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# Expression and secretion of a xylanase from the extreme thermophile, *Thermotoga* strain FjSS3B.1, in *Kluyveromyces lactis*

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**Abstract** The yeast *Kluyveromyces lactis* has been developed as a host for extracellular production of thermophilic hemicellulases by employing expression vectors based on the 2μ-like plasmid pKD1 of *Kluyveromyces drosophilarium*. A β-1,4-xylanase gene (*xynA*) from the extreme thermophile *Thermotoga* sp. strain FjSS3B.1 was fused in-frame with a synthetic secretion signal derived from the *K. lactis* killer toxin and expressed under control of the *K. lactis LAC4* (β-galactosidase) promoter. Correctly processed xylanase enzyme with full biological activity on oat spelts xylan was secreted during shake-flask cultivation of *K. lactis* transformants. The transcriptional activity of the *LAC4* promoter dramatically affected mitotic stability of the expression vector under nonselective conditions. However, one combination of host strain and expression plasmid showed higher stability and good yield and has been employed for scaled-up production of XynA and other thermostable hemicellulases in chemostat culture. XynA secreted by *K. lactis* is as thermostable as the native enzyme, having a half-life of 48 h at 90°C.

**Key words** Heterologous expression · Thermostable xylanase · Protein secretion · Plasmid stability

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## Introduction

Enzymatic prebleaching of kraft pulp with xylanases (endo-1,4-β-xylanase, EC 3.2.1.8) enhances lignin extraction (Viikari et al. 1994) and is an effective means of attaining the target paper brightness with a lower chlorine dosage (Kantilenen et al. 1993; Rattö et al. 1994; Tremblay and Archibald 1993). Most xylanases currently used commercially in the pulp and paper industry have optimal activity at between 30° and 55°C (Viikari et al. 1994), but it is anticipated that the process would be more economical if the enzymes were active and stable at the high temperatures (up to 130°C) that prevail during kraft pulping.

Extremely thermophilic bacteria have been examined as a potential source of thermostable biobleaching enzymes. *Thermotoga* sp. strain FjSS3B.1 was enriched from an intertidal hot spring on Savu-Savu beach in Fiji (Huser et al. 1986) and has been shown to produce a highly thermostable xylanase (Simpson et al. 1991). We isolated a xylanase gene, *xynA*, from a genomic library of this organism by screening for xylanase activity, and over-expressed the gene product in *Escherichia coli* (Saul et al. 1995). At its pH optimum of 6.3 the recombinant enzyme had a half-life of 12 h at 95°C (Saul et al. 1995). The amount of recombinant enzyme produced by *E. coli* was sufficient for preliminary laboratory tests but large quantities are now required in order to assess its effectiveness in enzymatic prebleaching under mill conditions. To simplify enzyme recovery, we wished to express XynA as an extracellular protein and chose the yeast *Kluyveromyces lactis* as an expression host due to its proven capacity for high-level secretion of foreign proteins (van den Berg et al. 1990; Fleer et al. 1991; Bergkamp et al. 1992). In a previous study we constructed an expression cassette comprising the promoter and terminator elements of the *K. lactis* β-galactosidase gene (*LAC4*), the secretion signal of the *K. lactis* killer toxin, and the *xynA* gene from *Dictyoglomus thermophilum* Rt46B.1 (Walsh and Bergquist 1997). Here, we describe the modification of this cassette to create a convenient episomal expression vector into which

foreign sequences can be inserted in a single step. We demonstrate the utility of this vector for high-level secretion of the *Thermotoga* xylanase in *K. lactis*.

## Materials and methods

### Strains and media

The *Escherichia coli* strain used for routine cloning manipulations was JM101 [F' *traD36 lacI*$^q$Δ*(lacZ)M15 proA*$^+$*B*$^+$*/ supE thi* Δ*(lac-proA,B)*]. *E. coli* was grown at 37°C in Luria broth, supplemented with ampicillin (60 μg/ml) or kanamycin (30 μg/ml) when these antibiotics were required for plasmid selection. The *K. lactis* wild-type strain CBS1065 was used as a host for gene expression. Transformation of CBS1065 via selection for resistance to G418 (Geneticin, Life Technologies, Gaithersburg, MD, USA) was performed according to Bianchi et al. (1987). Transformants were grown at 30°C in shake-flasks in YP medium (1% yeast extract, 2% bacto-peptone) containing either 2% glucose (YPD), 2% galactose (YPG), 2% glucose and 2% galactose (YPDG), or 2% glucose, 2% galactose, and G418 (200 μg/ml) for plasmid selection. The plasmid carrier state of transformed strains is indicated in square brackets, [ ].

### Plasmid construction

Plasmid pCWK1 was constructed according to the strategy summarized in Fig. 1. First, a 1.1-kb fragment containing the promoter of the *K. lactis LAC4* gene and the secretion signal from the *K. lactis* killer toxin was amplified by polymerase chain reaction (PCR) from plasmid pLAC-3 using the upstream primer KL1: 5′-AGGAAGCTT-CTTTCACTGAGATCC-3′ which contains a *Hin*dIII site (underlined) and the downstream primer KL2: 5′-GCCACGCGTTCTTTTACCTTGAACGAA-3′ which contains an *Mlu*I site (underlined). The PCR product was inserted into the *Sma*I site adjacent to the *LAC4* terminator region in pLAC-1 to generate pLAC-5. The *LAC4* promoter-terminator region was recovered from pLAC-5 on a 1.8-kb *Hin*dIII/*Sal*I fragment and inserted into *Hin*dIII/*Sal*I-digested pCXJ1 (Bianchi et al. 1987) to create pCW1. A 1.3-kb *Sal*I fragment containing the kanamycin resistance gene (Kan) of Tn903 was introduced into pCW1 at the unique *Sal*I site to create plasmid pCWK1 (Fig. 1).

The *xynA* gene of *Thermotoga* FjSS3B.1 was amplified by PCR from plasmid pNZ2824 (Saul et al. 1995) using the upstream primer TF1: 5′-GGTTCAACGCGTGT-TCCCTTGAGAGTG-3′ and the downstream primer TF2: 5′-CGCTGACGCGTTTATCTTTCCTTCAG-3′, so introducing *Mlu*I sites (underlined) at each end of the gene. Following digestion with *Mlu*I, the PCR product was inserted into *Mlu*I-digested pCWK1 to generate plasmid pCWK-xyn.

### Assays for xylanase activity

Xylanase activity was assayed quantitatively in yeast culture supernatants using oat spelts xylan (Sigma Chemical St. Louis, MO, USA) as the substrate. The enzymatic release of reducing sugar was determined by a modification of the method of Lever (1973). A 10-μl sample of culture supernatant diluted appropriately in 25 mM bis Tris propane, pH 6.5 at 85°C was mixed with 200 μl of xylan solution (0.25% oat spelts xylan in the same buffer) and incubated at 85°C for 15 min. The reaction was terminated by addition of 0.5 ml of colouring reagent (50 mM *para*-hydroxybenzoic acid hydrazide, 300 mM NaOH, 50 mM $Na_2SO_3$, 15 mM $CaCl_2$, 20 mM trisodium citrate). The mixture was boiled for 6 min and $A_{405}$ was measured in a Biotek 96-well plate reader. The concentration of reducing sugar present was determined by using D-xylose as the standard (concentration 0–200 nM). Enzyme activity was expressed in Xylanase Units (XUs) where 1 XU is the amount of enzyme required to release 1 mmol of reducing sugar from xylan per minute (Bailey et al. 1992).

The temperature optimum of *Thermotoga* XynA produced in *K. lactis* was determined by measuring reducing sugar release following incubations with substrate at temperatures ranging from 60° to 100°C. The thermal stability of XynA was determined by measuring residual enzyme activity after samples diluted in 25 mM bis Tris propane, pH 6.5, were incubated at the test temperature for periods of up to 24 h. All enzyme assays were performed in triplicate.

### Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis

Cells were removed from stationary phase yeast cultures by centrifugation and supernatant samples were mixed 4:1 by volume with 5 × loading buffer (250 mM Tris-HCl, pH 6.8, 500 mM dithiothreitol, 2.5% (wt/vol) SDS, 0.5% bromophenol blue, 50% (vol/vol) glycerol) and boiled for 5 min. Samples were subjected to electrophoresis in SDS-12% (wt/vol) polyacrylamide gels according to Laemmli (1970). Protein bands were visualized by staining with Coomassie brilliant blue G-250 and sized by comparison with high-range protein molecular weight standards (200–14.3 kDa, Life Technologies).

Protein bands with xylanase activity were localized in situ in 12% polyacrylamide gels containing 0.1% (wt/vol) oat spelts xylan. Following electrophoresis, SDS was removed by washing the gel in three changes of 25% isopropanol, 25 mM sodium acetate, pH 6.0, each for 30 min at room temperature. Proteins were renatured by soaking the gel overnight at 4°C in 25 mM bis Tris propane, adjusted to pH 6.5 at 85°C. The gel was then immersed in a fresh sample of the same buffer and incubated in a sealed container at 85°C for 30 min. Zones of xylanase activity were then visualized by the Congo Red assay of Teather and Wood (1982). The gel was stained in 0.5% Congo Red for 15 min and destained in 1 M NaCl until zones of clearing were visible.

### Protein microsequencing

N-terminal amino acid sequencing of the *Thermotoga* xylanase expressed in *K. lactis* was performed by C. Knight, School of Biological Sciences, University of Auckland, New Zealand. Supernatant from a culture of CBS1065[pCWK-xyn] was subjected to electrophoresis on a SDS-12% polyacrylamide gel and proteins were electrotransferred to ProBlott membrane (Applied Biosystems, Foster City, CA, USA) and stained with Coomassie Blue according to Matsudaira (1987). The XynA band was excised and sequenced using an Applied Biosystems Procise protein sequencer.

### Plasmid stability determination

The stability of plasmid pCWK-xyn in CBS1065 was determined as follows: individual transformed colonies that grew on selective plates were picked into 10 ml of medium and grown to stationary phase. Portions of the culture were then plated on nonselective YPD agar. After 2–3 days of incubation, colonies were transferred to YPD plates containing G418 (200 μg/ml). The proportion of cells that grew on the selective medium was defined as the plasmid stability. Two hundred colonies were transferred to selective plates for each culture sample tested.

## Results

### Construction of pCWK1, a general purpose secretion vector for *K. lactis*

We previously used a multistep cloning procedure to assemble a secretion cassette in which the *xynA* gene from *Dictyoglomus thermophilum* Rt46B.1 was fused in-frame with the secretion signal of the *K. lactis* killer toxin and expressed under the control of the *K. lactis LAC4* promoter and terminator elements (Walsh and Bergquist 1997). The secretion cassette was modified by removing the *Dictyoglomus xynA* gene and introducing a unique *Mlu*I site at the junction between the secretion signal and the *LAC4* terminator in order to create a more convenient vector that permits the foreign gene to be inserted in a single step. In the modified cassette, the 16-amino-acid secretion signal is followed by the dipeptide Lys-Arg, which is a potential cleavage site for the Kex1 endopeptidase of *K. lactis* (Tanguy-Rougeau et al. 1988), and an Thr-Arg pair encoded by the *Mlu*I restriction site (Fig. 1). The cassette was designed as a *Sal*I/*Hin*dIII fragment to facilitate its direct introduction into the corresponding sites in the *K. lactis* shuttle vector pCXJ1. The subsequent insertion of the Kan gene produced the vector pCWK1 (Fig. 1), which can

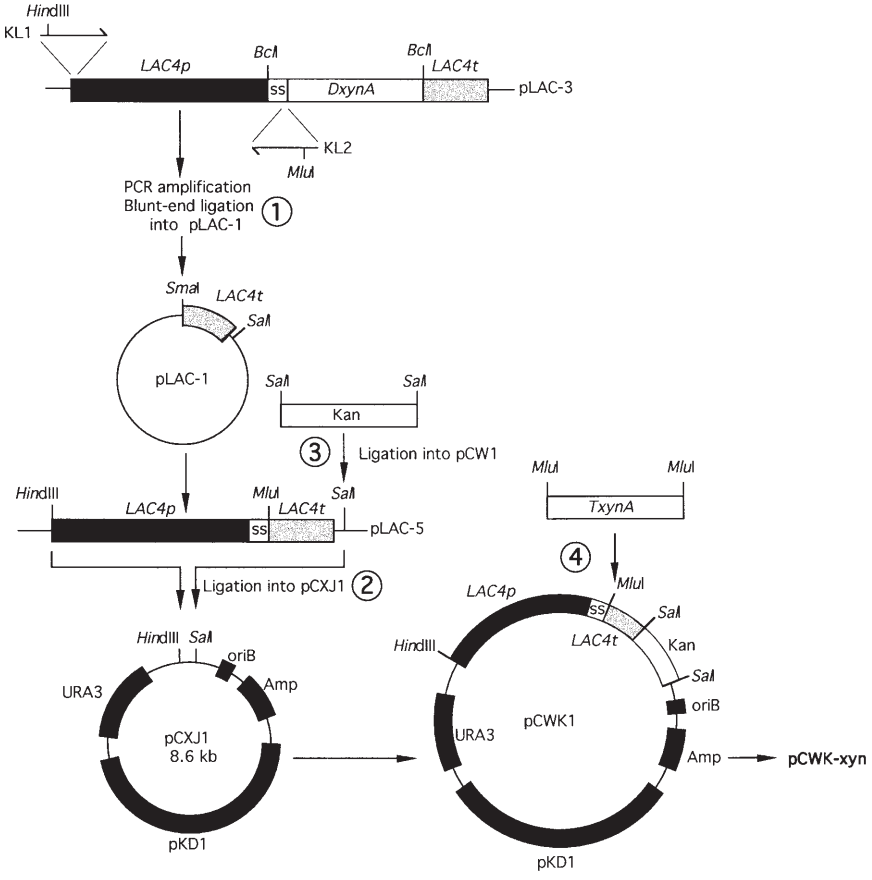


**Fig. 1.** Construction of vector pCWK-xyn. KL1 and KL2 are the polymerase chain reaction (*PCR*) primers used to amplify the *LAC4* promoter (*LAC4p*) and killer toxin secretion signal (*ss*) on a single fragment. Other abbreviations: *LAC4t*, terminator region of the *K. lactis LAC4* gene; *DxynA, xynA* gene from *Dictyoglomus thermophilum* strain Rt46B.1; *TxynA, xynA* gene from *Thermotoga* sp. strain FjSS3B.1; *oriB*, *E. coli* origin of replication; *Amp*, ampicillin resistance gene; *kan*, G418 resistance gene. *Circled numbers* indicate the order in which cloning manipulations were performed

be selected in both *uraA* and wild-type strains of *K. lactis* and can be maintained without the requirement for resident pKD1.

To allow cloning of the *Thermotoga xynA* gene in-frame with the *K. lactis* secretion signal in pCWK1, the coding region was amplified from a *Thermotoga* genomic clone using oligonucleotide primers containing *Mlu*I sites. The amino-terminal primer was designed so as to exclude from the amplified region the first 22 codons of *xynA* which encode the putative XynA signal sequence. The carboxy-terminal primer was designed to include the native *xynA* stop codon. Hence plasmid pCWK-xyn contains a fusion between the killer toxin secretion signal and the predicted mature XynA structural gene. This plasmid was introduced into *K. lactis* strain CBS1065.

## Effect of media composition on xylanase secretion and plasmid stability

*K. lactis* CBS1065[pCWK-xyn] was cultivated in YP medium containing either glucose, galactose, or glucose and galactose, and samples were withdrawn at daily intervals for determination of cell density and extracellular xylanase activity. This analysis revealed that stationary phase was reached in all media within 24 h but xylanase activity continued to increase after this point to reach maximal levels of 138, 54, and 184 XU/ml in YPD, YPG, and YPDG respectively after 5 days of growth. The proportion of cells remaining resistant to kanamycin was 98% in the YPD culture and 57% in the YPG culture (Fig. 2). Plasmid stability was monitored over three further culture cycles, representing a total of approximately 40 generations, by performing successive back-dilutions of stationary phase cultures into fresh media. At the end of this period, plasmid pCWK-xyn was retained by 77% of cells grown on glucose. However, plasmid pCWK-xyn was completely lost within 30 generations of growth on galactose (Fig. 2). By comparison, the control plasmid pCXJ-kan1 (Bianchi et al. 1987), which lacks the xylanase secretion cassette, was retained by 98% of CBS1065 cells after 40 generations of growth on YPD (Fig. 2).

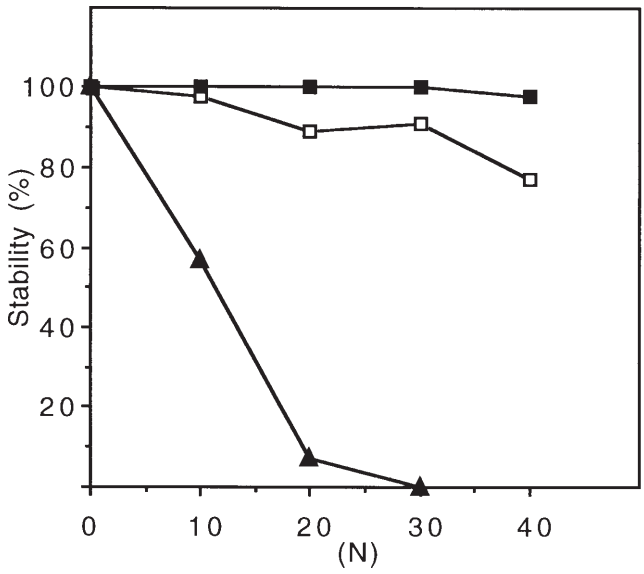


**Fig. 2.** Influence of the growth medium on plasmid stability. Mitotic stability is expressed as the percentage of cells remaining resistant to G418 (200 mg/ml) after growth in nonselective conditions for *N* generations. *Filled squares*, strain CBS1065[pCXJ-kan1] grown on YPD; *open squares*, strain CBS1065[pCWK-xyn] grown on YPD; *filled triangles*, strain CBS1065[pCWK-xyn] grown on YPG. YP, yeast extract/bactopeptone medium containing glucose (D) or galactose (G)

## Analysis of *K. lactis* culture supernatants by SDS-PAGE

We readily observed recombinant *Thermotoga* XynA as by far the most abundant protein in media used for cultivation of CBS1065[pCWK-xyn] by Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 3, lanes 2–4). By comparison, no major proteins were detected in culture supernatant from the control strain CBS1065[pCXJ-kan1] (Fig. 3, lane 1). The XynA protein has an apparent molecular mass of approximately 33 kDa, slightly less than the figure of 38 kDa predicted from the *xynA* nucleotide sequence cloned in pCWK-xyn. Densitometric comparison of the XynA band with a known amount of a 35-kDa *Dictyoglomus* xylanase expressed in *E. coli* (Gibbs et al. 1995) indicated that the *Thermotoga* enzyme is secreted to a level of 80 μg/ml in YPD medium. When this sample was fractionated in a polyacrylamide gel containing oat spelts xylan and stained for xylanase activity, we observed a single major zone of xylan hydrolysis which corresponded to the XynA protein (Fig. 3, lane 5).

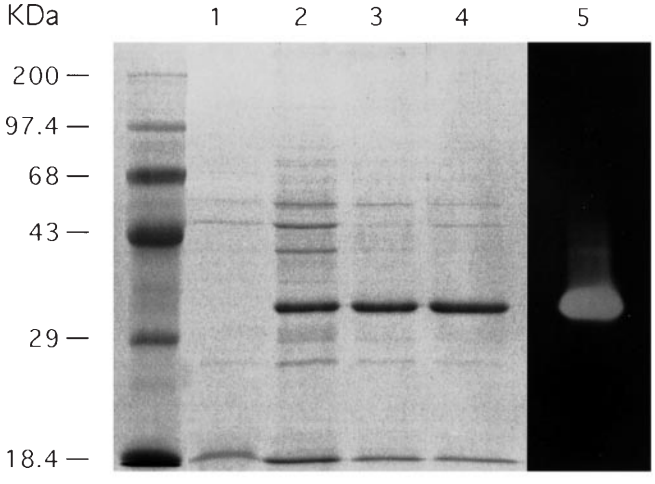


**Fig. 3.** Secretion of xylanase from *K. lactis*. Samples of culture supernatants (15 ml) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). *Lanes: 1*, CBS1065[pCXJ-kan1] grown on YPD; *2*, CBS1065[pCWK-xyn] grown on YPD; *3*, CBS1065[pCWK-xyn] grown on YPDG; *4* and *5*, CBS1065[pCWK-xyn] grown on YPDG + G418. *Lanes 1–4* were stained with Coomassie blue. *Lane 5* was proecessed for detection of xylanase activity

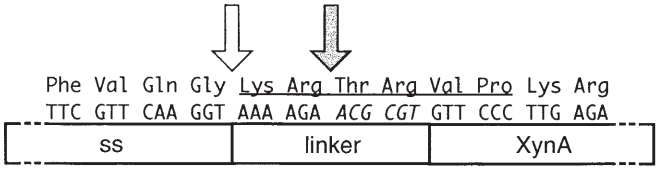


**Fig. 4.** Junction between the killer toxin secretion signal (*ss*) and the N-terminal sequence of XynA. The hypothetical cleavage sites recognized by the *K. lactis* signal peptidase (Gln-Gly) and the Kex1 endopeptidase (Lys-Arg) are indicated by *open* and *filled arrows* respectively. The experimentally determined N-terminal amino acid sequence of XynA expressed in *K. lactis* is *underlined*. The *Mlu*I cloning site is indicated in *italics*

N-terminal amino acid sequencing of XynA

The recombinant *Thermotoga* xylanase secreted from *K. lactis* was subjected to N-terminal amino acid sequencing in order to investigate how the protein had been processed. The first six residues from the N-terminus were Lys-Arg-Thr-Arg-Val-Pro-, which corresponds precisely to an internal region of the predicted signal peptide-XynA fusion shown in Fig. 4. This sequence indicates that the signal peptide was cleaved after the sequence Gln-Gly, which is presumed to be recognized by the *K. lactis* signal peptidase (Stark and Boyd 1986).

Effect of temperature on activity of XynA

The *Thermotoga* xylanase secreted by *K. lactis* showed maximal activity at 90°C and over 50% of maximal activity between approximately 76° and 96°C. The half-life of the enzyme at 90°C, pH 6.5, was approximately 48 h. These results are similar to those determined for the gene product expressed in *E. coli* (Saul et al. 1995).

## Discussion

*K. lactis* strains harbouring a pKD1-based expression vector efficiently secrete the recombinant *Thermotoga* xylanase at high levels. All strains examined were able to promote secretion of the xylanase with the same electrophoretic mobility as the control enzyme produced in *E. coli* (approximately 33 kDa). Recombinant xylanase is by far the most abundant protein present in transformed *K. lactis* culture supernatants and the *Thermotoga* XynA protein comprises over 95% of total proteins secreted from strain CBS1065.

Several parameters influence xylanase secretion levels, including plasmid architecture and composition of the growth medium. Significantly higher levels of extracellular enzyme were attained through *LAC4*-directed XynA expression on full pKD1 sequence vectors as compared to expression with other pKD1 ori plasmid derivatives that we have constructed (data not shown). The carbon source influenced secretion levels by modulating promoter strength, which in turn affects plasmid mitotic stability (see also Walsh and Bergquist 1997). In non-inducing glucose medium, *xynA* expression directed by *LAC4* is incompletely repressed in CBS1065, although plasmid stability remains high. Full induction of the *LAC4* promoter on galactose results in a drastic reduction in plasmid stability in strain CBS1065, and consequently, lower levels of xylanase secretion are observed than on glucose.

Our results show that the killer toxin secretion signal is processed by *K. lactis* signal peptidase to give cleavage after Gln-Gly in the killer toxin signal sequence and release of correctly processed XynA (Tanguy-Rougeau et al. 1988). Recombinant enzymes produced in *K. lactis* are biologically active as shown by activity gels (Walsh and Bergquist 1997; Walsh et al. 1997), and are not hyperglycosylated, as we found previously for expression of thermophilic enzymes in *Saccharomyces cerevisiae* (Donald et al. 1994). However, since one of our major aims is to produce sufficient enzyme for crystallization, even low levels of glycosylation may be a problem that will require further investigation. The XynA enzyme from *Thermotoga* produced in *K. lactis* has optimal activity at 90°C and a half-life comparable that reported for this enzyme produced in *E. coli* (Saul et al. 1995). Similarly, the *Dictyoglomus* XynA protein produced in *K. lactis* also was shown to have identical biochemical characteristics to its counterpart produced in *E. coli* (Walsh and Bergquist 1997). Strain CBS1065 combines good secretion levels on nonselective media (up to 120 μg/ml in shake-flask culture) with high plasmid stability on YEP Glucose and thus this recombinant is suitable for scaled-up production of *Thermotoga* xylanase in chemostat culture. The next step involves the scale-up of the expression system in fermentors for the production of substantial amounts of enzyme for X-ray crystallography and other basic research studies and for larger-scale bleaching trials. It will be necessary to develop an appropriate induction strategy to minimize the effects of plasmid instability.

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