194 had only *Hin*dIII restriction site, the entire 5.0 kb insert of pAX278 was ligated with pC194 and this newly constructed recombinant plasmid was named pCX174. Fig. 4 shows agarose gel electrophoresis pattern of pC194, pCX174 and pAX278. In order to introduce plasmid pCX174 into alkalophilic *Bacillus* sp. K-17, optimal conditions for transformation were obtained (data not shown). Generally it has been reported that specific activity of the most *E. coli* and *Bacillus* carrying an hybrid plasmid was lower than that of a donor strain, but the recloning of the whole 5.0kb *Hin*dIII fragment into *Bacillus* sp. K-17 resulted in the 2-fold increasing of the enzyme activity. The most of β -xylosidase, which is an extracellular enzyme in the donor strain, was also secreted into the medium.

β -Xylosidase activity of *E. coli* and *Bacillus* sp. K-17 harboring recombinant plasmid

The comparison of β -xylosidase activities produced by transformants of *Bacillus* sp. K-17 and *E. coli* JM109 is shown in Table 2. As shown in Table 2, the most of β -xylosidase was secreted into the medium, although β -xylosidase from *Bacillus pumilus* (13, 14) and *Bacilus subtilis* (15, 16) was reported to be cellular or cell bounded enzyme. There are pos-

Table 2. Distribution and activity of the β-xylosidase in *E. coli*[a] and *Bacillus* sp. K17[a] carrying recombinant plasmids.

Strains and plasmids	β-xylosidase activity (mU)		
	Extracellular (%)	Periplasmic (%)	Intracellular (%)
E. coli (pAX278)	280 (93)	20 (7)	0
E. coli (pAK208)	380 (90)	58 (10)	0
E. coli (pKHR212)	930 (91)	180 (9)	0
Bacillus sp. K17 (pCX174)	1040	ND[b]	ND
Bacillus sp. K17	510	ND	ND

a. *E. coli* and *Bacillus* sp. K17 were aerobically grown in LB+*p*NPX (2mM) for 24hr and 48hr at 37°C and 45°C, respectively
b. ND: not determined

sibilities that β-xylosidase gene itself posseses an uncleaved signal sequence, and that β-xylosidase produced from transformants is fused with other secretion protein and passed through the cell membrane (17, 18). To make this problem clear, more precise experiments are required, such as identification of the mRNA, the secretion mechanism of β-xylosidase, the DNA sequencing of β-xylosidase gene and so on. In Table 2, the enzyme activities of *E. coli* harboring plasmid pAK208 or pKHR212 were increased about 1.3 and 3.3 times than that of pAX278, respectively. For plasmid pAK208, it seems that the increasing of enzyme activity was caused by reduced size of 5.0kb insert DNA. Especially, β-xylosidase activity of alkalophilic *Bacillus* sp. K-17 harboring pCX174 was enhanced about 2.0 times than that of *Bacillus* sp. K-17 which is a gene source strain. It was shown that β-xylosidase gene from *Bacillus* sp. K-17 was better expressed in gene source strain than in *E. coli*.

Characteristics of enzyme purified from transformants

Characteristics of enzyme produced from both *E. coli* (pAK208 or pKHR212), *Bacillus* sp. K-17 (pCX174) and *Bacillus* sp. K-17 were compared in Table 3. In the results, optimal temperature of β-xylosidase from transformants was 45°C and was stable at 40°C for 30min. Optimal pH was 7.0. Molecular weight was 51,000 by 7.5% SDS-polyacrylamide gel electrophoresis. The above results were consistent with the value obtained from the purified β-xylosidase of *Bacillus* sp. K-17 (3). Thus, we could know that β-xylosidase produced from transformant was derived from alkalophilic *Bacillus* sp. K-17.

Table 3. Properties of β-xylosidase produced by *E. coli* JM109 carrying pAK208 and pKHR212.

	E. coli (pAK208)	*E. coli* (pKHR212)	*Bacillus* sp. K17
Optimal pH	7	7	7
Optimal temp.	45°C	45°C	45°C
pH stability	6-10	6-10	6-10
Thermal stability	40°C (30min.)	40°C (30min.)	40°C (30min.)
Molecular weight	51,000	51,000	51,000
Km value	0.43mM *p*NPX	0.43mM *p*NPX	0.45mM *p*NPX

요 약

최초로 구축된 β-xylosidase 유전자 함유 5.0 kb *Hin*dIII 절편을 포함하는 pAX 278의 insert 크기를 줄이기 위하여 subcloning을 행한 결과, 1.4 kb *Eco*RI-*Xba*I 절편이 subcloning 되었으며 이를 pAK 208로 명하였다. Plasmid pAX 208에 의해 형질전환된 대장균은 β-xylosidase 활성이 pAX 278의 경우보다 약 1.3배 증가하였고 또한, β-xylosidase의 활성을 증가시키기 위하여 강력한 *tac*-promoter를 가진 pKK 223-3 plasmid vector를 이용하여 대장균에 cloning 및 발현시켰을 때, pAX 278을 가진 대장균 형질전환체와 유전자 source 균인 호알칼리성 *Bacillus* 속 K-17에 비하여 각각 약 3.3배 및 1.8배의 효소활성 증가를 나타내었다. 전체 5.0 kb *Hin*dIII 절편을 cloning하여 *Bacillus* 속 K-17에 발현시킨 균주는 각각 3.7배 및 2.0배의 β-xylosidase 활성증가를 보였다. 각 형질전환체로부터 정제된 β-xylosidase의 효소학적 특성은 *Bacillus* 속 K-17과 거의 일치하였다.

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