Short Communication

Functional expression of *Burkholderia cenocepacia* xylose isomerase in yeast increases ethanol production from a glucose–xylose blend

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**ABSTRACT**

This study presents results regarding the successful cloning of the bacterial xylose isomerase gene (*xylA*) of *Burkholderia cenocepacia* and its functional expression in *Saccharomyces cerevisiae*. The recombinant yeast showed to be competent to efficiently produce ethanol from both glucose and xylose, which are the main sugars in lignocellulosic hydrolysates. The heterologous expression of the gene *xylA* enabled a laboratorial yeast strain to ferment xylose anaerobically, improving ethanol production from a fermentation medium containing a glucose–xylose blend similar to that found in sugar cane bagasse hydrolysates. The insertion of *xylA* caused a 5-fold increase in xylose consumption, and over a 1.5-fold increase in ethanol production and yield, in comparison to that showed by the WT strain, in 24 h fermentations, where it was not detected accumulation of xylitol. These findings are encouraging for further studies concerning the expression of *B. cenocepacia* *xylA* in an industrial yeast strain.

**1. Introduction**

The development of an efficient and cost-effective technology for the production of ethanol from lignocellulosic wastes has been a challenge due to the additional necessary steps for the processing of these recalcitrant materials in comparison to the ethanol from sugar cane (sucrose) and corn (starch), which are well-established commercial technologies in Brazil and the US, respectively. The sugar cane biomass (bagasse and straw) would be the obvious choice to increase ethanol productivity in Brazil due to its availability at industrial scale in the proximity of existing ethanol production plants (Stambuk et al., 2008).

Irrespective of the feedstock used to make the hydrolysates, glucose and xylose are often the predominant sugars (van Maris et al., 2006). Wild-type (WT) *Saccharomyces cerevisiae*, the preferred organism used for ethanol production, ferments glucose, the dominant sugar in all plant hydrolysates, at high rates, but is unable to ferment xylose (Fig. S1, Supplementary material). Efficient alcoholic fermentation of xylose by this yeast requires the introduction of heterologous enzymes to provide conversion of xylose into xylulose without causing cofactor imbalances. One of the best results reported to date was achieved with the cloning and expression of xylose isomerase (*XI*) from the fungus *Piromyces* sp. into *S. cerevisiae* (Cai et al., 2012). However, co-production of xylitol and low ethanol production rates remain problematic when fermenting with recombinant *S. cerevisiae*. The present work reports the cloning and successful expression of a *XI* from the prokaryote *Burkholderia cenocepacia* in *S. cerevisiae*. The recombinant *XI* showed high activity and enabled the genetically modified yeast to ferment xylose anaerobically, without xylitol accumulation, in a medium containing a glucose–xylose blend similar to that found in the sugar cane bagasse hydrolysates.
2. Methods

2.1. Bioinformatics analysis

Nucleotide and predicted amino acid sequences of the XIs were obtained from GenBank and compared with sequences of the fungus Piromyces sp using the BLAST algorithm (National Center for Biotechnology Information) and the program CLC Workbench 3.2 (http://workbench.sdsc.edu). Sequences were aligned to plot the phylogenetic tree using CLC Workbench.

2.2. Strains and plasmid construction

PCR was used to generate a 1,339-bp DNA fragment containing the full-length xylA, with primers xylA-F (5'-ATAAGCTTATGCGTA TTTGACAATATTCC-3') and xylA-R (5'-TATGGATCTCCACGCAACC CGTAgATC-3') from WT B. cenocepacia J2315 (ATCC BAA-245). The amplified xylA-containing DNA fragment was introduced into the HindIII/BamHI sites within the phosphoglycerate kinase (PGK) expression cassette in plasmid YEpPGK (Watanabe et al., 2007) to obtain YEpPGK-xylA. Finally, the engineered plasmid harboring xylA under the control of PGK promoter and URA3 as selectable marker was used to transform haploid strain BY4741 (MATa his3 leu2 met15 ura3) acquired from Euroscarf, Germany, using the lithium acetate protocol. Transformants were selected on synthetic (S) medium (0.67% yeast nitrogen base without aminoacids, 0.01% histidine, 0.01% leucine, 0.01% methionine and 2% agar) supplemented with 2% glucose.

3. Results and discussion

The isomerization of xylose to xylulose by XI is considered a promising approach to allow ethanol fermentation of xylose by S. cerevisiae due to its one-step conversion and cofactor-independent catalysis. Initially, we screened gene and protein databases for a putative XI, using the XI sequence from the fungus Piromyces sp. as reference. Piromyces XI was selected as a “bait” sequence because it has been considered to have the highest activity when expressed in S. cerevisiae (Cai et al., 2012). We selected XIs from 6 bacteria of different phylogenetic affiliations that exhibited nucleotide sequence identities ranging from 42% to 63% to xylA from the Piromyces sp. (Fig. 1). The level of identity found among the predicted protein sequences reflected that obtained in the gene alignment (Fig. 2). The best results were obtained with Xanthomonas oryzae, X. campestris, X. axonopodis and B. cenocepacia. XI from B. cenocepacia, which has not been characterized to date, was selected to be inserted into a high-copy number yeast expression vector and cloned in a laboratory yeast strain. Attempts to express xylA from some Xanthomonas failed to produce an active enzyme in S. cerevisiae (Brat et al., 2009).

To evaluate the ability of xylA-containing yeast to metabolize xylose, cells were cultivated at 28 °C aerobically on synthetic medium supplemented with 2% xylose using the WT strain as control (Fig. S2, Supplementary material). Pre-cultures of both strains were grown in synthetic medium containing 2% xylose or 2% glucose as carbon source, harvested after an overnight growth and used to inoculate fresh medium containing 2% xylose. As expected, WT cells did not grow on xylose. It was observed that xylA cells grew rapidly on xylose when pre-grown in a glucose-containing medium. However no growth was detected when cells were pre-grown on xylose, probably because the cloned xylA is under the control of a strong promoter, PGK1. This gene codes for 3-phosphoglycerate kinase, a glycolytic enzyme. Klimacek et al. (2010), observed that increasing the intracellular concentration of fructose-6-phosphate (F6P) is of relevance to improve xylose consumption. According to the authors, higher levels of F6P could lead to increased levels of glycolytic intermediates, affecting transcription of glycolytic and ethanologenic enzymes and, consequently, xylose catabolism. Another possible explanation is that xylose metabolism would be favored by pre-growth on glucose due to the induction of expression of hexose transporters involved in xylose uptake. At high glucose concentrations (above 1%), the expression of transporters exhibiting low affinity for glucose, such as Hxt1, is activated. This transporter showed the highest xylose uptake capacity among Hxts (Salheimo et al., 2007), suggesting that activation of its expression, as occurred under pre-growth in SD 2%, might improve xylose utilization.

Next, the XI activity was determined in crude extracts obtained from WT and recombinant yeasts, as well as in a crude extract obtained from the bacterium B. cenocepacia, as a control. Both organisms, S. cerevisiae and B. cenocepacia, were grown at standard conditions (in SD 2% at 28 °C/160 rpm and in LB medium at 37 °C/250 rpm, respectively) and collected at exponential growth phase. XI activity was assayed spectrophotometrically on the basis of NADH consumption (Brat et al., 2009). The heterologous expression of XI from B. cenocepacia led to significantly high activities in S. cerevisiae (Fig. S3, Supplementary material). The activity of XI found in recombinant S. cerevisiae corresponded to 75% of the activity measured in the crude extracts from B. cenocepacia, demonstrating that heterologous expression of xylA from B. cenocepacia in yeast resulted in active XI.

Fermentation experiments to compare the performance of the engineered S. cerevisiae cells expressing B. cenocepacia xylA to that of WT strain were carried out. Cells were pre-grown in SD 2% until mid-log growth phase and then transferred to a fermentation medium (3.0% glucose, 1.0% xylose, 0.4% (NH4)2SO4 and 0.4% KH2PO4) containing glucose and xylose at the same concentration and proportion found in sugar cane hydrolysates. Fermentation was carried out at 30 °C/pH 5.0 with initial cell concentration of 1.5 mg/mL. At the beginning and after 24 h of each fermentation, aliquots were harvested, centrifuged and the supernatants collected. Concentrations of glucose, xylose and xylitol were determined by HPLC (Shimadzu), using Li Chrospher 100 NH2 column (Merck) as stationary phase and 80% acetonitrile-20% H2O as mobile phase at a flow rate of 1.5 mL/min. Ethanol was evaluated as described by

Fig. 1. Phylogenetic tree of sequences of XI genes from bacteria Xanthomonas axonopodis, X. oryzae, X. campestris, Rhizobium etli, R. leguminosarum and B. cenocepacia and the fungus Piromyces.
It was observed that recombinant cells produced almost 2-fold more ethanol than WT cells (4.2 \times 7.8 \text{ g ethanol/L}, Fig. S4, Supplementary material). Glucose consumption was equivalent for both strains (around 13 g glucose/L), while ethanol/L, Fig. S4, Supplementary material). Glucose consumption was equivalent for both strains (around 13 g glucose/L), while...
xylose consumption by the engineered strain was 5-fold higher (0.5 × 2.5 g xylose/L), which could account for its higher ethanol production levels. The ethanol yield showed by the recombinant yeast (0.51 g ethanol/g sugar) was 45% higher than that of the WT strain. Besides, it is noteworthy that no accumulation of xylitol was observed in the recombinant cells.

To prove that expression of *B. cenocepacia* XI in *S. cerevisiae* is sufficient to enable the yeast to ferment xylose anaerobically, fermentation was conducted using xylose as the sole carbon source (4% xylose). The results showed an ethanol productivity of 0.05 g/g cell/h, a specific xylose consumption rate of 0.19 g/g cell/h and no xylitol production. Kuyper et al. (2003) showed that the expression of XI from *Piromyces* in the laboratorial strain CEN.PK113-7D resulted in a *S. cerevisiae* strain which displayed an ethanol productivity of 0.04 g/g cell/h and a xylose consumption rate of 0.12 g/g cell/h, besides producing low xylitol levels, in a medium supplemented with 2% xylose. The ethanol yield on 4% xylose obtained by the engineered yeast from this study was low (0.23 g ethanol/g xylose), which could be due to the lack of glucose to fully activate the *PCK1* promoter used in the *xylA* construction as well as to increase the carbon flux through glycolysis. Indeed, upon the use of increased glucose concentrations it was observed a corresponding increase on ethanol yields and productivities: 0.35 g ethanol/g sugar and 0.11 g ethanol/g cell/h in a 2% glucose-2% xylose blend and 0.51 g/g sugar and 0.19 g/g cell/h in a 3% glucose-1% xylose blend. Taken together, these results suggest that the introduction of *xylA* from *B. cenocepacia* into *S. cerevisiae* resulted in an efficient xylose-fermenting yeast, capable of anaerobically metabolizing glucose-xylose blends with high ethanol yield and without accumulation of xylitol.

*S. cerevisiae* has several advantages for ethanol production from lignocellulosic materials: it efficiently produces ethanol from hexoses, does not require oxygenation, has low optimum pH and a relatively high tolerance to ethanol and inhibitors produced during chemical hydrolysis of lignocellulose (Stambuk et al., 2008). The disadvantage of this yeast is that it cannot ferment xylose, which could be overcome by recombinant DNA technology. In the last 20 years, several studies have been performed in an attempt to

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**Fig. 2.** (continued)
develop a \textit{S. cerevisiae} strain able of anaerobically ferment xyllose with high ethanol yields and productivities, without xylitol accumulation. It is noteworthy that the ethanol yield measured for the modified laboratorial yeast strain from this work, presenting a single genetic modification, was higher than those observed for strains which were submitted to extensive metabolic and evolutionary engineering procedures. Although 100% of the theoretical yield has been obtained (Fig. S4, Supplementary material), it is important to note that the calculations were made considering sugars consumption. If all initial sugars concentration would be fully converted to ethanol, 2.5-fold more ethanol would be produced. However, even the recombinant yeast expressing \textit{B. cenocepacia} xylA had not reached its maximum fermentation potential, these findings provide an excellent starting point for further improvement of xyllose fermentation by industrial yeast strains.

Cai \textit{et al.} (2012) compared the fermentation performance of 30 recombinant \textit{S. cerevisiae} strains, able to convert xyllose to xylulose through either the heterologous expression of xyllose reductase (XR) and xylitol dehydrogenase (XD) genes or heterologous expression of a XI gene. In this review, the best result was achieved with the recombinant strain RWB218, carrying the XI of the fungus \textit{Piromyces}. Strain RWB218 carries the XI of the fungus \textit{Piromyces}, besides several additional genetic modifications (overexpression of XKS1, TAL1, TKL1, RKI1, and RPE1 and deletion of GRE3) and evolutionary adaptation. As a result, it showed the highest ethanol production and xyllose consumption rates, besides no synthesis of xylitol. With RWB218 cells, a 24 h fermentation of a 2% glucose and 2% xyllose blend resulted in 0.43 g ethanol/g sugar and 0.2 g ethanol/g cell/h (van Maris \textit{et al.}, 2007). Similar ethanol conversion yield was achieved by an engineered \textit{S. cerevisiae} strain overexpressing \textit{Piromyces} XYL3 (integrated in the genome). \textit{P. stipitis} XYL3 and all genes of the non-oxidative pentose phosphate pathway, besides being submitted to a three-stage process of xylose adaptation (Zhou \textit{et al.}, 2012). Although most of the recombinant \textit{S. cerevisiae} strains carrying heterologous XR–XD genes produced considerable amount of xylitol at a high yield, the strain F106KR, containing a mutation in XR that altered the preference of NADH to NADPH as well as several other genetic modifications, showed a high capacity to produce ethanol from high xyllose concentrations (Xiong \textit{et al.}, 2011). The yields of F106KR from 100 g/L glucose and 100 g/L xyllose in 72 h were 0.42 g ethanol/g and 0.07 g xylitol/g.

The recombinant strain developed in this study showed an equally good fermentation performance, if not better, since it presented the same ethanol productivity and a yield 28% higher in comparison to the RW2B18 strain. Therefore, it is a good reason to consider that the sole heterologous expression of \textit{B. cenocepacia} XI is advantageous in comparison to the seven modifications in the xylose adapted RW2B18. Since chromosomal integration is necessary for strains to be used under industrial conditions, XI pathway from \textit{B. cenocepacia} is a better choice unless all genetic modifications performed in strain RWB218 could be chromosomally integrated. The sole heterologous expression of \textit{B. cenocepacia} XI would be advantageous due to the higher stability of the recombinant yeast strain.

Interestingly, the recombinant yeast from this study did not accumulate xylitol. It is generally agreed that the introduction of XI and deletion of the endogenous reductase encoded by GRE3 results in a strong decrease in xylitol formation leading to full conversion of xyllose to xylulose. However, only the \textit{Piromyces} sp. XI was able to avoid xylitol production – this phenotype was attributed to a highly active fungal XI upon its expression in yeast. The recombinant strain expressing \textit{B. cenocepacia} xylA showed no xylitol accumulation and a fermentation performance comparable to yeasts expressing the fungal XI and deficient in GRE3, even though the XI activity was 20-fold lower than that of the yeast bearing the \textit{Piromyces} sp. XI gene. These data suggest that the heterologous XI from \textit{B. cenocepacia} is more efficient to convert xyllose to xylulose than the endogenous \textit{S. cerevisiae} aldose reductase, which converts xyllose to xylitol.

4. Conclusions

The recombinant bacterial enzyme exhibited significant activity in \textit{S. cerevisiae} and enabled yeast to metabolize xyllose, improving ethanol production without xylitol accumulation. The ethanol yield obtained by the strategy used in this work was equivalent to strains submitted to extensive metabolic and evolutionary engineering. We envision that the strategy of engineering \textit{S. cerevisiae} with xylA from \textit{B. cenocepacia} is very promising to make ethanol from biomass hydrolysate economically viable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.10.014.

References


