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Constitutive Coexpression of *Bacillus* Endoxylanase and *Trichoderma* Endoglucanase Genes in *Saccharomyces cerevisiae*

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Abstract The endoxylanase (GenBank Access No. U51675) of *Bacillus* spp. and endoglucanase (GenBank Access No. AY466436) of *Trichoderma* spp. were separately inserted downstream of the yeast constitutive *ADH1* promoter, resulting in three different plasmids (pAGX1, pAGX2, and pAGX3) according to the transcription direction of two genes. When the yeast transformants, *S. cerevisiae* SEY2102 harboring each expression plasmid, were grown on YPD medium, the total activities of the enzymes were approximately 3.01 unit/ml, 3.24 unit/ml, and 7.56 unit/ml for endoxylanase and 0.60 unit/ml, 0.54 unit/ml, and 0.39 unit/ml for endoglucanase, in the following order: the pAGX1, pAGX2, and pAGX3. More than 70% of the endoxylanase and endoglucanase activities was found in the extracellular media.

Keywords: Coexpression, endoglucanase, endoxylanase, *S. cerevisiae*, xylan

Cellulose and xylan are the major components of plant biomass. Cellulose, a polymer of the β-D-1,4-linked glucose unit, is the major polysaccharide constituent of plant cell wall and one of the most plentiful organic compounds in the biosphere [16, 18]. Many fungi and bacteria capable of utilizing cellulose as a carbon source have been identified [15, 20, 22, 25]. Cellulase 1 (E.C. 3.2.1.4; endoglucanase) catalyzes the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, thereby hydrolyzing cellulose to cellobiose [15, 20, 22, 25]. Endoglucanase has been shown to be nonspecific, releasing reducing sugars from amorphous phosphoric acid-swollen cellulose, hydroxyethyl cellulose, and carboxymethyl cellulose, as well as xylans [31].

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Moreover, xylan is a major component of the cell wall of monocots and hardwoods, representing up to 35% of the dry cell weight of these plants [1, 25]. Unlike cellulose, xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains [2, 26]. The xylanolytic enzyme system carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-endoxylanase (E.C. 3.2.1.8), β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterase. All these enzymes act cooperatively to convert xylan into its constituent sugars. A number of bacterial and fungal species are able to utilize xylan as a carbon source [1, 4, 9, 29]. The degradation of cellulose and xylan occurring in nature is carried out mainly by microorganisms. There are considerable interests in enhancement of the degradation properties because of their potential application in waste treatment, ruminal digestion, and paper manufacturing [2, 19, 28].

The yeast *Saccharomyces cerevisiae* is an attractive host for the production of recombinant derived proteins, including those of medical and food importance, since it is nonpathogenic and free of endotoxins for man and has been grown on an industrial scale for centuries [3, 11]. To increase the ability of yeast to hydrolyze different polysaccharide substrates present in plant raw materials, several heterologous genes coding for hydrolytic enzymes have been expressed in this organism [10, 13, 17, 26].

Numerous cellulose genes have been cloned from *Trichoderma* spp. [8, 12, 27]; several endoxylanase genes have also been cloned from *Bacillus* spp. [7]. Moreover, the endoxylanase and endoglucanase have been expressed and characterized in *E. coli* or yeast [7]. However, the coexpression of both endoxylanase and endoglucanase in *S. cerevisiae* has not yet been reported. In this study, we first report the coexpression of *Bacillus* endoxylanase and

Trichoderma endoglucanase in *S. cerevisiae*. Expression of both the genes in yeast was obtained with the aid of 2μ-based multicopy plasmids, which were constructed in three combinations with each different direction of transcription, using the constitutive alcohol dehydrogenase 1 (*ADH1*) promoter.

E. coli DH5α strain was used for all bacterial transformations and plasmid preparations. 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. the yeast host strain used in this work was *S. cerevisiae* SEY2102 (*MATα ura3-52 leu2-112 his4-519 suc2-Δ9*). For the simultaneous production of *Bacillus* endoxylanase and *Trichoderma* endoglucanase in *S. cerevisiae*, the ORFs of the endoxylanase (*xynA*; 642 bp; 213 amino acids; GenBank Access No. U51675) [7] and endoglucanase (*egl6*; 1,254 bp; 417 amino acids; GenBank Access No. AY466436) [23] genes were constructed under the control of yeast *ADH1* promoter [30], and resulted in the pAEDX-1 [5] and pVT-C4 [23] plasmids, respectively. The pVT-C4 plasmid was digested with SphI enzyme and eluted a 2.2-kb fragment. This fragment was ligated with a partial-digested pAEDX-1 vector. The resulting coexpression plasmids (Fig. 1), pAGX1, pAGX2, and pAGX3, were transformed into *E. coli* DH5α strain. The pAGX1 and pAGX2 plasmids were in the same direction of transcription, whereas the pAGX3 plasmid was in the opposite direction of transcription. Unfortunately, we could not obtain another plasmid for each different direction of transcription.

Transformation of *S. cerevisiae* was carried out by the lithium acetate method [6]. For selection the transformants, we used YNBCAD (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid, and 2% dextrose) plates. Yeast colonies producing endoxylanase and endoglucanase were detected on YPD (1% yeast extract, 2% peptone, 2% dextrose) plates containing 0.5% oat spelts xylan or 0.5% carboxymethylcellulose (CMC) by the Congo-red staining method [24], respectively. *S. cerevisiae* cells harboring plasmid were precultured at 30°C in 10 ml of YNBCAD medium. Second preculture was done in a 500-ml baffled-flask containing 50 ml of YPD medium and cultured in a fermentor containing 1,000 ml of YPD medium. The culture pH was controlled at 5.5 with 50% NH_4OH and 3 N HCl.

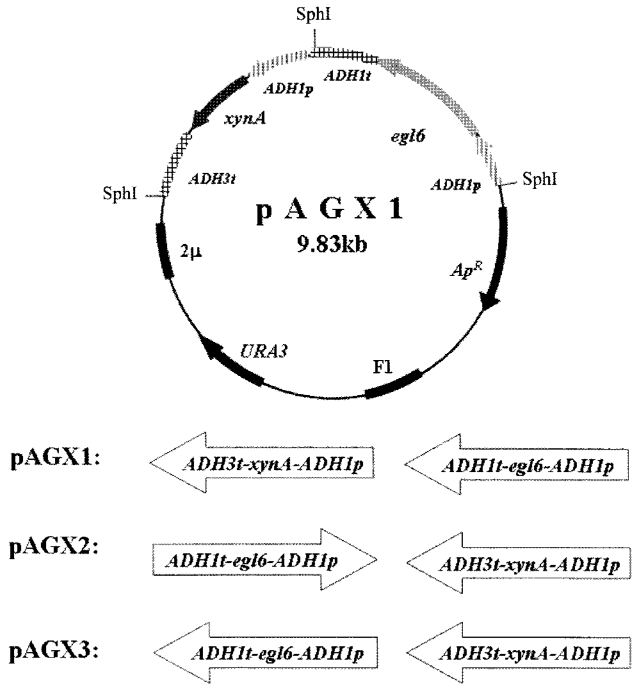

Fig. 1. Schematic diagram of plasmids pAGX1, pAGX2, and pAGX3.

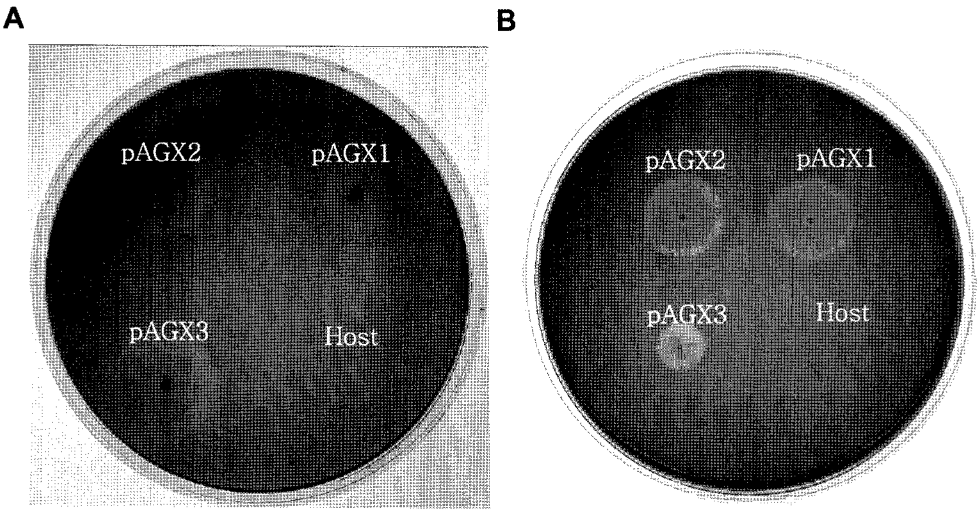

Fig. 2. Active staining of endoxylanase and endoglucanase coexpressed in yeast transformants that were grown on YPD medium containing xylan (**A**) and CMC (**B**), respectively.

The dissolved oxygen level was maintained above 30% of air saturation by automatically adjusting the agitation speed in the range of 300 to 600 rpm.

The yeast cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). The yeast culture broth was centrifuged, and then the supernatant was used for the measurement of extracellular endoxylanase and endoglucanase activities. The periplasmic and cytoplasmic fractions of yeast were obtained by treatment of Zymolyase 100T (Seikagaku Kogyo, Japan) and glass beads [23]. The residual concentration of glucose was measured by the dinitrosalicylic acid method [21]. The endoxylanase activity was determined by measuring the reducing sugar released from oat spelts xylan [21]. One unit of endoxylanase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar per min at 60°C. Quantitative assay of endoglucanase used CMC at 50°C and was measured according to the reducing sugar method. One unit of endoglucanase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min at 50°C.

To investigate the expression levels of endoxylanase (*xynA*) and endoglucanase (*egl6*), *S. cerevisiae* harboring the coexpression plasmid was cultured in a fermentor containing YPD medium. The endoxylanase and endoglucanase genes were successfully coexpressed in yeast transformants. As shown in Fig. 2, the *S. cerevisiae* harboring the pAGX3 plasmid had the most enzyme activity for endoxylanase by the Congo-red staining method. However, this also had the least enzyme activity for endoglucanase, except for the host strain. We have also studied the cell growth and the activities of intracellular and extracellular enzymes among the transformants after 48 h cultivation on YPD medium. *S. cerevisiae* SEY2102 harboring the pAGX1 plasmid was grown on YPD medium and the results are shown in Fig. 3A. The total activities of the enzyme reached about 3.01 unit/ml for endoxylanase and 0.60 unit/ml for endoglucanase after 48 h cultivation on YPD medium (Fig. 3A). The expression profile of extracellular endoxylanase in *S. cerevisiae* SEY2102 was accelerated in proportion to cell growth from 12 h to 30 h, and then it leveled off. The secretion efficiencies of endoxylanase reached at 79% and endoglucanase reached at 73% in *S. cerevisiae* SEY2102. When the yeast transformant, *S. cerevisiae* SEY2102 harboring the pAGX2 vector, was grown on YPD medium, the total activity of the enzyme was about 3.24 unit/ml for endoxylanase and 0.54 unit/ml for endoglucanase after 48 h cultivation on YPD medium (Fig. 3B). The expression profile of extracellular endoxylanase in *S. cerevisiae* SEY2102 was accelerated for cell growth from 12 h to 48 h. The secretion efficiencies of endoxylanase reached at 73% and endoglucanase reached at 76% in *S. cerevisiae* SEY2102. When the yeast transformant, *S. cerevisiae* SEY2102 harboring the pAGX3 vector was grown on YPD medium, the total activity of the enzyme was about 7.56 unit/ml for endoxylanase and 0.39 unit/ml for endoglucanase after 48 h cultivation on YPD medium (Fig. 3C). The expression profile of extracellular endoxylanase in *S. cerevisiae* SEY2102 was accelerated for cell growth from 12 h to 48 h. The major activities of endoxylanase and endoglucanase were found in the extracellular medium. The secretion efficiencies of endoxylanase reached at

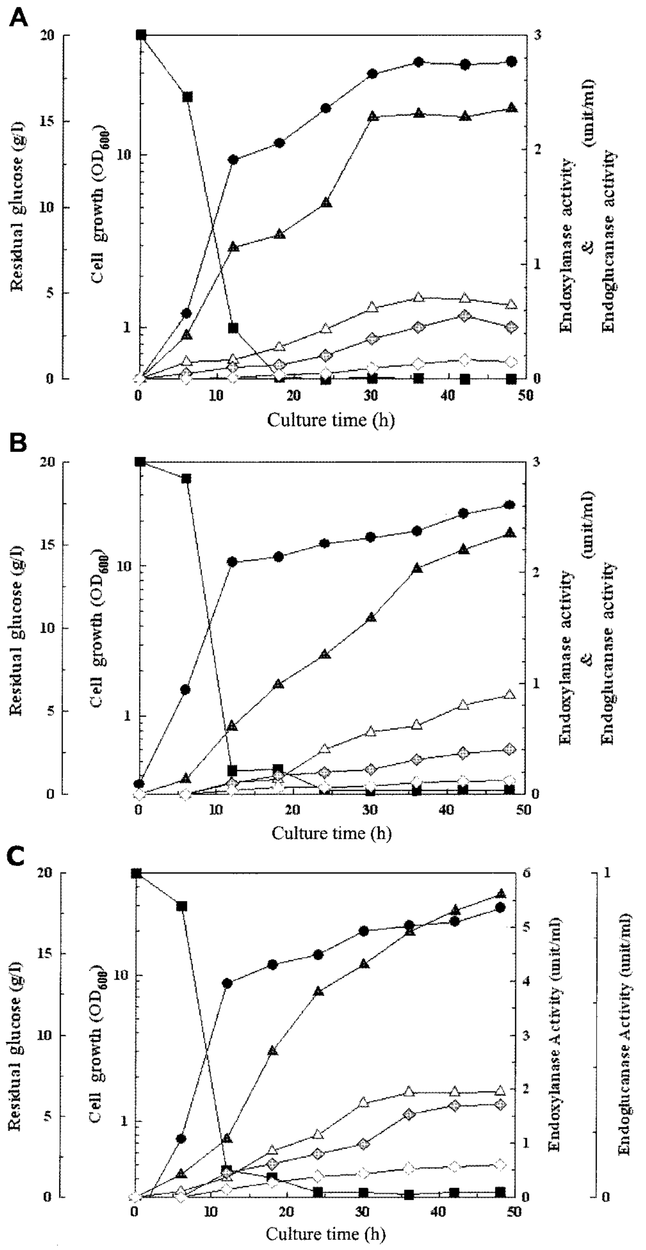

Fig. 3. Cell growth and coexpression of endoxylanase and endoglucanase in batch cultures of *S. cerevisiae* SEY2102 containing pAGX1 (**A**), pAGX2 (**B**), or pAGX3 (**C**) on YPD medium, respectively.

Symbols: (●), Cell growth; (▲), Extracellular endoxylanase activity; (△), Intracellular endoxylanase activity; (◆), Extracellular endoglucanase activity; (◇), Intracellular endoglucanase activity; (■), Residual glucose.

Table 1. Comparison of cell growth, endoxylanase activity, and endoglucanase activity in *S. cerevisiae* SEY2102/pAGX1, pAGX2, and pAGX3 after 48 h cultivation on YPD medium.

Plasmid	Cell growth (OD_{600})	Endoxylanase activity[a] (unit/ml)		Secretion efficiency	Endoglucanase activity[b] (unit/ml)		Secretion efficiency
		medium	cell	(%)	medium	cell	(%)
pAGX1	34.7	2.36	0.65	79	0.45	0.15	73
pAGX2	25.5	2.35	0.89	73	0.41	0.13	76
pAGX3	28.8	5.60	1.96	74	0.29	0.10	74

[a]One unit of endoxylanase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar per min at 60°C.
[b]One unit of endoglucanase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min at 50°C.

74% and endoglucanase reached at 75% in *S. cerevisiae* SEY2102. In this study, all the residual concentration of reducing sugar was depleted from the medium at around 18 h and 24 h. These results are shown in Table 1. We have previously reported the expression of endoxylanase [5] and endoglucanase [23] in *S. cerevisiae* SEY2102, respectively. In the previous study, the total activities of the enzymes were approximately 9.8 unit/ml for endoxylanase and 1.0 unit/ml for endoglucanase [5, 23]. When these data were compared with total activities of the enzymes by the coexpression system in this study, these data have higher values than those of the coexpression system. However, both enzymes produced by the coexpression system can be directly used to apply in ruminal digestion, waste treatment, fuel production, and paper manufacturing.

In this paper, we describe the simultaneous expression of *Bacillus* endoxylanase and *Trichoderma* endoglucanase in combination in *S. cerevisiae*. Expression of both genes in yeast was obtained with the aid of 2μ-based multicopy plasmids constructed in three combinations with each different direction of transcription, using the constitutive alcohol dehydrogenase 1 (*ADH1*) promoter. In the case of the coexpression system, it has been observed that the expression profiles of genes have a different manner, as the direction of transcription has the same or opposite direction [10, 14]. In this study, both *xynA* and *egl6* in pAGX1 and pAGX2 have the opposite direction from the 2μ-origin, but both genes in each plasmid have the same direction of transcription. The pAGX2 has the different direction from the 2μ-origin, for the endoglucanase, whereas the endoxylanase is located at the same direction from the 2μ-origin. We suspected that the binding of RNA polymerase to the *ADH1* promoter domain upstream of *xynA* and *egl6* ORFs had been affected by the RNA polymerase from the proceeding gene, and then the expression level of the following gene may be suppressed, for example of the following gene, *xynA* in the pAGX1 and *egl6* in the pAGX3.

Consequently, the results of this study can be used to develop or to produce effective feedstuff additives with higher ruminal digestibility and efficient biocatalysts for waste treatment, biofuel, and paper manufacturing. Moreover, the possibility to control separately the expression level of endoxylanase and endoglucanase in *S. cerevisiae* will provide us with wider biotechnological applications of the yeast coexpression system.

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