SIMULTANEOUS BIOCONVERSION OF GLUCOSE AND XYLOSE TO ETHANOL BY RECOMBINANT YEAST

by

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To my lifelines: Amma, Achan and Shijo,

for their unconditional love, support

and faith through thick and thin...

CERTIFICATE

This is to certify that the thesis entitled **"Simultaneous Bioconversion of Glucose and Xylose to Ethanol by Recombinant Yeast"** being submitted by **Ms. Anjali Madhavan** to the Indian Institute of Technology Delhi for the award of the degree of **'Doctor of Philosophy'**, is a record of the bonafide research work carried out by her, which has been prepared under our supervision in conformity with the rules and regulations of the "Indian Institute of Technology Delhi". The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

Prof. V. S. Bisaria

Dr. Aradhana Srivastava

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Anjali Madhavan

ABSTRACT

Lignocellulosic biomass from agricultural and agro-industrial residues represents one of the most important renewable resources that can be utilized for the biological production of ethanol. *Saccharomyces cerevisiae* is widely used for the commercial production of ethanol from sucrose or starch-derived glucose. While the glucose derived from biomass can be fermented to ethanol efficiently by *S. cerevisiae*, the major pentose sugar D-xylose remains unutilized. Nevertheless, D-xylulose, the keto isomer of xylose, can be fermented slowly by the yeast.

In most naturally occurring xylose-assimilating yeasts and fungi, the conversion of xylose to xylulose is mediated by two-step redox reactions catalyzed by the NADPH-dependent xylose reductase followed by the NAD^+-dependent xylitol dehydrogenase, with xylitol as the pathway intermediate. However, when expressed in *S. cerevisiae*, the different cofactor specificities of these two enzymes lead to considerable accumulation of xylitol as byproduct under anaerobic conditions. On the other hand, bacteria and a few fungi can directly isomerize xylose to xylulose in a single step via the divalent metal ion dependent enzyme xylose isomerase (XylA). However, bacterial XylA are mostly thermophilic in nature and inactive or misfolded when expressed in *S. cerevisiae*. Other bottlenecks in xylose fermentation include low activity of the Pentose Phosphate pathway enzymes, such as xylulokinase, and competitive inhibition of xylose transport into the cell cytoplasm by glucose.

The approach in the present study was, therefore, to incorporate a functional pathway for the efficient metabolism of xylose in yeast *S. cerevisiae* in order to overcome the potential bottlenecks during the fermentation of xylose in the presence of glucose.

The first step in developing such recombinant xylose-utilizing yeast was to introduce the gene for xylose isomerase (*xylA*) in *S. cerevisiae*, capable of producing functional enzyme during heterologous expression. The anaerobic fungus *Orpinomyces*, found in the rumen of herbivorous animals to enable the digestion of cellulosic and hemicellulosic plant material, was selected as the source of the *xylA* gene so that a recombinant enzyme could be produced in yeast which was compatible with the eukaryotic and mesophilic conditions encountered in *S. cerevisiae*. Therefore, the *Orpinomyces xylA* gene was cloned and expressed constitutively in *S. cerevisiae* under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter. Cell extract of the recombinant strain, grown on glucose, exhibited up to 1.91 IU mg^{-1} protein of specific xylose isomerase activity in the absence of xylose induction. Further characterization revealed that the recombinant enzyme was a homodimer with a subunit of molecular mass 49 kDa. The pH optimum of the enzyme was 7.5, while the low temperature optimum at 37 °C was the property that differed significantly from the majority of the reported thermophilic xylose isomerases. Furthermore, by expression of the *xylA* gene, the recombinant strain exhibited growth on xylose as the sole carbon source under aerobic conditions.

Secondly, in an attempt to improve the slow rate of xylulose utilization observed during the growth of the *xylA* expressing *S. cerevisiae* strain, the endogenous xylulokinase (*XKS*) gene was overexpressed constitutively in the recombinant yeast, along with *xylA*. The higher XKS activity resulted in a 1.5-fold increase in the specific growth rate during aerobic cultivation on xylose; however, the overall xylose consumption and biomass production were significantly reduced. Nevertheless, the

overexpression of *XKS* increased the anaerobic ethanol productivity from xylose by a factor of 1.7.

A third gene *SUT1*, coding for a xylose-transporting sugar permease from the natural xylose-assimilating yeast *Pichia stipitis*, was also expressed constitutively, in addition to *XKS* and *xylA*, in order to overcome the poor kinetics of xylose transport into the cell. Expression of *SUT1* resulted in a 1.5-fold increase in the ethanol productivity from xylose. Notably, ethanol was the major product of xylose fermentation with a yield of 0.39 g (g xylose consumed)$^{-1}$ and in comparison, the xylitol yield of 0.08 g (g xylose consumed)$^{-1}$ was considerably lower.

The recombinant strain was adapted for enhanced growth on xylose by serial culture in xylose containing minimal medium under aerobic conditions. After repeated batch cultivations, a strain was isolated which grew with a higher specific growth rate of 0.133 h^{-1}. The adapted strain (ADAP8) could ferment 20 g l^{-1} of xylose to ethanol with a yield of 0.37 g (g xylose consumed)$^{-1}$ and productivity of 0.026 g l^{-1} h^{-1}. Raising the fermentation temperature from 30 to 35 °C resulted in a substantial increase in ethanol yield [0.43 g (g xylose consumed)$^{-1}$] and productivity (0.07 g l^{-1} h^{-1}) with a significant reduction in the xylitol yield. By the addition of a sugar complexing agent, such as sodium tetraborate, further improvement in ethanol yield and productivity as well as a reduction in xylitol accumulation was achieved. Furthermore, ethanol production from xylose was demonstrated successfully in complex medium containing yeast extract and peptone. The anaerobic fermentation of xylose at 35 °C with the adapted strain, performed in complex medium containing a fairly low but optimal concentration of borate, gave the highest concentration of ethanol (7.63 g l^{-1}) and ethanol productivity

(0.191 g l^{-1} h^{-1}) compared to all the conditions tested. The ethanol yield of 0.48 g (g xylose consumed)$^{-1}$ observed was 94 % of the theoretical maximum.

Moreover, under these conditions, the simultaneous fermentation of a mixture of glucose and xylose was also achieved. During fermentation of a mixture of 50 g l^{-1} glucose and 20 g l^{-1} xylose, the highest ethanol concentration, yield and productivity of 33.20 g l^{-1}, 0.48 g (g sugars consumed)$^{-1}$ and 0.83 g l^{-1} h^{-1}, respectively, were achieved after 40 h. A substantial reduction in the production of xylitol was observed in the presence of borate, with the accumulation of only 0.95 g l^{-1} xylitol at the end of fermentation. However, the rate of glucose consumption was considerably higher than that for xylose. The recombinant xylose-utilizing strain was also evaluated for its ability to ferment the sugars derived from detoxified corn fiber hydrolysate. After 48 h of fermentation, 11.67 g l^{-1} of ethanol could be produced from the overlimed hydrolysate with a yield of 0.45 g (g total sugars consumed)$^{-1}$ and low xylitol accumulation. The present study is, by far, the first report for the successful fermentation of a lignocellulosic hydrolysate by a xylose isomerase-expressing recombinant *S. cerevisiae* strain.

CONTENTS