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Expression and Secretion of a Thermostable Bacterial Xylanase in *Kluyveromyces lactis*

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The *xynA* structural gene from the extremely thermophilic anaerobe *Dictyoglomus thermophilum* Rt46B.1 was fused in frame with the secretion signal of the *Kluyveromyces lactis* killer toxin in episomal expression vectors based on the *Kluyveromyces* plasmid pKD1. XynA was secreted predominantly as an unglycosylated 35-kDa protein which comprised up to 90% of the total extracellular proteins and reached a concentration of 130 μg/ml in shake-flask cultures grown under selective conditions.

The kraft process is widely used in the paper manufacturing industry for the production of pulp and involves alkaline sulfate cooking of wood chips to remove around 90% of the lignin. Enzymatic prebleaching of kraft pulp with endo-1,4-β-xylanases (EC 3.2.1.8) enhances lignin extraction (28) and is an effective means of attaining the target brightness with a lower chlorine dosage (13, 27) or of increasing the final brightness of pulp produced by totally chlorine-free bleaching procedures (13, 15). We isolated a xylanase gene, *xynA*, from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1, which has shown promise as a bleach-boosting aid. To simplify enzyme recovery, we wished to produce XynA as an extracellular protein and chose the yeast *Kluyveromyces lactis* as an expression host, because of its proven capacity for high-level secretion of foreign proteins (3, 9, 26).

Escherichia coli JM101 [F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/*supE thiD*(*lac-proAB*)] was used for routine cloning manipulations and was grown as described previously (11). *K. lactis* MD2/1 was transformed to uracil prototrophy by the lithium acetate procedure (12). Transformants were selected on SD minimal agar medium (0.67% yeast nitrogen base without amino acids, 40 mg of L-lysine per ml, 40 mg of L-arginine per ml, 2% glucose, 2% agar) and grown at 30°C in shake flasks containing three different media: SD medium, SD medium supplemented with 1% Casamino Acids (SDC), and YPD medium (1% yeast extract, 2% Bacto Peptone, 2% glucose). Wild-type strains CBS1065 and CBS2359 were transformed via selection for resistance to G418 (Geneticin; Life Technologies, Gaithersburg, Md.) as described by Bianchi et al. (4). Transformants were grown in shake flasks containing four different media: YPD, YP medium containing 2% galactose (YPG), YP medium containing 2% glucose and 2% galactose (YPDG), and YP medium containing 2% glucose, 2% galactose, and G418 at 200 μg/ml for plasmid selection (YPDG+G418).

The enzymatic release of reducing sugar by the secreted supernatant xylanase was determined as previously described (11) and was expressed in xylanase units (XU [2]).

The structures of the *K. lactis* shuttle vectors used in this study are shown in Fig. 1. Plasmid pSPGK1 contains the replication origin of the *Kluyveromyces* plasmid pKD1 (5) and a promoter-terminator cassette derived from the *Saccharomyces cerevisiae PGK* gene (21). A synthetic secretion signal derived from the pre-region of the *K. lactis* killer toxin α-subunit (24) is situated downstream of the *PGK* promoter and is followed immediately by a unique *Eco*RI site (Fig. 1A). Plasmid pCXJ-kan (4) contains the entire sequence of pKD1 and the kanamycin resistance gene (Kan^r) of Tn*903*, which confers resistance to G418 in yeast. Plasmid pCXJ1 was derived from pCXJ-kan by recircularization following removal of the Kan^r gene from a *Sal*I fragment. An expression cartridge in which the promoter and terminator regions of the *K. lactis LAC4* gene flank the secretion signal *xynA* fusion region from pSPGK-xyn was constructed in pUC19 and transferred to pCXJ1 according to the strategy summarized in Fig. 1B (16).

Plasmid pSPGK-xyn contains an in-frame fusion between the mature *xynA* structural gene and the secretion signal of the *K. lactis* killer toxin. The fusion was created at codon Met-30 in the full-length XynA amino acid sequence in order to exclude the predicted bacterial signal sequence encoded by residues 1 to 29 (11). The *K. lactis* secretion signal in pSPGK-xyn is followed by the dipeptide Lys-Arg, which is a potential cleavage site for the Kex1 endopeptidase of *K. lactis* (25), and an Ile-Arg pair partly encoded by the *Eco*RI cloning site. In this recombinant plasmid, *xynA* expression is under the control of the *S. cerevisiae PGK* promoter, which also functions in *K. lactis* (10, 19).

Plasmid pCXJK-xyn was constructed to allow regulated expression of *xynA* under the control of the promoter from the *K. lactis LAC4* gene, which is induced up to 100-fold by galactose and lactose (7). In order to ensure inclusion of all essential regulatory elements from the 5′ flanking region of *LAC4*, we amplified a 1.1-kb fragment containing all three UAS elements which potentially interact with the *trans*-acting LAC9 protein (16). The expression cartridge was designed as a *Sal*I-*Hin*dIII fragment to facilitate direct introduction into the corresponding sites in pCXJ1. The Kan^r gene was added to allow plasmid selection in wild-type strains, and the final recombinant plasmid was introduced into *K. lactis* CBS1065 and CBS2359.

Proteins secreted from recombinant *K. lactis* strains were visualized by Coomassie blue staining following sodium dodecyl sulfate-polyacrylamide gel electrophoresis of culture supernatants, as shown in Fig. 2. Media used for cultivation of MD2/1(pSPGK-xyn) (Fig. 2, lane 1) and CBS1065(pCXJK-xyn) (Fig. 2, lanes 2 to 5) contained a prominent protein band with an apparent molecular mass of 35 kDa which comigrated with

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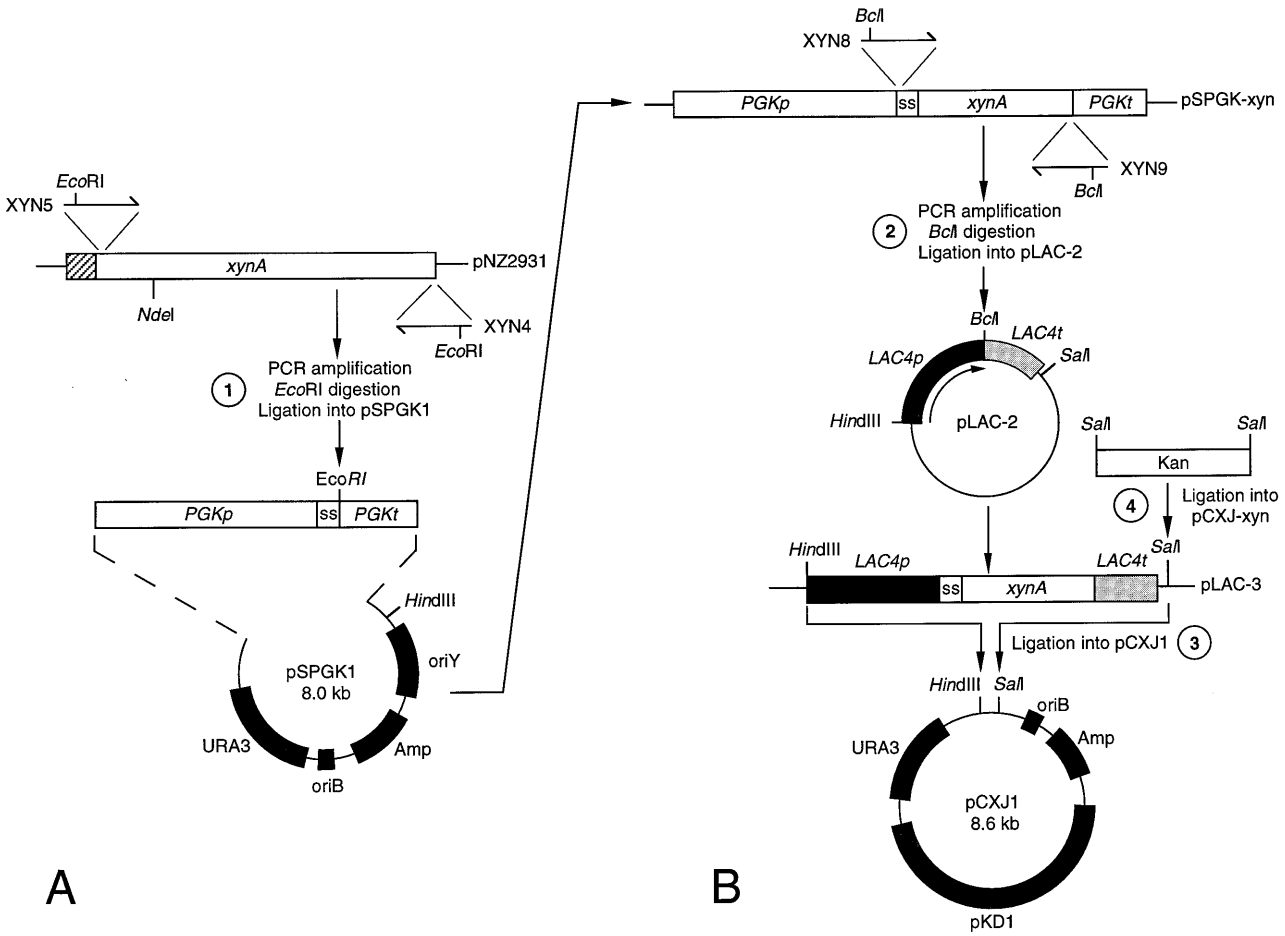

FIG. 1. Construction of xylanase expression vectors. (A) Construction of plasmid pSPGK-xyn. The hatched region of *xynA* encodes the predicted signal peptide. XYN4 and XYN5 are the PCR primers used to amplify *xynA*. ss, secretion signal of the *K. lactis* killer toxin; *PGKp* and *PGKt*, promoter and terminator regions, respectively, of the *Saccharomyces cerevisiae PGK* gene; oriY, replication origin of plasmid pKD1; Amp, ampicillin resistance gene; oriB, *E. coli* origin of replication. The positions of *Nde*I and *Hin*dIII restriction sites used to orientate the *xynA* gene in pSPGK-xyn are shown. (B) Construction of plasmid pCXJK-xyn. XYN8 and XYN9 are the PCR primers used to amplify the secretion signal-*xynA* fusion. The plasmid pLAC-1 was constructed by ligating the *K. lactis LAC4* terminator region on an *Eco*RI-*Kpn*I fragment into *Eco*RI-*Kpn*I-digested pUC19, which had its single *Sma*I site deleted. A 1.1-kb fragment containing the *LAC4* promoter region was amplified from *K. lactis* genomic DNA by a PCR with the introduction of a *Hin*dIII site and a *Bcl*I site by way of the primers. The PCR product was ligated into the *Sma*I site in the proximal portion of the *LAC4* terminator region to generate the plasmid pLAC-2. *LAC4p* and *LAC4t* are the promoter and terminator regions, respectively, of the *K. lactis LAC4* gene. Circled numbers indicate the order in which cloning manipulations were performed.

Dictyoglomus XynA expressed in *E. coli* (Fig. 2, lane 7) but which was not produced by the control strain CBS1065(pCXJ-kan1) (Fig. 2, lane 6). Densitometric scanning of the gel revealed that XynA comprised over 90% of total extracellular proteins in the CBS1065(pCXJK-xyn) YPDG+G418 culture. The concentration of the recombinant xylanase was estimated to be 130 μg/ml by comparison with a known amount of *Dictyoglomus* XynA expressed in *E. coli*. This figure is in good agreement with an estimate of 150 μg/ml based on the level of xylanase activity in the CBS1065(pCXJK-xyn) YPDG+G418 sample (95 XU/ml) and the specific activity of the *E. coli*-derived enzyme (630 XU/mg), which indicates that the *E. coli*- and *K. lactis*-derived xylanase preparations have approximately the same specific activity.

When proteins secreted from CBS1065(pCXJK-xyn) were stained for xylanase activity (Fig. 2, lane 8), we detected a zone of intensive xylan hydrolysis corresponding to the major 35-kDa XynA protein and a zone of less intense activity which coincided with a minor 38-kDa component detected by Coomassie blue staining (Fig. 2, lane 5).

The N-terminal sequence of the *Dictyoglomus* xylanase secreted by *K. lactis* was determined with supernatant from a culture of CBS1065(pCXJK-xyn) by methods described elsewhere (20) to investigate how processing of XynA had occurred. The sequence of the first six residues from the N terminus of the major 35-kDa protein was Lys-Arg-Ile-Arg-Met-Glu-, which corresponds precisely to an internal region of the predicted signal peptide-XynA fusion. This sequence is consistent with cleavage of the signal peptide after the sequence Gln-Gly presumed to be recognized by the *K. lactis* signal peptidase (24). Therefore, this protein is an XynA variant which contains an N-terminal extension of four nonnative amino acids comprising the Lys-Arg pair and the Ile-Arg pair created during cloning of *xynA* into the *Eco*RI site of pSPGK1. However, these additional residues do not appear to affect enzyme activity, since the temperature optimum, pH profile, thermostability, and specific activity of the yeast-derived enzyme are almost identical to those reported for XynA expressed in *E. coli*, which has a native N terminus beginning at Met-30 (11). The major form of XynA secreted from *K. lactis*

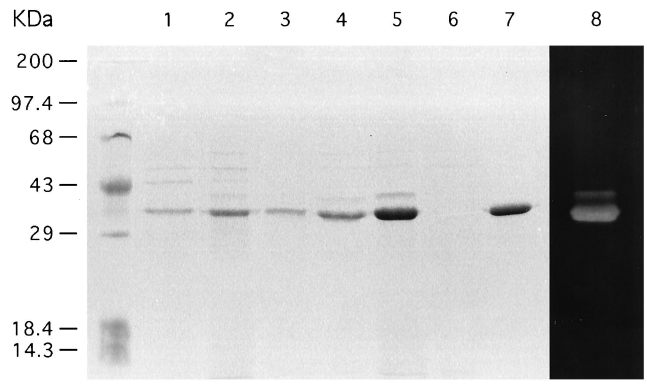

FIG. 2. Secretion of xylanase from recombinant strains of *K. lactis*. Samples of culture supernatants (15 ml) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). Lanes: 1, MD2/1(pSPGK-xyn) grown in YPD medium; 2, CBS1065(pCXJK-xyn) grown in YPD medium; 3, CBS1065 (pCXJK-xyn) grown in YPG medium; 4, CBS1065(pCXJK-xyn) grown in YPDG medium; 5 and 8, CBS1065(pCXJK-xyn) grown in YPDG+G418 medium; 6, CBS1065(pCXJ-kan 1) grown in YPDG medium; 7, 2 mg of *Dictyoglomus* XynA produced in *E. coli.* Lanes 1 to 7 were stained with Coomassie blue. Lane 8 was processed for detection of xylanase activity as described by Lüthi et al. (18).

comigrated with XynA expressed in *E. coli* and therefore was not glycosylated. The minor 38-kDa form detected by zymogram analysis may result from glycosylation at the single potential site, ^{114}Asn-Gln-Thr, which would be consistent with an increase in molecular mass of about 2 kDa per core glycosylated site (23).

The fraction of plasmid-containing cells in cultures of *K. lactis* transformants was measured by comparing growth on nonselective and selective plates. For MD2/1 cultures, individual colonies were transferred from YPD plates to SD minimal plates lacking uracil. Colonies from CBS1065 and CBS2359 cultures were transferred from YPD plates to YPD plates containing 200 μg of G418 per ml. Two hundred colonies were transferred to selective medium for each culture sample analyzed. In addition, the influence of the growth medium on xylanase secretion and plasmid stability was evaluated for recombinant *K. lactis* strains in shake-flask cultures. Three media were utilized for strain MD2/1 (pSPGK-xyn): a selective minimal medium (SD), a richer semidefined medium (SDC) which is still selective for the plasmid *URA3* marker, and a rich nonselective medium (YPD). Maximal cell density was reached after 24 to 40 h, depending on the growth medium, but xylanase activity continued to increase after this point and reached maximal levels of 3.1, 8.6, and 10.4 XU/ml in the SD, SDC, and YPD media, respectively, after 5 days of growth. No xylanase activity was detected by either assay in a culture of MD2/1 cells harboring the parent vector pSPGK1. The proportion of plasmid-containing cells remaining at the end of the cultivation period was 46% in SD medium and 42% in SDC medium but only 5% in YPD medium, indicating that pSPGK-xyn is mitotically unstable in the absence of selection.

K. lactis strains harboring pCXJK-xyn were grown in YP medium containing either glucose or galactose as the sole carbon source in order to investigate the effect of *LAC4* promoter strength on xylanase production and plasmid stability. For strains CBS1065(pCXJK-xyn) and CBS2359(pCXJK-xyn), the maximal levels of extracellular xylanase activity reached after 5 days of growth in YPD medium were 28.3 and 14.0 XU/ml, respectively. Surprisingly, enzyme activity levels reached only 10.3 XU/ml for both strains grown in YPG medium. We examined the proportion of plasmid-containing cells remaining in YPD and YPG stationary-phase cultures, which had undergone approximately 10 generations of growth. Plasmid retention in CBS1065 was 92% on glucose but only 26% on galactose. In CBS2359, the plasmid was also highly stable on glucose (88%) but was completely lost during growth on galactose. The retention of pCXJK-xyn after 10 generations of growth in YPDG medium was 72% in CBS2359 and 48% in CBS 1065, and we found that xylanase activity was higher in YPDG medium than in either YPD or YPG medium. The maximal levels attained in this medium were 32 and 55 XU/ml in CBS1065 and CBS2359, respectively. The addition to YPDG medium of 200 μg of G418 per ml for plasmid selection promoted a further increase in xylanase activity to 95 XU/ml in both strains.

Our results show that the mitotic stability of recombinant vectors and the level of xylanase secretion were strongly influenced by the transcriptional activity of the promoter used to drive expression of *xynA*. In nonselective rich medium, expression from the strong *S. cerevisiae PGK* promoter resulted in loss of the pSPGK-xyn plasmid at a rate of approximately 10% per generation. This figure is significantly higher than the 1 to 3% loss rate reported for vectors designed for expression of human serum albumin (9) and interleukin-1β (10) in *K. lactis* under the control of the same promoter. Plasmid instability is likely to be a major factor contributing to the lower level of product secretion observed for the recombinant xylanase (15 μg/ml) compared to human serum albumin (300 μg/ml) (9) or interleukin-1β (40 μg/ml) (10) in comparable shake-flask experiments. However, plasmids containing the entire sequence of pKD1, such as those used by Fleer et al. (9, 10), are inherently more stable than plasmids such as pSPGK-xyn, which contain only the pKD1 replication origin (4). The stability of pSPGK-xyn was improved by the use of either minimal or semidefined selective media, but in both cases, xylanase secretion was lower than in YPD medium. These results are in agreement with earlier reports describing higher productivity of secreted recombinant products in rich media due to decreased product proteolysis and increased cell yield (6, 29).

The *xynA* gene was placed under the control of the regulated *LAC4* promoter on a plasmid containing the entire sequence of pKD1 in an effort to improve plasmid stability and xylanase productivity in rich media. We found that the *LAC4* promoter was incompletely repressed by glucose in both wild-type strains transformed with pCXJK-xyn, and the level of enzyme secretion from CBS1065 was actually threefold greater than that from MD2/1(pSPGK-xyn). This relatively high basal expression of *xynA* on glucose was tolerated without any significant reduction in the mitotic stability of the expression vector compared to that of the parent plasmid pCXJ-kan1. However, full induction of the *LAC4* promoter on galactose resulted in a dramatic reduction in plasmid stability, and we observed no improvement in xylanase secretion compared to the levels attained on glucose. The highest concentration of secreted xylanase (130 μg/ml) was reached in YPDG medium containing G418 for plasmid selection. This result represents an improvement in the volumetric productivity of *Dictyoglomus* XynA of over 300-fold compared to that of the *E. coli* expression system described by Gibbs et al. (11).

A variety of prokaryotic and eukaryotic organisms have been used as expression hosts in attempts to improve the production of xylanases for biotechnological applications. *S. cerevisiae* has been employed successfully for extracellular production of xylanases from other fungi (17, 22), but a xylanase from the thermophilic bacterium *Caldicellulosiruptor saccharolyticus* was secreted to a level of only 10 μg/ml in this yeast (8). In commercial prebleaching procedures, xylanases are typically used

at a charge equivalent to approximately 120 XU per g of pulp (1). Hence, at the highest secretion levels achieved so far, 1 ml of culture supernatant from *K. lactis* contains sufficient enzyme to treat about 0.8 g of pulp, which is below commercially viable production levels. Optimization of growth and induction parameters in a fed-batch fermentation process should allow further improvements in xylanase productivity. An important consideration will be minimization of plasmid instability. Our results suggest that one possible strategy would be initial growth of pCXJK-xyn transformants to high cell density on glucose followed by moderated induction on a mixture of galactose and glucose. The development of integrant strains with a number of copies of the xylanase gene is another alternative strategy which may allow a higher level of thermostable enzyme production.

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