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Expression of *xylA* genes encoding xylose isomerases from *Escherichia coli* and *Streptomyces coelicolor* in the methylotrophic yeast *Hansenula polymorpha*

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Abstract

The thermotolerant methylotrophic yeast *Hansenula polymorpha* is able to ferment xylose to ethanol at high temperatures. *H. polymorpha* xylose reductase and xylitol dehydrogenase are involved during the first steps of this fermentation. In this article, expression of bacterial *xylA* genes coding for xylose isomerases from *Escherichia coli* or *Streptomyces coelicolor* in the yeast *H. polymorpha* was shown. The expression was achieved by integration of the *xylA* genes driven by the promoter of the *H. polymorpha* glyceraldehyde-3-phosphate dehydrogenase gene (*HpGAP*) into the *H. polymorpha* genome. Expression of the bacterial xylose isomerase genes restored the ability of the *H. polymorpha* Δ*xyl1* mutant to grow in a medium with xylose as the sole carbon source. This mutant has a deletion of the *XYL1* gene encoding xylose reductase and is not able to grow in the xylose medium. The *H. polymorpha* Δ*xyl1*(*xylA*) transformants displayed xylose isomerase activities, which were near 20% of that of the bacterial host strain. The transformants did not differ from the yeast wild-type strain with respect to ethanol production in xylose medium.

Keywords: *xylA* gene; Xylose isomerase; Expression in methylotrophic yeast; *Hansenula polymorpha*; Xylose fermentation

1. Introduction

Fuel ethanol production from the renewable plant material, lignocellulose, has a great economic and ecological significance [1,2]. A simultaneous saccharification and fermentation (SSF) process, that combines enzymatic hydrolysis of pretreated lignocellulose by cellulases and hemicellulases with fermentation of the produced hexoses and pentoses to ethanol, potentially is one of the most efficient ways to convert lignocellulosics to ethanol [3,4]. But SSF technology is not developed so far. One of prerequisites for development of this technology is obtaining microorganisms able to actively ferment the abundant sugar from lignocellulose, xylose, at elevated temperatures. Recent studies have indicated that the thermotolerant methylotrophic yeast *Hansenula polymorpha* ferments xylose, glucose and cellobiose to ethanol at elevated temperatures (37–40 °C) under semi-aerobic conditions [5]. Yeasts and most of the filamentous fungi that are able to ferment xylose can carry this out using NAD(P)H-dependent xylose reductase and NAD^+-dependent xylitol dehydrogenase. This results in a redox imbalance causing a low efficiency of the fermentation of xylose and accumulation of large

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amounts of xylitol instead of ethanol [1,2]. One of the ways to bypass the redox imbalance bottleneck for efficient xylose fermentation is to express the bacterial xylose isomerase gene in yeast [1,2,6]. There have been several attempts to express bacterial xylose isomerase genes in *Saccharomyces cerevisiae* but the results were unsuccessful because of folding problems and low activities of the isomerases [7–9]. Only the mutant xylose isomerase from the thermophilic bacterium *Thermus thermophilus* and the corresponding enzyme from the anaerobic fungus *Piromyces* sp. functioned efficiently in *S. cerevisiae*. This has enabled the isolation of yeast strains that ferment xylose effectively [10–13].

In this paper, we report on expression of bacterial *xylA* genes from *Escherichia coli* and *Streptomyces coelicolor*, coding for xylose isomerases, in the methylotrophic yeast *H. polymorpha*. The expression was achieved by integration of the *xylA* genes driven by the promoter of the *H. polymorpha* glyceraldehyde-3-phosphate dehydrogenase gene (*HpGAP*) into *H. polymorpha* genome. Expression of the xylose isomerase genes restored the ability of the *H. polymorpha* Δ*xyl1* mutant to grow in a medium with xylose as sole carbon source. The mutant has a deletion of the *XYL1* gene encoding the xylose reductase and is not able to grow in the xylose medium. The *H. polymorpha* Δ*xyl1*(*xylA*) transformants displayed xylose isomerase activities. They did not differ from the yeast wild-type strain with respect to ethanol production in xylose medium.

2. Materials and methods

2.1. Strains and media

H. polymorpha CBS4732s *leu2-2*, deficient in β-isopropyl malate dehydrogenase [14], was used as a parental strain for isolation of the Δ*xyl1* mutant (see Section 2.3). This *leu2-2* strain was kindly provided by Dr. K. Lahtchev (Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria). Yeast strains and transformants were cultured at 37 °C in a minimal medium consisting of 0.67% YNB without amino acids (Difco, Detroit, MI, USA), 4% xylose or 2% glucose. For the CBS4732s *leu2-2* strain leucine (40 mg l^{-1}) was supplemented into the medium.

The *E. coli* DH5α strain (Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169) was used as a host for propagation of plasmids. Genomic DNA of this strain was used as a source for isolation of the fragment carrying the *xylA* ORF. DH5α was grown at 37 °C in LB medium as described previously [15]. Transformed *E. coli* cells were maintained on a medium containing 100 mg l^{-1} of ampicillin or erythromycin.

The *S. coelicolor* A3(2) strain was used as a source for isolation of the genomic DNA fragment harbouring the *xylA* ORF of this species. This strain was kindly provided by Dr. V. Fedorenko (Department of Genetics and Biotechnology, National University of Lviv, Ukraine). The *S. coelicolor* strain was cultured in TSB (Tryptic Soy Broth) medium (Sigma, St. Louis, MO, USA) at 28 °C.

2.2. Molecular-biology techniques

Genomic DNA of *E. coli*, *S. coelicolor* and *H. polymorpha* was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Restriction endonucleases and DNA ligase (Fermentas, Vilnius, Lithuania) were used according to the manufacturer's specifications. Plasmid isolation from *E. coli* was performed with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega). DNA fragments were separated on a 0.8% agarose (Fisher Scientific, Fairlawn, NJ, USA) gel in 1 × TAE [15]. Isolation of fragments from the gel was carried out with the DNA Gel Extraction Kit (Millipore, Bedford, MA, USA). Amplification of the *E. coli* or *S. coelicolor xylA* ORFs was done with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specification. PCRs were performed in GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Transformation of the yeast *H. polymorpha* by electroporation was carried out as described previously [16].

2.3. Isolation of the H. polymorpha Δxyl1 mutant

The *H. polymorpha* Δ*xyl1* mutant was obtained by deletion of an internal part of the *XYL1* gene ORF. A deletion cassette was constructed for this. The cassette, as a part of the plasmid p19L2LRA that is a derivative of the vector p19L2 [17], is shown in Fig. 1. Information on the *H. polymorpha XYL1* gene (*HpXYL1*) sequence was obtained from the *H. polymorpha* genome database, Rhein Biotech GmbH (Düsseldorf, Germany) (orf76, Hp_contig37). Primers K17for (CCC AAG CTT GTA GGC GTT CTT TAG TCT TC) and K18rev (CAT GCA TGC GTG ATA ATT GTT CCA TAG C) were designed and used for isolation of the ~1.1-kb 5′ fragment of the *HpXYL1*. This fragment consists of a 5′ uncoding region and an initial part of the *HpXYL1* ORF. Primers K19for (CGC GTC GAC CCT GGA ACG AAA TTC CAA C) and K20rev (CGC GGA TCC CAG CAC TTT CCG TTT GTG A) were designed and used for isolation of the ~1.5 kb 3′ fragment of the *HpXYL1*. The fragment consists of a short final part of the *HpXYL1* ORF and a 3′ uncoding region (Fig. 1). *Hin*dIII, *Sph*I, *Sal*I and *Bam*HI restriction sites were incorporated into K17for, K18rev, K19for and