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Expression of *Aureobasidium pullulans xynA* in, and Secretion of the Xylanase from, *Saccharomyces cerevisiae*

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A previous report dealt with the cloning in *Escherichia coli* and sequencing of both the cDNA and genomic DNA encoding a highly active xylanase (XynA) of *Aureobasidium pullulans* (X.-L. Li and L. G. Ljungdahl, Appl. Environ. Microbiol. 60:3160–3166, 1994). Now we show that the gene was expressed in *Saccharomyces cerevisiae* under the *GAL1* promoter in pYES2 and that its product was secreted into the culture medium. *S. cerevisiae* clone pCE4 with the whole open reading frame of *xynA*, including the part coding for the signal peptide, had xylanase activity levels of 6.7 U ml^{-1} in the cell-associated fraction and 26.2 U ml^{-1} in the culture medium 4 h after galactose induction. Two protein bands with sizes of 25 and 27 kDa and N-terminal amino acid sequences identical to that of APX-II accounted for 82% of the total proteins in the culture medium of pCE4. These proteins were recognized by anti-APX-II antibody. The results suggest that the XynA signal peptide supported the posttranslational processing of *xynA* product and the efficient secretion of the active xylanase from *S. cerevisiae*. Clones pCE3 and pGE3 with inserts of cDNA and genomic DNA, respectively, containing only the mature enzyme region attached by a Met codon had low levels of xylanase activity in the cell-associated fractions (1.6 U ml^{-1}) but no activity in the culture media. No xylanase activity was detected in clone pGE4, which was the same as pCE4, except that pGE4 had a 59-bp intron in the signal peptide region. A comparison of the *A. pullulans* and *S. cerevisiae* signal peptides demonstrated that the XynA signal peptide was at least three times more efficient than those of *S. cerevisiae* invertase or mating α-factor pheromone in secreting the heterologous xylanase from *S. cerevisiae* cells.

The fungus *Aureobasidium pullulans* Y-2311-1 has been shown to produce high levels of very active xylanase (16, 19, 20). Unfractionated extracellular xylanase preparations from this fungus have been successfully used for the bleaching of kraft pulp (28). Two (APX-I and APX-II) of the four extracellular xylanases from this fungus have been purified, and their properties have been reported (17, 18, 22). These two enzymes shared almost identical amino acid sequences for the first 45 amino acids at their N termini, and both reacted to antiserum raised against APX-II, although they had distinct apparent molecular masses and pI values. An 895-bp cDNA sequence (*xynA*) for APX-II was isolated from a cDNA library (21). An open reading frame that encodes a polypeptide of 221 amino acids and that has an internal region identical to the N-terminal amino acid sequence of APX-II was identified. A putative secretion signal peptide containing 34 amino acids was identified at the N terminus of this polypeptide. Amplification of the genomic DNA of *xynA* by PCR and sequencing analysis revealed that a 59-bp intron was located in the DNA region encoding the signal peptide.

The yeast *Saccharomyces cerevisiae* has been widely used as a host organism for the production of such heterologous proteins as enzymes, structural proteins, hormones, interferons, and cytokines (3, 11–13, 23). Unlike bacteria, *S. cerevisiae* does not produce endotoxins, and products of yeast cells are considered safe for uses in pharmaceutical and food products. Another advantage of using *S. cerevisiae* as a host organism for heterologous protein production is that large-scale fermentation and downstream processing of the organism and its products are readily established, since this organism is one of the most commonly used species for industrial processes. Genetic manipulation of *S. cerevisiae* is done routinely, and this organism has several advantages over bacteria in that it carries out posttranslational modifications during the translocation of proteins through the endoplasmic reticulum and the cell membrane. These modifications may include proper folding, glycosylation, disulfate bond formation, and proteolysis. Proteins secreted by yeast cells are protected from aggregation and protease degradation, and they are easily purified, since *S. cerevisiae* secretes only a few proteins. The secretion of proteins is facilitated by hydrophobic short signal peptides at the N-terminal regions of protein precursors. Several secreted yeast proteins and peptides, including invertase and mating α-factor pheromone (α-factor), have such signal peptides. These signal peptides are cleaved off by specific peptidases during the secretion process. A number of heterologous proteins are often retained in periplasmic space or secreted into the culture medium at low yields when they are fused to these yeast signal peptides (5, 7, 23). In this paper, we report the expression of *A. pullulans xynA* in, and secretion of its product from, *S. cerevisiae*. A comparison of the levels of secreted xylanase by the use of different signal peptides revealed that the signal peptide of XynA was more efficient in directing the secretion of the heterologous xylanase than those of yeast invertase and α-factor.

MATERIALS AND METHODS

Strains, plasmids, and genes. *Escherichia coli* TOP10, *S. cerevisiae* INSC1 (*MATα his3-Δ1 leu2 trp1-289 ura3-52*), and plasmid pYES2 were purchased from Invitrogen Corp. (San Diego, Calif.). pYES2 possesses ampicillin and tetracycline resistance genes for selection in *E. coli*, a *URA3* gene for high-copy-number maintenance and selection in a *ura* mutant of *S. cerevisiae*, and a *GAL1* promoter sequence. cDNA of *xynA* from *A. pullulans* was cloned by cDNA library construction and screening as well as PCR amplification (21). The nucleotide se-

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TABLE 1. Oligonucleotide primers used for the amplification of PCR products for *xynA* expression

Primer	Sequence[a]
PF3	.....CACACAAGCTTATGGCCGGTCCCGGTGGCATCAA
PF4	.....CACAGAAGCTTGATCACATCCATTCAAACAAT
PIN	.....ACAGAAGCTTATATGATGCTTTTGCAAGCCTTCCTTTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCAGCCGGTCCCGGTGGCATCAACT
PAF	.....ACAGAAGCTTAAAGAATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCATCCTCCGCATTAGCTGCCGGTCCCGGTGGCATCAACT
PR	.....CCTTCGGATCCTAGCAAGGTGTCTGACATTTA

[a] All primers are written from 5′ to 3′. *Hin*dIII and *Bam*HI sites are underlined and double underlined, respectively.

quence of the full open reading frame was previously determined by automatic DNA sequencing procedures (21).

Construction of plasmid cassettes. Plasmid pYES2 was digested with *Hin*dIII and *Bam*HI. The digested plasmid was dephosphorylated with calf intestinal alkaline phosphatase and purified with the GenecleanII kit (Bio 101, Inc., La Jolla, Calif.). On the basis of the nucleotide sequence of *xynA*, forward (PF3 and PF4) and reverse (PR) PCR primers (Table 1) were synthesized and used to amplify various regions of *xynA*. PF3 corresponded to the first six amino acids of the mature xylanase, whereas PF4 was 19 bp upstream of the start codon of *xynA*. To insert the xylanase gene into pYES2 multiple cutting sites, PF3 and PF4 had a *Hin*dIII site attached and PR had a *Bam*HI site attached. With genomic DNA isolated from *A. pullulans* and its cDNA library being used as templates, *xynA* regions were amplified by 25 cycles of PCR on a 480 thermocycler (Perkin-Elmer Co., Norwalk, Conn.). *pfu* polymerase from Stratagene Cloning Systems, La Jolla, Calif., was used for the amplification. For the fusing of the nucleotide sequence encoding the signal peptides of invertase and α-factor of *S. cerevisiae* (Table 2) to the DNA region encoding the mature xylanase, two long oligonucleotides (PIN and PAF) were designed and synthesized (Table 1). With these two oligonucleotides being used as forward primers, PR being used as the reverse primer, and *A. pullulans* genomic DNA being used as the template, these two yeast signal peptides were fused to XynA mature enzyme during the PCR amplification. Each cycle included 1 min at 54°C for annealing, 1 min at 94°C for denaturation, and 2 min at 72°C for extension. PCR products were purified with the GenecleanII kit and digested with *Hin*dIII and *Bam*HI overnight. DNA fragments were purified and concentrated with the GenecleanII kit before they were ligated to prepared pYES2 with T4 ligase.

Transformation of *E. coli* and propagation of plasmids. Ligation reactions for the transformation of *E. coli* TOP10 (Invitrogen Co.) were performed as described by Sambrook et al. (26). Transformants were plated out on Luria-Bertani plates containing 100 μg of ampicillin per liter. Colonies were picked and grown overnight in Luria-Bertani medium containing ampicillin. Plasmids were purified with the spin column kit from Qiagen (Chatsworth, Calif.). Restriction digestion and nucleotide sequencing were done to verify the presence, orientations, and sequences of the inserts.

Transformation of *S. cerevisiae*. A single colony of yeast strain INVSc1 was grown to an optical density at 600 nm (OD_{600}) of 1.3 in YPD medium, pH 6.5, containing 2% (wt/vol) peptone-Y, 1% (wt/vol) yeast extract-Y, and 2% (wt/vol) dextrose. Cells were harvested by centrifugation (5,000 × *g* for 5 min) at 4°C and washed twice with sterile H_2O and twice with 1 M sterile sorbitol. Cells were resuspended in 2 ml of 1 M sorbitol. Approximately 5 μg of plasmids was used to transform 40 μl of prepared yeast cells with an electroporator (Bio-Rad Laboratories, Hercules, Calif.). Transformants were grown on Sabouraud dextrose (2% dextrose, wt/vol) agar medium lacking uracil and containing 1 M sorbitol and incubated for 3 days at 30°C.

Induction of gene expression. Sabouraud-raffinose (4% [wt/vol]) medium (200 ml) without uracil in 500-ml flasks was inoculated with single colonies of different transformants and shaken (250 rpm) at 30°C for about 48 h. The growth of transformants was monitored by measuring the OD_{600} periodically. When the OD_{600} of the cultures reached 2, sterile galactose was added to the cultures. Samples were collected before and periodically after the addition of galactose. Cells were harvested by centrifugation (5,000 × *g* for 5 min) at 4°C. All samples were kept at −20°C until analyzed.

Isolation of RNA from *S. cerevisiae* and Northern (RNA) blot analysis. After 4 h of induction with galactose, recombinant cells were harvested by centrifugation (5,000 × *g* for 5 min) and washed with H_2O at 4°C. Wet cells (1 g) were subjected to RNA isolation with the total RNA isolation kit (Promega Corp., Madison, Wis.). RNA samples (10 μg) were separated on a formaldehyde agarose (1.5%, wt/vol) gel as described by Sambrook et al. (26) and transferred to a nylon membrane with the Turboblotter (Schleicher & Schuell, Inc., Keene, N.H.). The full-length cDNA of *A. pullulans xynA* was labelled with digoxigenin by PCR in a manner similar to that for the amplification of the expression cassettes. With this DNA sequence being used as a hybridization probe, xylanase-specific mRNA bands on the membrane were detected as described by Li and Ljungdahl (21).

Enzyme and protein assays. Remazol brilliant blue R-D-xylan (Sigma Chemical Co., St. Louis, Mo.) (0.2%, wt/vol) in 50 mM sodium acetate buffer, pH 4.5, was used as a substrate for the routine xylanase assay, as modified from that of Biely et al. (2). Reactions were carried out at 30°C for 15 min. Activity units cannot be directly calculated by this procedure. Thus, a xylanase assay using birchwood xylan (22) as the substrate was used to calibrate and convert the absorption readings into activity units. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of xylose equivalent per min. Protein was measured with the bicinchoninic acid reagent from Pierce (Rockford, Ill.).

SDS-PAGE, N-terminal amino acid sequencing, and Western blot (immunoblot) analysis. Yeast clones were grown in raffinose medium to an OD_{600} of 2 and grown further for 6 h after the addition of galactose. Cells were removed by centrifugation. Supernatants were concentrated by tangential-flow ultrafiltration (Amicon, Inc., Beverly, Mass.) against a membrane (YM3) having an apparent molecular-weight cutoff of 3,000. Concentrated samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide (12%, wt/vol) gel electrophoresis (PAGE) as described by Laemmli (15). After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes at 4°C in 20 mM Tris-HCl (pH 8.3), 20% (vol/vol) methanol, and 0.1% SDS with a Mini Trans-Blot cell (Bio-Rad Laboratories). Transfer was done for 1 h at a constant 80 V. The membranes were subjected to Western blot analysis with antiserum against *A. pullulans* APX-II (22) and the immunoblot kit from Bio-Rad Laboratories. The protein bands on the membranes were also visualized by staining with Coomassie brilliant blue R-250 for 5 min, which was followed by destaining twice in a solution containing 10% (vol/vol) acetic acid and 40% (vol/vol) methanol for 5 min each. Dried membranes were scanned with a Hewlett-Packard II_c scanner, and the images were analyzed with the Gel-Pro package (Media Cybernetics, Silver Spring, Md.). The protein bands were also cut out and subjected to amino-terminal amino acid sequencing with a 477A gas-phase sequencer (Applied Biosystems Inc., Foster City, Calif.).

RESULTS AND DISCUSSION

Regions of genomic and cDNA sequences of *xynA* from *A. pullulans* were amplified with the oligonucleotides shown in Table 1. For the expression of the mature enzyme, a Met codon was added to PF3 as a translation initiation codon. DNA fragments were separated on agarose gels, excised after being visualized by ethidium bromide staining, and purified. These fragments were cloned into pYES2 after they were digested

TABLE 2. Signal peptides used for the secretion of *xynA* product from *S. cerevisiae*

Peptide	Sequence	Gene	Organism	Reference
SPXY	MKFFATIAALVVGAVAAPVAEAEAEASSPMLIER	*xynA*	*A. pullulans*	21
SPIN	MLLQAFLFLLAGFAAKISA	*SUC2*[a]	*S. cerevisiae*	4
SPAF	MRFPSIFTAVLFAASSALA	*MFα*[a]	*S. cerevisiae*	14

[a] *SUC2* and *MFα* represent genes of *S. cerevisiae* invertase and α-factor, respectively.

TABLE 3. Cell density and xylanase activity of *S. cerevisiae* clones expressing *A. pullulans xynA* 4 h after galactose induction

Clone	Cell density (OD_{600})	Activity (U ml^{-1})	
		Cell associated	Extracellular
pGE3	3.26	1.60	0
pGE4	3.29	0	0
pCE3	3.15	1.68	0
pCE4	3.22	6.7	26.2

with *Hin*dIII and *Bam*HI. Ten colonies were picked, and plasmid DNA was purified after *E. coli* cells were grown overnight in Luria-Bertani medium. The presence of inserts with proper sizes was detected by restriction analysis. For each construct, at least two plasmids were sequenced to confirm fidelity during PCR amplification. Plasmids without mutations were used for the transformation of yeast cells. Four yeast clones containing PCR sequences amplified from genomic and cDNA samples were obtained and tested for xylanase production (Table 3). Positive yeast colonies were grown in raffinose-Sabouraud medium to an OD_{600} of 2 and induced for the expression of the xylanase gene by the addition of galactose. Clones pGE3 and pCE3 contained DNA sequences amplified from genomic and cDNA templates, respectively, encoding the mature enzyme region. After 4 h of galactose induction, similar levels (1.6 and 1.68 U ml^{-1}) of xylanase activity were detected in cell-associated preparations (Table 3). No activity was detected in the medium of these two cultures. No activity was detected in either cell-associated or extracellular fractions of pGE4 containing genomic DNA of the whole open reading frame. In contrast to the low xylanase levels in pCE3, pGE3, and pGE4, high levels of xylanase activity were detected in both cell-associated (6.7 U ml^{-1}) and extracellular (26.2 U ml^{-1}) fractions of pCE4. The only difference between pGE4 and pCE4 was that pGE4 had the 59-bp-long intron whereas pCE4 did not. Apparently, the intron was a barrier against the production of the active xylanase by pGE4. This inability to produce the active xylanase could be due to either the lack of proper splicing or instability of the transcript in the presence of the intron. Without splicing, the open reading frame could not be read through during the translation. Therefore, no active enzyme was synthesized. It has been noted that no internal recognition signal sequence in the *xynA* intron was found to match the ones commonly found for *S. cerevisiae* introns, although the 5′ and 3′ ends of the intron matched those of *S. cerevisiae* introns (21). Similarly, no proper splicing occurred when *Cryptococcus albidus* xylanase (24) and *Aspergillus awamori* glucoamylase (12) genomic DNA sequences were cloned into *S. cerevisiae*. Nucleotide substitutions of the internal recognition sequence of the *xynA* intron by site-directed mutagenesis might allow *S. cerevisiae* to splice out the intron.

Surprisingly, the activity levels for pCE3 and pCE4 were very different, especially for the extracellular fractions (Table 3). Apparently, the difference was caused by the 34-amino-acid signal peptide of XynA. It seems that the signal peptide not only led to the secretion of the enzyme but also increased the overall levels of active enzyme synthesis. These results can be explained either by the fact that the signal peptide was needed for a high level of enzyme synthesis or that the newly synthesized enzyme was rapidly degraded in the absence of the signal peptide.

A time course study was done with pCE4. Cell-associated and extracellular activity levels and cell density after the addition of galactose were measured (Fig. 1). The culture had an OD_{600} of 2.0 when galactose was added. This value increased to 3.78 after 24 h, with a sharp increase during the first 5 h after induction. The cell-associated xylanase level increased to its highest level (8.1 U ml^{-1}) after 2.5 h of induction and then slowly declined to about 6 U ml^{-1} and stayed low. In contrast, the level of extracellular xylanase continuously increased for 24 h, although a fast increase was observed during the first 4 h. After 24 h of induction, the extracellular xylanase level was 36 U ml^{-1}. It constituted more than 85% of the total xylanase found in the culture medium and the cell-associated fractions. This high percentage of secretion was extraordinary, considering that most proteins mediated by other signal peptides are confined to the cell wall or periplasmic space, with only a small percentage being secreted (7, 9, 24). Assuming that the recombinant xylanase has the same specific activity (2,000 U mg^{-1}) as APX-I and APX-II have (18, 22), the estimated concentration of xylanase was 20 μg ml^{-1}. This yield might be greatly increased by replacing the *GAL1* promoter with constitutive promoters, integrating the expression cassette into chromosomal loci, or deleting the 3′ untranslated sequence of *xynA* (8).

Culture media from pCE4 before and after galactose induction were concentrated and subjected to SDS-PAGE and Western blot analyses (Fig. 2). The protein-banding patterns of lanes loaded with these two samples showed that two protein bands corresponding to molecular masses of 25 and 27 kDa (Fig. 2A) appeared after galactose induction. Thus, one of the bands had the same mass as APX-II (25 kDa), whereas the other band represented a protein with a slightly higher molecular mass. Both bands had sizes larger than that of APX-I (20 kDa). Analysis of the scanned image revealed that these two bands accounted for 82% (by weight) of the total extracellular proteins recovered. To find out whether the retention of the signal peptide or excessive glycosylation of the secreted enzymes caused the small shift from APX-I and APX-II, we determined the N-terminal amino acid sequences of the heterologously produced extracellular xylanases after they were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. N-terminal amino acid sequences for the 25-kDa band (AGPGGINYVQNY) and the 27-kDa band (AG PGGINYVQNYNGNLG) were obtained, and they were iden-

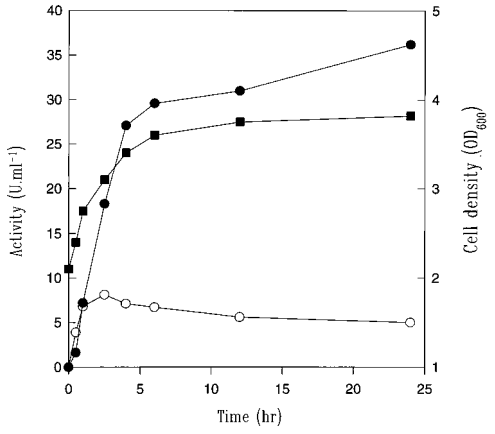

FIG. 1. Time course of xylanase production of pCE4 after galactose induction. One single colony was used to inoculate raffinose-Sabouraud medium. After growth to an OD_{600} of 2, sterile galactose was added. Samples were withdrawn after the addition of galactose at time points shown in the figure. OD_{600} (■), intracellular (○), and extracellular (●) xylanase activities were determined. Culture conditions, preparation of samples, and the enzyme assay are described in Materials and Methods.

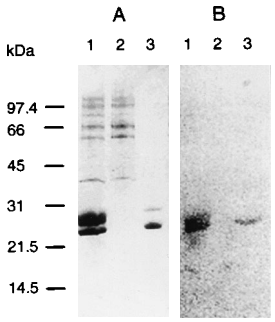

FIG. 2. Scan images of SDS-PAGE (A) and Western blot (B) analyses. Culture media of pCE4 before (lane 2) and 6 h after galactose induction (lane 1) were concentrated about 100-fold and then analyzed by SDS-PAGE and Western blotting as described in Materials and Methods. Partially purified APX-II (22) was also used as a positive control (lane 3). Protein molecular standard markers from Bio-Rad Laboratories are listed on the left.

tical to that of the APX-II from *A. pullulans* (22). There is no Asn-X-Ser/Thr sequence motif which is required for N-linked glycosylation in this xylanase (21). Carbohydrate staining of the protein bands on the polyvinylidene difluoride membrane revealed that both the 25- and 17-kDa bands were glycosylated (data not shown). The results suggest that the xylanases secreted from *S. cerevisiae* were more heavily glycosylated than those originally produced by *A. pullulans* and that the xylanases had different levels of O-linked glycosylation. The heavy glycosylation might explain why the 27-kDa band did not bind well to the APX-II antiserum (Fig. 2B). Excessive glycosylation has been reported for a number of secreted recombinant proteins from yeast cells (12, 25, 27). The results show that the signal peptide of *A. pullulans* XynA was recognized by the yeast secretory pathway. A 24-amino-acid *A. awamori* glucoamylase signal peptide with an Arg-24 was recognized and removed when the gene was expressed in, and its product was secreted from, *S. cerevisiae* (12). Mutation studies have demonstrated that the trypsin-like endopeptidase coded for by the yeast *KEX2* gene and responsible for the maturation of α-factor pheromone in yeast cleaved the peptide bond after Arg-24. Basic dipeptides Lys-Arg in yeast α-factor (1) and *A. awamori* glucoamylase (12) and Arg-Arg in human albumin (6) are the signal motifs for endopeptidase cleavage. It is not certain that the *KEX2* endopeptidase is also responsible for the processing of *A. pullulans* XynA in yeast cells, since the dipeptide Glu-Arg at the C terminus of the XynA signal peptide only has one basic residue. It should be pointed out that before endopeptidase cleavage, internal cleavage of the signal peptides by another signal peptidase was necessary for secretion of yeast invertase, α-factor, and *A. awamori* glucoamylase. This peptidase requires an Ala residue at the amino side of the cleavage. Such a cleavage might have occurred during XynA secretion, since there are several Ala residues on the carboxyl side after the hydrophobic core of the signal peptide.

TABLE 4. Cell density and extracellular xylanase activity of *S. cerevisiae* clones with different signal peptides 6 h after galactose induction

Clone	Cell density (OD_{600})	Activity (U ml^{-1})
pCE4	3.41	28.6
pCE3	3.48	0.34
pIN1	3.40	12.6
pAF1	3.38	9.7

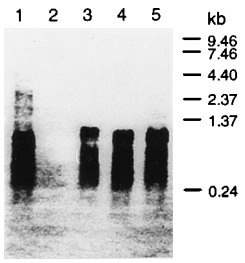

FIG. 3. Scan image of Northern blot analysis for total RNA isolated from *S. cerevisiae* clones pCE3 (lane 1), pYES2 (lane 2), pCE4 (lane 3), pIN1 (lane 4), and pAF1 (lane 5). RNA molecular standard markers from Boehringer Mannheim (Indianapolis, Ind.) are listed on the right.

To compare the efficiency of xylanase secretion directed by the XynA signal peptide with those directed by invertase and α-factor, which are the two most commonly used signal peptides for heterologous protein secretion from *S. cerevisiae*, we synthesized the oligonucleotides (PIN and PAF) encoding the two signal peptides and the first seven amino acids of the mature XynA (Tables 1 and 2). With PIN, PAF, and PR being used as primers and *A. pullulans* genomic DNA being used as template, the signal peptides for the invertase and α-factor genes were fused in frame to the mature xylanase region of *xynA* by PCR amplification. After the PCR products were digested with *Hin*dIII and *Bam*HI, inserted in predigested pYES2, and sequenced to confirm the lack of mutation during the PCR amplification, INVSc1 was transformed with the constructs (pIN1 and pAF1) as well as with pCE4 and pCE3. Single colonies of these transformants were tested for extracellular xylanase production. The four cultures had similar cell densities 6 h after galactose induction (Table 4). Xylanase activity levels in the culture media, however, were very different, with levels of 28.6, 0.34, 12.6, and 9.7 U ml^{-1} for pCE4, pCE3, pIN1, and pAF1, respectively. Total RNA samples were prepared from these four different clones from cells obtained 6 h after galactose induction. Northern blot analysis revealed that the samples had similar levels of xylanase-specific mRNA (Fig. 3), although the sizes of the mRNA bands between the clones were slightly different because of the presence of the signal peptide regions in pCE4, pIN1, and pAF1. It was also noticed that these heterologous mRNA species were not stable in *S. cerevisiae*, since a high percentage of incomplete mRNA was observed (Fig. 3). The low level of xylanase activity in the culture medium of pCE3 could be caused by leakage of intracellular xylanase out of dead cells. The results indicate that the XynA signal peptide is more capable than the two yeast signal peptides of translocating the heterologous xylanase out of the yeast cells. The differences in the supernatant xylanase levels for these clones were related to the efficiency of secretion rather than transcription or translation. The hydrophobic region in the *A. pullulans* XynA signal peptide is longer than those in the signal peptides of yeast invertase, α-factor (Table 2), and *A. awamori* glucoamylase (12). The difference in length may be related to secretion efficiency, as has been demonstrated for prokaryotic signal peptides secreting proteins from *Bacillus subtilis.*

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REFERENCES

1. **Achstetter, T., and D. H. Wolf.** 1985. Hormone processing and membrane-bound proteinases in yeast. EMBO J. **4:**173–177.
2. **Biely, P., D. Mislovicová, and R. Toman.** 1985. Soluble chromatogenic substrates for the assay of endo-1,4-β-xylanases and endo-1,4-β-glucanases. Anal. Biochem. **144:**142–146.
3. **Boreau, A., S. Durand, and R. Morosoli.** 1992. Secretion of a *Cryptococcus albidus* xylanase in *Saccharomyces cerevisiae*. Gene **116:**109–113.
4. **Carlson, M., R. Taussig, S. Kustu, and D. Botstein.** 1983. The secreted form of invertase in *Saccharomyces cerevisiae* is synthesized from mRNA encoding a signal sequence. Mol. Cell. Biol. **3:**439–447.
5. **Chaudhuri, B., K. Steube, and C. Stephan.** 1992. The pro-region of the yeast prepro-α-factor is essential for membrane translocation of human insulin-like growth factor 1 *in vivo*. Eur. J. Biochem. **206:**793–800.
6. **Collins, S. H.** 1990. Production of secreted proteins in yeast, p. 61–77. *In* T. J. R. Harris (ed.), Protein production by biotechnology. Elsevier Applied Science, New York.
7. **Das, R. C., and J. L. Shultz.** 1987. Secretion of heterologous proteins from *Saccharomyces cerevisiae*. Biotechnol. Prog. **3:**43–48.
8. **Demolder, J., W. Fiers, and R. Contreras.** 1992. Efficient synthesis of secreted murine interleukin-2 by *Saccharomyces cerevisiae*: influence of 3′-untranslated regions and codon usage. Gene **111:**207–213.
9. **Emr, S. D., R. Schekman, M. C. Flessel, and J. Thorner.** 1983. An MFα1-SUC2 (α-factor-invertase) gene fusion for study of protein localization and gene expression in yeast. Proc. Natl. Acad. Sci. USA **80:**7080–7084.
10. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1991. Domains in microbial β-1,4-glycanase: sequence conservation, function, and enzyme families. Microbiol. Rev. **55:**303–315.
11. **Hitzeman, R. A., F. E. Hagie, H. L. Levine, D. V. Goeddel, G. Ammerer, and B. D. Hall.** 1981. Expression of a human gene for interferon in yeast. Nature (London) **293:**717–722.
12. **Innis, M. A., M. J. Holland, P. C. McCabe, G. E. Cole, V. P. Wittman, R. Tal, K. W. K. Watt, D. H. Gelfand, J. P. Holland, and J. H. Meade.** 1985. Expression, glycosylation, and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. Science **228:**21–26.
13. **Kniskern, P. J., A. Hagopian, D. L. Montgomery, C. E. Carty, P. Burke, C. A. Schulman, K. J. Hofmann, F. J. Bailey, N. R. Dunn, L. D. Schultz, W. M. Hurni, W. J. Miller, R. W. Ellis, and R. Z. Maigetter.** 1991. Constitutive and regulated expression of the hepatitis B virus (HBV) preS2+S protein in recombinant yeast, p. 65–75. *In* R. T. Hatch, C. Goochee, A. Moreira, and Y. Alroy (ed.), Expression systems and processes for rDNA products. American Chemical Society, Washington, D.C.
14. **Kurjan, J., and I. Herskowitz.** 1982. Structure of a yeast pheromone gene (*MF*α): a putative α-factor precursor contains four tandem copies of mature α-factor. Cell **30:**933–943.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Leathers, T. D.** 1986. Color variants of *Aureobasidium pullulans* overproduce xylanase with extremely high specific activity. Appl. Environ. Microbiol. **52:** 1026–1030.
17. **Leathers, T. D.** 1988. Amino acid composition and partial sequence of xylanase from *Aureobasidium*. Biotechnol. Lett. **10:**775–780.
18. **Leathers, T. D.** 1989. Purification and properties of xylanase from *Aureobasidium*. J. Ind. Microbiol. **4:**341–348.
19. **Leathers, T. D., R. W. Detroy, and R. J. Bothast.** 1986. Induction and glucose repression of xylanase from a color variant strain of *Aureobasidium pullulans*. Biotechnol. Lett. **8:**867–872.
20. **Leathers, T. D., C. P. Kurtzman, and R. W. Detroy.** 1984. Overproduction and regulation of xylanase in *Aureobasidium pullulans* and *Cryptococcus albidus*. Biotechnol. Bioeng. Symp. **14:**225–250.
21. **Li, X.-L., and L. G. Ljungdahl.** 1994. Cloning, sequencing, and regulation of a xylanase gene from the fungus *Aureobasidium pullulans* Y-2311-1. Appl. Environ. Microbiol. **60:**3160–3166.
22. **Li, X.-L., Z.-Q. Zhang, J. F. D. Dean, K.-E. L. Eriksson, and L. G. Ljungdahl.** 1993. Purification and characterization of a new xylanase (APX-II) from the fungus *Aureobasidium pullulans* Y-2311-1. Appl. Environ. Microbiol. **59:**3212–3218.
23. **Marten, M. R., and J.-H. Seo.** 1991. Engineering studies of protein secretion in recombinant *Saccharomyces cerevisiae*, p. 77–95. *In* R. T. Hatch, C. Goochee, A. Moreira, and Y. Alroy (ed.), Expression systems and processes for rDNA products. American Chemical Society, Washington, D.C.
24. **Moreau, A., S. Durand, and R. Morosoli.** 1992. Secretion of a *Cryptococcus albidus* xylanase in *Saccharomyces cerevisiae*. Gene **116:**109–113.
25. **Penttilä, M. E., L. Andre, M. Saloheimo, P. Lehtovaara, and J. K. Knowles.** 1987. Expression of two *Trichoderma reesei* endoglucanases in the yeast *Saccharomyces cerevisiae*. Yeast **3:**175–185.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Vainio, A. E. I., H. T. Torkkeli, T. Tuusa, S. A. Aho, B. R. Fagerström, and M. P. Korhola.** 1993. Cloning and expression of *Hormoconis resinae* glucoamylase P cDNA in *Saccharomyces cerevisiae*. Curr. Genet. **24:**38–44.
28. **Yang, J. L., G. Lou, and K.-E. L. Eriksson.** 1992. The impact of xylanase on bleaching of kraft pulps. TAPPI J. **75:**95–101.