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SHORT CONTRIBUTION

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Secretory production of *Aspergillus oryzae* xylanase XynF1, *xynF1* cDNA product, in the basidiomycete *Coprinus cinereus*

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Abstract The signal peptide of *Aspergillus oryzae* endo-(1,4)-β-xylanase XynF1 contains a C-terminal serine-arginine that directs efficient secretion of the enzyme into the culture medium. In the basidiomycete *Coprinus cinereus*, however, there is little secretion of XynF1 into the culture medium. Modification of the C-terminal sequence of the signal peptide to lysine-arginine resulted in efficient secretion of *C. cinereus* XynF1, suggesting the presence of a KEX2-like protease in this fungus.

Introduction

Basidiomycete fungi are able to degrade lignocellulose, composed of cellulose, hemicellulose and lignin, which is the most abundant renewable organic resource on earth. Thus, fungal strains that constitutively secrete large amounts of lignolytic enzymes are of great interest with respect to the efficient utilization of lignocellulosic resources.. We have recently succeeded in constructing the chromosome-integrating vectors pLC1 and pLC2, which allow the expression of foreign genes in basidiomycete fungi (Ogawa et al. 1998). By using these vectors, the basidiomycete *Pleurotus ostreatus* manganese peroxidase (MnP) cDNA (designated *Po.mnp*c) (Asada et al. 1995) was efficiently expressed in the rapidly growing basidiomycete *Coprinus cinereus* (Ogawa et al. 1998). The *Coprinus cinereus* transformants obtained showed significantly high lignin decolorization and degradation activities. We have also produced *C. cinereus* strains that secrete *Bacillus subtilis* endo-(1,4)-β-xylanase (designated endo-xylanase) under control of the signal sequence of *Po. mnp*c. The *B. subtilis* endo-xylanase produced in the *C. cinereus* transformants were fully active at pH 7–8, but only partially active at around pH 5, which is the pH of the culture supernatant of filamentous fungi. This led us to attempt to produce *C. cinereus* strains that secrete large amounts of *Aspergillus oryzae* endo-xylanase XynF1, which has a pH optimum of 5 (Kitamoto et al. 1998, 1999). However, XynF1 was poorly secreted by *C. cinereus* into the culture medium. In the present study, the C-terminal serine-arginine sequence of the signal peptide of XynF1 was modified to lysine-arginine, resulting in efficient secretion of XynF1 into the culture medium. Application of XynF1-producing *C. cinereus* strains to the utilization of lignocellulosic resource is discussed.

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Materials and methods

Strains and media

C. cinereus monokaryotic strain LT2-44 (*trp1–1*, *trp1–6*) (Ogawa et al. 1998) was used as the recipient in transformation experiments. MYG and MY media (Ogawa et al. 1998) and generation medium (Ogawa et al. 1998) were used for growth of *C. cinereus* strains and generation of LT2-44 protoplasts, respectively. Construction, propagation, and amplification of recombinant plasmids were carried out in *Escherichia coli* JM109 (Yanisch-Perron et al. 1985). *E. coli* was cultured in Luria-Bertani medium.

Plasmid construction

The 1.0-kb *A. oryzae xynF1* cDNA (*Ao.xynF1*c), cloned between the *Pst*I and *Nde*I sites of pUC18 (Kitamoto et al. 1998; Kitamoto et al., unpublished results), was isolated by digestion with *Pst*I and *Nde*I

and both termini were blunted by Klenow fragment. The resulting fragment was ligated to *Eco*RI linker [5′-d(CCGAATTCGG)-3′] and then digested with *Eco*RI. This digest was inserted into the *Eco*RI site of chromosome-integration vector pLC2′, yielding the recombinant plasmid pLC2′-Ao.xynF1 (Fig. 1). pLC2′ is the derivative of pLC2 (Ogawa et al. 1998); pLC2 contains a *Bam*HI cloning site between the basal promoter and terminator of the basidiomycete *Lentinula edodes priA* gene, and pLC2′ contains an *Eco*RI cloning site in place of *Bam*HI. The 1.0-kb *Ao.xynF1*c was digested with *Sal*I and *Nde*I, yielding the 0.9-kb signal-sequence-depleted *Ao. xynF1*c. Both termini were blunted and ligated to the aforementioned *Eco*RI linker. The *Eco*RI digest was inserted in-frame into the *Eco*RI site between the *P. ostreatus* MnP cDNA (*Po.mnp*c) signal sequence and the *priA* terminator of pLC2-Bs.xyn′ (Kikuchi et al. 1999) after the signal-sequence-depleted *B. subtilis* endo-xylanase gene had been removed by digestion with *Eco*RI. The resulting recombinant plasmid was named pLC2-Ao.xynF1′ (Fig. 1). The in-frame rejoined sequence of *Po.mnp*c and signal-depleted *Ao.xynF1*c was named *Ao.xynF1′*c, which encodes a fusion protein containing the lysine-arginine sequence as the C-terminus of the signal peptide. As noted in the Introduction, wild-type *A. oryzae* XynF1 has a serine-arginine sequence as the C-terminus of the signal peptide. This sequence was changed to lysine-arginine by PCR using a primer designed to create the amino acid substitution. This point-mutated *Ao.xynF1* cDNA sequence (named *Ao.xynF″*c) was inserted into the *Eco*RI site of pLC2′, yielding the recombinant plasmid pLC2′-Ao.xynF1″ (Fig. 1). In addition to the lysine-arginine mutation, a cysteine residue was inserted between the N-terminal second and third amino acids of the mature XynF1 enzyme by PCR-mutagenesis. This double- point-mutated *Ao.xynF1* cDNA sequence (named *Ao.xynF1‴*c) was inserted into *Eco*RI site of pLC2′, yielding the recombinant plasmid pLC2′-Ao.xynF1‴ (Fig. 1).

Transformation of *C. cinereus*

C. cinereus LT2-44 was transformed by the polyethylene glycol method according to the procedure previously reported (Ogawa et al. 1998).

Southern- and Northern-blot analyses

DNA isolation and Southern-blot analysis were done according to the method previously reported (Ogawa et al. 1998). Northern-blot

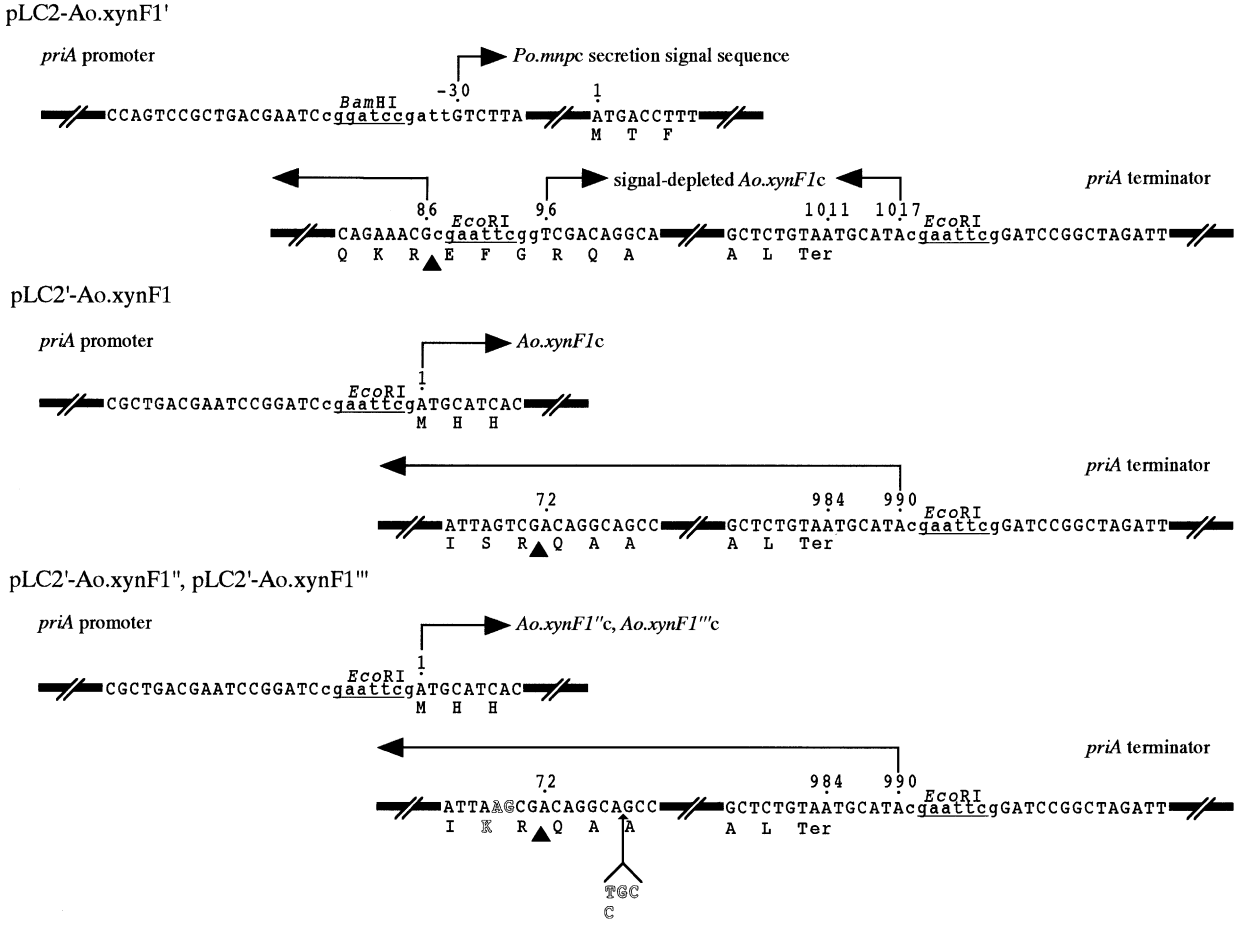

Fig. 1 Simplified structural features of the *Aspergillus oryzae* xylanase-XynF1-expressing plasmids pLC2′-Ao.xynF1, pLC2-Ao. xynF1′, pLC2′-Ao.xynF1″, and pLC2′-Ao.xynF1‴. The nucleotide sequences at the fusion junction between the *Lentinula edodes priA* promoter, *A. oryzae xynF1* cDNA (and its derivative cDNAs), and the *priA* terminator were shown. *Arrowheads* indicate the amino acid sequences of the suggested cleavage site of an endopeptidase responsible for generation of mature XynF1 enzyme. In the nucleotide sequence of pLC2-Ao.xynF1′ (*top*), *Po.mnp*c is *Pleurotus ostreatus* manganese peroxidase (MnP) cDNA. pLC2′-Ao. xynF1″ (*bottom*) carries *Ao.xynF1″*c, which contains a single amino-acid replacement (serine-arginine to lysine-arginine) at the C-terminal sequence of the signal peptide; pLC2′-Ao.xynF1‴carries *Ao.xynF1‴*c, which contains both the amino acid replacement at the C-terminus of the signal peptide and an inserted cysteine residue between the N-terminal second and third amino acids of mature XynF1 enzyme. For further details, see text

analysis of total cellular RNA was done as described previously (Hori et al. 1991).

Activity staining of xylanase in native polyacrylamide gel

A flask containing 20-ml of MY medium was inoculated with three (6×6×1-mm) cubes of MYG/agar colonized with *C. cinereus* mycelium and cultivated at 30°C for 14 days with shaking. Ten ml of the culture supernatant was lyophilized after dialysis against 10 mM Tris-HCl buffer (pH 7.0), and all dried material was dissolved in 1 ml phosphate-buffered saline (PBS; 10 mM Na_2HPO_4/1.7 mM KH_2PO_4(pH 7.4), 0.14 M NaCl, 2.7 mM KCl). Ten μl of the tenfold-concentrated culture supernatant was electrophoresed in native 10% polyacrylamide gel containing 0.5% beech wood xylan (Sigma) in Tris-glycine buffer (25 mM Tris-HCl (pH 8), 250 mM glycine). After electrophoresis, the gel was placed in 50 mM sodium acetate buffer (pH 5.0) and incubated 50°C for 1 h, and then stained with Congo-red followed by decolorization. Xylanase activity was detected as a decolorized band (Chaudhary and Deobagkar 1997). Five μl of the culture supernatant of the high-XynF1-producing *A. oryzae* strain (Kitamoto et al. 1999) grown in GP medium (Kitamoto et al 1999) at 30°C for 4 days was also analyzed as a reference.

Assessment of xylan degradation

Xylanase activity of the culture supernatant of *C. cinereus* was assayed according to the method previously reported (Kikuchi et al. 1999), except that 50 mM sodium acetate buffer (pH 5.0) was used in place of 50 mM sodium citrate buffer (pH 7.0).

Results

Co-transformation of the *C. cinereus* Trp$^-$ auxotrophic strain LT2-44 with pCc1001 and pLC2′-Ao.xynF1, pLC2-Ao.xynF1′, pLC2′-Ao.xynF1″, or pLC2′-Ao.xynF1‴

pLC2′-Ao.xynF1 contains *A. oryzae* endo-xylanase XynF1 cDNA (*Ao.xynF1*c) between the basal promoter and terminator of *L. edodes priA* (Fig. 1). pLC2-Ao.xynF1′ contains *Ao.xynF1′*c, which was constructed by fusing the signal-sequence-depleted *Ao.xynF1*c in frame just after the basidiomycete *P. ostreatus mnp*c signal sequence (Fig. 1). The C-terminus of the signal peptide of *P. ostreatus* MnP (Asada et al. 1995), *Phanerochaete chrysosporium* (basidiomycete fungus) lignin peroxidases (LiPs) (Naidu and Reddy 1990; Naidu et al. 1990; Ritch et al. 1991; Ritch and Gold 1992; Zhang et al. 1991), and *L. edodes* endo-xylanase (Lee et al., unpublished data) has been reported to be lysine-arginine. Therefore, although the C-terminal signal-peptide sequence of the expressed product of *Ao.xynF1*c is serine-arginine, pLC2′-Ao.xynF1″, containing *Ao.xynF1″*c, the expressed product of which has lysine-arginine as the C-terminal signal-peptide sequence, was constructed (Fig. 1). The N-terminal third amino acid of the mature (secreted form) *P. chrysosporium* LiPs and *P. ostreatus* MnP has been reported to be cysteine (Ritch et al. 1991: Asada et al. 1995). Similarly, pLC2′-Ao.xynF1‴, containing *Ao.xynF1‴*c, the expressed product of which has a cysteine residue inserted between the N-terminal second and third amino acids of the mature XynF1 enzyme, in addition to a mutation of the C-terminal sequence of the signal peptide to lysine-arginine, was also constructed (Fig. 1).

pLC2′-Ao.xynF1, pLC2-Ao.xynF1′, pLC2′-Ao.xynF1″, or pLC2′-Ao.xynF1‴was introduced into the *C. cinereus* LT2–44 (*trp1–1, trp1–6*) genome by co-transformation with pCc1001, carrying the *C. cinereus TRP1* (Binninger et al. 1987), and selecting for Trp$^+$ colonies. About ten Trp$^+$ transformants were obtained by introduction of each of the four recombinant plasmids (and pCc1001). The *Eco*RI digests (20 μg each) of total DNAs isolated from those Trp$^+$ transformants were subjected to Southern-blot hybridization using the ^{32}P-labeled 0.9-kb signal-depleted *Ao.xynF1*c fragment as probe. Trp$^+$Xyn$^+$ transformants showing a single hybridization band at the 1.0-kb or 0.9-kb position were selected (Fig. 2A). Two, one, two, and one Trp$^+$Xyn$^+$ transformants obtained by introduction of pLC2′-Ao.xynF1, pLC2-Ao.xynF1′, pLC2′-Ao.xynF1″, or pLC2′-Ao.xynF1‴ were named TF2–10 (XynF1) (lane 3), TF2–6(XynF1) (lane 4), TF2–1(XynF1′) (lane 2), TF2–14 (XynF1″) (lane 5), TF2–15(XynF1″) (lane 6) and TF2–21 (XynF1‴) (lane 7), respectively. The control Trp$^+$ transformant, obtained by introduction of pCc1001 alone, showed no signal for the probe(lane 1).

To ensure equal loading of the DNA preparations and to estimate the copy numbers of the *Ao.xynF1*c (and *Ao.xynF1′*c, *Ao.xynF1″*c, or *Ao.xynF1‴c*) sequence on the chromosomes of the six Trp$^+$Xyn$^+$ transformants, *Xho*I +*Sal*I digests (20 μg each) of total DNAs of these transformants were hybridized with a ^{32}P-labeled 1.3-kb *Xho*I-*Sal*I genomic fragment containing *C. cinereus ras* (*Cc.ras*), a single copy of which is present on the chromosome (Ishibashi and Shishido 1993) (Fig. 2B). The specific ^{32}P activity of the *Cc.ras* probe was almost the same as that of the 0.9-kb *Ao.xynF1*c probe. The six

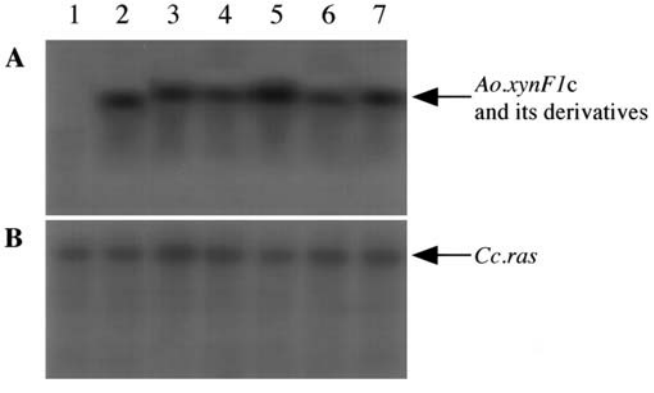

Fig. 2A, B Southern-blot analysis of restriction endonuclease digests of total cellular DNAs prepared from the six *Coprinus cinereus* Trp$^+$Xyn$^+$ transformants and the control Trp$^+$ transformant. **A** *Eco*RI digests were hybridized with the ^{32}P-labeled probe of 0.9-kb signal-depleted *Ao.xynF1*c fragment. **B** The *Xho*I+*Sal*I digests were hybridized with the ^{32}P-labelled probe of 1.3-kb *Cc.ras*. *Lanes* : *1* Control Trp$^+$ transformant, *2* TF2–1(XynF1′), *3* TF2–10(XynF1), *4* TF2–6(XynF1), *5* TF2–14(XynF1″), *6* TF2–15 (XynF1″), *7* TF2–21(XynF1‴)

Trp^+Xyn^+ transformants and control Trp^+ transformant each gave one faint signal of similar intensity at around 1.3 kb (Fig. 2B, lanes 1–7). The negatives of the autoradiographic patterns shown in Fig. 2A, B yielded a densitometric measurement of the intensities of the *Ao.xynF1*c (and *Ao.xynF1*′c, *Ao.xynF1*″c or *Ao.xynF1*‴*c*) and *Cc.ras* bands. TF2–10(XynF1), TF2–6(XynF1), TF2–1(XynF1′), TF2–14(XynF1″), TF2–15(XynF1″), and TF2–21(XynF1‴) were considered to carry five, three, five, five, three, and five copies on their chromosomes, respectively.

Transcriptional expression of *Ao.xynF1*c or its derivative sequences in the *C. cinereus* Trp^+Xyn^+ transformants

Transcriptional expression of *Ao.xynF1*c or its derivative sequences in the *C. cinereus* Trp^+Xyn^+ transformants was investigated by Northern-blot analysis. Total RNA blots (20 μg each) of TF2–10(XynF1), TF2–6(XynF1), TF2–1(XynF1′), TF2–14(XynF1″), TF2–15(XynF1″), and TF2–21(XynF1‴) were hybridized with the aforementioned signal-depleted *Ao.xynF1*c probe. As shown in Fig. 3A, all of the RNA blots gave a clear signal, the intensity of which coincided with that of the Southern blot, while no signal was obtained from the RNA blot of the control Trp^+ transformant. To ensure equal loading and transfer of RNA samples, the Northern blots hybridized with the *Ao.xynF1*c probe were put into boiling 0.1% SDS where they remained until reaching room temperature. After confirming complete removal of the *Ao.xynF1*c probe by autoradiography, the resulting Northern blots were re-hybridized with the *Cc.ras* probe. The 1.3-kb *ras* signal was detected in each RNA blots, and the signal intensities were similar.

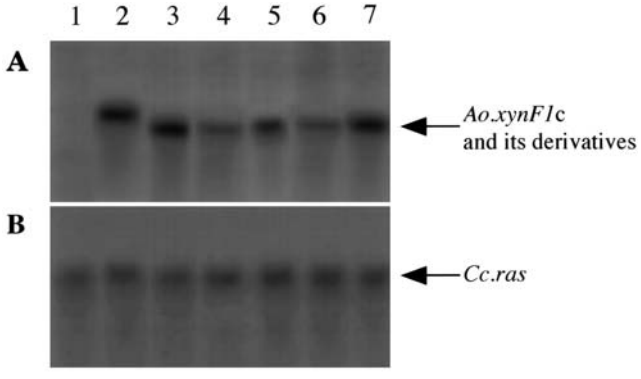

Fig. 3A, B Northern-blot analysis of total RNAs prepared from the six *C. cinereus* Trp^+Xyn^+ transformants and the control Trp^+ transformant. The ^{32}P-labeled probes were a 0.9-kb signal-depleted *Ao.xynF1*c fragment (**A**) and 1.3-kb *Cc.ras* (*B*). *Lanes*: *1* Control Trp^+ transformant, *2* TF2–1(XynF1′), *3* TF2–10(XynF1), *4* TF2–6(XynF1), *5* TF2–14(XynF1″), *6* TF2–15(XynF1″), *7* TF2–21(XynF1‴)

The xylan-degrading activities of the culture supernatants of TF2–10(XynF1), TF2–1(XynF1′), TF2–14(XynF1″) and TF2–21(XynF1‴)

TF2–10(XynF1), TF2–1(XynF1′), TF2–14(XynF1″), TF2–21(XynF1‴), and control Trp^+ transformants were then assayed. The strains were cultured in 10 ml of MY medium in L-shaped tubes at 30°C with shaking. As mentioned above, the four Trp^+Xyn^+ transformants of *C. cinereus* each carried five copies of *Ao.xynF1*c or its derivative sequences on their chromosomes. Triplicate tubes were prepared and used for cultivation. After the indicated times (see Materials and methods), 0.1-ml aliquots were removed from each of the five cultures and centrifuged. The supernatants (50 μl each) were assayed for xylan-degrading activity. The average values and SDs for xylanase activity shown in Fig. 4 indicated that the supernatant of TF2–10(XynF1), unexpectedly, showed a very low level of xylanase activity, almost the same as that of the control Trp^+ transformant. By contrast, the supernatants of TF2–1(XynF1′), TF2–14(XynF1″), and TF2–21(XynF1‴) clearly showed high levels of xylanase activity. Their supernatants obtained from 18-day cultures contained levels of xylanase activity eight to nine times as high as that of control Trp^+ transformant. The growth rates of TF2–10(XynF1), TF2–1(XynF1′), TF2–14(XynF1″), TF2–21(XynF1‴), and control Trp^+ transformants were about the same. The dry weights of the mycelial cells after 18 days of 10-ml cultivation of TF2–10(XynF1), TF2–1(XynF1′), TF2–14(XynF1″), TF2–21(XynF1‴), and control Trp^+ transformant were 193, 206, 202, 197 and 190 mg, respectively. To analyze the xylanase protein(s) secreted from TF2–10(XynF1), TF2–1(XynF1′), TF2–14(XynF1″), and control Trp^+ transformants, culture supernatants from these four transformants and from the high-XynF1-producing *A. oryzae* strain (Kitamoto et al. 1999) were subjected to native polyacrylamide gel electrophoresis and xylanase-activity staining. TF2–21(XynF1‴) was not analyzed. As shown in Fig. 5, although the xylanase-activity bands of intrinsic (*C. cinereus*) and *A. oryzae* XynF1 could not be clearly separated from each other, the culture supernatants of TF2–1(XynF1′) (lane 2) and TF2–14(XynF1″) (lane 4) contained large amounts of XynF1 xylanase and also *C. cinereus* xylanase, while the culture supernatant of TF2–10(XynF1) (lane 3) contained only *C. cinereus* xylanase, similar to the control Trp^+ transformant (lane 1). Taken together, the results show that *A. oryzae* XynF1, with a C-terminal signal-peptide sequence of serine-arginine, is poorly secreted by *C. cinereus* into the culture medium. However, modification of the C-terminal sequence of the signal peptide to lysine-arginine led to efficient secretion of the enzyme into the culture medium.

Discussion

In the present study,*C. cinereus* strains carrying high copy numbers of *A. oryzae* endo-xylanase XynF1 cDNA on

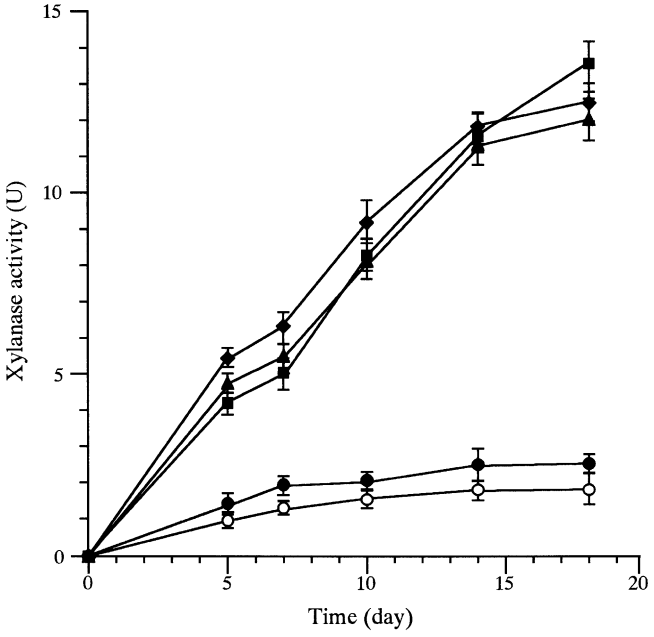

Fig. 4 Time course of xylanase production during cultivation of TF2-10(XynF1) (*closed circles)*, TF2–1(XynF1′) (*closed squares*), TF2–14(XynF1″) (*closed diamonds*), TF2–21(XynF1‴) (*closed triangles*), control Trp^+ transformant (*open circles*). Units (U) of xylanase activity are shown and were determined using culture supernatants (50 μl each). Average values and SDs are presented

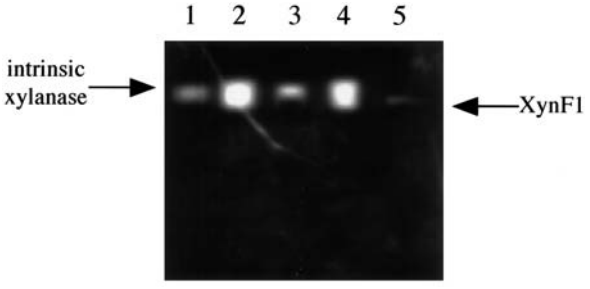

Fig. 5 Activity staining of xylanases contained in the culture supernatants of the control Trp^+ transformant (*lane 1*), TF2–1 (XynF1′) (*lane 2*), TF2–10(XynF1) (*lane 3*), TF2–14(XynF1″) (*lane 4*) and high-XynF1-producing *A. oryzae* strain (*lane 5*)

their chromosomes were produced. The cDNAs were modified to encode a C-terminal signal sequence of lysine-arginine, resulting in the secretion of large amounts of XynF1 enzyme into the culture media. Insertion of cysteine residues between the N-terminal second and third amino acids of mature XynF1 did not result in increased secretion of the enzyme. Therefore, it can be concluded that the lysine-arginine sequence is responsible for efficient secretion. In addition, these results strongly suggest that a KEX2-like protease (Broekhuijsen et al. 1993) is involved in the processing of XynF1 protein in *C. cinereus*.

So far, we have succeeded in isolating approx. 30% of the total cellulose from rice straw, one of the most common forms of plant biomass waste, by using molecular-genetically bred *C. cinereus* strains producing large amounts of *P. ostreatus* MnP and *B. subtilis* endo-xylanase, together with the mushroom *Elfvingia applanata*. *C. cinereus* strains secreting large amounts of *A. oryzae* XynF1 (pH optimum of 5) were used in place of *C. cinereus* efficiently secreting *B. subtilis* endo-xylanase (full activity at pH 7–8). With this approach, cellulose recovery increased up to 42%, suggesting that the more efficient degradation of rice-straw xylan was due to the full activity of the modified XynF1 secreted by *C. cinereus* (optimum pH~5) Thus, the high-XynF1-producing *C. cinereus* strains will be of great value in the utilization of lignocellulosic resources.

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