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2-Deoxyglucose as a Selective Agent for Derepressed Mutants of *Pichia stipitis*†

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Abstract

The glucose analog 2-deoxyglucose (2-DOG) has been used to obtain mutants derepressed for pentose metabolism. Some researchers have used 2-DOG alone whereas others have used it in the presence of a glucose-repressible carbon source. We examined both methods and screened mutant strains for improved use of xylose in the presence of glucose. *Pichia stipitis* mutants selected for growth on D-xylose in the presence of 2-DOG used xylose from a 1:1 glucose:xylose mixture more rapidly than did their parents. One of these mutants, FPL-DX26, completely consumed xylose in the presence of glucose and produced 33 g/L ethanol in 45 h from 80 g/L of this sugar mixture. Mutants selected for growth on 2-DOG alone did not show significant improvement. Selection for growth on D-xylose in the presence of 2-DOG has been useful in developing parental strains for further genetic manipulation.

Terms for Kinetic Factors: μ, Specific growth rate (/h); Rs, volumetric sugar uptake rate (g/L/h); Qs, specific sugar uptake rate (g/g/h); R_pe, volumetric ethanol production rate (g/L/h); Q_pe, specific ethanol production rate (g/g/h); Ye, ethanol yield (g/g).

Index Entries: *Pichia stipitis*; mutation; selection; 2-deoxyglucose (2-DOG); fermentation; ethanol; glucose repression.

Introduction

Sugar mixtures mainly D-glucose, D-xylose, and L-arabinose obtained from hydrolysates of cellulose and hemicellulose are potential feedstocks

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for the production of ethanol from biomass *(1–5)*. Such hydrolysates are not normally used for food production and are therefore less subject to price fluctuations than is grain. However, commercial utilization of hemicellulose hydrolysate will require a new generation of fermentative organisms. Among xylose fermenting yeasts, both *Candida shehatae* and *Pichia stipitis* can convert xylose and glucose into ethanol, but xylose fermentation and arabinose assimilation are slow or blocked in the presence of glucose owing to catabolite repression *(6–10)*. This results in lower ethanol productivity and yield *(11,12)*.

The presence of glucose likewise represses utilization of maltose and other sugars by *Saccharomyces cerevisiae*, but cells that are resistant to the nonmetabolizable D-glucose analog 2-deoxyglucose (2-DOG) show enhanced fermentation *(13–16)*. Carbon catabolite-derepressed yeast mutants can be obtained by selecting for growth on a glucose-repressible carbon source in the presence of 2-DOG. *S. cerevisiae* cells resistant to 2-DOG show enhanced fermentation on glucose-repressible carbon sources. The exact mechanism by which this selection works is not known, but it is believed to be owing to the loss of hexokinase II *(14)*. In *Kluyveromyces lactis*, mutants devoid of hexokinase showed relief from carbon-catabolite repression of several enzymes *(17)*. Because 2-DOG cannot be metabolized beyond phosphorylation, wild-type cells take it up and phosphorylate it to 2-DOG 6-phosphate, which then represses pathways that metabolize other sugars such as maltose, sucrose, or galactose. Pardo et al. *(18)* previously obtained mutants of *P. stipitis* defective in carbon-catabolite repression by selecting for resistance to 2-DOG as a sole carbon source.

We were interested in knowing whether 2-DOG could be used to obtain carbon-catabolite derepressed mutants of *P. stipitis* for use in further strain development. Random mutagenesis often introduces traits that decrease complex, multi-step metabolic processes such as fermentation. To assure that growth and fermentative activities were maintained, we screened for high-fermentation rates on a glucose:xylose mixture after each round of mutant selection *(19)*. In a previous study we showed that *P. stipitis* mutant FPL-061 showed a high-fermentative rate despite its diminished growth capacity *(20,21)*. We then used 2-DOG in the presence or absence of D-xylose to select for fast-growing mutants that would ferment a mixture of glucose and xylose. The best strain from this screening process, FPL-DX26 (= NRRL Y-21304), was subsequently used as the parent for further genetic development *(22)*.

Materials and Methods

Yeast Strains

Pichia stipitis FPL-061 (NRRL Y-21301) is a mutant that was obtained from *P. stipitis* CBS 6054 (=NRRL Y-11545, ATCC 58785) through selective enrichment and plating on L-xylose and xylitol in the presence of the respiratory inhibitors antimycin A (AA) and salicyl hydroxamic acid (SHAM)

(20,21). These strains were grown and maintained on fresh plates of yeast extract peptone xylose (YPX) agar (*see* Media) for 48 h at 30–32°C.

Media

Five different media in this study employed 10 g/L yeast extract plus 20 g/L peptone and 20 g/L agar (YP) plus various carbon sources. These media are designated as follows: 20 g/L dextrose (YPD); 20 g/L xylose (YPX); 1, 5, 10, or 20 g/L 2-DOG (YP 2-DOG), and 20 g/L xylose plus 1, 5, or 10 g/L 2-DOG (YPX 2-DOG). Sporulation media consisted of malt extract, 50 g/L and agar, 20 g/L; minimal medium agar consisted of yeast nitrogen base without amino acids and ammonium sulphate (YNB, Difco, Detroit, Michigan), 1.7 g/L plus 5.0 g/L $(NH_4)_2SO_4$, and 20 g/L agar.

The fermentation medium contained 1.7 g/L filter-sterilized YNB (Difco) plus 2.27 g/L urea and 6.56 g/L peptone as nitrogen sources (2x nitrogen). Arabinose, glucose, xylose, and a mixture of various concentrations of xylose and glucose were autoclaved in water and added to the medium after they had cooled to room temperature. The final pH was 4.0–4.5.

Ethyl Methane Sulfonate Mutagenesis

Yeast cultures (from 2-d-old YPD agar plates) were grown in YPD liquid medium at 30°C, with shaking. Aliquots containing 2×10^8 cells each were dispensed into tubes after washing twice with sterile water. Cells were spun for 5 s in a microfuge, then resuspended in 1 mL of 0.1 *M* sodium phosphate buffer, pH 7.0. Ethyl methanesulfonate (EMS) 0, 10, 20, and 30 µL was added and the cells were incubated with shaking for 1 h at 30°C. Cells were pelleted, washed with 1 mL of sterile water followed by two washings with 50 g/L sodium thiosulphate to inactivate the EMS. Cells were finally resuspended in 1 mL of 150 g/L glycerol and stored at –70 to –90°C.

Resistance to 2-DOG

Aliquots of cell suspensions (25 µL) containing $\approx 1 \times 10^7$ colony forming units (CFU) were plated on sporulation media and incubated for 48–96 h. Cells were periodically examined for the formation of asci-containing hat-shaped spores (2/ascus). In order to kill the vegetative cells, the spore suspension was quickly mixed with diethyl ether at 4°C. Immediately (within 15–30 s), aliquots were plated onto solid medium containing 20 g/L 2-DOG or a combination of 20 g/L xylose with 1, 5, or 10 g/L 2-DOG. Colonies from both kind of plates were picked and maintained on YPX agar plates before screening by microfuge fermentation *(19)*. The stability of resistance to 2-DOG by the mutant and parental strains was also repeatedly scored similarly by plating and direct streaking onto solid medium containing 20 g/L 2-DOG or a combination of 20 g/L xylose with 1, 5, or 10 g/L 2-DOG. Growth of minute colonies was evaluated by examining plates under a dissecting microscope.

Inoculum Preparation

One loopful of cells from a 48-h-old YPX plate was inoculated into 50 mL of culture medium containing 40 g/L xylose in replicate 125-mL Erlenmeyer flasks and cultivated with shaking at 100 rpm for 24 h at 25–27°C. The cultures were centrifuged at 3.4×10^3g for 10 min, suspended in 10 mL distilled water, and used as the inoculum. The optical density (OD) of each cell suspension was measured and adjusted to a constant value by dilution with water.

Microfuge Fermentation of Mixed Sugars

Mutants resistant to 2-DOG were screened for mixed sugar fermentation consisting of 32 g/L xylose and 32 g/L glucose in 1.0 mL of fermentation medium containing YNB plus urea and peptone in microfuge tubes in a variable-tilt fermentation rack *(19)*. Triplicate samples were analyzed.

Shake-Flask Fermentation

The conditions employed for shake-flask fermentations were as follows. Triplicate Erlenmeyer flasks (125 mL) each containing 50 mL of the fermentation medium containing nitrogen supplements and with 70–80 g/L of glucose, xylose, and equal amounts of xylose and glucose were inoculated with a standardized mass of cells (3 g/L) shaken at 100 rpm, and incubated at 25–27°C. For some experiments, arabinose (80 g/L) was fermented separately. The fermentation was monitored for 3–10 d by removing 1.3 mL samples for sugar and ethanol analyses.

Analytical Methods

Cell densities were measured at 525 nm after diluting cells to between 0.05 and 0.5 OD. An OD of 1.0 was equivalent to 0.21 mg dry weight of cells/mL. Ethanol was separated by gas chromatography (GC) *(23)*. Glucose, xylose, arabinose, xylitol, and other fermentation byproducts were determined by high-performance liquid chromatography (HPLC) *(24)*.

Results

Selection for Resistance to 2-DOG

P. stipitis CBS 6054 grew slowly on agar plates with 20 g/L 2-DOG (YP 2-DOG). However, in the presence of xylose (YPX 2-DOG), CBS 6054 grew faster in 3 d of incubation at various concentrations of 2-DOG. In contrast, FPL-061, a first-generation mutant of CBS 6054, showed poor growth after 5 d in the presence of YP-2-DOG or YPX 2-DOG. In our experiments, *P. stipitis* FPL-061 was used as the parent for all selections.

By plating mutagenized, sporulated cell suspensions onto YPX 2-DOG and YP 2-DOG, we obtained 58 mutants of *P. stipitis* FPL-061 that grew on 2-DOG in the presence or absence of xylose. Of these, 32 mutants were from

2-DOG alone (designated D) and 26 were from 2-DOG plus xylose (designated DX).

Screening for Ethanol Production by 2-DOG Resistant Mutants

We initially used the microfuge technique *(19)* to screen these putative mutants for increased ethanol production from mixtures of xylose and glucose. Many of the mutants selected for ability to grow on 2-DOG alone did not use all of the glucose provided. This resulted in strains with lower product yields. Only a small fraction of the mutants obtained by selection on 2-DOG alone (6.2%) produced more ethanol than the parent. A larger fraction (15%) of the DX mutants performed better than the parent. These improved strains were examined further in triplicate shake-flask cultures. The results obtained with four DX series and two D series strains are summarized in Table 1. *P. stipitis* DX-series mutants generally showed higher sugar-uptake rates than the D-series mutants. FPL-DX26 began to use xylose while glucose was still present and showed no lag in ethanol production; FPL-D17, showed slower consumption of glucose and essentially no xylose was utilized until the glucose was gone. FPL-DX26 consumed arabinose slowly but did not ferment.

Growth Characteristics of P. stipitis *and Mutants on 2-DOG*

Our initial studies of 2-DOG resistance focused on characterizing the fermentative activities of the mutant strains. Later we noted that the parent of FPL-DX26, FPL-061, was sensitive to 2-DOG, but the wild-type strain showed significant growth on 2-DOG or xylose in the presence of 0.1% 2-DOG. As can be seen in Fig. 1, all three of the strains could grow on YP with or without xylose in the presence of 0.1% 2-DOG. However, xylose greatly stimulated growth (c.f. A with A'). This was particularly true for FPL-DX26. At higher concentrations of 2-DOG, growth of FPL-061 was essentially blocked in the absence of xylose, but showed slight growth in its presence. We also observed occasional 2-DOG-resistant mutants with CBS 6054 on YP + 0.1% 2-DOG and with FPL-061 on YPX + 0.5% 2-DOG.

Because the mutant frequency was so high, we wanted to know whether we were selecting variants during the plating process. We therefore did a dilution-plating experiment and compared the total viable count on YP with growth on each of the selective media. As shown in Fig. 2 and in Table 2, essentially all the cells of CBS 6054 grew on YPX + 0.1% 2-DOG but showed only minute colonies on YP + 2% 2-DOG. The results with FPL-061 were similar, but smaller colonies were observed on YPX + 0.1% 2-DOG and only about 20% of the CFU would grow on YP + 2% 2-DOG. FPL-DX26 showed good growth on YPX + 0.1% 2-DOG, but likewise only about 15% of the cells could grow on YP + 2% 2-DOG. Occasional mutants arose from CBS 6054 and FPL-061 that were much more resistant to 2-DOG. When cells from these 2-DOG resistant colonies were again plated on the same medium, the appearance of the colonies and their number were consistent.

Table 1
Mixed-Sugar Fermentation of Various *Pichia stipitis* Strains Selected for Growth in the Presence of 2-Deoxyglucose[a]

Strain[b]	Kinetic factor					
	μ	R_s	Q_s	R_{pe}	Q_{pe}	Ye
CBS 6054[c]	a0.038 ± 0.005	a0.56 ± 0.20	a0.23 ± 0.08	a0.28 ± 0.04	a0.11 ± 0.04	a0.35 ± 0.04
FPL-061[c]	a0.033 ± 0.003	a0.63 ± 0.12	a0.25 ± 0.10	a0.30 ± 0.04	a0.12 ± 0.05	a0.48 ± 0.11
FPL DX17	a0.028 ± 0.005	b0.96 ± 0.03	a0.46 ± 0.16	a0.36 ± 0.03	a0.17 ± 0.01	a0.39 ± 0.01
FPL DX19	a0.035 ± 0.003	b0.94 ± 0.04	a0.36 ± 0.15	a0.35 ± 0.02	a0.14 ± 0.02	a0.37 ± 0.01
FPL DX21	b0.026 ± 0.003	b0.83 ± 0.03	a0.41 ± 0.14	a0.35 ± 0.03	a0.17 ± 0.02	a0.40 ± 0.02
FPL DX26	b0.026 ± 0.002	b1.00 ± 0.02	b0.50 ± 0.12	b0.45 ± 0.02	b0.23 ± 0.01	b0.45 ± 0.01
FPL D17	a0.023 ± 0.007	a0.67 ± 0.08	a0.34 ± 0.08	a0.32 ± 0.03	b0.18 ± 0.02	a0.48 ± 0.05
FPL D18	a0.037 ± 0.006	a0.71 ± 0.08	a0.25 ± 0.09	a0.34 ± 0.04	a0.12 ± 0.03	a0.48 ± 0.06

[a]Rates were calculated from data at 61 h. Numbers marked with same letters are not statistically different.
[b]Strains designated DX were obtained from YPX 2-DOG; strains designated as D were obtained from YP 2-DOG.
[c]Parental strains.

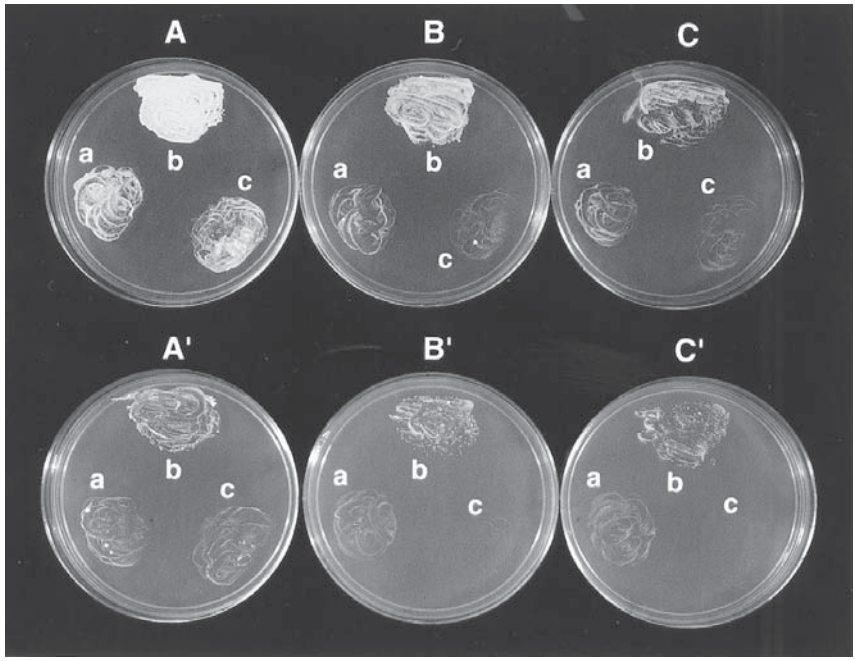

Fig. 1. Growth of wild-type and mutant strains of *P. stipitis* at 72 h on yeast extract peptone (YP) agar with various concentration of 2-DOG in the presence or absence of 2% xylose (X) **(A)** 0.1% 2-DOG; **(B)** 0.5% 2-DOG; **(C)** 1% 2-DOG. **(A')** 0.1% 2-DOG + X; **(B')** 0.5% 2-DOG + X; **(C')** 1% 2-DOG + X. (a) FPL-061; (b) FPL-DX26; (c) CBS 6054.

This experiment showed that resistance to 2-DOG arose with high frequency from the CBS 6054, FPL-DX26, or FPL-061 genetic backgrounds. The mutant FPL-DX26 showed a consistently high resistance to 0.1% 2-DOG in the presence of D-xylose, but only a fraction of the cells would grow on 2% 2-DOG in the absence of D-xylose.

Fermentation of Sugars by P. stipitis *Strains*

Among 58 independently isolated 2-DOG-resistant mutant colonies, *P. stipitis* FPL-DX26 was identified as the best fermentative strain. The glucose, xylose, and mixed-sugar fermentation was started with 3 g/L cells on dry weight basis. The cell growth of CBS 6054, FPL-061, and FPL-DX26 in glucose fermentation at 45 h was 10 g/L compared to 6.5 g/L in xylose fermentation. However, in mixed-sugar fermentation, cell growth was similar to glucose rather than xylose (data not shown). After 45 h fermentation, the growth rate of CBS 6054 in glucose and mixed sugar was slightly higher than that of FPL-061. The growth rate of FPL-DX26 was reduced after 45 h compared to that of CBS 6054 and FPL-061. However, in xylose fermentation the growth rate of all three strains was almost similar (data not shown).

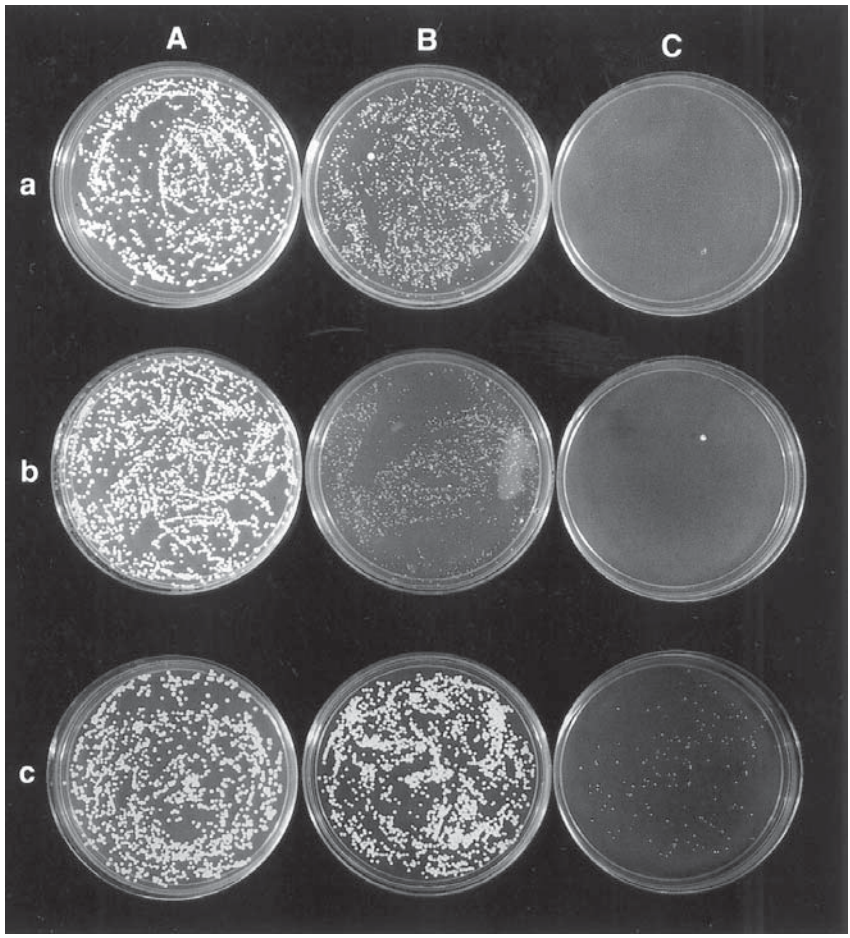

Fig. 2. Colony forming units at 93 h of growth of wild-type and mutant strains of *P. stipitis* on YP agar in the presence and absence of 2% xylose (X) and 2-DOG. **(A)** YP; **(B)** YP + X+ 0.1% DOG; **(C)** YP +2% 2-DOG. (a) CBS 6054; (b) FPL-061; (c) FPL-DX26. Note that the minute colonies of CBS 6054 and FPL-061 could be clearly observed and counted only under a dissecting microscope.

Table 2
Dilution Plating of *Pichia stipitis*
and Mutant Strains in Presence of 2-Deoxyglucose

	CFU		
Strain	YP	YP + 2% DOG	YPX + 0.1% DOG
CBS 6054[a]	1044 ± 81[b]	1016 ± 56[c]	1083 ± 151[d]
FPL-061[e]	962 ± 50[b]	211 ± 33[c]	1014 ± 113[c]
FPL-DX26[e]	974 ± 17[b]	142 ± 27[f]	1097 ± 100[d]

[a]Parental strain. [b]Growth in 44 h. [c]Growth in 168 h. [d]Growth in 72 h. [e]Mutant strain. [f]Growth in 93 h.

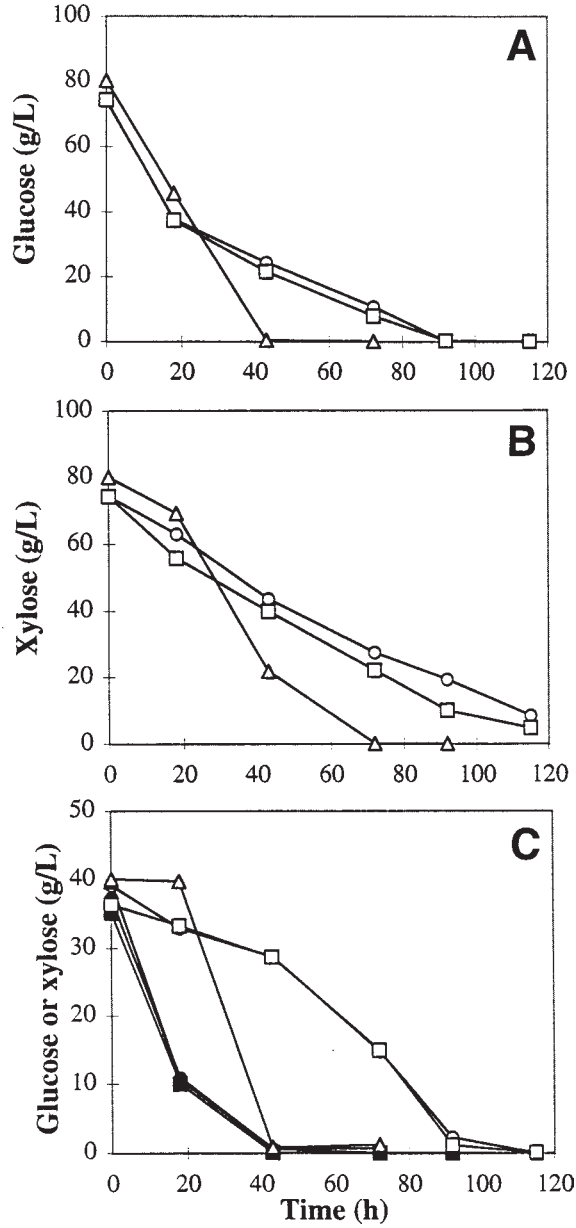

Fig. 3. Fermentation of D-glucose, D-xylose, and glucose + xylose mixture by *P. stipitis* CBS 6054, FPL-061, and FPL-DX26. Symbols: CBS 6054 (○); FPL-061 (□); FPL-DX26 (△). In sugar mixture, glucose-CBS 6054 (●); glucose-FPL-061 (■); glucose-FPL-DX26 (▲); xylose-CBS 6054 (○); xylose-FPL-061 (□); xylose-FPL-DX26 (△).

In FPL-DX26 strain, 8% glucose was consumed in 40 h compared to xylose (8%), which was consumed in 65 h (Fig. 3). The sugar mixture (8% of 1:1 glucose and xylose) was completely consumed in 45 h of fermentation. In mixed-sugar fermentation, FPL-DX26 utilized glucose and xylose

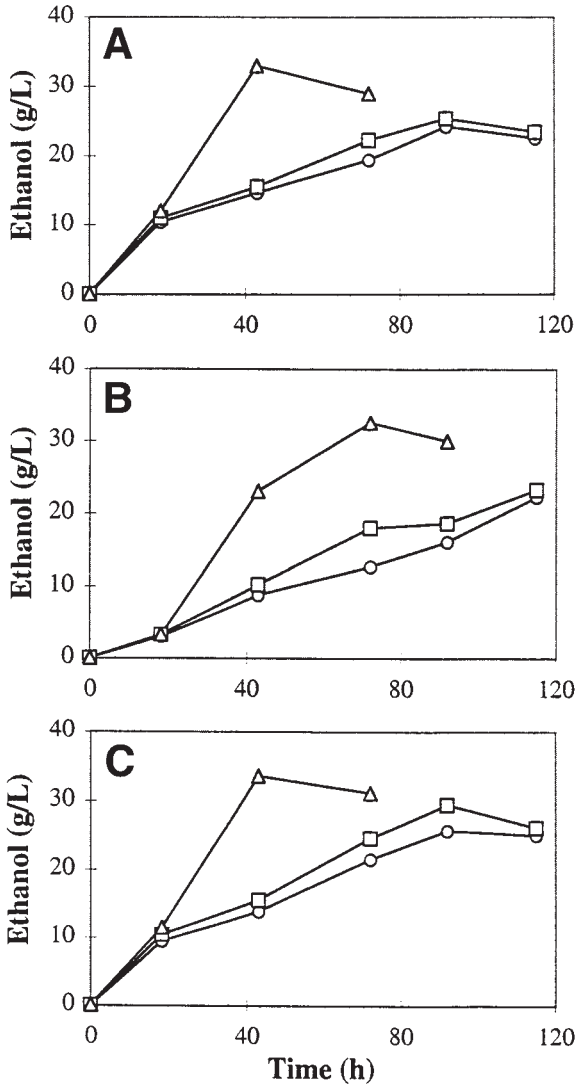

Fig. 4. Ethanol production during fermentation of D-glucose, D-xylose, and glucose + xylose mixture by *P. stipitis* CBS 6054, FPL-061, and FPL-DX26. **(A)** glucose; **(B)** xylose; **(C)** glucose and xylose. Symbols: CBS 6054 (○); FPL-061 (□); FPL-DX26 (△).

simultaneously after 20 h; the volumetric sugar utilization and ethanol production rates were higher, resulting in a much higher specific ethanol-production rate. In the CBS 6054 and FPL-061 strains, glucose was completely consumed in 90 h; xylose was never completely consumed even after 5 d (Fig. 3). In mixed-sugar fermentation, xylose was utilized at the end of glucose fermentation; and complete utilization of these sugars by CBS 6054 and FPL-061 strains took 120 h. However, FPL-061 utilized the sugars slightly faster than did CBS 6054.

Ethanol production during sugar fermentation is shown in Fig. 4. The ethanol production rate in FPL-DX26 in both glucose and mixed-sugar fermentation was faster than xylose and produced a maximum of 32–33 g/L ethanol in 40 h (Fig. 4A,C). On xylose, FPL-DX26 produced a maximum of 32 g/L ethanol at 65 h of fermentation (Fig. 4C). In contrast, in CBS 6054 and FPL-061 strains, the ethanol production rate was slower in glucose, xylose, and mixed-sugar fermentation (Fig. 4A–C).

Discussion

Previous studies by Pardo et al. *(18)* indicated that carbon catabolite-derepressed mutants could be obtained by selecting for growth on 2-DOG alone. These researchers showed that *P. stipitis* produces a L-rhamnose dehydrogenase, which is repressed by glucose. Six out of eight mutants capable of growing on 2% 2-DOG showed that L-rhamnose dehydrogenase synthesis is insensitive to glucose. The ability of these strains to use D-xylose in the presence of glucose or the fermentative capacities was not reported. Our results showed that strains derepressed for xylose utilization in the presence of glucose were obtained only after growth on xylose in the presence of 2-DOG. The difference between our results and those of Pardo et al. *(18)* could be attributed to differences in the criteria used to identify mutants, in parental *P. stipitis* strain (NRRL 5568 vs Y-21301) or in method of cell preparation. In Pardo et al.'s work *(18)*, cells grown on glycerol before plating onto 2-DOG. We chose growth on 2-DOG as a positive selection system for carbon-catabolite derepression. We observed that different *P. stipitis* strains had different capacities to grow on 2-DOG as a sole carbon source. Growth on 2-DOG may require a form of respiration that FPL-061 is deficient in generation of nicotinamide adenine dinucleotide phosphate and reduced form (NADP or NADPH) *(14,15)*. This strain had been selected for resistance to the respiration inhibitors SHAM and AA, and it showed diminished cell yield as compared to its parent, CBS 6054 *(21)*. The inability of many of our 2-DOG resistant mutants to use all of the glucose provided might be attributable to a loss of hexokinase *(17)* without a concomitant derepression of xylose metabolism. The FPL-DX26 mutant showed an greater capacity for using xylose in the presence of glucose than did its parent, FPL-061, even though both strains used glucose at the same rate. These results suggest that the mutational event giving rise to FPL-DX26 was a derepression of the xylose-assimilation pathway.

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