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High-Yield Shake-Flask Fermentation of Xylose to Ethanol

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ABSTRACT

Methods of achieving high-yield fermentation of D-xylose to ethanol in shake flasks were investigated using the microaerophilic yeast *Pichia stipitis* NRRL Y-7124. Ethanol yields based on consumed xylose approached the theoretical maximum of 0.51 g/g at high culture volumes. Although volumetric productivities decreased from 0.46 g/L-h at 100 mL to 0.15 g/L-h at 225 mL, ethanol yields on consumed xylose of 0.50 g/g were achieved in 250-mL DeLong culture flasks at culture volumes of 200 and 225 mL.

Index Entries: D-xylose; ethanol; fermentation; shake flask; *Pichia stipitis*.

INTRODUCTION

The overall mission of NREL's Ethanol from Biomass project is to develop a process for producing low-cost fuel ethanol from abundant and inexpensive lignocellulosic materials, such as agricultural and forestry residues, herbaceous crops, and hardwoods. Five carbon sugars (pentoses), most importantly D-xylose, represent roughly one-third of the total carbohydrate sugars available in hardwood feedstocks (*1*). Hinman et al. (2) performed an economic analysis of ethanol production from lignocellulosic biomass, and concluded that the fermentation of xylose to ethanol at high yield and high final ethanol concentration is essential to achieve economic ethanol production.

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Numerous microorganisms are capable of fermenting xylose to ethanol (3). The yeast *Pichia stipitis* was chosen as a candidate for experimentation because it ferments D-xylose at high ethanol yield (ethanol yields exceeding 0.40 g/g, which Jefferies [4] suggests is the minimum yield necessary for commercialization of xylose to ethanol fermentation) and low coproduct (primarily xylitol) formation rates (3). For reasons that are somewhat unclear, this organism requires small quantities of molecular oxygen to achieve high-yield ethanol production. Control of aeration is essential, because high levels of aeration lead to extensive cell mass production and concomitant low ethanol yield. Skoog and Hahn-Hägerdah (5) showed that at pH 5.5 yields of 0.48 g/g can be achieved with *P. stipitis* at aeration rates lower than 1 mmol O_2/L-h; yield decreased to 0.25 g/g during strictly anaerobic fermentation. Guebel et al. (6) observed a maximum in volumetric productivity of 0.5–0.6 g/L-h (pH 5.5) at slightly higher aeration rates of 3–5 mmol O_2/L-h; however, yields of only 0.21–0.28 g/g were achieved. Although no oxygen transfer rates were provided, experiments by du Preez et al. (7) conducted at 0.13 vvm suggest that at pH 4.5, ethanol yield and volumetric productivity are maximized at values near 0.43 g/g and 0.85 g/L-h, respectively. Clearly, strict control of aeration and pH is necessary for this organism to ferment xylose at high ethanol yield. We have previously shown that increasing shake-flask culture volume enables yields up to 0.40 g/g to be achieved (8). Although oxygen transfer rate calculations are not presented here (this will be the subject of a future publication), the experiment reported herein shows that this approach can be used to achieve ethanol yields approaching the theoretical maximum.

METHODS

Two hundred and fifty milliliter DeLong culture flasks containing a pH 4.5 phosphate buffer solution (30 g/L monobasic potassium phosphate) were autoclaved. After cooling, 10-fold concentrated solutions of filter-sterilized Difco yeast nitrogen base (YNB, 67 g/L) and xylose (300 g/L) were added to each of the flasks at 10% of the final fluid (culture) volume. Final culture volumes were varied from 100 to 225 mL in 25-mL increments. Duplicate flasks were run for each permutation of the experiment.

P. stipitis NRRL Y-7124, a xylose-fermenting yeast, was plated from a slant stored at 4°C. A single colony was used to inoculate a 250-mL DeLong culture flask containing 100 mL buffered YNB medium as described above. The inoculum flask was placed on an incubated rotary shaker operation at 29°C and 150 rpm. After approx 24 h of aerobic growth (cell concentration of 4.3 g dry cell wt/L), this seed culture was used to inoculate the experimental flasks at a level of 1% (v/v).

Inoculated flasks, capped with Morton closures, were placed on a rotary shaker operating at 29°C and 150 rpm. Periodically, flasks were sampled (3 mL) for pH and optical density. The pH was determined using a calibrated Orion model 720A pH meter. Optical density (OD) was determined by measuring absorbance at 600 nm using a Milton Roy Spectronic 20D spectrophotometer. Samples were diluted as needed to fall in the linear range of this instrument (determined to be 0.0–0.6). Vials of filtered supernatant were prepared from each of the samples for subsequent analysis by high-performance liquid chromatography (HPLC). Samples were analyzed for residual sugar, products, and byproducts on a Hewlett-Packard 1090L HPLC equipped with a Bio-Rad HPX-87H organic acid analysis column operating at 65°C and a Hewlett-Packard 1047A refractive index detector. The mobile phase was 0.01*N* sulfuric acid at a flow rate of 0.6 mL/min. Final dry cell wt (DCW) measurements were made by centrifuging 10 mL of fermentation broth for 10 min at 4°C and 9000 rpm. After the supernatant was poured off, the pellet was resuspended in deionized water, and the centrifugation step repeated. Washed pellets were resuspended in deionized water and placed into dried, pretared aluminum pans. The pans were dried to constant weight in an 80°C oven. OD values were converted to cell concentrations using correlation factors developed from final DCW and final OD measurements.

Concentration profiles represent the average of data obtained from duplicate shake-flask fermentations. Deviations between duplicate flasks and averaged profiles were $<5\%$. Product yield was calculated by dividing final ethanol concentration by the concentration of xylose consumed. Volumetric productivity was calculated by dividing final ethanol concentration by time of fermentation. Specific productivity was calculated by dividing volumetric productivity by average cell mass concentration.

RESULTS AND DISCUSSION

Experiments were conducted to determine the ability of *P. stipitis* to ferment xylose at high ethanol yield. Given that this yeast requires small amounts of oxygen, the culture volume was varied to provide different levels of oxygen limitation. Product yield, volumetric productivity, and specific productivity, calculated from substrate and product concentrations, are presented below.

Figure 1 shows averaged concentration profiles obtained from flasks containing culture volumes of 100, 175, and 225 mL. Final cell mass concentrations decrease with increasing culture volume, from 3.1 g DCW/L at 100 mL culture volume to 0.7 g DCW/L at 225 mL. The rate of fermentation slows with increasing culture volume. Overall xylose consumption

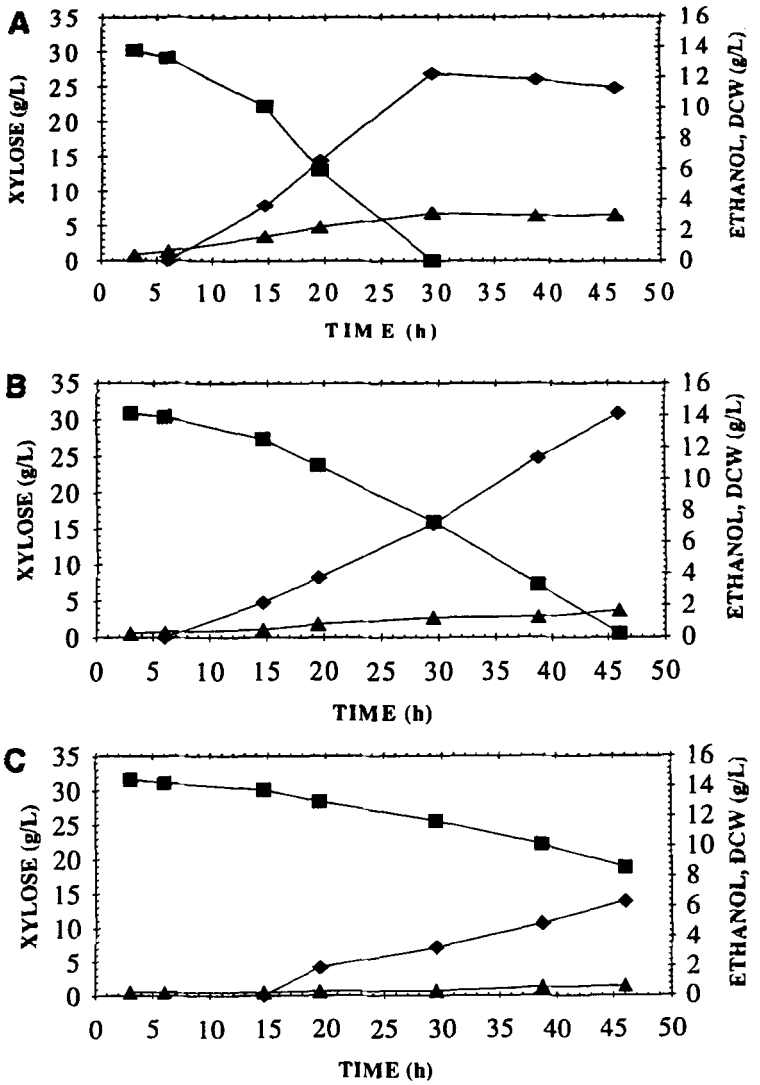

Fig. 1. Shake-flask fermentation of xylose at culture volumes of 100 mL (A), 175 mL (B), and 225 mL (C). Xylose (■), ethanol (◆), and dry cell wt (▲) concentration profiles are shown.

and ethanol production rates decrease from 1.1 g/L-h and 0.46 g/L-h, respectively, at 100 mL, to 0.3 g/L-h and 0.15 g/L-h, respectively, at 225 mL. As Fig. 2 shows, yield increases in an essentially linear fashion from 0.41 g/g at 100-mL culture volume to a value of 0.50 g/g at 200 mL. Product yield levels off above 200-mL culture volume.

Increases in shake flask culture volume enable increases in yield by reducing final cell mass concentrations and thereby increasing the amount of substrate available for fermentative conversion to ethanol. Results show that near-theoretical yields can be achieved by limiting cell growth. Flasks containing the highest culture volumes, 200 and 225 mL, achieved yields

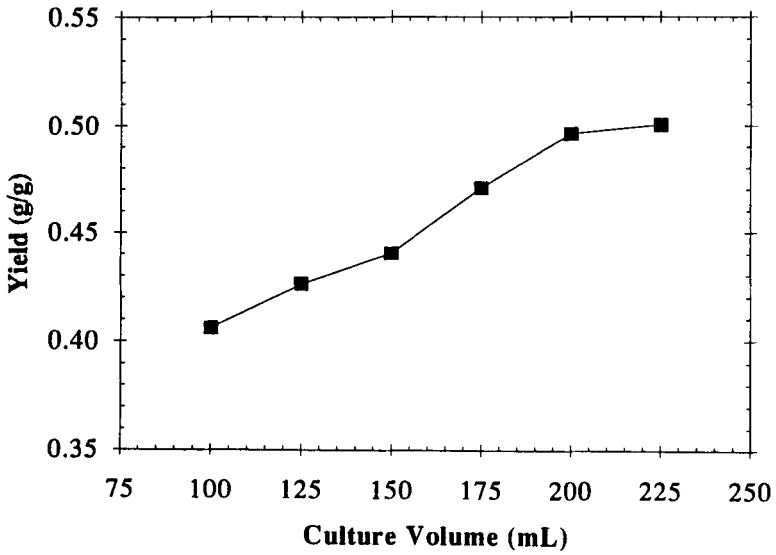

Fig. 2. Yield vs culture volume.

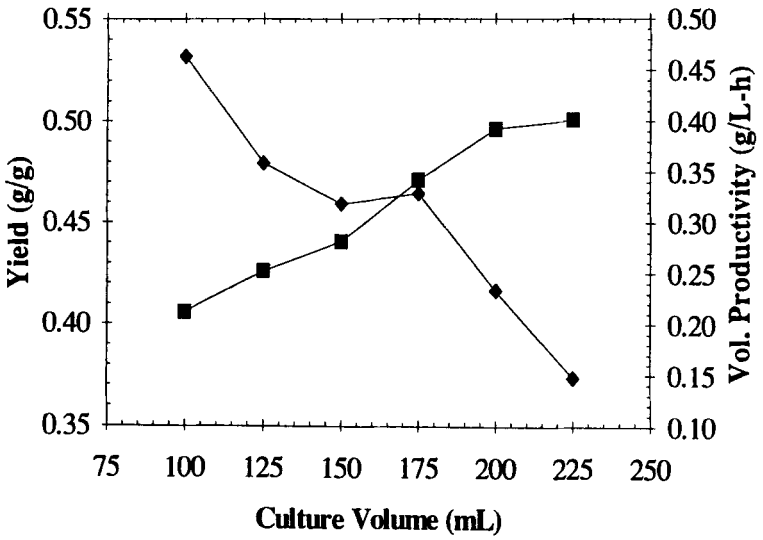

Fig. 3. Yield (■) and volumetric productivity (◆) vs culture volume.

of 0.50 g/g. However, as Fig. 3 shows, a significant trade-off exists between yield and volumetric productivity. Although yield increases from 0.41 g/g at 100-mL culture volume to 0.50 g/g at 225-mL culture volume, volumetric productivity decreases from 0.46 g/L-h at 100 mL to 0.15 g/L-h at 225 mL.

Final cell mass concentration and specific productivity are plotted as a function of culture volume in Fig. 4. Final cell mass concentration falls from 3.1 g DCW/L at 100 mL to 0.7 g DCW/L at 225 mL. In contrast, specific productivity gradually rises from 0.26 g/g-h at 100 mL to a maximum of 0.34 g/g-h at 175 mL. This is similar to the trend in specific productivity that Skoog and Hahn-Hägerdal (5) observed with decreasing aeration

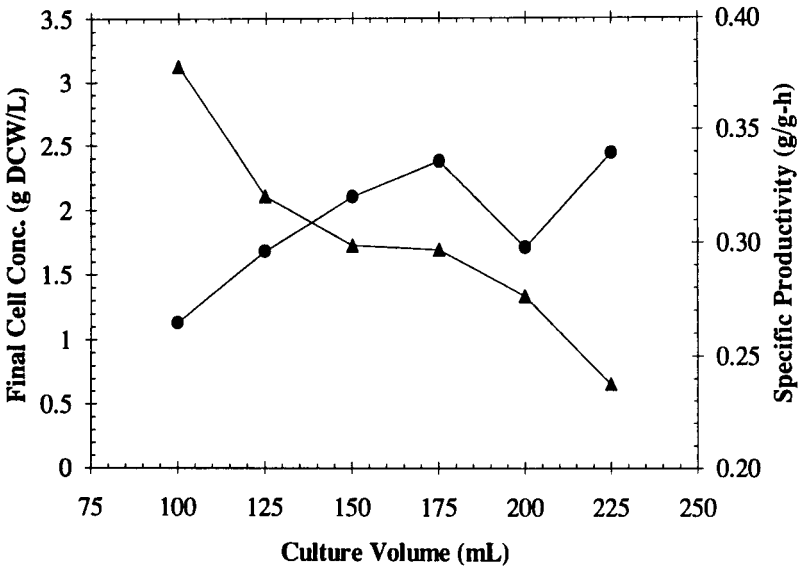

Fig. 4. Final dry cell wt (▲) and specific productivity (●) vs culture volume.

rate. It is difficult to interpret the trend in specific productivity in the current experiment at culture volumes above 175 mL, but the data suggest that the principal reason for volumetric productivity decreasing with increasing culture volume is reduced cell concentration. Given the substantial compromise that exists between high-yield and high-volumetric productivity fermentations, scale-up of this process will require technoeconomic assessments to determine profitable conditions of operation.

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