Continuous ethanol production from wheat straw hydrolysate by recombinant ethanologenic Escherichia coli strain FBR5

Badal C. Saha · Michael A. Cotta

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Abstract Continuous production of ethanol from alkaline peroxide pretreated and enzymatically saccharified wheat straw hydrolysate by ethanologenic recombinant Escherichia coli strain FBR5 was investigated under various conditions at controlled pH 6.5 and 35°C. The strain FBR5 was chosen because of its ability to ferment both hexose and pentose sugars under semi-anaerobic conditions without using antibiotics. The average ethanol produced from the available sugars (21.9–47.8 g/L) ranged from 8.8 to 17.3 g/L (0.28–0.45 g/g available sugars, 0.31–0.48 g/g sugar consumed) with ethanol productivity of 0.27–0.78 g L⁻¹ h⁻¹ in a set of 14 continuous culture (CC) runs (16–105 days). During these CC runs, no loss of ethanol productivity was observed. This is the first report on the continuous production of ethanol by the recombinant bacterium from a lignocellulosic hydrolysate.

Keywords Continuous culture · Ethanol · Recombinant ethanologenic Escherichia coli · Wheat straw hydrolysate · Alkaline peroxide pretreatment · Enzymatic hydrolysis

Introduction

Ethanol is a renewable oxygenated fuel. In the USA, the production of fuel ethanol from corn starch reached 10.6 billion gallons in 2009. Various agricultural residues (corn stover, wheat straw, barley straw, rice straw, sugar cane bagasse), processing by-products (corn fiber, rice hulls), and energy crops (switchgrass, miscanthus) are available as low-cost feedstock for conversion to fuel ethanol. The production of ethanol from lignocellulosic biomass generally involves four steps—feedstock pretreatment, enzymatic saccharification, fermentation, and product recovery. Furthermore, any lignocellulosic biomass, upon pretreatment and enzymatic saccharification, generally produces a mixture of sugars such as glucose, xylose, arabinose, and galactose (Saha 2004).

The utilization of all the sugars generated from lignocellulosic hydrolysate is essential for the economical production of ethanol (Saha 2003). The conventional ethanol fermenting yeast (Saccharomyces cerevisiae) or bacterium (Zymomonas mobilis) cannot ferment multiple sugar substrates to ethanol (Bothast et al. 1999). A major technical hurdle to converting lignocellulose to ethanol is developing an appropriate microorganism for the fermentation of a mixture of sugars such as glucose, xylose, arabinose, and galactose (Bothast et al. 1999). A number of recombinant microorganisms such as Escherichia coli, Klebsiella oxytoca, Z. mobilis, and S. cerevisiae have been developed over the last 25 years with a goal of fermenting mixed sugars to ethanol (Alterthum and Ingram 1989, Ohta et al. 1991, Zhang et al. 1995, Ho et al. 1998). Our research unit has developed a recombinant E. coli (strain FBR5) that can ferment mixed multiple sugars to ethanol (Dien et al. 2000). The strain carries the plasmid pLOI297, which contains the genes for pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh) from Z. mobilis necessary for efficiently converting pyruvate into ethanol (Alterthum and Ingram 1989). This plasmid also contains the genes for ampicillin and tetracycline resistance. It selectively maintains...
the plasmid when grown anaerobically and is capable of fermenting both hexose and pentose sugars to ethanol. This recombinant *E. coli* FBR5 strain was based on the host NZN111 (Bunch et al. 1997). The IdhA and pfl chromosomal defects make the host incapable of fermentative growth on carbohydrates in the absence of the genes carried by the plasmid (Dien et al. 2000).

In our previous papers, we have reported on the production of ethanol from wheat straw by dilute acid, lime and alkaline peroxide pretreatments, enzymatic saccharification, and batch fermentations of the hydrolysates using this recombinant *E. coli* strain FBR5 (Saha et al. 2005a, 2008; Saha and Cotta 2006, 2007a). We also have evaluated the batch productions of ethanol by this recombinant strain from pretreated rice hulls and barley straw (Saha et al. 2005b; Saha and Cotta 2007b, 2010). The yields of ethanol in these studies varied between 0.37 and 0.50 g/g available sugars depending on the type of pretreatment used. The fermentation time also varied greatly from 17 to 114 h, which was also highly dependent of the type of pretreatment. In order to produce ethanol industrially, the fermentative microorganism needs to be robust. We were interested in studying the long-term performance of this recombinant bacterium using lignocellulosic hydrolysate as feedstock. Recently, Martin et al. (2006) reported that stable ethanol yields of about 80–85% of the theoretical were obtained on glucose (50 g/L) or xylose (50 g/L) over 26 days at dilution rates of 0.075 h⁻¹ for glucose and 0.045 h⁻¹ for xylose under chemostat conditions using the recombinant *E. coli* FBR5. However, to our knowledge, no studies have been done using a lignocellulosic hydrolysate as feedstock. In this paper, we report on the continuous production of ethanol from alkaline peroxide pretreated and enzymatically saccharified wheat straw hydrolysate by the recombinant *E. coli* FBR5 strain.

**Materials and methods**

**Materials**

Wheat straw was purchased from a local farmer. It was dried in a forced-air oven at 55°C for 24 h and milled in a hammer mill to pass through a 1.27-mm screen. The milled wheat straw was stored at room temperature. It contained 41.77±0.50% cellulose and 33.42±0.33% hemicelluloses, which made up to the total carbohydrate content of 75.19±0.83% on dry matter basis. The detailed composition of the wheat straw used was reported in a previous paper (Saha et al. 2008). Celluclast 1.5 L (cellulase) and Novozym 188 (β-glucosidase) were purchased from Brenntag Great Lakes, Milwaukee, WI, USA. ViscoStar 150 L (hemicellulase) was supplied by Dyadic Corp., Jupiter, FL, USA. Aminex HPX 87P column (300×7.8 mm), Aminex HPX 87H column (300×7.8 mm), de-ashing cartridge (30×4.6 mm), Carbo-P micro-guard cartridge (30×4.6 mm), and Cation H micro-guard cartridge (30×4.6 mm) were purchased from Bio-Rad Laboratories, Inc., Hercules, CA, USA. Membrane filter unit (0.2 mm) was purchased from Nalge Company, Rochester, NY, USA. All other chemicals used were of standard analytical grades.

**Enzyme assays**

The cellulase activity in terms of filter paper activity was assayed and expressed as filter paper unit (FPU) by the procedure described by Ghose (1987). Carboxymethyl cellulase (CMCase), β-glucosidase, xylanase, β-xylosidase, α-L-arabinofuranosidase, and ferulic acid esterase activities were assayed by the procedures described previously (Saha et al. 2008). All activities were expressed in terms of international units (IU, micromoles of the product formed per minute). The activity level of each enzyme in the three commercial enzyme preparations (Celluclast, Novozym 188, and ViscoStar) used in this study has been reported previously (Saha et al. 2008). Celluclast had 34±2 FPU, 1.045±93 U CMCase, 21±0 U β-glucosidase, 1,227±57 U xylanase, 37±3 U β-xylosidase, 9±0 U α-L-arabinofuranosidase, and 0±0 U ferulic acid esterase activities. These activity units for Novozym 188 were 0.0±0.0, 48±2, 581±6, 47±0, 2±0, 13±0, and 0.3±0.1, respectively. For ViscoStar 150 L, these unit values were 10±1, 1,135±18, 42±1, 13,878±216, 35±0, 17±0, and 0±0, respectively.

Preparation of alkaline peroxide-pretreated and enzymatically saccharified wheat straw hydrolysate

The alkaline peroxide pretreatment of wheat straw was carried out by the optimized procedure described previously (Saha and Cotta 2006). Briefly, milled wheat straw was slurried in water (86 g/L) containing H₂O₂ (2.15%, v/v) and adjusted to pH 11.5 using 10 M NaOH and shaken in an incubator at 250 rpm at 35°C for 24 h. The pretreated wheat straw was adjusted to pH 5.0 using concentrated HCl prior to enzymatic saccharification.

The enzymatic saccharification of the alkaline peroxide-pretreated wheat straw was performed by shaking gently (100 rpm) at 45°C after adjusting the pH to 5.0 with HCl and adding a cocktail of three commercial enzyme preparations (cellulase, β-glucosidase, and hemicellulase) at each enzyme dose of 0.15 mL/g straw for 120 h. Samples (1 mL) were withdrawn and kept at −20°C before analysis. The liquid portion after separating it from the residual solids was used as feedstock.
Salt removal from the wheat straw hydrolysate

Salts were removed from the alkaline peroxide-pretreated and enzymatically saccharified wheat straw hydrolysate (WSH) by electrodialysis by the procedure described in detail previously (Qureshi et al. 2008). The salt concentrations of the hydrolysate before and after electrodialysis were 21.72 and 1.98 g/L, respectively. There was some sugar loss (3–10%) during the electrodialysis. An appropriate quantity of each individual sugar (glucose, xylose, and arabinose) was added to the desalted hydrolysate in order to make the sugar composition equal to the original hydrolysate to determine the effect of salt concentration on the fermentation rate.

Bacterial strain and preparation of inoculum

Recombinant E. coli strain FBR5 was provided by Bruce S. Dien (USDA-ARS, NCAUR, Peoria, IL, USA) and was maintained in glycerol vials at −80°C for use as a working stock. It was plated on Luria broth (LB, 10 g tryptone, 5 g yeast extract, and 5 g NaCl) containing 4.0 g xylose and 20 mg tetracycline solidified with 15 g agar per liter (pH 6.5). Plates were incubated at 35°C. Cells from a single well-isolated colony were inoculated into a 125-mL flask containing 100 mL of LB with 20 g xylose and 20 mg tetracycline per liter. Cultures were incubated at 37°C and 100 rpm for 24 h. This grown culture was used as seed culture for fermentation experiments.

Batch and continuous fermentation experiments

The batch fermentation experiments were carried out in pH-controlled 500-mL fleakers with a working volume of 350 mL at pH 6.5 and 35°C essentially as described previously (Bothast et al. 1994), except that 4 M NaOH was used instead of 4 M KOH for pH control. A diagram of the fleaker system was presented previously by Beall et al. (1991). Rubber fleaker caps were drilled to allow the insertion of a pH probe, CO₂ vent, sampling needle, and a port for the addition of base. A magnetic stirrer was located beneath the water bath and maintained at approximately 100 rpm. No attempts were made to exclude oxygen. Continuous culture (CC) experiment was conducted with a 500-mL fleaker fermenter with feed input and output outlets and automatic pH control and a working volume of 240 mL under semi-anaerobic conditions at pH 6.5 and 35°C. WSH was used as substrate. The medium was prepared by dissolving 10 g tryptone and 5 g yeast extract in the hydrolysate (per liter). It was adjusted to pH 7.5 with 10 M NaOH in order to lower the need for the volume of 4 M NaOH addition to control the pH to 6.5. The feed was then filter-sterilized using a membrane filter unit. The inoculum size was 10% (v/v). After 16 h of batch fermentation, filter-sterilized substrate was added to the fleaker fermenter at the feeding rate specified for each individual CC experiment. A schematic diagram of the reactor setup used in this study is shown in Fig. 1. The influent was fed by a peristaltic pump with multiple heads, and the effluent was taken out from the reactor outlet using the same peristaltic pump at the same rate of the influent feed and collected in a reservoir in sterile environment. Samples were withdrawn periodically, centrifuged to remove cells, and kept at −20°C prior to high-pressure liquid chromatography (HPLC) analysis. The optical density (A660nm) of the effluent was monitored immediately after withdrawal. The reactor performance was monitored by quantifying unutilized sugars and fermentation products (ethanol, succinate). Base consumption and pH were recorded. The reactor was operated continuously for a specific period (16–105 days). Duplicate parallel fermentation experiments were also run for reproducibility and comparison.

Calculation of fermentation parameters

The volumetric productivity of ethanol was calculated from the 24-h ethanol production data based on the ethanol content of the effluent. In the figures, the average value of each parameter of two parallel experiments at a particular time point was calculated as the mean value. The averages for Table 1 were calculated from the average value of all the time points for each CC run first, and then the average of the two average values for two parallel runs was calculated. The commercial enzyme preparations used for enzymatic saccharification contained small quantities of glucose, fructose, and sorbitol which were converted to ethanol by
the recombinant *E. coli* FBR5. For simplification purpose, the quantities of ethanol and succinic acid produced from these additional substrates present in the WSH were subtracted from the measured ethanol and succinic acid yields in each case.

**Analytical procedures**

Sugars, ethanol, succinic acid, and acetic acid were analyzed by HPLC (Saha and Bothast 1999). The separation system consisted of a solvent delivery system (P2000 Pump, Spectra-Physics, San Jose, CA, USA) equipped with an autosampler (717, Waters Chromatography Division, Millipore Corp., Milford, MA, USA), a refractive index detector (410 Differential Refractometer, Waters), a dual λ absorbance detector (2487, Waters), and a computer software-based integration system (Chromquest 4.0, Spectra-Physics). Two ion-moderated partition chromatography columns (Aminex HPX-87P with de-ashing and Carbo-P micro-guard cartridges, Aminex HPX 87H with Cation H micro-guard cartridge) were used. The Aminex HPX-87P column was maintained at 85°C, and the sugars were eluted with Milli-Q filtered water at a flow rate of 0.6 mL/min. The Aminex HPX-87H column was maintained at 65°C, and the sugars, organic acids, and ethanol were eluted with 10 mM HNO₃ prepared using Milli-Q filtered water at a flow rate of 0.6 mL/min. Peaks were detected by refractive index or UV absorption (277 nm) and were identified and quantified by comparison to retention times of authentic standards (glucose, xylose, arabinose, galactose, ethanol, succinic acid, and acetic acid). Cell growth of the bacterium was monitored by measuring the optical density of the appropriately diluted culture broth at 660 nm and was multiplied by a conversion factor 0.45 to obtain dry cell weight by liter. The cell dry weight was determined from duplicate 10-mL samples. Each sample was centrifuged, washed with distilled water twice, and dried for 24 h at 105°C.

**Results**

Effect of dilution rate on continuous production of ethanol from alkaline peroxide-pretreated and enzymatically saccharified wheat straw hydrolysate

The production of ethanol from WSH by the recombinant *E. coli* FBR5 was investigated in CC for 105 days at controlled pH 6.5 and 35°C at a dilution of 0.04 h⁻¹. Two parallel CC (CC-1 and CC-2) experiments were run under identical conditions using the same feedstock. The time courses of the production of ethanol and succinic acid, growth (cell mass), and residual xylose concentration are shown in Fig. 2 for CC-1 and CC-2, respectively. The total sugar concentration in the feed was 44.1 g/L (glucose, 24.9 g/L; xylose, 16.7 g/L; arabinose 2.5 g/L) for both CCs. During the CC runs, the average total residual sugar concentrations were 4.7±2.9 and 5.2±3.5 g/L, of which xylose contents were 3.6±2.4 and 4.0±2.7 g/L for CC-1 and CC-2, respectively. The averages (in grams per liter) of ethanol and succinic acid produced and cell mass were 17.3±1.5, 2.3±0.5, and 1.96±0.55, respectively, for CC-1 (Table 1). For CC-2, these values (in grams per liter) were 16.7±1.9, 2.3±0.7, and 1.98±0.71, respectively. The ethanol productivity values were 0.69±0.06 and 0.67±0.07 gl⁻¹ h⁻¹ for CC1 and CC-2, respectively (Table 1). On average, about 10.7% and 11.8% of total sugars (21.6% and 24% of xylose) were left unutilized during the CC runs for CC-1 and CC-2, respectively. Glucose was almost completely utilized in both cases.

In order to force the culture to more completely utilize xylose, the feed rate was reduced by half (dilution rate, 0.02 h⁻¹) and the CC experiments were run for 83 days.

![Fig. 2](image-url)
Again, two parallel CC (CC-3 and CC-4) experiments were run under identical conditions using the same feedstock. The total sugar concentration in the feed was 47.8 g/L (glucose, 26.7 g/L; xylose, 18.8 g/L; arabinose, 2.3 g/L) for both CCs. The average total residual sugar concentrations were 5.4±2.8 and 2.8±2.4 g/L, of which xylose contents were 3.6±1.9 and 1.8±1.7 g/L for CC-3 and CC-4, respectively. The averages (in grams per liter) of ethanol and succinic acid produced and cell mass were 13.3±4.3, 2.4±0.5, and 1.62±0.75, respectively, for CC-3. For CC-4, these values (in grams per liter) were 15.0±4.2, 2.2±0.4, and 1.60±0.70, respectively (Table 1). These data clearly indicate that decreasing the dilution rate to half (from 0.04 to 0.02 h⁻¹) did not help the bacterium to utilize all available sugars in the feedstock, particularly xylose.

Continuous production of ethanol at half feedstock concentration

The CC (CC-5 and CC-6) experiments were then performed at the same dilution rate (0.04 h⁻¹) of CC-1 and CC-2 for 17 days, but using 0.5x WSH to see the effect of total sugars as well as individual sugars, particularly xylose concentration on the substrate utilization and ethanol production by the recombinant E. coli FBR5. The total sugar in the feed was 22.2 g/L (glucose, 12.7 g/L; xylose, 8.2 g/L; arabinose 1.3 g/L) for both CCs. The results of ethanol and succinic acid production, cell growth, and residual xylose content are presented in Table 1. Almost stable productions of ethanol were obtained in both cases. During the CC runs, the average total residual sugar concentrations were 0.4±0.8 and 0.3±0.6 g/L, of which
xylose contents were 0.3±0.6 and 0.2±0.5 g/L for CC-5 and CC-6, respectively. The averages (in grams per liter) of ethanol and succinic acid produced and cell mass were 9.4 ±0.4, 2.3±0.8, and 1.30±0.27, respectively, for CC-5. For CC-6, these values (in grams per liter) were 9.5±0.4, 2.5±0.9, and 1.46±0.25, respectively (Table 1). The sugar utilization improved from the previous CC runs. There was very little or almost no sugars left unutilized during these 2 CC runs. However, the average succinic acid production level remained very similar to previous CC runs, even though the feedstock contained only half of the sugars. In fact, the succinic acid production shows an increasing trend in both cases of CC-5 and CC-6, even though the ethanol yield remained the same (Fig. 3).

Continuous production of ethanol at half feedstock concentration by stepwise increase of dilution rate

The CC (CC-7 and CC-8) experiments were performed using the half substrate concentration as mentioned above, but the dilution rate was increased from 0.04 to 0.06 h⁻¹ after 4 days, then to 0.08 h⁻¹ after 5 days, and the experiments were run for another 6 days. The total sugar concentration in the feed was 22.1 g/L (glucose, 12.5 g/L; xylose, 8.5 g/L; arabinose, 1.1 g/L) for both CC runs. As shown in Fig. 4, steady-state production of ethanol by the recombinant E. coli FBR5 was achieved during the period studied. During the CC runs, the average total residual sugar concentrations were 0.4±0.9 and 0.2±0.2 g/L, of which xylose concentrations were 0.3±0.7 and 0.1±0.1 g/L for CC-7 and CC-8, respectively. The averages (in grams per liter) of ethanol and succinic acid produced and cell mass were 10.0±0.5, 1.6±0.3, and 1.38±0.39, respectively, for CC-7. For CC-8, these values (in grams per liter) were 10.2±0.2, 1.7±0.3, and 1.63±0.36, respectively (Table 1). These data indicate that stable production of ethanol was achieved by a stepwise increase of dilution rates over the period studied in both CC cases. Also, the succinic acid production level remained almost steady during the whole period.
Continuous production of ethanol starting at half feedstock concentration and then stepwise increase to full feedstock concentration

Two CC (CC-9 and CC-10) experiments were run starting at half feedstock concentration (0.50x) and then increasing the feed concentration to about 0.66x, 0.86x, and 1.00x (total sugars, 43.7 g/L; glucose, 24.4 g/L; xylose, 17.2 g/L; arabinose, 2.1 g/L) after 4, 4, and 4 days, respectively. These were then run for another 5 days. The results are presented in Fig. 5. Along with increasing the feed concentration, the ethanol concentration increased to the level of CC-1 and CC-2 and the residual xylose concentration continued to increase; at 1.00x feed concentration, it became very similar to the CC-1 and CC-2 runs. The average ethanol production levels were 9.6±0.4, 12.4±0.1, 15.7±0.6, and 17.8±0.4 g/L at feedstock concentration levels of 0.5x, 0.66x, 0.86x, and 1.00x, respectively, for CC-9. For CC-10, these values were 9.9±0.4, 12.9±0.3, 15.7±0.4, and 18.0±0.3 g/L, respectively. However, the average succinic acid production levels were 1.6±0.4, 1.9±0.2, 2.3±0.1, and 1.5±0.1 g/L at feedstock concentration levels of 0.5x, 0.66x, 0.86x, and 1.00x, respectively, for CC-9. For CC-10, these values were 1.4±0.3, 1.5±0.1, 1.5±0.1, and 1.4±0.1 г/L, respectively. The average residual xylose concentrations were 0.5±0.6, 0.4±0.1, 1.4±0.3, and 5.7±1.3 g/L at feedstock concentrations of 0.5x, 0.66x, 0.86x, and 1.00x, respectively, for CC-11. For CC-12, these values were 0.2±0.3, 0.6±0.1, 2.9±0.9, and 6.7±1.9 g/L, respectively. These results indicate that concentration of sugars in the feedstock mattered most for their full utilization.

Continuous culture of desalted wheat straw hydrolysate

The CC (CC-11 and CC-12) experiments were run for 26 days using the desalted WSH by the recombinant E. coli FBR5 at the same dilution of 0.04 h\(^{-1}\) as in CC-1 and CC-2 in order to find out whether the presence of salts in the feedstock has an effect on the incomplete utilization of available sugars, particularly xylose. The total sugar content in the desalted hydrolysate was 45.7 g/L (glucose, 25.1 g/L; xylose, 18.0 g/L; arabinose, 2.6 g/L). The results of ethanol and succinic acid production, cell mass, and residual xylose content in the effluent fermentation broth are presented in Fig. 6 for CC-11 and CC-12. The averages (in grams per liter) of ethanol and succinic acid produced, cell mass, and residual xylose content for CC-11 were 19.2±1.6, 1.5±0.3, 1.71±0.38, and 4.0±2.3, respectively (Table 1). For CC-12, the corresponding values (in grams per liter) were 19.4±1.5, 1.6±0.3, 1.73±0.57, and 3.9±1.8. The data indicate that there was about a 9.6% (on an average basis) improvement in the production of ethanol, but there was no improvement in the utilization of total sugars from desalted WSH. On average, about 12.5% and 12.3% of total sugars (22.2% and 21.7% of xylose) were left unutilized during the CC runs for CC-11 and CC-12, respectively.

The CC (CC-13 and CC-14) experiments were then run for 17 days at about half feedstock concentration (glucose, 12.9 g/L; xylose, 7.9 g/L; arabinose, 1.1 g/L; total sugars, 21.9 g/L) using desalted hydrolysate under the same conditions of CC-11 and CC-12. The time course data (not shown) indicate that the sugars were all utilized and ethanol concentration remained almost the same during the period studied. The average total residual sugar concentrations were 0.05±0.1 and 0.03±0.03 g/L of which xylose contents were 0.3±0.8 and 0.0±0.01 g/L for CC-13 and CC-14, respectively. The averages (in grams per liter) of ethanol and succinic acid produced and cell mass were 8.8±0.6, 3.1±1.0, and 1.34±0.28, respectively, for CC-13 (Table 1). For CC-14, these values (in grams per liter) were 9.8±0.4, 2.0±0.6, and 1.78±0.13, respectively. However,
The succinic acid production showed a very slow increasing trend in both cases. The effluent of the first CC run was passed through a second reactor at the same dilution rate (0.04 h$^{-1}$) at a controlled pH of 6.5 and 35°C. The results indicate that about 75–100% residual xylose was utilized by the strain and that the ethanol production increased by 3–5% (data not shown).

A summary of the fermentation activity of the recombinant E. coli FBR5 from the WSH in CC is given in Table 1. The average mass balance for all CC runs accounted for 101±3% considering the amount of sugars present in the feedstock, sugars left unutilized in the effluent, growth (cell mass) of the bacterium, and the products (ethanol, succinic acid) obtained except for CC-3 and CC-4 runs. For these two CC runs, the average mass balance was about 74.3±0.6%.

Inhibitory effects of salt and ethanol on batch production of ethanol

The effects of ethanol addition on growth, sugar utilization, and ethanol production by the recombinant E. coli strain FBR5 from WSH before and after salt removed by electrodialysis in batch fermentation are presented in Table 2. The data show that there were 26% inhibition of growth (cell mass) and 20% inhibition of ethanol production using the hydrolysate as such compared to desalted feedstock. With the 1.5% ethanol addition, there was 29% inhibition of ethanol production in the case of desalted feedstock. With the same concentration of ethanol addition, the inhibition of ethanol production was 69% for the no-salt-removed hydrolysate. Thus, there is a synergistic interaction of both salt and ethanol on the inhibition of growth and ethanol production by the recombinant E. coli FBR5.

Discussion

To our knowledge, this is the first study on the continuous culture of recombinant E. coli FBR5 using lignocellulosic hydrolysate as a substrate. A series of CC experiments were carried out using WSH in order to assess the performance

Table 2 Effects of salts and ethanol on growth and ethanol production by the recombinant E. coli FBR5 from alkaline peroxide-pretreated and enzymatically saccharified wheat straw hydrolysate

<table>
<thead>
<tr>
<th>Wheat straw hydrolysate</th>
<th>Fermentation time (h)</th>
<th>Residual sugars* (g/L)</th>
<th>Ethanol produced (g/L)</th>
<th>Ethanol productivity (g l$^{-1}$ h$^{-1}$)</th>
<th>Cell mass (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desalted</td>
<td>16$^b$</td>
<td>0.0±0.0</td>
<td>19.8±0.3</td>
<td>1.24</td>
<td>3.91±0.04</td>
</tr>
<tr>
<td>No salt removed</td>
<td>24</td>
<td>8.3±1.0</td>
<td>15.9±0.3</td>
<td>0.66</td>
<td>2.90±0.22</td>
</tr>
<tr>
<td>Desalted+1.5% ethanol</td>
<td>24</td>
<td>7.1±0.1</td>
<td>14.0±0.0</td>
<td>0.58</td>
<td>2.04±0.24</td>
</tr>
<tr>
<td>No salt removed+1.5% ethanol</td>
<td>24</td>
<td>28.3±1.0</td>
<td>5.7±0.1</td>
<td>0.24</td>
<td>0.87±0.27</td>
</tr>
<tr>
<td>Desalted+2.5% ethanol</td>
<td>24</td>
<td>17.6±0.3</td>
<td>8.8±0.4</td>
<td>0.37</td>
<td>1.53±0.34</td>
</tr>
<tr>
<td>No salt removed+2.5% ethanol</td>
<td>24</td>
<td>33.5±0.1</td>
<td>3.7±0.5</td>
<td>0.15</td>
<td>0.55±0.01</td>
</tr>
</tbody>
</table>

The data presented are averages of two individual experiments

*Initial total sugars, 41.5±0.5 g/L

$^b$Fermentation was complete within 16 h
of the recombinant bacterium. These CC experiments were performed under semi-anaerobic conditions without using any antibiotics in the medium. For each CC experiment, two parallel CC runs were made under identical conditions using the same WSH. The data presented are averages of two titters obtained at the same time point. Even though these parallel CC runs showed very similar patterns of sugar utilization and product (ethanol, succinic acid) formation, these CC experiments were run for a long time, and there should obviously be some variations between the two individual runs for each time point. The recombinant bacterium was able to produce ethanol at a consistent level over a period of up to 16–105 days tested with apparently no loss of ethanol productivity. These results clearly indicate that the recombinant bacterium was able to maintain the plasmid pLOI297 under semi-anaerobic condition even in the absence of antibiotics during the period studied. There were no microbial contaminations observed during these studies. However, it is desirable to achieve an almost complete conversion of the sugars present in the hydrolysate in order to be economic. In our previous batch culture studies, we have observed that the recombinant *E. coli* strain FBR5 utilized glucose first, then arabinose, and finally xylose at a slower rate, even though it could completely utilize xylose (Saha et al. 2005a, b; Saha and Cotta 2006, 2007a, b, 2008, 2010). Similarly, in CC (nos. 1–4) experiments, most of the glucose was utilized but about 10–22% xylose remained in the effluent despite the dilution rate reduction from 0.04 to 0.02 h\(^{-1}\). However, about 75–100% of residual xylose in the effluent was utilized by the strain in the second CC run, with an average increase of ethanol production from 3% to 5% in the case of CC-1. Alkaline peroxide pretreatment after neutralization for enzymatic hydrolysis generally generates salts (Qureshi et al. 2008). We have observed salts from the WSH by electrolydialysis and found that there were similar levels of residual xylose in the CC experiments 11 and 12 (Fig. 6) like CC experiments 1 and 2 (Fig. 2). This indicates that salt alone was not responsible for inhibiting complete xylose utilization. Martin et al. (2006) reported that with 50 g/L xylose feed, complete substrate utilization could not be achieved by the recombinant *E. coli* FBR5 at the dilution rate of 0.082 h\(^{-1}\). The dilution rate was lowered to 0.045 h\(^{-1}\) after sampling on day 4 whereupon the residual xylose concentration fell rapidly to around 8 g/L before declining to a negligible amount after 17 days. Govindaswamy and Vane (2010) studied the multistage continuous culture fermentation of glucose–xylose mixtures (2:1, 30 g/L glucose and 15 g/L xylose) to fuel ethanol in a medium containing yeast extract and peptone using genetically engineered *S. cerevisiae* strain 42A-LNH-ST. They reported that at a dilution rate of 0.05 h\(^{-1}\), xylose (15 g/L) consumption was only 37% in the first-stage 1-L reactor. At the same flow rate, xylose consumption rose to 69% after subsequently passing through 3- and 1-L reactors in series, primarily due to longer residence time in the 3-L reactor (0.0167-h\(^{-1}\) dilution rate). In our CC experiments, xylose utilization varied between 78% and 100% in the first stages of all runs. Complete utilization of residual xylose was observed in the second-stage CC run tested only with the effluent of the CC-1 run. These data indicate that recombinant *E. coli* strain FBR5 can utilize xylose much more efficiently than the recombinant *S. cerevisiae* strain 42A-LNH-ST, even though we have used lignocellulosic biomass hydrolysate as a substrate instead of using pure glucose–xylose mixture.

In our studies, the bacterium was able to utilize all the sugars completely when diluted WSH (0.5x) was used as a feedstock (Fig. 3). Also, similar results were obtained when diluted desalted WSH (0.5x) was used as a feedstock (CC-13 and CC-14, data not shown). It showed the same pattern of substrate utilization when the dilution rate was changed from 0.04 to 0.06 and 0.08 h\(^{-1}\) (Fig. 4). However, when the feedstock concentration was increased stepwise from 0.5x to 1.0x concentration (total sugars, 43.7 g/L; glucose, 24.4 g/L; xylose, 17.2 g/L; arabinose, 2.1 g/L), the sugar utilization pattern changed to the original CC runs (nos. 1 and 2) using the feedstock concentration of 1.0x (total sugars, 44.1 g/L; glucose, 24.9 g/L; xylose, 16.7 g/L; arabinose, 2.5 g/L; Fig. 5). Thus, sugar concentration in the hydrolysate plays an important role in the utilization of xylose by the bacterium.

A disadvantage of recombinant *E. coli* strain FBR5 is that the fumarate reductase enzyme apparently functions in strain FBR5 since none of the four subunit genes were disrupted, allowing the production of considerable quantities of succinic acid (Dien et al. 2000; Martin et al. 2006). The fumarate reductase is only expressed anaerobically. Relatively stable succinic acid production (1.4–3.1 g/L) was observed in all these CC experiments performed. Similar quantities (1–3 g/L) of succinic acid were produced by the recombinant *E. coli* FBR5 over the course of CC experiments using 50 g/L glucose or xylose as feedstock (Martin et al. 2006).

It is evident from Table 2 that the ethanol productivity by the recombinant bacterium was decreased by 36% when the additional ethanol concentration was increased from 1.5% to 2.5%. Dien et al. (2000) reported that the recombinant *E. coli* FBR5 produced a maximum of 39–42 g/L ethanol from xylose (95 g/L) within 60 h with ethanol yields of 86–92% of theoretical in batch culture. Another study indicates that the maximum ethanol that could be produced by the *E. coli* FBR5 from xylose in batch culture was 43.5 g/L (Qureshi et al. 2006). In our CC experiments using undiluted WSH, it produced around 20 g/L ethanol. The data presented in Table 2 show that ethanol at this concentration slows down
the fermentation. Moreover, the combined effect of salt and ethanol is more detrimental to the fermentation (Table 2). Martin et al. (2006) reported that the maximum ethanol concentration achievable in CC is frequently limited by the ethanol tolerance of the organism. Asghari et al. (1996) reported that genetically engineered ethanologenic E. coli strain K011 produced about 45 g/L ethanol from hemicellulose hydrolysates of agricultural residues (bagasse, corn stover, and corn hulls).

Lawford and Rousseau (1996) investigated the factors contributing to the loss of ethanologenicity of E. coli B recombinants pLOI297 and K011 employing glucose- or xylose-limited chemostat cultures. These two strains were created by genetically engineering E. coli B (ATCC 11303) carrying the Zymomonas genes for pyruvate decarboxylase and alcohol dehydrogenase II on a multicopy plasmid pLOI297 and a chromosomal pet integrant of strain 11303, designated as strain K011. Both recombinants carry markers for antibiotic resistance. However, both recombinants exhibited rapid loss of ethanologenicity in chemostat cultures with glucose even when the selection pressure was imposed by the inclusion of antibiotics in the feed medium. Under xylose limitation, the plasmid-bearing recombinant strains appeared to be stabilized by antibiotics. These authors concluded that based on an average cost for large bulk quantities of antibiotics at $55/kg and an amendment level of 40 mg/L, the estimated economic impact regarding the potential stabilization by antibiotics in a plant operating in batch mode, the antibiotic cost can be $0.29/gal of ethanol for antibiotic amendment of all fermentation media. Dien et al. (1998) constructed a series of FBR strains using parental strains that have been mutated so that they could not grow fermentatively. These mutants carry pfl and ldh mutations that block the ability of the strains to reduce pyruvate and recycle NADH/\(^{\text{H}^{+}}\) generated from glycolysis. Transforming these strains with plasmid pLOI297, which encoded the PET operon, restored fermentative viability. Dien et al. (2000) produced the strain FBR5 based on the host NZN111 (Bunch et al. 1997). This host does not require amino acids for growth, and its pfl mutation, which was introduced by genetic recombination, is associated with a chloramphenicol resistance marker. The strain FBR5 selectively maintained the plasmid when grown anaerobically. The culture was serially transferred ten times in anaerobic culture with sugar-limited medium containing xylose, but no selective antibiotic. An average of about 95% cells maintained plasmid pLOI297 in anaerobic culture. Thus, the strains used by Lawford and Rousseau (1996) are different from strain FBR5. In fact, the stable nature of E. coli strain FBR5 without antibiotics is advantageous for industrial production of ethanol from a lignocellulosic hydrolysate as the antibiotic use will not only add extra cost but also create environmental problems.

Martin et al. (2006) mentioned that if the E. coli FBR5 would have lost the plasmid, the revertant strain would simply be the host strain NZN111. This host strain does not produce ethanol (Bunch et al. 1997, Dien et al. 2000). In conclusion, the recombinant E. coli FBR5 performed very well over the periods studied with respect to stability and viability without any antibiotics. The information is very important for the use of the recombinant bacterium for continuous production of ethanol from lignocellulosic hydrolysates. At present, the continuous fermentation is used in some grain-based ethanol plants for reducing the production cost (Martin et al. 2006). Based on the results of these CC experiments, we are planning to study the production of ethanol by the recombinant strain E. coli FBR5 at the pilot-scale (100 L) level using wheat straw as a feedstock in order to evaluate the performance of the strain for large-scale ethanol production from lignocellulosic hydrolysates.

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References


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