APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1996, p. 1036–1044 Vol. 62, No. 3
0099-2240/96/$04.00+0

Expression of a *Trichoderma reesei* β-Xylanase Gene (*XYN2*) in *Saccharomyces cerevisiae*

DANIËL C. LA GRANGE, ISAK S. PRETORIUS, AND WILLEM H. VAN ZYL*

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

Received 14 April 1995/Accepted 21 December 1995

The *XYN2* gene encoding the main *Trichoderma reesei* QM 6a endo-β-1,4-xylanase was amplified by PCR from first-strand cDNA synthesized on mRNA isolated from the fungus. The nucleotide sequence of the cDNA fragment was verified to contain a 699-bp open reading frame that encodes a 223-amino-acid propeptide. The *XYN2* gene, located on *URA3*-based multicopy shuttle vectors, was successfully expressed in the yeast *Saccharomyces cerevisiae* under the control of the alcohol dehydrogenase II (*ADH2*) and phosphoglycerate kinase (*PGK1*) gene promoters and terminators, respectively. The 33-amino-acid leader peptide of the Xyn2 β-xylanase was recognized and cleaved at the Kex2-like Lys-Arg residues, enabling the efficient secretion and glycosylation of the heterologous β-xylanase. The molecular mass of the recombinant β-xylanase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 27 kDa. The construction of *fur1 ura3 S. cerevisiae* strains allowed for the autoselection of the *URA3*-based *XYN2* shuttle vectors in nonselective complex medium. These autoselective *S. cerevisiae* strains produced 1,200 and 160 nkat of β-xylanase activity per ml under the control of the *ADH2* and *PGK1* promoters in rich medium, respectively. The recombinant enzyme showed highest activity at pH 6 and 60°C and retained more than 90% of its activity after 60 min at 50°C.

Xylan is a major component of the cell walls of monocots and hardwoods, representing up to 35% of the dry weight of these plants (31). This polymer is second only to cellulose in natural abundance and represents a major reserve of fixed carbon in the environment. Unlike cellulose, xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains. Hydrolysis of the xylan backbone is catalyzed by endo-β-1,4-xylanases (EC 3.2.1.8) and β-D-xylosidases (EC 3.2.1.37) (5). Endo-β-xylanases act on xylans and xylo-oligosaccharides, producing mainly mixtures of xylo-oligosaccharides. β-D-Xylosidases hydrolyze xylo-oligosaccharides to D-xylose (5). Many bacterial and fungal species are able to utilize xylans as a carbon source. Interest in the enzymology of xylan hydrolysis has recently increased because of the application of β-xylanases in biobleaching (27, 42) and in the food (23) and animal feed (45) industries. Several microbial sources have been investigated for β-xylanase production, and strains of the fungus *Trichoderma* have been shown to secrete large amounts of efficient xylan-degrading enzymes (48).

The yeast *Saccharomyces cerevisiae* can neither utilize nor degrade xylan (18), but it possesses a number of attributes that render it an attractive host for the expression and production of β-xylanases (32). It is a unicellular fungus which provides for posttranslational processing such as endoproteolytic cleavage and glycosylation. *S. cerevisiae* normally secretes few proteins in low abundance, so that a secreted heterologous protein is produced separate from the majority of yeast proteins. Furthermore, it has complete GRAS (generally regarded as safe) status, which allows for its use in the food industry.

Trichoderma reesei is a filamentous mesophilic fungus that is well known for its cellulolytic and xylanolytic enzymatic activities (29). The two major inducible endo-xylanases secreted by this fungus are Xyn1 and Xyn2 (44). They are both relatively small protein molecules with molecular masses of 19 and 21 kDa, respectively. The isoelectric points of Xyn1 and Xyn2 are 5.2 and 9.0, respectively. The pH optima of Xyn1 (pH 3.5 to 4.0) and Xyn2 (pH 4.5 to 5.5) differ, but the proteins produce similar hydrolysis end products (44). Xyn2 represents more than 50% of the total xylanolytic activity of *T. reesei* cultivated on xylan and, together with Xyn1, accounts for more than 90% of the xylan-degrading ability of this fungus (43).

In this paper, we describe the molecular cloning of the *T. reesei XYN2* gene in *S. cerevisiae*. Expression of the *XYN2* gene in *S. cerevisiae* was obtained with the aid of multicopy plasmids, using two different *S. cerevisiae* promoter-terminator expression cassettes derived from the inducible alcohol dehydrogenase II (*ADH2*) gene (35) and the constitutive 3-phosphoglycerate kinase (*PGK1*) gene (15). The enhanced production of recombinant β-xylanase in nonselective complex medium, without the risk of losing the episomal vector, was obtained by disrupting the uracil phosphoribosyltransferase (*FUR1*) gene (19) in the β-xylanase-producing *S. cerevisiae* strains. This step ensured autoselection of the *URA3*-bearing expression plasmids in rich growth medium.

MATERIALS AND METHODS

Strains and media. The genotypes of the microbial strains and plasmids used in the present study are summarized in Table 1. *S. cerevisiae* Y294 was cultivated on either rich YPD medium (1% yeast extract, 2% peptone, 2% glucose) or selective, buffered synthetic complete (SC) medium (2% glucose, 0.67% yeast nitrogen base [Difco] containing amino acid supplements, 20 mM succinate [pH 6.0]), respectively (33). *T. reesei* QM 6a was cultivated in basal medium [0.3% oat spelts xylan (Sigma), 0.4% KH_2PO_4, 1% $(NH_4)_2HPO_4$, 1% peptone, 0.3% yeast extract] (25). Both these organisms were cultured in 1-liter Erlenmeyer flasks containing 100 to 200 ml of medium at 30°C on a rotary shaker at 150 rpm.

Recombinant plasmids were constructed and amplified in *Escherichia coli* JM101 cultivated at 37°C in Luria-Bertani liquid medium or on Luria-Bertani agar (38). Ampicillin for selecting and propagating resistant bacteria was added to a final concentration of 100 μg/ml.

DNA manipulations and plasmid constructions. Standard protocols were followed for DNA manipulations (38). The enzymes for DNA cleavage and ligation and phosphorylated synthetic linkers were purchased from Boehringer Mann-

* Corresponding author. Mailing address: Department of Microbiology, University of Stellenbosch, Victoria St., Stellenbosch 7600, South Africa. Phone: 27-21-8083160. Fax: 27-21-8083611. Electronic mail address: whvz@maties.sun.ac.za.

TABLE 1. Microbial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
S. cerevisiae Y294	α *leu2-3,112 ura3-52 his3 trp1-289*	This laboratory
T. reesei QM 6a	Type culture	ATCC 13631
E. coli JM101	r_K' m_K' *supE thiD*(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*Z* ΔM15)	38
Plasmids		
YEpC-PADH2a	*bla LEU2 ADH2*$_{PT}$	30
pUC8	*bla tet*	46
pUC19	*bla tet*	49
pBL1	*bla ADH2*$_P$	This laboratory
pBL2	*bla ADH2*$_T$	This laboratory
YEp352	*bla URA3*	14
pDLG1	*bla URA3 ADH2*$_{PT}$	This work
pJC1	*bla URA3 PGK1*$_{PT}$	8
pDLG5	*bla URA3 ADH2*$_P$*-XYN2-ADH2*$_T$	This work
pDLG6	*bla URA3 PGK1*$_P$*-XYN2-PGK1*$_T$	This work
pPE	*bla TRP1 FUR1*	19
pDF1	*bla fur1*::*LEU2*	This work
YEp13	*bla LEU2*	7

heim and used as recommended by the supplier. Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Benson (4).

The construction of plasmid pDLG1 is summarized in Fig. 1A. A 1.4-kb *Eco*RI-*Sal*I fragment containing the *S. cerevisiae ADH2* promoter region was recovered from plasmid YEpC-PADH2a (30) and ligated to plasmid pUC8, which had been linearized with *Eco*RI and *Sal*I. The unique *Eco*RV site upstream of the *ADH2* promoter region and the *Hin*cII site (also the *Sal*I site) in the pUC8 moiety were subsequently changed to a *Bam*HI and *Bgl*II site, respectively, through the addition of synthetic linkers to create plasmid pBL1. The *ADH2* terminator region was recovered from YEpC-PADH2a as a 0.83-kb *Sma*I-*Bcl*I fragment and cloned into the *Sma*I and *Bam*HI sites of plasmid pUC8. The *Sma*I site was cleaved and changed to a *Bgl*II site through the addition of a synthetic *Bgl*II linker to generate plasmid pBL2. The *ADH2* promoter region was subsequently isolated as a 1.4-kb *Eco*RI-*Bgl*II fragment from plasmid pBL1 and subcloned into the *Eco*RI and *Bgl*II sites of plasmid pBL2. The following synthetic oligodeoxyribonucleotides were annealed to generate a polylinker with the unique restriction sites *Eco*RI, *Bgl*II, and *Xho*I: XBR-1 (5′-CCTCGAGGAGATCTGGAATTCC-3′) and XBR-2 (5′-GGAATTCCAGATCTCCTCGAGG-3′). The *Bgl*II site between the *ADH2* promoter and terminator regions was cleaved, and the protruding ends were filled in with DNA polymerase I (Klenow fragment). The *Eco*RI-*Bgl*II-*Xho*I polylinker was introduced in place of the *Bgl*II site. The 2.1-kb *Bam*HI-*Hin*dIII promoter-terminator fragment was isolated from the plasmid and cloned into the *Bam*HI and *Hin*dIII sites of the yeast-*E. coli* shuttle vector YEp352 (14). The unique *Eco*RI site in YEp352 was cleaved, the protruding ends were filled in with Klenow fragment, and the blunt ends were religated prior to the introduction of the *ADH2* fragment to ensure that the *Eco*RI within the polylinker remains unique. The final *ADH2* yeast expression vector was named pDLG1. Plasmids pDLG1 and pJC1 (8) were used as expression vectors for *XYN2*.

Plasmid pDF1 was constructed as follows. The 2.2-kb *Pst*I-*Eco*RI fragment containing the *S. cerevisiae FUR1* gene was isolated from plasmid pPE (19) and ligated to pUC19, predigested with *Pst*I and *Eco*RI. The *FUR1* gene was cleaved at the unique *Acc*I and *Cla*I sites, the overhanging ends were filled in with DNA polymerase I (Klenow fragment), and a *Sal*I synthetic linker was added to generate a 240-bp *Acc*I-*Cla*I deletion within the *FUR1* open reading frame. The *LEU2* gene was isolated from plasmid YEp13 (7) as a 2.2-kb *Sal*I-*Xho*I fragment and cloned into the *Sal*I site created within the *FUR1* open reading frame to construct a *fur1*::*LEU2* disruptive allele. The resulting integrating plasmid was designated pDF1.

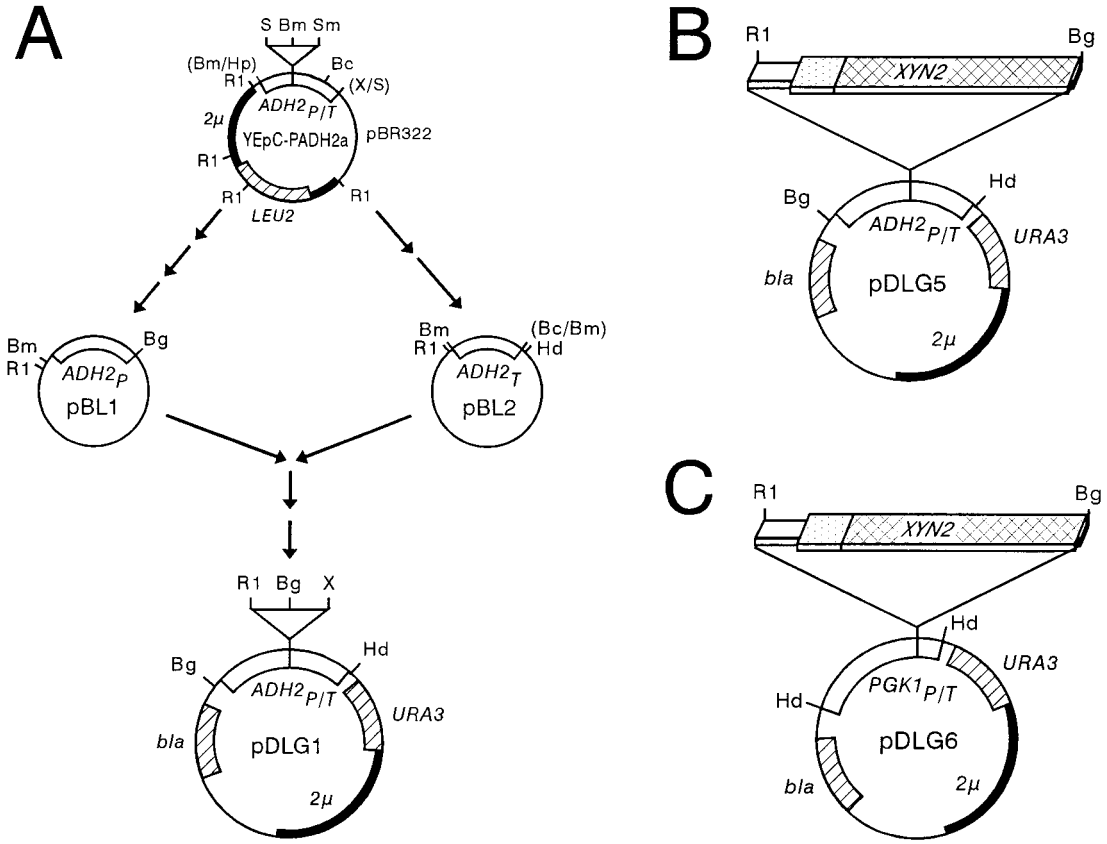

FIG. 1. Schematic summary of the construction of plasmid pDLG1 (A) and plasmid maps of the *XYN2*-containing plasmids pDLG5 (B) and pDLG6 (C). The *XYN2* gene is indicated by cross-hatched boxes, the selectable markers (*LEU2*, *URA3*, and *bla*) are indicated by hatched boxes, the *ADH2* and *PGK1* promoter and terminator sequences are indicated by open boxes, and the 2μm yeast origin of replication and pBR322/pUC8 sequences are indicated by thick and thin lines, respectively. The restriction sites indicated are *Bam*HI (Bm), *Bcl*I (Bc), *Bgl*II (Bg), *Eco*RI (R1), *Hin*dIII (Hd), *Hpa*I (Hp), *Sal*I (S), *Sma*I (Sm), and *Xho*I (X).

RNA isolation and first-strand cDNA preparation. One liter of *T. reesei* QM 6a culture was prepared in oat spelts basal medium for 48 h at 30°C. The fungal mycelia were harvested through cheesecloth and frozen under liquid nitrogen. The frozen mycelia were ground into a fine powder with a mortar and pestle and suspended in a mixture of 15 ml of phenol and 10 ml of ice-cold STE buffer (100 mM NaCl, 250 mM Tris-HCl [pH 7.2], 10 mM EDTA), and total cellular RNA was isolated as described previously (22). The poly(A)-containing RNA fraction was purified with the aid of an Oligotex-dT mRNA midi kit (Stratagene). First-strand cDNA synthesis was carried out with 100 ng of poly(A) RNA by using a first-strand cDNA synthesis kit (Boehringer Mannheim) as specified by the supplier.

PCR amplification. The *T. reesei XYN2* gene was isolated from a first-strand cDNA mix by PCR with the two oligonucleotides DLG1L (5′-GCATGAATTC GCCAAACCTGAACAACCC-3′) and DLG1R (5′-GCATAGATCTCCCTTT AGCTGACGGTGA-3′). These primers were based on the sequence of the *T. reesei XYN2* gene, as published by Törrönen et al. (44). DNA was amplified in 50-μl reaction mixtures (0.25 μM each primer, reaction buffer IV, 1 mM $MgCl_2$, 500 μM each deoxynucleoside triphosphate, 2 μl of template DNA [± 20 ng of first-strand cDNA-mRNA hybrid], 2.5 U of *Taq* polymerase [Advanced Biotechnology]) under mineral oil with a Biometra Trio Thermoblock TB1 (Biometra Biomedizinische Analytik, Göttingen, Germany) (17, 37). Denaturation, annealing, and polymerization were carried out for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, respectively, for 29 cycles. The amplified DNA fragment was digested with *Bgl*II and *Eco*RI and ligated to pDLG1 predigested with the same restriction enzymes. The resulting plasmid, pDLG5 (Fig. 1B), was transformed into *S. cerevisiae* Y294 by the dimethyl sulfoxide-lithium acetate method described by Hill et al. (13).

Screening for β-xylanase activity. Transformants were screened for xylan-degrading ability after being plated on SC^{-Ura} medium or SC medium (after disruption of the *FUR1* gene) containing 0.2% of 4-*O*-methyl-D-glucurono-D-xylan–remazol brilliant blue R (RBB)–xylan (Sigma) and 2% galactose as the carbon source (6, 10). β-Xylanase cleaves RBB-xylan into a colorless product.

Subcloning and sequencing of *XYN2*. The β-xylanase gene from pDLG5 was cloned into pUC8, and four deletion subclones were constructed with appropriate restriction enzymes. The nucleotide sequence of the *XYN2* gene was determined by sequencing both strands of the cloned cDNA inserts by the radioactive, dideoxy chain termination method (39) with T7 DNA polymerase (Pharmacia) as specified by the manufacturer. The sequence data obtained were analyzed with the GENEPRO software package (Hoefer Scientific Instruments, San Francisco, Calif.).

Southern and Northern hybridizations. Total DNA was isolated from *S. cerevisiae* strains (16), digested with *Nsi*I and *Nco*I, separated on a 1% agarose gel, and blotted to a Hybond-N membrane (Amersham International). Southern hybridizations were carried out by the method described by Southern (41), modified as described by Sambrook et al. (38). Northern (RNA) hybridizations were carried out as described by Sambrook et al. (38). For both Southern and Northern hybridizations, single-stranded DNA fragments were labelled with a random-primed DNA-labelling kit (Boehringer Mannheim) involving [α-^{32}P] ATP as specified by the manufacturer.

Protein preparation and gel electrophoresis. Protein preparations were performed on ice. The supernatant of a 200-ml culture of the recombinant *S. cerevisiae* strain was separated from the cellular mass by centrifugation for 5 min at 4,000 × *g*. The supernatant was filtered and concentrated in a Diaflo Ultrafilter PM10 concentrator (Amicon Division of W. R. Grace and Co., Danvers, Mass.) and subsequently precipitated with 2 volumes of ice-cold acetone for 1 h. The intracellular protein fraction was extracted after the cells were washed twice in 5 ml of extraction buffer [200 mM Tris (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol] and was ultimately suspended in 0.4 ml of the same buffer. Cells were ruptured with ca. 0.2 to 0.3 ml of ice-cold acid-washed glass beads (0.45 μm diameter) in a 2-ml microcentrifuge tube by vortexing six times for 30 s with 60-s intervals on ice. Glass beads and cell debris were removed by centrifugation at 13,500 × *g* for 60 min. The supernatant was subsequently precipitated by the same procedure as with the extracellular fraction. Both protein fractions were resuspended in 0.1 ml of HEPES buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 8.0], 5 mM EDTA [pH 8.0], 7 mM β-mercaptoethanol) after centrifugation at 13,500 × *g* for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% (wt/vol) polyacrylamide was performed by the method of Laemmli (21). Intracellular and extracellular protein fractions were boiled for 3 min and applied to the gel. Proteins were visualized by Coomassie brilliant blue staining (38).

Protein deglycosylation. The supernatant of a 200-ml culture of the recombinant *S. cerevisiae* was separated from the cellular mass by centrifugation for 5 min at 4,000 × *g* and filtration through a 0.22-μm-pore-size membrane. The recombinant protein of 27 kDa (Xyn2 protein) was separated from the other proteins secreted by *S. cerevisiae* (larger than 33 kDa) by using a Diaflo Ultrafilter PM30 concentrator (Amicon Division of W. R. Grance and Co.), and subsequently precipitated from the filtrate with 2 volumes of acetone for 1 h. The protein fraction was collected by centrifugation at 13,500 × *g* for 60 min at 4°C and resuspended in 0.25 ml of endoglycosidase F incubation buffer (50 mM sodium acetate buffer [pH 5], 20 mM EDTA, 1% 2-mercaptoethanol). The deglycosylation reaction was initiated by adding 0.2 U of endoglycosidase F (Sigma) to 40 μl of denatured protein (boiled for 5 min at 100°C). The reaction mixture was incubated at 37°C for 12 h, after which the sample was denatured again and 0.2 U of fresh endoglycosidase F was added. After a further 16 h of incubation, the reaction mixture was denatured again and SDS-PAGE on 15% (wt/vol) polyacrylamide was performed (21). Untreated Xyn2 protein was treated in the same manner, but no endoglycosidase F was added.

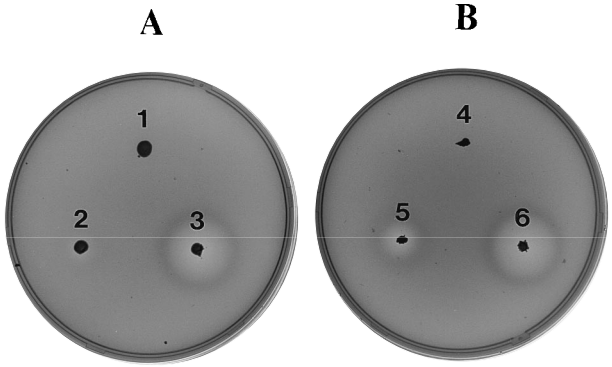

FIG. 2. Recombinant β-xylanase-producing *S. cerevisiae* strains. The colonies at the top of plates A and B are *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG1) (colonies 1 and 4); those at the bottom left are *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) (colonies 2 and 5); and those at the bottom right are *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) (colonies 3 and 6). Both plates contain SC medium and 0.2% RBB-xylan supplemented with 2% glucose (A) or 2% galactose (B) as the sole carbon source. Colonies degrading RBB-xylan are surrounded by pale clearing zones. The plates were photographed after 30 h of incubation at 30°C.

Enzyme activity assays. Endo-β-1,4-xylanase activity was assayed by the method described by Bailey et al. (1) with 1% birchwood glucuronoxylan (Sigma) as the substrate at 60°C. Appropriate dilutions of the cell-free culture solution in 50 mM sodium citrate buffer (pH 6.0) were used as the enzyme source. The amount of released sugar was determined by the dinitrosalicylic acid method described by Miller et al. (26). Thermostability was tested by heating enzyme samples for different times at various temperatures, and the activity was assayed at 60°C for 5 min as described above. Assays at different pH values were performed as described above, except that the buffers used were 50 mM citrate (pH 3.0), 50 mM citrate phosphate (pH 4.0 to 7.0), and 50 mM phosphate (pH 8.0), respectively (11).

RESULTS

Cloning of the *T. reesei XYN2* gene. The *T. reesei XYN2* gene was isolated from first-strand cDNA prepared from *T. reesei* by using sequence-specific PCR primers. The PCR product was cloned into pDLG1 (creating pDLG5 [Fig. 1B]) under the control of the inducible *ADH2* promoter and transformed into *S. cerevisiae* Y294. β-Xylanase-producing yeast colonies were identified by plating on selective SC medium containing RBB-xylan. The β-xylanase gene was isolated from pDLG5 and cloned into pJC1 (creating pDLG6 [Fig. 1C]) under the control of the constitutive *PGK1* promoter. This plasmid was also introduced into *S. cerevisiae* Y294. The *FUR1* gene of the above-mentioned yeast strains was disrupted to create strains *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) and *S. cerevisiae* Y294 (*fur1*::*LEU2* pDLG6), respectively. Both these recombinant yeast strains, together with *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG1) as a control, were plated on SC medium containing 2% glucose or 2% galactose as the carbon source, respectively (Fig. 2A and B). *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) showed smaller clearing zones, because the *ADH2* promoter is strongly repressed by 2% glucose but less so by 2% galactose.

Nucleotide sequence of the *XYN2* gene. The nucleotide and deduced amino acid sequences of *T. reesei XYN2* are presented in Fig. 3. A 780-bp DNA fragment encoding a 223-amino-acid propeptide has been cloned and expressed in *S. cerevisiae*. The 108-bp intron present in the *T. reesei* genomic *XYN2* gene was eliminated through the first-strand cDNA step. The hydropho-

```
                                                                    -104  aattcgccaaacc  -91

-90  tgaacaaccccagcacctgaacagtcatacaacccctccaagcccaaaagacacaacaactcctactagccgaagcaagaagacatcaac   -1

1    ATGGTCTCCTTCACCTCCCTCCTCGCCGGCGTCGCCGCCATCTCGGGCGTCTTGGCCGCTCCCGCCGCCGAGGTCGAACCCGTGGCTGTG    90
1    M  V  S  F  T  S  L  L  A  G  V  A  A  I  S  G  V  L  A  A  P  A  A  E  V  E  P  V  A  V     30

91   GAGAAGCGCCAGACGATTCAGCCCGGCACGGGCTACAACAACGGCTACTTCCACTCGTACTGGAACGATGGCCACGGCGGCGTGACGTAC   180
31   E  K  R  Q  T  I  Q  P  G  T  G  Y  N  N  G  Y  F  H  S  Y  W  N  D  G  H  G  G  V  T  Y     60

181  ACCAATGGTCCCGGCGGGCAGTTCTCCGTCAACTGGTCCAACTCGGGCAACTTTGTCGGCGGCAAGGGATGGCAGCCCGGCACCAAGAAC   270
61   T  N  G  P  G  G  Q  F  S  V  N  W  S  N  S  G  N  F  V  G  G  K  G  W  Q  P  G  T  K  N     90
                                    ↑

271  AAGGTCATCAACTTCTCGGGCAGCTACAACCCCAACGGCAACAGCTACCTCTCCGTGTACGGCTGGTCCCGCAACCCCCTGATCGAGTAC   360
91   K  V  I  N  F  S  G  S  Y  N  P  N  G  N  S  Y  L  S  V  Y  G  W  S  R  N  P  L  I  E  Y    120
             ↑

361  TACATCGTCGGGAACTTTGGCACCTACAACCCGTCCACGGGCGCCACCAAGCTGGGCGAGGTCACCTCCGACGGCAGCGTCTACGACATT   450
121  Y  I  V  G  N  F  G  T  Y  N  P  S  T  G  A  T  K  L  G  E  V  T  S  D  G  S  V  Y  D  I    150
                                 ↑

451  TACCGCACGCAGCGCGTCAACCAGCCGTCCATCATCGGCACCGCCACCTTTTACCAGTACTGGTCCGTCCGCCGCAACCACCGCTCGAGC   540
151  Y  R  T  Q  R  V  N  Q  P  S  I  I  G  T  A  T  F  Y  Q  Y  W  S  V  R  R  N  H  R  S  S    180

541  GGCTCCGTCAACACGGCGAACCACTTCAACGCGTGGGCTCAGCAAGGCCTGACGCTCGGGACGATGGATTACCAGATTGTTGCCGTGGAG   630
181  G  S  V  N  T  A  N  H  F  N  A  W  A  Q  Q  G  L  T  L  G  T  M  D  Y  Q  I  V  A  V  E    210

631  GGTTACTTTAGCTCTGGCTCTGCTTCCATCACCGTCAGCTAAagggagatct  682
211  G  Y  F  S  S  G  S  A  S  I  T  V  S  *              223
```

FIG. 3. Nucleotide and deduced amino acid sequences of the *XYN2* gene from *T. reesei* QM 6a. The hydrophobic leader sequence is underlined, the dibasic residues (Lys-Arg) are double underlined, and the PCR primers sequences used are indicated in boldface type. Residues marked with arrows are potential targets for N glycosylation. The nucleotide sequence data are available from GenBank nucleotide sequence databases (accession number U24191).

bic leader of the recombinant propeptide produced by *S. cerevisiae* is presumably proteolytically processed by the KEX2 protease, producing a 190-amino-acid mature protein (Fig. 3).

Identification and confirmation of *fur1* disruptions. The *FUR1* gene of *S. cerevisiae* Y294 strains was disrupted with the *fur1*::*LEU2* allele to generate *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG1), *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5), and *S. cerevisiae* Y294 (*fur1*::*LEU2* pDLG6), respectively. The *fur1*::*LEU2* disruptive allele was isolated as a 3.27-kb *Nco*I-*Nsi*I linear DNA fragment for subsequent transformation of *S. cerevisiae* strains to replace the chromosomal *FUR1* gene through gene replacement (34) with the *fur1*::*LEU2* allele (Fig. 4A). Leu^+ yeast transformants were selected for on SC^{-Leu} medium. To confirm the *FUR1* gene disruption, *Nsi*I-*Nco*I-digested genomic DNA from *S. cerevisiae* Y294, *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5), and *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) were subjected to Southern hybridization analysis with a 1.33-kb *Nsi*I-*Nco*I *FUR1* fragment from pPE as an α-^{32}P-labelled probe. In *S. cerevisiae* Y294, a DNA fragment of 1.33 kb was radioactively highlighted, and in *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) and *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6), DNA fragments of 3.27 kb were highlighted, corresponding to the *FUR1* and *fur1*::*LEU2* alleles, respectively (Fig. 4B). *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG1) without the β-xylanase gene was analyzed in the same way (data not shown).

Northern blot hybridization and SDS-PAGE analysis of cloned proteins. Northern analysis of the mRNA produced by the recombinant *S. cerevisiae* strains showed that high levels of *XYN2* mRNA were produced by *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) when cultivated for 48 h in YPD with 2% galactose as the carbon source (Fig. 5A). Much lower levels of mRNA were present in *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) cultivated for 48 h in YPD with 2% glucose as the carbon source. On the SDS-polyacrylamide gel, a new protein band with an estimated molecular mass of 27 kDa was visible for *S. cerevisiae* Y294 (*fur1*::*LEU2* pDLG5) (the more active clone), as well as for *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6). The high level of *XYN2*

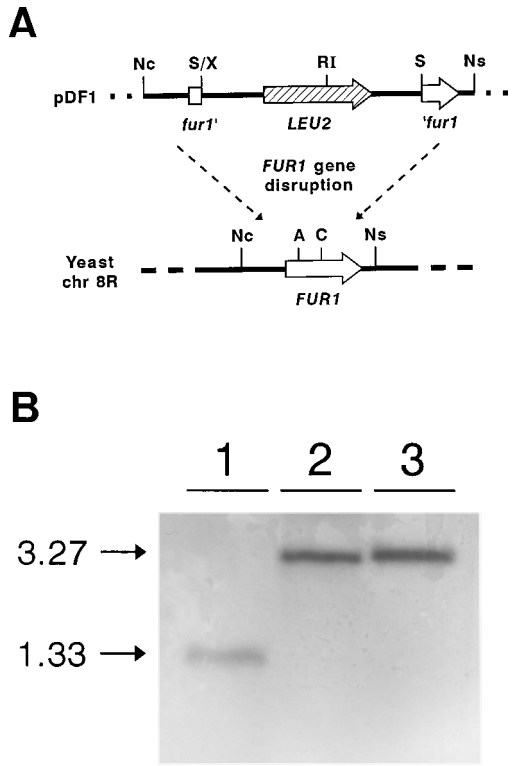

FIG. 4. (A) Schematic representation showing the disruption of the *FUR1* gene on the genome of β-xylanase-producing *S. cerevisiae* strains. The enzymes used were *Acc*I (A), *Cla*I (C), *Eco*RI (RI), *Nco*I (Nc), *Nsi*I (Ns), *Sal*I (S), and *Xho*I (X). (B) Southern blot analysis showing the genomic disruption of *FUR1*. Lanes: 1, *S. cerevisiae* Y294 with the wild-type *FUR1* gene; 2, *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5); 3, *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6). The genomic DNA was hybridized to an α-^{32}P-labelled 1.33-kb *FUR1* DNA fragment.

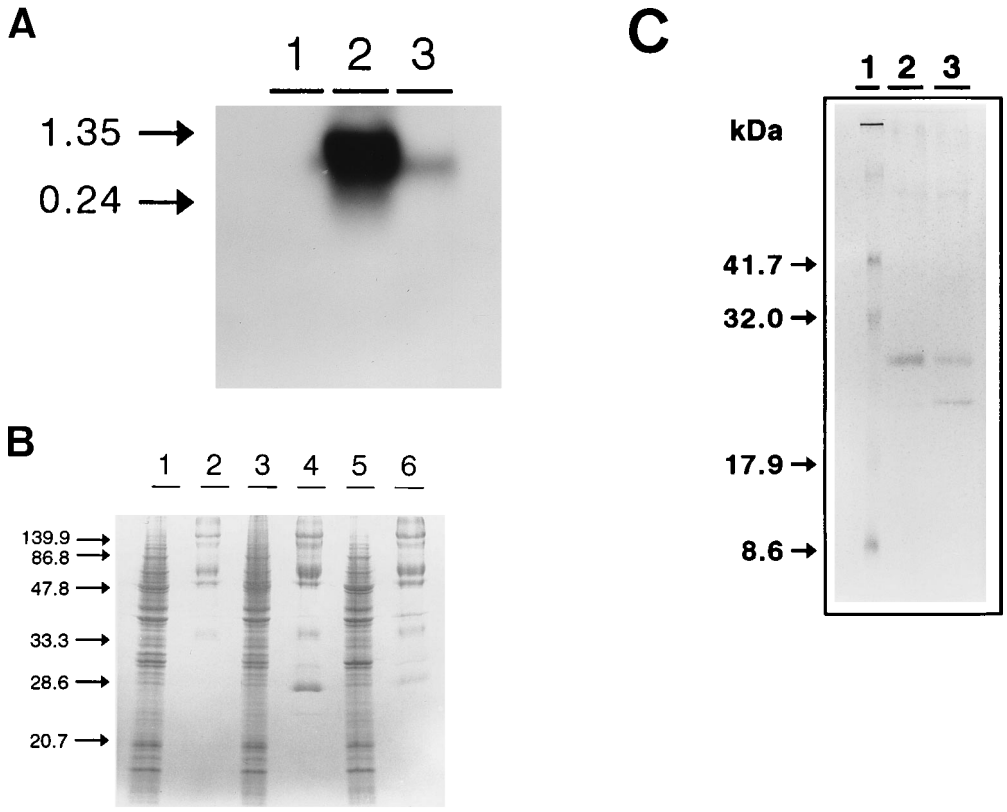

FIG. 5. (A) Northern blot analysis of total RNA isolated from *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG1) (lane 1), *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) (lane 2), and *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) (lane 3). Molecular sizes are indicated in kilobases. The 0.7-kb *XYN2* DNA fragment was used as a α-^{32}P-labelled probe. (B) SDS-PAGE of the Xyn2 protein produced by *S. cerevisiae* Y294 strains. Lanes: 1 and 2, *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG1); 3 and 4, *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5); 5 and 6, *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6). Lanes 1, 3, and 5 contain the intracellular protein fractions, and lanes 2, 4, and 6 contain the extracellular fractions. The band corresponding to the β-xylanase enzyme is at ca. 27 kDa. (C) SDS-PAGE of endoglycosidase F-treated Xyn2 protein produced by *S. cerevisiae* Y294. Lanes: 1, protein molecular markers; 2, untreated extracellular fraction of *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5); 3, endoglycosidase F-treated extracellular fraction of *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5).

mRNA produced by *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) probably leads to higher levels of enzyme compared with those in *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) (Fig. 5B). Treatment of the 27-kDa protein species with endoglycosidase F generated a new protein species of 21 kDa that corresponds to the molecular mass of native Xyn2 xylanase isolated from *T. reesei* (44) (Fig. 5C).

β-Xylanase activity. The β-xylanase-producing yeast strains and *T. reesei* QM 6a were analyzed for their ability to secrete biologically active β-xylanases over 125- and 250-h periods, respectively (Fig. 6). For optimal production of β-xylanase, *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) was cultured on YPD medium with 0.8% glucose (15), *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) was cultured on YPD medium with 0.8% galactose, and *T. reesei* was cultured on basal medium containing 0.3% oat spelts xylan (25). Lower concentrations of glucose and galactose were used to reduce the reducing-sugar background at the start of the growth curve, enabling the assessment of β-xylanase activity in the culture supernatants. The highest β-xylanase activity for *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) and *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) was recorded after ca. 70 and ca. 80 h, respectively (Fig. 6A and B). *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) showed a peak β-xylanase activity of 160 nkat/ml, and *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) showed a activity of 1,200 nkat/ml. β-Xylanase activity was detected earlier in *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6), as a result of the constitutive *PGK1* promoter. It also reached a higher cell density in a shorter period than in *S. cerevisiae* Y294 (*fur1*::*LEU2* pDLG5), probably because glucose is preferred to galactose as a carbon source for *S. cerevisiae* growth. In *T. reesei* QM 6a, β-xylanase activity was detected after about 65 h, with the highest levels measured at ca. 450 nkat/ml after about 180 h (Fig. 6C).

Effects of pH and temperature on β-xylanase activity. The recombinant β-xylanase activity peaked between pH 4 and 6, with the highest activity measured at pH 6 in 50 mM citrate buffer (Fig. 7A). The optimum temperature for this enzyme was at 60°C (Fig. 7B). Although the highest β-xylanase activity was measured at 60°C, the enzyme is not stable at this temperature (Fig. 7C). The β-xylanase activity decreased by more than 50% after a 5-min incubation at 60°C; less than 10% activity could be measured after a 10-min incubation (Fig. 7D). However, the recombinant enzyme is relatively stable at 50°C; more than 90% activity remained after 30 min at this temperature (Fig. 7C).

DISCUSSION

mRNA was isolated from the xylanolytic fungus *T. reesei*, and the *XYN2* gene encoding the main β-xylanase, Xyn2, was amplified with the aid of sequence-specific PCR primers. The DNA sequence was verified and compared with the DNA sequence published by Törrönen et al. (44) and with the DNA sequences available in the GenBank database (release 87.0, 13

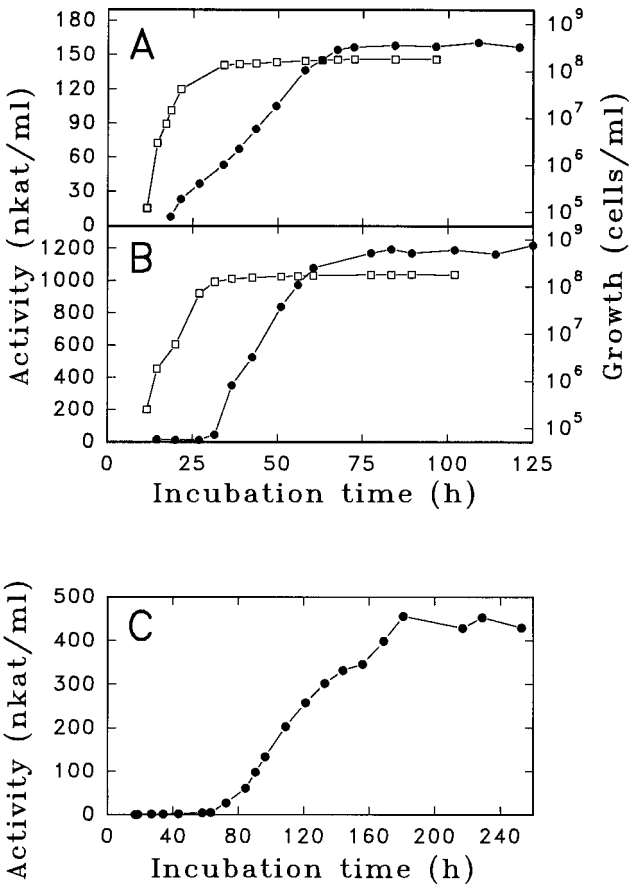

FIG. 6. Time course of extracellular β-xylanase activity produced by *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6) (A), *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) (B), and *T. reesei* (C) in rich medium. The β-xylanase activities were assayed by the method of Bailey et al. (1), as described in the text. Yeast cell counts were determined with the aid of a hemocytometer.

March 1995). The nucleotide sequence of the cDNA fragment containing the *T. reesei* QM 6a *XYN2* gene (Fig. 3) is 99% identical to that reported by Törrönen et al. for the *T. reesei* Rut C-30 *XYN2* DNA sequence (GenBank accession number S51973) (44), differing for only six base pair substitutions and three base pair insertions. The three C-G insertions are at nucleotides 29, 55, and 59 of the *T. reesei* QM 6a *XYN2* DNA sequence, resulting in amino acids 10 to 19 in the proprotein being different from (and more hydrophobic than) those in the proprotein encoded by the *T. reesei* Rut C-30 *XYN2* gene. The cDNA sequence of the *T. reesei* QM 6a *XYN2* gene is identical to the corresponding nucleotide sequence of *T. reesei* VTT-D-79125 (GenBank accession number S67387) reported by Saarelainen et al. (36), except for three base pair substitutions that occurred within the coding region of the *XYN2* gene. These three base pair substitutions also correspond to similar base pair substitutions when compared with the *T. reesei* Rut C-30 *XYN2* nucleotide sequence, suggesting that they are misincorporations by the *Taq* DNA polymerase during the PCR amplification of the *T. reesei* QM 6a *XYN2* cDNA fragment (12). The *XYN2* nucleotide sequences of both *T. reesei* QM 6a and *T. reesei* VTT-D-79125 are probably identical if misincorporation during the PCR amplification is taken into account.

Both *T. reesei* Rut C-30 and *T. reesei* VTT-D-79125 are mutants isolated in mutant selection programs at the U.S. Army Natick Laboratories, Natick, Mass. (24); Rutgers University, Rutgers, N.Y. (40); and VTT-D Technical Research Center, Espoo, Finland (3), that started with *T. reesei* QM 6a as the wild-type strain (Fig. 8). Our sequence data suggest that no mutations were introduced into the *XYN2* gene of *T. reesei* VTT-D-79125 during the three γ-irradiation steps, four *N*-nitro-*N*′-nitro-*N*-nitrosoguanidine steps, and one diethyl sulfate mutagenesis step that were performed prior to its isolation. However, the *XYN2* gene of *T. reesei* Rut-C-30 most probably acquired the three C-G deletion mutations during any of the UV radiation or two *N*-nitro-*N*′-nitro-*N*-nitrosoguanidine mutagenesis steps used in the mutant selection program at Rutgers University, resulting in a less hydrophobic proprotein when compared with *T. reesei* QM 6a and *T. reesei* VTT-D-79125.

The *XYN2* gene was inserted between a yeast promoter and transcription terminator on multicopy episomal plasmids to achieve high levels of gene expression (32). For this purpose, we used the *S. cerevisiae ADH2* and *PGK1* promoter-terminator cassettes. Transcription of *ADH2* is almost undetectable when *S. cerevisiae* is grown on fermentable sugars such as glucose or galactose but is derepressed to a level representing about 1% of total soluble cellular protein when the yeast is grown on nonfermentable carbon sources or fermentable sugars at concentrations lower than 1% (32). The level of *XYN2* expression under the control of the *ADH2* promoter was higher when the yeast transformants were grown on galactose instead of glucose as the carbon source (Fig. 2). The *PGK1* promoter is a strong constitutive promoter which can be induced to a level of expression that constitutes 4 to 10% of the total soluble protein depending on the growth conditions (15). The difference between the two promoters used can clearly be seen in Fig. 2. The *ADH2* promoter is strongly repressed when the cells are plated on glucose, resulting in a large difference in the size of the clearing zones on the plate containing glucose.

Translation in yeasts can be modulated at the level of initiation by four aspects of mRNA structure: (i) the primary sequence or context surrounding the AUG codon; (ii) the position of the AUG codon, i.e., whether it is first; (iii) secondary structure both upstream and downstream of the AUG codon; and (iv) leader length (20). The leader sequence of the *T. reesei XYN2* gene is similar to that of most *S. cerevisiae* genes (20). Therefore, initiation of translation of *XYN2* would be effective in *S. cerevisiae*. This degree of similarity between *S. cerevisiae* and *T. reesei* might be because both these organisms belong to the subdivision *Ascomycotina.*

High-level secretion of heterologous gene products in *S. cerevisiae* is mediated by the hydrophobic N-terminal extension of the polypeptide, the signal/leader sequence. The leader peptide is responsible for translocation of the protein to the endoplasmic reticulum, from where the protein is transported through specialized secretory organelles, modified, processed, and often glycosylated prior to its release by the plasma membrane into the culture medium. The *T. reesei* QM 6a *XYN2* leader peptide has all the characteristics required for processing in *S. cerevisiae*. It is hydrophobic and possesses the dibasic residues Lys-Arg (Fig. 3), which are presumably recognized and cleaved on the carboxyl side by the KEX2 protease of *S. cerevisiae* (32).

However, the Xyn2 β-xylanase secreted by *S. cerevisiae* has a different molecular mass from that of the *T. reesei* enzyme (Table 2). The mature β-xylanase produced by *T. reesei* has a molecular mass of 21 kDa as deduced from the amino acid sequence. The molecular mass determined by SDS-PAGE is almost the same, indicating a virtual absence of carbohydrates (44). The molecular mass of the enzyme secreted by *S. cerevisiae* is 27 kDa as estimated by SDS-PAGE. There is thus a

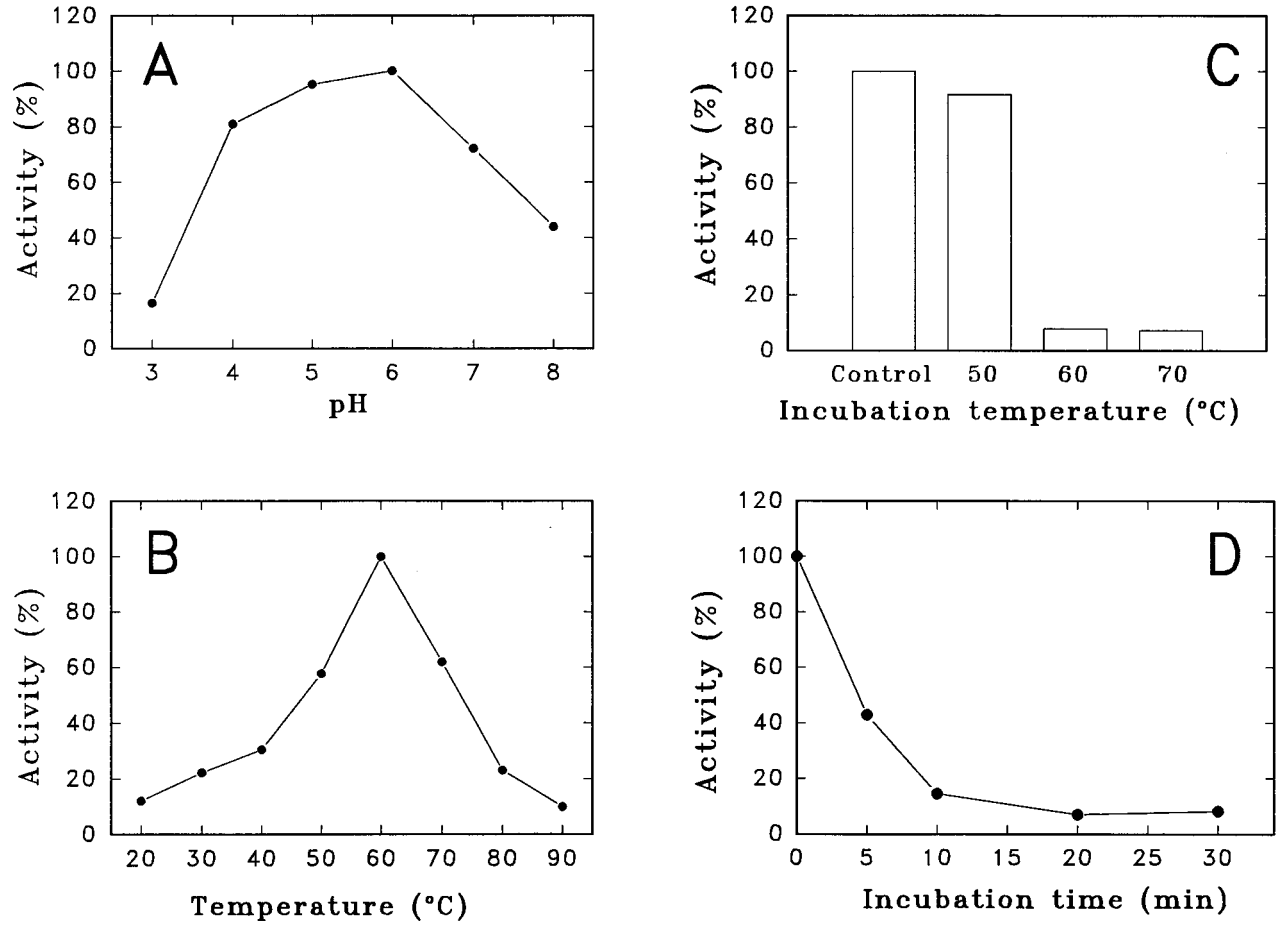

FIG. 7. Effect of pH (A) and temperature (B) on the activity of Xyn2. The highest activity was measured at pH 6 and at 60°C, respectively. The buffers used in the enzyme reactions were 50 mM citrate (pH 3), 50 mM citrate phosphate (pH 4.0 to 7.0), and 50 mM phosphate (pH 8.0). (C) The temperature stability of Xyn2 was determined after preincubating the enzyme in the absence of the substrate for 30 min at 50, 60, and 70°C. (D) The thermostability of Xyn2 at 60°C was determined by preincubating the enzyme at this temperature in the absence of the substrate for 5, 10, 20, and 30 min before determining its activity. The β-xylanase activity prior to the preincubations at different temperatures was taken as 100%.

6-kDa difference in the molecular masses of the β-xylanases secreted by *S. cerevisiae* and *T. reesei*. This is caused by N-glycosylation of the β-xylanase secreted by *S. cerevisiae* (32), because *S. cerevisiae* tends to "hyperglycosylate" heterologous proteins. Treatment of the recombinant Xyn2 protein with endoglycosidase F generated a new protein species with a molecular mass of 21 kDa (Fig. 5C), which corresponds to that of native β-xylanase produced by *T. reesei*. However, this large, glycosylated protein (22% sugar content) was efficiently secreted and passed through the yeast cell wall into the culture medium without any effect on the growth rate. The pH and temperature optima of the recombinant β-xylanase produced by *S. cerevisiae* compare well with those of the native enzyme produced by *T. reesei* (Table 2). Similar hyperglycosylation was observed with the cellobiohydrolases (CBHI and CBHII) of *T. reesei* when expressed in *S. cerevisiae* (28).

The *XYN2* gene is expressed from an episomal plasmid, and the yeast must be kept under selective conditions to ensure vector stability. However, the use of selective synthetic medium is not ideal for the production of high levels of heterologous proteins. We therefore genetically altered the recombinant yeast strains to allow autoselection for the episomal plasmids. The *FUR1* gene of *S. cerevisiae* encodes uracil phosphoribosyltransferase, which catalyzes the conversion of uracil into uridine 5′-phosphate in the pyrimidine salvage pathway (19). If this gene were disrupted, *S. cerevisiae* Y294 would not be able to utilize uracil from the extracellular medium and would therefore not be viable, unless it possessed a complementing functional *URA3* gene to synthesize uridine 5′-phosphate de novo. In this case, the *URA3* gene is the yeast selectable marker on the YEp352-based vectors used for the expression of *XYN2*. After the *FUR1* gene in the β-xylanase-producing *S. cerevisiae* strains was disrupted, these strains could be cultured in YPD medium or any other complete synthetic medium without the risk of losing the episomal plasmid. *S. cerevisiae* could grow to much higher cell densities when cultivated on YPD medium; therefore, β-xylanase activity was considerably higher. In *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG5) and *S. cerevisiae* Y294(*fur1*::*LEU2* pDLG6), β-xylanase activity increased 24- and 3-fold, respectively, when the cells were cultured on YPD medium instead of selective SC medium (data not shown). The time needed for β-xylanase production to start also decreased substantially.

The highest total β-xylanase activity obtained in shake flasks for the hyperproducing mutant *T. reesei* VTT-D-86271 (Rut C-30) was 5,400 nkat/ml (2). The total activity obtained for the most active recombinant *S. cerevisiae* strain (1,487 nkat/ml) compares well with that of *T. reesei* Rut C-30 if one takes into

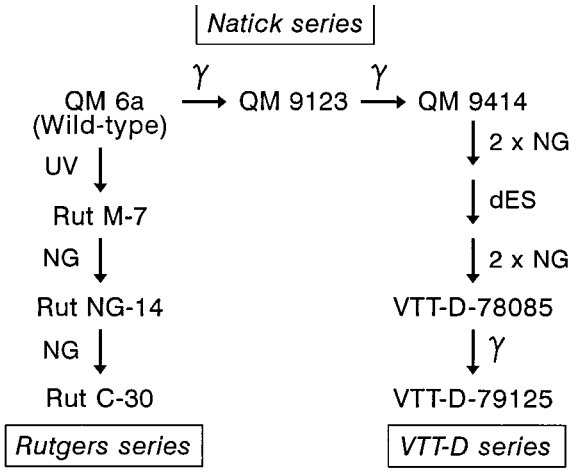

FIG. 8. Lineage of *T. reesei* Rut C-30 and VTT-D-79125 mutants, derived from the wild-type *T. reesei* QM 6a. Mutagenic agents: γ, gamma irradiation; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; dES, diethyl sulfate.

account that the *T. reesei* culture supernatant contains the complete battery of enzymes involved in xylan degradation. These include β-xylosidases that degrade the short xylo-oligosaccharides produced by the β-xylanases and debranching enzymes like α-glucuronidases. The cumulative and synergistic action of all these enzymes would therefore lead to larger amounts of reducing sugars being produced compared with a culture with only β-xylanase activity.

Although β-xylanase production by recombinant organisms, such as *S. cerevisiae*, may not be as high as that in organisms such as the above-mentioned *T. reesei* strain, it might still be worthwhile to consider its use for industrial enzyme production. First, *S. cerevisiae* cannot degrade cellulose, xylan, or any of the other polymers in wood; therefore, β-xylanases produced by this organism are pure and completely free of any contaminating cellulases. This is especially important for use in the paper industry. Currently, commercial β-xylanase preparations marketed for pulp treatment include Pulpzyme HA from *T. reesei* (47). This is a relatively crude β-xylanase preparation with residual cellulolytic activity and requires careful control of process parameters to avoid damage to fibers. Second, *S. cerevisiae* can be cultivated on a variety of relatively inexpensive culture media without the need for xylan to induce β-xylanase production. Furthermore, industrial-scale fermentation technology for *S. cerevisiae* is well established.

Future research in our laboratory will be directed toward the development of a more effective expression system for the production of β-xylanases in *S. cerevisiae*. The addition of a β-xylosidase gene and/or gene(s) encoding debranching enzyme(s) will also be considered.

TABLE 2. Molecular characteristics of Xyn2 produced by *T. reesei* and a recombinant Xyn2 produced by *S. cerevisiae*

Property	Value for Xyn2 from:		Reference(s)[a]
	T. reesei	*S. cerevisiae*	
Mol mass (kDa)	20–21	27.5	42, 44
Optimum pH	5	6	42, 44
Optimum temp (°C)	56–60[b]	60	9
Temp stability (60 min) (°C)	NA[c]	50[d]	
No. of N-glycosylation sites	3	3	44
Glycosylation	No	Yes	44

[a] References for *T. reesei* data only.
[b] Temperature optimum of β-xylanases produced by *T. reesei*, not only Xyn2.
[c] NA, not available.
[d] More than 90% of activity remained.

ACKNOWLEDGMENTS

We acknowledge Elizabeth J. Lodolo for the construction of plasmids pBL1 and pBL2.

This work was supported by a grant from National Chemical Products (NCP) (Pty) Ltd.

REFERENCES

1. **Bailey, M. J., P. Biely, and K. Poutanen.** 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. **23:**257–270.
2. **Bailey, M. J., J. Buchert, and L. Viikari.** 1993. Effect of pH on production of xylanase by *Trichoderma reesei* on xylan- and cellulose-based media. Appl. Microbiol. Biotechnol. **40:**224–229.
3. **Bailey, M. J., and K. M. H. Nevalainen.** 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulose. Enzyme Microb. Technol. **3:**153–157.
4. **Benson, S. A.** 1984. A rapid procedure of isolation of DNA fragments from agarose gels. BioTechniques **2:**66–67.
5. **Biely, P.** 1985. Microbial xylanolytic systems. Trends Biotechnol. **3:**286–290.
6. **Biely, P., D. Mislovicova, and R. Toman.** 1988. Remazol brilliant blue-xylan: a soluble chromogenic substrate for xylanases. Methods Enzymol. **160:**536–541.
7. **Broach, J. R., J. N. Strathern, and J. B. Hicks.** 1979. Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene **8:**121–133.
8. **Crous, J. M., I. S. Pretorius, and W. H. van Zyl.** 1995. Cloning and expression of an *Aspergillus kawachii* endo-1,4-β-xylanase gene in *Saccharomyces cerevisiae*. Curr. Genet. **28:**467–473.
9. **Dekker, R. F. H.** 1983. Bioconversion of hemicellulose: aspects of hemicellulase production by *Trichoderma reesei* QM 9414 and enzymic saccharification of hemicellulose. Biotechnol. Bioeng. **25:**1127–1146.
10. **Farkas, V., M. Lìsková, and P. Biely.** 1985. Novel media for detection of microbial producers of cellulase and xylanase. FEMS Microbiol. Lett. **28:**137–140.
11. **Gomori, G.** 1959. Preparation of buffers for use in enzyme studies. Methods Enzymol. **1:**138–146.
12. **Higuchi, R.** 1990. Recombinant PCR, p. 177–183. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
13. **Hill, J., K. A. Ian, G. Donald, and D. E. Griffiths.** 1991. DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res. **19:**5791.
14. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:**163–167.
15. **Hitzeman, R. A., F. E. Hagie, J. S. Hayflick, C. Y. Chen, P. H. Seeburg, and R. Derynck.** 1982. The primary structure of the *Saccharomyces cerevisiae* gene for 3-phosphoglycerate kinase. Nucleic Acids Res. **10:**7791–7808.
16. **Hoffman, C. S., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:**267–272.
17. **Innis, M. A., and D. H. Gelfand.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
18. **Jeffries, T. W.** 1983. Utilization of xylose by bacteria, yeasts, and fungi. Adv. Biochem. Eng. **27:**1–32.
19. **Kern, L., J. De Montigny, R. Jund, and F. Lacroute.** 1990. The *FUR1* gene of *Saccharomyces cerevisiae*: cloning, structure and expression of wild-type and mutant alleles. Gene **88:**149–157.
20. **Kozak, M.** 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. **266:**19867–19870.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Laing, E., and I. S. Pretorius.** 1992. Synthesis and secretion of an *Erwinia chrysanthemi* pectate lyase in *Saccharomyces cerevisiae* regulated by different combinations of bacterial and yeast promoter and signal sequences. Gene **121:**35–45.
23. **Maat, J., M. Roza, J. Verbakel, H. Stam, M. J. Santos da Silva, M. Bosse, M. R. Egmond, M. L. D. Hagemans, R. F. M. V. Gorcom, J. G. M. Hessing, C. A. M. J. J. van der Hondel, and C. V. Rotterdam.** 1992. Xylanases and their application in bakery, p. 349–360. *In* J. Visser, G. Beldman, M. A. Kusters-van Someren, and A. G. J. Voragen (ed.), Xylans and xylanases. Elsevier Science, Amsterdam.
24. **Mandels, M., J. Weber, and R. Parizek.** 1971. Enhanced cellulase production by a mutant of *Trichoderma viride*. Appl. Microbiol. **21:**152–154.
25. **Matsuo, M., and T. Yasui.** 1984. Purification and some properties of β-xy-

losidase from *Trichoderma viride*. Agric. Biol. Chem. **48:**1845–1852.
26. **Miller, G. L., R. Blum, W. E. Glennon, and A. L. Burton.** 1960. Measurement of carboxymethylcellulase activity. Anal. Biochem. **2:**127–132.
27. **Nevalainen, H., A. Harkki, M. Penttilä, M. Saloheimo, T. T. Teeri, and J. K. C. Knowles.** 1990. *Trichoderma reesei* as a production organism for enzymes for the pulp and paper industry, p. 593–599. *In* T. K. Kirk and H. M. Chang (ed.), Biotechnology in pulp and paper manufacture. Butterworth-Heinemann, Boston.
28. **Penttilä, M., L. André, P. Lehtovaara, M. Bailey, T. T. Teeri, and J. K. C. Knowles.** 1988. Efficient secretion of two fungal cellobiohydrolases by *Saccharomyces cerevisiae*. Gene **63:**103–112.
29. **Penttilä, M., T. T. Teeri, H. Nevalainen, and J. K. C. Knowles.** 1990. The molecular biology of *Trichoderma reesei* and its application to biotechnology, p. 85–102. *In* J. F. Peberdy, C. E. Caten, J. E. Ogden, and J. W. Bennett (ed.), Applied molecular genetics of fungi. Cambridge University Press, Cambridge.
30. **Price, V. L., W. E. Taylor, W. Clevenger, M. Worthington, and E. T. Young.** 1990. Expression of heterologous proteins in *Saccharomyces cerevisiae* using the *ADH2* promoter. Methods Enzymol. **185:**308–318.
31. **Puls, J., and J. Schuseil.** 1993. Chemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis, p. 1–27. *In* M. P. Coughlan and G. P. Hazlewood (ed.), Hemicellulose and hemicellulases. Portland Press, London.
32. **Romanos, M. A., C. A. Scorer, and J. J. Clare.** 1992. Foreign gene expression in yeast: a review. Yeast **8:**423–488.
33. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Rothstein, R. J.** 1983. One step gene disruption in yeast. Methods Enzymol. **101:**202–211.
35. **Russell, D. W., M. Smith, V. M. Williamson, and E. T. Young.** 1983. Nucleotide sequence of the yeast alcohol dehydrogenase II gene. J. Biol. Chem. **258:**2674–2682.
36. **Saarelainen, R., M. Paloheimo, R. Fagerström, P. L. Suominen, and K. M. H. Nevalainen.** 1993. Cloning, sequencing and enhanced expression of the *Trichoderma reesei* endoxylanase II (pI 9) gene *xln2*. Mol. Gen. Genet. **241:**497–503.
37. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
40. **Sheir-Neiss, G., and B. S. Montenecourt.** 1984. Characterization of the secreted cellulases of *Trichoderma reesei* wild type and mutants during controlled fermentations. Appl. Microbiol. Biotechnol. **20:**46–53.
41. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
42. **Tenkanen, M., J. Buchert, J. Puls, K. Poutanen, and L. Viikari.** 1992. Two main xylanases of *Trichoderma reesei* and their use in pulp processing, p. 547–550. *In* J. Visser, G. Beldman, M. A. Kusters-van Someren, and A. G. J. Voragen (ed.), Xylans and xylanases. Elsevier Science, Amsterdam.
43. **Törrönen, A., A. Harkki, and J. Rouvinen.** 1994. Three-dimensional structure of endo-1,4-β-xylanase II from *Trichoderma reesei*: two conformational states in the active site. EMBO J. **13:**2493–2501.
44. **Törrönen, A., R. L. Mach, R. Messner, R. Gonzalez, N. Kalkkinen, A. Harkki, and C. P. Kubicek.** 1992. The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Bio/Technology **10:**1461–1465.
45. **Van Paridon, P. A., J. C. P. Boonman, G. C. M. Selten, C. Geerse, D. Barug, P. H. M. De Bot, and G. Hemke.** 1992. The application of fungal endoxylanase in poultry diets, p. 371–378. *In* J. Visser, G. Beldman, M. A. Kusters-van Someren, and A. G. J. Voragen (ed.), Xylans and xylanases. Elsevier Science, Amsterdam.
46. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
47. **Wong, K. K. Y., and J. N. Saddler.** 1992. *Trichoderma* xylanases, their properties and application, p. 171–186. *In* J. Visser, G. Beldman, M. A. Kusters-van Someren, and A. G. J. Voragen (ed.), Xylans and xylanases. Elsevier Science, Amsterdam.
48. **Wong, K. K. Y., and J. N. Saddler.** 1992. *Trichoderma* xylanases, their properties and application. Crit. Rev. Biotechnol. **12:**413–435.
49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.