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Fermentation of Sugars in Orange Peel Hydrolysates to Ethanol by Recombinant *Escherichia coli* KO11

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ABSTRACT

The conversion of monosaccharides in orange peel hydrolysates to ethanol by recombinant *Escherichia coli* KO11 has been investigated in pH-controlled batch fermentations at 32 and 37°C. pH values and concentration of peel hydrolysate were varied to determine approximate optimal conditions and limitations of these fermentations. Very high yields of ethanol were achieved by this microorganism at reasonable ethanol concentrations (28–48 g/L). The pH range between 5.8 and 6.2 appears to be optimal. The microorganism can convert all major monosaccharides in orange peel hydrolysates to ethanol and to smaller amounts of acetic and lactic acids. Acetic acid is coproduced in equimolar amounts with ethanol by catabolism of salts of galacturonic acid.

Index Entries: Orange peel; enzymatic hydrolysis; ethanol; galacturonic acid fermentation; recombinant *E. coli* B.

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INTRODUCTION

Production of orange and grapefruit juice generates large amounts of residues (*1–3*). These residues, mainly peel and segment membranes, are rich in soluble and insoluble carbohydrates, and can serve as a feedstock for production of value-added fuels and chemicals by enzymatic hydrolysis and biological conversion processes. Previous investigations of enzymatic hydrolysis of orange peel have indicated that the reaction proceeds readily when a mixture of commercial pectinase and cellulase enzymes is used (*4–10*). These crude preparations also contain additional hemicellulolytic activities (*4,5*). The enzymatic hydrolysis releases not only glucose by hydrolysis of cellulose, galacturonic acid from pectin and arabinose, galactose and xylose from both pectin and hemicelluloses, but it also releases large amounts of glucose and fructose entrained in the peel tissues. Relatively concentrated solutions of these sugars have been produced by enzymatic hydrolysis of concentrated peel solids (*5*). However, the hydrolysates contain inhibitory compounds, primarily residual limonene, which must be removed by filtration, steam stripping, or aeration (*5,11–17*) before anaerobic fermentations can proceed. Purified peel hydrolysates were successfully fermented to ethanol by the yeast *Saccharomyces cerevisiae* (*5*), but relatively low conversion yields were observed. Low yields were caused by a lack of anaerobic fermentation of five carbon sugars, and galacturonic acid by this yeast and other industrial yeast strains in general (*18*). Therefore, we initiated investigations of fermentability of orange peel hydrolysates by recombinant bacteria *E. coli* KO11 and *Klebsiella oxytoca*, strain E2, which were developed for the conversion of five- and six-carbon sugars to ethanol (*19–24*). Results of fermentation of filtered orange peel hydrolysates by *E. coli* KO11 are presented in this article. During the course of our investigations, we also discovered a new pattern of galacturonic acid fermentation by *E. coli* KO11, where this bacterium ferments salts of galacturonic acid with the formation of ethanol, acetate, and carbon dioxide. A preliminary account of these investigations has been recently published (*25*).

MATERIALS AND METHODS

Substrates, Enzymes, and Reagents

Valencia orange (*Citrus sinensis* cv. Valencia) peel mixed with small amounts of segment membranes was obtained from the processing line at a local plant and stored frozen. Commercial pectinase (Pectinex Ultra SP), cellulase (Celluclast 1.5L), and β-glucosidase (Novozym 188) preparations were purchased from NOVO Laboratories Inc. (Danbury, CT). Assays and specific hydrolytic activities of these preparations were described in previous publications (*4,5*).

Batches of peel hydrolysate were prepared by hydrolysis of ground (approximate particle size 1–3 mm) peel with a mixture of all three enzymes for 24 h at 45°C. Ground peel was placed in a plastic carboy, mixed with diluted enzyme solution, and the carboy was rotated in a horizontal position at 10–15 rpm using a laboratory roller-type jar mill base (US Stoneware Co., Mahwah, NJ). Initial concentration of total dry peel solids was approx 23 wt% and enzyme loading was approx 0.4 international filter paper units (IFPU) of cellulase and 12.4 pectinase U/g of total peel solids (*5*). The enzyme mixture was supplemented with 1.6 mg of β-glucosidase protein/g of total peel solids. No pH control was utilized during enzymatic hydrolysis; therefore, the pH changed from an initial value of 4.3 to 3.3 at the end of hyrolysis owing to formation of galacturonic acid. Residual insoluble solids were removed by filtration through 1.2-μm glass fiber filter (Cole Parmer Instrument Co., Niles, IL), and the filtrate was partially neutralized by an addition of calcium carbonate until pH = 5.8 was reached. Excess calcium carbonate and a small amount of precipitate that formed during neutralization were removed by a second filtration through glass fiber filter and the clarified filtrate was sterilized by filtration through 0.45-μm membrane filter. Tryptone and yeast extract (media supplements) were procured from Difco Laboratories (Detroit, MI), and D-galacturonic acid was purchased from Sigma Chemical Co. (St. Louis, MO).

Organism

Recombinant *E. coli* strain KO11 has been described previously (*19–23*). The culture was maintained and inocula prepared as described previously (*19–23*).

Fermentation Equipment

All fermentations were conducted in magnetically stirred bioreactors (Multigen™ Model 2000 fermentor, New Brunswick Scientific Co., Edison, NJ) equipped with pH, temperature, and agitation controls.

Culture Media and Fermentations

The culture media contained filter-sterilized peel hydrolysate prepared as described above that was diluted with water to a desired starting concentration of sugars and supplemented with 2.5 g/L of yeast extract and 2.5 g/L of tryptone broth to approximately a quarter strength Luria broth recommended previously (*19,21*) for fermentations with this bacterium. The cultures also contained 40 mg/L of chloramphenicol to aid the maintenance of inserted ethanologenic genes. All media supplements were sterilized by filtration through a 0.45-μm filter, and the pH of complete media was adjusted to a desired initial pH value by addition of sodium hydroxide. Galacturonic acid fermentations contained 1–7 wt%

galacturonic acid neutralized to pH = 6.5 by addition of sodium hydroxide and both supplements described above. Control fermentations containing only yeast extract and tryptone were also conducted at pH = 6.5 and 37°C. The fermentations of peel hydrolysate were performed at pH values ranging from 5.6 to 6.5, at 32 and 37°C and without aeration. The pH of the cultures was maintained by addition of 2*N* NaOH and 1*N* HCl, respectively.

Analytical Procedures

Neutral monosaccharides released by enzymatic treatment of orange peel were separated and determined by ion-moderated partition chromatography of deionized hydrolysates and refractive index detection as described previously (*4*). Organic acids (i.e., galacturonic, acetic, formic, and lactic) and ethanol were separated and determined by ion-moderated partition chromatography using an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA) and refractive index detection as described previously (*4,26*). Initial total peel solids were determined by drying aliquots of ground peel at 70°C to constant weight. The residual insoluble peel solids were determined by filtering aliquots of peel hydrolysates through 1.2-μm glass fiber filters and drying as described above. Microbial cell mass was determined by filtering aliquots of cultures through 0.45-μm filters and drying as described above. Cultures were also examined by microscopic observations.

RESULTS AND DISCUSSION

During initial investigations of fermentation of orange peel hydrolysates by recombinant bacterium *E. coli* KO11, we observed, in addition to expected utilization of five- and six-carbon neutral sugars, an unexpected utilization of galacturonic acid. Additional experiments (*25*) demonstrated that this bacterium ferments dilute (2%) solutions of galacturonic acid salts, according to the following equation:

$$\underset{\text{galacturonic acid}}{C_6H_{10}O_7} \xrightarrow{\textit{E. coli}\ \text{KO11}} \underset{\text{ethanol}}{C_2H_6O} + \underset{\text{acetic acid}}{C_2H_4O_2} + \underset{\text{carbon dioxide}}{2\,CO_2} \quad (1)$$

Although the yields of ethanol are not as high as during fermentation of neutral sugars, this new fermentation can have a significant impact on the biological conversion of hydrolysates from orange peel and other pectin-rich plant tissues to ethanol. Therefore, we decided to investigate additional variables of this fermentation using orange peel hydrolysates

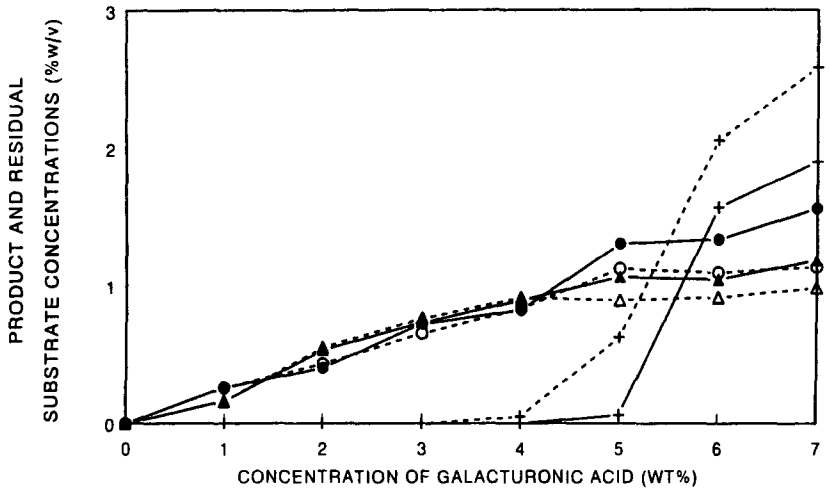

Fig. 1. Fermentation of galacturonic acid to ethanol and acetic acid by *E. coli* KO11 at 37°C and pH = 6.5. Solid lines 72-h samples, dashed lines 48-h samples. △, ▲ = ethanol; ○, ● = acetic acid; + = residual galacturonic acid.

as a substrate. First we conducted an initial study of the effects of galacturonic acid concentration on product formation at 37°C and pH = 6.5, because relatively concentrated solutions of galacturonic acid and other sugars can be produced by enzymatic hydrolysis of orange peel (*4,5*). The results of galacturonic acid fermentations are summarized in Fig. 1. High yields of ethanol and acetic acid (as sodium salt) were observed after 48 h fermentations of 1–4% galacturonic acid solutions. Five percent galacturonic acid solution could be fermented in 72 h, but declining yields were observed for fermentations of 6 and 7% galacturonic acid solutions where only part of the substrate was utilized (Fig. 1). The inhibition of galacturonic acid fermentation at concentrations higher than 4 or 5% did not appear to be caused by accumulation of ethanol, which reached concentrations well below the levels obtained during fermentations of peel hydrolysates (*see* Fig. 2). However, the decline in galacturonic acid utilization could be caused by ionic effects of sodium galacturonate or by end product inhibition owing to accumulation of sodium acetate. Acetic acid has been reported to be strongly inhibitory to recombinant *E. coli* strains, and its salts are inhibitory as well (*27*).

The fermentation of peel hydrolysates at 37°C was investigated in the next series of experiments. Undiluted peel hydrolysates had the following concentrations of monomeric sugars (% w/v, standard deviations are shown in parenthesis): glucose 5.28 (0.49), fructose 2.99 (0.29), arabinose 1.17 (0.20), galactose 0.73 (0.05), and galacturonic acid 3.78 (0.31). Average concentration of total neutral sugars was 10.17 wt% and of total sugars (including galacturonic acid) 13.95%. Sucrose, a well-known component

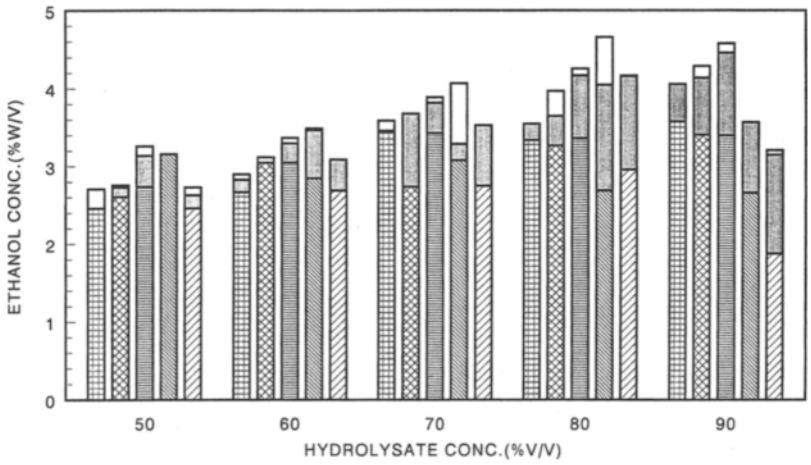

Fig. 2. Final ethanol concentrations after 24-, 48-, and 72-h batch fermentations of diluted peel hydrolysate by *E. coli* KO11 at 37°C. Fermentation pH values: ▦ = 6.5, ▩ = 6.2, ▤ = 6.0, ▧ = 5.8, ▨ = 5.6. Coded areas correspond to ethanol concentrations after 24 h, lightly shaded areas correspond to ethanol concentrations after 48 h, and unshaded areas correspond to ethanol concentrations after 72 h.

of peel juice (*1,3–5*) was not detected in peel hydrolysates, because it was hydrolyzed to glucose and fructose during enzymatic hydrolysis. We have detected invertase activity in both pectinase and β-glucosidase preparations (data not shown). We also detected formation of xylose during enzymatic hydrolysis, but its concentration was too low for accurate quantitative determination by the refractive index detector we used. It must be noted at this point that disaccharides, higher oligosaccharides, and other components potentially fermentable by recombinant *E. coli* were probably also present in peel hydrolysates, but those components could not be separated and determined during investigations reported here.

The hydrolysates were supplemented with complex nutrients of Luria broth (tryptone and yeast extract) as recommended in previous publications (*19,21*). Lower than recommended (quarter-strength) concentration of these nutrients was selected in preliminary experiments that indicated that this concentration of supplements was sufficient to stimulate fermentation of peel hydrolysates, and control fermentations using only yeast extract and tryptone as a carbon source yielded a low ethanol concentration (approx 0.2 wt%). Results of control fermentations and previous investigations (*21*) indicate that recombinant *E. coli* appears to produce significant amounts of ethanol by fermentation of amino acids as well as sugars.

Peel hydrolysates were diluted to several discrete concentrations (50, 60, 70, 80, and 90% of initial peel hydrolysate concentration) in order to investigate the effects of initial sugar concentration on fermentation per-

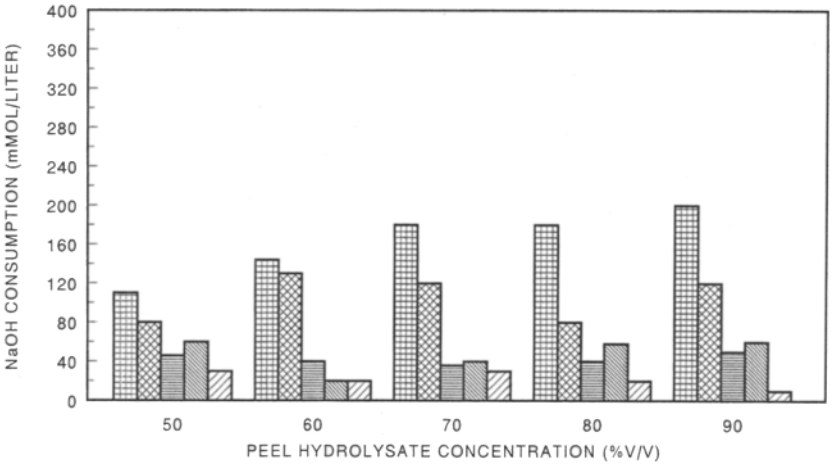

Fig. 3. Base sodium hydroxide consumption during 72-h batch fermentations of diluted peel hydrolysate by *E. coli* KO11 at 37°C. Fermentation pH values are coded as in Fig. 2.

formance at each pH value. Batch fermentations were then performed at pH = 5.6, 5.8, 6.0, 6.2, and 6.5, because previous publications (*28,29*) indicated that the recombinant *E. coli* can ferment sugar solutions at pH values lower than optimal pH, which is approx 6.5–7.5. Fermentation at lower pH values should improve outgassing of carbon dioxide, which is coproduced with ethanol, and decrease consumption of base necessary for maintenance of required pH values.

The final ethanol concentrations reached after 24, 48, and 72 h of these fermentations are summarized in Fig. 2. There is a trend toward increasing final ethanol concentration for each initial concentration of hydrolysate as the pH of fermentation is decreased from 6.5 to the 5.8–6.0 range, where highest final concentrations of ethanol have been observed. The major reason for this increase in ethanol concentration is a decrease in dilution of cultures by the addition of 2*N* sodium hydroxide. Consumption of 2*N* sodium hydroxide during these fermentations is summarized in Fig. 3. Although there is some scatter in the data and cultures also consumed small amounts of acid (1*N* HCl) during later stages of fermentation, a significant decrease in sodium hydroxide consumption is evident when pH of fermentations has been decreased from 6.5 to 6.0 or lower. The high consumption of base during these fermentations could be caused by formation of bicarbonate and imperfect outgassing of carbon dioxide, by formation of organic acids, or by a combination of both effects. The pK_a of carbonic acid is 6.35 at 25°C (*30*). Therefore increasing concentrations of base are required to maintain higher pH values in equilibrium with gaseous phase containing a given concentration of carbon dioxide (*29,30*). In order to address this issue, we performed a simple comparison between base added to the fermentations in the form of salt of galacturonic acid

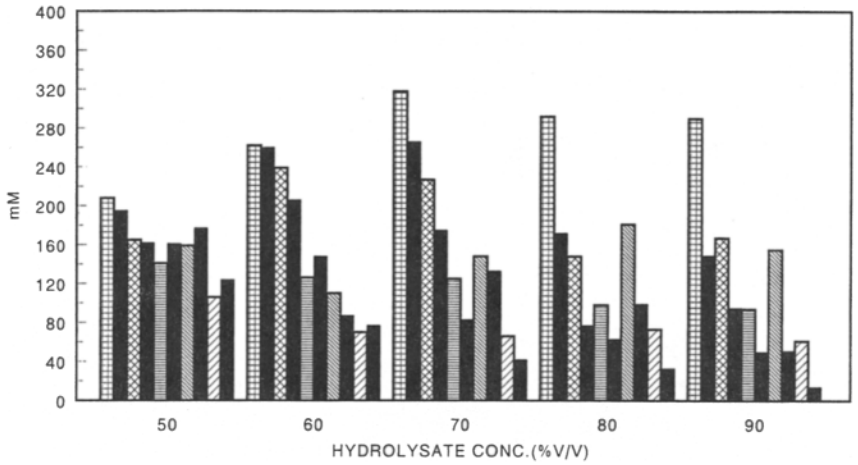

Fig. 4. Comparison of base consumption and acid production during 72-h batch fermentations of diluted peel hydrolysate by *E. coli* KO11 at 37°C. Fermentation pH values are coded as in Fig. 2. First bars (coded) correspond to sum of base utilization (galacturonic acid salt and sodium hydroxide). Second bars (black) correspond to sum of acid production (acetic acid and lactic acid).

and as sodium hydroxide and a sum of organic acid produced during these fermentations. The results are summarized in Fig. 4. These results show that the base was consumed mainly for neutralization of organic acids (acetic, lactic, and occasionally traces of formic), except when more concentrated hydrolysates were fermented. Incomplete outgassing of carbon dioxide appears to occur under these conditions.

Although results reported in Fig. 2 represent actual ethanol concentrations achieved in these fermentations, they are not directly related to the efficiency of the fermentations because of corrections that must be applied. The yields of ethanol provide a clearer measure of the efficiency of sugar utilization and conversion. Ethanol yields, corrected for base addition and for ethanol produced by fermentation of media supplements and introduced with the inoculum, are summarized in Fig. 5. Theoretical yields were calculated using the following conversion factors: 0.511 g of ethanol/g of all five- and six-carbon neutral sugars (*19,20*) and 0.237 g of ethanol/g of galacturonic acid (*5*), respectively. These results show that monosaccharides in peel hydrolysates were converted to ethanol in very high yields in the pH range of 5.8–6.5 and at substrate concentrations equal to 50–70% of the concentration in initial hydrolysate. These concentrations of peel hydrolysate correspond to total carbohydrate concentrations of 6.99–9.77 wt%, respectively. A decrease in the yield of ethanol was evident for all fermentations conducted with more concentrated (80 and 90% of original) peel hydrolysates, but this decrease was most pronounced for fermentations performed at pH =5.6 and 5.8. Concentrations of residual sugars after 72 h fermentations at 37°C are summarized in Fig. 6. Residual galacturonic acid is highlighted, because it represents

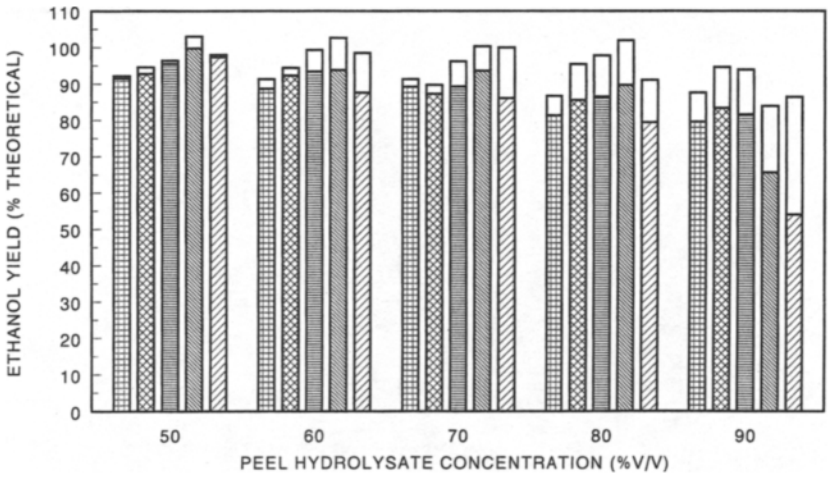

Fig. 5. Ethanol yields after 72-h batch fermentations of diluted peel hydrolysate by *E. coli* KO11 at 37°C. Fermentation pH values are coded as in Fig. 2. Coded areas correspond to ethanol yields calculated on initial content of monosaccharides. Unshaded ares correspond to ethanol yields calculated on the basis of monosaccharides utilized during the fermentations.

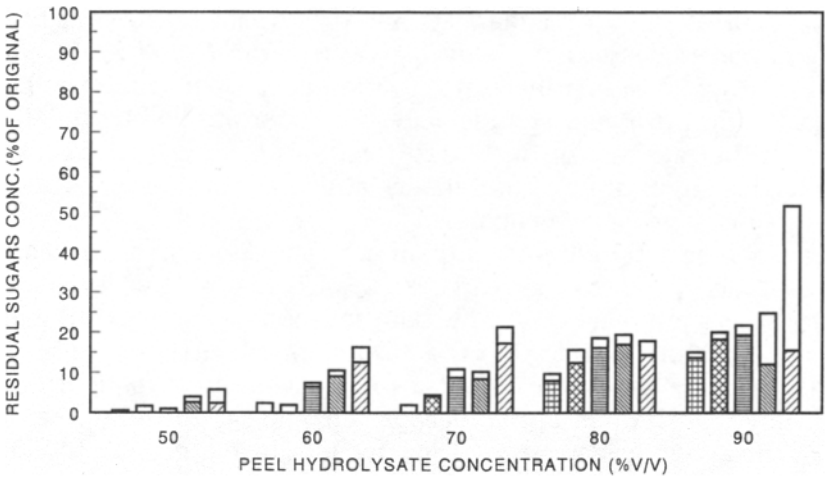

Fig. 6. Concentrations of residual sugars after 72-h batch fermentations of diluted peel hydrolysate by *E. coli* KO11 at 37°C. Fermentation pH values are coded as in Fig. 2. Unshaded areas correspond to residual sugar concentrations calculated as a percentage of initial total monosaccharide concentrations. Shaded areas correspond to residual concentrations of galacturonic acid calculated on the same basis.

the bulk of unfermented monosaccharides, except in two fermentations using 90% peel hydrolysate at pH = 5.6 and 5.8, where all sugars were poorly utilized.

The data in Fig. 6 indicate that, in peel hydrolysates at pH = 6.5, *E. coli* KO11 can only ferment lower concentrations (2.7–3%) of galacturonic

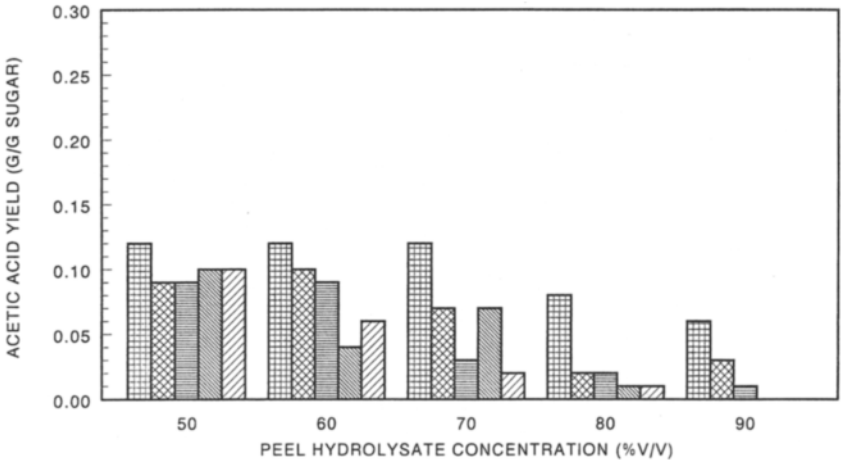

Fig. 7. Yields of acetic acid ($Y_{P/S}$ g acetic acid/g total monosaccharides) after 72-h batch fermentations of peel hydrolysate by *E. coli* KO11 at 37°C. Fermentation pH values are coded as in Fig. 2.

acid than those observed in fermentations of pure galacturonic acid (*see* Fig. 1). Utilization of this sugar becomes progressively worse as the pH of fermentation is decreased. Below pH = 6.5, the *E. coli* KO11 can only utilize approx 2.0% starting concentration of galacturonic acid. Detailed studies using pure galacturonic acid as a substrate will be necessary to elucidate the reasons for decreased tolerance of *E. coli* KO11 fermentations to this sugar at lower pH values and with complex substrates containing abundant neutral sugars.

Ethanol formation was accompanied by a production of small amounts of acetic, lactic, and formic acids. Yield of conversion of total sugars to acetic acid is shown in Fig. 7. The main source of acetic acid is the fermentation of galacturonic acid, but the production of small amounts of acetic and lactic acids has been reported for fermentations of neutral sugars (*19–23*) by this strain of *E. coli,* because enzymes involved in the production of these two acids were not inactivated during genetic construction of this strain (*19*). Conversion of sugars to lactic acid was lower than for acetic acid and ranged from 0 to 0.02 g of lactic acid/g of sugar consumed at pH = 5.6 to 0.03–0.07 g of lactic acid/g of sugar at pH = 6.5 (data not shown). There seems to be a trend toward higher levels of lactic acid formation as fermentation pH is increased from 5.6 to 6.5, but low concentrations of lactic acid made accurate determination of its concentrations and yields dificult. Traces of formic acid were also formed during fermentations at pH = 6.5, but this product was not detected at lower pH values. Dry cell mass yields were in the range of 0.03–0.07 g cells/g of total monosaccharides consumed. These values are very similar to cell mass yields reported by other investigators (*19,23*) for this strain of *E. coli.*

Although we did not conduct kinetic studies during the investigations reported here, we sampled the fermentations at 24-h intervals (*see* Fig. 2). Preliminary analysis of the results (data not shown) indicates that the fermentations of most diluted (50 and 60% of original concentration) peel hydrolysate were completed in 24–48 h at all pH values, whereas fermentations of more concentrated peel hydrolysates required 48–72 h for their completion. The rates of utilization of individual sugars also appear to be different, decreasing in order glucose > fructose > arabinose > galactose > galacturonic acid. A similar relationship between the rates of utilization of glucose, xylose, galactose, and arabinose has been reported previously (*20,22*) for other biomass hydrolysates. We also performed a set of fermentations of peel hydrolysate at the same concentrations as described above, pH = 5.6, 5.8, and 6.0 and at 32°C. Preliminary results of these fermentations do not show significant improvements in ethanol yields and concentrations when the temperature of fermentations has been decreased from 37 to 32°C.

CONCLUSIONS

Our preliminary investigations indicate that the recombinant *E. coli* KO11 is a promising microorganism for the conversion of complex mixtures of sugars in orange peel hydrolysates to ethanol. Relatively concentrated (2.8–4.8 wt%) ethanol solutions have been produced in 48–72 h by batch fermentations of diluted peel hydrolysates with this microorganism at 32 and 37°C and pH = 5.8–6.5. Optimal pH for these fermentations appears to be in the range of 5.8–6.2, but serious decline in performance is evident for fermentations conducted at pH = 5.6. Current investigations confirm our initial discovery (*25*) that *E. coli* KO11 can ferment salts of galacturonic acid to ethanol, acetic, and carbon dioxide. Since galacturonic acid is an important component of total sugars in all hydrolysates of pectin-rich plant tissues, its utilization has to be addressed in any potential process for a conversion of these tissues to ethanol or other value-added products. However, our preliminary investigations also indicate that fermentation of this sugar in peel hydrolysates is subject to catabolite repression or inhibition, since lower (< 2%) concentration of galacturonic acid have been fermented to completion than when pure galacturonic acid was used as a substrate. Fermentation of all major sugars in peel hydrolysates by recombinant *E. coli* KO11 has a significant impact on the yield of ethanol from hydrolyzed orange processing residues. Utilization of *E. coli* KO11 increases ethanol yields to levels approximating theoretical maximum and 25–35% over yields observed during fermentations of the same hydrolysates with the yeast *Saccharomyces cerevisiae* (*5*). The high ethanol yields appear to be caused in part by fermentation of other components of peel hydrolysate than monosaccharides by *E. coli* KO11. Similar

high yields of ethanol were reported by other investigators (*19–22*) for fermentations of other complex substrates by this microorganism. However, our investigations also indicate that *E. coli* KO11 has difficulties with fermentation of concentrated peel hydrolysate and may not be able to produce concentrations of ethanol much higher than those reported in Fig. 2. Adaptation of this strain to higher concentrations of peel hydrolysate may be required for improved performance, or fermentations of peel hydrolysate by *E. coli* KO11 may have to be coupled to yeast fermentations using other substrates to achieve higher concentrations of ethanol and improve efficiency of ethanol recovery.

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