0273-2289/04/114/0403–0416/$25.00

Production of Ethanol from Cellulosic Biomass Hydrolysates Using Genetically Engineered *Saccharomyces* Yeast Capable of Cofermenting Glucose and Xylose

Miroslav Sedlak and Nancy W. Y. Ho*

Laboratory of Renewable Resources Engineering (LORRE), Purdue University, 500 Central Drive, West Lafayette, IN 47907, E-mail: nwyho@ecn.purdue.edu

Abstract

Recent studies have proven ethanol to be the ideal liquid fuel for transportation, and renewable lignocellulosic materials to be the attractive feedstocks for ethanol fuel production by fermentation. The major fermentable sugars from hydrolysis of most cellulosic biomass are D-glucose and D-xylose. The naturally occurring *Saccharomyces* yeasts that are used by industry to produce ethanol from starches and cane sugar cannot metabolize xylose. Our group at Purdue University succeeded in developing genetically engineered *Saccharomyces* yeasts capable of effectively cofermenting glucose and xylose to ethanol, which was accomplished by cloning three xylose-metabolizing genes into the yeast. In this study, we demonstrated that our stable recombinant *Saccharomyces* yeast, 424A(LNH-ST), which contains the cloned xylose-metabolizing genes stably integrated into the yeast chromosome in high copy numbers, can efficiently ferment glucose and xylose present in hydrolysates from different cellulosic biomass to ethanol.

Index Entries: Ethanol; *Saccharomyces* yeasts; hydrolysate; corn stover; corn fiber; xylose; glucose; glycerol; xylitol.

Introduction

Recent studies have proven ethanol to be an ideal liquid fuel for transportation and renewable lignocellulosic biomass to be an attractive feedstock for ethanol fuel production by fermentation *(1,2)*. The major fermentable sugars from hydrolysis of lignocellulosic biomass, such as rice and wheat straw, sugarcane bagasse, corn stover, corn fiber, softwood, hardwood, and grasses, are D-glucose and D-xylose except that softwood

*Author to whom all correspondence and reprint requests should be addressed.

also contains substantial amounts of mannose. Economically, it is essential that both glucose and xylose present in lignocellulosic biomass can be fermented to ethanol in order for these renewable resources to be used as feedstock for ethanol fuel production.

The naturally occurring *Saccharomyces* yeasts have been proven to be safe, effective, and user-friendly microorganisms for the large-scale production of industrial ethanol from various traditional feedstocks such as starch and cane sugar. The fermentable sugars present in those feedstocks are glucose and fructose. However, these yeasts cannot metabolize xylose. We successfully developed genetically engineered recombinant *Saccharomyces* yeasts that can effectively coferment glucose and xylose to ethanol *(3,4)*. This was accomplished by the cloning and overexpression of three xylose-metabolizing genes: xylose reductase (XR), xylitol dehydrogenase (XD), and xylulokinase (XK) genes in yeast. Subsequently, we further developed stable *Saccharomyces* yeasts that contain multiple copies of XR, XD, and XK stably integrated into the yeast chromosomes *(5,6)*. Even more important, we developed a convenient system for testing and converting virtually any *Saccharomyces* yeast, wide type or mutants, to glucose/xylose cofer-menting yeast. As such, we can screen the best yeast for cofermentation of sugars from lignocellulosic biomass to ethanol. In recent years, we have tested more than 10 different strains of yeast for glucose/xylose cofermentation and converted three of the best ones to stable recombinant yeasts with high efficiencies in cofermenting glucose and xylose to ethanol. They are 1400 (LNH-ST) *(5)*, 259 (LNH-ST) *(7)*, and 424A (LNH-ST) *(8)*. These three yeasts can all fer-ment 8% glucose and 4% xylose to nearly 6% ethanol in 30–40hrs *(5,7,8)*.

However, the cost-effective conversion of various types of lignocellulosic biomass to fermentable sugars with as little toxic inhibitory byproducts as possible remains a challenge for the production of cellulosic ethanol (ethanol produced from lignocellulosic biomass). Furthermore, the ability of individual recombinant yeast to tolerate the inhibitors present in the hydrolysates is also an important factor for cost-effective production of cellulosic ethanol. The effective production of cellulosic ethanol from hydrolysates of various lignocelluloses with our 1400 (LNH-32) or 1400 (LNH-ST) yeast has previously been reported *(6,7,9,10)*. In this article, we examine the effectiveness of our stable recombinant *Saccharomyces* yeast 424A (LNH-ST) in cofermenting cellulosic sugars (glucose and xylose from hydrolysates) derived from different lignocellulosic biomass to ethanol. We also compare the effectiveness of 424A(LNH-ST) with those recombinant yeasts developed by others in converting sugars from cellulosic hydrolysates to ethanol.

Materials and Methods

Microorganisms and Growing Conditions

The genetically engineered *Saccharomyces cerevisiae* 424A (LNH-ST) was used for fermentation of lignocellulosic hydrolysates to ethanol. *S. cerevisiae* 424A (LNH-ST) was constructed by integrating multiple copies of XD, XR, and XK into the chromosomes of *S. cerevisiae* ATCC 4124 accord-

ing to the technology reported by Ho and colleagues *(3–6)*. The 424A (LNH-ST) strain was maintained in liquid YEPX medium (20 g/L of Difco peptone; Becton Dickinson, St. Louis, MO), 10 g/L of Difco yeast extract (Becton Dickinson), and 20 g/L of xylose (Sigma, St. Louis, MO). Fresh seed cultures were prepared by inoculating the respective seed cultures in 100 mL of YEPX (for 424A [LNH-ST]) in 300-mL baffled Erlenmeyer flask equipped with a sidearm (Bellco). The cultures were incubated in a shaker at 30°C and 200 rpm and grown aerobically overnight. The following morning, when the cultures reached OD 350–400 Klett unit (KU) (OD 400 KU corresponds to a cell mass concentration of 9 g dry wt/L), the flasks containing the seed cultures were stored in a refrigerator at 4°C.

Lignocellulosic Hydrolysates

The corn fiber– and corn stover–based hydrolysates (hydrolysates A and B, respectively) were prepared by the following procedure. Two hundred grams of corn fiber or 100 g of corn stover was mixed with 800 mL of 1% sulfuric acid. The mixture was divided into four 2-L flasks and hydrolyzed for 1 h in an autoclave (SG-120 Scientific Gravity Sterilizer; Amsco Century) at 121°C. The hydrolysate was filtered (Whatman No. 1, 125-mm diameter). The filtrate was adjusted to pH 10.0 using $Ca(OH)_2$ and kept at room temperature overnight, followed by adjusting the pH to 6.0 using H_3PO_4. The precipitation that appeared was removed by centrifugation and the supernatant in some cases was concentrated (two- to three-fold) using a vacuum evaporator. The resulting liquid was used for fermentation.

Hydrolysates C–G were supplied by others as follows: hydrolysates C and D from Laboratory Renewable Resources Engineering, Purdue University; hydrolysate E from Department of Chemical Engineering, Purdue University; hydrolysates F and G from Tennessee Valley Authority, Public Power Institute, Muscle Shoals, AL.

Fermentation

For ethanol production, 8 mL of seed cultures was used to inoculate 100 mL of YEPD (YEP plus 2% glucose, for ethanol fermentation) in a 300-mL baffled Erlenmeyer flask equipped with a sidearm. The cultures were incubated in a shaker at 30°C and 200 rpm and grown aerobically overnight. By the next morning, they reached OD 350–400 KU. The yeast was harvested by centrifugating (J-21 Beckman) at 5000 rpm for 5 min at room temperature. The supernatant was discarded, and the cells were transferred into a 300-mL baffled Erlenmeyer flask containing 100 mL of lignocellulosic hydrolysate supplemented with 10 mL of 10% yeast extract. The initial cell mass concentration prior to fermentation in each experiment was 8.5–9.0 g dry wt/L. The flasks were then sealed with Saran Wrap to allow fermentation to be carried out under largely anaerobic conditions. The cultures were placed in a shaker and incubated as just described. One milliliter of the fermentation mixture was removed at proper intervals to serve as a sample for monitoring fermentation.

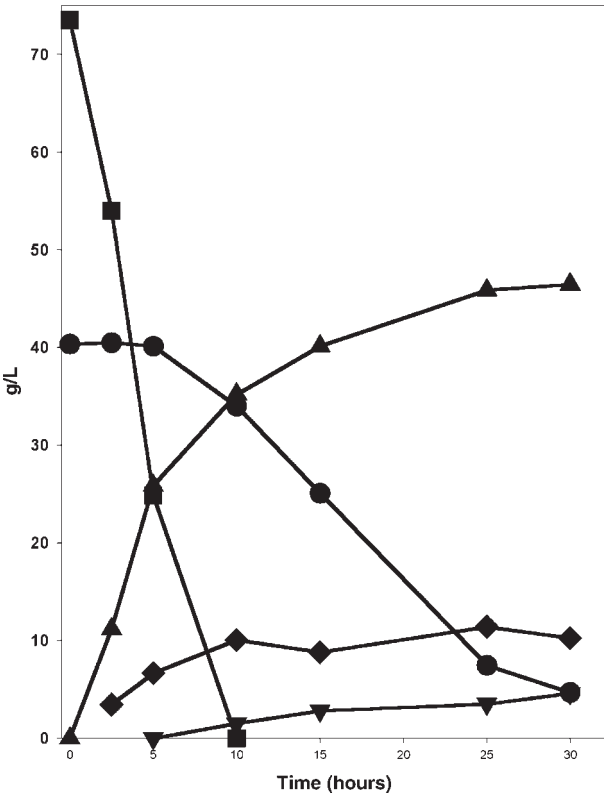

Fig. 1. Fermentation of standard sugar mixture (glucose and xylose) by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol; (◆) glycerol; (▼) xylitol.

Analysis of Sugars and Fermentation Products

Xylose, glucose, acetic acid and their fermentation products such as xylitol, glycerol, ethanol, and lactic acid were analyzed as we reported previously *(3)* by high-performance liquid chromatography using an HPX-87H (8×300 mm; Bio-Rad, Hercules, CA) equipped with an autoinjector (Hitashi model AS-4000), an isocratic liquid pump (Hitashi model L-6000), an RI detector (Hitashi model L-3350), and a computing integrator (Hitashi model D-2500). One-milliliter samples were centrifuged and the supernatant was collected. Ten microliters of the 10-fold diluted sample was used for analysis.

Results

Fermentation of Standard Sugar Mixture

Our standard sugar mixture (Std. Mix) for fermentation contained YEP, and 70 g/L of pure glucose and 40 g/L of pure D-xylose. Glucose in Std. Mix was completely fermented in 10 h. Higher rates of xylose fermentation started when glucose concentration dropped below 2.5%. Of the xylose 88.5% was fermented in 30 h (Fig. 1). The production of xylitol dur-

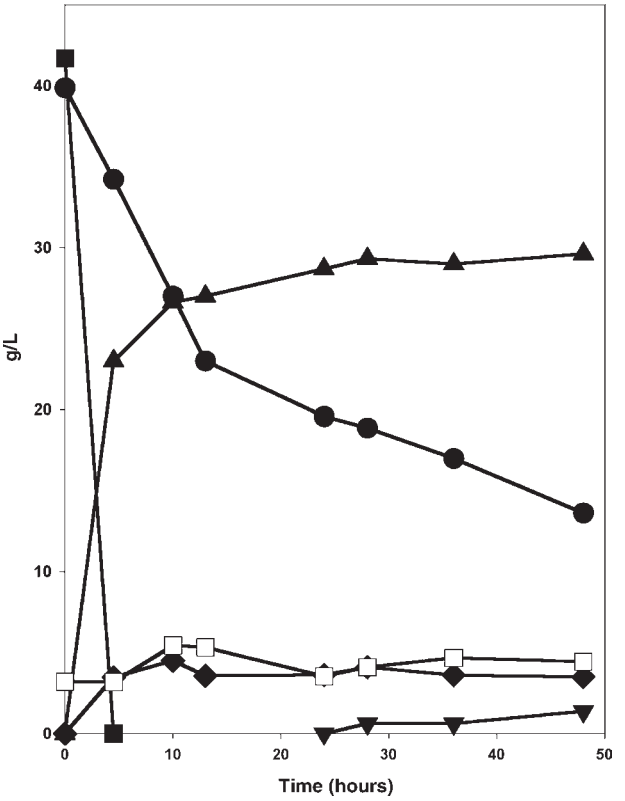

Fig. 2. Fermentation of hydrolysate A (corn fiber–based hydrolysate) by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol; (◆) glycerol; (▼) xylitol; (□) acetic acid.

ing fermentation was very low, only 4.6 g/L (Table 1). The maximum glycerol concentration reached approx 11 g/L (Fig. 1). The amount of ethanol produced was 46.5 g/L, and the ethanol yield from consumed sugar was 0.43 g/g (Table 1).

Fermentation of Lignocellulosic Hydrolysates Method

Hydrolysate A prepared from corn fiber as described earlier contained 41.7 g/L of glucose, 39.9 g/L of xylose, 21 g/L of arabinose, and 3.2 g/L of acetic acid. The glucose in hydrolysate A was fermented completely in 4.5 h. Of the xylose 42.3% was fermented in 13 h. Afterward only 23.6% of the remaining xylose was fermented in 35 h. One hundred percent of the glucose and 65.9% of the xylose was fermented in 48 h (Fig. 2). The production of ethanol was 29.6 g/L in 48 h. The yield of ethanol from consumed sugars reached 85% of the theoretical yield. The yeast did not consume arabinose or acetic acid. The production of xylitol was very low; only 5.3% (1.4 g/L) of the consumed xylose was converted to xylitol (Table 1).

Table 1
Fermentability of Different Lignocellulosic Hydrolysates by *S. cerevisiae* 424A(LNH-ST)[a]

Hydrolysate	G (g/L)	X (g/L)	T (g/L)	CX (%)	Xyl (g/L)	E (g/L)	$Y_{E/CS}$ (g/g)	$Y_{E/T}$ (g/g)	$TY_{E/CS}$ (%)	$TY_{E/TS}$ (%)
A	41.7	39.9	81.6	65.9	1.4	29.6	0.44	0.36	85.4	36.3
B	4.0	17.9	21.9	83.2	0.0	9.0	0.48	0.41	93.4	80.6
C	37.2	39.8	77.0	70.9	0.5	29.8	0.46	0.39	89.5	76.0
D	32.0	18.1	50.1	69.4	0.0	22.0	0.49	0.44	96.7	86.1
E	4.0	26.7	30.7	98.5	0.0	12.8	0.42	0.42	82.8	81.6
F	6.9	26.9	33.8	72.7	0.0	9.8	0.37	0.29	72.6	56.5
G	25.5	17.0	42.5	80.0	0.0	15.1	0.39	0.36	75.7	69.6
Std. Mix	73.5	40.4	113.9	88.5	4.6	46.5	0.43	0.41	83.5	80.0

[a]G, glucose; X, xylose; T, total fermentable sugar; CX, consumed xylose; Xyl, xylitol; E, ethanol; $Y_{E/CS}$, g of ethanol/g of consumed sugars; $Y_{E/T}$, g of ethanol/g of initial total fermentable sugars; $TY_{E/CS}$, theoretical ethanol yield of consumed sugar; $TY_{E/TS}$, theoretical ethanol yield of initial total fermentable sugar; Std. Mix, a mixture of pure glucose (7%) and xylose (4%). The fermentation time for hydrolysates A, and C–G was 48 h. The fermentation time for hydrolysate B was 24 h and for Std. Mix was 30 h. The initial cell mass concentration was 8.5–9 g dry wt/L.

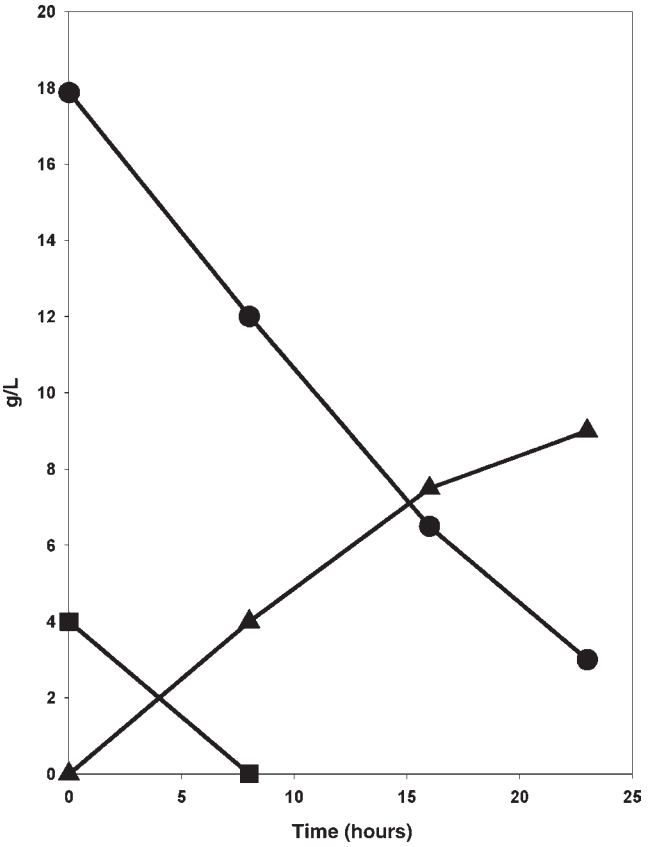

Fig. 3. Fermentation of hydrolysate B (corn stover based hydrolysate) by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol.

Hydrolysate B from corn stover contained 4 g/L of glucose, 17.9 g/L of xylose, 5 g/L of arabinose, and 2.5 g/L of acetic acid. Glucose was readily fermented; eighty-three percent of xylose was fermented in 23 h. The production of ethanol by fermentation of the corn stover hydrolysate was 9 g/L (Fig. 3). The yield of ethanol from consumed sugars reached 93% of theoretical yield. We did not observe xylitol production and acetic acid consumption.

Hydrolysates C contained 37.2 g/L of glucose, 5.5 g/L of xylose, 15.3 g/L glycerol of, and 11.5 g/L of lactic acid. This hydrolysate was not optimized for xylose recovery although there is a possibility for higher xylose recovery from the original feedstock (R. Hendrickson, personal communication). For this reason, we added xylose to a final concentration of 39.8 g/L to evaluate how effectively our yeast ferments the hydrolysate in case the process will be optimized for xylose recovery. Glucose was fermented

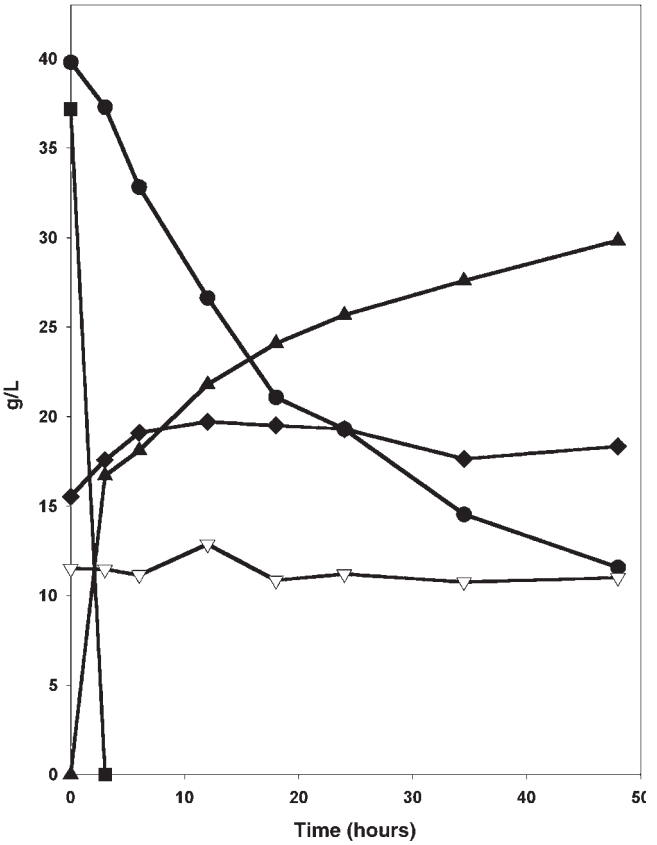

Fig. 4. Fermentation of hydrolysate C (corn fiber based hydrolysate) by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol; (◆), glycerol; (▽), lactic acid.

in 3 h. Of the xylose 70.9% was fermented in 48 h (Fig. 4). The ethanol produced during 48 h of fermentation of this hydrolysate was 29.8 g/L. The yield of ethanol from consumed sugars reached 89.5% of the theoretical yield, and xylitol production was below 0.5 g/L (not shown).

Hydrolysates D contained 32 g/L of glucose, 18.11 g/L of xylose, 6.5 g/L of arabinose, and 1.5 g/L of acetic acid. Glucose was readily fermented; in addition, 69.4% of the xylose was fermented in 48 h. The ethanol was produced from fermentation of this hydrolysate was 22 g/L (Fig. 5). The yield of ethanol from consumed sugars reached 96.7% of the theoretical yield. Xylitol production was not detected in this fermentation.

Hydrolysates E contained 4 g/L of glucose, 26.7 g/L of xylose, and 3.4 g/L of arabinose. Glucose was completely consumed in 3 h, and 98.5% of xylose was fermented in 48 h. The ethanol produced by fermentation of this hydrolysate in 48 h was 12.8 g/L (Fig. 6). The yield of ethanol from con-

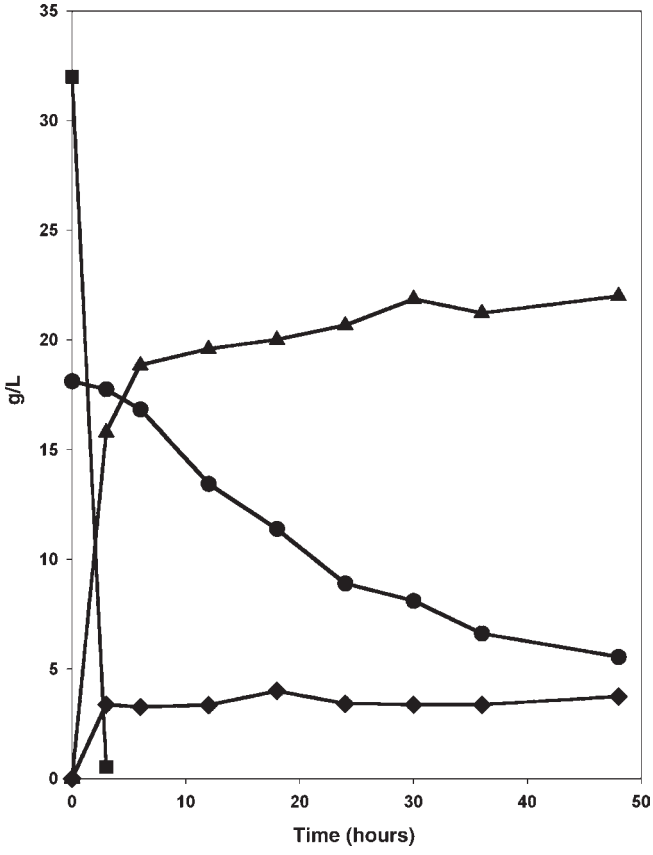

Fig. 5. Fermentation of hydrolysate D (corn stover based hydrolysate) by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol; (◆), glycerol.

sumed sugars reached 82.8% of the theoretical yield. There was no xylitol production. Arabinose was partially converted into arabitol (not shown).

Hydrolysate F contained 6.9 g/L of glucose, 26.9 g/L of xylose, 1 g/L of arabinose, and 3 g/L of acetic acid. One hundred percent glucose and 72.7% xylose were consumed during fermentation of this hydrolysate. Ethanol production in 48 h was 9.8 g/L (Fig. 7), and the yield of ethanol from consumed sugars reached 72.6% of the theoretical yield. Arabinose and acetic acid were not utilized during fermentation (not shown), and no xylitol production was detected. Hydrolysate G contained 25.5 g/L of glucose, 17 g/L of xylose, and 2.5 g/L of acetic acid. The glucose was fermented in 3 h and 80% of xylose was consumed in 48 h (Fig. 8). During fermentation of this hydrolysate,15.1 g/L of ethanol was produced. The yield of ethanol from consumed sugar reached 75.7% of the theoretical yield. Acetic acid was not utilized during fermentation (not shown), and no xylitol production was detected.

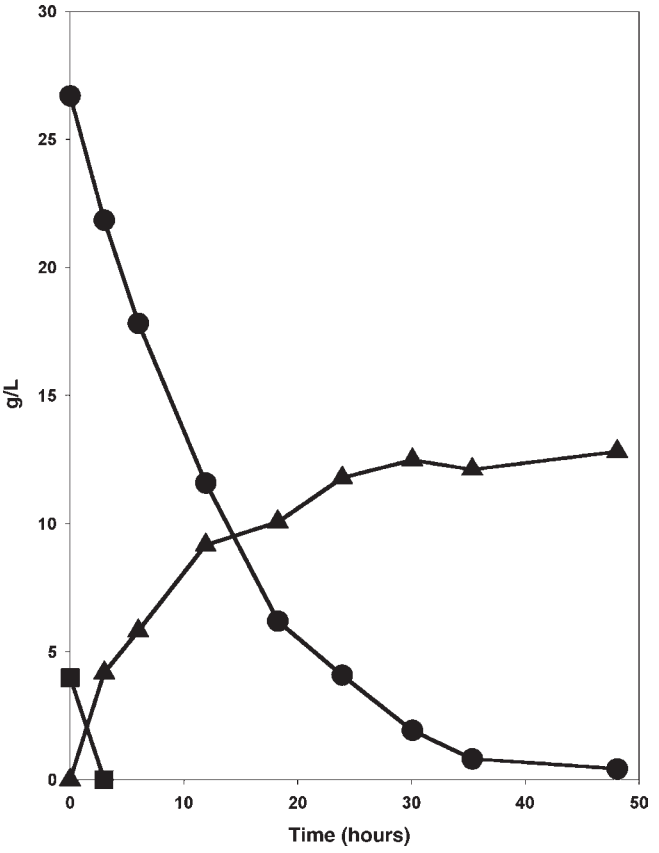

Fig. 6. Fermentation of hydrolysate E by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol.

Discussion

We have confirmed that our strain 424A(LNH-ST), recombinant glucose/xylose cofermenting yeast carrying multiple copies of XD, XR, and XK integrated into its chromosomes, is capable of effectively fermenting not only mixtures of pure glucose and xylose (Fig. 1) but also glucose and xylose present in lignocellulosic hydrolysates to ethanol. In fermentation of a mixture of pure glucose (7%) and xylose (4%), 424A(LNH-ST) fermented 100% glucose and 88.5% xylose in 30 hours with an ethanol yield of 0.41 g/g of initial total sugar and 80% of the ethanol theoretical yield of initial total sugar (Fig. 1 and Table 1). This strain also had very low production of xylitol; only 14% of consumed xylose was converted into xylitol. These results are comparable with data obtained previously using our recombinant *Saccharomyces* strain 1400 (pLNH32) *(3)*.

However, the efficiency of fermentation of hydrolysates by 424A(LNH-ST) was also largely dependent on how the hydrolysates were prepared.

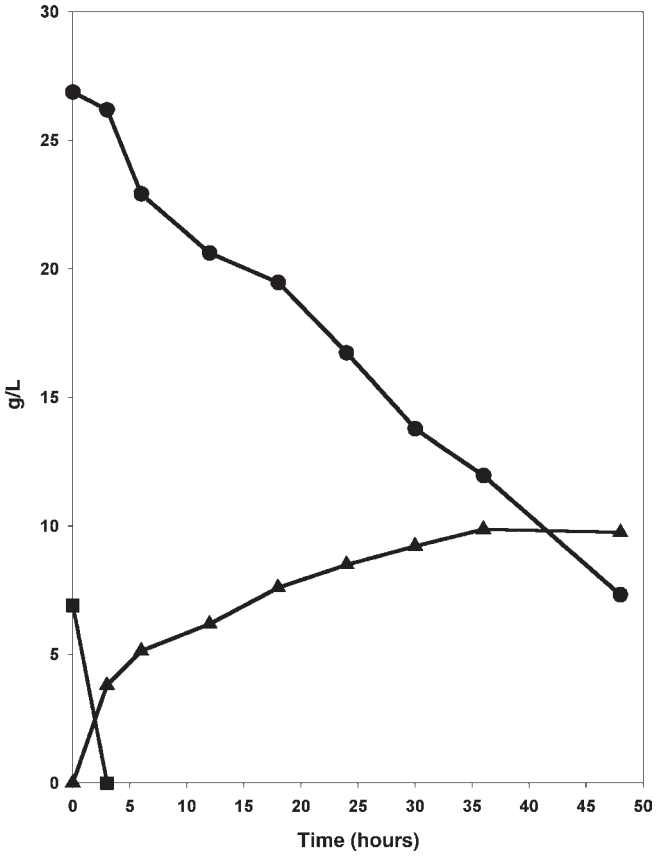

Fig. 7. Fermentation of hydrolysate F by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol.

Nevertheless, in all cases, all glucose was fermented within the first 3 h and >65% of xylose was consumed in 48 h (Figs. 1–9 and Table 1). The ethanol yield was >75% of the theoretical yield of consumed sugar (Table 1). In addition, the percentage of theoretical ethanol yield of initial total sugar was high with the exception of hydrolysate A from corn fiber, which was prepared in our laboratory as described in Materials and Methods. This demonstrates that our process for preparing hydrolysates from corn fiber needs to be improved. We observed that fermentation of most hydrolysates by our yeast produced very little xylitol except in the fermentation of the corn fiber based hydrolysates, which was still no more than 2–5% of consumed xylose (Table 1). One possible explanation might be that xylose in the hydrolysates was metabolized slower, so xylitol could be completely converted to ethanol.

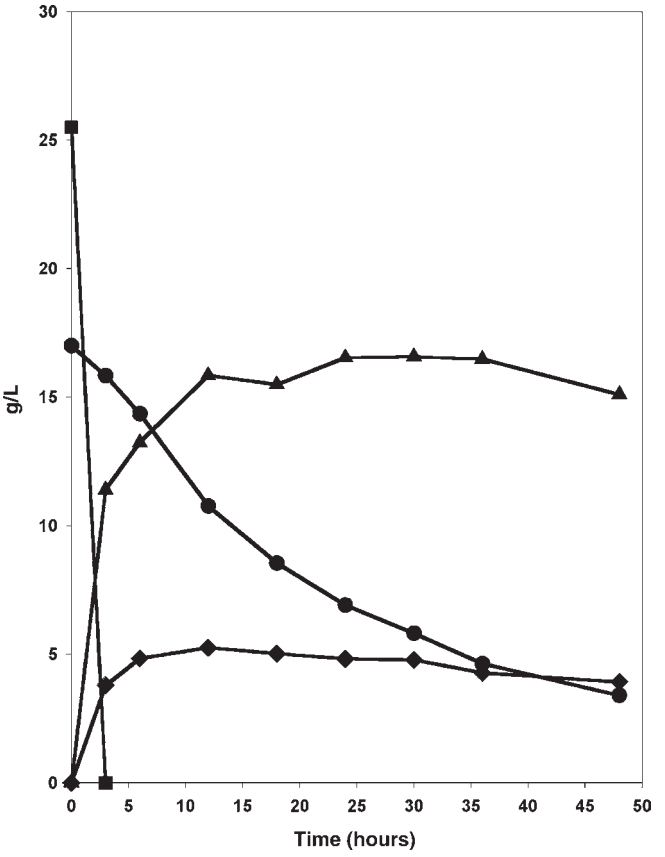

Fig. 8. Fermentation of hydrolysate G by *S. cerevisiae* 424A(LNH-ST). (■) Glucose; (●) D-xylose; (▲) ethanol; (◆), glycerol.

Now others have used our approaches (i.e., by cloning three genes, XR, XD, and XK, into the yeast rather than just XR and XD) to develop their recombinant *Saccharomyces* yeast for cofermenting glucose and xylose to ethanol. Our recombinant yeasts are still far more effective in cofermenting glucose and xylose to ethanol. For example, Johansson et al. *(11)* used their recombinant *Saccharomyces* strains to ferment their standard sugar mixture (39 g/L of xylose, 5.7 g/L of glucose 3.5 g/L of mannose, 3.1 g/L of galactose 1.7 g /L of arabinose, 0.16 g/L of hydroxymethylfurfural, and 0.7 g/L of furfural) and sugars present in lignocellulosic hydrolysate. One strain was able to metabolize xylose but it converted xylose mostly to xylitol; approx 78% (estimated from the published figures, designated as est.) of xylose was converted to xylitol and the percentage of theoretical ethanol yield of initial total sugar was just 19% (est.) with an estimated ethanol yield of 0.1 g/g of initial total sugar. Another of their strains produced lower

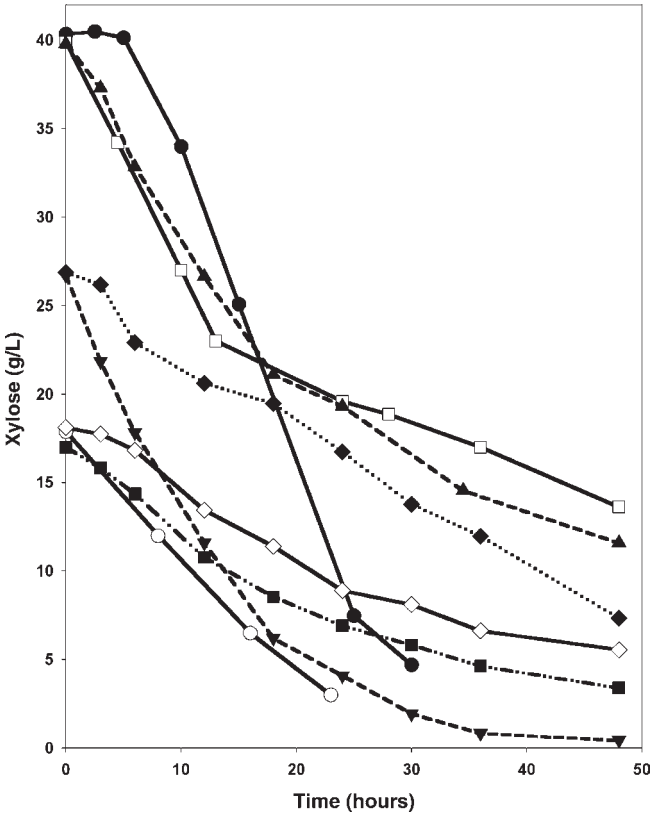

Fig. 9. Xylose consumption by *S. cerevisiae* 424A(LNH-ST) during fermentation the different hydrolysates. (●——●) Std. mixture; (□——□) hydrolysate A; (○——○) hydrolysate B; (▲– – –▲) hydrolysate C; (◇——◇) hydrolysate D; (▼– – –▼) hydrolysate E; (◆• • •◆) hydrolysate F; (■• •—• •■) hydrolysate G.

amounts of xylitol; just 6% (est.) of xylose was converted to xylitol, but the strain consumed only 23% of xylose in 65 h with an initial concentration of xylose of 39 g/L. The percentage of theoretical ethanol yield of the initial total sugar was 25% (est.) with an estimated ethanol yield of 0.13 g/g of initial total sugar. In addition, the fermentation time was 65 h or more. The sugar consumption was even slower during fermentation of their birch wood hydrolysate *(11)* with the same sugar concentration as for their standard sugar mixture.

Zadivar et al. *(12)* reported fermentation of a pure glucose/xylose mixture using their xylose-fermenting *S. cerevisiae*, but xylose mainly converted to xylitol with ethanol only as a minor product *(12)*. Furthermore, the time needed for completion of the fermentation exceeded 100 h with an initial xylose concentration of 50 g/L.

Martin et al. *(13,14)* used their genetically engineered *S. cerevisiae (11)* for fermentation of sugars presented in sugarcane bagasse hydrolysates, but very little xylose utilization was observed *(13,14)*. It is now generally acknowledged *(15–17)* that cloning three genes, the XR, XD, and XK, is needed to make the *Saccharomyces* yeast ferment xylose, as we recommended nearly a decade ago *(3)*.

Why our recombinant yeasts are superior to those developed by others using the same principles of construction is most likely owing to a combination of factors, including how our genes were modified, how our genes were integrated into the yeast chromosomes, and how our host yeast strain for cloning was selection.

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