Cofermentation of Glucose, Xylose, and Arabinose by Genomic DNA–Integrated Xylose/Arabinose Fermenting Strain of *Zymomonas mobilis* AX101

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Abstract

Cofermentation of glucose, xylose, and arabinose is critical for complete bioconversion of lignocellulosic biomass, such as agricultural residues and herbaceous energy crops, to ethanol. We have previously developed a plasmid-bearing strain of *Zymomonas mobilis* (206C[pZB301]) capable of cofermenting glucose, xylose, and arabinose to ethanol. To enhance its genetic stability, several genomic DNA–integrated strains of *Z. mobilis* have been developed through the insertion of all seven genes necessay for xylose and arabinose fermentation into the *Zymomonas* genome. From all the integrants developed, four were selected for further evaluation. The integrants were tested for stability by repeated transfer in a nonselective medium (containing only glucose). Based on the stability test, one of the integrants (AX101) was selected for further evaluation. A series of batch and continuous fermentations was designed to evaluate the cofermentation of glucose, xylose, and L-arabinose with the strain AX101. The pH range of study was 4.5, 5.0, and 5.5 at 30°C. The cofermentation process yield was about 84%, which is about the same as that of plasmid-bearing strain 206C(pZB301). Although cofermentation of all three sugars was achieved, there was a preferential order of sugar utilization: glucose first, then xylose, and arabinose last.

Index Entries: Recombinant *Zymomonas*; genomic integration; cofermentation; continuous fermentation; xylose; arabinose; ethanol productivity; acetic acid.

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Introduction

Lignocellulosic feedstocks are composed predominantly of cellulose, hemicellulose, and lignin and are naturally resistant to chemical and biologic conversion. Because the feedstock can represent >40% of all process costs, an economical biomass-to-ethanol process critically depends on the rapid and efficient conversion of all of the sugars present in both its cellulose and hemicellulose fractions. While many microorganisms can ferment the glucose component in cellulose to ethanol, efficient conversion of the pentose sugars in the hemicellulose fraction, particularly xylose and arabinose, has been hindered by the lack of a suitable biocatalyst. Xylose is the predominant pentose sugar derived from the hemicellulose of most hardwood feedstocks, but arabinose can constitute a significant amount of the pentose sugars derived from various agricultural residues and other herbaceous crops, such as switchgrass, that are being considered for use as dedicated energy crops. Whereas arabinose makes only 2–4% of the total pentoses in hardwoods, arabinose represents 10–20% of the total pentoses in many herbaceous crops *(1)*. Arabinose contents can be as high as 30–40% of the total pentoses in corn fiber, a byproduct of corn processing.

A sensitivity analysis of process costs has shown that higher ethanol yields and final concentrations are the most important factors influencing the production cost, with increased volumetric productivity being an important, but secondary, target *(2)*. In addition, there are substantially greater savings in capital and operating costs to be gained by the use of a cofermentation process in which the hexose and pentose sugars derived from both the cellulose and hemicellulose fractions are simultaneously fermented to ethanol in a single operation. Unfortunately, the yeast commonly used in simultaneous saccharification and fermentation does not ferment pentose sugars. Furthermore, the microorganisms that can ferment pentose sugars such as xylose are typically sensitive to glucose catabolite repression that would result in low productivities and long fermentation times in a cofermentation process.

Zymomonas is well recognized for its ability to produce ethanol rapidly and efficiently from glucose-based feedstocks, and comparative performance trials have shown that *Z. mobilis* can achieve 5% higher yields and up to five-fold higher volumetric productivity when compared with traditional yeast fermentations *(3)*. *Z. mobilis* has demonstrated ethanol yields of up to 97% of theoretical and ethanol concentrations of up to 12% (w/v) in glucose fermentations *(4)*. These notably high yields have been attributed to lower biomass formation during fermentation. Yeast produces 2 mol of adenosine triphosphate (ATP)/mol of glucose through the Embden-Meyerhoff-Parnas pathway, while *Z. mobilis* ferments glucose through the Entner-Doudoroff pathway and produces only 1 mol of ATP/mol of glucose *(5)*. *Z. mobilis's* facilitated diffusion sugar transport system *(6)*, coupled with its highly expressed pyruvate decarboxylase and alcohol dehydrogenase genes *(7)*, enables rapid and efficient conversion of glucose to ethanol.

Despite these potential advantages, fermentation processes based on the use of *Z. mobilis* have yet to be commercialized in the United States for fuel ethanol production from starch-based feedstocks. *Z. mobilis* also demonstrates many of the essential traits that we are seeking in an ideal biocatalyst for fuel ethanol production from lignocellulosic feedstocks, including high ethanol yield and tolerance, high specific productivity, ability to ferment sugars at low pH, and considerable tolerance to the inhibitors found in lignocellulosic hydrolysates. Whereas *Z. mobilis* may become an important ethanol-producing microorganism from glucose-based feedstocks, its substrate utilization range is restricted to the fermentation of glucose, sucrose, and fructose. As such, wild-type strains are not naturally suited for fermentation of the xylose found in lignocellulosic feedstocks because they lack the essential xylose assimilation and pentose metabolism pathways.

We have developed several integrated combined xylose/arabinose-fermenting *Z. mobilis* strains by introducing genes of pentose assimilation and pentose phosphate pathways into genomic DNA. In this article, we discuss cofermentation of xylose and arabinose by one of the single recombinant *Z. mobilis* strains AX101 in batch and continuous fermentations. We also compare the results of batch cofermentation to control xylose/arabinose utilizing plasmid-bearing strain 206C(pZB301).

Materials and Methods

Microorganisms and Media

Genomic DNA–integrated xylose/arabinose fermenting strains of *Z. mobilis* and a plasmid-bearing strain 206C(pZB301) as a control were used for cofermentation studies. Rich medium ([RM], 10 g/L of yeast extract and 2 g/L of KH$_2$PO$_4$) *(8)* supplemented with the desired sugar (glucose, D-xylose, L-arabinose, or mixture, [GXA]) at concentrations of 40 g/L for glucose and xylose and 20 g/L for arabinose was used for batch studies. For continuous fermentation, either a mixture of 40:40:20 g/L or 80:40:1.5 g/L for GXA was used. In later cases, the feed stream was prepared with clarified corn steep liquor (CSL) as a nutrient source that was also supplemented with different amounts of acetic acid. The clarified CSL was prepared by diluting whole CSL (supplied by Iogen, Otawa, Canada) with deionized water at a ratio of 1:4 and then centrifuging and filter sterilizing with a 0.2-μ filter (Gelman).

Growth Measurement

Growth was measured as optical density(OD) at 600 nm (Spectronic 601; Milton Roy) using deionized water as a blank. Dry cell mass was calculated by using a correlation factor of 0.3 (1 OD at 600 nm/g of cell mass), which was previously determined in our laboratory for the *Zymomonas* strains. This correlation factor was also verified by Dr. H. Lawford of the University of Toronto, Canada (final technical report of subcontract, 1999).

Cultivation of Inoculum

One milliliter of thawed frozen stock culture was inoculated into 400 mL of sterile RM medium containing 20 g/L of glucose plus 10 g/L each of xylose and arabinose. The inoculum culture was incubated on a rotary shaker at 150 rpm 30°C for 14 h or until an OD of 2 to 3 at 600 nm was reached. The grown culture was centrifuged for 10 min at 3838*g* in 250-mL centrifuge bottles. The centrifuged cells were concentrated into 20 mL of RM medium and used to inoculate the fermentors.

Stability Test

Frozen stock culture was transferred into RMGXA (20:10:10 g/L). Overnight grown culture was transferred into an RMG (20 g/L) tube containing 10 mL of medium to an initial OD of 0.02 at 600 nm. After each 10 generations, the culture was transferred again into the same medium. At the end of 40 generations, the grown culture was also tested for cofermentation of glucose, xylose, and arabinose in a 125-mL shake flask containing 100 mL of RMGXA (20:10:10 g/L). The process was continued for 160 generations.

Fermentations

Batch fermentations were carried out for 120 h in BioStat-Q fermentors (B. Braun Biotech, Allentown, PA) with a 500-mL working volume. The fermentors were inoculated from concentrated cells to achieve an initial OD at 600 nm of 0.2. The pH range studied was 5.0–5.5 and was controlled by titrating with 2 *N* KOH. The temperature was kept at 30°C and the revolutions per minute was controlled at 300. Initial sugar concentration, pH, and temperature varied between fermentations depending on the desired conditions. All the sugars used were reagent grade. Samples were taken periodically throughout the course of the fermentations and analyzed for sugars, ethanol, and byproducts by high-performance liquid chromatography. Continuous fermentations were carried out in MultiGen fermentors (New Brunswick Scientific) with a 300-mL working volume. The pH values studied were 4.5, 5.0, and 5.5. Temperature was kept at 30°C for all studies. The ethanol process yield (Y_p) was calculated based on the final concentration of ethanol produced per mass of initial total sugar added to the medium. The concentrations were not corrected for dilution caused by titration.

Results and Discussion

Stability Test

A total of 12 chromosomal integrated xylose/arabinose fermenting strains of *Z. mobilis* were engineered in our laboratory. After preliminary evaluation, four strains (AX1, AX23, AX101, and G8) were selected for further stability testing and cofermentation studies. Figure 1 shows the
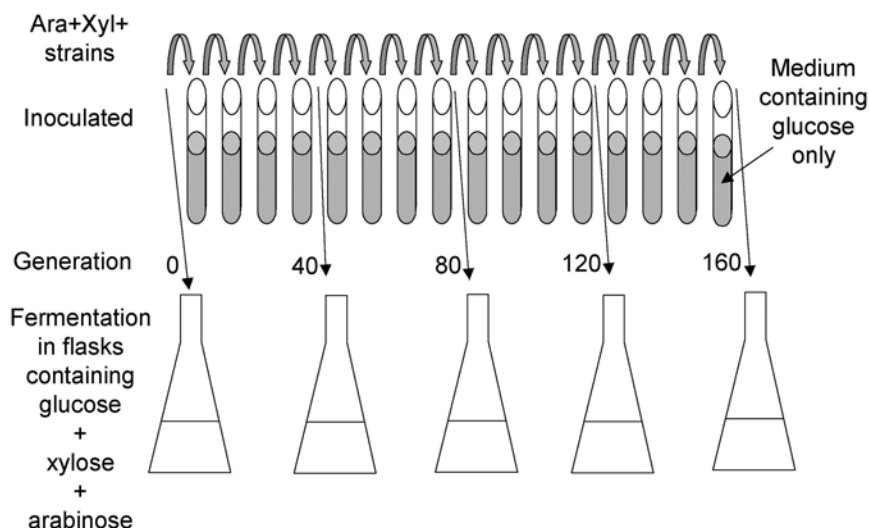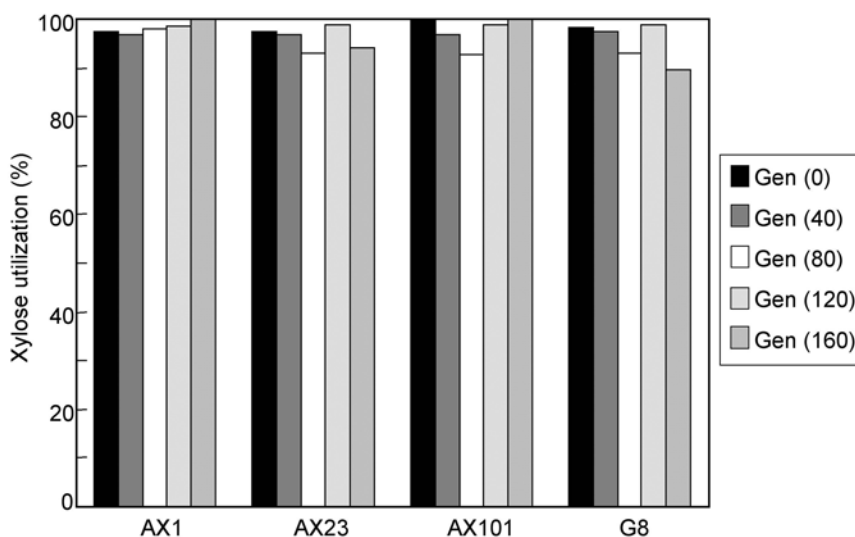
Fig. 1. Stability test protocol.

Fig. 2. Final xylose utilization at different generation for four strains tested for stability.

protocol used for stability testing. Final xylose and arabinose utilization (%) for four strains during stability testing is shown in Figs. 2 and 3, respectively. After 160 generations, xylose utilization remained above 95% for all four strains but the percentage of arabinose utilization decreased except for strain AX101. Based on these stability test results (Figs. 2 and 3), strain AX101 was selected for further evaluation.
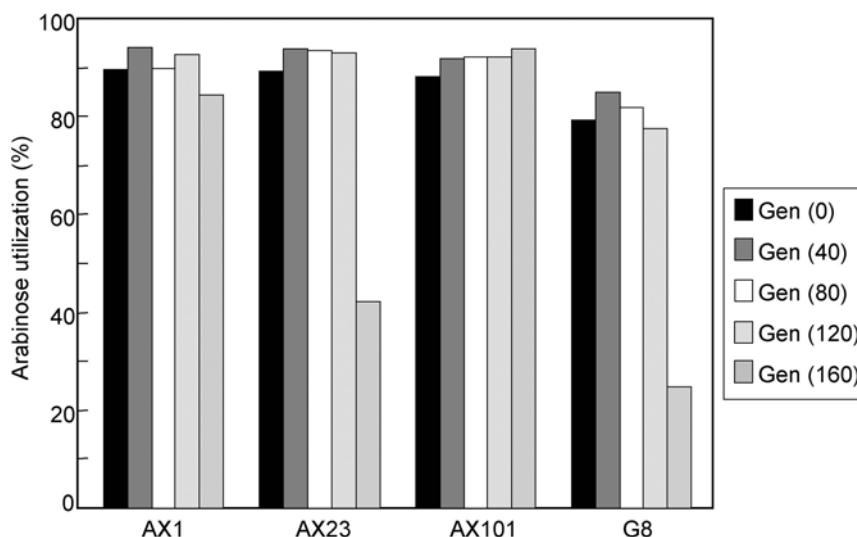
Fig. 3. Final arabinose utilization at different generation for four strains tested for stability.

Batch Fermentation:

Cofermentation of mixed sugars of glucose, xylose, and arabinose in RM media by strain AX101 was evaluted at pH 5.0 and 5.5 and at 30°C. Examples of growth profile and byproduct formation on sugar mixtures of glucose, xylose, and arabinose (40:40:20 g/L) at pH 5.0 and 5.5 and 30°C are shown in Figs. 4 and 5, respectively. Xylitol, lactate, glycerol, and acetate were the detected by-products. As a control, plasmid-bearing strain 206C-(pZB301) was run under similar conditions. The growth and byproduct formation pattern for this strain at pH 5.5 is shown in Fig. 6. Comparison of Figs. 5 and 6 shows that the newly integrated strain AX101 performed as well as the plasmid-bearing strain 206C(pZB301). The process yield for both strains under the tested conditions was about 84%. The noticeable differences were in by-product xylitol formation and cell mass. The newly integrated strain produced higher amounts of xylitol (3.5 g/L) than the plasmid-bearing strain (1.6 g/L), and strain AX101 produced slightly higher cell mass with an OD of 6.4 vs 5.7. At the pH tested, the cofermentation of the above mentioned sugar mixture (total of 100 g/L) by both strains was almost completed in 48 h, with a final ethanol concentration of about 42 g/L and volumetric productivity of 0.6 g/(L·h).

Although cofermentation of all three sugars was achieved, there was a preferential order of sugar utilization: glucose first, then xylose, and arabinose last. The highest cell density obtained had an OD of about 6.5 at pH 5.5 and 6.2 at pH 5.0 at 600 nm equivalent to 1.95 and 1.86 g of dry cell mass/L, respectively. The cell density was slightly higher at pH 5.5. The highest cell density occurred at the completion of glucose utilization, after
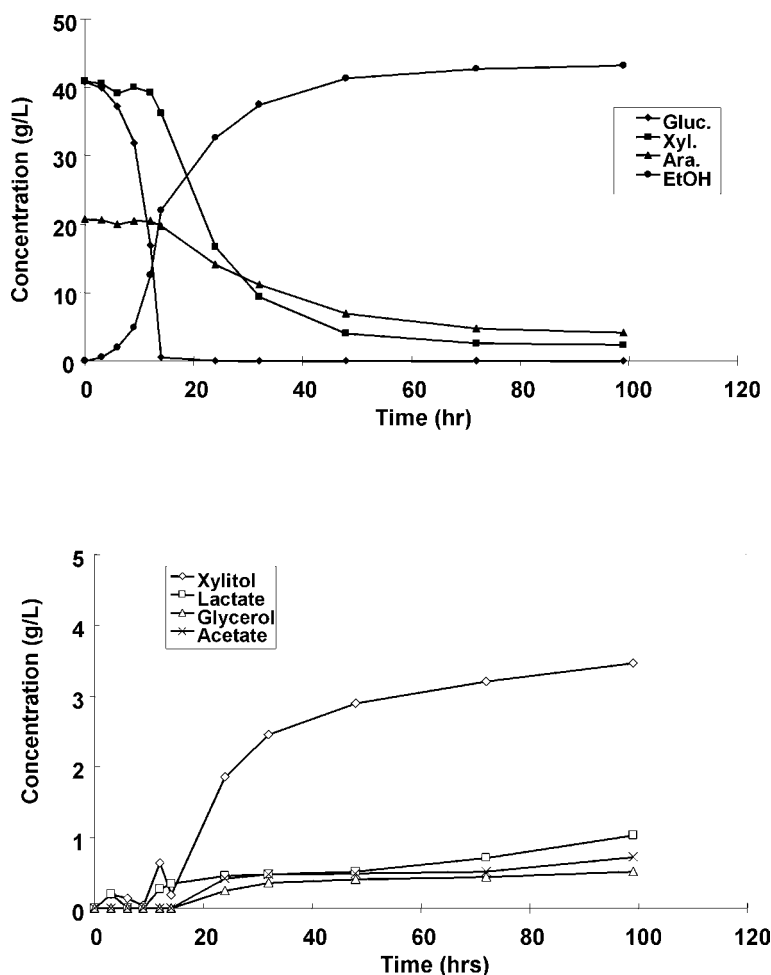
Fig. 4. Growth and by-products formation profile for strain AX101 grown on pure sugar RMGXA (40:40:20 g/L) at pH 5.0 and 30°C.

which it appears that uncoupling of growth and fermentation took place, and the cell density did not increase with further utilization of xylose and arabinose. It is also likely that at the end of cofermentation relatively high concentrations of ethanol inhibited arabinose utilization. As a result, the process yield in batch fermentation was below theoretical value.

The kinetic parameters for batch fermentations at pH 5.0 and 5.5 and 30°C are given in Table 1. The maximum specific growth rate (μ_m) was calculated from the exponential phase of the growth (OD vs time) pattern on mixed sugar. The values of the overall specific sugar uptake (q_s) and specific ethanol production rate (q_p) of mixed sugar fermentation were calculated using the following formula:

$$q_s = (1/x_{av})(\Delta s/\Delta t) \text{ and } q_p = (1/x_{av})(\Delta p/\Delta t)$$
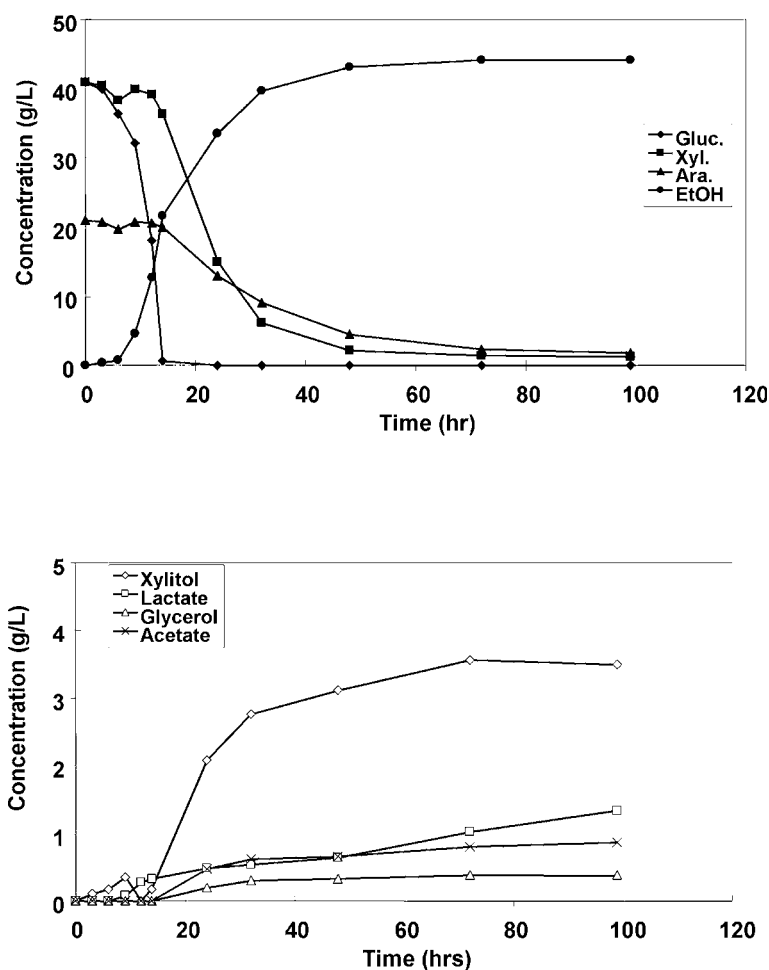
Fig. 5. Growth and by-products formation profile for strain AX101 grown on pure sugar RMGXA (40:40:20 g/L) at pH 5.5 and 30°C.

Table 1
Kinetic Parameters for Batch Fermentations
for Strains AX101 and 206C(pZB301) at 30°C

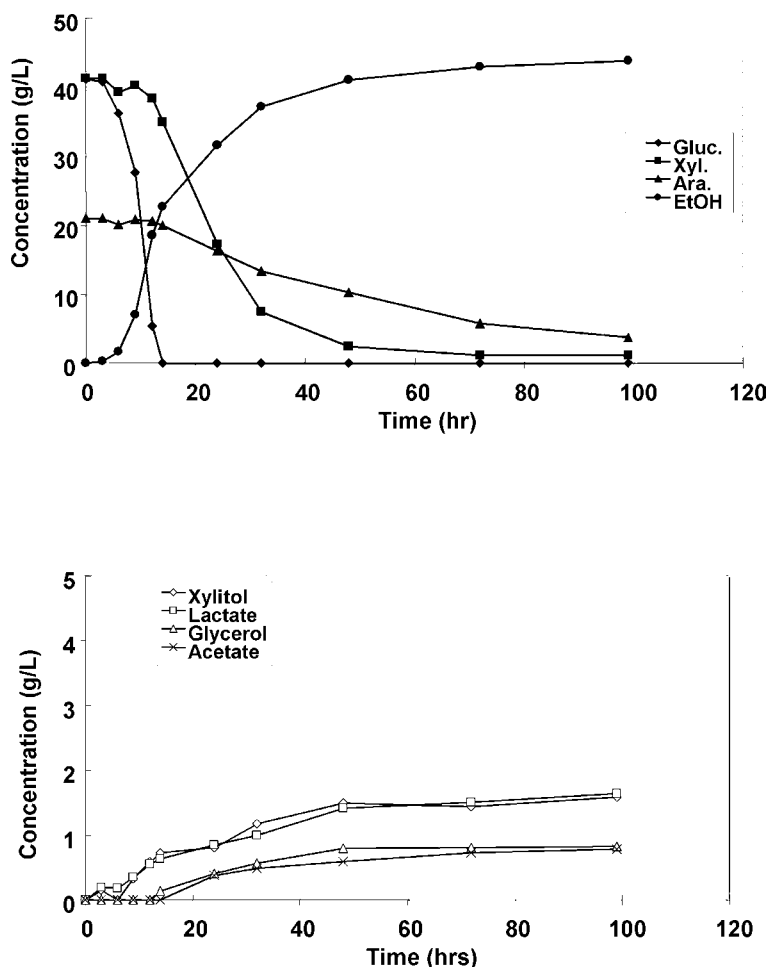| Kinetic parameter	AX101		206C(pZB301)	
	pH 5.0	pH 5.5	pH 5.0	pH 5.5
μ_{max} (h^{-1})	0.34	0.34	0.34	0.34
q_s (g sugar/[g cell·h])	0.72	0.71	0.72	0.79
q_p (g ethanol/[g cell·h])	0.32	0.32	0.33	0.35
Q_p (g ethanol/[L·h])	0.59	0.61	0.60	0.60
Process yield (%)	82.6	84.1	83.3	83.0

Fig. 6. Growth and by-products formation profile for strain 206C(pZB301) grown on pure sugar RMGXA (40:40:20 g/L) at pH 5.5 and 30°C.

in which Δs and Δp are the changes in the sugar and ethanol concentrations, respectively, over the time period Δt *(9)*; and x_{av} is the average of the cell mass concentration over Δt.

Overall volumetric ethanol productivity, Q_p was calculated by

$$Q_p(\text{g}/[\text{L·h}]) = \text{Final ethanol (g/L)}/t_f$$

in which t_f is the time at which maximum ethanol was produced.

As Table 1 shows, all the kinetic parameters for both strains were equal except that the plasmid-bearing strain 206C(pZB301) had a slightly higher specific sugar uptake rate at pH 5.5. The values of specific sugar utilization and product formation for both strains were similar to the values reported by Joachimsthal et al. *(9)* on *Z. mobilis* CP4(pZB5). The process yield was

lower than that of CP4(pZB5) owing to incomplete utilization of arabinose by strain AX101.

Continuous Fermentation:

Strain AX101 was further evaluated under continuous fermentations with two different feed streams of industrial interest. First, a batch fermentation with a growth medium composition similar to the feed stream was started, and after almost full glucose utilization (1 or 2 d), the feed flow was started. In a first attempt, the fermentations were run at pH 4.5, 5.0, and 5.5 at 30°C with a sugar feed concentration of 40 g/L of glucose, 40 g/L of xylose, and 20 g/L of arabinose. In a second attempt, the strain was evaluated at pH 5.5 at 30°C with a sugar feed concentration of 80 g/L of glucose, 40 g/L of xylose, and 15 g/L of arabinose in the presence of variable amounts of acetic acid. In both cases, the dilution rate was started at a low value of about 0.015–0.02 (1/h). After at least four cycles of steady state in which xylose and arabinose concentrations remained the same in successive days and at least 80% of the input xylose was utilized, the dilution rate was increased or experimental conditions were changed. The continuous run with a feed stream of 40 g/L of gluose, 40 g/L of xylose, and 20 g/L of arabinose was started at pH 5.5 with a dilution rate of 0.02 (1/h). After the xylose concentration fell below the 80% level and steady state was achieved, the dilution rate was increased gradually to 0.04 (1/h). At this point, the pH was decreased to 5.0 and the dilution rate lowered to 0.028 (1/h). The continuous growth profile was studied up to a dilution rate of 0.035 (1/h) when the pH was decreased to 4.5. At pH 4.5, the growth profile was studied at two different dilution rates of 0.02 (1/h) and 0.028 (1/h). The overall results including by-product formation are shown in Fig. 7. With all three pH values, the ethanol process yield was about 84%, and the remaining xylose and arabinose concentrations, after steady state was achieved, were about 3 and 8 g/L, respectively. As Fig. 7 shows, unlike with batch fermentation, xylitol production was minimal (about 0.5 g/L).

There was some decline in biomass concentration at this low dilution rate that could be owing to substrate uptake for maintenance energy requirements. There was also wall growth during continuous fermentation that affected accurate measurement of the OD and, as a result, cell mass measurement. Because of this decline in cell mass, the values of the specific sugar utilization and maintenance energy requirement (m_e) were not accurate. Figure 8 is a Pirt plot of q_s and q_p versus D for chemostat culture at pH 5.5. As the regression analysis of the data show, the m_e value was negative, –0.59 (g/[g·h]), owing to less cell mass measured because of wall growth. Therefore, comparison of these data with those of other published data *(10,11)* on different variants of *Zymomonas* was not possible. Volumetric productivity, Q_p, was about 1–1.9 g of ethanol/(L·h) at the range of dilution rates studied at all three pH values. Consequently, lowering the pH to 4.5 did not have any effect on the volumetric productivity.
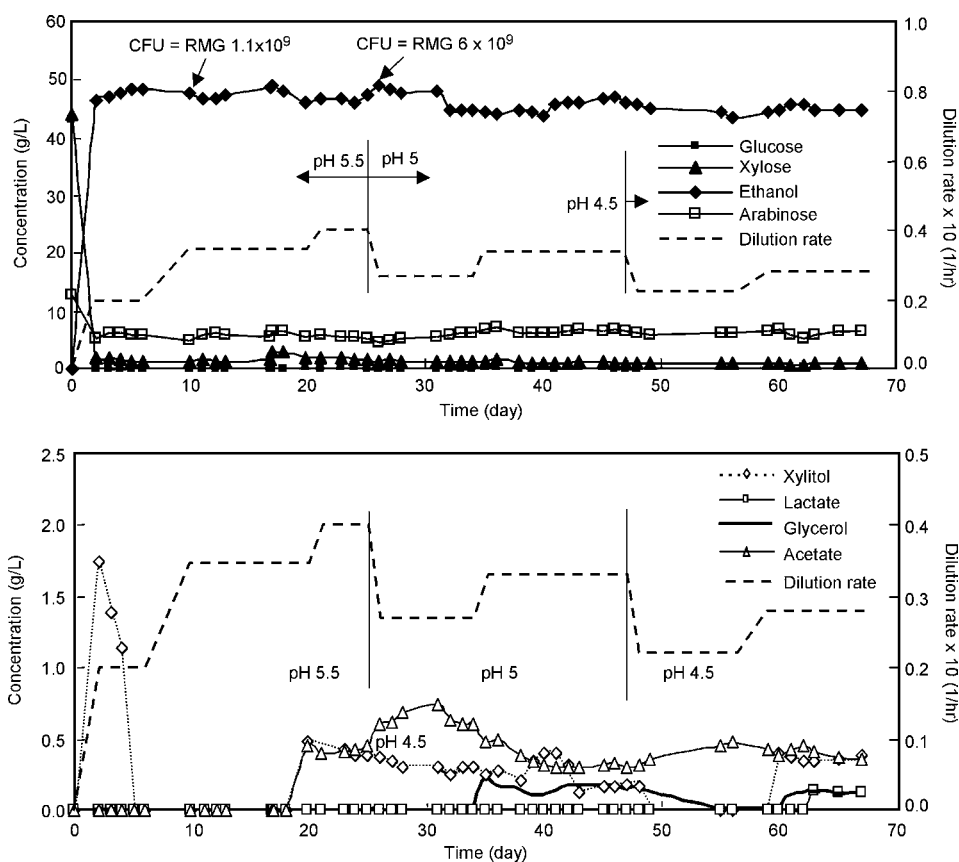
Fig. 7. Continuous growth and by-products formation profile for strain AX101 grown on RMGXA (40:40:20 g/L) at pH 4.5, 5.0, and 5.5 and 30°C.
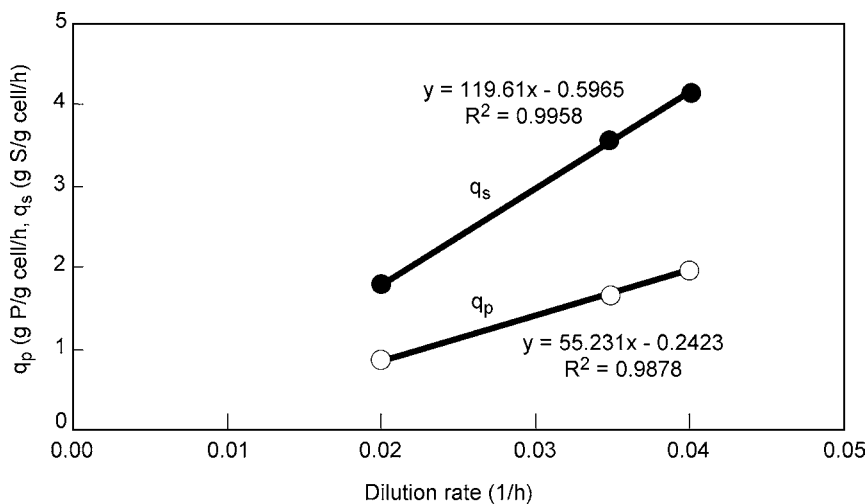

Fig. 8. Pirt plot of specific product formation and substrate utilization for strain AX101 grown under continuous fermentation at pH 5.5 and 30°C.
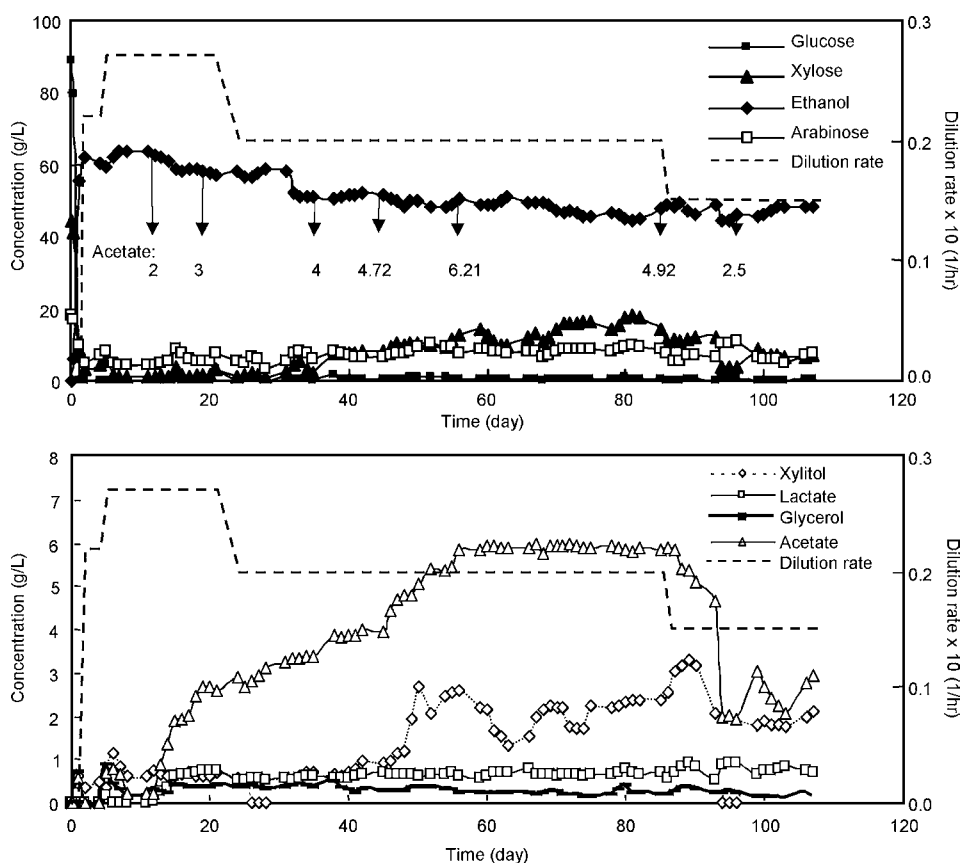
Fig. 9. Continuous growth and by-products formation profile for strain AX101 grown on cCSLGXA (80:40:15 g/L) supplemented with acetic acid at pH 5.5 and 30°C.

Toon et al. *(12)* conducted a comprehensive study of cofermentation of glucose and xylose by recombinant *Saccharomyces* yeast strains. Comparison of cofermentation data from the present work with those of Toon et al. *(12)* shows that strain AX101 achieved higher yields (70% process yield for *Saccharomyces* vs 84% for AX101). In addition, comparison of data from the present work with previously reported data on glucose/xylose fermentations by a recombinant strain of *E. coli (13)* shows that strain AX101 can achieve comparable maximum ethanol concentration, ethanol process yield, and productivity.

Continuous Fermentation with Higher Feed Stream in Presence of Acetic Acid

In a second attempt, strain AX101 was evaluated in continuous fermentation with a high-sugar feed stream containing variable amounts of acetic acid. Figure 9 shows that the continuous growth and byproduct pro-

file using the high sugar feed stream containing 80 g/L of glucose, 40 g/L of xylose, and 15 g/L of arabinose in the presence of acetic acid varied between 2 and 6 g/L. The dilution rate varied between 0.015 and 0.035 (1/h). The same criteria as mentioned before were used for changing experimental conditions including dilution rate. As Fig. 9 shows, below 4.5 g/L of acetic acid, the performance of the strain AX101 was not affected, which could be owing to gradual adaptation of the strain to acetic acid. In a previous study, we have shown that *Zymomonas* is adaptable to inhibitors such as acetic acid *(10)*. When the concentration of acetic acid exceeded 4.5 g/L, the xylose concentration in the fermentor increased, ethanol production decreased, and xylitol production increased. This unfavorable performance was the result of toxicity of acetic acid, a substance known as an energetic uncoupler *(14)*. The ethanol concentration remained at about 48 g/L, and volumetric productivity was in the range of 0.73–1.6 g of ethanol/(L·h) at acetic acid concentrations <4.5 g/L at all dilution rates studied. These values of volumetric productivities are comparable with those of adapted *rZymomonas* developed in our laboratory *(10)*. The ethanol process yield was about 84% with an acetic acid concentration <4.5 g/L. By increasing the acetic acid concentration above 4.5 g/L, xylose and arabinose started accumulating in the broth and ethanol yield and productivity decreased. Again, because of wall growth it was not possible to calculate specific sugar utilization and maintenance energy requirement (m_e).

Conclusion

Several stable genomic DNA-integrated strains of *Z. mobilis* capable of cofermenting glucose, xylose, and arabinose were developed. Strain AX101 was stable up to 160 generations on nonselective media containing only glucose. Although cofermentation of all three sugars was achieved, there was a preferential order of sugar utilization: glucose first, then xylose, and arabinose last. The process yield for strain AX101 was similar to the control plasmid-bearing strain 206C (pZB301). Xylitol, lactate, glycerol, and acetate were the detected by-products.

The values of specific sugar utilization and product formation were similar to those of other *rZ. mobilis* reported previously by others. Ethanol productivity of strain AX101 was inhibited by acetic acid in continuous fermentation. This unfavorable performance was the result of the toxicity of acetic acid, a substance known as an energetic uncoupler. The overall results, indicate that the higher ethanol process yield (Y_p) and low pH performance with minimal by-product formations makes strain AX101 attractive for industrial processes.

Acknowledgment

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