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Molecular Cloning and Expression of the *Trichoderma harzianum* C4 Endo-β-1,4-Xylanase Gene in *Saccharomyces cerevisiae*

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An endo-β-1,4-xylanase (β-xylanase) from *Trichoderma harzianum* C4 was purified without cellulase activity by sequential chromatographies. The specific activity of the purified enzyme preparation was 430 units/mg protein on D-xylan. The complementary DNA (cDNA) encoding β-xylanase (*xynII*) was amplified by PCR and isolated from cDNA PCR libraries constructed from *T. harzianum* C4. The nucleotide sequence of the cDNA fragment contained an open reading frame of 663 bp that encodes 221 amino acids, of which the mature protein is homologous to several β-xylanases II. An intron of 63 bp was identified in the genomic DNA sequence of *xynII*. This gene was expressed in *Saccharomyces cerevisiae* strains under the control of *adhI* (alcohol dehydrogenase I) and *pgkI* (phosphoglycerate kinase I) promoters in 2 μ-based plasmids, which could render recombinants able to secrete β-xylanase into the media.

Keywords: *Trichoderma harzianum*, endo-β-1,4-xylanase, *Saccharomyces cerevisiae*

Cellulose and hemicellulose are the main components of plant cell walls and account for about 50% of all plant biomass [6, 13]. Many studies have focused on methods to improve the utilization of plant biomass. To utilize plant biomass, the components of plants such as cellulose, hemicellulose, and lignin must first be dismantled and broken down into constituent sugars capable of being utilized by organisms harboring corresponding enzymes. This process is catalyzed by cellulolytic and xylanolytic enzymes.

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Xylan is a major component of hemicellulose and an abundant natural polysaccharide [3]. Recently, there have been industrial interests in xylan and its hydrolytic enzymes as a supplement in animal feed; in the manufacture of bread, food, and drinks; in textiles; in bleaching of cellulose pulp; and for ethanol and xylitol production [3]. The main enzymes involved in xylan degradation are β-xylanase, which hydrolyzes the xylose backbone, and β-xylosidase, which hydrolyzes xylobiose and short xylooligosaccharides [1]. Filamentous fungi, particularly *Aspergillus* and *Trichoderma*, are known to be effective producers of both xylanolytic and cellulolytic enzymes. We have isolated and characterized the cellulase system of *T. harzianum* C4 from cellulosic wastes in Korea [16]. Hydrolytic enzymes from *T. reesei* have been well characterized, but little is known about them in *T. harzianum*.

In this paper, we report the purification of a β-xylanase from *T. harzianum* C4 without contamination with cellulolytic enzymes, making it suitable for use in the manufacture of high-quality pulp. The cDNA encoding β-xylanase was cloned and heterologously expressed in *S. cerevisiae* to facilitate its use in animal feed and the production of bread.

Materials and Methods

Strains and Media

T. harzianum C4 was cultivated in Mandels and Andreotti medium [9] with 1% glucose, 0.25% carboxylmethyl cellulose, 0.25% avicel, and 0.5% birchwood xylan at 26°C. *E. coli* XL1-Blue cells were used for construction of recombinant plasmids and preparations, and cultivated in Luria–Bertani media (Difco, U.S.A.). For the heterologous production of β-xylanase, different genetic backgrounds of *S. cerevisiae* strains SEY2102 (*Matα*, *ura*3-52, *leu*2-3, *leu*2-112, *his*4-419, *suc*2-Δ9) or YNN27 (*Matα*, *trp*-289, *ura*3-52, *his*3, *gal*2, *gal*10), and plasmids pVT-103-u (*adhI* promoter and terminator, *ura*3, amp^R, 2 μm *ori*) or YEp-IPT (*pgk* promoter and terminator, *ura*3, amp^R,

2 μm *ori*), were used. *S. cerevisiae* was cultivated in either YPD rich medium (Difco, U.S.A.) for the production of β-xylanase or YNBCAD (0.5% casamino acid, 0.67% yeast nitrogen base without amino acid, 2% glucose) for the selection of transformants.

Purification of β-Xylanase from *T. harzianum* C4 and Assay of its Enzymatic Activity

An 8-l volume of a 6-day culture of *T. harzianum* C4 was filtered through Whatman No. 1 paper. The filtrate was precipitated with ammonium sulfate between 20% and 80% saturation. The precipitate was dissolved in 50 mM sodium acetate buffer, pH 5.0, and dialyzed twice against the same buffer at 4°C. The dialyzed solution was fractionated by gel filtration chromatography with Sephacryl S-200, equilibrated and eluted with 50 mM acetate buffer, pH 5.0. The fraction containing β-xylanase was diluted with a 25 mM acetate buffer, pH 5.0, and applied to a column packed with carboxymethyl-Sepharose CL-6B equilibrated with dilution buffer. The column was eluted with dilution buffer with a linear NaCl gradient from 0 to 0.5 M. The fraction containing β-xylanase activity was diluted with an equal volume of 4 M NaCl and applied to the column with phenyl–Sepharose equilibrated with 2 M NaCl, and 25 mM acetate buffer, pH 5.0. The column was washed with the same buffer and eluted with 25 mM acetate buffer, pH 5.0, with a decreasing linear gradient from 2 to 0 M NaCl. The activity of β-xylanase was assayed by measuring the release of reducing sugars from 1% birchwood xylan (Sigma, U.S.A.) [14]. The activity of endoglucanase was quantified by using carboxymethyl-cellulose as a substrate according to the previous reducing sugar method. One unit was defined as the amount of enzyme releasing 1 μmole of reducing sugars per minute. The amount of protein was determined by the Lowry method using bovine serum albumin as a standard [8].

Preparation of Genomic DNA, mRNA, and cDNA of *T. harzianum* C4

Mycelia of *T. harzianum* C4, grown as previously described in the methods, were harvested and ground in liquid nitrogen. Genomic DNA was extracted with buffer (1.4 M NaCl, 100 mM Tris-Cl, 20 mM EDTA, 3% CTAB, and 1% β-mercaptoethanol) and total RNA was also extracted with buffer (0.18 M Tris, 4.5 mM EDTA, and 1% SDS). After phenol extractions, genetic material was precipitated by the addition of ethanol. The poly(A)-containing messenger RNA was purified with an Oligotex-dT mRNA mini kit (Qiagen, U.S.A.). cDNA synthesis was carried out with a cDNA synthesis kit (Takara, Japan), and a cDNA library was prepared with a cDNA PCR library kit (Takara, Japan) according to the manufacturer's instructions.

Cloning of cDNA and Genomic DNA of *T. harzianum* C4 β-Xylanase Gene (*xynII*)

At first, the partial cDNA of *xynII* was amplified from the generated cDNA library by PCR with the *xyn* C1 primer (5'-GACAGTGATG GAAGCAGA-3') based on the C-terminal sequence of the *T. reesei* β-xylanase gene (*xynI*) [18] and the CA primer (5'-CGTGGTACCA TGGTCTAGAGT-3') located in the CA adaptor for cDNA library. Then, completed cDNA was amplified with the *xyn* P1 primer (5'-ACATggatccGAACAACAACTTGAAACCATG-3') at the N-terminal sequence of partial cDNA and the RA primer (5'-CTGATCTAGAC CTGCAGGCTC-3') in the oligo-dT–RA primer used for RNA reverse transcription. The *xyn* P1 primer included a cleavage site for BamHI (indicated in lowercase) to facilitate subsequent cloning of the fragment. The complete cDNA was amplified and cloned into the pGEM T-easy vector (Promega, U.S.A.), resulting in pTXC1. Genomic DNA containing *T. harzianum* C4 *xynII* was amplified by PCR with the *xyn* N1 primer (5'-TACTGGAACGATGGCCAC-3') and the C1 primer based on cDNA and cloned into the pGEM T-easy vector (pTXG1). Comparison of nucleotide sequences revealed the presence of an intron.

Construction and Characterization of *S. cerevisiae* Transformants with a *T. harzianum* C4 β-Xylanase Gene (*xynII*)

The *T. harzianum* C4 *xynII* from pTXC1 isolated by digestion with BamHI and PstI was subcloned under an *adh*1 promoter in pVT103-U with compatible ends. The resulting plasmid pYPX1 was transformed into *S. cerevisiae* SEY2102 and YNN27 by electroporation. Transformants were selected by complementation of *ura*3 as a selection marker on YNBCAD containing 0.02% histidine for SEY2102, and 0.02% histidine and tryptophan for YNN27. The *xynII* cDNA was also under transcription of a *pgk*1 promoter in YEp-IPT containing the *trp*1 as a selection marker using BamHI and SacI. The resulting plasmid pYYX1 was transformed into YNN27 by electroporation and selected on YNBCAD containing 0.02% histidine and uracil. Transformants exhibiting high activity were selected on plates containing 0.5% birchwood xylan by Congo-red staining [15]. Among them, transformants secreting the highest activity in YPD media were selected. Secretion efficiencies were calculated by measuring the activities in media and cell lysate with glass beads in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% TritonX-100, 0.1% SDS, 1 mM PMSF). Plasmid stabilities were analyzed by calculation of the ratio of the colonies on YPD plate and a selection plate after cultivation for 72 h in YPD media. Purified β-xylanase from *T. harzianum* C4 and secreted β-xylanase from *S. cerevisiae* transformant were used to examine the optimum temperature and pH of this enzyme.

The GenBank accession number of the sequence reported in this paper is EU821597.

Results

Purification of Endo-β-1,4-Xylanase from *T. harzianum* C4

Crude culture filtrates of *T. harzianum* C4 grown on modified Mandels' media contained high concentrations of cellulases and β-xylanase [16], which were concentrated by precipitation with ammonium sulfate. Chromatography of the concentrate on Sephacryl S-200 resulted in one major peak between the CMC–cellulase peaks (Fig. 1A). The β-xylanase peak was diluted and further purified by chromatography on CM–Sepharose. β-Xylanase bound to CM–Sepharose was eluted in one major peak with a gradient of 0.15 to 0.25 M NaCl (Fig. 1B), but the β-xylanase activity was still contaminated with endoglucanase activity. Upon analysis with SDS–PAGE, the major band showed a protein of 22.5 kDa, which contained a little contamination (data not shown). The β-xylanase was further purified to homogeneity by chromatography on phenyl–Sepharose (Fig. 1C) by the

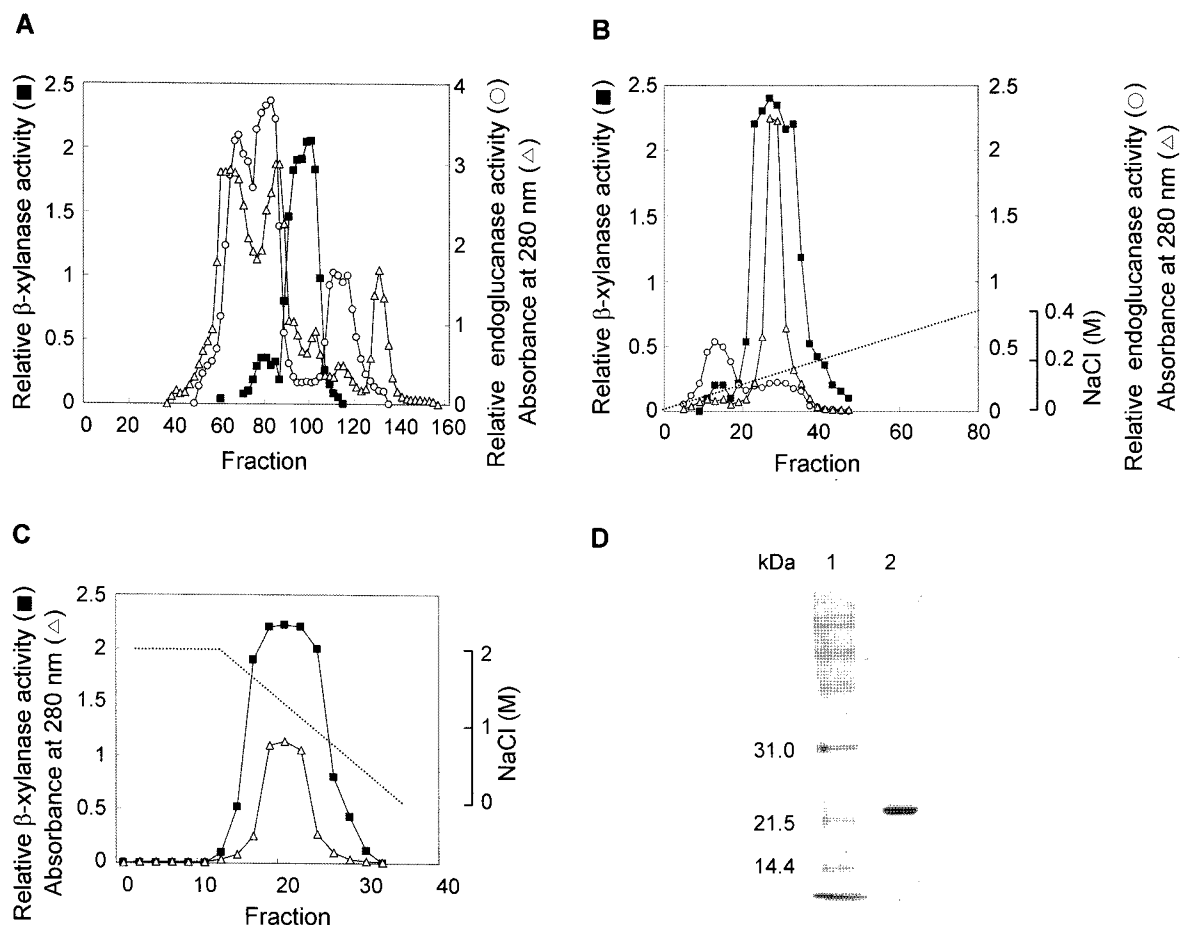


Fig. 1. Purification of β-xylanase with sequential chromatographies of Sephacryl S-200 (**A**), CM–Sepharose CL6B using a linear NaCl gradient (**B**), and phenyl–Sepharose using a decreasing gradient of NaCl (**C**). **D**. SDS–PAGE of the purified β-xylanase. Lane 1 contained protein standards and lane 2 was loaded with 5 μg of purified β-xylanase.

criteria of SDS–PAGE (Fig. 1D) and isoelectric focusing, from which its molecular mass and pI were estimated to be 22.5 kDa and 9.4, respectively. The results for purification of β-xylanase from *T. harzianum* C4 are summarized in Table 1. Specific activity of the final enzyme preparation increased 4.8-fold and total activity was recovered by 33%.

cDNA of β-Xylanase (*xynII*) from *T. harzianum* C4

The *xyn* C1 primer from strictly identical C-terminals of several β-xylanase genes and the CA primer used for generation of the cDNA library were used to amplify the fragment containing the 5'-noncoding region and cDNA of β-xylanase. The second round of PCR was performed with the P1 primer specific to the 5'-terminal of the amplified cDNA and the RA primer, and the complete cDNA containing the 3'-noncoding region was amplified. The nucleotide sequence of the cDNA is shown in Fig. 2. The open reading frame of 663 bp started with the ATG codon at position 19 and terminated with the TAA codon at position 681, encoding a protein of 221 amino acids. The translated protein was identical to the secreted form of *T. harzianum* E58 XynII chain A [20], except for one glycine at position

Table 1. Purification of the endo-β-1,4-xylanase from *T. harzianum* C4.

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Fold	Recovery (%)
Culture filtrate	1,750	275	24,500	89	1	100
Precipitation with AS[a]	100	143	20,335	142	1.6	83
Sephacryl S-200	400	52.3	12,400	237	2.7	51
CM–Sepharose	240	28.2	10,200	362	4.1	42
Phenyl–Sepharose	60	19.1	8,200	430	4.8	33

[a]AS, ammonium sulfate from 20% to 80% saturation

47 in our protein and many related β-xylanases, which was replaced with an alanine in *T. harzianum* E58 XYNII chain A. Two glutamic acids, Glu 86 and Glu 177, in active sites of β-xylanase of *T. reesei* (XYNII) [19] were also conserved in the β-xylanase of *T. harzianum* C4. The N-terminal polypeptide of translated β-xylanase probably functions as a secretion signal. This suggestion is based on the observation that β-xylanase activity was measured in the media of transformants harboring its recombinant expression vector, even though the vector has no extra sequence for secretion other than what is coded for by the cDNA of the β-xylanase. This secretion signal might be cleaved by a dibasic protein-processing endopeptidase activity assumed to be present in filamentous fungi [2]. In support of this, the deduced amino acid sequence of this leading polypeptide had a single dibasic endopeptidase processing site [5].

Identification of an Intron Sequence in the *T. harzianum* C4 *xynII* Gene

PCR using P1 and C1 primers against genomic DNA amplified a fragment that contained an intron in addition to the coding region (Fig. 1). The *xynII* cDNA and genomic DNA sequences were compared and an intron of 63 bp was identified. The intron has splice donor, splice acceptor, and lariat branch acceptor sites that are the consensus splice signals for *T. reesei* (GTRxxx...xxCTRAx...xAG) [17].

Expression of the β-Xylanase from *T. harzianum* C4 in *S. cerevisiae*

Growth-associated expression of β-xylanase in *S. cerevisiae* was attempted by using constitutive promoters such as the *adh1* and *pgk1* promoters. The complete *xynII* cDNA was cloned into pVT-103-u (resulting plasmid pYPX1) under the control of the constitutive *adh1* promoter and transformed into *S. cerevisiae* SEY2102 and YNN27 to yield transformants PS and PY, respectively. The *xynII* cDNA was also cloned into YEp-IPT (resulting plasmid pYYX1) under the control of the constitutive *pgk1* promoter and transformed into *S. cerevisiae* YNN27, resulting in YY. Among transformants, 30 colonies were selected to examine the production of extracellular β-xylanase based on Congo-red staining

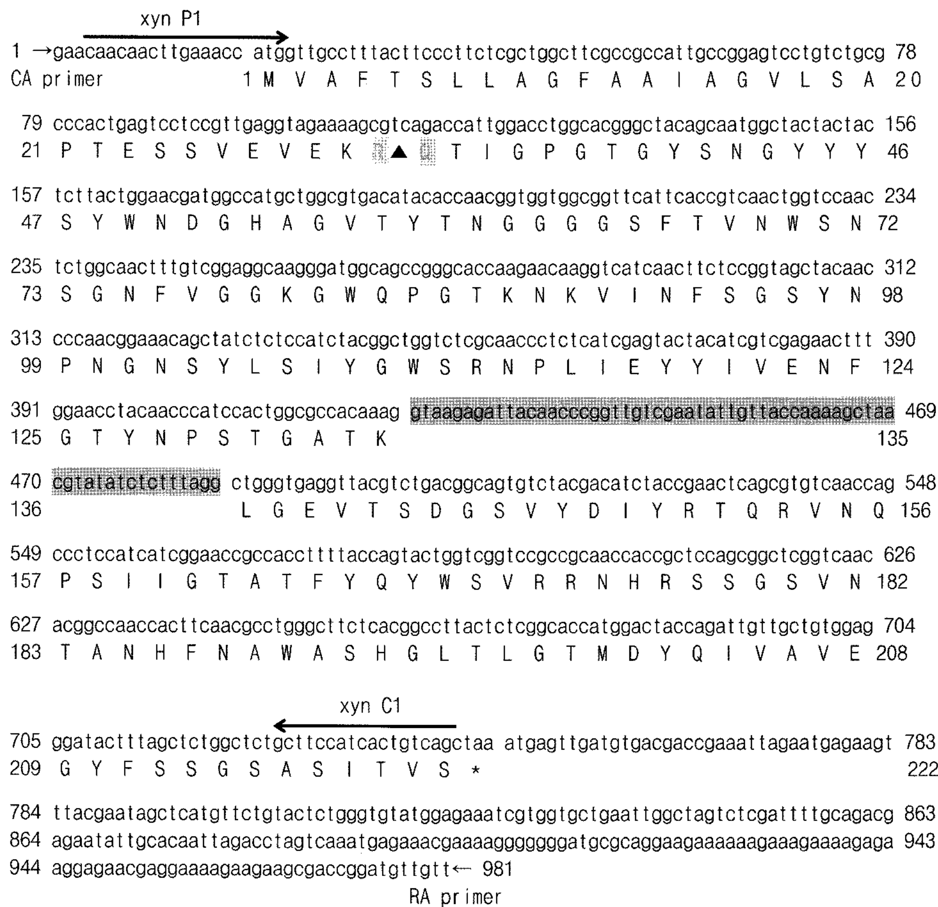


Fig. 2. Genomic DNA sequence for endo-β-1,4-xylanase and deduced amino acid sequence.
The putative cleavage site of the secretion signal propeptide is indicated by a triangle and shade. Glutamic acids in the active center are boxed [18] and an intron is indicated by shading. Primers used are indicated by arrows.

Table 2. Cell growth, plasmid stability and secretion efficiency of *S. cerevisiae* transformants grown on YPD medium for 72 h.

Transformants	Host strains	Promoters	Cell growth (OD_{600})	Plasmid stability (%)	β-xylanase activity (units/l)			Secretion efficiency (%)
					Lysate	Medium	Total	
PS5	SEY 2102	*adhl*	48.7	67	278	871	1,149	68
PY16	YNN 27	*adhl*	37.4	72	157	811	968	75
YY18	YNN 27	*pgkl*	22.6	78	71	556	627	82

results. Transformants PS5, PY16, and YY18 showed the highest β-xylanase activity after 72 h cultivation in 10 ml of YPD medium (data not shown). Each transformant of PS5, PY16, and YY18 was cultivated in 200 ml of YPD medium for 72 h and their activities were assayed every 24 h (Fig. 3). As expected, the enzyme activity in the media increased in proportion to cell growth. Secretion efficiencies of PS5, PY16 and YY18 were 68%, 75%, and 82%, respectively, which indicated the function of polypeptide leading mature form as secretion signal (Table 2). Plasmids of PS5, PY16, and YY18 were maintained at 67%, 72%, and 78%, respectively, after cultivation for 72 h in rich media (Table 2). The optimum temperature for β-xylanase from *S. cerevisiae* was 50°C, identical to that found for the original enzyme (Fig. 4). The recombinant β-xylanase had the highest activity at pH 5.5, whereas the highest activity of this enzyme from *T. harzianum* was seen at pH 5.1 in 0.2 M sodium acetate buffer (Fig. 4).

DISCUSSION

We purified cellulase-free β-xylanase from *T. harzianum* C4. The lack of cellulase activity is important in the textile and paper industries because it is important to be able to extract hemicellulose from natural fibers without damaging the cellulose. This process is called bleaching to enhance the brightness of paper by removal of lignin. For this purpose, several methods, such as fermentation conditions to favor production of β-xylanase and purification process, have been designed. Heterologous expression with a host without cellulase is another approach. In this respect, isolation of the β-xylanase gene represents an essential step in the engineering of efficient microorganisms. We isolated complete cDNA for β-xylanase II from *T. harzianum* C4, thereby expanding the pool of genetic material that can be used in the heterologous expression and protein engineering for novel properties.

To produce β-xylanase in this study, we used a plasmid-based expression vector containing *adh*1 and *pgk*1 promoters for constitutive expression in media containing glucose and ethanol. Although these two promoters transcribe downstream genes to a greater extent in the presence of glucose than ethanol [4], transformants can keep producing

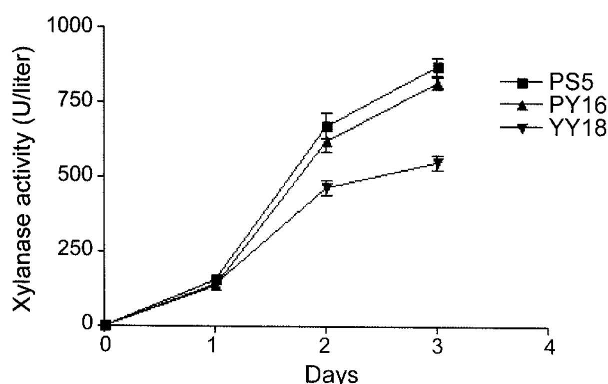
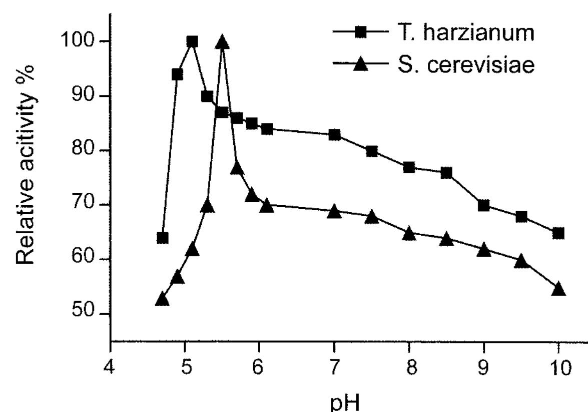


Fig. 3. β-Xylanase expression of *S. cerevisiae* SEY2102 (PS5, ADH1-XYNII), YNN27 (PY16, ADH1-XYNII), and YNN27 (YY18, PGK1-XYNII) after cultivation for 72 h in YPD medium.



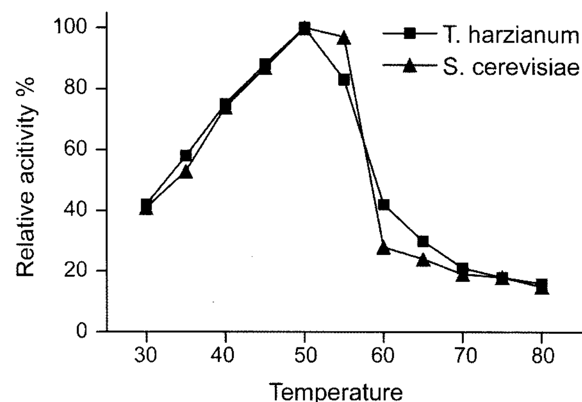


Fig. 4. Optimum pH and temperature (°C) of β-xylanase from *T. harzianum* C4 and *S. cerevisiae* transformant PS5.

the enzyme using ethanol after depletion of glucose, as has been observed for the expression of *E. coli* β-galactosidase under regulation of the *cyc1* promoter [7]. We used auxotrophic markers for selection of transformants, which was reported to have deleterious effects on heterologous protein production [12] and segregational instability in rich media [10]. Despite of these disadvantages and difference in protein machinery between fungi and yeasts, β-xylanase production by *S. cerevisiae* was not as high as it is in industrial production strains, it might be useful for specific purposes such as the biobleaching previously described [11]. Because the β-xylanase preparations from transformants were free of cellulases, it could be used for pulp bleaching. Other applications could include animal feeds and the production of bread where xylanases are used in combination with glucanases, pectinases, cellulases, proteases, and amylases. The β-xylanases in feed can use arabinoxylans as substrates, which improves the digestion of nutrients and consequently feed performance. Breakdown of the hemicellulose in wheat-flour by β-xylanases increases bread volume and allows greater absorption of water, resulting in dough that is easier to knead. Moreover, it is recommended in biscuit-making for making cream crackers lighter and improving the texture, palatability, and uniformity of the wafers. Therefore, cloning of the β-xylanase gene from *T. harzianum* C4 into *S. cerevisiae*, as reported in our study, may confer the above-described benefits if these transformed yeasts are used as probiotics in animal feed and starters in bread fermentation.

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References

1. Biely, P., C. R. Mackenzie, J. Plus, and H. Schneider. 1986. Cooperativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. *Bio/Technology* **4:** 731–733.
2. Calmels, T. P. G, F. Martin, H. Durand, and G. Tiraby. 1991. Proteolytic events in processing of secreted proteins in fungi. *J. Biotechnol.* **17:** 51–66.
3. Collins, T., C. Gerday, and G. Feller. 2005. Xylanases, xylanases families and extremophilic xylanases. *FEMS Microbiol. Rev.* **29:** 3–23.
4. Denis, C. L., J. Ferguson, and E. T. Young. 1983. mRNA levels for the fermentative alcohol dehydrogenase of *Saccharomyces cerevisiae* decrease upon growth on a nonfermentable carbon source. *J. Biol. Chem.* **258:** 1165–1171.
5. Goller, S. P., D. Schoisswohl, M. Baron, M. Parric, and C. P. Kubicek. 1998. Role of endoproteolytic dibasic protein processing in maturation of secretory proteins in *Trichoderma reesei. Appl. Environ. Microbiol.* **64:** 3202–3208.
6. de Vries, R. P., H. C. Kester, C. H. Polusen, J. A. Benen, and J. Visser. 2000. Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. *Carbohydr. Res.* **327:** 402–410.
7. Hardjito, L., P. F. Greenfield, and P. L. Lee. 1993. Recombinant protein production *via* fed-batch culture of the yeast *Saccharomyces cerevisiae. Enzyme Microbiol. Technol.* **15:** 120–126.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1952. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
9. Mandels, M. and R. E. Anderotti. 1978. The cellulose to cellulase fermentation. *Proc. Biochem.* **13:** 6–113.
10. Nacken, V., T. Achstetter, and E. Degryse. 1996. Probing the limits of expression levels by varying promoter strength and plasmid copy number in *Saccharomyces cerevisiae*. *Gene* **175:** 253–260.
11. Polizeli, M. L. T. M., A. C. S. Rizzatti, R. Monti, H. F. Terenzi, J. A. Jorge, and D. S. Amorim. 2005. Xylanases from fungi: Properties and industrial applications. *Appl. Microbiol. Biotechnol.* **67:** 577–591.
12. Pronk, J. T. 2002. Auxotrophic strains in fundamental and applied research. *Appl. Environ. Microbiol.* **68:** 2095–2100.
13. Uffen, R. L. 1997. Xylan degradation: A glimpse at microbial diversity. *J. Ind. Microbiol. Biotech.* **19:** 1–6.
14. Sengupta, S., M. L. Jana, D. Sengupta, and A. K. Naskar. 2000. A note on the estimation of microbial glycosidase activities by dinitrosalicylic acid reagent. *Appl. Microbiol. Biotechnol.* **53:** 732–735.
15. Shirahama, T. and A. S. Cohen. 1966. A Congo red staining method for epoxy-embeded amyloid. *J. Histochem. Cytochem.* **14:** 725–729.
16. Son, Y. J., O. J. Sul, D. K. Chung, I. S. Han, Y. J. Choi, and C. S. Jeong. 1997. Isolation and characterization of *Trichoderma* sp. C-4 producing cellulase. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 346–353.
17. Thomson, J. A. 1993. Molecular biology of xylan degradation. *FEMS Microbiol. Rev.* **104:** 65–82.
18. Torronen, A., R. L. Mach, R. Messner, R. Gonzalez, N. Kalkkinen, A. Harkki, and C. P. Kubicek. 1992. The two major xylanases from *Trichoderma reesei*: Characterization of both enzymes and genes. *Biotechnology* **10:** 1461–1466.
19. Torrone, A and J. Rouvine. 1995. Structural comparison of two major endo-1,4-xylanases from *Trichoderma reesei*. *Biochemistry* **34:** 847–856.
20. Yaguchi, M., C. Roy, D. C. Watson, F. Rollin, L. U. L. Tan, D. J. Senior, and J. N. Saddler. 1992. The amino acid sequence of the 20 kd xylanase from *Trichoderma harzianum* E58, pp. 435–438. *In* J. Visser (ed.), Xylans and Xylanases, Elsevier, Amsterdam.