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Simultaneous Fermentation and Isomerization of Xylose to Ethanol at High Xylose Concentration

Scientific Note

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INTRODUCTION

Fermentation of xylose found in the hemicellulose fraction of agricultural and hardwood biomass is a key to the economic production of ethanol from these feed stocks (*1*). Xylose is easily obtained by dilute acid prehydrolysis of biomass, but it is more difficult to ferment to ethanol than the glucose derived from the cellulose fraction. In yeast, xylose is initially converted to xylitol, and then to xylulose. The enyzmes that catalyze these conversions, xylose reductase and xylitol dehydrogenase, require the coenzymes $NADPH_2$ and NAD, respectively, for hydrogen transfer. It has been shown that the regeneration of NAD from the $NADH_2$ produced in the xylitol dehydrogenase reaction usually requires the presence of oxygen (*2*). Thus, although many yeasts can grow on xylose, they can ferment it poorly, if at all.

Some yeast strains have been found to ferment xylose in a microaerophilic or "oxygen-limited" ennvironment (*3–6*). The species used, *Pachysolen tannophilus, Candida shehatae,* and *Pichia stipitis,* require very

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low oxygen concentrations (*7,8*), low temperatures (≤30°C), and low substrate concentrations (≤5% xylose). In addition, the low ethanol tolerance of these strains and slow rates of fermentation, along with catabolism of ethanol in the presence of oxygen during or at the end of fermentation (*9–11*), limit commercial application of this approach.

It has also been found that converting xylose to the more fermentable xylulose, using the bacterial enzyme xylose isomerase, allows for totally anaerobic fermentation by ethanol-tolerant yeast strains, such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* or *Canada tropicalis* (*12–18*). Simultaneous fermentation and isomerization of xylose (SFIX) is preferred over isomerization prior to fermentation, because the ratio of xylulose to xylose is low at equilibrium (approximately 1:5) (*19*). The simultaneous process allows the total fermentation of xylose in a single step, since the yeast remove xylulose as the isomerization proceeds.

In a previous report (*20*), we found that SFIX fermentation of xylose was optimal when *S. pombe* (Y164) was used with a commercial xylose isomerase, Sweetzyme Q™, over a pH range from 5.5 to 6.0 and a temperature of 35–37°C. A xylose concentration of 6% (w/v), an amount that might be expected from the hydrolysis of numerous xylose feedstocks, was used.

In this report, initial xylose concentrations of 2–16% (w/v) were used to determine the effect of substrate concentrations on ethanol yield. These results were compared with glucose fermentations under the same conditions.

MATERIALS AND METHODS

Schizosaccharomyces pombe (Y164) was obtained from the Northern Regional Research Laboratories, USDA, Peoria, IL. Sweetzyme Q™, an immobilized xylose isomerase, was obtained as a gift from NOVO Industries. Fermentations were conducted in six New Brunswick Biogen F-1000 fermentation units with 1-L vessels (working volumes of 400–500 mL). These units provided temperature control; pH control was achieved using 1*M* KOH and Cole-Palmer J-7537 peristaltic pumps controlled with a computerized scanning/control unit (Fisher Scientific) that monitors and/or controls temperature and pH and automatically records pH/temperature data at preset intervals.

S. pombe cells were grown to late stationary phase at 30°C (2.2–2.7 OD_{600}, 1.1 mg/mL dry weight = 1 OD_{600}) in yeast extract–peptone medium (Difco, YEP), with 2% glucose. They were then centrifuged (8000 x *g*, 10 min) and resuspended in YEP medium with either filter-sterilized xylose and 10 g/L Sweetzyme Q™ xylose isomerase, or filter-sterilized glucose at the specified concentrations. In order to maintain anaerobiosis, the fermentations were sparged with nitrogen at the beginning, and a small amount of nitrogen was used when sampling to displace 5 mL of culture.

Ethanol concentration was measured using a Hewlett Packard gas-liquid chromatograph equipped witha flame-ionization detector and peak area integrator (model #5880A with a Porapak Q 80–100 column) operated at an oven temperature of 175°C, injection port at 225°C, and detector at 250°C, with isopropanol as an internal standard. Sugar concentrations (xylose, xylulose, xylitol, and glycerol) were measured by high-performance liquid chromatography with a Bio-Rad HPX-87C column at 60°C, Hewlett-Packard 1037A high-sensitivity refractive index detector, and a Shimadzu C-R3A integrating recorder (*21*) using mannitol as an internal standard. Filtered H_2O was used as eluant at 0.2 mL/min. Computer analysis of chromatographic data was performed using a Lotus 123™ spreadsheet. The spreadsheet was constructed to correct for calibration between sugar concentrations and peak area, internal standard control of injection volume, and dilution of the fermentation by bases used for pH control.

RESULTS AND DISCUSSION

It can be stated, in general, that fermentation of xylose and xylulose to ethanol by yeast strains is slower and less efficient than glucose fermentation. Xylose must be converted by at least six additional enzymes in the pentose phosphate pathway to intermediates of the glycolytic pathway. The levels of these enzymes, their requirements for induction, and the availability of required cofactors are thought to be major factors causing the inefficiency of xylose fermentation (*22,23*). When xylose levels exceed 4–6%, microaerophilic xylose fermentations are inhibited (*10*) either because of the factors mentioned above or because of ethanol toxicity. The experiments described in this report examine the effect of xylose concentration on SFIX fermentation.

Glucose fermentation by *S. pombe* at 35°C and pH 5.75, the temperature and pH used in SFIX fermentations, is rapid and efficient at all levels tested (2–16%). An ethanol concentration of 6.5% w/v was obtained from 16% glucose within 30 h, fermentations of glucose from 2 to 12% were all completed within 16 h. The average initial rate, determined by linear regression over the first 12 h of these fermentations, was 55 g/L/d (2.3 g/L/h) (Fig. 1).

In contrast to these results, SFIX conversion of xylose to ethanol by *S. pombe* is slower and less efficient. The average initial rate of xylose fermentation, 12g/L/d (0.5 g/L/h), is approximately 4.5× slower than the initial rate of glucose fermentation, and the time required to complete the xylose fermentations increases as the initial xylose concentration increases, to over 7 d for fermentation of 16% xylose (Fig. 2). Linear regression of the initial rates of SFIX fermentation indicate that, at xylose levels of less than 6%, the fermentations are apparently limited by insufficient levels of xylulose, whereas at levels over 10%, the rate of fermentation is reduced by other factors (Fig. 3). These effects on fermentation rate can be seen in

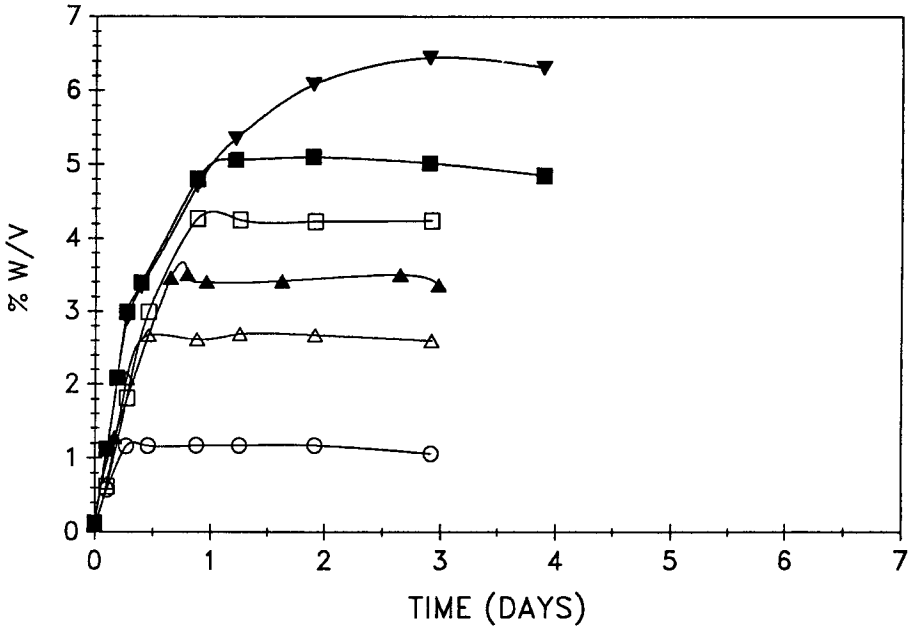

Fig. 1. Ethanol production in batch glucose fermentations performed at 35°C, pH 5.75, and initial substrate concentrations of 2%, 6%, 8%, 10%, 12%, and 16%. ○=2%; ●=4%; △=6%; ▲=8%; □=10%; ■=12%; ▼=16%.

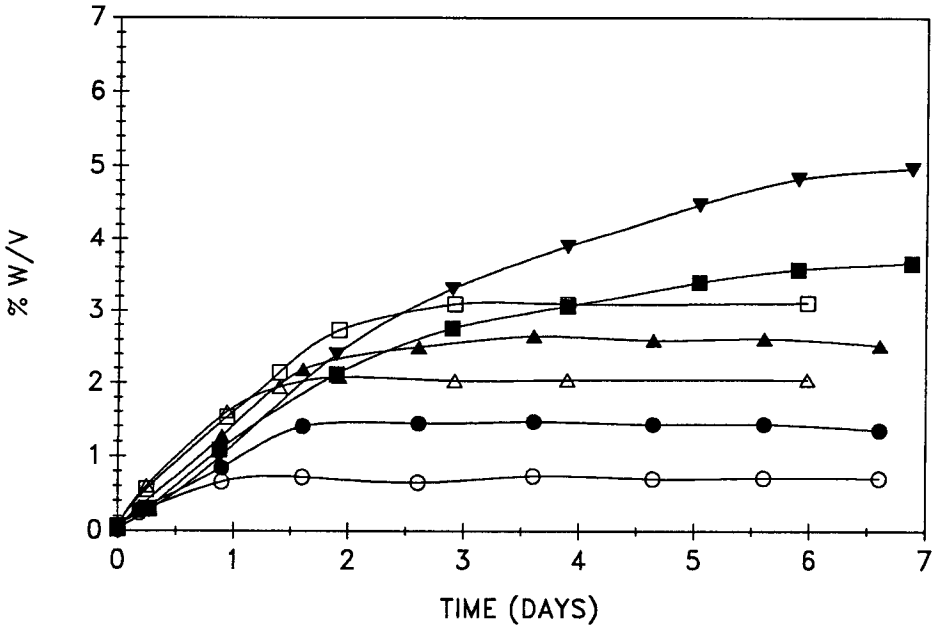

Fig. 2. Ethanol production in SFIX xylose fermentations performed at 35°C, pH 5.75, and initial substrate concentrations of 2%, 4%, 6%, 8%, 10%, 12%, and 16%. ○=2%; ●=4%; △=6%; ▲=8%; □=10%; ■=12%; ▼=16%.

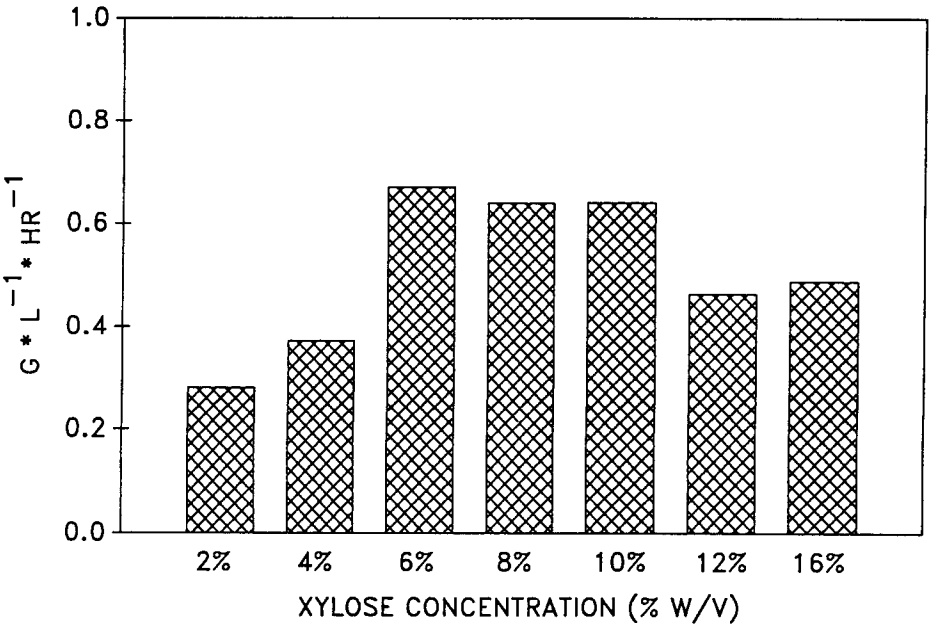

Fig. 3. Initial ethanol production rates in SFIX xylose fermentations.

the reduced rate of xylose utilization (Fig. 4) and xylulose uptake (Fig. 5). The increase in initial xylose concentration also caused increased byproduct (xylitol)/ethanol ratios (Fig. 6), which indicates that higher xylose or ethanol concentrations either inhibit or overload the ethanol production pathway.

Despite the difference in rate between glucose and SFIX xylose fermentation, final yields of ethanol in SFIX were impressive when compared to the microaerophilic fermentation by less ethanol-tolerant yeasts. Fermentation of 16% xylose resulted in a final ethanol concentration of 5.3% w/v, a yield of 65% of the theoretical limit. At the lower xylose concentrations that can be easily produced from pretreated biomass, i.e., less than 8%, yields of 70% of the theoretical production are obtained, and at these concentrations the fermentations require 2–3 d for completion.

In addition, a preliminary SFIX fermentation with recycling of the enzyme and yeast cells, using two consecutive runs, each at 6% xylose, was shown to allow for more cost-effective utilization of the biocatalysts (Fig. 7). Since this strain of *S. pombe* cells tend to flocculate, no centrifugation was required. A total of 41 g of ethanol was produced from 120 g of xylose in this experiment, whereas 37 g was found when 12% xylose was fermented in a single batch. This experiment, and the results presented in Fig. 5, also show that low rates of xylose fermentation are not the results of loss of xylose isomerase activity.

The major difference between fermentation of xylulose in SFIX fermentation and glucose fermentation is the uptake and conversion of xylulose to glycolytic intermediates by enzymes in the pentose phosphate

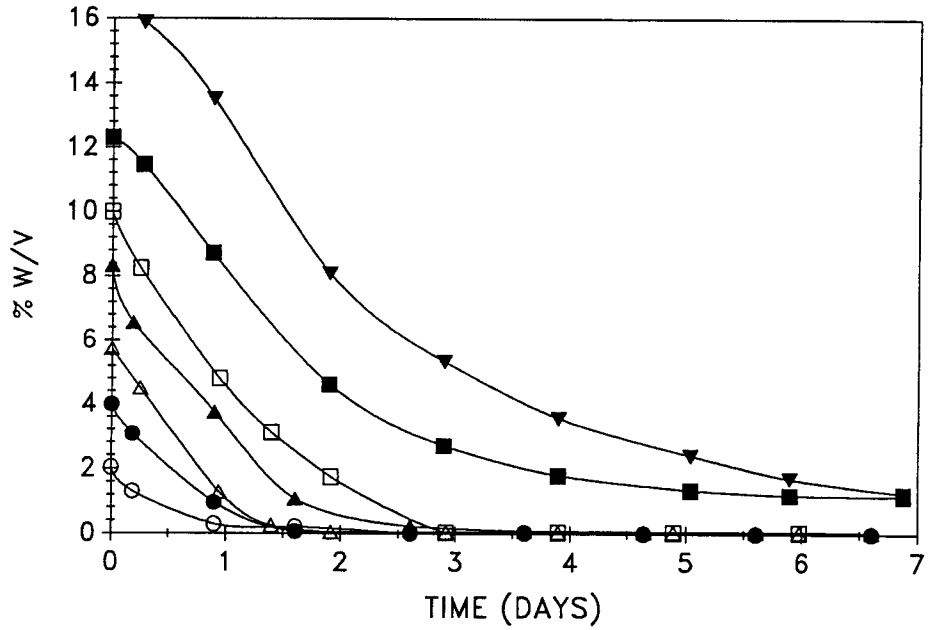

Fig. 4. Substrate utilization in SFIX fermentatioan of xylose. ○=2%; ●=4%; △=6%; ▲=8%; □=10%; ■=12%; ▼=16%.

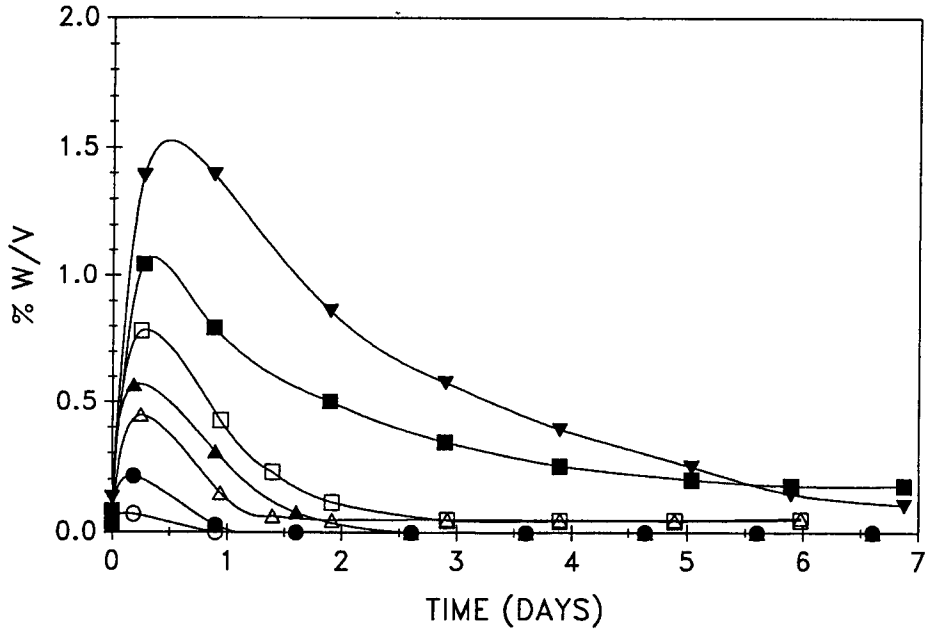

Fig. 5. Changes in xylulose concentration during SFIX fermentations. ○=2%; ●=4%; △=6%; ▲=8%; □=10%; ■=12%; ▼=16%.

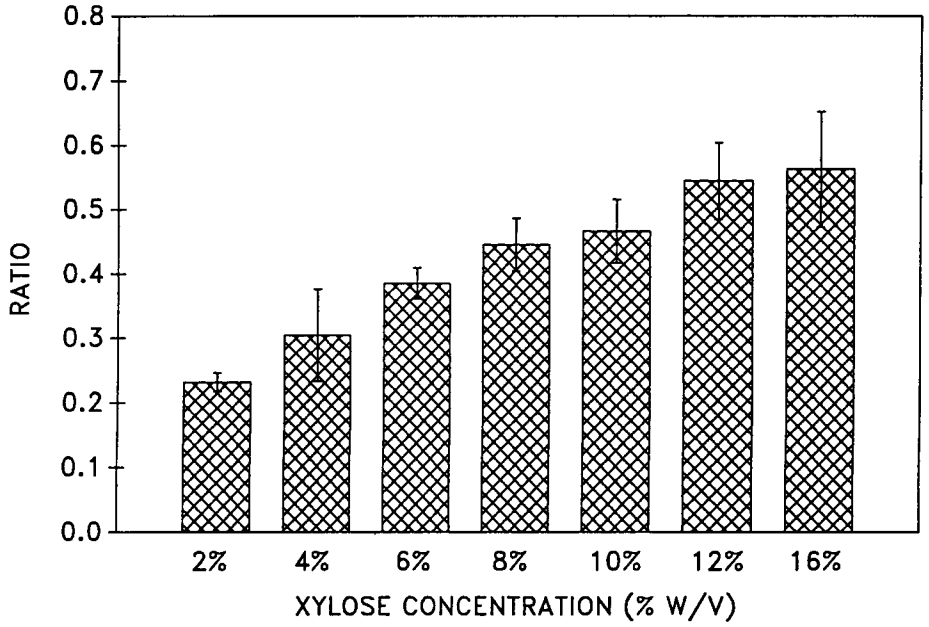

Fig. 6. Changes in xylitol/ethanol ratio with increasing initial xylose concentration in SFIX fermentations.

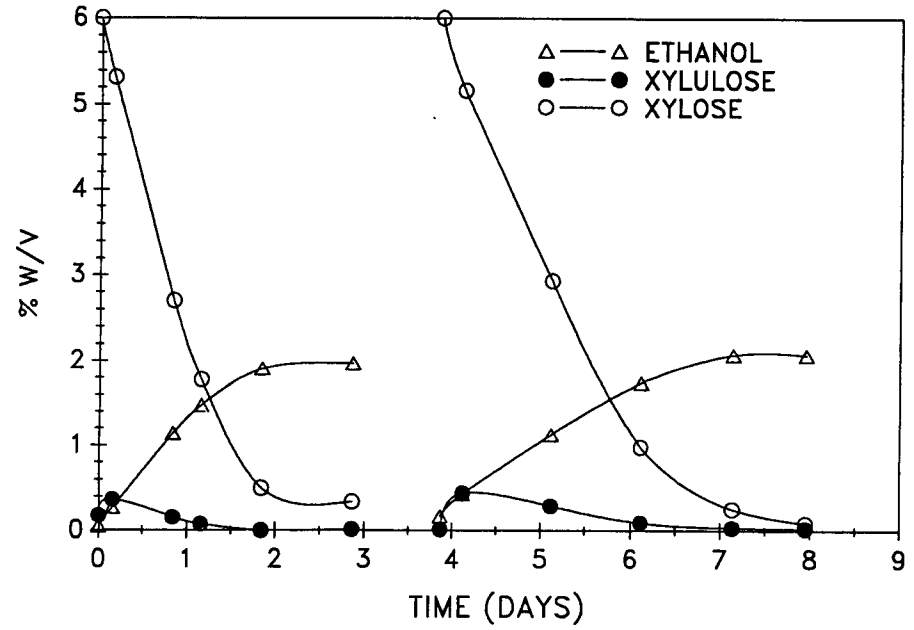

Fig. 7. Recycling of cells and enzyme in SFIX fermentation. ○=2%; ●=4%; △=6%; ▲=8%; □=10%; ■=12%; ▼=16%.

pathway. Thus, it is likely that one or more of these conversion steps are inefficient, as a result of either low enzyme levels, or inhibition of the enzymes (or their synthesis), by xylose, xylulose, or ethanol. Detailed biochemical studies are needed for elucidation of critical enzymatic steps that decrease the rate and yield of xylulose fermentation in *S. pombe.*

On the other hand, our current results show that the SFIX approach provides a significant improvement over current alternative systems for fermentation of xylose to ethanol, because it is faster and more tolerant to higher xylose and ethanol concentrations than microaerophilic fermentations with xylose-fermenting yeast strains. Recycling immobilized xylose isomerase enzyme and yeast, which appears to be feasible, can also significantly decrease biocatalyst costs.

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