APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1992, p. 1128–1133 Vol. 58, No. 4
0099-2240/92/041128-06$02.00/0

Conversion of Xylan to Ethanol by Ethanologenic Strains of *Escherichia coli* and *Klebsiella oxytoca*†

GERHARD BURCHHARDT AND L. O. INGRAM*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611

Received 23 September 1991/Accepted 30 January 1992

A two-stage process was evaluated for the fermentation of polymeric feedstocks to ethanol by a single, genetically engineered microorganism. The truncated xylanase gene (*xynZ*) from the thermophilic bacterium *Clostridium thermocellum* was fused with the N terminus of *lacZ* to eliminate secretory signals. This hybrid gene was expressed at high levels in ethanologenic strains of *Escherichia coli* KO11 and *Klebsiella oxytoca* M5A1(pLOI555). Large amounts of xylanase (25 to 93 mU/mg of cell protein) accumulated as intracellular products during ethanol production. Cells containing xylanase were harvested at the end of fermentation and added to a xylan solution at 60°C, thereby releasing xylanase for saccharification. After cooling, the hydrolysate was fermented to ethanol with the same organism (30°C), thereby replenishing the supply of xylanase for a subsequent saccharification. Recombinant *E. coli* metabolized only xylose, while recombinant *K. oxytoca* M5A1 metabolized xylose, xylobiose, and xylotriose but not xylotetrose. Derivatives of this latter organism produced large amounts of intracellular xylosidase, and the organism is presumed to transport both xylobiose and xylotriose for intracellular hydrolysis. By using recombinant M5A1, approximately 34% of the maximal theoretical yield of ethanol was obtained from xylan by this two-stage process. The yield appeared to be limited by the digestibility of commercial xylan rather than by a lack of sufficient xylanase or by ethanol toxicity. In general form, this two-stage process, which uses a single, genetically engineered microorganism, should be applicable for the production of useful chemicals from a wide range of biomass polymers.

Lignocellulosic materials represent the only cheap, renewable source of biomass capable of replacing petroleum as a feedstock for fuel and chemical production. Microbial conversion of the sugar residues present in wastepaper and yard trash from U.S. landfills could provide more than 10,000,000,000 gallons (1 gallon = 3.785 liters) of ethanol (16). Lignocellulose consists primarily of a mixture of cellulose, hemicellulose, and lignin. Cellulose is a homopolymer of glucose. Hemicellulose is a more complex heteropolymer containing primarily xylose but also significant amounts of arabinose, mannose, glucose, and galactose (9). Microorganisms have been developed which can efficiently ferment all sugars present as polymeric constituents of lignocellulose and produce ethanol concentrations above 40 g/liter (1, 14, 18, 19). These polymers must be hydrolyzed to their component sugars for fermentation. However, the development of improved methods for the hydrolysis of lignocellulose remains a major research goal.

Genetic engineering approaches for the addition of saccharifying traits to microorganisms for the production of ethanol have been directed at the secretion of high levels of enzymes into the medium either by using native signal sequences or by fusing these target genes to host signals (2). In this study, we have used xylan as a model substrate to develop a novel, alternative approach to hemicellulose hydrolysis. This approach involves the production of high cytoplasmic levels of thermostable enzymes within the ethanologenic microorganism during fermentation in a two-stage process for ethanol production.

* Corresponding author.

† Florida Agricultural Experiment Station publication number R-01832.

MATERIALS AND METHODS

Organisms and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria broth (15) supplemented with 50 g of xylose per liter. Transformants of *Escherichia coli* were selected on Luria agar plates containing 50 mg of ampicillin per liter or 40 mg of chloramphenicol per liter. Transformants of *Klebsiella oxytoca* M5A1 were selected on Luria agar containing 1,000 mg of ampicillin per liter or 20 mg of chloramphenicol per liter.

Recombinant clones were screened for xylanase activity by using microtiter plates which contained 4-methylumbelliferyl-β-D-cellobiopyranoside (100 mg/liter) (17). Similarly, expression of the *Butyrivibrio fibrisolvens xylB* gene encoding both xylosidase and arabinosidase activities was screened by incorporating 4-methylumbelliferyl-α-L-arabinofuranoside (20 mg/liter) into solid medium (22). Hydrolysis of these substrates by positive clones produced a fluorescent product (umbelliferone) which was readily detected under 340-nm UV.

Genetic procedures and recombinant techniques. Plasmid preparation, digestion with restriction enzymes, ligation, transformation, and gel electrophoresis were carried out by using standard procedures (20). *E. coli* DH5α was used as the host for all plasmid constructions. Polymerase chain reactions were performed with the TempCycler model 50 (Coy Laboratory Products Inc., Ann Arbor, Mich.) and a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) containing *Taq* polymerase. Amplification reaction mixtures contained 2 mM (each) deoxynucleoside triphosphate, 100 pmol of each primer, 20 ng of template, and 2.5 U of *Taq* polymerase in 100-μl total volumes. Products were isolated after 30 cycles of amplification (1 min at 94°C, 2 min at 47°C, and 1 min at 72°C; final extension for 3 min at 72°C).

Determination of enzymatic activities. Xylanase, xylopyranosidase, and arabinofuranosidase activities were measured in recombinants which had been grown overnight at 30°C.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
E. coli DH5α	*lacZ recA*	Bethesda Research Laboratories
E. coli KO11	*frd* Cm^r IPET	18
K. oxytoca M5A1 (pLOI555)	Cm^r PET	19
pCT1202	Ap^r *xynZ*$^+$	11
pLOI1001	Ap^r *xylB*$^+$	22
pLOI2000	Ap^r *xylB*$^+$	This study
pLOI2001	Ap^r *lacZ*::*xynZ*$^+$	This study
pLOI2002	Ap^r *lacZ*::*xynZ*$^+$	This study
pLOI2003	Ap^r *lacZ*::*xynZ*$^+$ *xylB*$^+$	This study

[a] IPET refers to the integration of the *Z. mobilis pdc* and *adhB* genes into the chromosome. PET refers to the presence of the *Z. mobilis pdc* and *adhB* genes on plasmid pLOI555.

Cells were harvested by centrifugation (7,000 × *g* for 10 min) and washed twice with phosphocitrate buffer (50 mM potassium phosphate and 12.5 mM citric acid [pH 6.3]) for the determination of xylanase activity or with phosphate buffer (5 mM sodium phosphate buffer containing 10 mM 2-mercaptoethanol [pH 6.8]) for the measurement of xylosidase and arabinosidase activities. Cells were disrupted by two passages through a French pressure cell at 20,000 lb/in^2. The resulting lysate was centrifuged (13,000 × *g* for 30 min, 4°C) to remove cell debris.

Xylosidase and arabinofuranosidase activities were assayed as described previously (22). Xylanase was measured by using the same procedure but with *p*-nitro-β-D-cellobioside as the substrate. Xylanase activity was also estimated by measuring the release of reducing sugars (3) from the hydrolysis of birchwood xylan (Sigma Chemical Co., St. Louis, Mo.). All activities are reported as nanomoles of product liberated per minute. Protein concentration was determined by the method of Bradford (5).

Separation of xylooligosaccharides by thin-layer chromatography. A mixture of xylooligosaccharides was prepared by the partial hydrolysis of 0.5% birchwood xylan with trifluoracetic acid (8). This solution was concentrated 10-fold under vacuum at room temperature, and 1-μl samples were used as the standards. Xylooligosaccharides were separated at room temperature by single development on unactivated Whatman silica gel 150A plates (Whatman, Inc., Clifton, N.J.) with a solvent composed of acetone, ethylacetate, and acetic acid (2:1:1). After drying, oligosaccharides were visualized with naphthylethylenediamine reagent as described by Bounias (4).

Fermentation experiments. Inocula for fermentations were prepared from freshly isolated colonies by overnight growth in unshaken flasks (30°C). Fermentations were inoculated to an initial optical density at 550 nm (OD_{550}) of either 0.1 or 0.3 and incubated at 30°C in stirred pH stats (350-ml working volume, pH 6.0) as described previously (1, 18, 19). Chloramphenicol or chloramphenicol and ampicillin were included in fermentation broths.

Xylan was fermented by a two-stage process in which xylan saccharification was carried out at elevated temperatures and then was followed by fermentation at 30°C. For the degradation of xylan, cells from previous 350-ml fermentations were harvested by centrifugation (7,000 × *g* for 10 min) after 48 h, resuspended in an equal volume of fresh Luria broth containing 4% xylan (pH 6.0), and incubated at 60°C for 65 h. Incubation at this temperature killed the cells and released active xylanase. After cooling to 30°C, the saccharified xylan in Luria broth was inoculated with recombinant organisms to provide an initial OD_{550} of 0.3 and to start the fermentation.

Samples were removed at various times to monitor xylooligosaccharides (thin-layer chromatography), cell growth (OD_{550}), and ethanol. Ethanol was measured by gas-liquid chromatography (7).

RESULTS

Construction of recombinant plasmids for the hydrolysis of xylan. Three plasmids were constructed for use in xylan fermentations in which the *xynZ* (xylanase) gene from the thermophile *Clostridium thermocellum* (11) and the *xylB* (xylosidase and arabinosidase) gene from *B. fibrisolvens* (22) were expressed singly and in combination as an operon (Fig. 1). The *xylB* gene was subcloned into the *Xba*I-to-*Pst*I region of the polylinker in pUC18 by inserting two fragments from pLOI1001, a 0.3-kbp *Xba*I-to-*Pst*I fragment containing the amino terminus with a ribosome binding site and a 2.4-kbp *Pst*I fragment containing the remainder of the coding region and the translational terminator. The resulting plasmid, pLOI2000, expressed the *xylB* gene from the *lac* promoter.

Previous studies by Grepinet et al. (11) have shown that xylanase expression was elevated in *lacZ* fusions in which a large segment encoding the secretion signal sequence and the amino terminus of the processed xylanase had been deleted. A very similar *lacZ*::*xynZ* fusion was constructed by blunt-end ligation of the Klenow-treated 2.4-kbp *Sty*I fragment from pCT1202 containing the amino-truncated *xynZ* gene into the Klenow-treated *Pst*I site of pUC18. Both reading frames were aligned in the resulting plasmid, pLOI2001.

It was necessary to remove the putative transcriptional terminator 30 bp downstream from the coding region of *lacZ*::*xynZ* and to add a new *Sst*I site to the 3′ end of the fusion gene prior to constructing a plasmid with both xylanase and xylosidase. These modifications were made by using the polymerase chain reaction. Plasmid pLOI2001 was used as the template, with 5′-GAATTCGAGCTCGGTAC-3′ as a primer for the 5′ end and 5′-GGGAGCTCCGGCATC ATTATCTG-3′ as a primer for the 3′ end. These primers allow amplification of a DNA fragment containing the ribosome binding site and the coding region for *xylB* (22) and include a new *Sst*I site on both ends. After *Sst*I digestion, this fragment was inserted into the *Sst*I polylinker sites of pUC18 and pLOI2000 to construct pLOI2001 and pLOI2003, respectively.

Expression of enzymes involved in xylan degradation. The expression of xylanase, xylosidase, and arabinosidase activities was initially examined with stationary-phase cells of the host organism used for constructions, strain DH5α (Table 2). Xylanase (*xynZ*) activity was reduced by 60% in clones harboring pLOI2003, which contains *xylB* downstream, compared with pLOI2001, which contains *xynZ* alone. However, xylosidase and arabinosidase (*xylB*) activities were similar in clones harboring pLOI2000, which contains *xylB* alone, and pLOI2003, which contains an upstream *xynZ* gene.

The plasmids pLOI2001 and pLOI2003 were transformed into the ethanologenic strain of *E. coli*, KO11, in which the genes for ethanol production are integrated into the chromosome. Expression was compared in stationary-phase cells from shake flasks and in cells from a pH stat at the end of fermentation (Table 2). Xylosidase and arabinosidase activ-

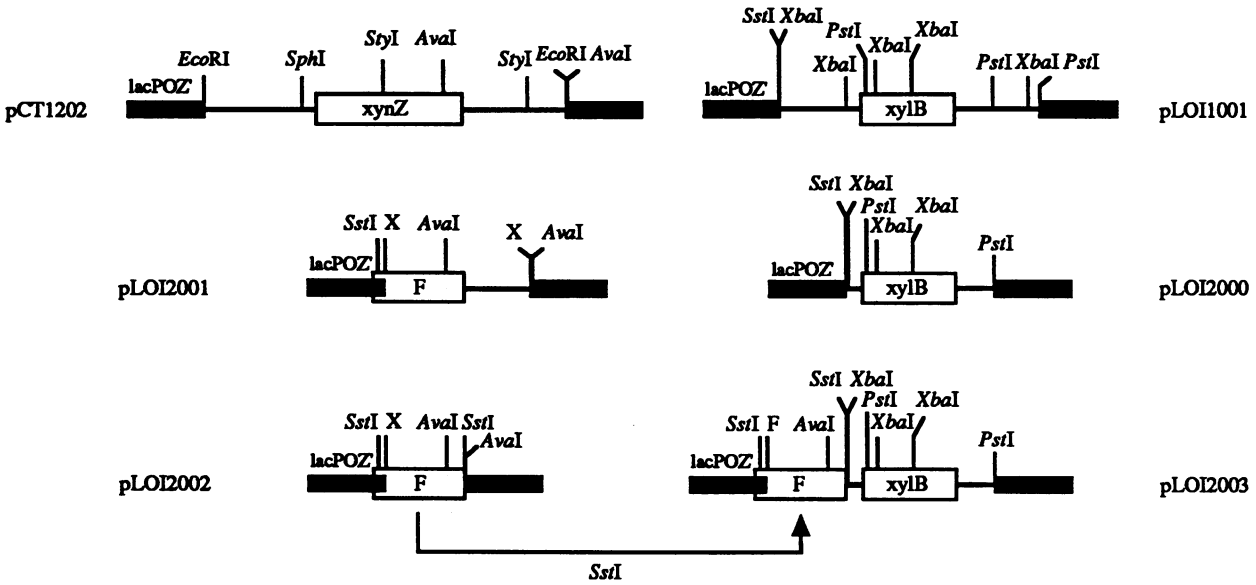

FIG. 1. Construction of recombinant plasmids containing *xynZ* and *xylB*. Coding regions are boxed. *C. thermocellum* and *B. fibrisolvens* DNA are represented by thin lines. Thick lines denote vector DNA. The sites of blunt-end ligation are marked by X's. F labels the coding region for the in-frame *lacZ::xynZ* fusion.

ities were equivalent to those observed with DH5α. Xylanase activity was lower in the ethanologenic strain, KO11. Again, xylanase activity was reduced in pLOI2003, in which *xylB* was present downstream.

Expression was also examined in *K. oxytoca* M5A1 harboring pLOI555 containing genes from the *Zymomonas mobilis* ethanol pathway. Although the type of replicon present in pLOI555 is unknown, it appeared quite stable in the presence of a second plasmid constructed from pUC18. Expression of arabinosidase and xylosidase activities was roughly equivalent to that observed with *E. coli*. An abundant native xylosidase was discovered in M5A1. Xylosidase activities were somewhat higher in cells from shake flasks than in cells from pH stats, while xylanase activities followed the opposite trend.

TABLE 2. Specific activities of recombinant enzymes for xylan degradation[a]

Strain and plasmid	Sp act (mU/mg of cell protein)			
	Xylosidase	Arabinosidase	Xylanase[b]	Xylanase[c]
E. coli DH5α	0	0	0	0
pLOI2000	1.2	2.2	0	0
pLOI2001	0	0	1.4	124
pLOI2003	1.5	2.5	0.5	48
E. coli KO11	0	0	0	0
pLOI2001	0	0	0.4	38
pLOI2003	1.3	2.4	0.3	25
pLOI2003[d]	1.1	1.9	0.3	47
pLOI2003[d,e]	ND	ND	0.8	93
K. oxytoca M5A1 (pLOI555)	53	0	0	0
pLOI2001	49	0	0.4	39
pLOI2003	56	2.6	0.2	24
pLOI2001[d]	30	0	0.7	80
pLOI2001[d,e]	ND	ND	1.8	144
pLOI2003[d]	38	2.9	0.4	58
pLOI2003[d,e]	ND	ND	0.8	144

[a] Unless otherwise indicated, cells were grown in shake flasks containing Luria broth with 5% xylose and harvested after 20 h (stationary phase), xylosidase and arabinosidase activities were determined at 30°C, and xylanase activities were measured at 45°C. ND, not determined.

[b] Xylanase activities were measured by using *p*-nitrophenyl-β-D-cellobioside as a substrate.

[c] Xylanase activities were measured by using 0.5% birchwood xylan as a substrate.

[d] Cells were harvested from pH stats (8% xylose in Luria broth [pH 6.0]) at the end of fermentation.

[e] Xylanase activities were determined at 60°C.

Under the assay conditions used, arabinosidase activity was 1.5 to 1.7 times higher than xylosidase activity, confirming previous reports (22). Xylanase activity measured at 45°C was approximately half that measured at 60°C, the optimal temperature for the native enzyme (10). On the basis of the data presented, specific activities computed from reducing sugar assays for the xylanase fusion are approximately 100-fold higher than estimates based on the hydrolysis of *p*-nitrophenyl-β-D-cellobioside, indicating a strong preference for a native substrate.

Hydrolysis of xylan at elevated temperatures by recombinant clones. Strain KO11(pLOI2003) was grown in a pH stat containing 8% xylose and tested as a source of enzymes for xylan hydrolysis. Cells were resuspended in an equal volume of fresh Luria broth containing 4% xylan and incubated at 45°C, the maximal temperature at which *B. fibrisolvens* xylosidase is stable, and at 60°C, the optimal temperature for *C. thermocellum* xylanase.

Although xylanase and xylosidase were produced as intracellular products, initial experiments indicated that these enzymes were readily released into the medium by incubation at 45 and 60°C. Samples were removed at various times, and digestion products were analyzed by thin-layer chromatography (Fig. 2). Xylose, xylobiose, xylotriose, and xylotetrose were clearly resolved, with xylobiose the dominant product. A comparison of the extent of digestion after 24 and 48 h clearly shows that less hydrolysis occurred at 45 than at 60°C, despite the instability of xylosidase at 60°C.

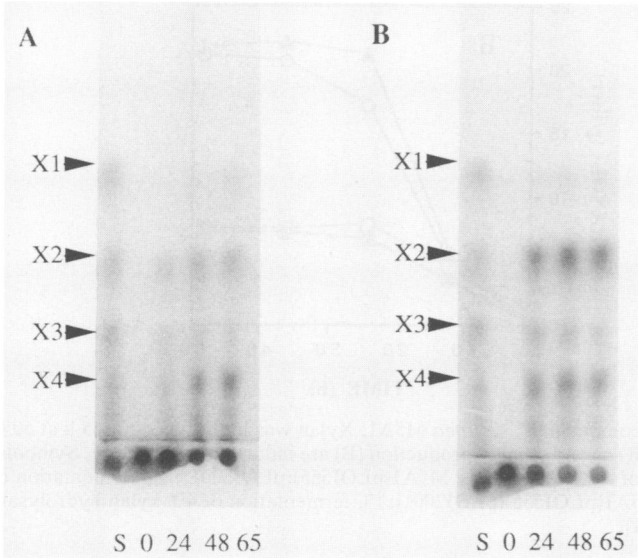

FIG. 2. Thin-layer chromatographic analysis of xylan hydrolysis. *E. coli* KO11(pLOI2003) was incubated at 45°C (A) or at 60°C (B) in Luria broth (pH 6.0) containing 4% birchwood xylan. A sample size of 1 μl was applied to each lane. The incubation times are given in hours beneath each lane. An acid hydrolysate of xylan was used as a standard (S) in the first lane.

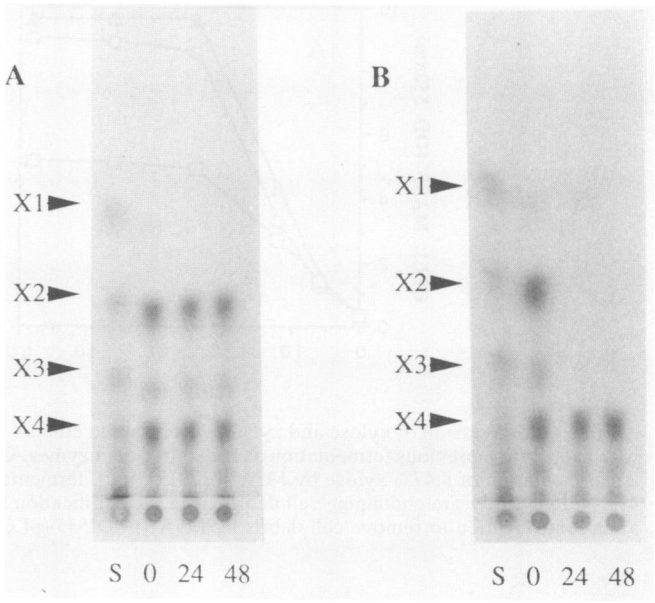

FIG. 3. Utilization of xylooligosaccharides for growth by recombinant *E. coli* KO11 and *K. oxytoca* M5A1. Xylan (4%) was hydrolyzed for 65 h by incubation with *E. coli* KO11(pLOI2003) in Luria broth (pH 6.0) and then centrifuged and sterilized by filtration. Samples were inoculated and incubated at 30°C to allow growth. Samples were removed at 24-h intervals, as indicated beneath each lane, and analyzed by thin-layer chromatography. A mixture of xylooligosaccharides was used as a standard (S) in the first lane. (A) *E. coli* KO11(pLOI2003); (B) *K. oxytoca* M5A1(pLOI555).

Although xylan digestion was incomplete even after 65 h at 60°C, xylanase remained quite active and was readily detected with *p*-nitrophenyl-β-D-cellobioside as a substrate. Xylosidase was rapidly inactivated at this temperature. No further changes in thin-layer profiles of xylan hydrolysate were observed after the addition of cell lysates containing both enzymes and incubation at 60°C for another 24 h. Thus, the xylooligosaccharide products appear to be the final products of Sigma birchwood xylan when this enzyme is used. Oxidation products or substitutions may block complete digestion of this commercial preparation.

Utilization of xylan hydrolysis products by recombinant strains of *E. coli* and *K. oxytoca*. Small-scale experiments were conducted to evaluate the extent to which xylooligosaccharides could be metabolized by *E. coli* KO11(pLOI2003) and *K. oxytoca* M5A1(pLOI555). These strains were inoculated into 1 ml of xylan hydrolysate (60°C for 65 h; supernatant after removal of cell debris by centrifugation) and incubated for 48 h at 30°C without agitation. Samples were removed and analyzed by thin-layer chromatography (Fig. 3). Only xylose was metabolized by the recombinant *E. coli* strain, despite the presence of active *B. fibrisolvens* xylosidase. Xylose, xylobiose, and xylotriose were all completely consumed by the ethanologenic *K. oxytoca* strain. On the basis of these results, derivatives of *K. oxytoca* were selected as superior for further studies of xylan conversion to ethanol.

Fermentation of xylose and xylan by derivatives of *K. oxytoca* M5A1. Fermentation of xylan was carried out as a two-stage process in which cells harvested from a previous fermentation were resuspended in Luria broth containing 4% xylan for saccharification (60°C for 65 h). Saccharified xylan was inoculated and fermented at 30°C (pH 6.0). Parallel experiments were conducted in which the cell debris had been removed by centrifugation before inoculation. Fermentations were also conducted with 4.47% xylose (equivalent to the xylose content of 4% xylan) for comparison (Fig. 4; Table 3).

As previously reported, M5A1(pLOI555) produced ethanol very efficiently (19). Xylose fermentation was essentially completed after 24 h, with 93% of the maximal theoretical yield. Both growth and ethanol production from xylose were reduced by the simultaneous presence of a second plasmid, either pLOI2001 or pLOI2003. Required fermentation times with pLOI2001 increased to approximately 36 h, with 91% of the theoretical yield. This decrease in ethanol productivity from monomeric sugar reflects the additional burden imposed by the recombinant enzymes.

Xylan hydrolysis (60°C for 65 h) was carried out with M5A1(pLOI555) containing either the hybrid xylanase gene (pLOI2001) or the xylosidase and hybrid xylanase gene (pLOI2003). Thin-layer profiles of these hydrolysates appeared identical to those of KO11(pLOI2003) shown in Fig. 3. No difference was found in the extent of xylan hydrolysis between the two M5A1 derivatives.

Growth and ethanol production in these hydrolysates were measured after inoculation with the respective organism used in saccharification (Fig. 4). Growth in clarified hydrolysate was only half that observed with equivalent levels of xylose monomer. Fermentations were essentially complete after 24 h and appeared slightly faster in clarified hydrolysates. Ethanol yields from xylan were approximately one-third of the maximal theoretical values, 7.7 to 7.9 g/liter.

Xylooligosaccharides in fermentation broth were monitored by thin-layer chromatography. The profiles at the end of fermentation were identical to that shown in Fig. 3B (48 h). Xylose, xylobiose, and xylotriose were completely metabolized, with xylotetrose and longer oligomers remaining.

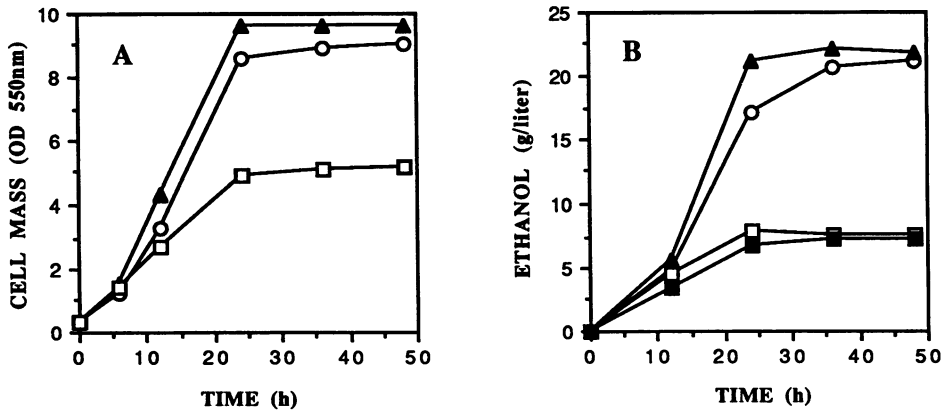

FIG. 4. Conversion of xylose and xylan hydrolysate to ethanol by recombinant *K. oxytoca* M5A1. Xylan was hydrolyzed for 65 h at 60°C with cells from a previous fermentation as the source of enzymes. Growth (A) and ethanol production (B) are indicated in the figure. Symbols: ▲, fermentation of 4.47% xylose by M5A1(pLOI555); ○, fermentation of 4.47% xylose by M5A1(pLOI555)(pLOI2001); ■, fermentation of 4% xylan hydrolysate containing cell debris from saccharification by M5A1(pLOI555)(pLOI2001); □, fermentation of 4% xylan hydrolysate after centrifugation to remove cell debris by M5A1(pLOI555)(pLOI2001).

DISCUSSION

Highly engineered strains of *K. oxytoca* M5A1 can be used both as a source of enzyme for polymer hydrolysis and for ethanol production from xylan. The presence of a native, intracellular xylosidase and the ability to transport and metabolize xylooligosaccharides have not been previously reported for this organism. *E. coli* isolates from nature which utilize a phosphotransferase system for cellobiose and an intracellular phosphocellobiase have been previously described (12, 13). Analogous systems for xylobiose and xylotriose metabolism may function in *K. oxytoca* M5A1. The presence of transport systems and enzymes for the metabolism of xylose monomers, dimers, and trimers in *K. oxytoca* M5A1 offers a considerable advantage over *E. coli*-based or *Saccharomyces cerevisiae*-based systems in the further development of biomass into ethanol processes.

Many of the previous problems in the genetic engineering of single organisms for polymer degradation and ethanol production are eliminated by using a two-stage process, with thermostable enzymes being produced as intracellular products during fermentation. The synthesis of proteins destined to be secreted at high levels often adversely affects normal cellular processes. This problem should be minimized by using N-truncated enzymes in which the secretory signal has been deleted for intracellular expression. Since hydrolysis time is a limiting factor in conversion, high levels of hydrolytic enzymes would be most advantageous at the very beginning of the conversion process to maximize the early release of sugars for rapid fermentation. In secretory processes, enzyme levels are initially low, with enzymes accumulating to reach maximal levels near the end of fermentation. However, by using the cells from previous fermentations as a source of enzymes, one achieves nearly maximal levels initially. Although the use of thermostable enzymes for hydrolysis offers additional process advantages for minimizing contamination and may provide higher rates of hydrolysis, a primary advantage is the simplicity of the release of intracellular enzymes from harvested cells.

Ethanol tolerance is frequently a problem in native organisms which degrade cellulose and xylose. Such organisms

TABLE 3. Ethanol production from xylose and xylan by recombinant strains of *K. oxytoca* M5A1(pLOI555)[a]

Substrate and second plasmid	Base (mM/g of sugar)[b]	Time (h)[c]	Ethanol yield		Theoretical yield (%)[d]	VP (g/liter/h)[e]	Cell yield (g/g of sugar)
			In g/liter	In g/g of substrate			
Xylose (4.47%)							
None (pLOI555 alone)	1.2	36	22.6	0.51	99	1.29	0.07
With pLOI2001	1.5	48	21.7	0.49	95	1.02	0.08
With pLOI2003	1.3	36	20.6	0.47	91	0.83	0.07
Xylan (4.0%)							
With pLOI2001[f]	0.7	24	7.7	0.19	34	0.37	0.04
With pLOI2003[f]	1.3	36	7.7	0.19	34	0.31	0.04
With pLOI2001[g]	1.3	60	7.8	0.20	34	0.28	ND
With pLOI2003[g]	1.1	60	7.9	0.20	35	0.30	ND

[a] Calculations are based on total amounts of substrate added. ND, not determined.
[b] Amount of base consumed to maintain a pH of 6.0 during fermentation.
[c] Time required to reach maximal ethanol concentration.
[d] Theoretical yields on a weight basis are 0.51 for xylose and 0.58 for xylan.
[e] VP, maximum volumetric productivity during batch fermentation.
[f] Hydrolysate after debris from saccharification was removed by centrifugation.
[g] Hydrolysate containing debris from saccharification.

typically produce fewer than 20 g of ethanol per liter (21). Although we have not achieved high-level ethanol production from xylan thus far, M5A1(pLOI555) is capable of producing at least 48 g of ethanol per liter from 100 g of xylose per liter (approximately 95% of the maximal theoretical yield).

The overall yield of ethanol from birchwood xylan was lower than anticipated, and this is primarily due to incomplete xylan hydrolysis. Undegraded xylooligosaccharides remained after the 65-h saccharification period. Although the level of xylanase produced by our constructs was below that produced by the optimal xylanase fusion constructs reported by Grepinet et al. (11), the level of enzymes per se did not appear limiting. Xylan (40 g/liter; approximately 0.3 mol of anhydroxylose) should be completely degraded into xylobiose equivalents after 24 h by hydrolysis a rate of 105 μmol/min. The cell yields from fermentation were approximately 4.5 g of cell protein per liter, with a xylanase activity of 144 μmol/min/g of cell protein providing a total activity of 648 μmol/min. However, xylooligosacchrides remained even after 65 h, despite the persistence of active xylanase. These levels of xylooligosaccharides were unchanged by the addition of fresh enzyme and further incubation for 24 h. Competitive inhibition of hydrolysis by xylooligosaccharides may contribute to incomplete digestion, and this was tested, in part, by hydrolysis at 45°C, a temperature at which xylanase remained active, as did the native enzymes in M5A1 which metabolized xylose, xylobiose, and xylotriose. The oligosaccharide profile under these conditions was identical to that observed after fermentation by M5A1 strains, with equivalent levels of the xylotetrose and longer oligosaccharides remaining undigested (data not shown). These oligosaccharides appear to limit digestion products, although the nature of the substitutents limiting digestion remains unknown. According to the manufacturer, birchwood xylan is 99% xylose. We speculate that this product may contain oxidized residues generated during storage or substitutions which have survived base extraction and purification (6).

Fermentative ethanol production in the United States is approximately 1 billion gallons (3.8 billion liters) per year (16). Microorganisms such as genetically engineered *K. oxytoca*, *E. coli*, *S. cerevisiae*, or *Z. mobilis* strains could be used to produce a variety of enzymes as coproducts with ethanol. At a minimal cell yield of 2 g of protein per liter of beer, the diversion of 5% of cell protein would produce 380,000 kg of enzymes as coproducts. These enzymes need not be limited to depolymerization of substrates for fermentation but could also include other enzymes for large markets such as the detergent industry, the food industry, and the wood-pulp industry and the development of new, biocatalyst-based industries for novel chemicals. Providing that large markets develop, the value of such enzymes could well exceed the current value of ethanol itself.

ACKNOWLEDGMENTS

We thank J.-P. Aubert and P. Beguin for generously sharing their plasmids containing the xylanase gene from *C. thermocellum*.

These studies were supported by grant FG05-86ER3574 from the Office of Basic Energy Science, U.S. Department of Energy; by grant 88-37233-3987 from the Alcohol Fuels Program, U.S. Department of Agriculture; and by a grant from the University of Florida Research Foundation provided by BioEnergy International (Gainesville, Fla.).

REFERENCES

1. **Beall, D. S., K. Ohta, and L. O. Ingram.** 1991. Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*. Biotechnol. Bioeng. **38:**296–303.
2. **Beguin, P.** 1990. Molecular biology of cellulose degradation. Annu. Rev. Microbiol. **44:**219–248.
3. **Bergmeyer, H. U., M. Grassl, and H.-E. Walter.** 1983. Reagents for enzymatic analysis, p. 151–152. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 3rd ed., vol. II. Verlag Chemie, Weinheim, Germany.
4. **Bounias, M.** 1980. N-(1-naphtyl)ethylenediamine dihydrochloride as a new reagent for nanomole quantification of sugars on thin layer plates by a mathematical calibration process. Anal. Biochem. **106:**291–295.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Chesson, A., A. H. Gordon, and J. A. Lomax.** 1983. Substituent groups linked by alkali-labile bonds to arabinose and xylose residues of legume, grass and cereal straw walls and their fate during digestion by rumen microorganisms. J. Sci. Food Agric. **34:**1330–1340.
7. **Dombek, K. M., and L. O. Ingram.** 1985. Determination of the intracellular concentration of ethanol in *Saccharomyces cerevisiae* during fermentation. Appl. Environ. Microbiol. **51:**197–200.
8. **Domer, L. W.** 1988. High performance thin-layer chromatography of starch, cellulose, xylan, and chitin hydrolyzates. Methods Enzymol. **160:**176–180.
9. **Eriksson, K.-E. L., R. A. Blanchette, and P. Ander.** 1990. Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, New York.
10. **Grepinet, O., M.-C. Chebrou, and P. Beguin.** 1988. Purification of *Clostridium thermocellum* xylanase Z expressed in *Escherichia coli* and identification of the corresponding product in the culture medium of *C. thermocellum*. J. Bacteriol. **170:**4576–4581.
11. **Grepinet, O., M.-C. Chebrou, and P. Beguin.** 1988. Nucleotide sequence and deletion analysis of the xylanase gene (*xynZ*) of *Clostridium thermocellum*. J. Bacteriol. **170:**4582–4588.
12. **Hall, B. G., and W. Faunce III.** 1987. Functional gene for cellobiose utilization in natural isolates of *Escherichia coli*. J. Bacteriol. **169:**2713–2717.
13. **Kricker, M., and B. G. Hall.** 1987. Biochemical genetics of the cryptic gene system for cellobiose utilization in *Escherichia coli K12*. Genetics **115:**419–429.
14. **Lawford, H. G., and J. D. Rousseau.** 1991. Fuel ethanol from hardwood hemicellulose hydrolysate by genetically engineering *Escherichia coli* B carrying genes from *Zymomonas mobilis*. Biotechnol. Lett. **13:**191–196.
15. **Luria, S. E., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28:**491–511.
16. **Lynd, L. R., J. H. Cushman, R. J. Nichols, and C. E. Wyman.** 1991. Fuel ethanol from cellulosic biomass. Science **251:**1318–1323.
17. **Millet, J., D. Petre, P. Beguin, O. Raynaud, and J.-P. Aubert.** 1985. Cloning of ten distinct DNA fragments of *Clostridium thermocellum* coding for cellulases. FEMS Microbiol. Lett. **29:**145–149.
18. **Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram.** 1991. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. **57:**893–900.
19. **Ohta, K., D. S. Beall, J. P. Mejia, K. T. Shanmugam, and L. O. Ingram.** 1991. Metabolic engineering of *Klebsiella oxytoca* strain M5A1 for ethanol production from xylose and glucose. Appl. Environ. Microbiol. **57:**2810–2815.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Tailliez, P., H. Girard, R. Longin, P. Beguin, and J. Millet.** 1989. Cellulose fermentation by an asporogenous mutant and an ethanol-tolerant mutant of *Clostridium thermocellum*. Appl. Environ. Microbiol. **55:**203–206.
22. **Utt, E. A., C. K. Eddy, K. F. Keshav, and L. O. Ingram.** 1991. Sequencing and expression of the *Butyrivibrio fibrisolvens xylB* gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl. Environ. Microbiol. **57:**1227–1234.