DNA microarray analysis of the expression of the genes encoding the major enzymes in ethanol production during glucose and xylose co-fermentation by metabolically engineered *Saccharomyces* yeast

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Abstract

Lignocellulosic biomass, which contains large amounts of glucose and xylose, is the new ideal feedstock for ethanol production used as renewable liquid fuel for transportation. The naturally occurring *Saccharomyces* yeasts traditionally used for industrial ethanol production are unable to ferment xylose. We have successfully developed genetically engineered *Saccharomyces* yeasts that can effectively co-ferment both glucose and xylose simultaneously to ethanol. Our engineered yeast contains three xylose metabolizing genes, the xylose reductase (XR), xylitol dehydrogenase (XD) and xylulokinase (XK) genes, fused to glycolytic promoters, on high copy plasmids or integrated into the yeast chromosome in multiple copies. Although our glucose/xylose co-fermenting yeasts are currently the most effective yeast for producing ethanol from cellulosic biomass, they still utilize glucose more efficiently than xylose. We believe that carefully analyzing gene expression during co-fermentation of glucose and xylose to ethanol, using our genetically modified strains, will reveal ways to optimize xylose fermentation. In this paper, we report our results on analyzing the expression of genes in the glycolytic and alcoholic fermentation pathways using microarray technology. We also report the results on the analysis of the activities of the selected enzymes for ethanol production during co-fermentation of glucose and xylose to ethanol by one of our effective glucose/xylose co-fermenting yeasts 424A(LNH-ST).

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1. Introduction

Recent studies have proven ethanol to be an ideal liquid fuel for transportation. Lignocellulosic biomass (corn stover, rice straw, sugarcane bagasse, corn fiber, softwood, hardwood, grasses, etc.) is the ideal renewable resource for the production of fuel ethanol by fermentation. The major fermentable sugars from the hydrolysis of lignocellulosic biomass are *d*-glucose and *d*-xylose. However, there is no known naturally occurring microorganism that can effectively ferment both glucose and xylose simultaneously. Therefore, we have developed genetically engineered *Saccharomyces* yeasts that not only metabolize xylose, but also have altered control mechanisms for sugar utilization. This was accomplished by cloning the xylose reductase gene (XR), xylitol dehydrogenase gene (XD) and xylulokinase gene (XK), fused to glycolytic promoters (known collectively as the KDR genes), on high copy plasmids or integrated into the yeast chromosome. This genetic manipulation can easily convert most *Saccharomyces* yeasts to xylose metabolizing yeasts, which can also co-ferment both glucose and xylose simultaneously as shown in Fig. 1. Our recombinant yeasts are the only *Saccharomyces* yeasts available today that are sufficient for industrial use in converting lignocellulosic sugars (sugars extracted from lignocellulosic biomass) to ethanol [1–5].

Our metabolically engineered glucose-xylose-co-fermenting *Saccharomyces* yeasts, such as 424A(LNH-ST) (with multiple copies of KDR genes stably integrated in the yeast chromosome) have exceeded all expectations in co-fermenting glucose and xylose. However, there is room for improvement.
for further improvement. For example, 424A(LNH-ST) still ferments glucose several times faster than xylose (Fig. 1). Our glucose-xylose-co-fermenting yeasts could be further improved to produce ethanol more efficiently by optimally expressing enzymes and proteins involved in metabolizing glucose and/or xylose.

We believe that by carefully and thoroughly analyzing gene expression patterns by our genetically modified strains during co-fermentation of glucose and xylose to ethanol will reveal ways to optimize xylose fermentation. The use of DNA array technology for transcriptome analysis, proteomics technology for quantitative mapping of protein expression, metabolome analysis for analyzing intracellular metabolite levels and bioinformatics for analyzing pathway structure and pathway fluxes can all be employed in this research effort.

In this paper, we analyze the expression of genes in the glycolytic pathway and genes encoding enzymes for alcoholic fermentation using microarray technology. We also report the results on the analysis of the activity of selected enzymes for ethanol production during co-fermentation of glucose and xylose to ethanol by our yeast 424A(LNH-ST).

2. Materials and methods

2.1. Media

Saccharomyces cerevisiae 424A(LNH-ST) was cultured in YEPX (1% yeast extract, 2% peptone, and 2% xylose) or YEPD (1% yeast extract, 2% peptone, and 2% glucose) at 30°C.
Fig. 2. K-mean clustering (by using Pearson correlation) of *S. cerevisiae* 424A(LNH-ST) during co-fermentation of glucose/xylose.

Fig. 3. Scatter plot of the expression profile of *S. cerevisiae* 424A(LNH-ST) at 2.5 h of co-fermentation of glucose/xylose.
2.2. Yeast strain

Genetically engineered *S. cerevisiae* 424A(LNH-ST) [4] containing multiple copies of three xylose-metabolizing genes, KDR, stably integrated into the host chromosome, is a derivative of the ATCC strain 4124 and was developed by the method described in Ho and Chen [5].

2.3. Conditions for co-fermentation glucose and xylose by *S. cerevisiae* 424A(LNH-ST)

*S. cerevisiae* 424A(LNH-ST) was inoculated directly from the agar plates into 5 ml of YEPX medium. The cultures were incubated in a shaker at 30°C at 200 rpm and grown aerobically overnight. The following morning, the cultures were transferred directly to 100 ml YEPX in a 300 ml Erlenmeyer flask equipped with a sidearm (Bellco) which allows for direct monitoring of the growth of yeast cultures by a Klett colorimeter. The cultures were incubated as described above until cell density reached 400 KU. This culture was stored in a refrigerator at 4°C and served as the seed culture. Eight milliliters of *S. cerevisiae* 424A(LNH-ST) from the seed culture were transferred directly to 100 ml of YEPD in a 300-ml Erlenmeyer flask equipped with a sidearm (Bellco). The cultures were incubated as described above until cell density reached 375–400 KU. At this point, 18 ml of (50%) glucose and 8 ml of (50%) xylose were added to each flask. The flask was then sealed with Saran wrap to allow fermentation to be carried out. However, it is important to note that the conditions described here for fermentation are not strictly anaerobic, and that yeast fermentation does not require strict anaerobic conditions. Samples for preparation of total RNA and samples for monitoring fermentation were taken at 0, 2.5, 5, 10, 15, and 25 h (30 h for monitoring fermentation and enzyme activities) during fermentation (Fig. 1).

2.4. Analysis of fermentation products

The substrate D-glucose and D-xylose and their fermentation products such as xylitol, glycerol and ethanol were analyzed by HPLC using the HPX 87H and HPX 87C columns (8 mm × 300 mm, Bio-Rad Laboratories, CA), an autoinjector (Hitachi, model AS-4000), an isocratic liquid pump (Hitachi, model L-6000), a RI detector (Hitachi, model L-3350), and a computing integrator (Hitachi, model D-2500). One milliliter samples were centrifuged, the supernatant was taken and diluted 10-fold with water and 10 µl of the diluted samples was injected.

Fig. 4. Scatter plot of the expression profile of *S. cerevisiae* 424A(LNH-ST) at 10 h of co-fermentation of glucose/xylose.
2.5. RNA preparation and hybridization to Affymetrix microarray

Gene expression was analyzed using Affymetrix Yeast Genome S98 GeneChips®. The co-fermentation of glucose/xylose by *S. cerevisiae* 424A(LNH-ST) was performed as described above in “Conditions for co-fermentation glucose and xylose by *S. cerevisiae* 424A(LNH-ST).”

Total RNA was extracted using the method described by Li et al. [6] at six time points, 0, 2.5, 5.0, 10, 15, and 25 h after the addition of the sugars. This experiment was performed four separate times, yielding 24 total RNA samples. The 24 RNA samples were individually purified using RNeasy columns (Qiagen). Samples were labeled using the Affymetrix recommended protocol (GeneChips® Expression Analysis Technical Manual, Affymetrix, Santa Clara, CA). Total RNA was converted to single-stranded cDNA using a primer containing a T7 promoter sequence attached to a sequence of 24 T residues. This was converted to double stranded cDNA. RNA containing biotinylated nucleotides was synthesized by in vitro transcription, using an Enzo BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). After fragmentation, control oligonucleotide and eukaryotic hybridization controls were added and each sample was hybridized to a Yeast Genome S98 GeneChips® for 17 h at 45°C with

![Fig. 5. The expression level of GLK1 (•), HXK1 (□), HXK2 (▲), PGI1 (■), FBA1 (△) and TPI1 (○) mRNA in *S. cerevisiae* 424A(LNH-ST) during co-fermentation of glucose and xylose.](image-url)
constant rotation. The unhybridized RNA was washed off and the bound RNA stained with phycoerythrin-conjugated streptavidin, followed by signal amplification using biotinylated anti-streptavidin antibody and a second staining; washing and incubation of these solutions were carried out on the Fluidics station. The 24 GeneChips® were scanned and analyzed using the dedicated scanner and Affymetrix Microarray Suite (MAS) version 5.0. The average intensity on each array was normalized by global scaling to a target intensity of 1000, and the data exported into GeneSpring v. 4.2 (Silicon Genetics, CA) for further analysis and visualization. For each probeset, MAS provides a measure of the expression level (“signal”), and also provides the level of confidence with which the sequence was detected.

To reduce non-random error, the biochemistry of cDNA synthesis was carried out in two batches of 12 samples each (experiments 1 and 2, then experiments 3 and 4). In vitro transcription, fragmentation and hybridization for the six samples of experiment 1 were carried out together. The in vitro transcription of experiment 2 was done as a second group, and experiments 3 and 4 as a third group; fragmentation and preparation of the hybridization cocktail for experiments 2–4 were done together, and each of these experiments was hybridized separately.

Fig. 6. The expression level of PFK1 (●), PFK2 (□), PGK1 (▲), ENO1 (○) and ENO2 (○) mRNA in S. cerevisiae 424/ALNH-ST during co-fermentation of glucose and xylose.
2.6. Preparation of crude extract and enzyme assays

The yeast cells were harvested at 0, 2.5, 5, 10, 15, and 30 h after glucose and xylose were added. Six milliliters culture was centrifuged for 5 min at 5000 × g, washed with ice-cold sterile water, and resuspended in 0.350 ml ice-cold 0.1 M phosphate buffer, pH 7.2. Cells were disrupted with a Mini-Beatbeater (Biospect-Product) by shaking with glass beads (diameter 0.5 mm) for 5 min in 45 s intervals, cooling the samples for 2 min between intervals. The cell suspension was centrifuged at 15,000 × g for 15 min. The supernatant was used in the enzymatic assay. The following enzyme activities were determined: pyruvate kinase [7], pyruvate decarboxylase [7], alcohol dehydrogenase [7], phosphoglycerate kinase [3] and glyceraldehyde-3-phosphate dehydrogenase [3]. One unit of enzyme activity is defined as 1 μmol substrate converted per minute per milligram of protein at 25 °C.

2.7. Protein estimation

Protein estimation was performed by the method of Bradford [8], using bovine serum albumin as a standard.

3. Results and conclusions

Microarrays generate huge datasets and require special methods for organization, presentation, and analysis. One of the methods is clustering, a mathematical approach...
that groups together apparently co-regulated genes. That is, genes are grouped together if their expression levels correlate with one another under different experimental conditions (Fig. 2). Another useful tool for examining the expression levels of genes is the scatter plot as demonstrated in Figs. 3 and 4. In these figures, the vertical position of each gene represents its expression level in the current experimental condition and the horizontal position represents its control strength (in this case, median expression level of this gene in all conditions).

In this paper, we present the expression profile of genes involved in glycolysis and alcoholic fermentation during glucose-xylose-co-fermentation with our yeast. Our results show that most of the glycolytic genes are highly expressed (except PYK2) but not significantly affected by the presence of high concentrations of glucose during anaerobic fermentation. For example, the expression levels of five out of seven genes in the upper part of glycolysis and seven out of nine genes in the lower part of glycolysis were slightly increased during 2.5–15 h co-fermentation (Figs. 5–7). However, the expression of GLK1 and HXK1 was decreased by the presence of glucose at the beginning of fermentation, but increased with the loss of glucose from the fermentation broth (Fig. 5). Also only two genes of the glycolysis pathway, ENO2 and HXK2, were activated by the presence of glucose (Figs. 5 and 6) and their levels of expression decreased after the first 5 h of fermentation. However, the decrease of the expression of these two genes is compensated by the
increase of the expression of ENO1 and HXK1 (Figs. 5 and 6). On the other hand, the decrease in the levels of expression of all ADH and PDC genes was significant after glucose was depleted from the fermentation broth (Fig. 8).

We also measured the specific activity of selected enzymes involved in glycolysis and alcoholic fermentation (Fig. 9). The specific activities of the enzymes (ADH, PDC, PGK, PYK and G3PDH) correlate nicely with the level of their mRNA expression (Figs. 6–9). The expression of the two-xylose metabolizing genes, XR and XD, cannot be measured by Yeast Genome S98 Array, but will be measured by QRT-PCR in the near future. The level of expression of XK was high, comparable with that of the glycolytic genes, and was not significantly affected by the presence of glucose (Fig. 7). These five enzymes were chosen for study because they all catalyze irreversible reactions in the glycolysis pathway or pathway for alcoholic fermentation. Comparing our previous work [1,3] with that of others [9,10], clearly demonstrates the crucial importance of cloning and over-expression of the xylulokinase gene in the Saccharomyces yeast in making the latter yeast able to effectively metabolize xylose both aerobically and anaerobically. The xylulokinase gene encodes the only enzyme that catalyzes an irreversible reaction in the yeast pentose pathway.

The results obtained from this study will guide us in developing new yeast with much improved capability to co-ferment glucose and xylose. We hope to achieve our goal partially through enhancing the expression of some of the genes encoding enzymes involved in glycolysis and alcoholic fermentation.
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