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**Studies on the Secretory Production of  
Heterologous Xylanases in the  
Basidiomycete *Coprinus cinereus***

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## **Chapter 1**

### **General introduction**

Filamentous fungi such as basidiomycetes, ascomycetes and hyphomycetes belong to the decomposers in ecological field (Whittaker, 1969), and they secrete many hydrolyzing enzymes. Filamentous fungi decompose a plant biomass which consisted of cellulose, hemicellulose and lignin (Fig. 1) and use them as nutrient sources. Cellulose is a polymerized D-glucose by  $\beta$ -1,4 linkage. Hemicellulose is a polymer of the saccharides except for glucose and its major component is xylan of which structure is shown in Fig. 2 (Coughlan and Hazlewood, 1993a). Lignin is a polyphenolic polymer and it forms very complex molecular networks. Cellulose and hemicellulose are degraded by the enzymes secreted from many fungi and also bacteria, but the lignin is only degraded by the enzyme of white-rot basidiomycete fungi (Miles and Chang, 1997).

The informations about the signal for secretion of the above enzymes in filamentous fungi, however, are very insufficient. The suggestion whether the secretion signal required by basidiomycetous fungi is the same as that required by other filamentous fungi is uncertain. So, the author attempted to obtain the information about the secretion signal. The author selected the basidiomycete *Coprinus cinereus* (*Nenagano-hitoyotake*) and endo ( $\beta$ -1,4) D-xylanase as the research targets from the following reasons. The growth rate of *C. cinereus* is very high among the basidiomycetous fungi (Bennett and Lasure, 1991). Transformation system has been already well established (Binniger et al., 1991). By using chromosome-integrating vectors pLC1 and pLC2 which were constructed by our laboratory, Ogawa et al. (1998) have succeeded in high level of secretory production in *C. cinereus* monokariotic strain of *Pleurotus ostreatus* (*Hiratake*) manganese (II) peroxidase (MnP) which is major enzyme involved in the lignin-degradation in *P. ostreatus* (Asada

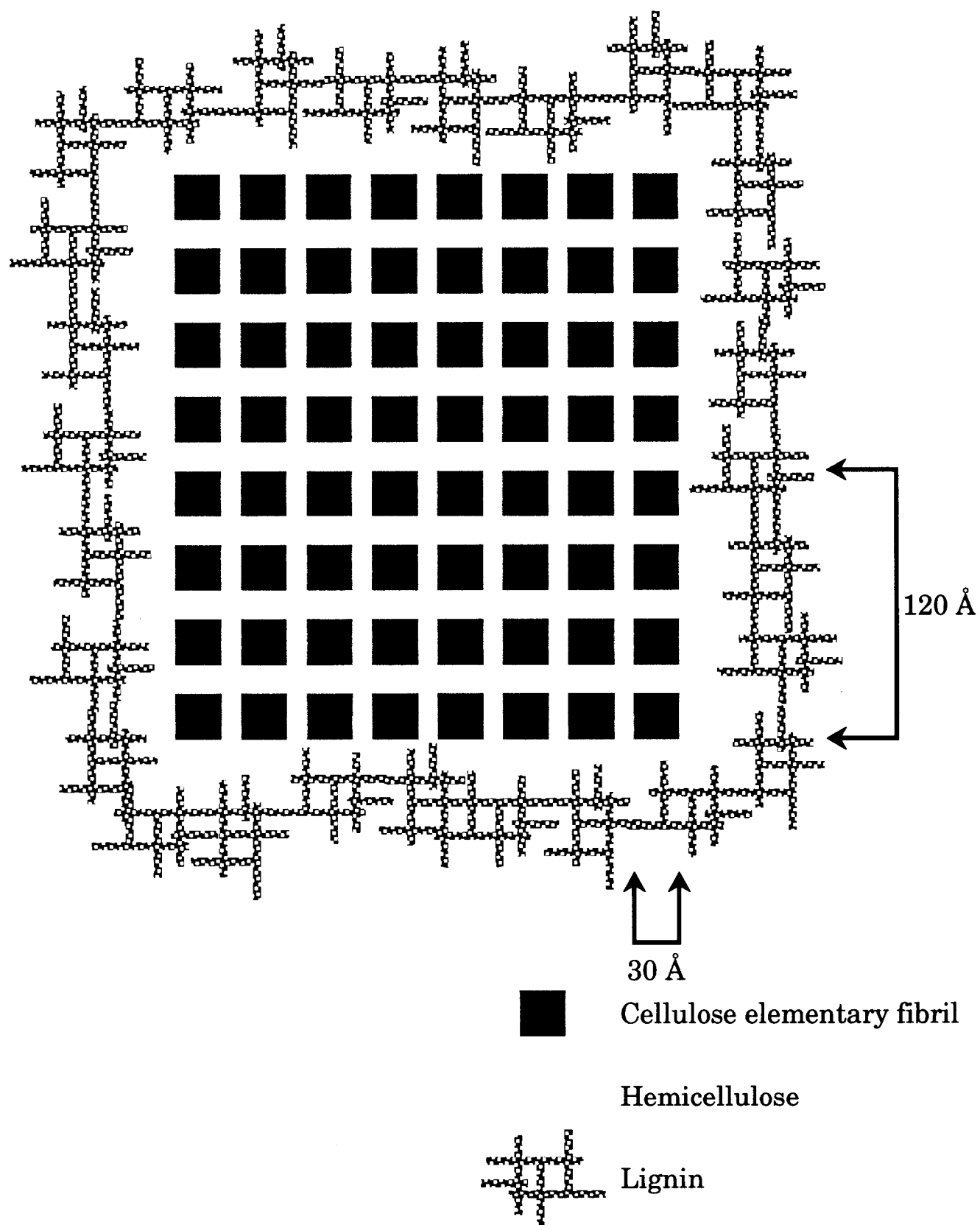


**Fig. 1.** A model structure of lignocellulose

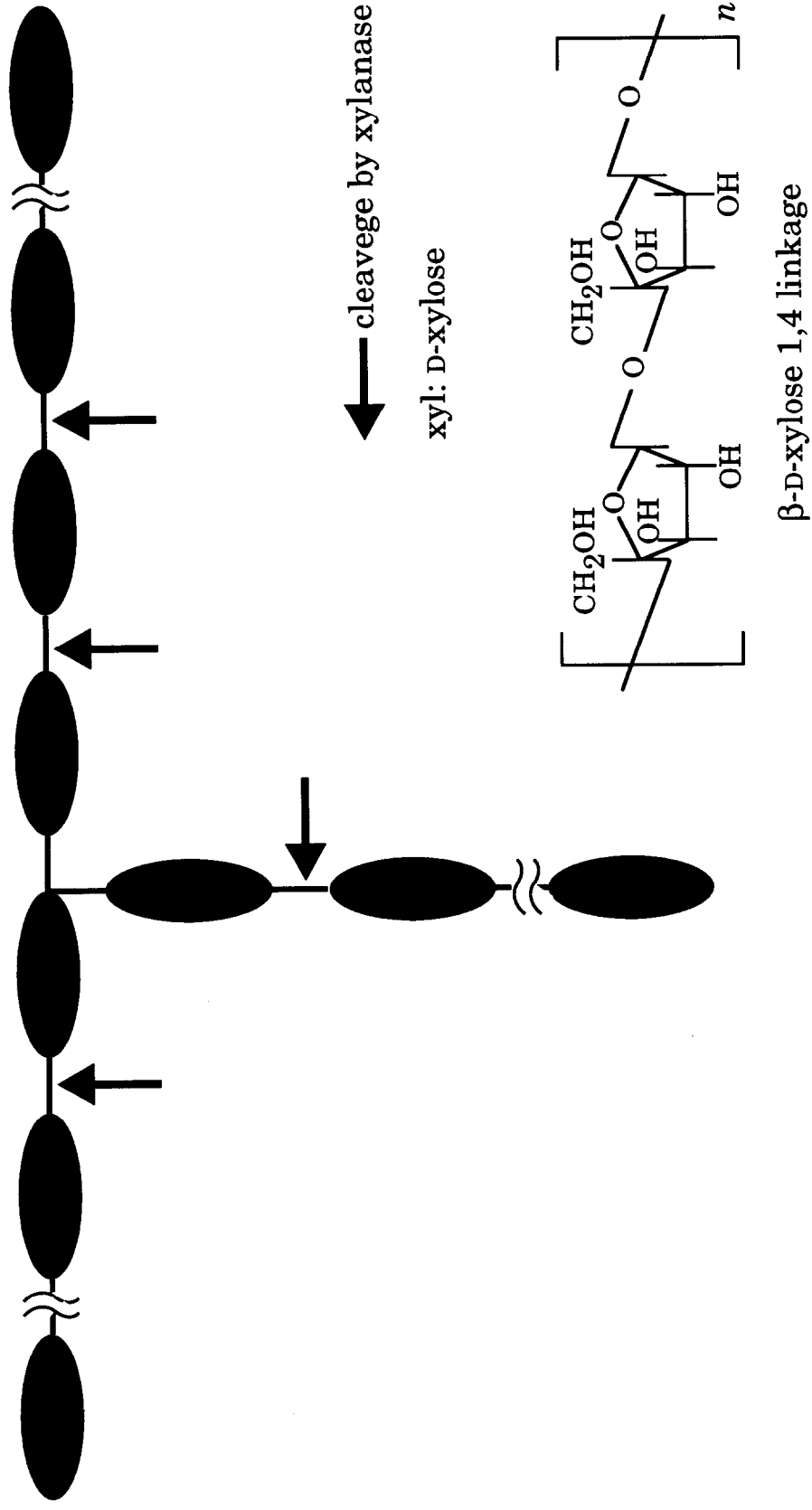


**Fig. 2.** Structure of xylan and cleavage of the  $\beta$ -D-xylose 1,4 linkage by xylanase

et al., 1995). If we succeed in production of the *C. cinereus* strains which produce a large amount of xylanase and use them together with the strain which produce a large amount of MnP, cellulose convertible into ethanol by fermentation must be isolated from waste plant biomass. This is meaningful and remarkable from the point of view of obtaining a source of energy from waste lignocellulosic materials.

In this thesis, the author studied expressions in *C. cinereus* of the gram-positive bacterium *Bacillus subtilis* xylanase gene (named *Bs.xyn*) and the hyphomycete *Aspergillus oryzae* (*Kiiko-kojikabi*) xylanase XynF1 cDNA (named *Ao.xynF1c*) and secretion of their expressed products under the control of the secretion signal from *P. ostreatus* MnP. The author then studied the secretion of *A. oryzae* xylanase XynF1 in *C. cinereus* by introducing the point-mutations into the signal sequence of *A. oryzae* XynF1. Finally the author attempted to isolate cellulose from waste plant biomass using the aforementioned molecular genetically-bred *C. cinereus* strains.

## **Chapter 2**

**Expression of heterologous xylanase gene or cDNA and secretion of their expressed products in *Coprinus cinereus***

## 2.1 Introduction

The author introduced bacterial *Bacillus subtilis* xylanase gene (*Bs.xyn*) and hyphomycete *Aspergillus oryzae xynF1* cDNA (*Ao.xynF1c*) into *Coprinus cinereus* and studied the secretion of their expressed productions. Two methods were examined. One is the use of the signal sequence of the basidiomycete *Pleurotus ostreatus* managanese (II) peroxidase (Po.MnP) and the other is the point-mutation of signal sequence of *Ao.xynF1c*.

The author used *C. cinereus* of which growth rate is very high and transformation system is well established as the target of molecular breeding.

## **2.2 Expression of *Bacillus subtilis* xylanase gene and secretion of its expressed product**

### **2.2.1 Materials and Methods**

#### **(i) Strains and media**

*Coprinus cinereus* monokariotic strain LT2-44 (*trp1-1*, *trp1-6*) (Binniger et al., 1987) was used as the recipient in transformation experiments. MYG medium (1% malt extract, 0.4% yeast extract, 0.4% glucose, pH5.6), containing 100  $\mu$ g tryptophan /ml when required or MY medium (1% malt extract, 0.5% yeast extract), were use for growth of *C. cinereus* strain. Regeneration medium (pH 6.0), used for *C. cinereus* LT2-44 protoplasts, containing 171 g sucrose, 5 g glucose, 2 g asparagine, 40  $\mu$ g thiamine, 1 g  $\text{KH}_2\text{PO}_4$ , 2.25 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 1.5 g  $\text{NH}_4\text{Cl}$ , 0.29 g  $\text{Na}_2\text{SO}_4$ , 0.25 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 5 g soluble starch /l. Construction, propagation and amplification of recombinant plasmids were carried out in *Escherichia coli* DH5 $\alpha$  and JM109 (Yanisch-Perronet al., 1985) *E. coli* was cultured in Luria-Bertani medium.

#### **(ii) Plasmid construction**

pLC1 (6.4 kb) is the pUC19 (Hanahan, 1983)-based vector carrying the *Lentinula edodes* (*Shiitake*) *ras* (*Leras*) gene promoter (2.5 kb) (Hori et al., 1991) (Fig. 3) and *priA* gene terminator (1.2 kb) (Kajiwaera et al., 1992) (Fig.4) and containing *Bam*HI cloning site between the promoter and the terminator (Yanai et al., 1996). pLC2 (5.6 kb) in the pBR322 (Watson, 1988)-based vector carrying the *priA* gene basal promoter (0.37 kb) (Fig. 3) and the terminator and it has the *Bam*HI cloning site. The

## ***Le.ras* promoter**

-500  
 GTGCAGCGTAAATCACCAACCATCAAGAG  
 -450  
 GGGTTCAATTTAGATGAGAGATGCATCGATGAGTGATATAACATGTCGAGTTGGAAAAGG  
 CCAAT box  
 -400  
 CGTGGCTTGAGACAACACGGACACAGCTTCTTGAACCAGAATAGGGAGCTTCGATGCAGC  
 GC box  
 -350  
 TGC GCACAGCCCCACACCCCCAGCCAGTGATACTATGTACTGTGATACTATAAAACCTGTA  
 CACCC box  
 TATA box  
 TAAAACCATTTTGCCGTGCTCGTCGTTGATTAAAGATCTTGGTCCTTGGCAAAATAAAATA  
 AT rich sequence  
 -250  
 AACCTCGTGCCCGAACACGTGAATCCAACCCCTCTTTTGGATCTCGGAATTCAACGCCTTT  
 -200  
 TTAGGGTTCTACCCGCTTTTCCCATCTTCCCCTTTATTCTTTGGTTGTCCTTCTTCTTCT  
 CT-rich sequence  
 -150  
 TCTTTTCCAAAAGACTTGAGGAGCCGACTGTTTCTGAATCTTCCTCTACTTTTGTACATC  
 -100  
 AACGCCTCCTCCTCCCGCGTCTTTAGAAAAGAAATAGGAAATTCAAAGAAATGGCTGGT  
 -50  
 M A G

## ***priA* promoter**

-500  
 CGGTGATGTCGGCGATATAGGCGCCAGCA  
 -450  
 ACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCGATCCG  
 -400  
 GAATCGTGGCCGTAAACCATGTGAACCAAGGTAGGGAGATTTTTTTTGCTGGGTCTGAATTT  
 -350  
 CAAGTTACTTTTACCTACTTTCAATCGCAGGCTTGCGTGTGTTGGGCGGAATGAACAGAG  
 GC box  
 -250  
 AGCTGCAGAACTCTTCGACAAATCATCCTGCAGTCATAATTACAAATTCCCGAACCTAG  
 -200  
 AGCGACATATTCATCAGCGGTCCAACGAAACCGCTTTGATCCTCTGATTTTGAGTAAAT  
 -150  
 TAGAGCCAATTACAGCATAAGGTACTGTTTCCCATGATGATAAACTTCATAATCAGGGTA  
 CCAAT box  
 -100  
 TATAAACTCGTTGTTTCGTTTTTATTGTTTCATCTCTCTCCCTCTCCTCTCTTGCACCAGT  
 CT-rich sequence  
 TATA box  
 -50  
 CCGCTGACGAATCTGTCCTGTCGCTCTTTTGAGACCCTTCTTTAGATTAAATGACTGTC  
 M T V

Promoter consensus sequence are underlined  
 ▼: Major transcription start point  
 ▽: Minor transcription start point

**Fig. 3.** Upstream sequences of *L. edodes ras* and *priA* genes

CTCGCGTAG<sup>1</sup>ATTGTGGCTCTCCATCATATCGACCCGGCAACGTTATAGATTCTACATCTA<sup>50</sup>  
 A L Ter  
 CTGATCGTACGGTCTTCAATCTTCCTATCATTTGCCCTACCTGTCTTTACTTCTCTTTTG<sup>100</sup>  
 ATTCCAATGCTTGACTTGTTGCGCGATGACTCGTGTCA<sup>150</sup>CAAATGCTATTTATCGAATGCA  
 TTCTTGAGTTTTTCCGATTTACCTTCTT<sup>200</sup>GGACGCAGAGTACATAGGTCTTTATCGTTTTA  
 GGCTTTGATCTCGATTGTTT<sup>250</sup>TGCATTATTACATCCGCGCGCTTCTACACAATTTCTAGTG  
 TCTGATAGTGTTCGCCAACTGTTGTTGTTCC<sup>300</sup>TTTTGTTCCCTCTCTACCGTTACACTTGCG<sup>350</sup>  
 TGTGTGCGTTTAATCTTAGTCTTGGTCTGTCCTTAGATTTCGTTGAAGCT<sup>400</sup>AGTGGATGACT  
 TCCTGTAAGCCTCGGCACAAATAATCTGAGTAAATAAT<sup>450</sup>TCTTAGTAGGCCAGTCCGATGC  
 AATAATCCTATCCTTAGATCCGGATAC<sup>500</sup>AC

Terminator consensus sequences are underlined

▼: polyA addition site

**Fig. 4.** Downstream sequence of *L. edodes priA* gene

pLC1 and pLC2 recombinant plasmids carrying the modified *Bacillus subtilis* endo ( $\beta$ -1,4-) D-xylanase gene (*Bs.xyn'*) (Fig. 5) were constructed as follows. The 1.2-kb *Bs.xyn* sequence was prepared from *B. subtilis* strain DB104 by polymerase chain reaction (PCR) method based on the nucleotide sequence previous determination for *B. subtilis* strain PAP115 (Paice et al., 1986) and the PCR product was entirely sequence to confirm the absence of artificial mutations. Sequencing was carried out in Li-cor model 4000 sequencer by the dideoxy chain-termination method of Sanger et al. (1977). The 1.2-kb *Bs.xyn* sequence was digested with *AluI*, obtaining the 0.58-kb *Bs.xyn* sequence lacking the signal sequence encoding the signal peptide (named signal-depleted *Bs.xyn*). The 0.58-kb signal-depleted *Bs.xyn* was inserted into *SmaI* site of pUC19, yielding recombinant plasmid pUBX1. The pLC1 carrying the *Pleurotus ostreatus mnpc* (*Po.mnpc*) (Fig. 6), i. e. pLC1-*Po.mnp* (Ogawa et al., 1998), was digested with *AccII*, obtaining the 0.6-kb fragment that contains a small part of *Leras* promoter sequence and *Po.mnpc* signal sequence. This 0.6-kb fragment was ligated to *EcoRI* linker (pd(CGAATTCTG)) and then digested with *EcoRI*. The *EcoRI* digest was inserted into the *EcoRI* site of pUBX1, yielding pUBX2. pUBX2 was digested with *BamHI*, obtaining the 0.7-kb fragment that contains the signal-depleted *Bs.xyn* preceded by *Po.mnpc* signal sequence. This modified 0.7-kb *Bs.xyn* sequence (Fig. 7) (named *Bs.xyn'*) was inserted into the *BamHI* site of pLC1 and pLC2 (these process was shown in Fig. 8), yielding pLC1-*Bs.xyn'* and pLC2-*Bs.xyn'* (Fig. 9). Plasmid pCc1001, used for co-transformation with pLC1-*Bs.xyn'* or pLC2-*Bs.xyn'*, is the pUC9 (Vieira and Messing, 1982)-based vector carrying the 6.5-kb *PstI* genomic fragment containing the *C. cinereus* tryptophan synthetase gene (*TRP1*) (Binniger et al. 1987).

-90  
ATCAAAAGCTTTGGCGTTAGTAATTAAGAA  
-35 sequence

TGTTTTAAATGTATACGAGTGCTACCTCAAAGTCGGAAAAAATATTATAGGAGGTAACAT -60  
1 -10 sequence SD sequence

ATGTTTAAGTTTAAAAAGAATTTCTTAGTTGGATTATCGGCAGCTTTAATGAGTATTAGC 60  
M F K F K K N F L V G L S A A L M S I S

AluI

TTGTTTTCGGCAACCGCCTCTGCAGCTAGCACAGACTACTGGCAAAATTGGACTGATGGG 120  
L F S A T A S A A S T D Y W Q N W T D G

GGCGGTATAGTAAACGCTGTCAATGGGTCTGGCGGGAATTACAGTGTTAATTGGTCTAAT 180  
G G I V N A V N G S G G N Y S V N W S N

ACCGGAAATTTTGTGTTGGTAAAGGTTGGACTACAGGTTCCGCAATTTAGGACGATAAAC 240  
T G N F V V G K G W T T G S P F R T I N

TATAATGCCGGAGTTTGGGCGCCGAATGGCAATGGATATTTAACTTTATATGGTTGGACG 300  
Y N A G V W A P N G N G Y L T L Y G W T

AGATCACCTCTCATAGAATATTATGTAGTGGATTTCATGGGGTACTTATAGACCTACTGGA 360  
R S P L I E Y Y V V D S W G T Y R P T G

ACGTATAAAGGTACTGTAAAAAGTGATGGGGGTACATATGACATATATACAACCTACACGT 420  
T Y K G T V K S D G G T Y D I Y T T T R

TATAACGCACCTTCCATTGATGGCGATCGCACTACTTTTACGCAGTACTGGAGTGTTTCGC 480  
Y N A P S I D G D R T T F T Q Y W S V R

CAGTCGAAGAGACCAACCGGAAGCAACGCTACAATCACTTTTCAGCAATCATGTGAACGCA 540  
Q S K R P T G S N A T I T F S N H V N A

TGGAAGAGCCATGGAATGAATCTGGGCAGTAATTGGGCTTACCAAGTCATGGCGACAGAA 600  
W K S H G M N L G S N W A Y Q V M A T E

GGATATCAAAGTAGTGGAAGTTCTAACGTAACACTGTGGTAA 642  
G Y Q S S G S S N V T L W Ter

AluI

CAGATCATCCTTAATCAGGCGTAGCTAACGGGCTGCTGATCGTTCCTTGAGAAGTTTTAT 702

AATCAATGATTATTAAAAATCGTTAGTAATG 732

Secretion signal sequence is shown by red letters

**Fig. 5.** Nucleotide sequence and deduced amino acid sequence of *Bacillus subtilis* xylanase (*Bs.xyn*) gene

1  
ATGACCTTTGCTTCGCTTTCTGCGCTCGTCCTTGTCTTCGCTGTGACTGTCCAAGTCGCT 60  
M T F A S L S A L V L V F A V T V Q V A  
  
CAAGCCGTATCTTTGCCTCAGAAACCGCGCAACTTGTGCTGGTGGACAAGTTACCGCCAAC 120  
Q A V S L P Q K R A T C A G G Q V T A N  
  
GCGGCCTGTTGTGTTCTGTTCCCGCTCATGGAAGACCTGCAGAAGAACCTGTTTCGACGAC 180  
A A C C V L F P L M E D L Q K N L F D D  
  
GGCGCATGCGGCGAAGATGCCCATGAAGCCCTTCGCTGACCTTCCACGACGCTATTGGA 240  
G A C G E D A H E A L R L T F H D A I G  
  
TTCTCTCCTTCCAGGGGTGTTATGGGAGGCGCCGATGGCTCTGTCATCACATTCTCCGAT 300  
F S P S R G V M G G A D G S V I T F S D  
  
ACTGAGGTCAACTTCCCAGCCAACCTCGGTATTGACGAGATCGTCGAGGCTGAGAAACCG 360  
T E V N F P A N L G I D E I V E A E K P  
  
TTCCTTGCAAGGCACAACATCTCCGAGGCGATTGTTGGTTCACTTCGCCGGCACCCCTCGCT 420  
F L A R H N I S A G D L V H F A G T L A  
  
GTTACCAACTGTCCTGGTGCTCCACGAATCCCGTTCTTCTTAGGTGCGCCCTCTGCCAAG 480  
V T N C P G A P R I P F F L G R P P A K  
  
GCCGCATCACCCATTGGATTGGTTCCGGAACCATTCGATACCATACAGATATCCTGGCC 540  
A A S P I G L V P E P F D T I T D I L A  
  
CGAATGGATGACGCTGGATTTCGTCTCTGTCGAGGTTGTCTGGCTTCTTTCCGCCCACTCT 600  
R M D D A G F V S V E V V W L L S A H S  
  
GTCGCTGCAGCTGACCATGTTGACGAAACTATTCTGGAACGCCGTTTCGACTCAACGCCA 660  
V A A A D H V D E T I P G T P F D S T P  
  
AACCTTTTCGATTCACAAATCTTCATCGAGACGCAACTCCGTGGAATTTCTTCCCAGGC 720  
N L F D S Q I F I E T Q L R G I S F P G  
  
ACTGGTGGGAATCACGGCGAAGTACAATCCCCACTCAAGGGTGAAATGAGACTCCAGTCA 780  
T G G N H G E V Q S P L K G E M R L Q S  
  
GATCACTTGTTTCGCTCGAGACGATAGGACATCCTGCGAATGGCAGTCCATGACTAATGAT 840  
D H L F A R D D R T S C E W Q S M T N D  
  
CAACAGAAGATCCAAGACCGCTTTTCCGACACACTGTTCAAGATGTCGATGCTTGGACAG 900  
Q Q K I Q D R F S D T L F K M S M L G Q  
  
AACCAGGACGCCATGATCGATTGCTCCGATGTCATCCCTGTCCCCGCTGCCCTTGTAACC 960  
N Q D A M I D C S D V I P V P A A L V T  
  
AAACCCCATCTCCCCGCCGGAAGAGTAAGACCGACGTTGAACAAGCCTGTGCCACCGGC 1020  
K P H L P A G K S K T D V E Q A C A T G  
  
GCCTTCCCAGCCCTCGGTGCTGACCCTGGCCCAGTCACCTCTGTTCTCGTGTCCACCT 1080  
A F P A L G A D P G P V T S V P R V P P  
  
GCGTAA 1086  
A Ter

Secretion amino acid signal sequence is shown by red letters

*Po.mnpc* nucleotide signal sequence is shown by blue letters: this sequence was used for *Bs.xyn'*

**Fig. 6.** Nucleotide sequence and deduced amino acid sequence of *P. ostreatus* manganese(II) peroxidase cDNA(*Po.mnpc*)

1  
ATGACCTTTGCTTCGCTTTCTGCGCTCGTCCTTGTCTTCGCTGTGACTGTCCAAGTCGCT 60  
M T F A S L S A L V L V F A V T V Q V A

*EcoRI*

CAAGCCGTATCTTTGCCTCAGAAACGCGAATTCGAGCTCGGTACCCCTAGCACAGACTAC 120  
Q A V S L P Q K R E F E L G T P S T D Y

TGGCAAAATTGGACTGATGGGGGCGGTATAGTAAACGCTGTCAATGGGTCTGGCGGGAAT 180  
W Q N W T D G G G I V N A V N G S G G N

TACAGTGTTAATTGGTCTAATACCGGAAATTTTGTGTTGGTAAAGGTTGGACTACAGGT 240  
Y S V N W S N T G N F V V G K G W T D G

TCGCCATTTAGGACGATAAACTATAATGCCGGAGTTTGGGCGCCGAATGGCAATGGATAT 300  
S P F R T I N Y N A G V W A P N G N G Y

TTAACTTTATATGGTTGGACGAGATCACCTCTCATAGAATATTATGTAGTGGATTTCATGG 360  
L T L Y G W T R S P L I E Y Y V V D S W

GGTACTTATAGACCTACTGGAACGTATAAAGGTACTGTAAAAAGTGATGGGGGTACATAT 420  
G T Y R P T G T Y K G T V K S D G G T Y

GACATATATACAACCTACACGTTATAACGCACCTTCCATTGATGGCGATCGCACTACTTTT 480  
D I Y T T T R Y N A P S I D G D R T T F

ACGCAGTACTGGAGTGTTCCGAGTCGAAGAGACCAACCGGAAGCAACGCTACAATCACT 540  
T Q Y W S V R Q S K R P T G S N A T I T

TTCAGCAATCATGTGAACGCATGGAAGAGCCATGGAATGAATCTGGGCAGTAATTGGGCT 600  
F S N H V N A W K S H G M N L G S N W A

TACCAAGTCATGGCGACAGAAGGATATCAAAGTAGTGGAAGTTCTAACGTAACAGTGTGG 660  
Y Q V M A T E G Y Q S S G S S N V T V W

TAA 663  
Ter

Secretion amino acid signal sequence is shown by red letters  
*Po.mnpc* nucleotide sequence is shown by blue letters

**Fig. 7.** Nucleotide and amino acid sequences of the modified *B. subtilis* xylanase gene (*Bs.xyn'*) of which signal sequence is replaced by that of *P. ostreatus* manganese (II) peroxidase cDNA (*Po.mnpc*)

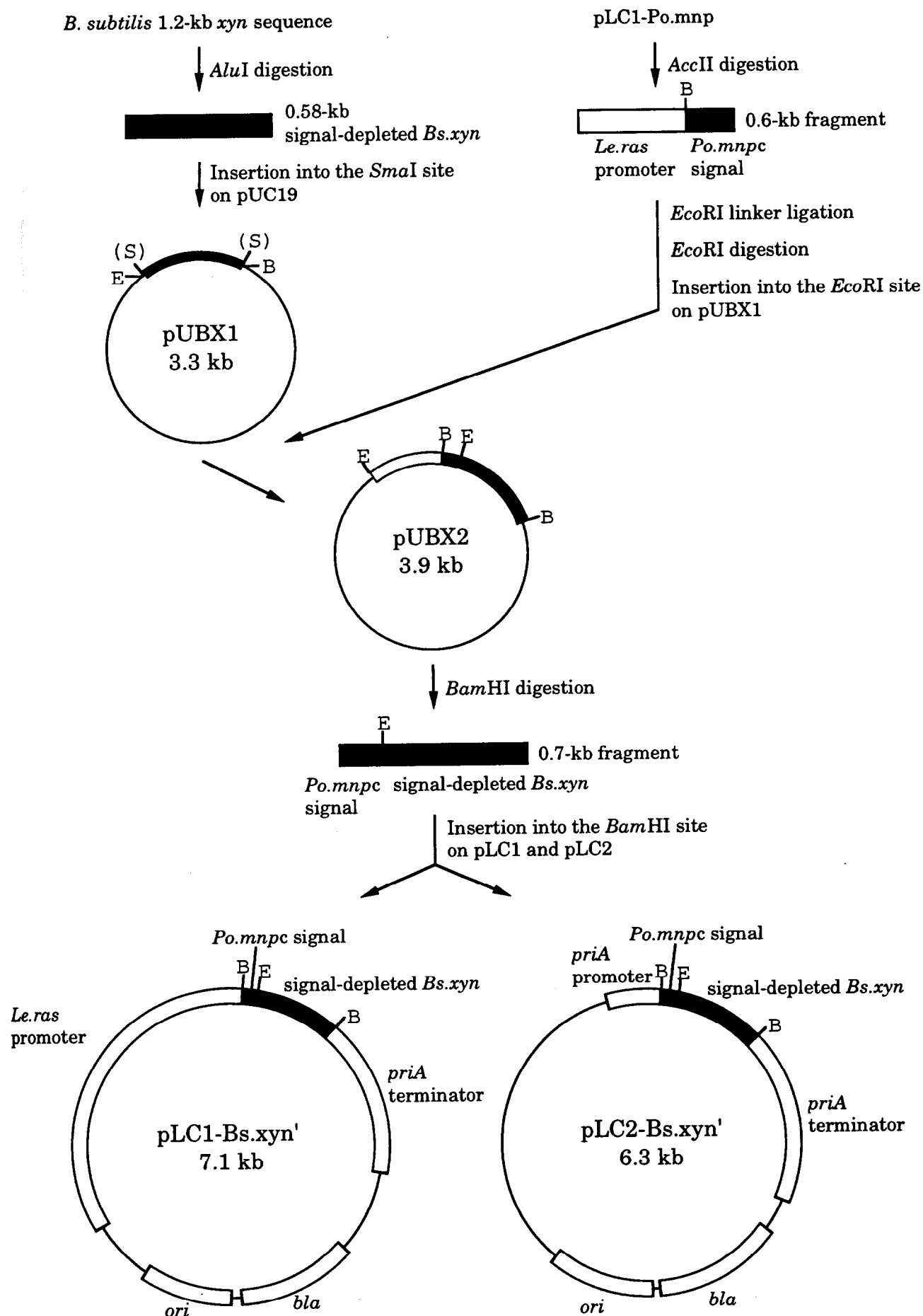


**Fig. 8.** Construction of *B. subtilis* xylanase expressing plasmids pLC1-Bs.xyn' and pLC2-Bs.xyn'

# pLC1-Bs.xyn'



# pLC2-Bs.xyn'

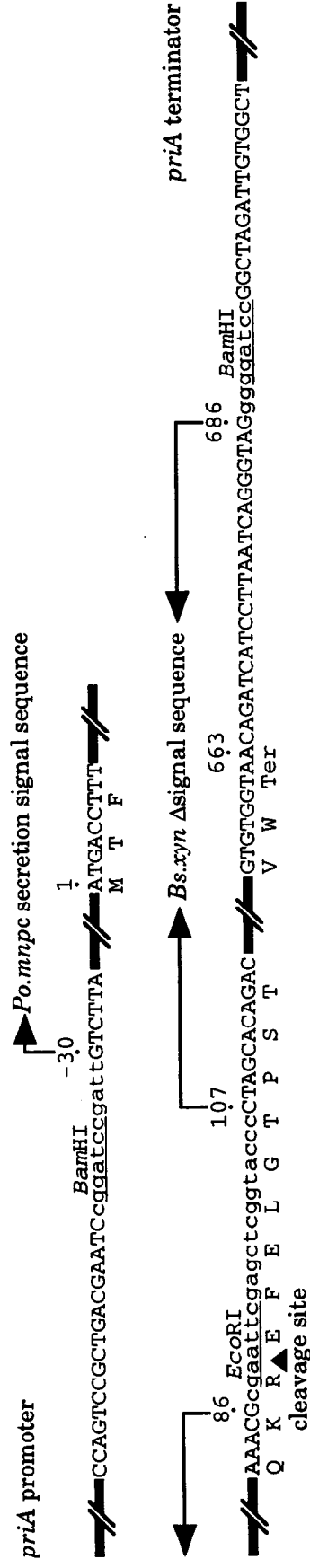


**Fig. 9.** The structural features of *B. subtilis* xylanase expressing sequence on plasmid pLC1-Bs.xyn' and pLC2-Bs.xyn'

### (iii) Transformation of *C. cinereus*

Oidia (approx.  $10^9$  cells) were harvested from slant cultures of LT2-44 and germinated at 30°C for 48 h without shaking in 80 ml MYG medium supplemented with tryptophan. The cells were harvested by centrifugation at 750 g for 5 min at room temperature and washed twice with 10 ml MM buffer (0.5 M mannitol in 50 mM maleate, pH 5.5). The washed cells were suspended in 1 ml the MM buffer containing 10 mg Novozyme 234 and 1 mg Chitinase-RS and incubated at 30°C for 2 h. The protoplasts were separated from cell debris by filtering through 60- $\mu$ m-pore nylon mesh, washed once with MM buffer and once 10 ml MMC (MM containing 50 mM  $\text{CaCl}_2$ ), and resuspended in 1 ml MMC to give  $(1-2) \times 10^8$  cells/ ml. Transformations were essentially carried out as described by Bininger et al. (1987). To 200  $\mu$ l protoplasts suspension were added 20  $\mu$ l plasmid DNA (10  $\mu$ g) in buffer containing 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and 50  $\mu$ l polyethylene glycol solution (25% PEG4000, 50 mM  $\text{CaCl}_2$  in 10 mM Tris-HCl pH 7.5) in 15-ml polypropylene conical tube. The tube was placed in ice for 20 min and then 2 ml PEG was added. The protoplasts were incubated at room temperature for 5 min. To regeneration medium containing 0.7% agar (kept molten at 50 °C) and poured over a layer of the regeneration medium containing 1.5% agar in 90-mm plastic plate. The plate was inverted and incubated at 30°C. Colonies were examined 10-18 days.

### (iv) DNA isolation and Southern-blot analysis

Genomic DNA of *C. cinereus* was isolated as described by Zolan and Pukkila (1986). Plasmid DNA was isolated from *E. coli* by alkaline lysis method and purified by centrifugation to

equilibrium in cesium chloride/ ethidium bromide gradients (Sambrook et al., 1989). The restriction enzymes digested genomic DNA (20  $\mu$ g each) were fractionated on a 1% agarose gel and transferred to nylon membrane by the method of Southern (1975). DNA probe labeling  $^{32}$ P, hybridization, and signal detection were done according to the method of Ishii et al. (1991).

(v) RNA isolation and Northern-blot analysis

Total cellular RNA was isolated from vegetative grown mycelia which was frozen by liquid nitrogen. The frozen mycelia were powdered and suspended in LETS buffer (0.1 mM LiCl, 10 mM EDTA, 10mM Tris-HCl pH7.4, 0.2% SDS) (Adams et al., 1997). An equal volume of water-saturated phenol was added and mixed. The mixture was incubated at 65°C for 30 min. After centrifugation, the supernatant was precipitated with ethanol. Northern-blot analysis was carried out according to the method of Sambrook et al. (1989). Hybridization and signal detection were done according to the procedures previously published Hori et al. (1991).

(vi) Protein preparation from medium and sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE)

A-100 ml sample of MY medium in SAKAGUCHI flask was inoculated with six each of disks (6 mm in diameter and 1 mm in thickness) of MYG/ agar colonized with *C. cinereus* mycelium and cultivation at 25°C for 18-days with shaking. The 10-ml of culture supernatants were frozen and dried, and suspended in 1-ml PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.7 mM KH<sub>2</sub>PO<sub>4</sub>, pH7.4) buffer. The suspension were precipitated with acetone and resuspended in 50  $\mu$ l PBS buffer. The samples resolved by

0.1%SDS 10%PAGE (Laemmli, 1970) and subsequently stained with Coomassie brilliant blue (Ausubel et al., 1987).

(vii) Assessment of xylanase degradation

A-100 ml sample of MY medium in SAKAGUCHI flask was inoculated with 6 pieces slice (6-mm diameter) of MYG/ agar colonized with *C. cinereus* mycelium and cultivation at 25°C for indicated periods with shaking. Xylanase activity of the culture supernatant was measured by a standard method using 3,5-dinitrosalicylic acid (DNS) (Miller, 1959). To 50  $\mu$ l of 50 mM sodium citrate buffer (pH7.0) containing 1% xylan, 50  $\mu$ l each of culture supernatants were added and then incubated at 37°C for 1 h. The pH optimum of endo ( $\beta$ -1,4-) D-xylanase produced by *B. subtilis* was 7-8 and the temperature optimum was 37-42°C. To the reaction mixtures, 200  $\mu$ l of DNS reagent was added and boiled for 5 min, followed by rapid cooling and centrifuging. The resulting supernatants were subjected to measurement of the absorbance at 500 nm. One unit of xylanase activity was defined as the amount of enzyme that produced a reducing sugar equivalent to 100 nmole of xylose under the conditions described above. The xylan-degrading reaction continued almost constantly for up to 2 h.

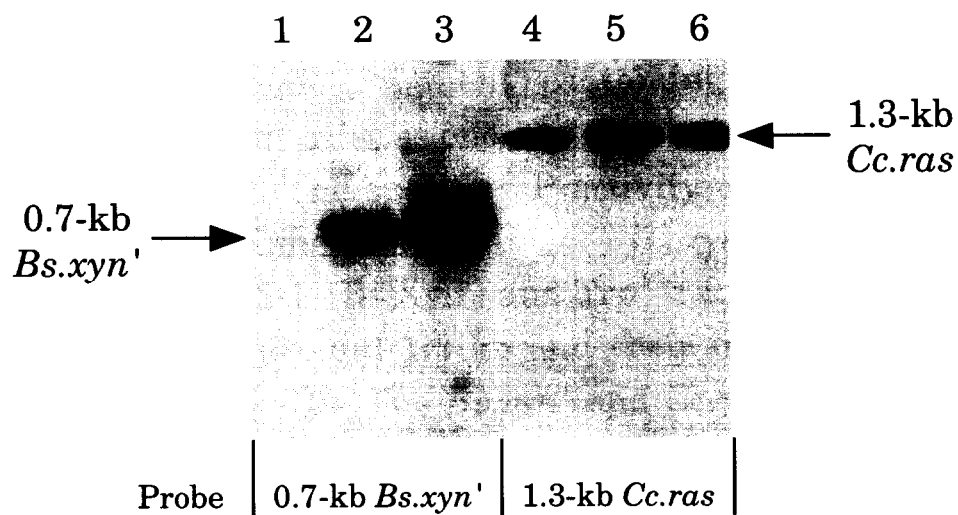
## 2.2.2 Results and Discussion

- (i) Co-transformation of *Coprinus cinereus* Trp<sup>-</sup> auxotrophic strain with pCc1001 and pLC1-Bs.xyn' or pLC2-Bs.xyn'

pLC1-Bs.xyn' and pLC2-Bs.xyn' were introduced into *C. cinereus* LT2-44 genome with pCc1001 and selected for Trp<sup>+</sup> transformants. A total of 26 and 32 Trp<sup>+</sup> colonies were obtained for pCc1001 plus pLC1-Bs.xyn' and pCc1001 plus pLC2-Bs.xyn', respectively. These transformants were screened for xylan degradation activity in 0.5% xylan containing MY (MYX) medium. For each plasmid, Trp<sup>+</sup> Xyn<sup>+</sup> transformants showing high xylan degradation activity, was obtained, named TF1-16(Bs.Xyn') or TF2-11(Bs.Xyn').

To ascertain the presence of the introduced DNA in the transformants, Southern-blot analysis was done using a <sup>32</sup>P-labelled 0.7-kb *Bam*HI fragment containing *Bs.xyn'* as a probe. In the DNA samples (20 µg each) of TF1-16(Bs.xyn') and TF2-11(Bs.xyn') without restriction enzyme digestion, specific hybridization signals were observed in the high molecular mass DNA region corresponding to the chromosomal DNA (data not shown). No specific hybridization signal was found in the DNA sample of Trp<sup>+</sup> transformant obtained by introduction of pCc1001 DNA alone.

All the genomic DNA samples were digested with *Bam*HI and subjected to Southern-blot analysis using *Bs.xyn'* probe. As shown in Fig. 10, TF1-16(Bs.Xyn') and TF2-11(Bs.Xyn') both gave one intense hybridization signal at the position corresponding to size (0.7 kb) of the probe (lane 2 and 3). No hybridization signal was observed in the case of the Trp<sup>+</sup> transformant (lane 1). These results suggest that the introduced TF1-16(Bs.Xyn') and TF2-11(Bs.Xyn') DNA had been integrated into chromosomal DNA of



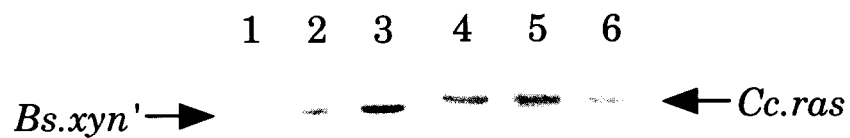
lane 1: *Bam*HI digest of Trp<sup>+</sup> transformant  
 lane 2: *Bam*HI digest of TF1-16(Bs.Xyn')  
 lane 3: *Bam*HI digest of TF2-11(Bs.Xyn')  
 lane 4: *Xho*I-*Sal*I digest of Trp<sup>+</sup> transformant  
 lane 5: *Xho*I-*Sal*I digest of TF1-16(Bs.Xyn')  
 lane 6: *Xho*I-*Sal*I digest of TF2-11(Bs.Xyn')

**Fig. 10.** Southern-blot analysis of the restriction endonuclease-digests of total DNA from TF1-16(Bs.Xyn'), TF2-11(Bs.Xyn') and Trp<sup>+</sup> transformant

the two transformants. To ensure equal loading of DNA preparations and to estimate the copy numbers of *Bs.xyn'* sequence on the chromosomes of TF1-16(*Bs.Xyn'*) and TF2-11(*Bs.Xyn'*), the DNA samples (20  $\mu$ g each) used in the above experiment were digested with both *Xho*I and *Sal*I and then subjected to Southern-blot analysis using the  $^{32}$ P-labelled 1.3-kb *Xho*I-*Sal*I genomic fragment containing the *C. cinereus ras* (*Ccras*) gene that exist as one copy on chromosome (Ishibashi and Shishido, 1993). The specific  $^{32}$ P activity of the *Ccras* probe was almost the same as that of *Bs.xyn'* probe. The TF1-16(*Bs.Xyn'*), TF2-11(*Bs.Xyn'*) and *Trp*<sup>+</sup> transformants all gave one faint signal at around 1.3 kb and their intensities were similar (lane 4-6 of Fig. 10). The *Bs.xyn'* and *Ccras* hybridization bands were cut from the membranes and their radioactivities were measured. TF1-16(*Bs.Xyn'*) and TF2-11(*Bs.Xyn'*) were estimated to carry about six and 10 copies of *Bs.xyn'* sequence, respectively, on their chromosomes.

#### (ii) Expression of *Bacillus subtilis* xylanase gene

Northern-blot analysis was done to investigate transcriptional expression of *Bs.xyn'* gene in the two *Trp*<sup>+</sup> *Xyn*<sup>+</sup> transformants. As shown Fig. 11, a clear signal of around 1.2 kb was detected in the RNA blots of TF1-16(*Bs.Xyn'*) and TF2-11(*Bs.Xyn'*) (lane 2 and 3), while the RNA blot of control *Trp*<sup>+</sup> transformant gave no signal (lane 1). To ensure equal loading and transfer on RNA samples, the Northern-blot hybridized with *Bs.xyn'* probe were put into 0.1% SDS and kept in it until room temperature. After confirming perfect removal of the *Bs.xyn'* probe by autoradiography, the resulting Northern blots were re-hybridized with  $^{32}$ P-labelled probe of the *Ccras* gene (Ishibashi and Shishido, 1993). The 1.2-kb *Ccras* signals were detected in all RNA blots and their intensities



probe | 0.7-kb *Bs.xyn'* | 1.3-kb *Cc.ras* |

lane 1: Trp<sup>+</sup> transformant  
 lane 2: TF1-16(*Bs.Xyn'*)  
 lane 3: TF2-11(*Bs.Xyn'*)  
 lane 4: Trp<sup>+</sup> transformant  
 lane 5: TF1-16(*Bs.Xyn'*)  
 lane 6: TF2-11(*Bs.Xyn'*)

**Fig. 11.** Northern-blot analysis of total RNA from TF1-16(*Bs.Xyn'*), TF2-11(*Bs.Xyn'*) and Trp<sup>+</sup> transformant

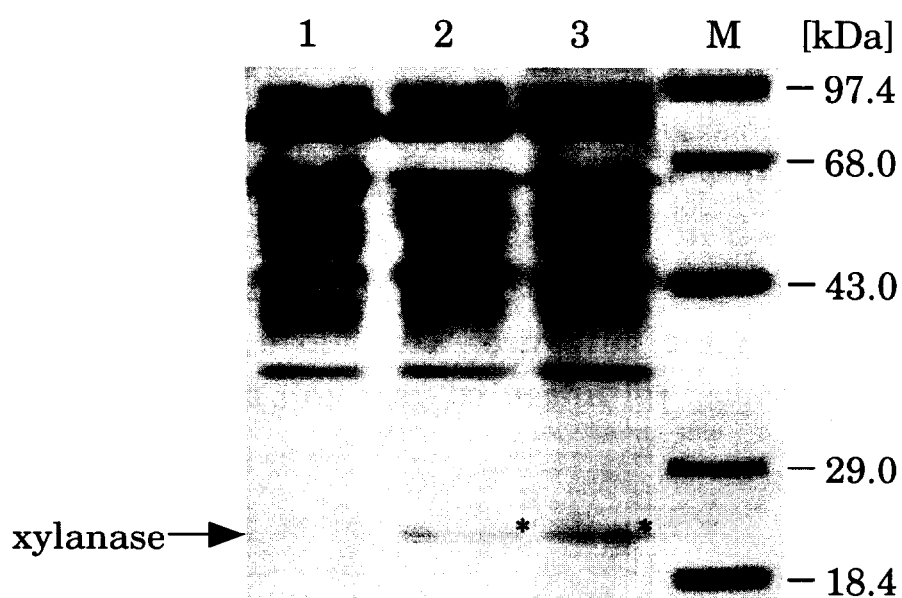
were similar (lane 4-6 of Fig. 11). The negatives of autoradiographic patterns pictured in Fig. 11 yielding a densitometric measurement of the intensities of *Bs.xyn'* and *Ccras* bands TF2-11(*Bs.Xyn'*) was considered to contain the *Bs.xyn'* transcript about 1.6 times as many as TF1-16(*Bs.Xyn*). These data coincide with the estimates of copy numbers of the *Bs.xyn'* sequence.

(iii) Secretion of the *Bacillus subtilis* xylanase gene product in *Coprinus cinereus*

The supernatants of the cultures in MY medium of TF1-16(*Bs.Xyn'*), TF2-11(*Bs.Xyn'*) and control  $\text{Trp}^+$  transformants were subjected to SDS-PAGE for analysis of the secreted proteins from the cells. As shown in Fig. 12, the culture supernatants of TF1-16(*Bs.Xyn'*) and TF2-11(*Bs.Xyn'*) (lane 2 and 3) gave a protein band around the expected position of the molecular mass of xylanase and the band was not detected in the culture supernatant of  $\text{Trp}^+$  control (lane 1). These data strongly suggested that the band is of xylanase. Based on the intensities of the xylanase band, TF2-11(*Bs.Xyn*) is considered to secrete larger amounts of xylanase into culture medium as compared with TF1-16(*Bs.Xyn*).

(iv) The xylan-degradation activities of the culture supernatants of TF1-16(*Bs.Xyn'*) and TF2-11(*Bs.Xyn'*)

TF1-16(*Bs.Xyn'*), TF2-11(*Bs.Xyn'*) and control  $\text{Trp}^+$  transformants were cultured in 100 ml of MY medium in SAKAGUCHI flask at 30°C with shaking. Triplicate flasks were prepared and used for cultivation. After indicated periods,



lane 1: Trp<sup>+</sup> transformant  
lane 2: TF1-16(Bs.Xyn')  
lane 3: TF2-11(Bs.Xyn')

**Fig. 12.** SDS-PAGE of total secreted protein from TF1-16(Bs.Xyn'), TF2-11(Bs.Xyn') and control Trp<sup>+</sup> transformant

aliquots were taken out from each of cultures and centrifuged. The supernatants were investigated for xylan-degrading activity. This is Fig. 13 pictured average values and S. D. s for xylanase activity indicated that the supernatants of TF1-16(Bs.Xyn') and TF2-11(Bs.Xyn') obtained from their 18-day cultures contain a xylanase activity seven or nine times high as that of control Trp<sup>+</sup> transformant (Fig.13 and Table 1). The growth rates of TF1-16(Bs.xyn') , TF2-11(Bs.xyn') and control Trp<sup>+</sup> transformant were almost the same. The dry weights of the mycelial cells after 18 days of 100-ml cultivation of TF1-16(Bs.Xyn'), TF2-11(Bs.Xyn') and control Trp<sup>+</sup> transformants were 680 mg, 652 mg and 604 mg, respectively.

By using heterologous protein expression vectors, the author have produced the *C. cinereus* strains that carry high copy numbers of *B. subtilis* xylanase gene on their chromosomes and secrete the bacterial xylanase efficiently under control of the signal sequence of the *P. ostreatus* manganese (II) peroxidase gene. These strains may be useful for isolation of cellulose from lignocellulosic resource.

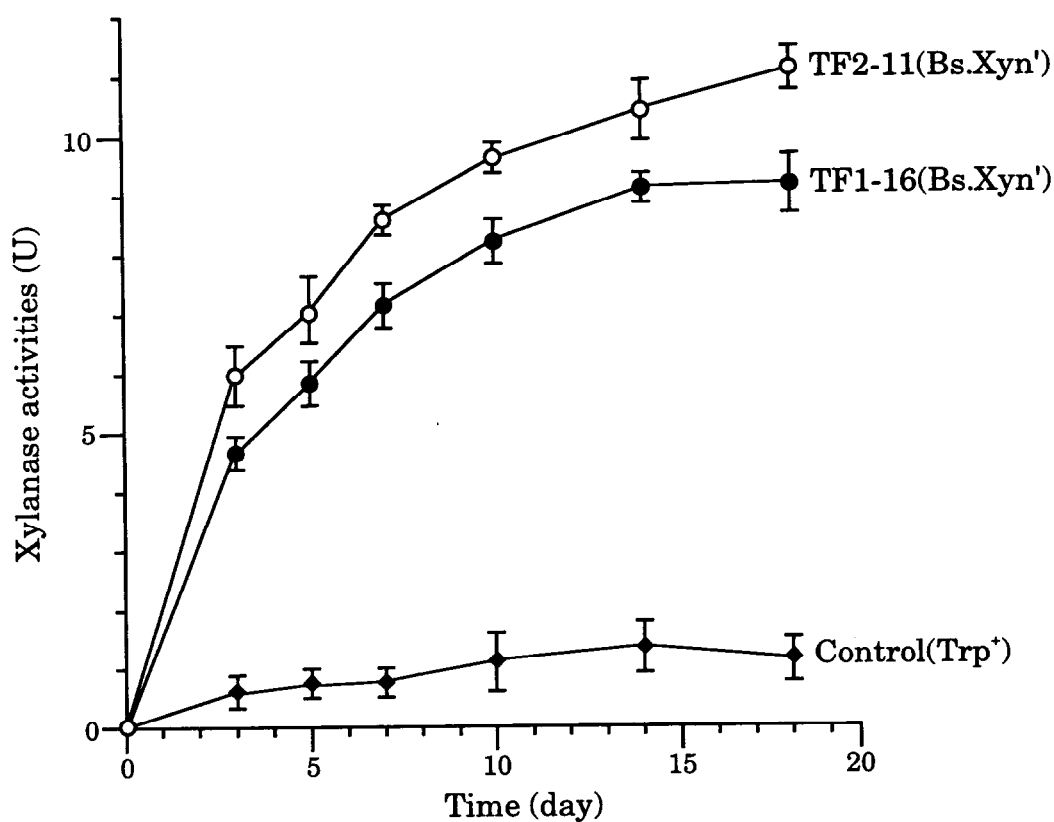


**Fig. 13.** Time course of xylanase production during cultivations of TF1-16(Bs.Xyn'), TF2-11(Bs.Xyn') and control Trp<sup>+</sup> transformant

Xylanase activity is shown by units (U), determined for the culture supernatants (50  $\mu$ l each). Average values and S. D. s are presented.

**Table 1.** Percentage of xylanase activities of the culture supernatants of TF1-16(Bs.Xyn') and TF2-11(Bs.Xyn') when compared with that of control Trp<sup>+</sup> transformant

| Time<br>(day)<br>Strain       | 3       | 5       | 7       | 10      | 14      | 18      |
|-------------------------------|---------|---------|---------|---------|---------|---------|
| Trp <sup>+</sup> transformant | 100     | 100     | 100     | 100     | 100     | 100     |
| TF1-16(Bs.Xyn')               | 870 ±10 | 820 ±19 | 620 ±25 | 718 ±24 | 723 ±19 | 730 ±33 |
| TF2-11(Bs.Xyn')               | 915 ±9  | 950 ±22 | 790 ±30 | 845 ±36 | 832 ±29 | 940 ±40 |

Xylanase activity of the culture supernatants of control Trp<sup>+</sup> transformant was taken as 100%. Average values and S. D. s are presented.

## 2.3 Secretion of *Aspergillus oryzae* xylanase XynF1 (*xynF1* cDNA product)

### 2.3.1 Materials and Methods

#### (i) Plasmid construction

The 1.0-kb *Aspergillus oryzae xynF1* cDNA (*Ao.xynF1c*) (Fig. 14) cloned into *Pst*I-*Nde*I site on pUC18 (Kitamoto et al., 1999) was isolated and its both termini were blunted by Klenow fragment and T4 polymerase. The resulting fragment was ligated to the *Eco*RI linker (pd(CCGAATTCGG)) and digested with *Eco*RI. This digest was inserted between the promoter and terminator of pLC2 of which *Bam*HI site was changed to *Eco*RI (pLC2E). This recombinant plasmid was named pLC2-*Ao.xynF1* (Fig.15 and Fig. 16).

The 1.0-kb *Ao.xynF1c* was digested with *Sal*I and *Nde*I to obtain the signal sequence-depleted *Ao.xynF1c*. Its both termini were blunted by Klenow fragment and ligated to the *Eco*RI linker (pd(CCGAATTCGG)). The 0.9-kb *Eco*RI digest was inserted into the *Eco*RI site between *P. ostreatus mnpc* signal sequence and *priA* terminator of pLC2-Bs.xyn', yielding pLC2-*Ao.xynF1* (Fig.16). This combinat sequence of *Po.mnpc* and signal-depleted *Ao.xynF1c* was named *Ao.xynF1'*c, which has a single open reading frame.

The C-terminus of secretion signals of the basidiomycete *P. ostreatus* MnPs, *Phanerochaete chrysosporium* Lignin peroxidases (LiPs) and *Lentinula edodes* xylanase, ascomycete *Trichoderma reesei* xylanases and xylan esterase, *Aspergillus niger* glucanase and glucoamylase, and hyphomycete *Penicillium* spp. xylanase and xylan esterase were reported to be -lysine-arginine/ (cleavage point) (Table 2). The C-terminus of the secretion signal of A.

1  
ATGCATCACCTTAAGGCACTTGCCAGTGGCACTCTCTTCGCCTCCCTCGCTTCGTCTGCG 60  
M H H L K A L A S G T L F A S L A S S A

*SalI*

GTTATTAGTTCGACAGGGCAGCCGCCAGCATCAATGATGCCTTTGTGCGCCCATGGAAAGAAG 120  
V I S R Q A A A S I N D A F V A H G K K

TACTTCGGTACCTGTTCCGACCAGGCTCTGCTGCAAAACAGTCAGAATGAGGCCATTGTC 180  
Y F G T C S D Q A L L Q N S Q N E A I V

CGGGCGGACTTTGGCCAGCTGACTCCCGAGAATAGCATGAAGTGGGATGCTCTTGAGCCG 240  
R A D F G Q L T P E N S M K W D A L E P

AGTCAGGGTAGCTTTAGCTTCGCTGGAGCTGATTTCTTGGCTGATTATGCTAAACTAAT 300  
S Q G S F S F A G A D F L A D Y A K T N

AATAAACTTGTGAGGGGGTCATACTTTGGTGTGGCACTCGCAACTCCCATCCTGGGTGCAG 360  
N K L V R G H T L V W H S Q L P S W V Q

GGTATCACCGACAAGGATACTTTGACTGAGGTAATTAAGAATCACATCACTACTATCATG 420  
G I T D K D T L T E V I K N H I T T I M

CAGAGATACAAGGGCCAGATCTATGCTTGGGACGTTGTCAACGAGATCTTCGATGAGGAC 480  
Q R Y K G Q I Y A W D V V N E I F D E D

GGCACTCTTCGTGACAGTGTCTTTTCCAGGTCCTAGGAGAGGACTTTGTCCGCATTGCC 540  
G T L R D S V F S Q V L G E D F V R I A

TTTGAGACCGCTCGTGAGGCGGATCCCAACGCTAAGCTATACATTAACGATTACAACCTTG 600  
F E T A R E A D P N A K L Y I N D Y N L

GATTCGGCAGACTACGCCAAGACCAAGGGAATGGTTAGCTATGTGAAGAAGTGGTTGGAT 660  
D S A D Y A K T K G M V S Y V K K W L D

GCTGGTGTTCATTTGACGGCATTGGCTCCCAGTCTCATTACAGTGCGAACGGCTTCCCC 720  
A G V P I D G I G S Q S H Y S A N G F P

GTTTCTGGCGCCAAAGGAGCTCTGACCGCGCTCGCCTCTACCGGTGTTTCTGAAGTCGCC 780  
V S G A K G A L T A L A S T G V S E V A

GTGACCGAACTTGATATCGAAGGAGCTAGCTCCGAGTCTTATCTCGAGGTGGTCAACGCC 840  
V T E L D I E G A S S E S Y L E V V N A

TGTCTCGACGTCAGCTCCTGCGTCGGTATCACTGTCTGGGGTGTATCCGACAAGGACTCG 900  
C L D V S S C V G I T V W G V S D K D S

TGGCGCTCCAGCACCTCCCCGTTGCTATTTGATAGCAATTACCAGGCCAAGGATGCGTAT 960  
W R S S T S P L L F D S N Y Q A K D A Y

AATGCTATCATCGATGCTCTGTAA 984  
N A I I D A L Ter

Secretion signal sequence is shown by red letters

**Fig. 14.** Nucleotide sequence and deduced amino acid sequence of *A. oryzae* xylanase cDNA (*Ao.xynF1c*)

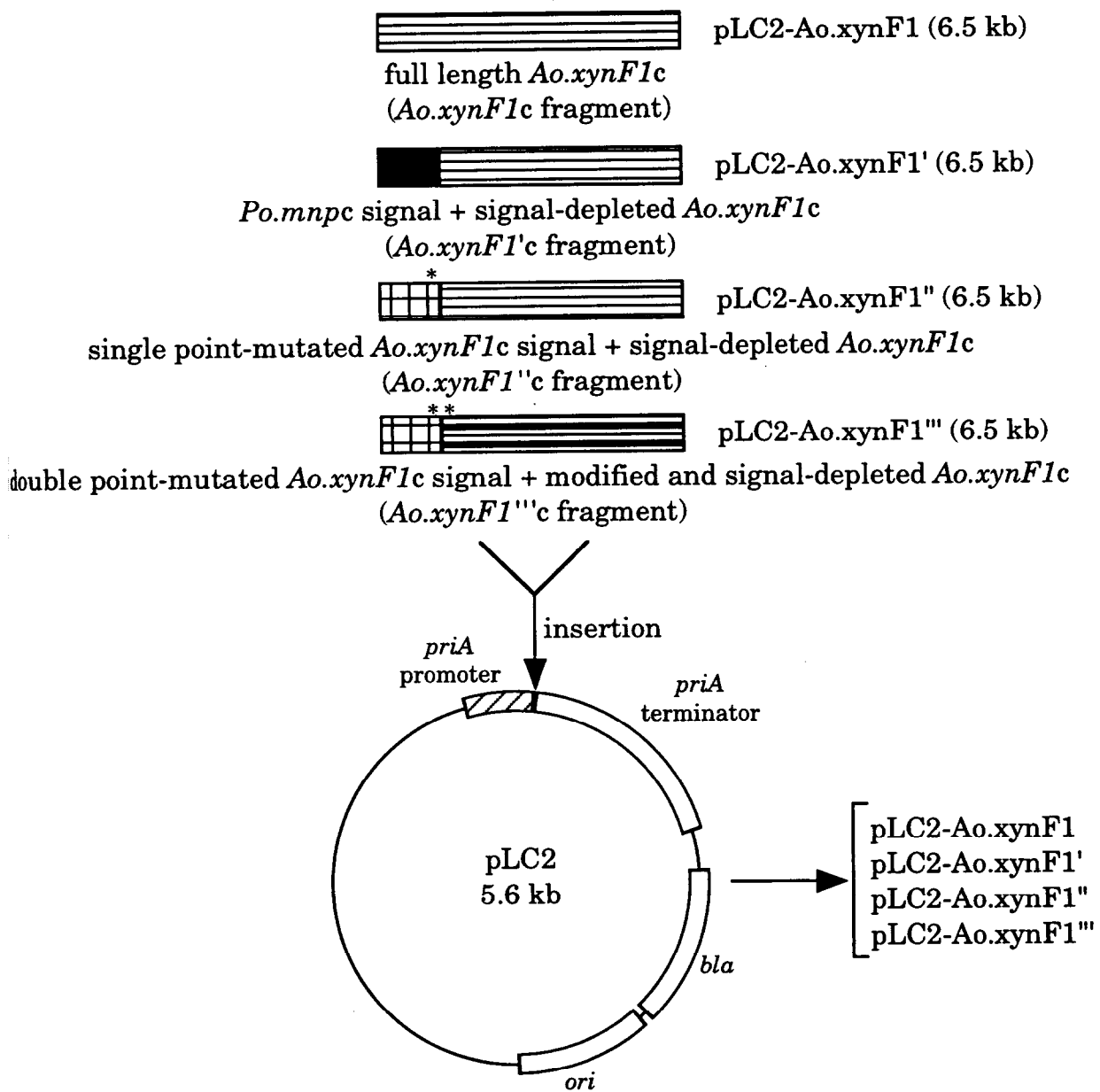


**Fig. 15.** Construction of the *A. oryzae* endo ( $\beta$ -1,4-) D-xylanase XynF1 expressing recombinant plasmids pLC2-Ao.xynF1, pLC2-Ao.xynF1', pLC2-Ao.xynF1'' and pLC2-Ao.xynF1'''

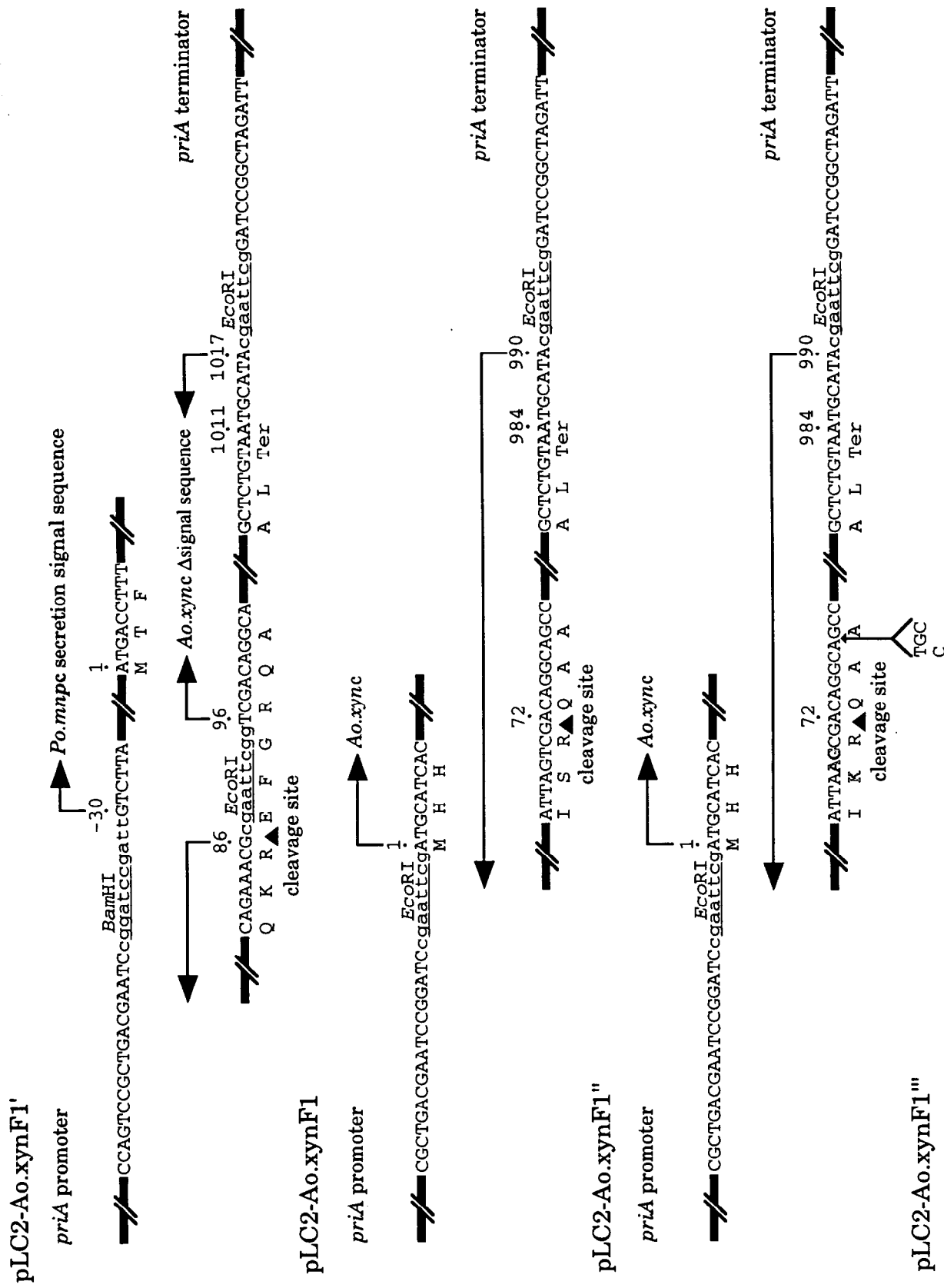


**Fig. 16.** The structure features of the *A. oryzae* xylanase expressing sequence of plasmid pLC2-Ao.xynF1', pLC2-Ao.xynF1, pLC2-Ao.xynF1'' and pLC2-Ao.xynF1'''

**Table 2.** Secretion signals with the C-terminus -Lys-Arg sequence in representative filamentous fungi

| Secretion signal sequence                | gene               | source                             | enzyme                | Reference                           |
|------------------------------------------|--------------------|------------------------------------|-----------------------|-------------------------------------|
| <b>Basidiomycetes</b>                    |                    |                                    |                       |                                     |
| MAFAKLSALVLTALGATVALGAPSLNKR/VTCAT       | <i>mnp2</i>        | <i>Pleurotus ostreatus</i>         | manganese peroxidase  | Giardina et al. (2000), unpublished |
| MAFKHFSLLVLLTLASQAVRGAVMKR/ATCAD         | <i>mnp3</i>        | <i>Pleurotus ostreatus</i>         | manganese peroxidase  | Irie et al. (2000), unpublished     |
| MSVKQFFVVLSTALFLSTVQGAAIEKR/ATCSN        | <i>lipJ</i>        | <i>Phanerochaete chrysosporium</i> | lignin peroxidase     | Stewart and Cullen (1999)           |
| MAFKQLFAAISLALSLSAANAAAVIEKR/ATCSN       | <i>LIG1</i>        | <i>Phanerochaete chrysosporium</i> | lignin peroxidase     | Brown et al. (1988)                 |
| MAFKQLVAAISLALSISAVQGAAVKEKR/ATCSN       | <i>GLG3</i>        | <i>Phanerochaete chrysosporium</i> | lignin peroxidase     | Naidu and Reddy (1990)              |
| MALKQLAAVALALSISQAAQGAAVKEKR/ATCSN       | <i>GLG6</i>        | <i>Phanerochaete chrysosporium</i> | lignin peroxidase     | Naidu et al. (1990)                 |
| MAFKQLFAAITVALSLTAANAADVKEKR/ATCAN       | <i>LiP2</i>        | <i>Phanerochaete chrysosporium</i> | lignin peroxidase     | Ritch and Gold (1992)               |
| MAYKSLFLALIAVTATALDVFDNSTEVIKGR/SIPNG    | <i>LIP6</i>        | <i>Phanerochaete chrysosporium</i> | lignin peroxidase     | Zhang et al. (1991)                 |
| MAFKKLLAVLTAALSLRAAQGAAVEKR/ATCSN        | <i>xyn11a</i>      | <i>Lentinula edodes</i>            | xylanase              | Lee et al. (2001), unpublished      |
| <b>Ascomycetes</b>                       |                    |                                    |                       |                                     |
| MVSFTSLLAASPSPSRASCRPAAEVESVAVEKR/QTIQP  | <i>xyl1</i>        | <i>Trichoderma reesei</i>          | endoxylanase          | Torronen et al. (1992)              |
| MVSFTSLLAGVAAISGVLAAPAAEVEVSVAVEKR/QTIQP | <i>xln2</i>        | <i>Trichoderma reesei</i>          | endoxylanase          | Saarelainen et al. (1993)           |
| MPSVKETLTLLLSQAFLATGSPVDGETVVKR/QCPAI    | <i>axe1</i>        | <i>Trichoderma reesei</i>          | acetyl xylan esterase | Margolles-Clark et al. (1996)       |
| MKFQSTLLLAAGGSALAVPHGPGHKR/ASVFE         | <i>eng1</i>        | <i>Aspergillus niger</i>           | endoglucanase         | Jiong et al. (2001)                 |
| MSFRSLLALSGLVCTGLANVISKR/ATLDS           | glucoamylase<br>G1 | <i>Aspergillus niger</i>           | glucoamylase          | Boel et al. (1984)                  |
| <b>Hyphomycetes</b>                      |                    |                                    |                       |                                     |
| MHSKFFAASLLGLGAAAIPLEGVMEKR/SCP AI       | <i>axe-2</i>       | <i>Penicillium purporogenum</i>    | acetyl xylan esterase | Gutierrez et al. (1998)             |
| MKSFLAYLLASVAVTGVMVPGEYHKRHDKR/QTITS     | <i>xynA</i>        | <i>Penicillium sp. 40</i>          | xylanase              | Kimura et al. (1999), unpublished   |

*oryzae* XynF1 was -serine-arginine/ (cleavage point). So the author changed it to be -lysine-arginine/ (cleavage point) by PCR method. The modified *Ao.xynF1* cDNA sequence (named *Ao.xynF1''c*) was amplified using the primer with the nucleotide mutations (Fig. 17). This mutated 1.0-kb fragment was inserted into *EcoRI* site of pLC2E, yielding the recombinant plasmid pLC2-*Ao.xynF1''* (Fig. 15 and Fig. 16). The *P. chrysosporium* LiPs have cysteine residue in third position downstream from the cleavage point (in the C-terminus of the mature LiPs) (Ritch Jr. et al., 1991). Thus the author prepared the further modified *Ao.xynF1* cDNA sequence: -lysine-arginine/ (cleavage point) glutamine-alanine-cysteine and it was named *Ao.xynF1'''c* (Fig. 17). This doubly mutated 1.0-kb fragment was inserted into *EcoRI* site of pLC2E, yielding the recombinant plasmid pLC2-*Ao.xynF1'''* (Fig. 15 and Fig. 16).

(ii) Southern- and Northern-blot analyses.

These analyses were done as described in Materials and Methods of section 2.2.

(iii) Assessment of xylan degradation

This was done as described in Materials and Methods of section 2.2.

(iv) Tricine-SDS-PAGE analysis

A-100 ml sample of MY medium in SAKAGUCHI flask was inoculated with six each of disks (6 mm in diameter and 1 mm in thickness) of MYG/ agar colonized with *C. cinereus* mycelium and cultivated at 25°C for 14-days with shaking. The 10-ml of the

<sup>1</sup>ATGCATCACCTTAAGGCACTTGCCAGTGGCACTCTCTTCGCCTCCCTCGCTTCGTCTGCG 60  
M H H L K A L A S G T L F A S L A S S A  
  
GTTATTAGTTCGACAGGCA GCCGCCAGCATCAATGATGCCCTTTGTCCGCCATGGAAGAAG...120  
V I S R/ Q A A A S I N D A F V A H G K K  
  
↑ AAG TGC Cys  
K Ao.xynF1'''c  
Ao.xynF1'''c and  
Ao.xynF1'''c

Secretion signal sequence is shown by red letters

**Fig. 17.** Nucleotide sequences and deduced amino acid sequences of the point-mutated *A. oryzae* xylanase cDNAs, *Ao.xynF1'''c* and *Ao.xynF1'''c*

culture supernatants were frozen and dried, and suspended in 1-ml PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.7 mM KH<sub>2</sub>PO<sub>4</sub>, pH7.4) buffer. The protein samples were analyzed by Tricine-(0.1%)SDS-(12.5%)polyacrylamid gel electrophoresis according to method of Schägger and Jagow (1987).

(v) Analysis of active staining

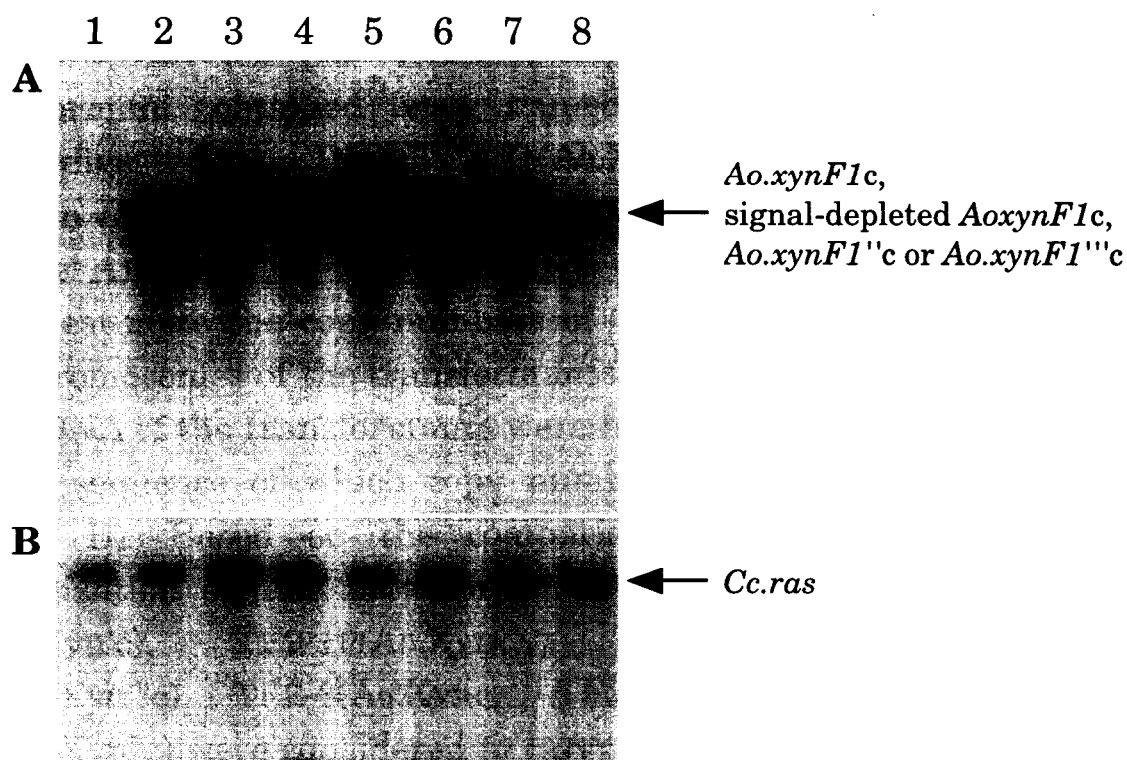
A-100 ml sample of MY medium in SAKAGUCHI flask was inoculated with six each of disks (6 mm in diameter and 1 mm in thickness) of MYG/ agar colonized with *C. cinereus* mycelium and cultivated at 25°C for 14-days with shaking. The 10-ml of culture supernatants were frozen and dried, and suspended in 1-ml PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.7 mM KH<sub>2</sub>PO<sub>4</sub>, pH7.4) buffer. The samples were electrophoresed in native 10% polyacrylamid gel containing 0.5% xylan. After electrophoresis gel was put into 50 mM sodium citrate buffer (pH6.0) at 50°C 1 h, and stained with Congo-Red and decolorized. The decolorized bands showed xylanase activity (Chaudhary and Deobagkar, 1997). An isoelectric point (IEP) electrophoresis was done in xylan-containing native gel. The range of pI analyzed was 3-8. After electrophoresis gel was treated as same as above. These two experiments were done according to the method of Lappalainen (1986).

### 2.3.2 Results and Discussion

- (i) Co-transformation of *Coprinus cinereus* Trp<sup>-</sup> auxotrophic strain with pCc1001 and pLC2-Ao.xyn, pLC2-Ao.xyn', pLC2-Ao.xyn'' or pLC2-Ao.xyn'''

pLC2-Ao.xynF1 was introduced into *C. cinereus* LT2-44 genome with pCc1001, and the transformants obtained, were screened by Southern-blot hybridization. The *Eco*RI-digested total DNA of the transformants were hybridized with <sup>32</sup>P-labelled 0.9-kb signal-depleted *Ao.xync* fragment as a probe. The transformants that carry the *Ao.xynF1c* sequence on their chromosomes were selected and they were named TF2-6(Ao.Xync) and TF2-10(Ao.Xync). As shown in Fig. 18, TF2-6(Ao.Xync) and TF2-10(Ao.Xync) gave a hybridization signal at the position corresponding to the size of 1.0-kb (lane 3, 4 of panel A). The pLC2-Ao.xynF1'c was introduced into *C. cinereus* genome with pCc1001 and the transformants were screened by Southern-blot analysis. The *Eco*RI-digested total DNA of the transformants were hybridized with 0.9-kb signal-depleted *Ao.xync* fragment as a probe. TF2-1(Ao.Xyn'c) selected showed an intense 0.9-kb signal corresponding to the size of probe (lane2 of panel A).

pLC-Ao.xynF1'' which contains a single point mutation in the C-terminus of the signal sequence (serine-arginine → lysine-arginine), was introduced into *C. cinereus* LT2-44 with pCc1001 and transformants were screened by Southern-blot analysis. The *Eco*RI-digested total DNA of transformants were hybridized with 0.9-kb signal-depleted *Ao.xynF1c* fragment as a probe. TF2-14(Ao.Xyn''c), TF2-15(Ao.Xyn''c) and TF2-17(Ao.Xyn''c) selected gave a hybridization signal at the position corresponding to the size (1.0 kb) of *Ao.xynF1''c* (lane5, 6 and 8 of panel A). pLC-Ao.xynF1''' was introduced into *C. cinereus* with pCc1001 and the



[A] *Eco*RI- digest  
 probe, 0.9-kb signal-depleted *Ao.xynF1c* fragment  
 [B] [*Xho*I + *Sal*I]-digest  
 probe, 1.3-kb *Cc.ras* sequence

lane 1: Trp<sup>+</sup> transformant  
 lane 2: TF2-1(*Ao.Xyn'*c)  
 lane 3: TF2-10(*Ao.Xync*)  
 lane 4: TF2-6(*Ao.Xync*)  
 lane 5: TF2-14(*Ao.xyn''c*)  
 lane 6: TF2-15(*Ao.Xyn''c*)  
 lane 7: TF2-21(*Ao.Xyn'''c*)  
 lane 8: TF2-17(*Ao.Xyn''c*)

**Fig. 18.** Southern-blot analysis of the restriction endonuclease-digests of total DNA from TF2-1(*Ao.Xyn'*c), TF2-6(*Ao.Xync*), TF2-10(*Ao.Xync*), TF2-14(*Ao.Xyn''c*), TF2-15(*Ao.Xyn''c*), TF2-17(*Ao.Xyn''c*), TF2-21(*Ao.Xyn'''c*) and Trp<sup>+</sup> transformant

transformant obtained was named TF2-21(Ao.Xyn<sup>'''</sup>c). The *Eco*RI-digested total DNA TF2-21(Ao.Xyn<sup>'''</sup>c) gave a signal at the position corresponding to the size (1.0 kb) of *Ao.xynF1*<sup>'''</sup>c (lane 7 of panel A).

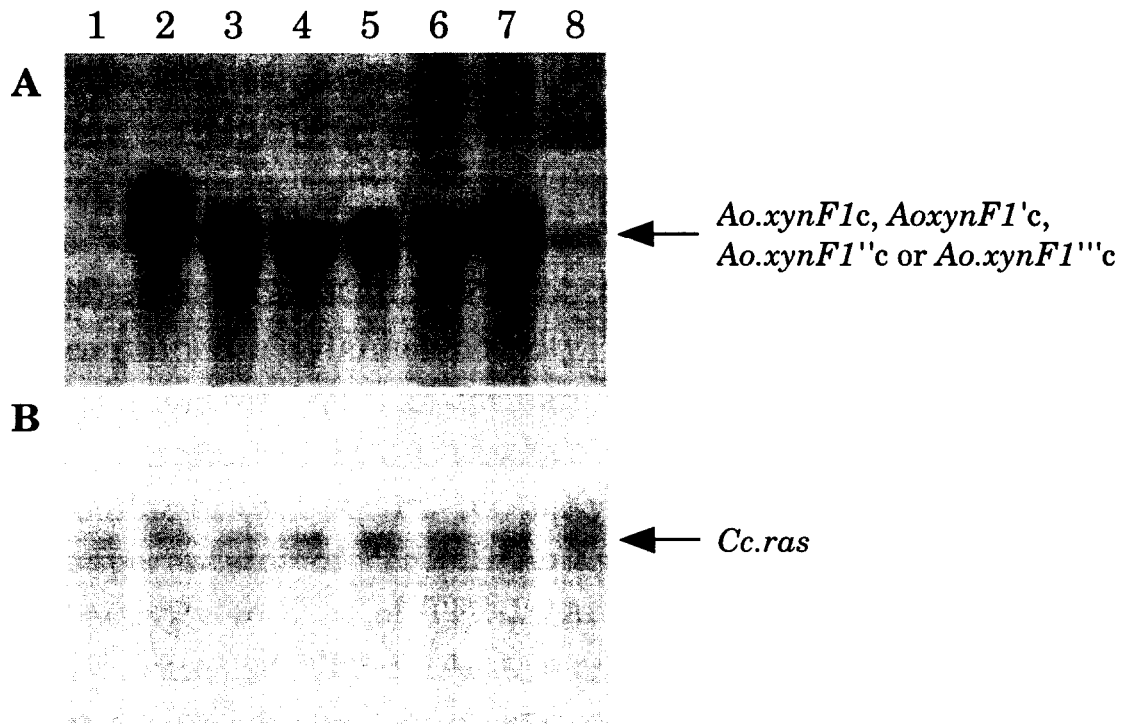
To estimate the copy numbers of the *Ao.xynF1* sequences on the chromosomes of the transformants, the [*Xho*I + *Sal*I]-digested total DNA of the transformants were hybridized with the probe of *Ccras* sequence of which copy number on chromosome is one (panel B). Based on the comparison of the intensities of hybridization signals between *Ccras* and the *Ao.xynF1*c. TF2-1(Ao.Xyn'<sup>c</sup>), TF2-10(Ao.Xync), TF2-14(Ao.Xyn''<sup>c</sup>), TF2-21(Ao.Xyn<sup>'''</sup>c), TF2-6(Ao.Xync), TF2-15(Ao.Xyn''<sup>c</sup>) and TF2-17(Ao.Xyn''<sup>c</sup>) were considered to carry 5, 5, 5, 5, 3, 3 and 1 copies on the chromosomes. The Trp<sup>+</sup> control was shown no signal for *Ao.xynF1*c (lane 1 of panel A).

(ii) Transcriptional expression of *Aspergillus oryzae* xylanase cDNA

Total RNA of TF2-1(Ao.Xyn'<sup>c</sup>), TF2-6(Ao.Xync), TF2-10(Ao.Xync), TF2-14(Ao.Xyn''<sup>c</sup>), TF2-15(Ao.Xyn''<sup>c</sup>), TF2-17(Ao.Xyn''<sup>c</sup>) and TF2-21(Ao.Xyn<sup>'''</sup>c) were hybridized with the probe of signal-depleted *Ao.xync* fragment. The results of Northern-blot analysis pictured in Fig. 19 showed that the signal intensities of Northern-blot were proportional to those of Southern-blot.

(iii) The xylan-degradation activities of the culture supernatants of TF2-1 (Ao.Xyn'<sup>c</sup>), TF2-10 (Ao.Xync) and TF2-14(Ao.Xyn''<sup>c</sup>)

TF2-1 (Ao.Xyn'<sup>c</sup>), TF2-10 (Ao.Xync) and TF2-14(Ao.Xyn''<sup>c</sup>) were cultured in 100 ml MY medium in shake flask at 25°C with



[A] probe, 0.9-kb signal-depleted *Ao.xynF1c* fragment  
[B] probe, 1.3-kb *Cc.ras* sequence

lane 1: Trp<sup>+</sup> transformant  
lane 2: TF2-1(Ao.Xyn'c)  
lane 3: TF2-10(Ao.Xync)  
lane 4: TF2-6(Ao.Xync)  
lane 5: TF2-14(Ao.xyn''c)  
lane 6: TF2-15(Ao.Xyn''c)  
lane 7: TF2-21(Ao.Xyn'''c)  
lane 8: TF2-17(Ao.Xyn''c)

**Fig. 19.** Northern-blot analysis of total RNA from TF2-1(Ao.Xyn'c), TF2-6(Ao.Xync), TF2-10(Ao.Xync), TF2-14(Ao.Xyn''c), TF2-15(Ao.Xyn''c), TF2-17(Ao.Xyn''c), TF2-21(Ao.Xyn'''c) and Trp<sup>+</sup> transformant

shaking. The culture supernatants were investigated for xylan degradation activity (Fig. 20). TF2-10(Ao.Xync) showed a low activity of xylanase similar to that of the control Trp<sup>+</sup> transformant (Fig. 20 and Table 3). The supernatants of TF2-1(Ao.Xyn'c) and TF2-14(Ao.Xyn''c) obtained from their 18-days cultures contained a xylanase activity around 10 times as high as that of the control Trp<sup>+</sup> transformant (Fig. 20 and Table 3). The culture supernatant of TF2-17(Ao.Xyn''c) showed a low xylanase activity similar to that of TF2-10(Ao.Xync) (data not shown). The culture supernatant of TF2-21(Ao.Xyn'''c) showed a similar xylanase activity to that of TF2-14(Ao.xyn''c) (data not shown). The growth rates of TF2-1 (Ao.Xyn'c), TF2-10 (Ao.Xync), TF2-14(Ao.Xyn''c), TF2-17(Ao.Xyn''c) and control Trp<sup>+</sup> transformant were almost the same. The dry weights of mycelia after 18 days of 100 ml cultivation of TF2-1(Ao.Xyn'c), TF2-10 (Ao.Xync), TF2-14(Ao.Xyn''c), TF2-17(Ao.Xyn''c) and control Trp<sup>+</sup> transformant were 617 mg, 580 mg, 608 mg, 540 mg and 523 mg, respectively.

(iv) Secretion of the *Aspergillus oryzae* xylanase cDNA expressed product in *Coprinus cinereus*

The concentrated culture supernatants of TF2-1(Ao.Xyn'c), TF2-10 (Ao.Xync) and TF2-14(Ao.Xyn''c) were subjected to Tricine-SDS-PAGE. As shown in Fig. 21, the culture supernatants of TF2-1(Ao.Xyn'c) and TF2-14(Ao.Xyn''c) gave a weak protein band at the position corresponding the expected size of xylanase.

The concentrated supernatants were electrophoresed in xylan-containing native gel, and then the gel was incubated in 50 mM sodium citrate buffer at 50°C for 3 hr to degrade the xylan in it. After staining with Congo-red and decolorization, two bands with xylanase activity were detected for the culture supernatants of

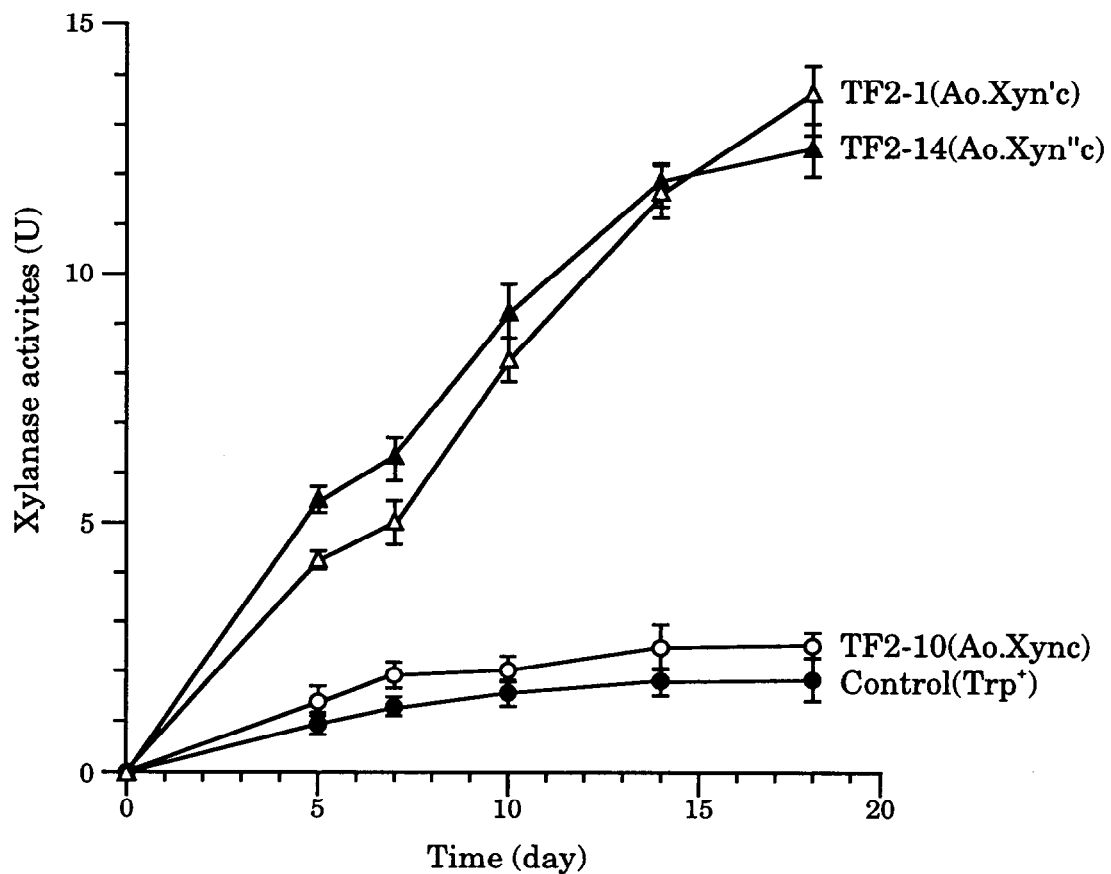


**Fig. 20.** Time course of xylanase production during cultivation of TF2-1(Ao.Xyn'c), TF2-10(Ao.Xync), TF2-14(Ao.Xyn''c) and control Trp<sup>+</sup> transformant

Xylanase activity is shown by units (U), determined for the culture supernatants (50  $\mu$ l each). Average values and S. D. s are presented.

**Table 3.** Percentages of xylanase activities of the culture supernatants of TF2-1(Ao.Xyn'c), TF2-10(Ao.Xync) and TF2-14(Ao.Xyn" c) when compared with that of control Trp<sup>+</sup> transformant

| Time<br>(day)<br>Strain       | 5      | 7      | 10      | 14      | 18       |
|-------------------------------|--------|--------|---------|---------|----------|
| Trp <sup>+</sup> transformant | 100    | 100    | 100     | 100     | 100      |
| TF2-10(Ao.Xync)               | 143 ±5 | 147 ±4 | 125 ±6  | 131 ±7  | 151 ±6   |
| TF2-1(Ao.Xyn'c)               | 471 ±9 | 403 ±7 | 645 ±9  | 764 ±22 | 1039 ±52 |
| TF2-14(Ao.Xyn" c)             | 575 ±5 | 566 ±8 | 718 ±13 | 798 ±30 | 976 ±36  |

Xylanase activity of the culture supernatants of control Trp<sup>+</sup> transformant was taken as 100%. Average values and S. D. s are presented.

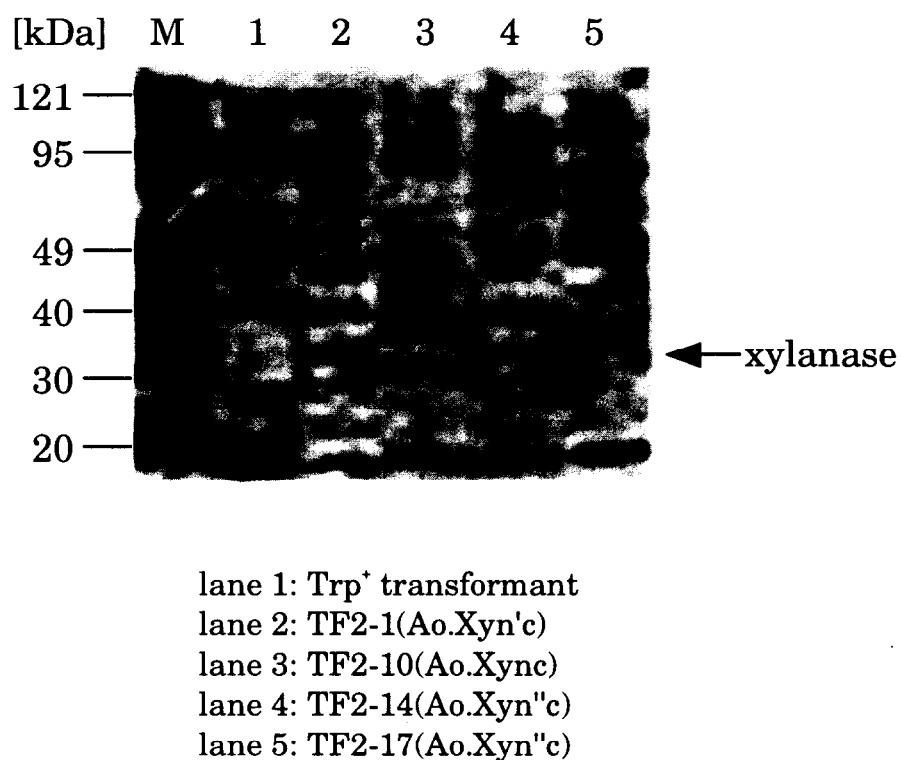
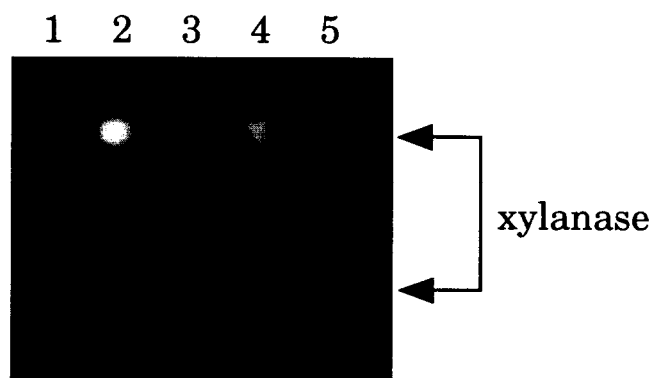


**Fig. 21.** Tricine-SDS-PAGE of a total secreted protein from TF2-1(Ao.Xyn'<sup>c</sup>), TF2-10(Ao.Xync), TF2-14(Ao.Xyn''<sup>c</sup>), TF2-17(Ao.Xyn''<sup>c</sup>) and control Trp<sup>+</sup> transformant

TF2-1(Ao.Xyn'c) (lane 2 of Fig. 22) and TF2-14(Ao.Xyn''c) (lane 4 of Fig. 22). The culture supernatants of TF2-10(Ao.Xync) (lane 3 of Fig. 22) and TF2-17(Ao.Xyn''c) (lane 5 of Fig. 22) showed one weaker band at the upper position. These results suggest that the two forms of the *A. oryzae* XynF1 protein may be present in the case of TF2-1(Ao.Xyn'c) and TF2-14(Ao.Xyn''c). These two forms are considered to be different each other from the points that (1) xylanase protein is monomer or oligomers, (2) xylanase protein is differently modified with sugar side chains, and (3) xylanase protein interacted with other enzyme proteins. The upper band observed in the case of TF2-10(Ao.Xync) and TF2-17 (Ao.Xyn''c) (as well as TF 2-1(Ao.Xyn'c) and TF2-14(Ao.Xyn''c)) are considered to also contain *C. cinereus* intrinsic xylanase protein. The xylanase is known to form the complex with other hydrolyzing enzymes such as cellulase via their catalytic domain (Ali et al., 1995 and Subramaniyan and Prema, 2000) and probably unknown functional domain (Ferreira et al., 1990) (see Fig. 23).

Isoelectric point (IEP) electrophoresis was done for analysis of xylanase protein. The culture supernatants of TF2-1(Ao.Xyn'c) and TF2-14(Ao.Xyn''c) formed a decolorized band (xylanase band) at around pI 4.2 (lane 4 and 5 of Fig. 24). The culture supernatants of TF2-10(Ao.Xync) and Trp<sup>+</sup> transformant gave no detectable xylanase band (lane 1 and 2 in Fig. 24). An intrinsic xylanase (around pI 7.0) was detected in the culture supernatant of Trp<sup>+</sup> transformant which cultured in the presence of xylan (lane 3 in Fig. 24) (Biely, 1985 and Coughlan and Hazlewood, 1993b). In TF2-1(Ao.Xyn'c) and TF2-14(Ao.Xyn''c), the heterologous xylanase Ao.XynF1 (pI 4.2) was produced xylan-non inducing condition.

These results suggest that *A. oryzae* XynF1 cDNA product, which has an intrinsic signal sequence (serine-arginine/ (cleavage



lane 1: Trp<sup>+</sup> control  
 lane 2: TF2-1(Ao.Xyn'<sup>c</sup>)  
 lane 3: TF2-10(Ao.Xync)  
 lane 4: TF2-14(Ao.Xyn"<sup>c</sup>)  
 lane 5: TF2-17(Ao.Xyn"<sup>c</sup>)

**Fig. 22.** Active staining of xylanase by the xylan containing native-PAGE of TF2-1(Ao.Xyn'<sup>c</sup>), TF2-10(Ao.Xync), TF2-14(Ao.Xyn"<sup>c</sup>), TF2-17(Ao.Xyn"<sup>c</sup>) and Trp<sup>+</sup> transformant

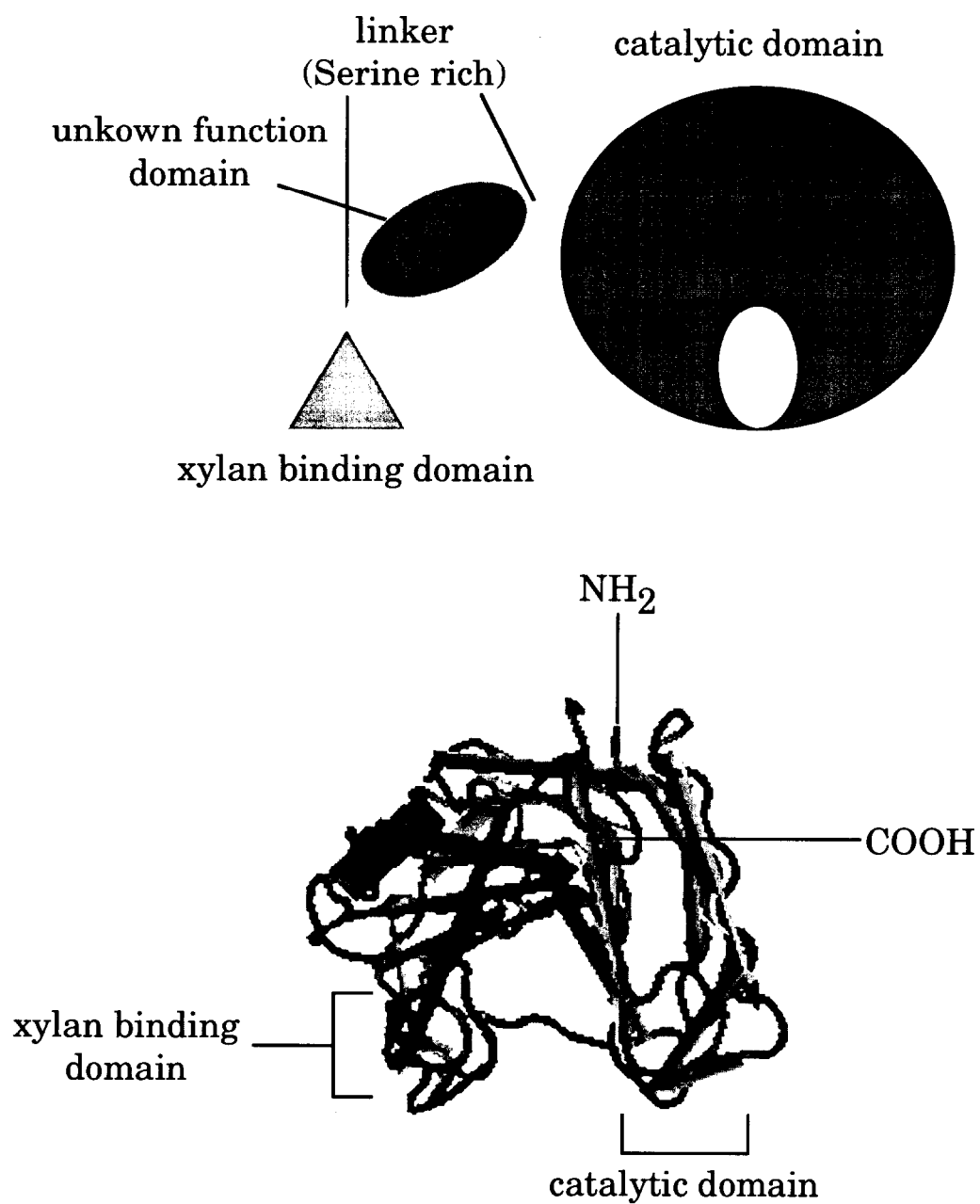
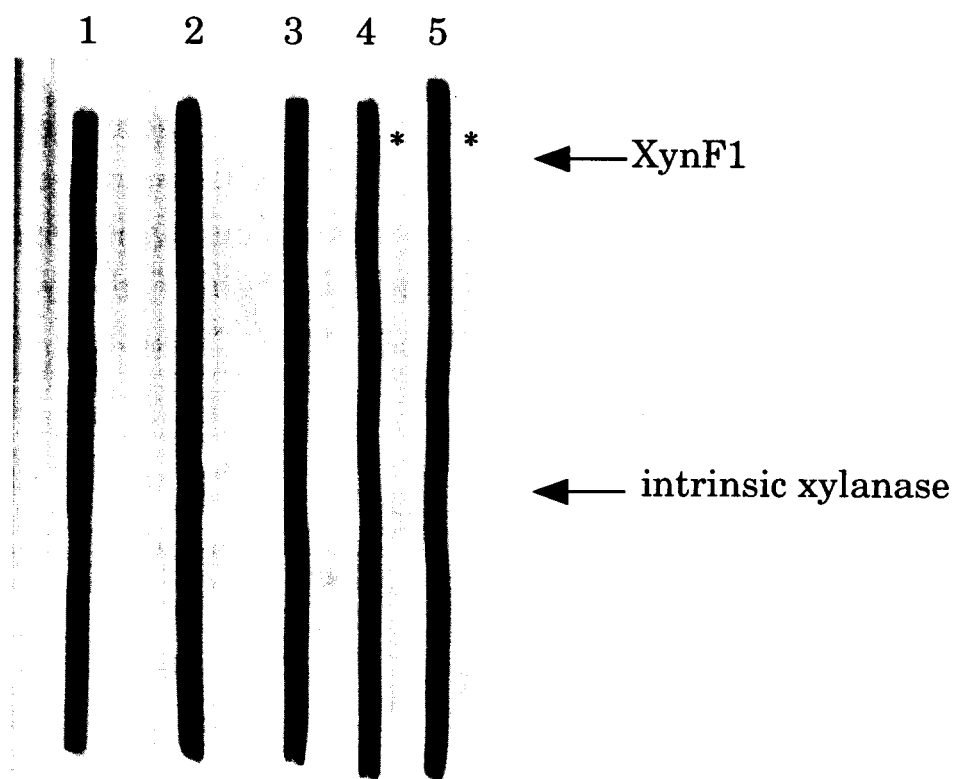


**Fig. 23.** Structures of xylanase protein



lane 1: TF2-10(Ao.Xync)  
 lane 2: Trp<sup>+</sup> transformant  
 ¶ lane 3: Trp<sup>+</sup> transformant  
 lane 4: TF2-1(Ao.Xyn'c)  
 lane 5: TF2-14(Ao.Xyn''c)

**Fig. 24.** Active staining of xylanase by the xylan-containing IEP electrophoresis of TF2-1(Ao.Xyn'c), TF2-10(Ao.Xync), TF2-14(Ao.Xyn''c) and Trp<sup>+</sup> transformant

¶ lane 3: xylanase production is induced by adding of xylan into medium

point)), dose not secreted into culture medium, but *A. oryzae* XynF1" cDNA product of which signal sequence has the C-terminus of lysine-arginine in place of serine-arginine dose secreted into culture medium. The addition of cysteine residue at the third amino acid position of mature XynF1 xylanase is not absolutely necessary for a secretion of XynF1 in *C. cinereus*. Fig 25 is the comparison of signal sequences among *A. oryzae* XynF1, *Pleurotus ostreatus* MnP which contained lysine-arginine/ (cleavage point), and *Phanerochaet chrysosporium* LiPs (Peberdy, 1994) which contained lysine-arginine/ (cleavage point) and cysteine residue at the third position of mature protein.

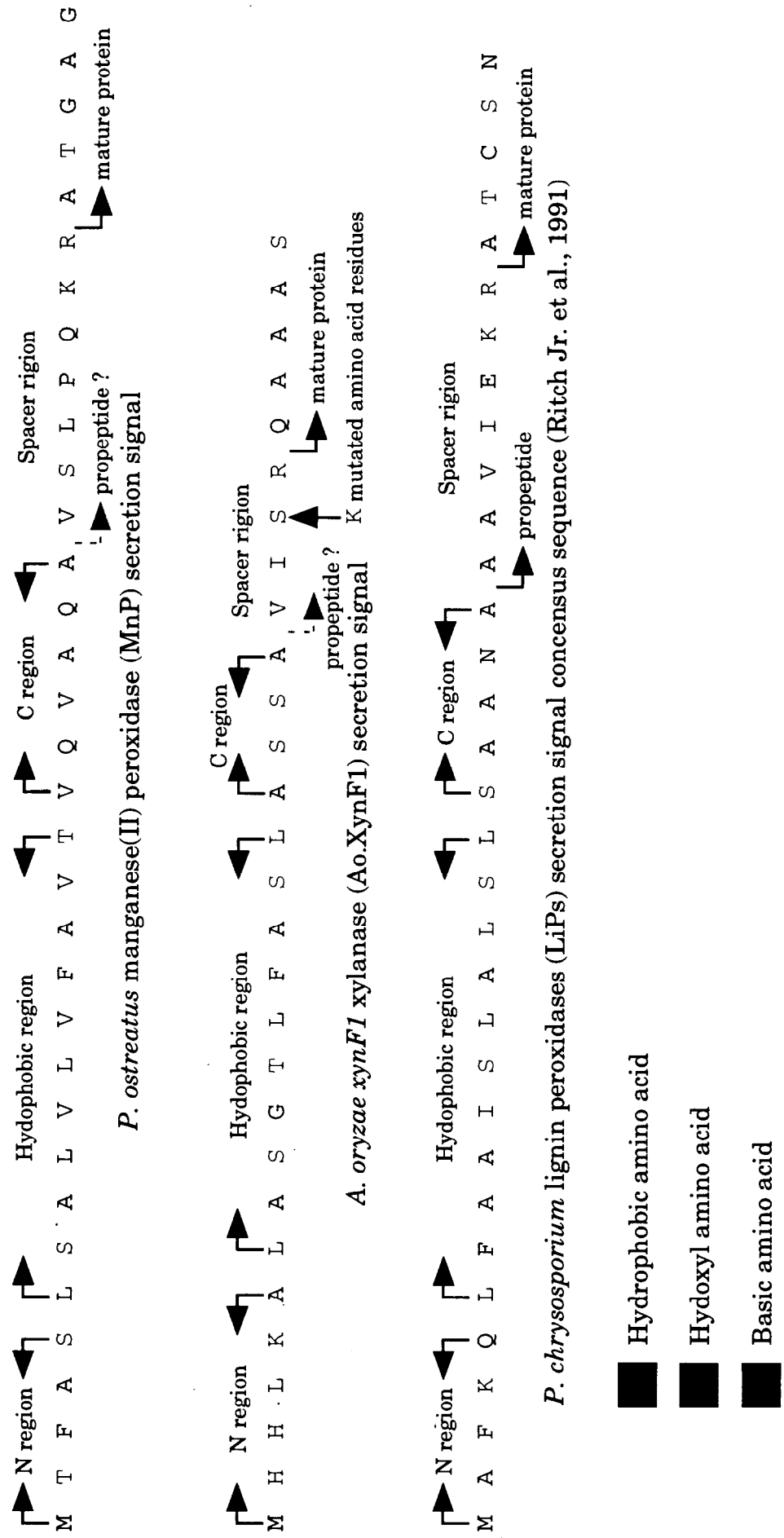


**Fig. 25.** Comparison of amino acid sequences among the secretion signal of *P. ostreatus* MnP, *A. oryzae* XynF1 and *P. chrysosporium* LiP

## **Chapter 3**

**Use of molecular-genetically improved *Coprinus cinereus* strains with high xylan- and lignin-degrading activities for an efficient isolation of cellulose from waste plant biomass**

### 3.1 Introduction

Lignocellulose, which consisted of cellulose, hemicellulose and lignin, is most abundant renewable organic resource on earth. Basidiomycete fungi intrinsically have a hydrolyzing activity of lignocellulose. The efficient isolation of cellulose, which is convertible into ethanol by fermentation, by using the molecular-genetically improved basidiomycete fungal strain from waste plant biomass is meaningful and remarkable from point of view of obtaining a source of energy. The author attempted to isolate cellulose from rice straw using the molecular-bred *C. cinereus* strains with high xylan- and lignin-degrading activities.

### 3.2 Materials and Methods

#### (i) Strains and medium

*Coprinus cinereus* monokaryotic transformants TF2-11(Bs.Xyn') (see chapter 2), TF2-7(Po.MnP) (Ogawa et al., 1998) and control Trp<sup>+</sup> transformant (see chapter 2) and *Ganoderma applanatum* (Kofuki-Sarunokoshikake) dikaryotic wild type strain isolated by our laboratory were used.

All strains were precultured in MYG/ agar at room temperature for one week. Dried rice straw cut into 5-mm lengths were added to 25 ml of 0.025% MnCl<sub>2</sub> solution in a 100-ml flask to give a final concentration of 0.5% (w/v). Three kind of rice straw were used; untreated, that treated with 0.1 N NaOH at room temperature for 2 h and that cultured by growing *G. applanatum* at 27°C for one week (see Fig. 26). The culture medium was inoculated with total six disks (6 mm in diameter and 1 mm in thickness) of the MYG/ agar growing of TF2-7(Po.MnP), TF2-11(Bs.Xyn) and control Trp<sup>+</sup> strain cultivated for 1, 2 and 3 weeks at 27°C with shaking. Triplicate flasks were prepared for each of the cultivation times, and used.

#### (ii) Measurement of cellulose in medium

After 1, 2 and 3 weeks, the cultures were centrifuged, the supernatants of culture were dialyzed overnight against 50 mM sodium citrate (pH 6.2) using a dialysis membrane, size 20 to remove a low molecular weight substance (see Fig. 26). The remaining culture supernatants were extensively treated with the crude cellulase preparation of Cellulase ONOZUKA R-10 that can convert cellulose to glucose with more than 95% efficiency. To 50  $\mu$ l of the dialyzed culture supernatant, 50  $\mu$ l (~35 units) of

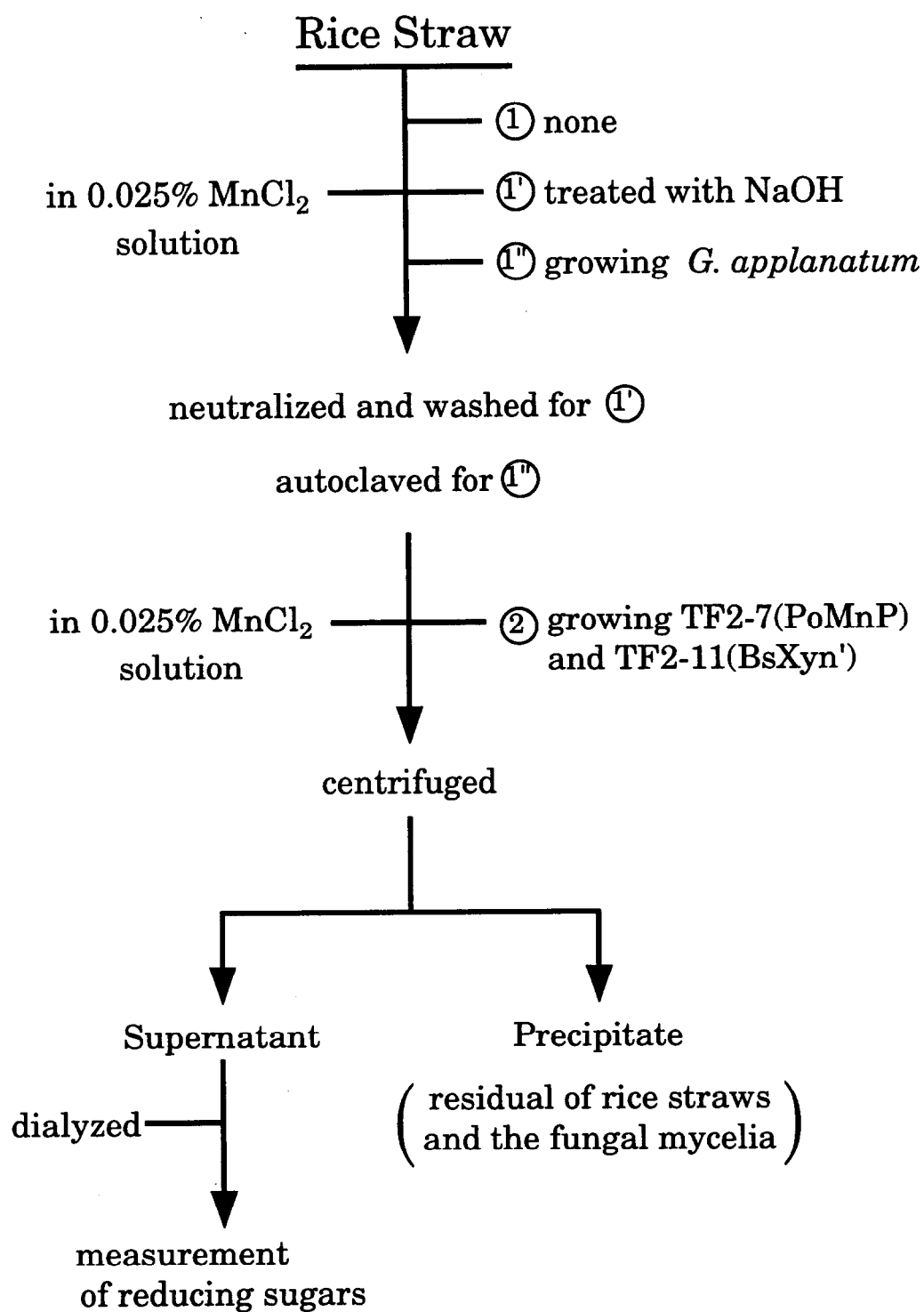


**Fig. 26.** Flow chart for examination of usefulness of the molecular-bred *Coprinus cinereus* strains to isolate cellulose from rice straw

Cellulase ONOZUKA R-10 was added and then incubated at 37°C for 16 h. One unit of cellulase activity was defined as the amount of enzymes that produced a reducing sugar equivalent to 1  $\mu$ mol of glucose under these conditions. To the reaction mixtures, 200  $\mu$ l of 3,5-dinitrosalicylic acid (DNS) reagent was added and boiled for 5 min, followed by rapid cooling and centrifuging. The resulting supernatants were measured for the absorbance at 500 nm. The percentage of the total cellulose contained in rice straw was estimated as follows. The holocellulose fraction (cellulose + hemicellulose) was obtained from the rice straw used for this study by the Klason method (Berrocal et al., 2000). This holocellulose was digested extensively with Cellulase ONOZUKA R-10 and the amount of glucose was measured for the digest, estimating the percentage of total cellulose contained in the rice straw to be 40% (Duke, 1983 unpublished) .

**Table 4.** Recoveries of cellulose from rice straw into the culture supernatants by using the molecular-genetically bred and non-bred strains of *Coprinus cinereus*

| Cultivation time<br>(week) | Mixed cultivation of TF2-7(Po.MnP)<br>and TF2-11(Bs.Xyn') |           |                      | Cultivation of control Trp <sup>+</sup> strain |            |                      |
|----------------------------|-----------------------------------------------------------|-----------|----------------------|------------------------------------------------|------------|----------------------|
|                            | none                                                      | NaOH      | <i>G. applanatum</i> | none                                           | NaOH       | <i>G. applanatum</i> |
| 1                          | 5.4 (±0.6)                                                | 16 (±1.1) | 18 (±0.8)            | 2.5 (±0.3)                                     | 4.3 (±0.9) | 5.4 (±0.8)           |
| 2                          | 7.1 (±0.5)                                                | 25 (±1.8) | 25 (±0.9)            | 2.9 (±0.5)                                     | 6.4 (±0.8) | 6.4 (±0.5)           |
| 3                          | 9.3 (±0.5)                                                | 29 (±2.2) | 29 (±1.1)            | 4.3 (±0.3)                                     | 8.6 (±0.8) | 7.5 (±0.7)           |

All the values are means of data from three independent experiment ±S. E. M. and indicated by percentage of cellulose recovered from the rice straw. The total cellulose contained in the rice straw was taken as 100%. For more details, refer to text.

the total cellulose from rice straw, one of the most popular waste plant biomasses, by using molecular-genetically bred *C. cinereus* strains together with an other mushroom, *G. applanatum*. This indicates the success in the conversion of approx. 12% of total weight of rice straw into non-precipitable forms of cellulose. This is meaningful and interesting from point of view of obtaining a source of energy from waste material and recycling of natural resources (Viikari et al., 1994 and Oliver and Schweizer, 1999).

## **Chapter 4**

## **Conclusion**

In this thesis, the following results and evidences were obtained in the basidiomycete *Coprinus cinereus*

1. By using the constitutive expression vector and the basidiomycete *Pleurotus ostreatus* manganese (II) preoxidase signal sequence, *Bacillus subtilis* xylanase gene product successfully secreted into culture medium.
2. *Aspergillus oryzae* xylanase cDNA (*xynF1c*) product (XynF1) did not secrete into culture medium, but it secreted by using the *P. ostreatus* manganese (II) preoxidase signal sequence, as like the case of *B. subtilis* xylanase.
3. The modified *A. oryzae* XynF1 of which signal sequence has the point-mutation in its C-terminus (serine-arginine \_ lysine-arginine) successfully secreted into medium.
4. The molecular-genetically bred *C. cinereus* strains with high xylan- and lignin-degrading activities were shown to be useful for isolation of cellulose from rice straw, one of the most popular wastes.

The author believes that these results and evidences will give valuable informations for the mechanism of protein secretion in basidiomycetous fungi, and utilization of lignocellulosic resource with molecular-bred *C. cinereus*.

## References

- Adams, A., Gottschling, D. E. Kaiser, C. A., and Stearns, T., "Methods in yeast genetics", a Cold Spring Harbor laboratory course manual, 1997 edn., Cold Spring Harbor Laboratory Press, New York (1998)
- Ali, B. R. S., Zhou, L., Graves, F. M., Freedman, R. B., Black, G. W., Gilbert, H. J. and Hazlewood, G. P., Cellulases and hemicellulases of the anaerobic fungus *Piromyces constitute* a multiprotein cellulose-binding complex and are encoded by multigene families., *FEMS Microbiol. Lett.*, **125**, 15-22 (1995)
- Asada, Y., Watanabe, A., Irie, T., Nakayama, T. and Kuwahara, M., Structures of genomic and complementary DNAs cloning for *Pleurotus ostreatus* manganese (II) peroxidase., *Biochem. Biophys. Acta*, **1251**, 205-209 (1995)
- Ausubel, F. M., Brent, R., Kingston R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K, "Current protocols in molecular biology", John Wiley and Sons, Inc. (1987)
- Bennett, J. W. and Lasure, L. L., "More gene manipulations in fungi", Academic press, Inc., California (1991)
- Berrocal, M., Ball, A. S., Huerta, S., Barrasa, J. M., Hernández, M., Pérez-Leblic, M. I., and Arias, M. E., Biological upgrading of wheat straw through solid-state fermentation with *Streptomyces cyaneus*., *Appl. Microbiol. Biotechnol.*, **54**, 764-771 (2000).
- Biely, P., Microbial xylanolytic system., *Trends Biotechnol.*, **11**, 286-290 (1985)
- Binninger, D. M., Skrzynia, C., Pikkila, P. J. and Casselton, L. A., DNA-mediated transformation of the basidiomycete *Coprinus cinereus*., *EMBO J.*, **6**, 835-840 (1987)
- Binninger, D. M., Chevanton, L. L., Skrzynia, C., Shubkin, C. D.

- and Pukkila, P. J., Targeted transformation in *Coprinus cinereus*., *Mol. Gen. Genet.*, **227**, 245-251 (1991)
- Boel, E., Hjort, I., Svensson, B., Norris, F. , Norris, K. E. and Fiil, N. P., Glucoamylases G1 and G2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs., *EMBO J.*, **3**, 1097-1102 (1984)
- Brown, A., Sims, P. F., Raeder, U. and Broda, P., Multiple ligninase-related genes from *Phanerochaete chrysosporium*., *Gene*, **73**, 77-85 (1988)
- Chaudhary, P. and Deobagkar, D. N., Purification and characterization of xylanases from *Cellulomonas* sp. N.C.I.M. 2353., *Biotechnol. Appl. Biochem.*, **25**, 127-133 (1997)
- Coughlan, M. P. and Hazlewood, G. P.,  $\beta$ -1,4-D-xylan-degradation enzyme system: biochemistry, molecular biology and applications., *Biotechnol. Appl. Biochem.*, **17**, 259-289 (1993a)
- Coughlan, M. P. and Hazelwood, G. P., "Hemicellulose and Hemicellulases", Portland press ltd., Cambridge (1993b)
- Ferreira, L. M. A., Durrant, A. J., Hall, J., Hazlewood, G. P. and Gilbert, H. J., Spatial separation of protein domains is not necessary for catalytic activity or substrate binding in a xylanase., *Biochem. J.*, **269**, 261-264 (1990)
- Gutierrez, R., Cederlund, E., Hjelmqvist, L., Peirano, A., Herrera, F., Ghosh, D. , Duax, W. , Jornvall, H. and Eyzaguirre, J., Acetyl xylan esteraseII from *Penicillium purpurogenum* is similar to an esterase from *Trichoderma reesei* but lacks a cellulose binding domain., *FEBS Lett.*, **423**, 35-38 (1998)
- Hanahan, D., Studies on transformation of *Escherichia coli* with plasmid., *J. Mol. Biol.*, **166**, 557-580 (1983)
- Hori, K., Kajiwara, S., Sato, T., Miyazawa, H., Katayose, Y. and Shishido, K., Cloning, sequence analysis and transcriptional

- expression of a *ras* gene of the edible basidiomycete *Lentinus edodes*., *Gene*, **105**, 91-96 (1991)
- Ishii S., Murakami, T. and Shishido, K., Gyrase inhibitors increase the content of knotted DNA species of plasmid pBR322 in *Escherichia coli*., *J. bacriol.*, **173**, 551-555 (1991)
- Ishibashi, O. and Shishido, K., Nucleotide sequence of *ras* gene from the basidiomycete *Coprinus cinereus*., *Gene*, **125**, 233-234 (1993)
- Jiong, H., Tamaki, H., Akiba, S., Yamamoto, K. and Kumagai, H., Cloning of a gene encoding a highly stable endo- $\beta$ -1,4-glucanase from *Aspergillus niger* and its expression in yeast., *J. Biosci. Bioeng.*, **92**, 434-441 (2001)
- Kajiwara, S., Yamaoka, K., Hori, K., Miyazawa, H., Sito, T., Kanno, T. and Shishido, K., Isolation and sequence of developmentally regulated putative novel gene, *priA*, from the basidiomycete *Lentinus edodes*., *Gene*, **114**, 1731-178 (1991)
- Kitamoto, N., Yoshino, S., Ohmiya, K. and Tsukagoshi, N., Purification and characterization of the overexpressed *Aspergillus oryzae* xylanase, XynF1., *Biosci. Biotechnol. biochem.*, **63**, 1791-1794 (1999)
- Laemmli, U. K., Cleavage of structural proteins during the assemble of the head of bacteriophage T4., *Nature*, **227**, 680-685 (1970)
- Lappalainen, A., Purification and characterization of xylanolytic enzymes from *Trichoderma reesei*., *Biotechnol. Appl. Biochem.*, **8**, 437-448 (1986)
- Margolles-Clark, E., Tenkanen, M., Soderlund, H. and Penttila, M., Acetyl xylan esterase from *Trichoderma reesei* contains an active-site serine residue and a cellulose-binding domain., *Eur. J. Biochem.*, **237**, 553-560 (1996)

- Miller, G. L, Use of dinitrosalicylic acid reagent for determination of reducing sugar., *Anal. Chem.*, **31**, 426-428 (1959)
- Milres, P. G. and Chang, S.-T., "Mushroom biology", Concise basics and current developments, World scientific, Farrer Road (1997)
- Naidu, P. S. and Reddy, C. A., Nucleotide sequence of a new lignin peroxidase gene *GLG3* from the white-rot fungus, *Phanerochaete chrysosporium*., *Nucleic Acids Res.*, **18**, 7173 (1990)
- Naidu, P. S., Zhang, Y. Z. and Reddy, C. A., Characterization of a new lignin peroxidase gene (*GLG6*) from *Phanerochaete chrysosporium*., *Biochem. Biophys. Res. Commun.*, **173**, 994-1000 (1990)
- Ogawa, K., Yamazaki, T., Hasebe, T., Kajiwarra, S., Watanabe, A., Asada, Y. and Shishido, K., Molecular breeding of the basidiomycete *Coprinus cinereus* strains with high lignin-decolorization and -degradation activities using novel heterologous protein expression vectors., *Appl. Microbiol. Biotechnol.*, **49**, 285-289 (1998)
- Oliver, R. P. and Schweizer, M., "Molecular fungal biology", Cambridge University press, Cambridge (1999)
- Paice, G. M., Bourbinnaiss, R., Desrochers, M., Jurasek, L. and Yaguchi, M., A xylanase gene from *Bacillus subtilis*: nucleotide sequence and comparison with *B. pumilus* gene., *Arch. Microbiol.*, **144**, 201-206 (1986)
- Ritch Jr., T. G., Nipper, V. J., Akileswaran, L., Smith, A. J., Pribnow, D. G. and Gold, M. H., Lignin peroxidase from the basidiomycete *Phanerochaete chrysosporium* is synthesized as a preproenzyme., *Gene*, **107**, 119-126 (1991)
- Ritch Jr., T. G. and Gold, M. H., Characterization of a highly expressed lignin peroxidase-encoding gene from the basidiomycete *Phanerochaete chrysosporium*., *Gene*, **118**,

73-80 (1992)

- Saarelainen, R., Paloheimo, M., Fagerstrom, R., Suominen, P. L. and Nevalainen, K. M., Cloning, sequencing and enhanced expression of the *Trichoderma reesei* endoxylanaseII (pI 9) gene *xln2*., *Mol. Gen. Genet.*, **241**, 497-503 (1993)
- Sambrook, J., Fritsch, E. F. and Maniatis, T., "Molecular cloning", a laboratory manual, 2 nd edn., Cold Spring Harbor Laboratory Press, New York (1989)
- Sanger, F., Nicklen, S. and Coulson, A. R., DNA sequencing with chain-terminating inhibitors., *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467 (1977)
- Schägger, H. and Von Jagow, G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa., *Anal . Biochem.*, **166**, 368-379 (1987)
- Southern, E. M., Detection of specific sequences among DNA fragments separated by gel electrophoresis., *J. Mol. Biol.*, **98**, 503-517 (1975)
- Stewart, P. and Cullen, D., Organization and differential regulation of a cluster of lignin peroxidase genes of *Phanerochaete chrysosporium*., *J. Bacteriol.*, **181**, 3427-3432 (1999)
- Subramaniyan, S. and Prema, P., Cellulase-free xylanases from *Bacillus* and other microorganisms., *FEMS Microbiol. Lett.*, **183**, 1-7 (2000)
- Torronen, A., Mach, R. L., Messner, R., Gonzalez, R., Kalkkinen, N., Harkki, A. and Kubicek, C. P., The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes., *Biotechnology*, **10**, 1461-1465 (1992)
- Vieira, J. and Messing, J., The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers., *Gene*, **19**, 259-268 (1982)

- Viikari, L., Kantelinen, A., Sundquis, J. and Linko, M., Xylanases is bleaching: from an idea to the industry., *FEMS Microbiol. Rev.*, **13**, 1335-350 (1994)
- Watson, N., A new revision of the sequence of plasmid pBR322., *Gene*, **70**, 399-403 (1988)
- Whittaker, R. H., New concepts of kingdoms of organisms., *Science*, **163**, 150-160 (1969)
- Yanai, K., Yonekura, K., Usami, H., Hirayama, M., Kajiwarra, S., Yamazaki, T., Shishido, K. and Adachi, T., The integrative transformation of *Pleurotus ostreatus* using bialaphos resistance as a dominant selectable maker., *Biosci. Biotechnol. Biochem.*, **60**, 472-475 (1996)
- Yanisch-Perron, C., Vieira, J. and Messing, J., Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors., *Gene*, **33**, 103-119 (1985)
- Zhang, Y. Z., Reddy, C. A. and Rasooly, A., Cloning of several lignin peroxidase (LIP)-encoding genes: sequence analysis of the *LIP6* gene from the white-rot basidiomycete, *Phanerochaete chrysosporium*., *Gene*, **97**, 191-198 (1991)
- Zolan, M. E. and Pikkila, P. J., Inheritance of DNA methylation in *Coprinus cinereus*., *Mol. Cell. Biol.*, **6**, 195-200 (1986)

# List of Papers

## Paper for Dissertation

Kikuchi, M., Ogawa, K., Yamazaki, T., Kajiwarara, S., Sugio, A. Nakamura, S., and Shishido, K., Secretional expression of a *Bacillus subtilis* xylanase gene in the basidiomycete *Coprinus cinereus*. *FEMS Microbiol. Lett.*, **178**, 277-282 (1999).

Kikuchi, M., Ogawa, K., Yamazaki, T., Kajiwarara, S., and Shishido, K., Use of molecular-genetically bred *Coprinus cinereus* strains for an efficient isolation of cellulose from rice straw. *Biosci. Biotechnol. Biochem.*, **66**, 199-201 (2002)

## Other Papers

Shishido, K., Ogawa, K., Kikuchi, M., Yamazaki, T., Hasebe, T., Kajiwarara, S., Watanabe, A., Asada, Y., Sugio, Y., and Nakamura, S., Construction of novel heterologous protein expression vectors and their use in molecular breeding of *Coprinus cinereus* strains with high lignin- and xylan-degrading activities. In "Proceedings of the Fourth Meeting on the Genetics and Cellular Biology of the Basidiomycetes", eds. Van Griensven L. J. L. D., and Visser, J., Mushroom Experimental Station, Horst, The Netherlands, pp.76-82 (1998)

Shishido, K., Ogawa, K., Matsuda, S., Kikuchi, M., Yamazaki, T., Kajiwarara, S., Tsukamoto, A., and Sugiura, J., Heterologous protein expression vectors and molecular breeding of basidiomycetous fungal strains with high lignin- and xylan-degradation activities. In "Abstracts of 20th Fungal Genetics Conference", California, USA, p.18, p.63 (1999)

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