0273-2289/91/2829—0221$03.20

Ethanol Production by Recombinant *Escherichia coli* Carrying Genes from *Zymomonas mobilis*

HUGH G. LAWFORD* AND JOYCE D. ROUSSEAU

Department of Biochemistry, University of Toronto
Toronto, Ontario, Canada M5S 1A8

ABSTRACT

Efficient utilization of lignocellulosic feedstocks offers an opportunity to reduce the cost of producing fuel ethanol. The fermentation performance characteristics of recombinant *Escherichia coli* ATCC 11303 carrying the "PET plasmid" (pLOI297) with the *lac* operon controlling the expression of pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) genes cloned from *Zymomonas mobilis* CP4 (Alterthum & Ingram, 1989) were assessed in batch and continuous processes with sugar mixtures designed to mimic process streams from lignocellulosic hydrolysis systems.

Growth was pseudoexponential at a rate (generation time) of 1.28 h at pH 6.8 and 1.61 h at pH 6.0. The molar growth yields for glucose and xylose were 17.28 and 7.65 g DW cell/mol, respectively (at pH 6.3 and 30°C), suggesting that the net yield of ATP from xylose metabolism is only 50% compared to glucose. In pH-stat batch fermentations (Luria broth with 6% sugar, pH 6.3), glucose was converted to ethanol 4–6 times faster than xylose, but the glucose conversion rate was much less than can be achieved with comparable cell densities of *Zymomonas*. Sugar-to-ethanol conversion efficiencies in nutrient-rich, complex LB medium were near theoretical at 98 and 88% for glucose and xylose, respectively. The yield was 10–20% less in a defined-mineral-salts medium. Acetate at a concentration of 0.1*M* (present in lignocellulosic hydrolysates from thermochemical processing) inhibited glucose utilization (about 50%) much more than xylose, and caused a decrease in product yield of about 30% for both sugars.

*Author to whom all correspondence and reprint requests should be addressed.

With phosphate-buffered media (pH 7), glucose was a preferred substrate in mixtures with a ratio of hexose to pentose of 2.3 to 1. Xylose was consumed after glucose, and the product yield was less (0.37 g/g). Under steady-state conditions of continuous culture, the specific productivity ranged from 0.76–1.24 g EtOH/g cell/h, and the maximum volumetric productivity, 2.5 g EtOH/L/h, was achieved with a rich complex LB medium (glucose) at pH 6.0 (30°C) and ethanol at 1.63% (v/v). Growth and fermentation were poor in a buffered-wood (aspen) "hemicellulose hydrolysate" containing 4% xylose and 0.1*M* acetate with added thiamine and mineral salts.

Index Entries: Fuel ethanol, recombinant *E. coli;* genetic engineering; xylose; lignocellulose; *Zymomonas* genes; hemicellulose hydrolysate.

INTRODUCTION

The fuel-ethanol industry was born out of the energy crisis of the 1970s and was developed on the existing technology base of the alcoholic-beverage industry, in which consumer acceptance, not process efficiency, was the only motivation for technological innovation. The present low cost of oil means that fuel alcohol derived from grain (starch) is economically feasible only because of government tax incentives or subsidies and the value of byproduct credits. However, growing environmental concern creates a renewed opportunity for fuel ethanol.

The fuel market potential for fermentation alcohol makes it by far the largest of existing biotechnology products. Unlike the low-volume, high-valued products produced by genetically engineered organisms in modern biotech industries (e.g., pharmaceuticals), the large-volume market for fuel ethanol demands that raw materials and processing costs be kept very low (*1–3*).

Our strategy for developing an economically competitive process for fuel-alcohol production combines modern advances in biotechnology and bioengineering to increase both the efficiency and the rate of bioconversion using cost-effective feedstocks, such as "waste" lignocellulosic materials (*4,5*). The engineering approach to improving process productivity involves operating the bioreactors in a continuous mode (rather than the more traditional batch mode) and at higher cell densities, with systems employing either cell retention or cell recycle; the biological approach addresses the performance characteristics of the biocatalyst, or process microorganism (*6*). The monopoly enjoyed by yeast is now being challenged by new alternative alcohol producers that are tailored to the specific requirements of the fuel-alcohol industry. Our patented, high-performance bacterial

bioconversion process technologies have been developed over the last 10 years and are based on *Zymomonas*, whose superior kinetic and yield characteristics have the potential to revolutionize the fuel-alcohol industry (for review, *see* ref. *7*). However, one limitation of *Zymomonas* is that it is restricted to utilizing only glucose, fructose, and sucrose, thereby limiting its usefulness to starch-based or sucrose (molasses) fermentations.

The increasing demand for ethanol as an environment-friendly fuel means that the industry must expand beyond its present starch- and yeast-based technology. The current practice of using carbohydrate sources that can be used alternatively as either food or feed, means that the fermentation feedstocks account for about two-thirds of the cost of producing ethanol (*7*). If there is to be a reduction in cost, then additional, lower-cost feedstocks are needed to supplement the present supply of surplus corn and cereal grains. Lignocellulosic materials (biomass) are the only renewable resource that is sufficiently abundant to produce the large volumes of ethanol needed to replace petroleum-based transportation fuels (*8*). The economic benefit to be derived from the efficient bioconversion of biomass feedstocks relates to the low cost associated with such lignocellulosic "waste" materials as agricultural residues (straw, corn stover), forestry wastes (sawdust, pulp mill residues), and certain solid municipal wastes (newsprint, paper and cardboard packing). However, lignocellulose remains recalcitrant to bioconversion because the yeast cultures presently employed in starch-based fermentations are unable to utilize the five-carbon pentose sugars that make up the hemicellulose component (10–40%) of biomass (*8,9*). Xylose (hemicellulose) conversion offers the potential to increase yields by 30% (*8*).

Genetic engineering represents another aspect of the biological strategy for process improvement (*10*). Using rDNA technology, *Escherichia coli* has been genetically transformed with the "PET operon" carrying pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) genes from *Z. mobilis* CP4 (*11,12*). *E. coli* can utilize all the sugar constituents of lignocellulosic biomass (both hexoses and pentoses), and expression of the *Zymomonas* genes in recombinant strains directs the flow of carbon away from the production of organic acids to the production of ethanol (*13*). However, with the exception of some preliminary reports (*11,12*), the physiological characteristics of these lab strains is largely unknown. The objective of this study was to quantitatively assess the fermentation performance of one of the more promising recombinant ethanologenic *E. coli* cultures, namely ATCC 11303 (pLOI297 (*12*), and to optimize operational parameters with respect to the identified key technoeconomic sensitivity factors of yield and productivity (*2*), with a view to assessing the potential for this type of organism, relative to that of various pentose-utilizing yeasts (*8,14–17*) and thermophilic bacteria (*24–26*), in expanding the raw-materials base of the fuel-ethanol industry.

MATERIALS AND METHODS

Organisms

Escherichia coli (Luria strain B) ATCC 11303 was obtained from the American Type Culture Collection (Rockville, MD) and *E. coli* ATCC 11303 (carrying the PET plasmid pLOI297) (*12*) was a gift from L. O. Ingram (University of Florida, Gainsville, FL).

Culture Media

Luria broth (*27*) consisted of tryptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L), and a fermentable sugar, either glucose or xylose (Sigma Chemical Co., St. Louis, MO). The sugars were autoclaved separately—xylose solutions were neutralized before autoclaving to minimize browning and decomposition. The defined-mineral-salts medium contained NH_4Cl (2.25 g/L), $MgSO_4$ (0.2 g/L), $FeSO_4 \cdot 7H_2O$ (0.5 mg/L), KH_2PO_4 (2.72 g/L), KOH (1.12 g/L), thiamine (0.05 mg/L), and citric acid (0.21 g/L). In the case of flask cultures, the pH was adjusted using NaOH, whereas for chemostat cultures, HCl was used initially to set the pH. In experiments using a buffered medium, the concentration of phosphate was increased to 0.2*M* (27.2 g KH_2PO_4/L). Different buffered media were solidified by the addition of agar (20 g/L), and selective media contained the antibiotics tetracycline and ampicillin (Sigma) at concentrations of 10 mg/L and 40 mg/L, respectively (*11*). Cultures were stored (−10°C) in LB/glycerol-citrate, and were plated on selective media. Inocula were prepared in media containing antibiotics, but fermentations (both batch and continuous) were conducted without added antibiotics.

Batch Fermentations

Fermentations were conducted both in batch mode either in 250-ml Erlenmyer flasks [100 mL of buffered media] in a temperature-controlled water bath or in MultiGen [model F2000] bench-top stirred-tank bioreactors [STR], and in continuous mode (in BioFlo C30 chemostats), both systems having agitation and pH (2*N* KOH) and temperature (30°C) control, and being manufactured by New Brunswick Scientific Co. (Edison, NJ).

Wood Hemicellulose Hydrolysate

"Prehydrolyzed" wood (aspen) was obtained from Bio-hol Developments (Toronto, Canada) and was prepared using a Wenger extruder and with SO_2 as catalyst (*19*). The hemicellulose component was completely hydrolyzed (process referred to as *prehydrolysis*) with the mass total of sugars/dry wood being 23% (predominantly xylose). Cellulose was only partially hydrolyzed under these conditions (*19*). The extruded wood material was slurried in boiling water and filtered. The filtrate was concentrated under vacuum to achieve a xylose concentration in the range of

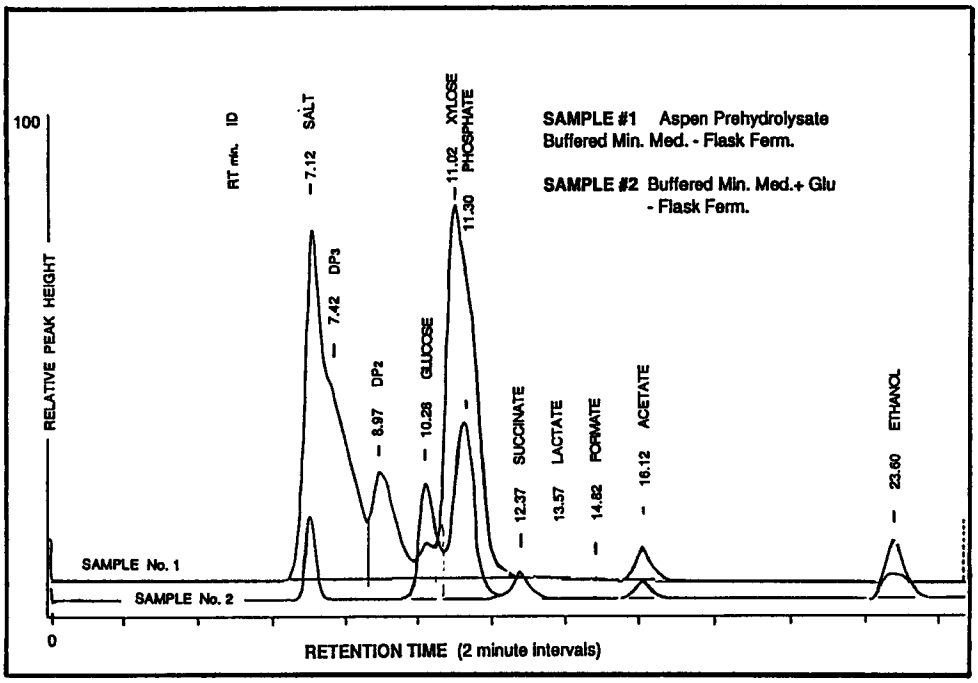

Fig. 1. Analysis of fermentation broth by HPLC—typical chromatograms. Details are given in Materials and Methods sections.

3–5% (w/v). Under these conditions the acetic acid is largely retained, but can be removed by steam-stripping under atmospheric conditions (*28*).

Analytical Procedures

The concentration of fermentation substrates (glucose, xylose, and wood hydrolysates) and products (ethanol and organic acids—acetic, succinic, lactic, and formic) were determined in cell-free supernatants using a HPLC equipped with a refractive-index monitor and computer-interfaced controller/integrator (Bio-Rad Labs, Richmond, CA). Separations were performed at 65°C on an aminex HPX-87H column (Bio-Rad Labs) at a flow rate of 0.6 mL/min of 6.7 m*M* phosphoric acid (injection vol 0.02 mL). Pure chemical standards were used to identify the various peaks.

Typical chromatograms from HPLC analysis of cell-free fermentation broths are shown in Fig. 1, with peaks identified and retention times (min) indicated. It is important to note that, in batch fermentations in which phosphate was used to buffer the media, the assay for fermentable sugars was complicated by the inorganic phosphate, which comigrates on the column with glucose and xylose. Oligomers that are presumed to be di- and trisaccharides (indicated as DP2 and DP3) were present in the aspen-wood prehydrolysate, but the predominant fermentable sugar is xylose. The concentrated APH contains about 40 g/L xylose, a slight amount of glucose, and about 0.1*M* acetate. Alternatively, a Yellow Springs model

Fig. 2. *E. coli* (Luria strain B ATCC 11303) and Ingram's recombinant strain carrying the "PET plasmid," pLOI297: Cultures were streaked on LB agar containing 2% glucose and incubated at 37°C for the same period of time before being photographed. (Left) The parent *E. coli* ATCC 11303 (Luria strain B); (Right) r *E. coli* ATCC 11303 (pLOI297)—this selective medium also contains antibiotics (tetracycline + ampicillin).

27 glucose analyzer (Yellow Springs, OH) was used to measure glucose, since glucose and xylose are difficult to quantitatively distinguish by HPLC under the conditions employed in this investigation.

Growth was measured turbidometrically at 550nm using a cuvet with a 1-cm light path in a Unicam SP1800 spectrophometer (Canlab, Toronto, Canada) and culture dry weight was measured by ultrafiltration, washing and drying the filter to constant weight under an infrared heat-lamp.

RESULTS AND DISCUSSION

Figure 2 is a photograph of agar plates (LB with 2% glucose) illustrating the very obvious phenotypic differences with respect to size and colony morphology between *E. coli* ATCC 11303 and the recombinant *E. coli* carrying the "PET operon" (*p*ortable *et*hanol production) constructed from cloned "alcohol-production" genes (*pdc* and *adhB*) from *Zymomonas mobilis* CP4 and expressed under the control of the *lac* promoter (*12*). Because this is not a color reproduction, it does not show that the recombinant culture is also distinctly more opaque and yellow in color when grown on a selective (with antibiotics), rich medium with a fermentable sugar (glucose). During the course of this investigation we routinely used this diagnostic method to screen for plasmid stability.

One of the recognized advantages of operating yeast-based fermentations at relatively low pH (range 4–5.5) is the decreased opportunity for bacterial infection. For this reason, we examined the effect of pH on the physiology of the recombinant bacterial culture, with a view to operating

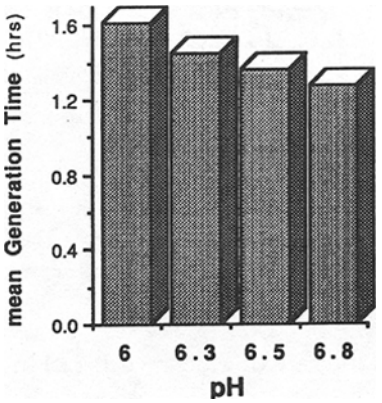

Fig. 3. Effect of pH on growth rate: Growth of *E. coli* ATCC 11303 (pLOI297) in STR pH-stat in Luria broth (2% glucose) with pH controlled by the addition of 2*N* KOH.

at a pH as low as possible. Figure 3 shows the effect of pH (over the range 6.0–6.8) on the growth rate of *E. coli* ATCC 11303 (pLOI297). The results of this preliminary investigation do not permit us to recommend an "optimal" operating condition with respect to pH. The effect of pH over a more extended range (<6.0) is the subject of ongoing studies.

In complex rich media, such as Luria broth (at very low glucose concentrations), *E. coli* grows exponentially, with a mean generation time on the order of 20–30 min (*13*). However, we observed that growth of the recombinant culture was not always truly exponential, but could be more properly described as pseudoexponential or linear. The latter was particularly true when higher sugar concentrations were employed (>3%). The doubling times observed for anaerobic growth in LB (glucose) medium for this PET-plasmid-bearing strain of *E. coli* B (Fig. 3) are significantly slower than those reported for another *E. coli* K12 strain (TC4), for which the mean generation times ranged from 38 to 48 min (*11*). Nevertheless, the growth rates observed in this study (at low sugar concentrations, <3%) were comparable to those for both *Zymomonas* and *Saccharomyces* (*6*). At pH 6.0, the doubling time for *Z. mobilis* is 1.4 h (*6*).

The pH over the range tested (6.8–6.0) also appeared to have very little effect on productivity at low glucose concentrations (Fig. 4). For glucose (1.5–2%), the observed values for the specific productivity (q_P 2.2 g EtOH/g cell/h) and volumetric productivity (Q_P 1.2 g EtOH/L/h) (Table 1) are comparable to the average values reported for batch fermentation in buffered LB medium and 12% glucose (*12*).

Figure 5 demonstrates that, whereas the growth rate is independent of glucose concentration, the final cell density (biomass concentration) is proportional to the amount of glucose. In plots of cell density vs sugar concentration (glucose or xylose), the dry cell mass was found to be directly proportional to the amount of sugar consumed, up to 3.3% (w/v). Beyond

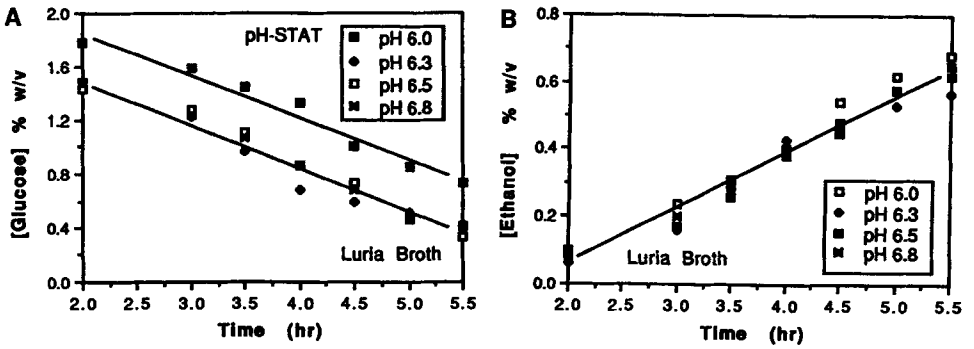

Fig. 4. Effect of pH on the rate of glucose utilization and ethanol production.

Table 1
Operational Parameters For Batch Fermentations
Glucose and Xylose Conversion by Recombinant *E. coli* ATCC 11303 (pLOI297)

	SUBSTRATE		[PRODUCT]	PRODUCTIVITY		YIELD	
Culture Medium	[S]	[Biomass]	[EtOH]	q_p	Q_p	$Y_{p/s}$	Conversion
Luria Broth	g/L	gDW/L	g/L	g P/g cell/hr	g P/L/hr	g P/g S used	Effic %
GLUCOSE	23	2.43	13.0	2.20	1.20	0.57	112
	58	3.50	29.0	1.43	1.60	0.50	98
	148	2.65	46.0	0.16	0.35	0.35	69
XYLOSE	40	2.04	17.5	0.27	0.36	0.44	86
	80	2.40	36.0	0.27	0.47	0.45	88

CONDITIONS: pH-stat batch culture with pH controlled at 6.3 by addition of KOH; temperature controlled at 30°C; cell density ([biomass]) was determined as dry weight at maximum OD_{550} (ie. onset of stationary-phase); q_p calculated as average over initial period of growth phase; Q_p calculated as final ethanol concentration divided by total time of fermentation; $Y_{p/s}$ calculated as amount of ethanol produced per mass of sugar consumed. S = fermentable sugar, either glucose or xylose. P = ethanol

that point it was constant at a maximum value of about 3.5 g/L in LB medium (results not shown). The cell yield in LB without fermentable sugar was 0.4 g DW/L. From the slope of these plots (not shown), the molar growth yields for glucose and xylose were determined to be 17.28 and 7.65, respectively. From a knowledge of sugar metabolism in *E. coli*, where lactic acid (or ethanol, in the case of the recombinant culture) is the major metabolic end product and very little acetic acid is produced, it has been generally assumed that the net molar yield of energy (ATP) from glucose is 2, whereas for xylose is it only five-sixths as much (1.67 mol

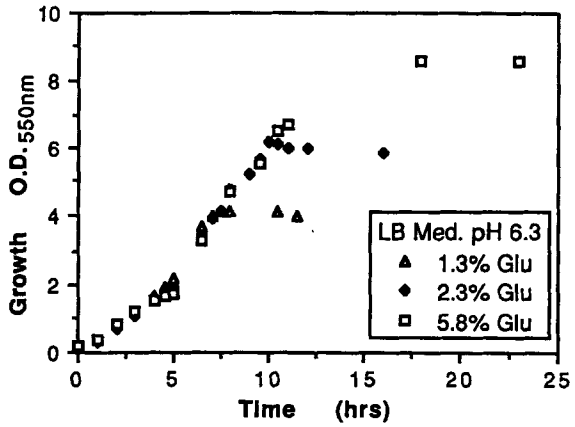

Fig. 5. Growth rate and cell yield of recombinant *E. coli:* Growth rate and cell yield are shown as a function of glucose concentration in LB medium (variable glucose) in pH-stat controlled at pH 6.3 (Temperature 30°C).

ATP/mol xylose). Assuming that growth yield with respect to ATP (Y_{ATP}) is constant for both sugars, it could be expected that the molar growth yield for each sugar would be proportional to the net yield of ATP for each sugar. In fact, this was not the case, and our observations on growth yields with glucose and xylose suggest that the net yield of energy (ATP) derived from C_5 sugars by this culture is only about one-half the amount obtained from the metabolism of C_6 sugars. To our knowledge, this has not been reported by others. This difference in energy yield could explain the slower rates observed with xylose compared to glucose (Fig. 7A, Table 1).

The performance of this culture was assessed at high sugar concentrations (15% glucose; 4 and 8% xylose) that are more realistic in terms of an industrial operation, and the results are represented in Fig. 6. The important operational parameters for productivity, final product concentration, and yield for these batch fermentations (pH controlled at 6.3 and temperature at 30°C) are summarized in Table 1. For the most part, these observations serve to confirm those made earlier by Alterthum and Ingram (*12*) in buffered LB media (only 12% glucose), with the notable exception of the batch with 15% glucose, which perhaps surpassed the tolerable limit of this culture. The observed q_p for xylose (0.27 g EtOH/g cell/h) was only about 20% of that previously observed (*12*).

In contrast to the biological approach to process improvement (including genetic engineering), the engineering approach involves the transformation of the batch fermentation to a more productive continuous process (*6*). Table 2 summarizes our preliminary chemostat studies, in which we explored the fermentation performance characteristics of *E. coli* ATCC 11303 (pLOI297) in both a complex and a defined-mineral-salts medium with glucose. Functional parameters determined under the steady-state conditions of continuous culture are more reliable than those estimated

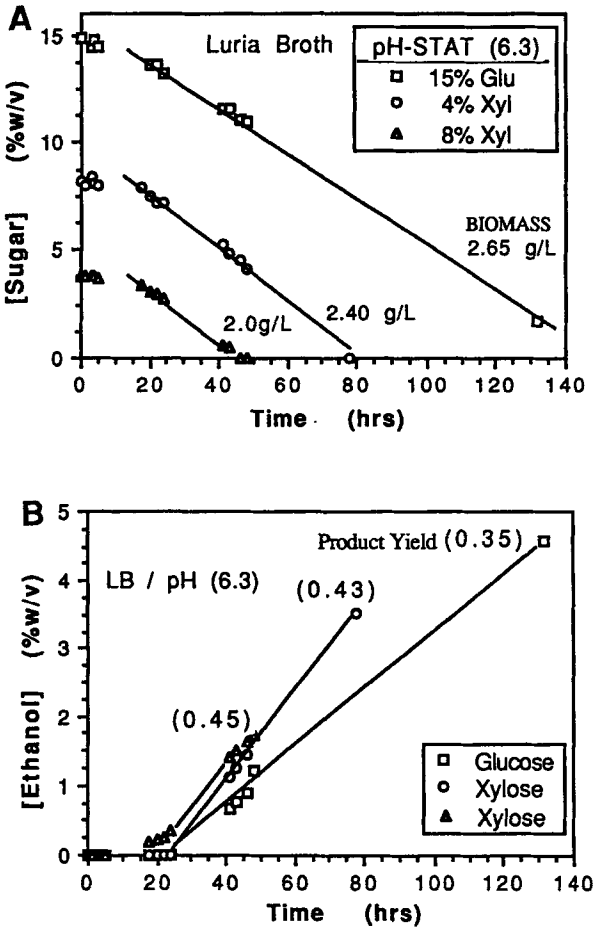

Fig. 6. Comparative rates of glucose and xylose utilization and ethanol production by recombinant *E. coli:* Batch fermentations were conducted in STR pH-stats controlled at pH 6.3 at a temperature of 30°C. The numbers in brackets in B represent the product yield based on sugar utilized.

from batch fermentations for which the environment is not kept constant. The objective of improved productivity has clearly not yet been achieved, since the values observed are comparable to those observed in batch fermentations both by us and by others (*12*). We were surprised to note that the best Q_p value was achieved at the lowest pH (Table 2, line 3). We have experienced a great deal of difficulty in operating the continuous-flow bioreactors under a condition of complete substrate utilization, even at relatively low sugar concentrations (<5%) and at dilution rates that were only 50% of the anticipated maximal rate (0.4/h), based on the observed specific growth rates at these pH values (Fig. 3). The experiments with a defined-salts-medium in which glucose is the sole source of carbon show that the ethanol yield is lower than that for the rich complex Luria broth (Table 2).

Table 2
Operational Parameters For Continuous Fermentations
Glucose Conversion by Recombinant E. coli ATCC 11303 (pLOI297)
with Complex and Defined Mineral Salts Media

		SUBSTRATE		[PRODUCT]	PRODUCTIVITY		YIELD	
Medium Comp.		[Glu]	Feedstock	[EtOH]	q_p	Q_p	$Y_{p/s}$	Conversion
Glucose	(pH)	g/L	Util. (%)	g/L	g P/g cell/hr	g P/L/hr	g P/g Glu	Effic (%)
COMPLEX								
Luria Broth	(6.3)	21	88	8.7	0.92	1.91	0.47	92
	(6.3)	38	94	12.7	0.69	1.27	0.35	69
	(6.0)	38	70	12.9	1.24	2.58	0.48	94
	(6.3)	50	68	16.6	0.76	1.66	0.49	96
DEFINED								
Mineral Salts	(6.8)*	36	81	12.0	0.86	0.96	0.42	82
	(6.3)	50	53	10.5	1.10	0.95	0.40	78
	(6.3)	50	13	2.9	0.97	0.58	0.45	88

CONDITIONS: continuous fermentations were conducted in NBS BioFlo C30 bench-top chemostats with pH (2N KOH), temp. control (30°C) and agitation (minimally at approx.150 RPM). Steady-state was assumed only after a minimum of 5 volume turnovers.
The *volumetric productivity*, Q_p was calculated as steady-state ethanol conc'n times the dilution rate; the *specific productivity*, q_p was obtained by dividing the value for Q_p by the steady-state biomass concentration; cell density (biomass) was determined as dry weight by microfiltration method; the *product yield*, $Y_{p/s}$ was calculated as the steady-state ethanol conc'n divided by the mass of sugar utilized.
No antibiotics were added to either the LB or minerals salts media. P = steady-state [ethanol].

* The mineral salts medium, defined in *Materials & Methods*, was modified by the addition of 0.01M phosphate and 1mM citric acid.

Again, our observations on yields with minimal media (Table 2, *see also* Figs. 8B [glu] and 8C [xyl]) provide evidence to confirm the suggestion made by Alterthum and Ingram that "the unusually high ethanol yields... (over 100% of theoretical values) may include ethanol derived from the catabolism of complex nutrients" (e.g., amino acids in tryptone) (*12*).

The hydrolysis of lignocellulosic feedstocks produces a mixture of hexoses and pentoses, and it was of interest to examine the behavior of this recombinant ethanologen in media (designed to mimic the $C_6 \cdot C_5$ ratio of complete hydrolysates) containing glucose and xylose as the sole carbon sources for growth and ethanol production. Figure 7A shows that, with buffered media (0.2*M* phosphate, initially at pH 7), the rate of xylose metabolism is about 50% of the rate of glucose metabolism and that glucose is a preferred substrate, since it disappears before the xylose is consumed. The yield of 0.37 (based on complete utilization of the sugars added after 72 h) is also lower in the case of the glucose/xylose (Fig. 8A). From studies with other pentose-fermenting yeasts and bacteria, lignocellulosic hydrolysates are also known to contain various inhibitory sub-

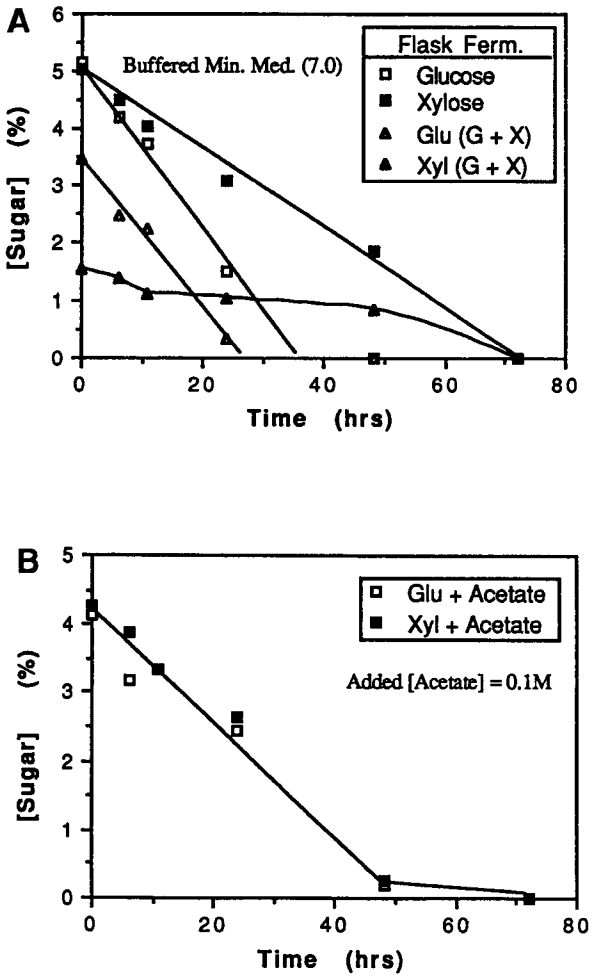

Fig. 7. (A) Comparative rates of glucose and xylose utilization: Glucose and xylose separately and as a mixture (Glu:Xyl=2.3:1) to mimic lignocellulosic hydrolysate. (B) Comparative effect of acetate on glucose and xylose utilization by r *E. coli:* The concentration of sodium acetate added was 8g/L (0.1*M*) to mimic concentration of aspen-wood prehydrolysate (APH). Fermentations were conducted in shake-flasks with buffered defined-salts medium, initially at pH 7.

stances, and acetic acid (a byproduct of thermochemical processing [*19,28*]) is recognized as one of these toxic compounds. Our assay of the concentrated aspen-wood hemicellulose hydrolysate (so-called prehydrolysate) showed it to contain about 0.1*M* acetate. The effect of this amount of acetate on the fermentation performance of r *E.coli* with 0.2*M* phosphate buffer is shown in Figure 7B. Whereas the rate of glucose metabolism is inhibited, the rate of xylose utilization appears little affected by this concentration of exogenous acetate (Fig. 7B). The effect of acetate on ethanol production is shown in Figs. 8B and 8C for glucose and xylose, respec-

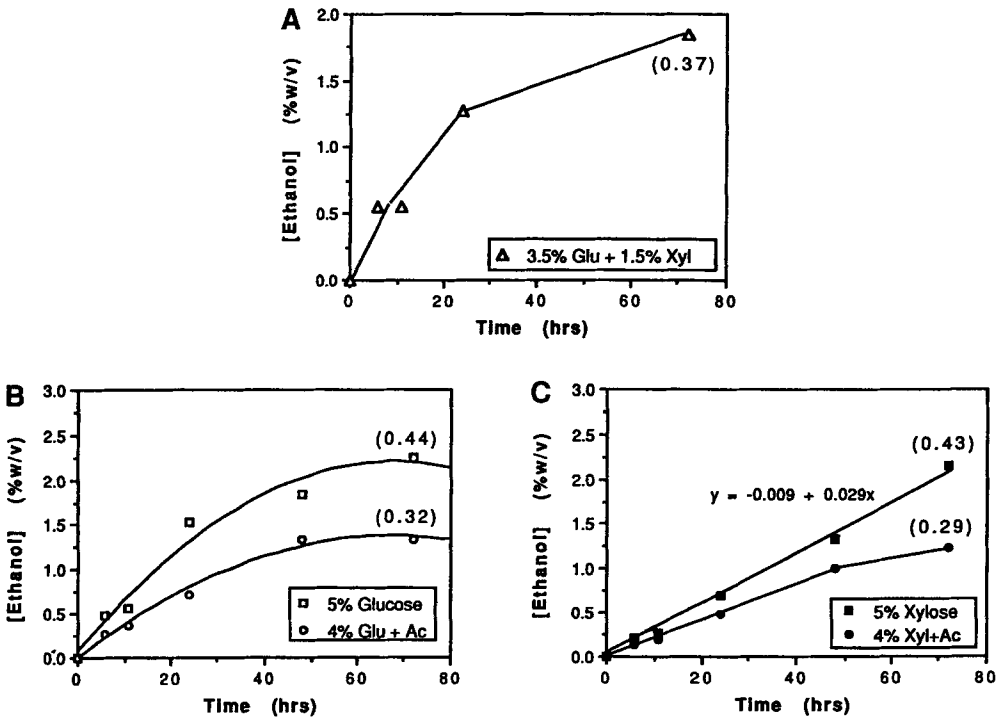

Fig. 8. (A) Ethanol production by recombinant *E. coli* from a mixture of glucose and xylose: Fermentation conditions were as described in Fig. 7. The value for $Y_{p/s}$ is shown in brackets. (B) Effect of acetate on ethanol production from glucose. (C) Effect of acetate on ethanol production from xylose.

tively). Acetate causes the product yield to be dramatically decreased with both sugars—a 28% reduction for glucose and 33% for xylose.

Figure 9 represents our first attempt at a "hemicellulose" fermentation with Ingram's recombinant *E. coli*. The conditions of this test case were not designed to be particularly favorable, since they were designed to model that of an industrial fermentation with lignocellulose prehydrolysate (*19*), with a minimum of added nutrients to reduce cost. The xylose concentration was about 4% (w/v) and the acetate was 0.1*M*—only thiamine (50 μg/L) and mineral salts (as in the "defined" medium) were added to the hemicellulose hydrolysate concentrate filtrate. The inoculation cell density was not high, and, therefore, the rather poor performance is not too surprising, considering the nonconducive nature of the test environment. Experiments are ongoing to test the effect of much higher initial cell loadings and immobilized cell systems with added nutrients.

This organism was not designed to compete with or replace the efficient yeasts that are presently employed in the starch-based fuel-ethanol industry. The specific productivity exhibited by this recombinant is better than that of *Saccharomyces* yeast, but the organism is neither as fast nor as

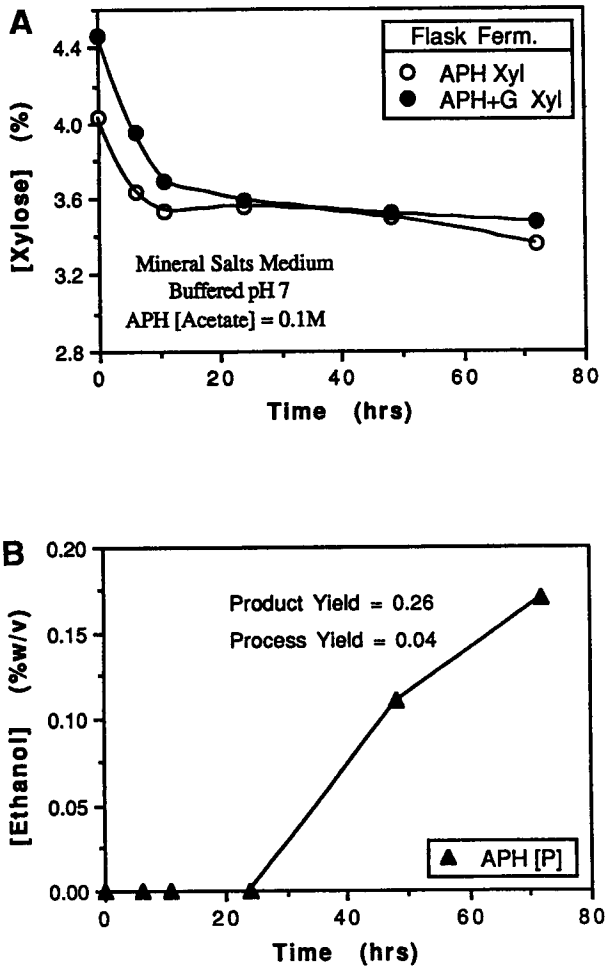

Fig. 9. Ethanol fermentation by r *E. coli* in wood hemicellulose hydrolysate: Flasks were inoculated at relatively low cell density (10% v/v). The defined-salts medium was initially buffered at pH 7. The xylose concentration of the aspen wood prehydrolysate (APH) (open circles) was about 40 g/L; closed circles represent an experiment with a small amount of added glucose (4 g/L). The concentration of acetic acid was 0.1*M*. The "process yield" is based on the amount of ethanol produced and the total amount of fermentable sugars.

efficient as *Zymomonas* (based on glucose) (*7*). However, since neither the yeast presently used for the production of fermentation alcohol nor Z. *mobilis* can convert pentose sugars to ethanol, this PET-plasmid-containing strain of *E. coli* (*12*) will find its competition only from those pentose-fermenting organisms that have been claimed to have potential for the bioconversion of lignocellulosic (biomass) feedstocks (*14–16*).

Our observations with Ingram's recombinant *E. coli* both confirm the claims already in the literature (*12*) and easily provide a justification for

further research with this and other related genetic constructs, since the pentose-utilizing yeasts and bacteria are all generally characterized by inferior yields (*8,14–17*) or conversion rates (productivities) (*20–22*), or, in the case of the thermophilic bacteria, by a low tolerance to ethanol (*24–26*). There are always, however, possible exceptions, as perhaps is the case with the "adapted" strains (R) of *P. stipitis* (*18,19,28,29*).

ACKNOWLEDGMENTS

This research was supported by the Dean's Fund (Faculty of Medicine) and by an operating grant from the Natural Sciences and Engineering Research Council of Canada. We are grateful to Bio-hol Developments for donating the processed lignocellulosic feedstocks and to Lonnie Ingram for the recombinant ATCC 11303 (pLOI297).

REFERENCES

1. Keim, C. R. (1989), *Trends Biotechnol.* **7,** 22–29.
2. Wright, J. D. (1988), *Chem. Eng. Progress,* **84,** 62–68.
3. Wyman, C. E. and Hinman, N. D. (1990), Proc. 11th Symp. on Biotech. for Fuels & Chemicals, *Appl. Biochem. Biotechnol.*, vol. **24/25,** 735–753.
4. Fein, J. E., Charley, R. C., Droniuk, R., Good, D., Hopkins, K., Lawford, G. R., Zawadzki, B., and Lawford, H. G. (1984), *Proc. Waterloo Biomass Conversion Symp.*, University of Waterloo, July, Waterloo, Ontario, Canada.
5. Lawford, G. R., Charley, R. C., Edamura, R., Fein, J. E., Hopkins, K., Potts, D., Zawadzki, B., and Lawford, H. G. (1984), *Proc. Bioenergy Specialists' Meeting in Waterloo,* October, Waterloo, Ontario, Canada.
6. Lawford, H. G. (1988), *Appl. Biochem. Biotechnol.* **17,** 203–209.
7. Lawford, H. G. and Ruggiero, A. (1989), *Bioenergy,* (Proc. 7th Cdn. Bioenergy R&D Seminar), Hogan, E., ed., NRCC, Ottawa, Canada, pp. 401–408.
8. Skoog, K. and Hahn-Hagerdal, B. (1988), *Enzyme Microb. Technol* **10,** 66–80.
9. Lynd, L. R. (1989), Proc. 11th Symp. on Biotech. for Fuels & Chemicals, *Appl. Biochem. Biotech.*
10. Ingram, L. O., Conway, T., Clark, D. P., Sewell, G. W., and Preston, J. F., (1987), *Appl. Environ. Microbiol.* **53,** 2420–2425.
11. Ingram, L. O. and Conway, T. (1988), *Appl. Environ. Microbiol* **54,** 397–404.
12. Alterthum, F. and Ingram, L. O. (1989), *Appl. Environ. Microbiol.* **55,** 1943–1948.
13. Lin, E. C. C. (1987), *Escherichia coli and Salmonella typhimurium,* vol. 1, Neidhradt, F. C., Ingraham, J. L., Low, F. B., Magasanik, B., and Schaechter, M., eds., Am. Soc. Microbiol., Washington, DC.
14. Kastner, J. R. and Roberts, R. S. (1990), *Biotechnol. Lett.* **12,** 57–60.
15. Jeffries, T. W. and Sreenath, H. K. (1988), *Biotechnol. Bioeng.* **31,** 502–506.
16. Slinger, P. J. R., Bothast, R.J., Okos, M. R., and Ladisch, M. R. (1985), *Biotechnol. Lett.* **7,** 431–436.

17. Beck, M. J. (1989), *Biotechnol. Bioeng.* **17,** 617–627.
18. Nicholson, C., Foody, B., Malloch, E., Fiander, H., Wayman, M., Parekh, S., and Parekh, R. (1989), *Bioenergy* (Proc. 7th Cdn. Bioenergy R&D Seminar), Hogan, E., ed., NRCC, Ottawa, pp. 385–388.
19. Gans, I., Potts, D., Matsuo, A., Tse, T., Holysh, M., and Assarsson, P. (1989), *Bioenergy,* (Proc. 7th Cdn. Bioenergy R&D Seminar), Hogan, E., ed., NRCC, Ottawa, Canada, pp. 419–423.
20. Delgenes, J.P., Moletta, R., and Navarro, J. M. (1988), *Biotechnol. Lett* **10,** 725–730.
21. Alexander, M. A., Chapman, T. W., and Jeffries, T. W. (1988), *Appl. Biochem. Biotechnol.* **17,** 221–229.
22. Alexander, M. A., Chapman, T. W., and Jeffries, T. W. (1988), *Appl. Microbiol. Biotechnol.* **28,** 478–486.
23. Gong, C-S., Claypool, T. A., McCracken, L. D., Maun, C. M., Ueng, P. P., and Tsao, G. T. (1983), *Biotechnol. Bioeng.* **25,** 300–305.
24. Lacis, L. S. (1989), PhD thesis, University of Toronto, Toronto, Canada.
25. Lacis, L. S. and Lawford, H. G. (1989), *Bioenergy,* (Proc. 7th Cdn. Bioenergy R&D Seminar) Hogan, E., ed., NRCC, Ottawa, Canada, pp. 411–415.
26. Liu, H-S., Hsu, H-W., and Sayler, G. S. (1988), *Biotechnol. Prog.* **4,** 40–46.
27. Luria, S. E. and Delbruck, M. (1943), *Genetics* **28,** 491–511.
28. Parekh, S. R., Parekh, R. S., and Wayman, M. (1987), *Process Biochem.* **22**(6), 85–91.
29. Parekh, S. R., Yu, S., and Wayman, M. (1986), *Appl. Microbiol. Biotechnol.* **25,** 300–304.