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Ethanol Production from Glucose/Xylose Mixes by Incorporating Microbes in Selected Fermentation Schemes

Scientific Note

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Index Entries: Ethanol; xylose; recombinant processes; comparison.

INTRODUCTION

Four microbial strains were incorporated into selected fermentation schemes to enhance the production of ethanol from mixes of predominantly glucose and xylose. Lesser amounts of mannose, galactose, and arabinose were included in substrates that provided sugar compositions much like various sources of acid pretreated biomass hydrolysates. The microbial strains were selected because of characteristics that could enhance ethanol production. The strains included an ethanol-producing *Escherichia coli* S17-1 with pLOI308-10, the result of insertion of genes coding for alcohol dehydrogenase II and pyruvate decarboxylase from the ethanologenic bacterium *Zymomonas mobilis* (*1*); *Pachysolen tannophilus* ($NO_3NO_{3-4}\times$ *eth* 2, a hybrid of the xylose-fermenting yeast, reported to be glucose negative) (*2*); *P. tannophilus* (NRRL Y-2460), a xylose-fermenting yeast; and a glucose-fermenting yeast, *Saccharomyces cerevisiae* (ATCC 24860). The strains were evaluated for the potential to ferment xylose as a sole carbon source, as well as a mixed sugar substrate at each of three concentration levels. In addition, each of the three strains with xylose-fermenting ability was tested in the mixed sugar substrate for its ability to produce ethanol in coculture with *S. cerevisiae* and in a sequential fermentation

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process, following an initial *S. cerevisiae* fermentation, for production of ethanol from glucose and mannose. The comparisons were made under a set of conditions favoring the xylose-fermenting yeasts, which were kept uniform for all the strains observed. The utilization of various sugars, the production of ethanol, and the production of biomass were monitored.

MATERIALS AND METHODS

Inoculum Preparation

The yeast species were maintained on YM agar (Difco Laboratories, Detroit, Michigan) slants. The *E. coli* strain was maintained on slants of LB agar with added glucose (*1*). Growth was transferred from 24-h slants of each species to an inoculum medium. The resulting cultures were incubated for 48 h at 30°C on a rotary shaker at 100 rpm. For both *Pachysolen tannophilis* m (hybrid strain, $NO_3NO_{3-4} \times$ eth 2) and *P. tannophilus* s (NRRL Y-2640), the inoculum medium was 20 g/L xylose plus 6.7 g/L yeast nitrogen base (YNB, Difco), adjusted to pH 4.5 with sodium hydroxide. The inoculum medium was the same for *S. cerevisiae*, except that a level of 20 g/L glucose was used. The inoculum medium for *E. coli* was LB broth with 20 g/L glucose, pH 7.

Inoculum cultures were centrifuged to recover cells. The spent medium was decanted, and the cells were resuspended in an equal amount of sterile, distilled water. Each fermentation flask received a sufficient volume of the resuspended cells, resulting in a 10% (by volume) inoculum.

Fermentation Test Media

All test media were supplemented with 2 g/L yeast extract, 2 g/L urea, and 0.5 g/L potassium dihydrogen phosphate. Xylose fermentation media contained concentrations of 50, 125, or 200 g/L xylose. Mixed sugar fermentation media contained 50, 125, or 200 g/L sugars composed of 46% glucose, 44% xylose, 4% mannose, 2% arabinose, and 4% galactose.

Fermentation

Fermentations were conducted in 100 mL vol at 30°C and 100 rpm on a rotary shaker. Sterile, disposable, 250 mL Erlenmeyer flasks were used as fermentation vessels.

Xylose fermentation media were inoculated with each of the four species for batch fermentations. Mixed sugar fermentation media were inoculated with: each of the species for batch fermentations, a combination of *S. cerevisiae* with each of the other three species for cocultures, and an initial inoculum of *S. cerevisiae*, followed by a second inoculum after 3 d of incubation, with one of the three remaining species for sequential fermentations. Samples were taken during the fermentations at 1-, 3-, 5-, 7-,

Table 1
Results of Fermentation of Xylose

Microorganism	Initial xylose, g/L	Maximum ethanol, g/L	Xylitol, g/L	g ethanol/ g sugar
P. tannophilus m	50	15.6	9.2	0.31
P. tannophilus s	50	11.8	10.2	0.24
E. coli	50	10.1	4.3	0.29
S. cerevisiae	50	1.2	2.5	0.10
P. tannophilus m	125	14.5	24.8	0.20
P. tannophilus s	125	20.2	38.3	0.19
E. coli	125	6.3	3.5	0.16
S. cerevisiae	125	7.0	19.1	0.13
P. tannophilus m	200	24.1	34.3	0.26
P. tannophilus s	200	12.1	34.1	0.17
E. coli	200	5.4	4.5	0.30
S. cerevisiae	200	7.8	28.7	0.17

and 10-d intervals. The fermentations were not pH controlled by the addition of base. The final pH of *E. coli* fermentations was between 5.5 and 6.

Analytical

Biomass was measured by gravimetric determinations of cell mass at the initiation of the fermentations and at the end of the time course observed. Sugars and organic components were determined by high performance liquid chromatographic methods (HPLC) (*3*).

RESULTS AND DISCUSSION

Xylose Fermentation

The yields of ethanol from xylose (50, 125, and 200 g/L), using *P. tannophilus* strains, were superior to the yield using *E. coli* under the set of conditions for this experiment (Table 1). Over the time course, *E. coli* lagged behind the *P. tannophilus* strains in initiation of sugar consumption and ethanol production (Fig. 1). This suggests that sugar consumption and ethanol production by *E. coli* would have reached greater levels if the fermentation time course had been extended. Additional tests with *E. coli* are needed to optimize conditions of culture to determine the potential of *E. coli*. Conditions of this experiment were those determined as best for the *P. tannophilus* strains.

There was production of xylitol from xylose by all the yeast strains (Table 1). There was a trend toward a greater ratio of xylitol to ethanol as

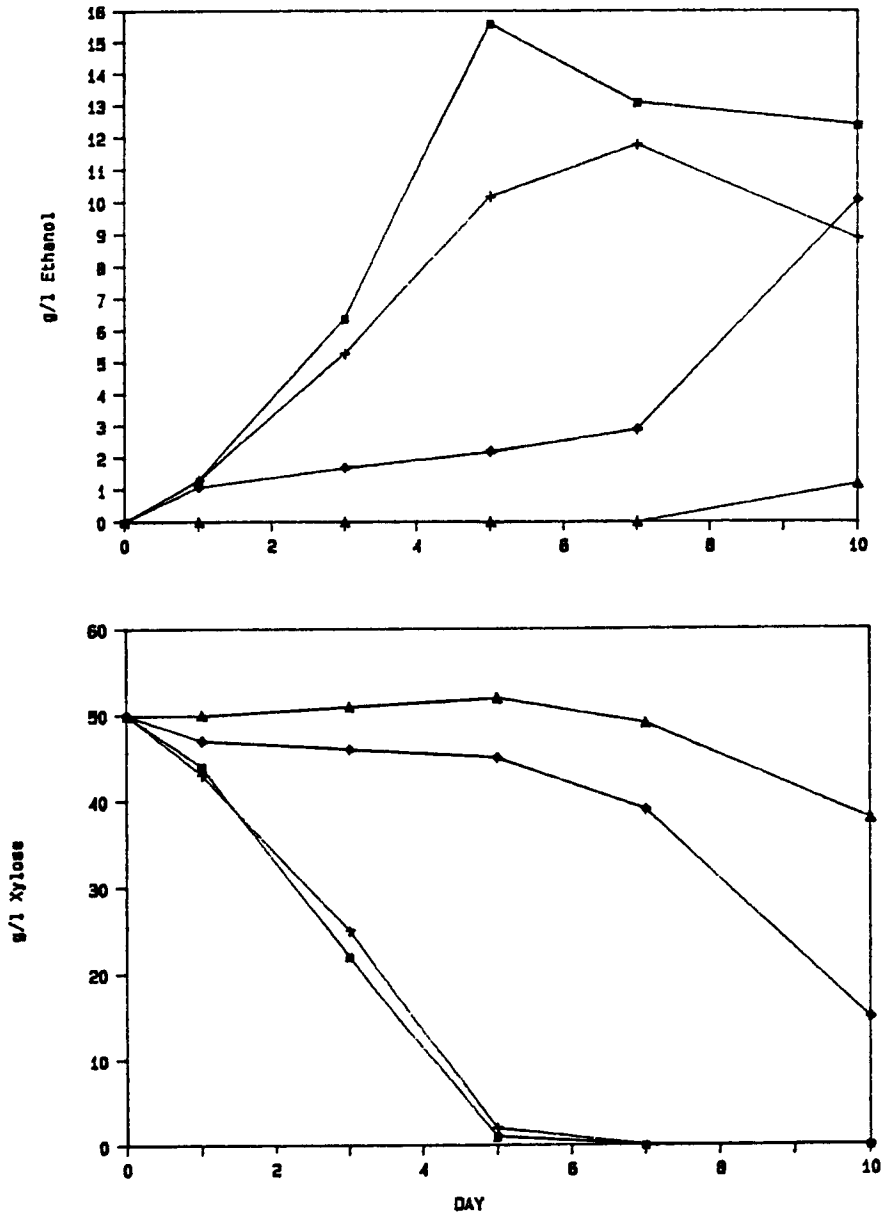

Fig. 1. Time courses of batch fermentations of 50 g/L xylose (square, *P. tannophilus* m; plus, *P. tannophilus* s; diamond, *E. coli*; triangle, *S. cerevisiae*).

the concentration of xylose in the feed increased. The identification of xylitol in the *E. coli* fermentations were unexpected. Little acetic acid and glycerol were produced by any of the strains.

Batch Fermentations of Mixed Sugars

The sugar composition of the mixed sugar fermentation media was typical of that obtained from the TVA dilute acid hydrolysis process. (If all

Table 2
Results of Fermentation of Mixed Sugars (Batch)

Microorganism	Initial sugars, g/L	Maximum ethanol, g/L	Xylitol, g/L	g ethanol/ g sugar
P. tannophilus m	50	20.5	2.1	0.42
P. tannophilus s	50	18.5	1.6	0.40
E. coli	50	16.0	1.1	0.33
S. cerevisiae	50	10.5	5.6	0.42
P. tannophilus m	125	29.9	2.7	0.41
P. tannophilus s	125	33.5	3.5	0.36
E. coli	125	33.6	1.6	0.40
S. cerevisiae	125	26.2	3.8	0.44
P. tannophilus m	200	42.5	3.8	0.48
P. tannophilus s	200	44.1	6.2	0.42
E. coli	200	44.1	3.0	0.45
S. cerevisiae	200	40.7	4.8	0.43

sugars were recovered from hardwood, the sugar mix would be about 69% hexose and 31% pentose.) There are nearly equal amounts of glucose and xylose. The nutrient source was typical of what is used in this laboratory with acid hydrolysates.

The yields of ethanol from sugar mixes (50, 125, and 200 g/L) in batch fermentations were somewhat greater with *P. tannophilus* strains when the substrate was 50 g/L sugars (Table 2). However, ethanol yield coefficients from 125 and 200 g/L sugars were similar for *E. coli* and the *P. tannophilus* strains. Time-course plots show that *E. coli* lagged behind the other strains in the initiation of sugar consumption and ethanol production (Fig. 2). Time-course data suggest that only ethanol concentrations that exceed those produced by *S. cerevisiae* are owing to ethanol production from xylose (Fig. 2). Additional tests are needed with *E. coli* under conditions optimum for the strain.

Time courses show that there was diauxic sugar utilization with *P. tannophilus* and *E. coli*. This pattern in *P. tannophilus* has been attributed to sugar transport problems (*4,5*). Diauxie may result from nutrient limitations as well. The nutrient requirements of *E. coli* in the application of ethanol production have not been determined. There is reason to consider sugar transport for future studies since the use of *E. coli* with biomass-derived materials could require exposure to a variety of sugars in many combinations.

There was little difference in the performance of the hexose-negative *P. tannophilus* m strain and the s strain (Table 2). The yields using *P. tannophilus* m of 15.6 g/L ethanol from 50 g/L xylose and 20.5 g/L ethanol from 50 g/L mixed sugars were 61 and 80%, respectively, of the theoretical yield.

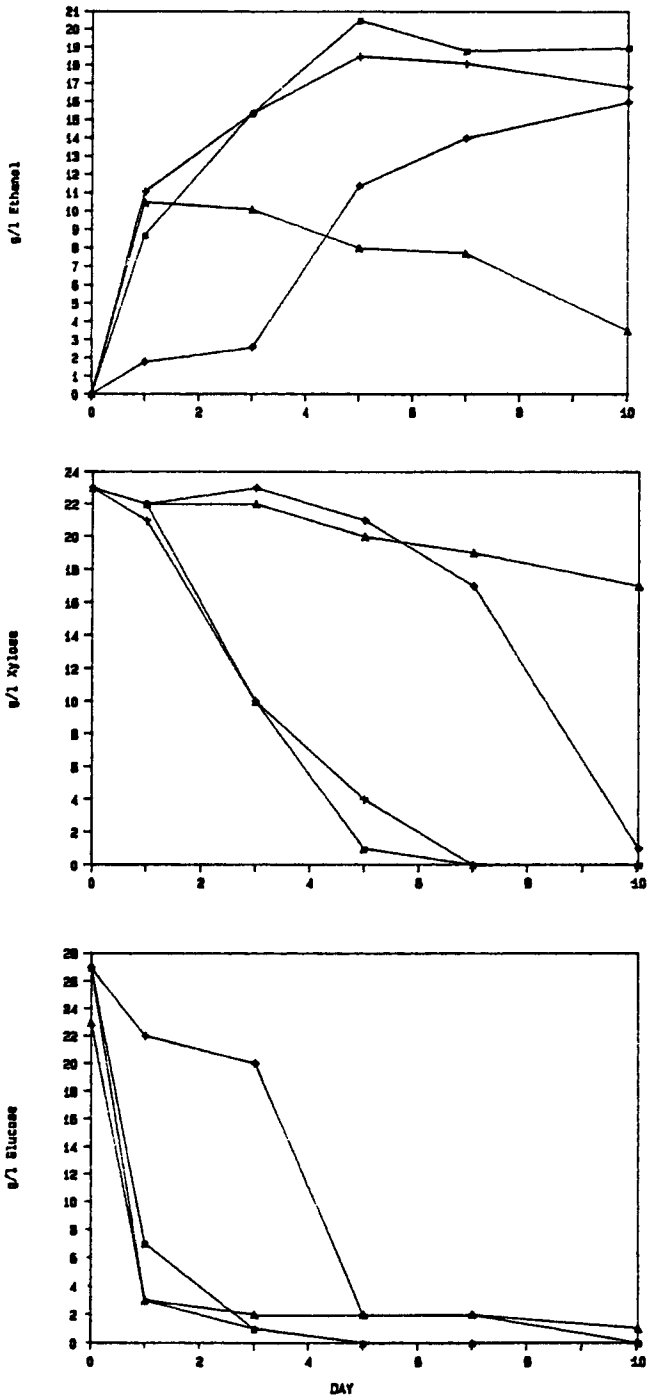

Fig. 2. Time courses of batch fermentations of 50 g/L sugars (square, *P. tannophilus* m; plus, *P. tannophilus* s; diamond, *E. coli*; triangle, *S. cerevisiae*).

Table 3
Results of Fermentation of Mixed Sugars
by Each Microorganism in Combination with *S. cerevisiae*

Microorganism	Initial sugars, g/L	Maximum ethanol, g/L	Xylitol, g/L	g ethanol/ g sugar
P. tannophilus m	50	11.9	4.8	0.41
P. tannophilus s	50	14.9	3.2	0.35
E. coli	50	10.3	5.2	0.43
P. tannophilus m	125	27.0	3.8	0.46
P. tannophilus s	125	30.7	3.8	0.44
E. coli	125	26.7	4.8	0.43
P. tannophilus m	200	43.3	4.3	0.45
P. tannophilus s	200	42.7	3.5	0.44
E. coli	200	41.2	4.6	0.43

Coculture

There was an initial fermentation of the glucose by the cocultures (Fig. 3). In the case of *P. tannophilus* s, there was xylose utilization and production of ethanol, which was probably derived from xylose (Table 3). The time-course data suggest that there was initial ethanol production from glucose by the *P. tannophilus* m and *E. coli* cocultures with *S. cerevisiae*. There followed a period of simultaneous consumption of xylose and ethanol, rather than ethanol production from xylose, as desired.

Sequential Fermentations

Results from sequential fermentations are given in Table 4. Only *P. tannophilus* s (Fig. 4) showed any indication of ethanol production from xylose after its introduction to the culture. Unlike coculture fermentations, sequential fermentations showed no simultaneous consumption of xylose and ethanol by *P. tannophilus* m and *E. coli* after their introduction into the fermentations.

The *P. tannophilus* m strain, despite claims that it is glucose negative, did not prove to be of greater benefit in cocultures and sequential fermentations than the s strain. The s strain provided somewhat better yields at 50 g/L sugars, in both coculture and sequential fermentation schemes, in comparison to the m strain. In coculture, 14.9 g/L ethanol was produced and 42.5 g/L sugars were consumed. In the sequential fermentation scheme, 11.6 g/L ethanol was produced and 48 g/L sugars were consumed using the s strain.

No benefits were achieved from coculture and sequential fermentation schemes in comparison to batch cultures, as a result of the combination of *S. cerevisiae* as a glucose fermenter with the yeasts and *E. coli*. It has

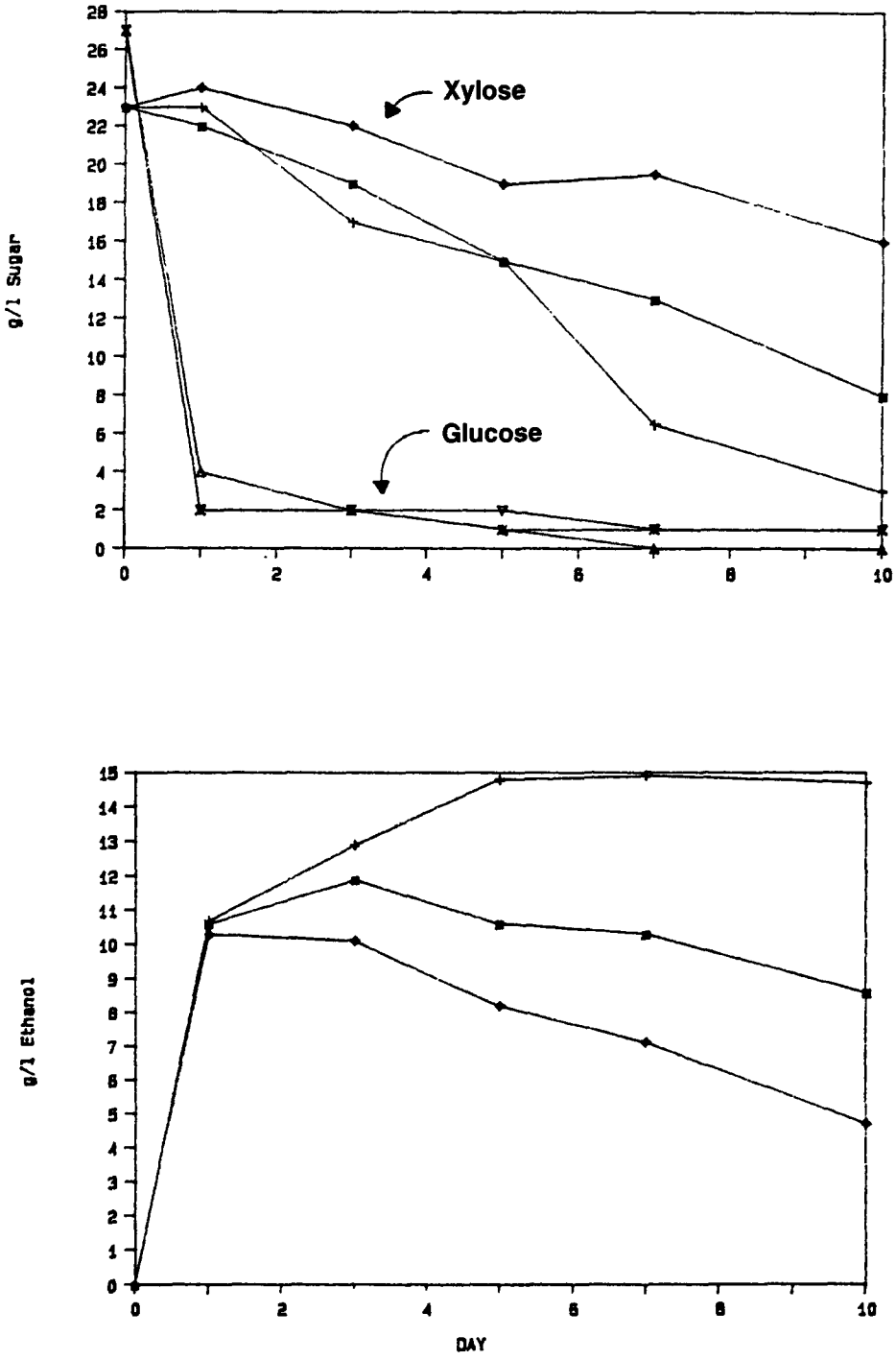

Fig. 3. Time courses of batch fermentations of 50 g/L sugars by coculture of each microorganism with *S. cerevisiae* (square, *P. tannophilus* m; plus, *P. tannophilus* s; diamond, *E. coli*).

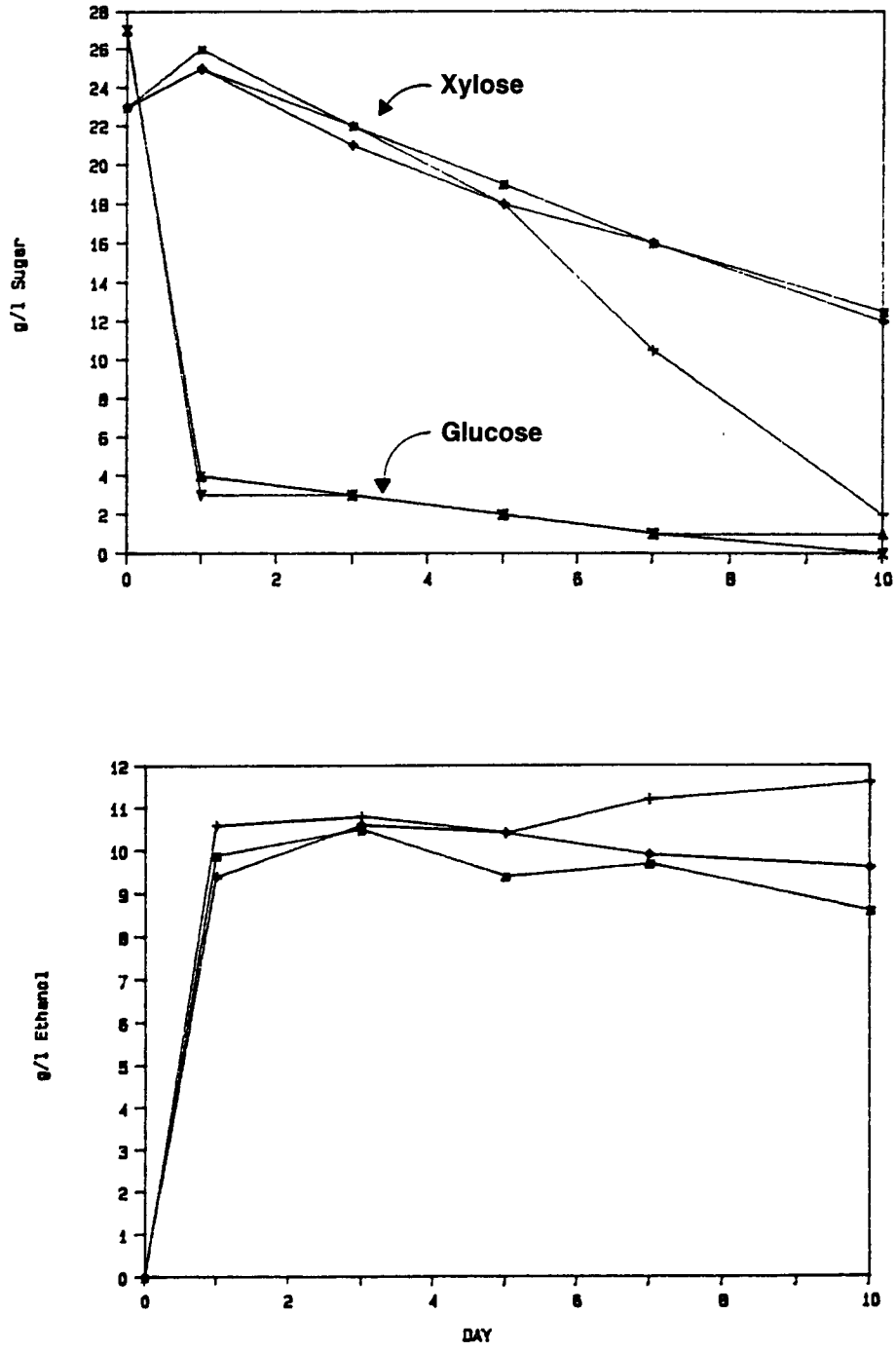

Fig. 4. Time courses of fermentations of 50 g/L sugars using a sequential fermentation scheme incorporating *S. cerevisiae* for initial glucose fermentation, followed by the addition of a second microorganism at d 3 (square, *P. tannophilus* m; plus, *P. tannophilus* s; diamond, *E. coli*).

Table 4
Results of Sequential Fermentation of Mixed Sugars Through Initial Glucose Fermentation with *S. cerevisiae* and the Addition of a Second Microorganism at 3 d

Microorganism	Initial sugars, g/L	Maximum ethanol, g/L	Xylitol, g/L	g ethanol/ g sugar
P. tannophilus m	50	10.5	4.9	0.38
P. tannophilus s	50	11.6	5.3	0.24
E. coli	50	10.6	4.0	0.41
P. tannophilus m	125	27.9	3.8	0.42
P. tannophilus s	125	28.3	5.6	0.50
E. coli	125	28.3	3.4	0.51
P. tannophilus m	200	44.4	3.1	0.42
P. tannophilus s	200	43.0	4.0	0.43
E. coli	200	40.0	4.6	0.41

been reported that, with the *P. tannophilus* s, there was little potential for increased ethanol yield in a sequential fermentation (*6*). In this work, the *P. tannophilus* s strain alone showed any potential for improved ethanol yields by such schemes.

The rapid formation of ethanol from glucose in the coculture and sequential fermentation schemes raises the possibility of inhibition of xylose fermentation by ethanol. Ethanol inhibition of *P. tannophilus* is reported to occur at ethanol concentrations around 20 g/L (*7*). The effect of ethanol on *E. coli* fermentation to ethanol is important for further study of the strain.

Additional work is already under way with the recombinant *E. coli* in this laboratory to optimize the production of ethanol, both from a sugar substrate and dilute acid hydrolysate of hardwood.

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