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Efficient Secretory Expression of Recombinant Endoxylanase from *Bacillus* sp. HY-20 in *Saccharomyces cerevisiae*

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The *XylP* gene, which encodes endoxylanase in *Bacillus* sp. HY-20, was subcloned, and two expression plasmids, pG-xylP and pGMF-xylP were constructed. These plasmids, which contain different signal sequences, *XylP* s.s and $MF\alpha_{opt}$ s.s, respectively, for the secretory expression of endoxylanase, were transformed into *Saccharomyces cerevisiae* SEY2102 and FY833, respectively. The recombinant endoxylanases were successfully expressed, with a total activity range of 23.7 - 70.1 unit/ml according to the expression system and host strain. The endoxylanase activity in SEY2102/pGMF-xylP reached a maximum of 88.1 unit/ml in baffled flask culture. Most of the recombinant endoxylanase was efficiently secreted in the extracellular fraction, and the $MF\alpha_{opt}$ s.s was more efficient for secreting endoxylanase in yeast than the *XylP* s.s. Therefore, the expression system developed in this study produces large extracellular amounts of endoxylanase using *S. cerevisiae* as the host strain, and it could be used in bioethanol production and industrial applications.

Key words : Endoxylanase, optimized MFα signal sequence ($MF\alpha_{opt}$ s.s), *GAL* promoter, secretory production, *Saccharomyces cerevisiae*

Introduction

Xylan is a major component of the cell walls of monocots and hardwoods, representing up to 35% of the dry cell weight of these plants, and is a complex polymer consisting of β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains [1, 28]. The hydrolysis of xylan is of considerable interest for various biotechnological applications, such as biobleaching, food, bio-fueling, effluent treatment, and agro-waste treatment [1, 9, 20, 26]. However, the biodegradation of xylan is a complex process that requires the coordinated action of several enzymes: β-1,4-endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.31), acetyl xylan esterase (EC 3.1.1.72), and phenolic acid esterase (EC 3.1.1.2) [1, 19]. All of these enzymes act cooperatively to convert xylan into its constituent sugars, and among these xylanolytic enzymes, the β-1,4-endoxylanase and β-xylosidase are the two main enzymes involved in the degradation of xylan. In this study, we focused on xylanase. The degradation of xylan is carried out mainly by microorganisms in nature. A number of bacterial and fungal species are able to utilize xylan as a carbon source [19, 29]. Among them, *Bacillus*, *Aspergillus*, and *Trichoderma* have been investigated for the production and cloning of xylanases [2, 14, 21]. Most microbial xylanases, which decompose primarily β-1,4-xylosic polysaccharide, are endo-fashion. Indeed, the genes encoding endoxylanase in these microorganisms have been expressed in yeast, *Saccharomyces cerevisiae* [6, 7, 10, 11, 13, 18], *Pichia pastoris* [15, 16], and *Kluyveromyces lacis* [22, 23], because of possibilities for the food-grade safety and large production of these enzymes in yeast. However, most previous studies focused on the characterization of the expressed enzyme(s), resulting in a lack of information about process development for the efficient secretion and production of recombinant xylanase itself. In the present study, we constructed two expression systems with different signal sequences to overexpress and secrete endoxylanase genes cloned from *Bacillus* sp. in *S.*

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cerevisiae. Secretory expressions of recombinant endoxylanase were investigated in yeast cells using specific signal sequences, and an optimal system for secretory production of endoxylanase was constructed.

Materials and Methods

Bacteria and yeast strains

Escherichia coli DH5α was used for the amplification and subcloning of the *XylP* gene (687 bp ORF) encoding the endoxylanase from *Bacillus* sp. HY-20 [14]. LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) agar medium containing ampicillin (50 mg/ml) was used for the selection of the transformants. *S. cerevisiae* SEY2102 (*MATα ura3-52 leu2-3 leu2-112 his4-519 suc2-△9*) [4] and FY833 (*MATa ura3-52 his3-△200 leu-△1 lys2-△202 trp1-△63*) [25] were used as the host strains for expression of the *XylP* gene.

Construction of the expression plasmids

For the secretory production of *Bacillus* endoxylanase in *S. cerevisiae*, the ORF of the endoxylanase gene was constructed under the control of inducible yeast *GAL10* promoter and fused to the codon-optimized leader sequence of *S. cerevisiae* mating factor *a* (MFa_{opt}) [24]. Because the endoxylanase from *Bacillus* sp. HY-20 is an extracellular enzyme, the ORF of *XylP* gene has its own signal sequence (peptide: MNLRKLRLLFVMCIGLTLILTAVPAHA, 27 amino acids). However, this signal sequence was not sufficient to completely secrete expressed endoxylanase from the cell. Therefore, we constructed two plasmids, pG-xylP and pGMF-xylP, containing *XylP* signal sequence and MFa_{opt} signal sequence, respectively. The *XylP* gene encoding the endoxylanase protein was subcloned into an expression vector, pGAL-MFα$_{opt}$-CALB14$_{opt}$ [24]. This vector contains the promoter of the *GAL10* gene for inducible expression, the *URA3* gene as a selective marker and the MFa_{opt} signal sequence (MFa_{opt} s.s). The pG-xylP plasmid was constructed by inserting a PCR product containing the 687 bp *XylP* gene in frame into the *Eco*RI-*Sal*I site of the pGAL-MFα$_{opt}$-CALB14$_{opt}$ vector (Fig. 1). The PCR product containing the *XylP* gene with a flanking *Eco*RI-*Sal*I site was generated using pET-28a(+)/xylP plasmid [14] as a template and forward (5-GCGAATTCATGAATTTAAGAAAA-3) and reverse primers (5-CTCGTCGACTTAGTTGCCAATAAAC-3′). The pGMF-xylP which contains MFa_{opt} s.s for secretory production of endoxylanase, was constructed by inserting a PCR product containing the 606 bp fragment without its own signal sequence (81 bp) of *XylP* gene in frame into the *Xba*I-*Sal*I site of the pGAL-MFα$_{opt}$-CALB14$_{opt}$ vector (Fig. 1B). The PCR product containing the *XylP* gene with a flanking *Xba*I-*Sal*I site was generated using pET-28a(+)/xylP plasmid as a template and forward (XylP-F, 5′-CTCTCTAGATAAGAGAAGAACCA TTACGAA-3′) and reverse primers (XylP-R, 5′-CTCGTC GACTTAGTTGCCAATAAAC-3′). PCR was carried out in 30 cycles of 30 sec at 94℃, 30 sec at 58℃ and 1 min at 72℃. PCR-amplified fragments were sequentially purified and cloned into the vector. The sequence of the original target for the removal of the MFα$_{opt}$ propeptide is KREAEAEA, which is cleaved after the dibasic peptide, K-R, by the yeast endopeptidase, *KEX2* [12, 24]. Thus, according to the design of the pGMF-xylP expression plasmid, the pro-segment of MFα$_{opt}$ s.s was expected to be cleaved after the dibasic residues Lys-Arg by a single *KEX2* endopeptidase in *S. cerevisiae*. The amino acid sequence of the junction region between MF α$_{opt}$ s.s and the *XylP* gene became KRRTITN, and the mature form of endoxylanase was thus able to be secreted via cleavage by the *KEX2* endopeptidase. The constructed plasmids were propagated in *E. coli* DH5α and isolated using a plasmid purification kit (GeneAll Biotechnology, Seoul, Korea). The junction between MFa_{opt} s.s and *XylP* was confirmed using an ABI 3730x1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Yeast transformation, media and culture condition of yeast transformants

The constructed plasmids, pG-xylP and pGMF-xylP, were transformed into *S. cerevisiae* SEY2102 and FY833, respectively, using high-frequency transformation methods [5]. Ura$^+$ transformants were selected on synthetic complete (SC) medium [6.7 g of yeast nitrogen base without amino acids (YNB), 20 g of dextrose, appropriate amino acids (20 mg histidine, 100 mg leucine, 30 mg lysine, 20 mg tryptophan), and 15 g of agar per liter]. In order to investigate the level of endoxylanase expression, *S. cerevisiae* SEY2102 and FY833 harboring pG-xylP and pGMF-xylP, respectively, were precultured at 30°C in 5 ml YPD (10 g yeast extract, 20 g peptone, 20 g dextrose per liter) medium. After a 16 h incubation, the cells were inoculated into 10 ml (for test tube culture) or 50 ml (for baffled flask culture) YPDG (10 g yeast extract, 20 g peptone, 10 g dextrose, 10 g galactose per liter) medium for galactose-inducible expression at 30℃

for 48 h. The growth of the transformants was periodically monitored by measuring OD_{600}. The supernatants obtained from each yeast transformant were used for an extracellular endoxylanase activity assay, and the intracellular fractions of yeast were obtained by treatment of Zymolyase 100T (Seikagaku Kogyo, Japan) and glass beads [13].

Assay of endoxylanase activity

The activity of endoxylanase toward xylan was measured based on the release of the reducing sugar equivalent using the 3,5-dinitrosalicylic acid (DNS) method [14, 17]. The enzyme solution was incubated at 50℃ for 15 min in 50 mM sodium phosphate buffer (pH 6.0) buffer and 1% birchwood xylan. After adding the DNS solution, the enzyme mixture was boiled for 5 min and cooled, and spectrophotometric activity measurements were subsequently carried out at 540 nm. The amount of liberated reducing sugar was measured using xylose as a standard. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmole reducing sugar per min under these assay conditions. The endoxylanase activity was also detected on YPDG plates containing 0.3% azo-birchwood xylan (Megazyme) in which white halos distinguish cells expressing endoxylanase.

Reverse transcription PCR (RT-PCR)

RT-PCR was performed to determine the *XylP* gene transcription level in the *S.cerevisiae* transformants. Total RNA of each strain was extracted according to the method of Chomczynski and Sacchi [3] with minor modification and 1 μg of total RNA was used to synthesize the cDNA using PrimeScript Reverse Transcriptase (TaKaRa BIO, Shiga, Japan) and oligo dT as primer in a 20 μl reaction system. The cDNA synthesis reaction conditions were 10 min at 30℃, 60 min at 42℃ and 15 min at 72℃. The synthesized cDNA was used as a template for PCR and XylP-F, XylP-R and ACT1-F (5′-ATCCAAGAGAGGTATCT-3′), ACT1-R (5′-CACACTTCATGATGGAG-3′) primer sets were used to amplify the *XylP* and *ACT1* genes, respectively. *ACT1* was used as an internal control.

Results and Discussion

Selection of signal sequence for secretory expression of recombinant endoxylanase

To investigate the expression levels of endoxylanase, *S. cerevisiae* harboring the pG-xylP and pGMF-xylP plasmids were randomly selected and the endoxylanase activity in these selected clones was analyzed by active staining on

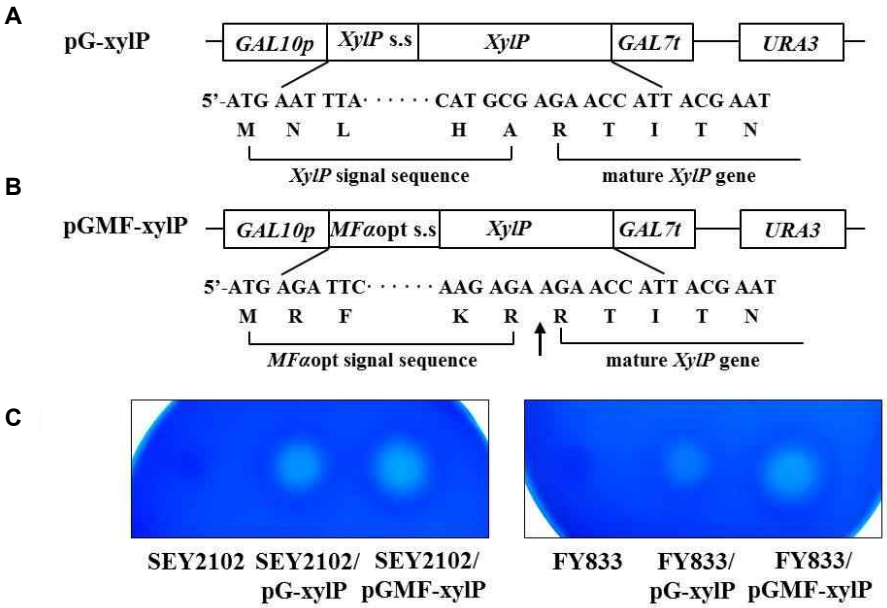

Fig. 1. Schematic of the endoxylanase expression plasmids, pG-xylP (A) and pGMF-xylP (B). The nucleotide sequence and deduced amino acid sequence of the junction site between the *XylP* signal sequence or $MF\alpha_{opt}$ signal sequence and the *XylP* gene are indicated, and the endopeptidase cleavage site is denoted by an arrow. Active staining of endoxylanase expressed in *S. cerevisiae* transformants possessing different signal sequence systems that were grown on YPDG medium containing 0.3% azo-birchwood xylan (C). These cells were incubated at 30℃ for two days and the active halos were detected.

azo-xylan plate. The azo-xylan was successfully degraded by the secreted recombinant endoxylanase and halos were evident around the *S. cerevisiae* transformants compared with the host strains, SEY2102 and FY833, which did not degrade the xylan (Fig. 1C). For the quantitative analysis of endoxylanase activity, test tube culture was conducted for 48 h on YPDG medium and cell growth, the activities of extracellular and intracellular endoxylanase among the transformants were investigated (Table 1). In *S. cerevisiae* SEY2102 harboring the pG-xylP and pGMF-xylP plasmids, the total activities of endoxylanase reached 26.8 unit/ml and 70.1 unit/ml, respectively, after 48 h of cultivation. In case of the *S. cerevisiae* FY833 harboring the pG-xylP and pGMF-xylP plasmids, the total activities of endoxylanase reached 23.7 unit/ml and 42.4 unit/ml, respectively. The specific activity was 1.3 fold higher when SEY2102 was used as the host strain compared to FY833. Furthermore, most endoxylanase activity was found in the extracellular medium, and secretion efficiency was increased by using $MF\alpha_{opt}$ s.s compared with the signal sequence of the *XylP* gene itself, suggesting that the $MF\alpha_{opt}$ s.s was a more efficient inducer of the secretory production of recombinant endoxylanase.

Analysis of the endoxylanase expression level by RT-PCR

However, the total expression level of recombinant endoxylanase was also increased in strain harboring pGMF-xylP despite the use of the same expression system (*Gal10* promoter and *Gal7* terminator) except signal sequence in pG-xylP and pGMF-xylP. Therefore, we performed RT-PCR to examine the level of *XylP* transcription in the yeast transformants. Total RNA was prepared from SEY2102/pG-xylP and SEY2102/pGMF-xylP and cDNA was subsequently synthesized. The cDNA was amplified using a specific primer set, and the transcription level was assessed. As shown in Fig. 2A, the transcription level of *XylP* gene in SEY2102/pG-xylP was reduced relative to that in SEY2102/pGMF-xylP. Thus, we inferred that the higher activity of total endoxylanase was a result of increased transcription, however, it should be more studied the relationship between signal sequence and transcription level.

Secretory production of recombinant endoxylanase by baffled-flask culture

The SEY2102/pGMF-xylP transformant exhibiting the highest level of *XylP* expression was chosen for the subsequent baffled-flask culture experiment. The SEY2102/pGMF-xylP transformant was grown on 50 ml YPDG medium and cell growth, reducing sugar and activities of extracellular and intracellular endoxylanase were investigated. The total endoxylanase activity reached 88.1 unit/ml after 48 h cultivation (Fig. 2B). The expression profile of extracellular endoxylanase was accelerated for cell growth and galactose induction from 18 h to 48 h. The secretion efficiency of endoxylanase reached a maximum at 92%. When another endoxylanase from *Bacillus* sp. was expressed in *S. cerevisiae*, the total activity of endoxylanase reached 9.8 unit/ml although a constitutive expression system was used [7]. However, when endo-1,4-β-xylanase C isolated from *Phanerochaete chrysosporium* was inducibly expressed in *P. pastoris* under the control of the alcohol oxidase I promoter, recombinant endoxylanase of 2.5 unit/ml was secretory produced by using α-factor signal peptide of *S. cerevisiae* [8]. Thermostable xylanase from hyperthermophilic bacterium *Thermotoga maritima* MSB8 was secretorily expressed in *K. lactis* and enzyme activity reached 49 unit/ml in the optimized medium [27]. When these data were compared with activity of the endoxylanase by the

Table 1. Cell concentration, endoxylanase activity, specific endoxylanase activity and secretion efficiency of each transformant after 48 h cultivation on YPDG medium

Transformants	DCW (mg-dry wt/ml)	Endoxylanase activity[a] (unit/ml)		Specific activity[b] (unit/mg-DCW)	Secretion efficiency[c] (%)
		medium	cell		
SEY2102/ pG-xylP	9.0	22	4.8	3.0	82
SEY2102/ pGMF-xylP	9.6	64	6.1	7.3	91
FY833/ pG-xylP	8.7	19.3	4.4	2.7	81
FY833/ pGMF-xylP	7.7	37.3	5.1	5.5	88

[a] One unit of endoxylanase was defined as the amount of enzyme that liberated 1.0 μmol of reducing sugar, equivalent to xylose, from xylan per minute under the conditions described in text.
[b] Specific activity was expressed in total units per milligram of dry cell weight (DCW).
[c] Secretion efficiency was indicated as the ratio (%) of extracellular (medium) activity for total (medium+cell) endoxylanase activity.

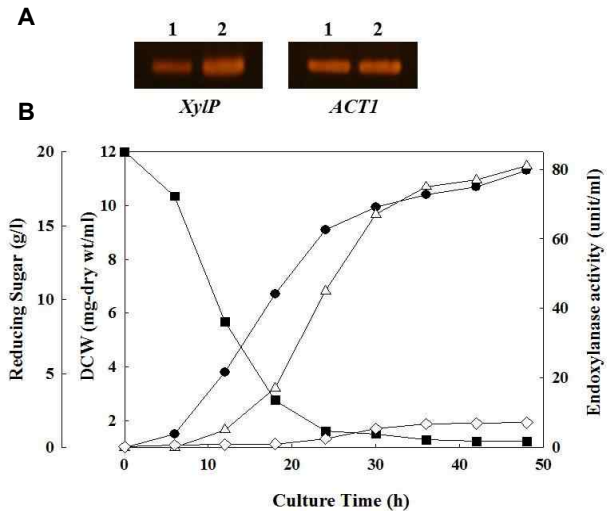

Fig. 2. Analysis of the transcription levels of endoxylanase genes using RT-PCR (A). Total RNA was isolated from *S. cerevisiae* SEY2102/pG-xylP and SEY2102/pGMF-xylP transformants. cDNA was synthesized from total RNA by RT-PCR. The *XylP* and *ACT1* (internal control) were PCR-amplified using each cDNA as template, and the products were visualized by agarose gel electrophoresis. Lane 1: SEY2102/pG-xylP; lane 2: SEY2102/pGMF-xylP transformant. Cell growth (dry cell weight, DCW), reducing sugar and endoxylanase activity in baffled flask culture of *S. cerevisiae* SEY2102 containing pGMF-xylP on YPDG medium (B). Symbols: (●), DCW; (■), Reducing sugar; (△), Extracellular endoxylanase activity; (◇), Intracellular endoxylanase activity.

secretory-expression system in this study, our expression values were higher than those of the other yeast expression system, suggesting that the expression system containing *XylP* gene and $MF\alpha_{opt}$ s.s was efficient for secretory production of recombinant endoxylanase in yeast.

In this study, we described the secretory expression of *Bacillus* endoxylanase in *S. cerevisiae*. For the efficient secretion of recombinant endoxylanase in extracellular medium, the signal sequence of the *XylP* gene and codon optimized MFa_{opt} signal sequence of *S. cerevisiae* were used. We found that the MFa_{opt} signal sequence was an efficient inducer of secretory production of recombinant endoxylanase compared to its own signal sequence. Therefore, the expression system developed in this study produces large extracellular amounts of endoxylanase enzyme using *S. cerevisiae* as the host strain. The endoxylanase produced using this system could be used extensively in industrial applications such as the production of effective feedstuff additives with higher digestibility, efficient biocatalysts for waste treatment, biofuel, and paper manufacture.

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초록 : 출아효모(*Saccharomyces cerevisiae*)에서 *Bacillus* sp. HY-20균주의 재조합 endoxylanase의 효율적 분비 발현

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Bacillus sp. HY-20균주 유래 endoxylanase를 코드하는 *XylP* 유전자를 효모에서 발현시키기 위해 두 개의 발현 플라스미드 pG-xylP와 pGMF-xylP를 구축하였다. 이들 플라스미드는 endoxylanase의 분비발현을 위해 각각 다른 분비서열인 *XylP* 유전자의 자체 분비서열(*XylP* s.s)과 최적화된 MFα 분비서열(MFα_{opt} s.s)을 가지고 있으며, *S. cerevisiae* SEY2102와 FY833균주에 형질전환되어 그 분비활성이 비교 조사되었다. 재조합 endoxylanase는 분비발현시스템과 숙주세포에 따라 23.7~70.1 unit/ml의 활성으로 효모 세포에서 성공적으로 발현되었고, 그 중 SEY2102/pGMF-xylP 형질전환주를 이용해 baffled-flask 배양을 실시한 결과 최대 88.1 unit/ml의 endoxylanase 활성을 보임을 확인하였다. 대부분의 재조합 endoxylanase는 세포 외 분획에 효율적으로 분비 생산되었으며, MF α_{opt} 분비서열이 *XylP* 유전자의 자체 분비서열보다 endoxylanase를 더 효율적으로 분비시킴을 확인할 수 있었다. 그러므로 본 연구에서 개발된 발현시스템은 효모를 숙주세포로 하여 많은 양의 세포 외 endoxylanase의 생산을 가능하게 하고, 바이오에탄올 생산 및 산업적 응용에도 유용하게 사용 될 수 있으리라 기대된다.