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Cloning and characterisation of the xylose transporter coding gene of *Debaryomyces hansenii* in *E. coli*

Clonación y caracterización del gen codificador del transportador de xylose de *Debranomyces hansenii* en *E. coli*

DE LA RIVA-DE LA RIVA, Gustavo Alberto*†, COLLI-MULL, Juan Gualberto and JUÁREZ-SALDAÑA, Eric

Instituto Tecnológico Superior de Irapuato (ITESI). Irapuato-Silao Highway km 12.5. El Copal, Irapuato, Guanajuato, Mexico.

ID 1st Author: *Gustavo Alberto, De La Riva-De La Riva*

ID 1st Co-author: *Juan Gualberto, Colli-Mull*

ID 2nd Co-author: *Eric, Juárez- Saldaña*

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Abstract

Xilitol is 5 C alcohols, and noncariogenic noncaloric sweetener usually obtained by a chemically reaction of D-xylose hydrogenation. An alternative to this reduction reaction is the use of highly polyol producing yeast from genus *Debaryomyces* and *Candida*. In our project we have amplified by PCR and cloned the gen encoding for a transmembranal transpoter of xylose. The cloned gene was sequenced and characterized for further transfer to Saccharomyces cerevisiae to study the capacity of transformed yeast to use xylose as a carbon source and its possible role in xylitol production.

***D. hansenii*, Xylose, Conveyor**

Resumen

El xilitol es un alcohol de 5 C, es un edulcorante no calórico, es obtenido por la reducción química del azúcar D-xilosa por hidrogenación. Una alternativa de la producción química es la utilización de levaduras altamente productoras de polioles del género *Debaryomyces* y *Candida*, aplicando ingeniera genética. En nuestro proyecto hemos amplificado por PCR y clonado en E. coli el gen codificador del transportador de xilosa, para posteriormente transferirlos expresarlos en cepas de Saccharomyces cerevisiae para estudiar la capacidad de utilizar xilosa como fuente de carbono y su posible rol en la producción de de xilitol.

***D. hansenii*, Xilosa, Transportador**

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* Correspondence to Author (Email: gudelariva@itesi.edu.mx)
† Researcher contributing first author.

Introduction

Fermentation processes have always been a field of permanent interest in biotechnological research and industrial biotechnology development. The production of ethanol and xylitol by fermentative processes is one of the areas where fermentative processes are constantly being improved for applications in both the food industry and biofuel production. Currently, fermentative processes are used to convert residues from primary forest products into industrial by-products such as ethanol and xylitol. To improve this type of process we sought to create genetically modified Saccharomyces cerevisiae strains capable of using xylose as a carbon source. These attempts have not resulted in more efficient processes because S. cerevisiae is a very efficient yeast in hexose fermentation processes, but incapable when it comes to using pentoses as a carbon source. For this reason, we tried to improve this situation by transferring the xylose transporter coding gene from *Debaryomyces hansenii* into S. cereviciae strains. This is a hylotrophic yeast that efficiently uses pentoses and xylose as a carbon source.

Xylitol is a major by-product in the production of ethanol from lignocellulosic by-products that is widely used in the food and pharmaceutical industries, as it has useful characteristics, including its use for the prevention of dental caries, as a sugar substitute for insulin-independent diabetics, and as a natural food sweetener (Makinen, 1992).

Xylitol is currently produced on an industrial scale by catalytic reduction (hydrogenation) of xylose obtained from wood sources such as white birch trees. *Candida intermedia* has been reported (Leandro et al; 2006) as a yeast with the ability to grow in xylose-rich media and to transport this pentose by two different transport systems: a high-affinity system, in which it is carried out by H^+ symport, and a low-affinity system by facilitated diffusion; both systems use glucose as substrate. *D. hansenii* has been described as a halophilic-halotolerant yeast (González-Hernández, J. C. et al., 2004, 2005), which can metabolise D-xylose to xylitol (Gírio et al., 2000). This characteristic is an interesting and potential biotechnological aspect due to the dietary and clinical characteristics of xylitol.

At the same time, a molecular approach will be followed to identify the genes involved in this process (GXF1, GXS1, XR). Our aim is to have basic knowledge of the xylose transport and metabolism systems in *D. hansenii*; and in the immediate future to establish and propose this technology for xylitol production. The enzyme xylose reductase (XR) is responsible for the first step in xylose metabolism in yeast (Chiang and Knight, 1960). In a reaction catalysed by this enzyme, xylose is reduced to xylitol which can be oxidised to xylulose by the enzyme xylitol dehydrogenase (XDH) or can be released into the environment, depending on the conditions of the culture medium (Kern et al., 1997; Ho et al., 1998). Studies on the extraction and purification of XR from yeast have been conducted with the aim of characterising the enzyme to implement better fermentation processes or to obtain a purified solution of XR to be used directly for the conversion of xylose to xylitol (Mayerhoff et al., 2001; Cortez et al., 2001).

Candida magnoliae is a yeast capable of growing and utilising xylose as a carbon source and, on the other hand, accumulating xylitol, preventing it from being transformed into D-xylulose. *D. hansenii* is a highly polyol-producing yeast that can have a potential use for the utilisation of lignocellulosic hydrolysates, which has been extensively studied for this bioconversion to take place.

We have designed a strategy for the study and characterisation of the xylose transporter gene of *D. hansenii*. PCR amplification of the complete gene and separate PCR amplification of the promoter and coding region fragments was performed. The amplified fragments were cloned into an E. coli vector and sequenced. We also included a strategy to manipulate these fragments for transfer to an ethanol-overproducing *Saccharomyces cerevisiae* strain isolated from industrial processes to evaluate their behaviour and fermentation efficiency in both ethanol and xylitol production.

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Materials and methods

Strains and culture media

Four different yeast strains were used, two of them from *D. hansenii* and *C. magnoliae*, which grow efficiently using xylose as carbon source and two *Saccharomyces cerevisiae*, one of them, *Saccharomyces cerevisiae* W303-1 Ura-, is an auxotrophic mutant to test the constructs to be tested and the other is the *Saccharomyces cerevisiae* strain ITM 2014 and is characterised by its high efficiency in fermentative ethanol production (Table 1). The layers were grown on various culture media, YPD being the most suitable.

It included yeast extract, usually 1% mass/volume ratio with water, 2% peptone, 2% glucose. When glucose was required, it was replaced by 2% xylose and called YPX. *E. coli* strain DH5 alpha was used for cloning. And grown on LB medium. When required, the medium was supplemented with 75 mg/l ampicillin, and/or 40 µg/l 5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 40 µg/l isopropyl-β-D-thiogalacpyranoside (IPTG).

Yeast kinetics in YPD and YPX media

A growth kinetics of the yeasts of interest for this project was performed to determine their behaviour both on glucose and xylose in the culture medium. These yeasts were grown in 50 mL batches of YPD (Yeast Extract, Peptone and Glucose 2%) or YPX (Yeast Extract, Peptone and Xylose 2%) media at 110 rpm and a temperature of 28°C. Samples were taken for 24 hours every 2 hours and optical density parameters were measured at 595 by spectrophotometry in visible light. The pH was also measured, the number of cells per mL of culture was determined (seeding dilutions every 4 hours).

Design and synthesis of primers for PCR amplification

Based on the comparative analysis of nucleotide sequence information of *C. intermedia and D. hansenii* from the NCBI GenBank and EMBL databases (www.ncbi.nlm.nih.gov; www.embl.de), different pairs of primers or oligonucleotides are designed to facilitate the cloning of the regulatory and coding regions of the genes from total DNA.

The characteristics of the primers are summarised and their purpose is summarised in the table below.

Extraction of total DNA from *Debaryomyces hansenii*

We established the optimal growth conditions for *D. hansenii* by using two methods: one based on a commercial extraction kit (Yeastar Genomic DNA kitTM, Zymo Research) and the other based on breaking with glass beads and detergent solution (Winston Solution: 2% Triton, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1mM EDTA).

PCR amplification of DNA fragments corresponding to the complete xylose transporter gene, its promoter region and coding region.

Conditions were optimised for separate amplification of the promoter region (1'-95°C, 1'-53°C, 1'-72°C), the coding region and its terminator (1'-95°C, 1'-53°C, 1'-72°C). Coding region and its terminator (1'-95°C, 1'-53°C, 2'-72°C) and the whole gene (1'-95°C, 1'-53°C, 3'-72°C). The reactions were carried out by standard Polymerase Chain Reaction methodology using DreamTaq™ (ThermoscietificTM). The amplified fragments were analysed by electrophoresis in TA buffer and purified by a commercial kit (ZymocleanTM Gel, Biosys TM).

Cloning in *E. coli* and sequencing

The purified fragments were ligated into a commercial TOPO vector (TOPOTM PCR Cloning, Life Science) and the ligation was transformed into E. coli DH5 alpha competent cells and selected in LB medium supplemented with 100 mg/l ampicillin, 40 µg/l 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 40 µg/l isopropyl-β-D-thiogalacpyranoside (IPTG).

And incubated at 37°C for 18 hours. Possible clones were analysed by restriction with the enzymes Xho1, Spe1 and Sac1 and by analytical PCR using the primers corresponding to each type of amplified fragment (Table 1). The selected E. coli clones were purified and sent for sequencing (T4 OligoTM).

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Results and Discussion

Optimisation of culture conditions. Yeast kinetics in YPD and YPX media

Optimal growth conditions for *Debaryomyces hansenii* and *Candida magnoliae* and the two *Saccharomyces cerevisiae* strains were established. Different rich media were tested including YNB (Yeast Nitrogen Base) and YPD (Yeast Extract, Peptone and Glucose or Xylose) medium. The most suitable medium proved to be the but in the case of *Saccharomyces cereviciae* strain W303-1 Ura-, growth, even on YPD, was always slower compared to the other strains.

Kinetics of *D. hansenii* and *S. cereviciae* yeasts in YPD and YPX media

It was verified that *Debaryomyces hansenii* and *Candida magnoliae* grow satisfactorily both in the presence of glucose (YPD medium) and in the presence of xylose (YPX) while *S. cereviciae* grows efficiently only in the presence of glucose as carbon source. With this experiment we verified that our *Debaryomyces hansenii* and *Candida magnoliae* strains do indeed possess the ability to internalise xylose.

First, the growth conditions were optimised for each of the four yeast strains that will be used throughout this project. The results show that the strains (Fig. 1).

The growth kinetics (Fig. 1) allowed us to determine the behaviour of both the strains that will be sources of the gene of interest and those that will be hosts of the heterologous gene, determining that the former have the capacity to internalise xylose and metabolise it and the latter cannot, although a certain basal level of xylose internalisation is observed. This is since the YPX medium has a certain number of hexoses that allow basal growth. From this result, we have developed a minimal medium, like M9, composed of salts and where we can completely manipulate the carbon source for each type of yeast we transform. In this way, the transformants that grow on xylose medium will only be those that have received and correctly expressed the gfx gene, which codes for a fully functional protein in the host.

Comparative study of xylose transporter protein sequences

We performed a comparative analysis of the known sequences of the xylose transporters (GenBank www.ncbi.nlm.nhi.gov) of *Candida intermedia* with the sequences corresponding to the two reported complete genomes of *D. hansenii*. The analysis allowed the determination of the region and predicted nucleotide sequence of these homologous genes. We used the *C. intermedia* PYCC 4715 genes gxf1 (glucose/xylose facilitator) and gxs1 (glucsa/xylose symporter, accession number AJ875406) as well as the reported *D. hansenii* genome sequences (*D. hansenii* MTCC 234 and *D. hansenii* CBS767). From this analysis we designed the primers used for PCR amplification (Table 2).

Amplification of the *D. hansenii* gfx gene by PCR and cloning into *E. coli* vector

We first standardised conditions for the extraction of total yeast DNA. As this is a novel yeast, we used the method of total DNA extraction with glass beads and Winston solution but introduced the use of ximolase and the columns of the commercial kit.

With the primers designed, PCR amplification was carried out to amplify the fragments corresponding to the promoter region, coding region, terminator, and complete gene (Figure 2), which were subsequently ligated into a commercial TOPO vector (TOPOTM PCR Cloning, Life Science) and transformed by electroporation into electrocompetent cells.

E. coli DH5 alpha cells. These were seeded onto plates of LBA xgal/IPTG medium and white colonies were selected, which indicated that these cells contained the DNA insert.

The selected clones were analysed by restriction with the enzymes Spe1, Sac1 and Xho1 and by analytical PCR, which gave certainty to the clones of the pPTX series, containing the promoter fragment, pRCTX containing the coding region fragment and PGTX containing the complete gene. The result was corroborated by sequencing of the cloned fragments and their subsequent comparison with the known sequences of the homologous genes.

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Further manipulation of this gene is aimed at cloning it into the plasmid vector of pYES and introducing it into S. cerevisiae W303-1A Ura-la allowing selection by auxotrophy and evaluating in this system the expression of the gene and the functioning of the recombinant protein in medium using xylose as a carbon source.

Subsequently, an integrative vector will be used to transform the S. cerevisiae ITM 2014 strain that is able to use both hexoses and xylose as a carbon source. This, together with the fact that it is a very efficient strain in ethanol production, offers the possibility of optimising industrial alcoholic fermentation processes in the mezcal and wine industries using various fruit substrates, biofuel production and xylitol production.

Conclusions

D. hansenii and *C. magnoliae* can use either hexoses (glucose) or pentoses (xyloses) as carbon source whereas *S. cerevisiae* only grows efficiently on substrates containing hexoses. If S. cerevisiae acquires the ability to internalise xylose more efficiently, this may result in optimisation of various industrial fermentation processes, including better utilisation of lignocellulosic hydrolysers, production of wines and mezcal, and the possibility of designing an alternative process for xylitol production.

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