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NOTES

Ethanolic Fermentation of Xylose with *Saccharomyces cerevisiae* Harboring the *Thermus thermophilus xylA* Gene, Which Expresses an Active Xylose (Glucose) Isomerase

MATS WALFRIDSSON,[1] XIAOMING BAO,† MIKAEL ANDERLUND,[1] GÖSTA LILIUS,[2] LEIF BÜLOW,[2] AND BÄRBEL HAHN-HÄGERDAL[1]*

Department of Applied Microbiology[1] and Department of Pure and Applied Biochemistry,[2] Lund Institute of Technology/Lund University, S-221 00 Lund, Sweden

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The *Thermus thermophilus xylA* gene encoding xylose (glucose) isomerase was cloned and expressed in *Saccharomyces cerevisiae* under the control of the yeast *PGK1* promoter. The recombinant xylose isomerase showed the highest activity at 85°C with a specific activity of 1.0 U mg^{-1}. A new functional metabolic pathway in *S. cerevisiae* with ethanol formation during oxygen-limited xylose fermentation was demonstrated. Xylitol and acetic acid were also formed during the fermentation.

D-Xylose, a major component of lignocellulosic biomass, can be fermented by bacteria, yeasts, and filamentous fungi (5, 6, 9, 20). Bacteria utilize xylose by first isomerizing xylose to xylulose, a process which is catalyzed by xylose isomerase (XI), before entering the pentose phosphate pathway. In xylose-fermenting yeasts, such as *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis*, xylose is first reduced to xylitol by xylose reductase (XR), which then is oxidized to xylulose by xylitol dehydrogenase (XDH). Baker's yeast, *Saccharomyces cerevisiae*, is not able to metabolize xylose due to the lack of XR and XDH activity. However, it can utilize the isomeric form xylulose, and because of the many positive properties of the organism with regard to alcoholic fermentation, several approaches to the metabolic engineering of *S. cerevisiae* for xylose utilization have been investigated. Introducing the *Pichia stipitis* NAD(P)H-dependent XR and NAD^+-dependent XDH into *S. cerevisiae* has resulted in a new metabolic pathway, allowing xylose to be fermented to ethanol (11, 21, 24). A major drawback with these recombinant strains is that the K_m of XR for NADPH is an order of magnitude lower than that for NADH. This leads to NADH accumulation from the oxidization of xylitol to xylulose, catalyzed by XDH. The redox imbalance results in the formation of by-products such as xylitol and glycerol. It has recently been shown that by-product formation can be overcome by overexpressing XDH 15 times in relation to XR (23). The redox problem could also be avoided by introducing the non-cofactor-requiring bacterial XI. XI genes (*xylA*) from several bacteria have been cloned into *S. cerevisiae*. These include the *xylA* genes from *Actinoplanes missouriensis* (1), *Bacillus subtilis* (1), *Clostridium thermosulfurogenes* (16), *Escherichia coli* (8, 17), and *Lactobacillus pentosus* (7). The XIs produced by the recombinant *S. cerevisiae* strains were, however, inactive. Improper protein folding, posttranslational modifications, inter- and intramolecular disulfide bridge formation, and the internal pH of yeast have been suggested as possible reasons (1).

In the present study, we describe the cloning and expression of the *Thermus thermophilus xylA* gene in *S. cerevisiae*. The temperature profile of the recombinant XI was investigated and product formation during xylose utilization of the *xylA*-containing *S. cerevisiae* under oxygen limitation was studied.

S. cerevisiae H158 [GPY55-15B (*MAT*α *leu2-3 leu2-112 ura3-52 trp1-289 his4-519 prb1 cir*$^+$)] was used as the host strain. Plasmids used were pUC19-XI, containing the *T. thermophilus xylA* gene, and the yeast expression vector pMA91 (15). Yeast strains were grown in a synthetic complete (SC) medium (19), supplemented with the appropriate amino acids as previously described (24). For selection of the transformants, leucine was omitted from the medium. The primers 5′-GCGC*TGATCA*TCTAGAATGTACGAGCCCAAACCG GAGCACAG-3′ (5′ primer) and 5′-GCTT*TGATCA*TCTAG ATCACCCCCGCACCCCCAGGAGGTACT-3′ (3′ primer) were used for PCR amplification of the *xylA* gene. Both primers contained restriction endonuclease sites for *Bcl*I (shown in italics) and *Xba*I (underlined). The PCR mixture contained PCR buffer with 2 mM $MgSO_4$ (Boehringer Mannheim), 0.8 mM deoxynucleoside triphosphates, 0.3 μM each primer, 0.2 μg of template (pUC19-XI), and 2.5 U of Pwo DNA polymerase (Boehringer Mannheim). A DNA thermal cycler (Perkin-Elmer Cetus) was used for amplification of the gene under the following conditions: melting temperature, 94°C (1 min); annealing temperature, 58°C (1 min); and polymerization at 72°C (1 min). Twenty-eight cycles were run with a subsequent polymerization period of 7 min at 72°C. The amplified DNA fragment was digested with *Bcl*I and ligated into the *Bgl*II site of pMA91, resulting in plasmid pBXI. The *Bgl*II site of pMA91 was placed between the phosphoglycerate kinase gene (*PGK1*) promoter and terminator. Yeast strain transformation was performed by the lithium acetate method (18). Cell extracts were prepared as previously described (24). The protein concentration was measured according to Bradford's method (2), with

* Corresponding author. Mailing address: Department of Applied Microbiology, Lund Institute of Technology/Lund University, P.O. Box 124, S-221 00 Lund, Sweden. Phone: 46 46 222 8428. Fax: 46 46 222 4203. Electronic mail address: Barbel.Hahn-Hagerdal@tmb.lth.se.

† Present address: Department of Microbiology, Shandong University, Jinan City 250100, People's Republic of China.

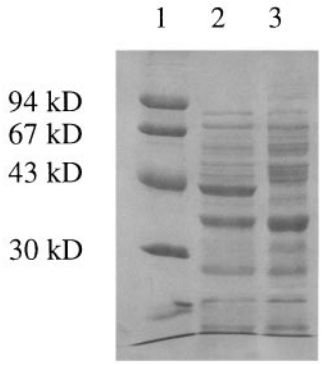

FIG. 1. Coomassie blue-stained SDS-PAGE gel. Lane 1, low-molecular-weight standard; lane 2, cell extract of *S. cerevisiae* transformed with the *T. thermophilus xylA* gene; lane 3, cell extract of the *S. cerevisiae* reference strain.

bovine serum albumin as the standard. XI activity in recombinant strain H158(pBXI) and reference strain H158(pMA91) was determined by measuring the conversion of fructose to glucose (4). The assay mixture (1,000 μl) contained 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.0), 400 mM fructose, 10 mM $MnCl_2$, and 100 μl of cell extract. After 10 min of incubation the reaction was stopped by adding 0.3 ml of 50% trichloroacetic acid. The glucose formed was measured according to the glucose-oxidase-based method (22) (Sigma Diagnostics, St. Louis, Mo.). The specific activity of D-xylose (glucose) isomerase was expressed as micromoles of converted substrate per milligram of protein per minute, equivalent to units per milligram. The activity of the recombinant XI was measured at temperatures between 22 and 95°C at pH 7. Proteins were analyzed by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (total monomer, 8.8 to 21.3%; cross-linker concentration, 2.6%) (13). The gel was stained with 0.1% Coomassie blue R-250 (Sigma) in 25% methanol and 10% acetic acid. Xylose fermentation with recombinant

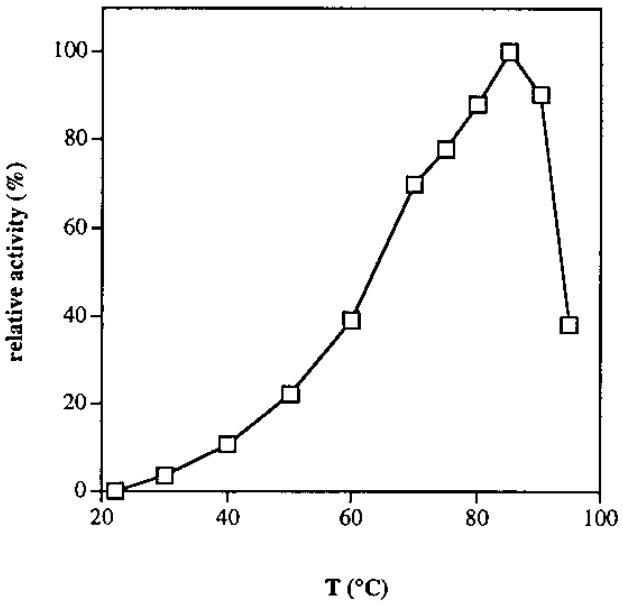

FIG. 2. Relative activity at different temperatures (measured at pH 7) of the recombinant XI.

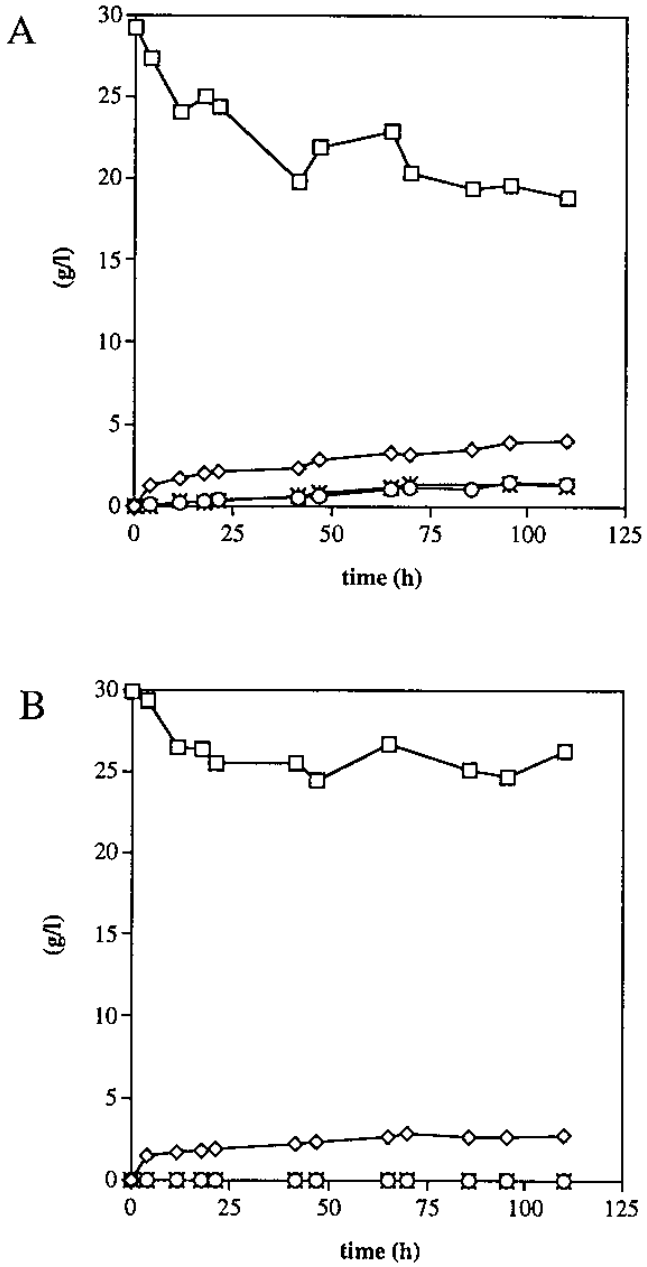

FIG. 3. Oxygen-limited cultivations of H158(pBXI) (A) and reference strain H158(pMA91) (B) in SC medium containing 30 g of xylose liter^{-1}. Symbols: □, xylose; ◇, xylitol; ○, acetic acid; ∗, ethanol.

strain H158(pBXI) and reference strain H158(pMA91) was studied under oxygen-limited conditions. Twenty-five-milliliter flasks were filled with 15 ml of SC-Leu medium (pH 5.5) containing 30 g of xylose liter^{-1}. The flasks were plugged with rubber stoppers, and the gas outlet was secured by inserting a cannula through the rubber stopper. Agitation was achieved with a magnetic stirrer. The fermentations were performed at 38°C. The initial cell mass concentrations were 15.6 g (dry weight) liter^{-1} for H158(pBXI) and 19.1 g liter^{-1} for H158 (pMA91). Concentrations of sugar substrates and fermentation products were determined by high-performance liquid chromatography (Varian, Sunnyvale, Calif.) with two columns (Aminex ion-exclusion HPX-87H; Bio-Rad, Richmond,

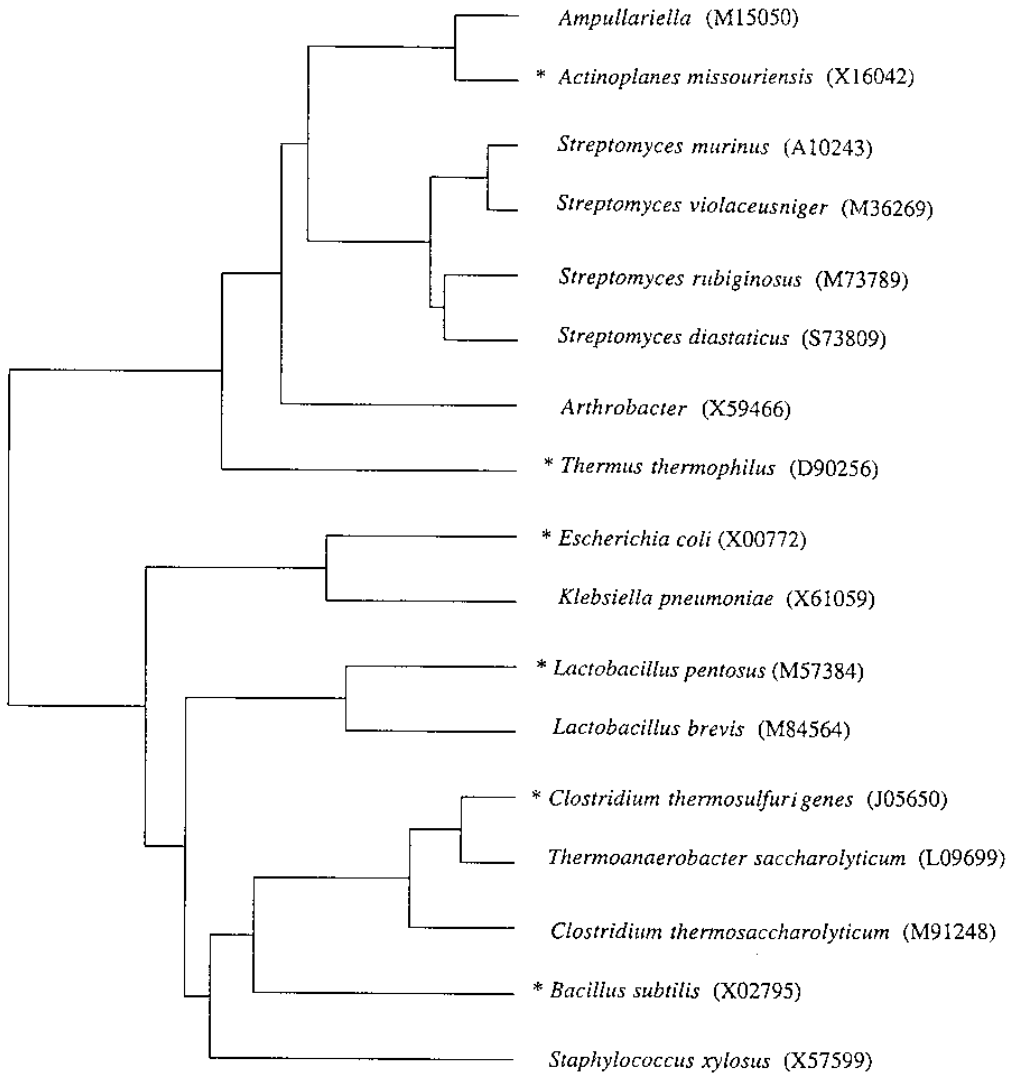

FIG. 4. Dendrogram showing several bacterial XI (*xylA*) genes. EMBL gene accession numbers are in parentheses. Asterisks indicate organisms whose *xylA* genes were cloned in *S. cerevisiae.*

Calif.) in series (14) at 65°C with 5 mM H_2SO_4 as the mobile phase. The compounds were detected with a refractive-index detector (410 differential refractometer; Waters, Milford, Mass.). The dendrogram (see Fig. 4) was created by the computer program PILEUP (version 8; Genetics Computer Group, Madison, Wis.).

Based on the published *T. thermophilus xylA* sequence (3), primers were designed for PCR amplification of the gene. The GTG start codon was changed to the more yeast-like ATG start codon when cloned in *S. cerevisiae*. The *xylA* gene was not sequenced after PCR amplification. For expression in *S. cerevisiae* the *xylA* gene was cloned between the phosphoglycerate kinase gene (*PGK1*) promoter and terminator in the multicopy yeast expression vector pMA91, resulting in pBXI, and transformed into *S. cerevisiae*. Production of XI in *S. cerevisiae* was seen in a Coomassie stained SDS-PAGE gel (Fig. 1). In the native form, *T. thermophilus* XI is a tetramer with a molecular mass of approximately 200 kDa (3). The molecular mass of the recombinant XI monomer was estimated to be 43 kD (Fig. 1), which is in reasonable agreement with the calculated molecular mass of 43.8 kDa. Cell extracts of H158(pBXI) were used in the determination of the XI activity at different temperatures (Fig. 2). The optimum temperature was at 85°C, where a specific activity of 1.0 U mg^{-1} was obtained. The activity decreased drastically at temperatures below 50°C. At 30 and 40°C, the activity was only 4 and 11% of the maximum, respectively. The optimum pH was 7 (data not shown). No XI activity was detected in reference strain H158(pMA91) (data not shown). Xylose consumption and product formation by H158(pBXI) and H158(pMA91) were studied under oxygen-limited conditions. The strains were cultivated in an SC-Leu minimal medium containing 30 g of xylose $liter^{-1}$ at 38°C (Fig. 3). H158(pBXI) consumed 10.4 g of xylose $liter^{-1}$ and produced 1.3 g of ethanol, 1.4 g of acetic acid, and 4 g of xylitol $liter^{-1}$. The reference strain consumed 3.6 g of xylose $liter^{-1}$ and produced 2.8 g of xylitol $liter^{-1}$. Neither ethanol nor acetic acid was produced by the reference strain. Growth, measured as dry weight at the beginning and end of the fermentations, was not possible under the cultivation conditions employed. The inability of the recombinant *S. cerevisiae* to grow on xylose (measured as increase in dry weight) is in accordance with the

observation that native *S. cerevisiae* does not grow on xylulose in minimal media (10).

The results show that the *xylA*-containing recombinant *S. cerevisiae* was able to convert xylose to xylulose with the aid of XI, resulting in the production of ethanol and acetic acid. The formation of xylitol, also observed in the reference strain, which does not carry the *xylA* gene, was due to an unspecific NADPH-dependent aldo-keto reductase present in *S. cerevisiae*, which converted xylose to xylitol until the cell's pool of reduced cofactors had been depleted (12). This aldo-keto reductase, with a K_m for xylose of 27.9 mM, has been purified from *S. cerevisiae*, and xylose reductase activities of 5 mU mg^{-1} were measured in both glucose- and ethanol-grown *S. cerevisiae* cultures, indicating constitutive expression (12). The metabolically engineered *S. cerevisiae* also formed acetic acid during xylose fermentation. Similarly, a shift in product formation, from ethanol to acetic acid, occurred in *S. cerevisiae* cometabolizing xylulose and glucose, compared with the situation when only glucose was fermented (10a).

The present investigation is the first successful attempt to express the procaryotic gene *xylA* for the enzyme XI in the eucaryote *S. cerevisiae*. The success in producing an active XI by expressing the *T. thermophilus xylA* gene in *S. cerevisiae* could be due to the relatedness between the two organisms. Taxonomically, *T. thermophilus* diverged from the eubacteria and might in many respects be more closely related to *S. cerevisiae* than are the eubacteria. Comparison of XI amino acid sequences from several bacteria has shown that regions with substrate-binding residues and catalytic residues are different in *T. thermophilus* (3). The homology in these regions is high for the other bacteria. A dendrogram of known bacterial *xylA* sequences shows that they are divided into two subgroups (Fig. 4). With *A. missouriensis xylA* being the only exception, *T. thermophilus xylA* belongs to a different subgroup from the *xylA* genes that have previously been cloned in *S. cerevisiae.*

In the construction of an efficient recombinant *S. cerevisiae* strain carrying the *xylA* gene it might be relevant to search for a mesophilic XI within the same subgroup as *T. thermophilus xylA*. Alternatively, the temperature optimum for the *T. thermophilus* XI could be lowered by mutagenesis. Ethanolic fermentation of xylose with *S. cerevisiae* containing the *xylA* gene could be further improved by deleting the *S. cerevisiae* aldo-keto reductase gene to prevent xylitol formation.

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