Fermentation of Sugarbeet Pulp for Ethanol Production Using Bioengineered *Klebsiella oxytoca* Strain P2

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ABSTRACT

The purpose of this study was to evaluate ethanol production from galacturonic acid and from sugarbeet pulp by bioengineered ethanologenic *Klebsiella oxytoca* strain P2. *K. oxytoca* P2 fermentation of galacturonic acid (20 g L^{-1}) produced a higher level of acetic acid (6.8 g L^{-1}) than of either ethanol (2.2 g L^{-1}) or succinic acid (1.6 g L^{-1}). Sugarbeet pulp fermentations were conducted with and without fungal enzyme supplementation. Without fungal enzyme supplementation, *K. oxytoca* P2 produced 5.4 g ethanol L^{-1} from pelleted pulp and 7.0 g ethanol L^{-1} from pressed pulp. Inclusion of fungal enzymes (60 mg cellulase and 30 mg pectinase/ g dw sbp) increased ethanol production to 15.5 g ethanol L^{-1} using pelleted pulp, while fermentation of pressed pulp produced 18.3 g ethanol L^{-1}. A preincubation step at 42°C and pH 5.0 before fermentation of the pressed beet pulp decreased viscosity, increased mixing, and increased ethanol production from 18.3 g L^{-1} at 120 hours of fermentation to 21 g ethanol L^{-1} by 96 hours of fermentation. At a conversion rate of 0.2 g ethanol/ g dry weight sugarbeet pulp, over 97 million gallons of ethanol could be produced in the United States from 1 x 10^6 tons (dry weight) of sugarbeet pulp using this process and *Klebsiella oxytoca* P2 as the biocatalyst.

Additional key words: biomass conversion, recombinant, alternative fuel

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Development of large scale fuel ethanol production from renewable resources is an attractive alternative to petroleum based fuel production, due to environmental concerns, limited fossil fuels, and an increased dependence on imported crude oil. Currently, agricultural residues, municipal waste paper, waste from the pulp and paper industry and other lignocellulosic residues could be used to produce approximately 100 billion gallons of fuel grade ethanol in the United States (Himmel et al. 1997). Processing of pectin-rich crops such as sugarbeet generates residues abundant in carbohydrates which can be fermented to ethanol (Grohmann and Bothast 1994). Over 400 million tons of sugarbeet are produced annually worldwide with 20 to 30 million tons produced in the United States alone (Clarke and Edye 1996). On a dry weight (d wt) basis, over 1.6×10^6 tons of sugarbeet pulp remain after extraction of sucrose. During sucrose extraction, a pressed form of sugarbeet pulp (75% moisture) is generated and is dried and pelletized (10% moisture) for sale as cattle feed (Coons 1971). Sugarbeet pulp is primarily composed of polymers of cellulose (20-24%), hemicellulose (25-36%), pectin (19-25%), and lignin (1-5.6%) on a dry weight basis (Bertin et al. 1988; Michel et al. 1988; Wen et al. 1988). High carbohydrate and low lignin content make sugarbeet pulp an attractive substrate for ethanol production, however, the polymers must be broken down into simpler carbohydrates. After reducing the polymers to monomeric carbohydrates, beet pulp contains primarily arabinose, glucose, and galacturonic acid, along with smaller amounts of other sugars (Micard et al. 1996; Table 1).

Table 1: Composition of Carbohydrates in Sugarbeet Pulp.

% Dry Weight of Total Solids	Polymeric Component	Major Monomeric Carbohydrate Yielded from Polymer	% Dry Weight of Total Solids
20-24	cellulose	glucose	21-24
25-36	hemicellulose	arabinose	20-25
		galactose	4-6
		xylose	1-3
		mannose	1-2
19-25	pectin or uronic acids	galacturonic acid	20-22
		rhamnose	2-3

(adapted from Bertin et al. 1988; Michel et al. 1988; Wen et al. 1988; Micard et al. 1996)

Klebsiella oxytoca was bioengineered by the addition of two genes involved in ethanol production, pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*), that were originally isolated from another bacterium, *Zymomonas mobilis* (Ohta et al. 1991). Insertion of these genes into the chromosome of *K. oxytoca* effectively diverted normal production of neutral end-products and mixed acids to ethanol as the major fermentation end product (Wood and Ingram 1992; Doran and Ingram 1993; Bothast et al. 1994; Doran et al. 1994; Ingram and Doran 1995). The bioengineered *K. oxytoca*, now designated strain P2, can produce ethanol from xylan, xylose, arabinose, and glucose (Ohta et al. 1991; Burchhardt and Ingram 1992; Bothast et al. 1994). This organism exhibits the native ability to transport and metabolize cellobiose, eliminating the need for exogenous cellobiase to degrade cellobiose into two glucose molecules (Wood and Ingram 1992). *K. oxytoca* P2 does not posses the ability to degrade the polymers found in plant biomass to monomeric form. However, previous studies have shown that *Klebsiella oxytoca* P2 can be used as a biocatalyst for the simultaneous saccharification and fermentation of cellulosic substrates such as sugar cane bagasse (Doran et al. 1994) and mixed office waste paper (Brooks and Ingram 1995). *K. oxytoca* P2 provides cellobiase activity, however, fermentations must be supplemented with commercially available enzymes for the plant polymers to be broken into smaller carbohydrates. During simultaneous saccharification and fermentation, the simple sugars released via enzymatic activity are consumed by the fermenting microorganism, thus relieving end-product inhibition of the enzymes.

The purpose of this study was to evaluate ethanol production from galacturonic acid and from sugarbeet pulp in pressed and pelletized forms by bioengineered ethanologenic *K. oxytoca* strain P2. Fermentations were conducted with and without fungal enzyme supplementation. A preincubation step with substrate and enzymes together before inoculation with *K.oxytoca* P2 was compared with fermentations where all components were added in one step (simultaneous saccharification and fermentation).

MATERIALS AND METHODS

Klebsiella oxytoca Strain P2.

K. oxytoca strain P2 was provided by Dr. Lonnie Ingram (University of Florida) and has been previously described (Ohta et al. 1991; Wood and Ingram 1992). The bacterial strain was maintained on Luria Bertani (LB) medium containing per liter: 20 g glucose, 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 40 mg chloramphenicol solidified with 15 g agar. *K. oxytoca* P2 was prepared for fermentation experiments as previously described (Beall et al. 1991; Doran et al. 1994). Essentially, plates were

incubated at 30 C for 24 hours. A single isolated colony was used to inoculate a 500 ml flask containing 400 ml LB medium supplemented with 5 % (w/v) glucose. Inocula were incubated for 18 to 24 hours at 30 C without agitation. Cells were then harvested by centrifugation and resuspended in fermentation medium at an initial cell density of 330 mg dry weight per liter.

Substrate preparation.

An aqueous solution of D-galacturonic acid was filter sterilized and added to autoclaved LB medium for a final concentration of 20 g galacturonic acid L^{-1}. Fermentations were also conducted in LB medium with no carbohydrate addition.

Sugarbeet pulp was provided by Monitor Sugar Company (Bay City, Michigan). Sugarbeet pulp pellets were ground in a coffee mill in order to reduce particle size. The moisture content was determined by drying the sugarbeet pulp in tared aluminum weighing dishes in a gravity convection oven for 24 h at 105 C. After 24 h, the dishes were cooled in a dessicator, and weighed. Moisture was reported as the gravimetric moisture content. All moisture determinations were performed in triplicate for each experiment. Pelleted and pressed sugarbeet pulp were sterilized by autoclaving at 121 C for 20 minutes as a slurry in approximately 100 ml water.

Fermentation Experiments.

Fermentations were conducted in modified 500 mL fermenters with a total working volume of 350 mL essentially as previously described (Beall et al. 1991). Distilled water and double strength complex nutrients (LB) were sterilized by autoclaving at 121 C for 20 minutes. Initial pH was adjusted with 1 N HCl while 2M KOH was used to maintain pH during the fermentation process. Fermentations of (20 g L^{-1}) galacturonic acid were conducted at 30 C and pH 6.0 for 77 hours. Fermentations with sugarbeet pulp (106 g d wt L^{-1}) were maintained at 35 C and pH 5.5 for 168 hours. A pH of 6.0 at 30 C was shown to be optimal for ethanol production from monomeric carbohydrates using the bioengineered *Escherichia coli* strain KO11 and *Klebsiella oxytoca* strain P2 (Beall et al., 1991; Bothast et al., 1994). Fungal enzymes used in the study have an acidic pH optimum and work best at 40 C. Previous studies with crystalline cellulose and sugar cane bagasse (Doran and Ingram 1993; Doran et al., 1994) found that a pH of 5.5 at 35 C was the best condition for optimizing ethanol production and fungal enzymatic activity in the same fermentation vessel.

Fungal Enzymes.

Commercially available fungal enzyme preparations, Pectinex SP (pectinase) and Celluclast 1.5-L (cellulase) were provided by Novo Nordisk (Franklinton, NC). Enzyme preparations were sterilized by filtration. Enzyme loads for Pectinex and Celluclast were 30 mg and 60 mg per g dry weight of sugarbeet pulp, respectively.

Analysis by gas chromatography (GC).

Samples (2 ml) were removed at appropriate intervals and measured for ethanol concentration (g L^{-1}) using a Perkin Elmer 8500 gas chromatograph equipped with a DB624 column (J & W Scientific) and a flame ionization detector. An internal standard of isopropanol and external ethanol standards were used in analysis. Oven temperature was 55 C and detector temperature was 250 C . Ethanol yields were corrected for dilution by addition of acid and base during fermentations and determined on the basis of total substrate (dry weight) initially present. All results represent averages from three or more fermentations.

Analysis by high performance liquid chromatography (HPLC).

Samples containing maximum ethanol concentrations from fermentation of galacturonic acid (ascertained by GC) were analyzed using a Waters high performance liquid chromatograph with ultraviolet detection (210 nm). The liquid chromatograph was equipped with an Aminex HPX-87H 300 mm x 7.8 mm column operated at a temperature of 22 C. External organic acid standards included oxalic acid, citric acid, malic acid, succinic acid, formic acid, and acetic acid. Quantitation was made by peak area measurement with external standards.

Electron Microscopy.

Fermentation samples were fixed for six hours at room temperature in 0.1 M phosphate buffer containing 5% glutaraldehyde (pH 7.2). Samples were rinsed in phosphate buffer and washed with distilled water. The samples were subjected to ethanol dehydration in a graded series (30, 70, 95, 100, 100, 100%). Samples were dried using liquid Peldri II (1 part 100% ethanol : 1 part Peldri II) for 1 hour followed by 100% Peldri II for an additional hour. The samples were allowed to solidify and Peldri II was sublimed in a low vacuum chamber overnight. Dry specimens were mounted on stubs, sputter coated with 30 nm of gold and photographed using a JEOL-840A scanning electron microscope.

RESULTS

Fermentation of galacturonic acid.

Klebsiella oxytoca P2 fermentation of galacturonic acid (20 g L^{-1}) produced a higher level of acetic acid (6.8 g L^{-1}) than of either ethanol (2.2 g L^{-1}) or succinic acid (1.6 g L^{-1}), with no lactic acid detected. Approximately 1 g ethanol L^{-1} was obtained from fermentation of LB medium with no additional carbohydrates, indicating additional ethanol production from galacturonic acid (t-test, $p<0.001$).

Ethanol production from sugarbeet pulp.

Ethanol production from sugarbeet pulp (106 g dw L^{-1}) with and without a fungal enzyme mixture is summarized in Table 2. Without fungal enzyme supplementation, *K. oxytoca* P2 produced a maximum of 5.4 g ethanol L^{-1} from pelleted pulp and 7.0 g ethanol L^{-1} from pressed pulp. Inclusion of fungal enzymes (60 mg cellulase/ g dw sbp, 30 mg pectinase/ g dw sbp) increased ethanol production from both forms of pulp (ANOVA, $p<0.001$). Fermentation of pelleted pulp with added enzymes by *K. oxytoca* P2 produced a maximum of 15.5 g of ethanol L^{-1} while fermentation of pressed pulp with the same enzyme loading produced 18.33 g ethanol L^{-1} (Table 2). Since the largest ethanol levels were obtained using pressed pulp, further fermentation experiments were conducted with this substrate.

Effects of preincubation step.

Optimal conditions for maximum enzymatic activity of Pectinex SP and Cellulclast 1.5 were determined to be 45-50 C, pH 4.0-5.0 by Novo Nordisk, Franklinton, NC. These conditions are more extreme than those favoring the growth and activity of *K. oxytoca* P2 (Doran and Ingram 1993; Ingram et al. 1997). Therefore, we examined effects of partial enzymatic saccharification prior to fermentation. A preincubation step was performed at 42 C and pH 5.0 before fermentation of the pressed beet pulp was initiated with *K. oxytoca* strain P2. After 24 hours, the temperature and pH were adjusted to 35 C and 5.5 and *K. oxytoca* strain P2 was added to the fermentation vessel. This preincubation step decreased viscosity and increased mixing (observed) and increased ethanol production from 18.3 g L^{-1} at 120 hours of fermentation to 21 g ethanol L^{-1} by 96 hours of fermentation (Figure 1 and Table 2).

Fermentations of beet pulp conducted in water.

The use of LB medium in large scale fermentations is economically unfeasible. Therefore, fermentations were conducted in water to determine

Table 2: Ethanol production by *Klebsiella oxytoca* strain P2 from D-galacturonic acid (20 g L^{-1}, 30 C, pH 6.0), or pelleted or pressed sugar beet pulp (106 g dwt L^{-1}, 35 C, pH 5.5), with and without fungal enzymes.

Substrate	Replicates	Enzymes[¶]	Average KOH added (ml L^{-1})	Maximum Ethanol (g L^{-1}) ± SD[††]	Time to Max ethanol (hours)	Ethanol Yield[#] (g ethanol/ g substrate)
Gal[†]	6	-	18.5	2.2 ± 0.2	24	0.12
Pellet[‡]	3	-	4.3	5.4 ± 0.2	48	0.05
Pellet[‡]	3	+	42.4	15.5 ± 1.0	48	0.15
Pressed[§]	3	-	3.7	7.6 ± 1.0	96	0.06
Pressed[§]	3	+	36.6	18.3 ± 1.0	96	0.18
Pressed[§] (enz. pre)	4	+	18.5	21.0 ± 0.5	96	0.20

[†] Gal=galacturonic acid (20 g L^{-1})
[‡] Pellet=Ground Pellets (10% moisture), (106 g dry weight L^{-1})
[§] Pressed=Pressed pulp, (75% moisture), (106 g dry weight L^{-1})
[¶] (-)=None; (+)=Celluclast (60 mg/g dw sbp), Pectinex (30 mg/g dw sbp)
[#] Corrected for dilution by base addition
[††] Standard Deviation

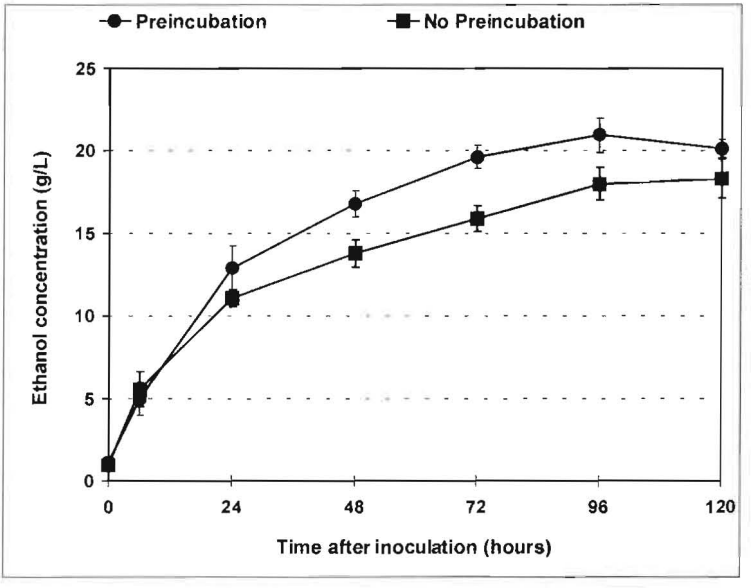

Figure 1. Effects of substrate preincubation with Pectinex SP (30 mg/g dw sbp) and Celluclast 1.5-L (60 mg/ g dw sbp) on ethanol production from sugarbeet pulp (106 g dw, L^{-1}) using *Klebsiella oxytoca* P2 as biocatalyst. Substrate preincubation with enzymes at 42°C, pH 5.0 for 24 hours before titration to pH 5.5 and inoculation.with *Klebsiella oxytoca* P2 (●), compared with the same enzyme load with no preincubation (■). Experiments were performed in triplicate and error bars denote standard deviations.

the amount of ethanol produced if supplemental LB medium was not added to provide supplemental nutrients. Fermentations were conducted using a preincubation step and ethanol production was compared to parallel experiments conducted in LB medium. Comparable yields of ethanol were obtained under both fermentation conditions at all sampling times, indicating that sugarbeet pulp contains sufficient nutrients to sustain fermenting bacteria (t-test, $p>0.05$) (Figure 2).

Ultrastructural Analysis.

Samples of pressed sugarbeet pulp were removed and analyzed during partial saccharification and fermentation stages. Following 24 hours of enzymatic treatment and the additional 72 hours of fermentation, the pulp

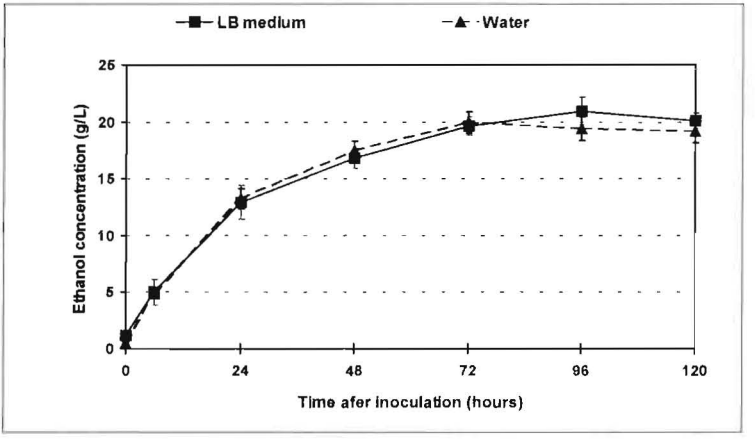

Figure 2. Ethanol production from fermentation of pressed sugarbeet pulp (75% moisture, 106 g dw sbp L^{-1}) conducted in LB medium (■) or sterile water only (▲), with preincubation of pulp and fungal hydrolytic enzymes (cellulase, 60 mg/ g dw sbp; pectinase, 30 mg/g dw sbp). Experiments were performed in triplicate and error bars denote standard deviations.

experienced a dramatic reduction in particle size compared to that observed at the start of the preincubation step (Fig. 3A, C, E). After 24 hours of enzymatic hydrolysis, much of the ragged surface of the pulp was removed as sugars were hydrolyzed (Figs. 3B, D). Seventy-two hours of fermentation by *K. oxytoca* P2 produced a relatively smooth pulp surface (Fig. 3F). This polishing effect is consistent with previous investigations of cellulose (Din et al. 1991; Doran et al. 1994).

DISCUSSION

Sugarbeet pulp is a potential substrate for ethanol production using genetically engineered ethanologenic *K. oxytoca* P2. Sugarbeet pulp is especially attractive for fuel production because it is an abundant domestic renewable resource. *K. oxytoca* P2 can produce ethanol from glucose and arabinose (Ohta et al. 1991; Burchhardt and Ingram 1992; Bothast et al. 1994), two of the predominant carbohydrates found in sugarbeet pulp. This study demonstrates that *K. oxytoca* P2 can also produce ethanol from galacturonic acid, another major constituent of sugarbeet pulp. However, *K. oxytoca* P2 produced lower levels of ethanol and higher levels of acetate and succinate

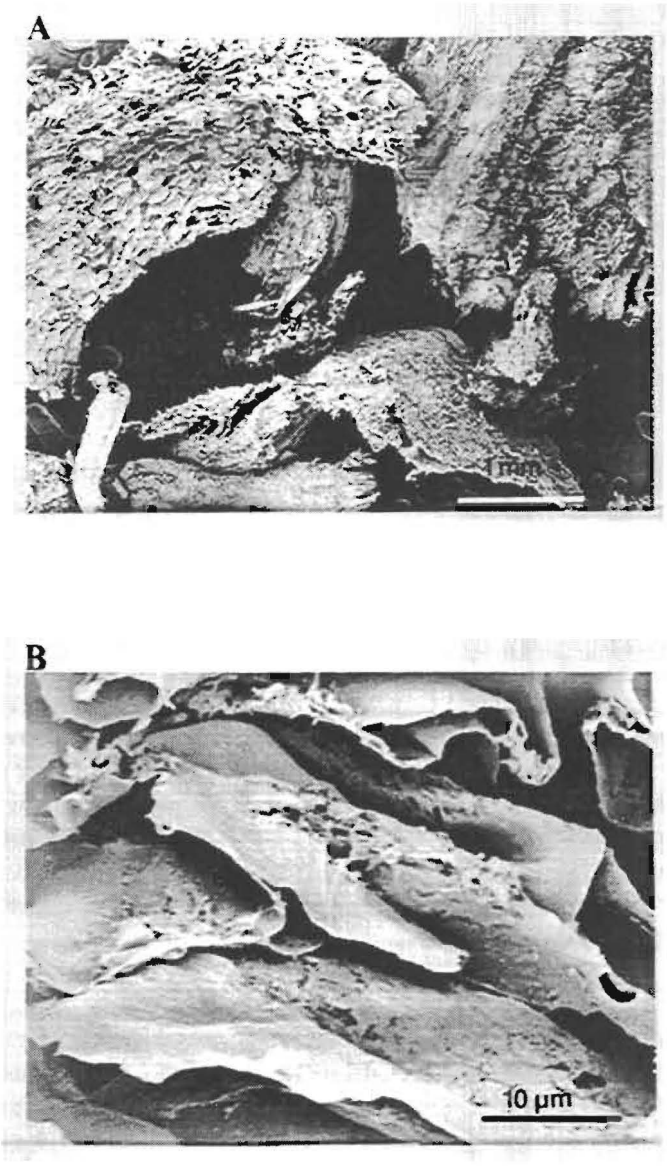

Figure 3 (A, B). Scanning electron micrographs of saccharification and fermentation samples. Sugarbeet pulp prior to enzymatic treatment at 20X and 2000X, (A, B) respectively.

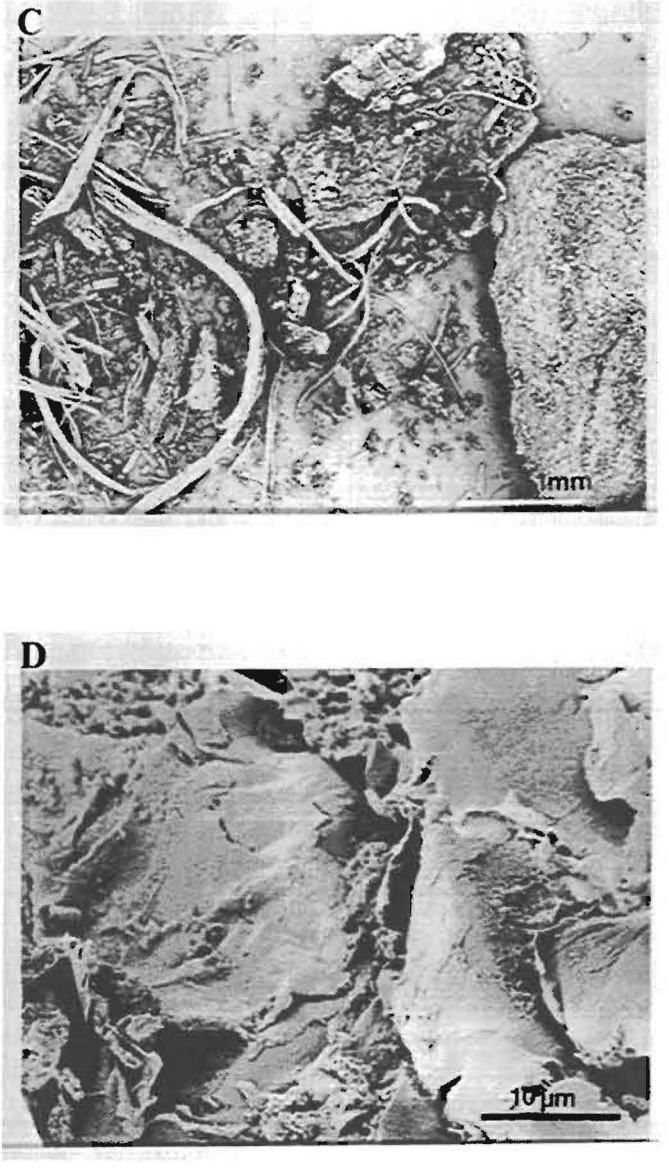

Figure 3 (C, D). Scanning electron micrographs of saccharification and fermentation samples. Sugarbeet pulp after 24 hours of enzymatic hydrolysis at 20X and 2000X, (C, D) respectively.

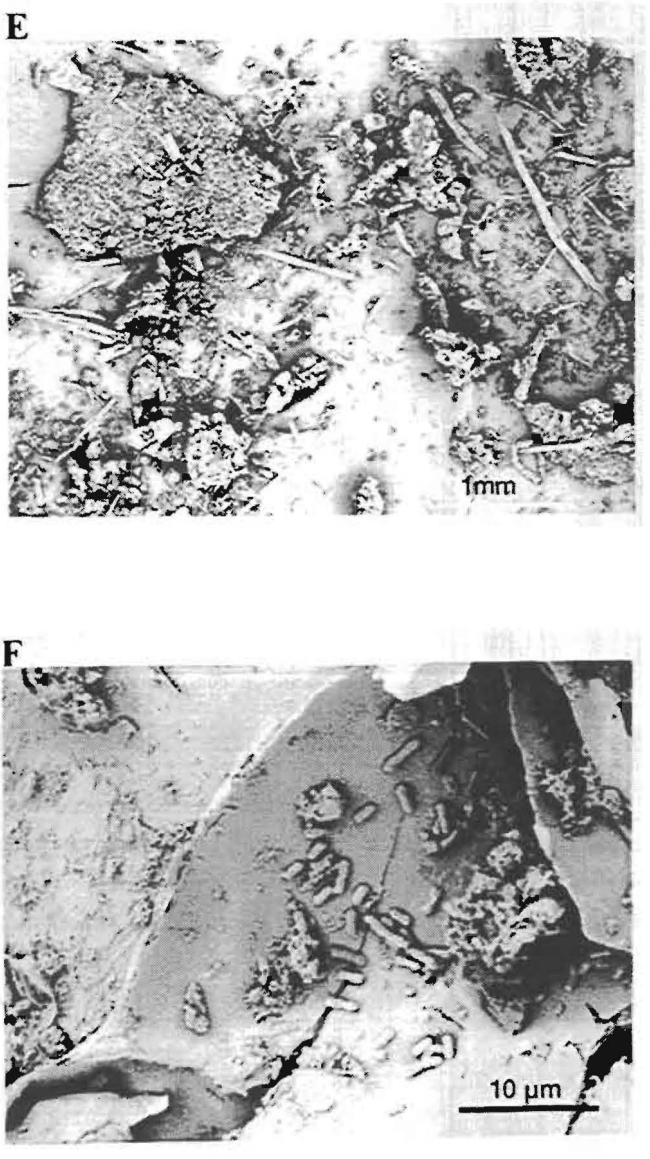

Figure 3 (E, F). Scanning electron micrographs of saccharification and fermentation samples. Sugarbeet pulp following 72 hours of fermentation by *Klebsiella oxytoca* P2 at 20X and 2000X (E, F) respectively. *Klebsiella oxytoca* cells are visible in F.

than those obtained in studies conducted using ethanologenic recombinant *E. coli* KO11 (Grohmann et al. 1994). Utilization of galacturonic acid may be a limiting factor in ethanol production from sugarbeet pulp using *K. oxytoca* P2 as the biocatalyst.

The discovery that comparable levels of ethanol are produced from pelleted and pressed forms of sugarbeet pulp presents an economic advantage. Drying and pelletizing pulp currently expends 25-40% of the total annual fuel energy costs for many sugar refineries (Coons 1971), making pressed pulp the substrate of choice. No mechanical pretreatment was required for comparable levels of ethanol from pressed pulp. Fermentations by *K. oxytoca* P2 using sugar cane bagasse with a 24-hour preincubation period and Genencor Spezyme CE produced only 7 g of ethanol/L after 168 hours (Doran et al. 1994). In this study, fermentations conducted under similar conditions produced 21 g of ethanol/L in 96 hours. Furthermore, additional nutrient supplementation was not required for ethanol production from beet pulp. This finding supports claims by researchers that the high content of mineral nutrients in pectin-rich substrates often makes the addition of nutritional supplementation unnecessary (Grohmann and Bothast 1994). In addition, the use of water in large-scale fermentations would reduce overall costs in ethanol production from biomass because the use of LB medium in large-scale fermentations is not currently economically feasible. At a conversion rate of 0.2 g ethanol/ g dry weight sugarbeet pulp, over 97 million gallons of ethanol could be produced in the United States from 1×10^6 tons (dry weight) of sugarbeet pulp using this process and *Klebsiella oxytoca* P2 as the biocatalyst.

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LITERATURE CITED

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.

Bertin, C., X. Roman and J-F. Thibault. 1988. Structure and properties of sugarbeet fibers. J. Sci. Food Agric. 44: 15-29.

Bothast, R.J., B.C. Saha, A.V. Flosenzier and L.O. Ingram. 1994. Fermentation of l-arabinose, oxytoca d-xylose, and d-glucose by ethanologenic recombinant *Klebsiella strain* P2. Biotechnol. Lett. 16: 401-406.

Brooks, T.A. and L.O. Ingram. 1995. Conversion of mixed waste office paper to ethanol by genetically engineered *Klebsiella oxytoca* strain P2. Biotechnol. Prog. 11: 619-625.

Burchhardt, G. and L.O. Ingram. 1992. Conversion of xylan to ethanol by ethanologenic strains of *Escherichia coli* and *Klebsiella oxytoca*. Appl. Environ. Microbiol. 58: 1128-1133.

Clarke, M.A. and L.A. Edye. 1996. Sugarbeet and sugarcane as renewable resources, Pages 229-247. *In*: Fuller G., McKeon T.A., Bills D.D. (eds.) Agricultural materials as renewable resources: non-food and industrial applications, ACS Symposium Series 647, American Chemical Society, Washington, DC.

Coons, F.F. 1971. Dried pulp, pages 587-595. *In*: McGinnis RA (ed) Beet-sugar technology, 2nd ed, Beet Sugar Development Foundation.

Din, N., N. Gilkes, B. Tekant, R. Miller, Jr., R.A.J. Warren and D.G. Kilburn. 1991. Non-hydrolytic disruption of cellulose fibers by the binding domain of a bacterial cellulase. Biotechnol. 9: 1096-1099

Doran, J.B. and L.O. Ingram. 1993. Fermentation of crystalline cellulose to ethanol by *Klebsiella oxytoca* containing chromosomally integrated *Zymomonas mobilis* genes. Biotechnol. Prog. 9: 533-538.

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.

Grohmann, K., E.A. Baldwin, B.S. Buslig and L.O. Ingram. 1994. Fermentation of galacturonic acid and other sugars in orange peel hydrolysates by the ethanologenic strain of *Escherichia coli.* Biotechnol. Lett. 16: 281-286.

Grohmann, K. and R.J. Bothast. 1994. Pectin-rich residues generated by processing of citrus fruits, apples, and sugarbeets: enzymatic hydrolysis and biological conversion to value-added products. Pages 372-390. *In*: Himmel M.E., Baker J.O., Overend R.P. (eds) Enzymatic conversion of biomass for fuels production, ACS Symposium Series 566, American Chemical Society, Washington, DC.

Himmel, M.E., W.S. Adney, J.O. Baker, R. Elander, J.D. McMillan, R.A. Nieves, J.J. Sheehan, R. Thomas, T.B. Vinzant and M. Zhang. 1997. Advanced bioethanol production technologies: a perspective. Pages 2-45. *In*: Saha BC, Woodward J (eds). Fuels and chemicals from biomass, ACS Symposium Series 666, American Chemical Society, Washington, DC.

Ingram, L.O. and J.B. Doran. 1995. Conversion of cellulosic materials to ethanol. FEMS Microbiol. Lett. 16: 235-241.

Ingram, L.O., X. Lai, M. Moniruzzaman, B.E. Wood and S.W. York. 1997. Fuel ethanol production from lignocellulose using genetically engineered bacteria. Pages 57-73. *In*: Saha BC, Woodward J (eds) Fuels and chemicals from biomass, ACS Symposium Series 666, American Chemical Society, Washington, DC.

Micard, V., C.M.G.C. Renard and J-F. Thibault. 1996. Enzymatic saccharification of sugar-beet pulp. Enz. Microbial Technol. 30: 162-170.

Michel, F., J-F. Thibault and J-L. Barry. 1988. Preparation and characterization of dietary fiber from sugarbeet pulp. J. Sci. Food Agric. 42: 77-85.

Ohta, K, D.S. Beall, J.P. Mejia, K.T. Shanmugam and L.O. Ingram. 1991. Metabolic engineering of *Klebsiella oxytoca* M5A1 for ethanol production from xylose and glucose. Appl. Environ. Microbiol. 57: 2810-2815.

Wen, L.F, K.C. Chang, G. Brown and D.D. Gallagher. 1988. Isolation and characterization of hemicellulose and cellulose from sugarbeet pulp. J. Food Sci. 53: 826-829.

Wood, B.E. and L.O. Ingram. 1992. Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant *Klebsiella oxytoca* containing chromosomally integrated *Zymomonas mobilis* genes for ethanol production and plasmids expressing thermostable cellulase genes from *Clostridium thermocellum*. Appl. Environ. Microbiol. 58: 2103-2110.