Ethanol Production from Sugar Beet Pulp Components by Genetically Engineered Bacteria Joy B. Doran* , Jennifer Cripe, and Misty Sutton. Central Michigan University, Dept. of Biology, Mt. Pleasant, MI 48859.

Abstract. Studies were conducted using glucose, xylose, arabinose, and galacturonic acid to evaluate the feasibility of sugar beet pulp as a substrate for fermentation processes using genetically engineered ethanol producing bacteria. Using beet pulp to produce ethanol may help provide an added value for a beet processing co-product. Ethanol may be sold as an alternative fuel, or used by the processing plant itself to reduce energy costs. Galacturonic acid and arabinose are not fermentable to ethanol by conventional yeast traditionally used in corn based fermentation. Recombinant ethanol producing bacteria have been developed in the laboratory of Dr. L. O. Ingram (University of Florida) by inserting a functional ethanol pathway into enteric bacteria, diverting pyruvate metabolism during fermentation from a mixture of acids to ethanol. *Erwinia chrysanthemi* EC16 containing the alcohol genes on plasmid pLOI555 secreted enzymes which appeared to aid in the conversion of sugar beet pulp to ethanol.

Introduction. Concerns about dependence on foreign crude oil and increasing environmental awareness have rekindled an interest in renewable energy sources. Using ethanol or ethanol-blended fuels will help reduce this dependence on foreign crude oil, increase market opportunity for agricultural crops and provide benefits to the environment in reduced carbon dioxide and carbon monoxide emissions. "Oxygenated fuels", such as ethanol blends , are mandated in certain areas of the United States to reduce hazardous emissions. Increasing usage of oxygenates as fuel additives provides an impetus for a significant expansion of fuel ethanol production (Sheehan, J. J., 1994). Cellulosic materials potentially available from energy crops, agricultural and industrial wastes, and conventional forestry could provide ethanol equivalent to current liquid transportation fuel needs in the United States (Lynd et al., 1991). Fuel ethanol manufacturing is the largest industrial market for corn, with about 95% of all ethanol manufacturing being corn based (Bothast et al., 1994). Plant biomass is a complex mixture of cellulose (glucose), hemicellulose (predominantly xylose and other pentoses), starch, pectin and uronic acids along with varying amounts of protein and smaller amounts of other components. It is not known whether there exists in nature an organism capable of degrading all of these plant components and metabolizing them to produce ethanol, however, none has been described to date. The bacterium *Escherichia coli* has the native ability to metabolize all sugars which are components of cellulose, hemicellulose, starch, and pectin, producing a mixture of acids and a small amount of ethanol. *Escherichia coli* has been genetically engineered to produce ethanol from pentose and hexose sugars by inserting genes from *Zymomonas mobilis* encoding alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) (Ingram et al., 1987). A comparison of yeasts and bacteria using dilute acid hydrolysates of corn cobs concluded that the recombinant bacterium *E. coli* strain KO11 was superior to other pentose-fermenting microorganisms in ethanol productivity, ethanol yield, and resistance to inhibitors generated during acid hydrolysis (Hahn-Hagerdal et al., 1994). Sugar beet pulp contains high concentrations of carbohydrates (Table 1) that may be converted to ethanol by genetically engineered bacteria. Conventional yeasts used in corn

based fermentations are unable to metabolize arabinose and galacturonic acid (pectin) to ethanol (Grohmann et al., 1994).

Table1 Composition of sugar beet pulp expressed as% dry weight of total solids.

Component	Bertin et al., 1988	Michel et al., 1988	Wen et al., 1988
Cellulose	20.0	22-24	24.0
Hemicellulose	25.0	26-32	36.4
Pectin or Uronic Acids	25.0	21-23	19.6
Lignin	1.8	1-2	5.6
Protein	8.0	7-8	nr
Ash	8.4	7-12	12.2
Other	7.2	1-16	2.2
Total	95.4	84-96	97.8

A second bacterium, *Erwinia chrysanthemi* EC 16 was genetically engineered to produce ethanol and CO_2 as primary fermentation products from cellobiose, glucose and xylose (Beall and Ingram, 1993). This organism has the native ability to secrete enzymes to aid in the solubilization of plant biomass, including pectin methylesterases and pectate lyases for degradation of pectin. Because of the high pectin content in beet pulp, this study was conducted to determine the feasibility of using the recombinant *Erwinia chrysanthemi* EC16 pLOI555 as the biocatalyst in an ethanol production process with beet pulp as the substrate.

Materials and Methods. Bacterial strains. *Escherichia coli* strain KO11 (Ingram et al., 1987) and *Erwinia chrysanthemi* EC 16 pLOI555 (Beall and Ingram, 1993) have been described previously and were generously provided by Dr. Lonnie Ingram (Univ. of Fl). Strains were maintained on Luria Broth (containing per liter: 20 g glucose, 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 40 mg of chloramphenicol) or medium solidified with agar. Preparation of inocula for fermentations was essentially as described (Doran et al., 1994). Fermentations using pure carbohydrates were conducted in modified 500-ml Fleakers (fermentation vessels from Fisher Scientific). Carbohydrates were filter sterilized and added to autoclaved Luria broth (121^{o}C for 15 min.). Sugar beet pulp was sterilized by autoclaving, as well.

Analyses. Samples were removed for measurement of ethanol by gas-liquid chromatography (Beall et al., 1991). Ethanol yields ere corrected for dilution by base added to maintain fermentation pH and computed on the basis of carbohydrate initially present. No adjustment was made to correct for unused carbohydrate nor for the production of cell mass. All results were the averages of 2 or more experiments.

Results and Discussion. Fermentations conducted with *Erwinia chrysanthemi* EC 16 pLOI555 using glucose, xylose, arabinose, cellobiose, and galacturonic acid are summarized in Table 2. This recombinant is able to utilize all carbohydrates tested to produce ethanol. Grohmann and coworkers (1994) evaluated the recombinant *E. coli* strain KO11 in fermentations with galacturonic acid and orange peel hydrolysates and

found equimolar concentrations of ethanol and acetate produced. Ethanol yields for *Erwinia chrysanthemi* EC16 pLOI555 using the same concentration of galacturonic acid are very close to those of *E.coli* strain KO11. Ethanol yield was calculated for *E. coli* using g ethanol produced/ g sugar consumed (0.19), while the yield for *E. chrysanthemi* EC16 pLOI555 was calculated using g ethanol produced/g carbohydrate initially present (0.16). It is not clear at this time if the recombinant *Erwinia* is also producing equimolar amounts of acetate.

Table 2. Ethanol production from pure carbohydrates using Erwinia chrysanthemi EC16 pLOI555 at pH 6.0 and 30°C.

Carbohydrate (g/liter)	Maximum Ethanol (g/liter)	Ethanol Yield (g/g carbohydrate initially present)	Reference
Glucose (80)	28.6	0.37	This Study
Glucose (100)	45.7	0.48	Beall and Ingram, 1993
Cellobiose (100)	49.4	0.52	Beall and Ingram, 1993
Xylose (80)	40.7	0.55	Beall and Ingram, 1993
Xylose (100)	39.0	0.41	Beall and Ingram, 1993
Arabinose (80)	26.7	0.34	This Study
Arabinose (100)	30.9	0.32	This Study
Galacturonic Acid (20)	3.2	0.16	This Study

Conversion of pure carbohydrates to ethanol using the recombinant *Erwinia* strain indicated efficient utilization of the sugars present in sugar beet pulp. Since this strain produces enzymes to degrade plant tissue, fermentations were conducted using *E. chrysanthemi* EC16 pLOI 555 and the non-biomass degrading *E. coli* strain KO11 to access the contribution of *Erwinia* enzymes. Using 80 g dry weight /liter of pelletized sugar beet pulp (ground to reduce particle size) there was a difference in the amount of ethanol produced for *E. coli* KO11 (2.0 g /liter) versus that produced by the recombinant *Erwinia* (7.1 g/liter). Studies are underway to determine the minimum amount of fungal enzymes that will be required for a biomass conversion process to produce fuel ethanol using sugar beet pulp as the substrate and recombinant bacteria as the biocatalysts.

This study was funded in part by Research Excellence Funds from the State of Michigan and Central Michigan University. The authors wish to thank student researchers Terri Kuehnlein, Ernie Riley, Brian Foster, Rich West, and Lisa VanOmmeren for assistance with the study.

References Cited

1. Beall, D. S. and L. O. Ingram. (1993) Genetic engineering of soft-rot bacteria for ethanol production from lignocellulose. *J.Indus.Microbiol.* 11: 151-155.

2. Bothast, R. J., B. C. Saha, A. V. Flosenzier, and L. O. Ingram. (1994) Fermentation of L-arabinose, D-xylose, and D-glucose by ethanologenic recombinant *Klebsiella* strain P2. *Biotechnol. Lett.* 16: 401-406.
3. Doran, J. B., H. C. Aldrich, and L. O. Ingram. (1994) Saccharification and fermentation of sugar cane bagasse by *Klebsiella oxytoca* P2 containing chromosomally integrated genes encoding the *Zymomonas mobilis* ethanol pathway. *Biotechnol.Bioeng.* 44: 240-247.
4. Grohmann, K., E.A. Baldwin, B. S. Buslig, and L. O. Ingram. Fermentation of galacturonic acid and other sugars in orange peel hydrolysates by the ethanologenic strain of *Escherichia coli*. *Biotechnol. Lett.* 16: 281-286.
5. Hahn-Hagerdal, B., H. Jeppsson, L. Olsson, and A. Mohaghegi. (1994) An interlaboratory comparison of the performance of ethanol producing microorganisms in a xylose rich acid hydrolysate. *Appl. Microbiol.*41: 62-72.
6. Ingram, L. O., T. Conway, D.P. Clark, G. W. Sewell, and J. F. Preston. (1987) Genetic engineering of ethanol production in *E. coli* . *Appl. Environ.Microbiol.* 53: 2420-2425.
7. Lynd, L. R., J. H. Cushman, R. J. Nichols, C. E. Wyman. (1991) Fuel ethanol from cellulosic biomass. *Science*: 251: 1318-1323.
8. Sheehan, J. J., (1994) Bioconversion for production of renewable transportation fuels in production, ACS Symposium Series 566 (Himmel, M. E., J. O Baker and R. P. Overend, eds) pp 1-53, Am .Chem. Soc. , Washington, DC.