Effect of pretreatment of soy insoluble fiber and SSCF with Saccharomyces cerevisiae and Escherichia coli KO11 on ethanol production in an integrated corn-soy biorefinery

J. K. Sekhon  
Iowa State University, jasreen@iastate.edu

D. Maurer  
Iowa State University, dmaurer@iastate.edu

K. A. Rosentrater  
Iowa State University, karosent@iastate.edu

T. Wang  
Iowa State University, tongwang@iastate.edu

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Effect of pretreatment of soy insoluble fiber and SSCF with *Saccharomyces cerevisiae* and *Escherichia coli* KO11 on ethanol production in an integrated corn-soy biorefinery

Sekhon J. K.¹, Maurer D.², Rosentrater K. A.¹,²,*, Wang T.¹, Jung S.¹,³.

Department of Food Science and Human Nutrition, Center for Crops Utilization Research, Iowa State University, Ames IA, USA¹

Department of Agricultural and Biosystems Engineering, Iowa State University, Ames IA, USA²

Department of Food Science and Nutrition, California Polytechnic State University, San Luis Obispo, CA, USA³

Presenter*

Written for presentation at the 2015 ASABE Annual International Meeting New Orleans, Louisiana July 26 – 29, 2015

Abstract

Soy insoluble fiber (IF), co-product of enzyme-assisted aqueous extraction process (EAEP) of soybeans, is rich in carbohydrate and protein. It can be used to enhance ethanol production in an integrated corn-soy biorefinery, which integrates components from soybean processing into corn-based ethanol processing. However, cornstarch and IF have unique carbohydrate compositions that require different treatments for optimal fermentation. The present study investigated the effect of pretreatment method [soaking in aqueous ammonia (SAA), liquid hot water (LHW), and enzymatic hydrolysis], simultaneous saccharification and co-fermentation (SSCF) with *Saccharomyces cerevisiae* and *Escherichia coli* KO11, and scaling up from bench scale (150 mL) to pilot scale (60 L) on ethanol production in IF fermentation. Untreated IF was added to integrated corn-soy fermentation and the effect of SSCF was evaluated. It was demonstrated that enzymatic hydrolysis with enzyme cocktail of pectinase, cellulase and xylanase, is the best pretreatment method to maximize ethanol production in IF fermentation with an added advantage of adding enzymes to the fermentation slurry at the SSF step. Ethanol yield almost doubled when SSCF of IF was performed with *E. coli* KO11 due to conversion of arabinose and xylose into ethanol. Addition of untreated IF to dry-grind corn fermentation increased ethanol production rate, but low ethanol tolerance of *E. coli* KO11 was a limiting factor in achieving SSCF with *S. cerevisiae* and *E. coli* KO11. Michaelis-Menten equation accurately predicted *E. coli* KO11 growth kinetics by Hanes-Woolf linearization.

Keywords. Bioethanol, enzyme-assisted aqueous extraction of soybeans, pretreatment of soy fiber, dry-grind corn fermentation, *Escherichia coli* KO11.
Introduction

Enzyme-assisted aqueous extraction process (EAEP) is an environmentally friendly alternative to chemical and mechanical extraction of oil from soybean (Campbell & Glatz, 2009; de Moura, Maurer, Jung, & Johnson, 2011; Jung, Maurer, & Johnson, 2009; Lamsal & Johnson, 2007; Li, Zhang, Wang, Jiang, & Sui, 2013). In addition to being a sustainable solution to conventional oil extraction methods, EAEP entails safer operation, less initial capital investments, higher oil recovery, and production of variety of products. EAEP of soybeans is a 4-step process comprising of mechanical pretreatment, enzyme-assisted aqueous extraction, separation of cream and co-products, and demulsification of the cream fraction to recover free oil (de Moura, et al., 2011). Cell wall disruption and particle size reduction by flaking and extrusion are critical steps to achieve high oil and protein extraction efficiencies. Other key parameters are pH, solid-to-liquid ratio, and temperature (Jung, de Moura, Campbell, & Johnson, 2011). Although 97% of oil recovery can be achieved by the EAEP of soybeans, considerable amounts of co-products, skim (protein-rich) and IF (carbohydrate-rich), are produced in the process. To realize maximum potential of the EAEP of soybeans, skim and IF must be utilized such as in dry-grind corn fermentation.

Over 14 billion gallons of bioethanol was produced in the U.S. in 2014, which met ~10% of the gasoline demand (RFA, 2015). In addition, biofuel industry produced an estimated 39 million metric tons of feed, making the renewable fuels sector a major animal feed processing segments in the U.S. Incorporating soy skim and IF, rich in protein and carbohydrates respectively, in corn fermentation would not only enhance ethanol production but also produce high quality animal feed (high in protein and essential amino acids).

While carbohydrate in corn are mainly starch (~70%), in IF they are mainly cellulose, hemicellulose and lignin. These different carbohydrate compositions of corn and IF could be a limiting factor to ethanol production in an integrated corn-soy fermentation. However, it has been demonstrated that soy IF is relatively easy to saccharify (after extrusion and proteolysis step in EAEP) and a potential lignocellulosic feedstock for ethanol production (Karki, Maurer, Box, Kim, & Jung, 2012; Karki, Maurer, & Jung, 2011a; Karki, Maurer, Kim, & Jung, 2011b). Sekhon, Jung, Wang, Rosentrater, & Johnson (2015) reported significant increase in ethanol production rate and ethanol yield, and decrease in fermentation times when soy skim and IF were added to dry-grind corn fermentation.

A wide variety of processes for the production of ethanol from cellulosic biomass has been studied and is under development (Cardona & Sanchez, 2007; Ojeda, Sanchez, El-Halwagi, & Kafarov, 2011; Piccolo & Fabrizio, 2009; Teixeira, Linden, & Schroeder, 1999; Wyman et al., 2005; Xu & Lad, 2007). Among them, three methods are widely applied: conventional separated hydrolysis and fermentation, simultaneous saccharification and fermentation (SSF) and
Simultaneous saccharification and co-fermentation (SSCF). In the present study we evaluated (1) separate pretreatment of IF followed by SSF with cellulase and *S. cerevisiae*, (2) SSF of IF with cocktail of fiber hydrolyzing enzymes, pectinase, cellulase and xylanase, and *S. cerevisiae*, and (3) SSCF of IF with *S. cerevisiae* fermentation followed by *E. coli* KO11 fermentation.

IF is composed of mainly cellulosic material, therefore pretreatment is necessary to breakdown cellulose and hemicellulose to monomeric sugars (hexoses and/or pentoses), which can then be fermented into ethanol (Hendriks & Zeeman, 2009). Various physical, chemical and biological pretreatment methods have been studied for cellulosic biomass (Hendriks & Zeeman, 2009; Mosier et al., 2005; Ortiz & Quintero, 2014; Tomas-Pejo, Alvira, Ballesteros, & Negro, 2011; Wan, Zhou, & Li, 2011; Wyman et al., 2005a; Wyman et al., 2005b; Yoo, Alavi, Vadlani, & Behnke, 2012). Karki et al. (2011a) evaluated several pretreatment methods for soy IF (high power ultrasound, ammonium hydroxide, sodium hydroxide, and sulfuric acid) and found alkali treatment with ammonium hydroxide to be the best method. In the present study we evaluated the effect of soaking in aqueous ammonia (SAA), liquid hot water (LHW) treatment, and enzymatic hydrolysis (using pectinase, cellulase and xylanase) on ethanol production. Alkali pretreatment is a low temperature treatment, which hydrolyzes intermolecular ester bonds cross-linking hemicelluloses and other components such as lignin. Though sugar degradation in this treatment is significantly lower than acid treatment, the application is hindered by high costs of alkalis and production of toxic byproducts (Talebnia, Karakashev, & Angelidaki, 2010). LHW is a hydrothermal treatment conducted at high temperature (~170-230°C) and high pressure (>5 MPa) (Talebnia et al., 2010). This method has potential owing to simplicity, low generation of inhibiting byproducts and high yields (Hamelinck, Van Hooijdonk, & Faaij, 2005). Enzymatic hydrolysis is yet another preferred method over acid and alkali pretreatment methods because it is environmentally-friendly, has higher conversion efficiency, uses moderate non-corrosive conditions and has low energy requirements (Bon & Ferrara, 2007). However, the rate of cellulose hydrolysis by cellulase enzymes depends on crystallinity of cellulose and its degree of polymerization. Further, the matrix polysaccharides and lignin prevent the enzymes from reaching cellulose (Ballesteros et al., 2006). Therefore, to improve hydrolysis a physical pretreatment of lignocellulosic material before enzymatic hydrolysis or using cocktail of enzymes that will enhance cellulase activity may be required.

The monomeric hexoses produced from the pretreatment of lignocellulosic material such as soy IF can be easily fermented to ethanol by *S. cerevisiae*, which can only convert hexoses (glucose, galactose) but not pentoses (xylose, arabinose) into ethanol. Therefore, in order to convert pentoses to ethanol in the integrated corn-soy slurry either a modified strain of *S. cerevisiae* or other organism such as *E. coli* KO11 can be used. It has been found that bacterial strains (*E. coli* KO11 and *Z. mobilis* AX101) were more effective at fermenting ethanol from cellulosic sugars compared to modified yeast strain (*S. cerevisiae* 424A) (Lau, Gunawan, Balan, & Dale, 2010).
Different strains of *E. coli* have been successfully used to convert cellulosic biomass such as brown microalgae (Wargacki et al., 2012), switch grass (Bokinsky et al., 2011), sugarcane bagasse (Geddes et al., 2011), corn stover (Jin, Balan, Gunawan, & Dale, 2012), etc. into ethanol and higher alcohols. In the present study we evaluated SSCF of IF by *S. cerevisiae* fermentation followed by *E. coli* KO11 fermentation as a potential method to maximize ethanol production from corn-soy slurry. Therefore, specific objectives of the study were (1) to determine the effect of different pretreatment methods on ethanol production from IF (2) to determine the effect of SSCF of IF by *S. cerevisiae* fermentation followed by *E. coli* KO11 fermentation (3) to predict the effect of ethanol concentration on *E. coli* KO11 kinetics in IF and corn-soy fermentation using Michaelis-Menten equation.

**Materials and Methods**

**Materials**

Soy IF was produced as a co-product of EAEP of soybeans in the pilot plant of the Center for Crops Utilization Research, Iowa State University, Ames, IA according to de Moura et al. (2011). Composition of IF was 14.4% solids, which contained 2.5% (db) oil, 11.5% (db) protein, 4.0% (db) ash and 82% (db) carbohydrates, respectively. IF samples were stored at -14°C until used. Yellow dent corn harvested in 2013 was procured from Honeyville (West Chester, OH). The corn was ground using a Fitz Mill (Model DAS 06, Fitzpatrick Co., Elmhurst, IL) according to Sekhon et al. (2015). The particle retention (%) on each sieve was 17.8, 17.3, 17.6, 12.9, 11.2, 9.2, 7.7 and 0.03%, respectively. The particles per gram were 119,335 and surface area (cm²/gram) was 116. Composition of corn was 89.9% (db) solids, 5.1% (db) oil, 8.6% (db) protein, 1.1% (db) ash and 74.6% (db) carbohydrates. Ground corn was then stored at 4°C until further processing.

Soy fiber hydrolyzing enzymes, cellulase (powder; activity 75,000 CU/g; optimum pH 4-6, temperature 30-70°C), xylanase (powder; activity 75,000 XU/g; optimum pH 4-6, temperature 40-65°C) and pectinase (liquid; activity 3,500 ENDO-PG/g; optimum pH 2-5, temperature 40-65°C) were donated by Bio-Cat (Troy, VA). Peptone N-Z Soy BL 7 enzymatic hydrolysate, d-glucose, chloramphenicol, and antibacterial lactrol were procured from Sigma (St. Louis, MO), BactoTM tryptone (pancreatic digest of casein) and BactoTM yeast extract (extract from autolyzed yeast cells) from Becton, Dickson and Company (Sparks, MD), sodium chloride (crystalline) from Fisher Scientific (Fair Lawn, NJ), and *Escherichia coli* KO11 (ATCC® 55124™) was procured from ATCC (Manassas, VA). Corn liquefaction and saccharification enzymes, α-amylase (liquid) and glucoamylase (liquid) (Spirizyme Excel XHS, Novozyme, Franklinton, NC), and antibacterial chlorine dioxide (commercial grade) were procured from Lincolnway Energy LLC, Ames, IA. Dry yeast (*Saccharomyces cerevisiae*; Ethanol Red) was procured from Fermentis, a division of Lesaffre Yeast Corp., Headland, AL. Ammonium sulfate and ammonium hydroxide were procured from Fisher Scientific.
Compositional analyses

Proximate analyses

Total solids (%) were estimated by drying 1 g of sample overnight at 110°C in a forced air oven (Precision by Thermo Scientific, Winchester, VI); oil content (%) was estimated by using the Mojonnier acid hydrolysis method (AOAC method 922.06); protein content (%) was estimated by using the Dumas combustion method with Nitrogen conversion factor of 6.25 (Vario MAXCN Elementar Analysensysteme GmbH, Hanau, Germany); and ash content (%) was determined by heating dried samples in a furnace (1400 Barnstead|Thermolyne Corporation, Dubuque, IA) at 550°C for 5 h (AOAC 923.03). Total carbohydrate content (%) was calculated by subtracting oil, protein, and ash contents from 100%. All analyses were performed in duplicates.

Carbohydrate composition and lignin content

The samples were analyzed according to NREL CAT Standard Procedure NREL/TP-510-42618 to estimate structural carbohydrates composition and lignin content (acid soluble and acid insoluble). All analyses were performed in duplicates.

IF Treatment

Effect of SSCF on ethanol production in IF fermentation

SSCF of IF was performed according to Sekhon et al. (2015). The method is stated here briefly.

Yeast-extract peptone (YP) media: YP media was prepared by dissolving 1 g yeast extract and 2 g peptone in 100 mL deionized (DI) water. Dry yeast (0.16 g) was allowed to soak in the YP media for 10 min before adding to 10 mL aliquots of IF slurry.

Escherichia coli KO11 inoculum: E. coli KO11 inoculum was prepared by aseptically transferring one stock vial (1 mL) of E. coli KO11 into LB broth (prepared by mixing 0.50 g tryptone, 0.25 g yeast extract, 0.50 g sodium chloride and 1.00 g glucose in 0.05 L DI water and autoclaved at 121°C for 15 min) and 1 mL of chloramphenicol (40 mg/L). The flask was incubated overnight (15-16 h) in a shaker incubator (Lab-line Orbit Shaker Bath model 3540) at 37°C at 150 rpm. After incubation, the cell culture was aseptically transferred to a sterilized centrifuge tube and centrifuged at 9,000×g for 10 min. The supernatant was aseptically discarded and the pellet was resuspended in 10 mL of sterile saline solution and centrifuged again to remove ethanol and unused glucose. The supernatant was again discarded and the pellet was resuspended in 10 mL of fresh LB broth (with no glucose). The resuspended E. coli KO11 in LB broth (in 10 mL aliquots) was used as inoculum for saccharification and fermentation of IF and for the integrated corn-soy fermentation process.

SSCF with S. cerevisiae and E. coli KO11: SSCF of IF was performed in a 4-L double-jacketed round-bottom bioreactor. Slurry (3000 g as-is) was prepared by adding IF (2% glucan loading;
1822.5 g of IF-55 g glucan loaded), 0.05 M citrate buffer (699.9 mL), pectinase, cellulase and xylanase (PCX; each enzyme was added at 5% of soy solids), YP media (200 mL), *S. cerevisiae* (3.2 g), lactrol (0.16 g) and water (233.7 mL). Simultaneous saccharification and fermentation (pH 4.5) was carried out at 37°C for 48 h at 150 rpm. After 48 h, the pH of the fermentation broth was adjusted to 7.0 with sodium hydroxide and then inoculated with 10 mL of *E. coli* KO11. The slurry was fermented at 37°C for 24 h at 150 rpm. At the end of a 72 h saccharification and fermentation period, the slurry was separated by centrifuging at 3000×g. Proximate composition of the fermented IF was 14.3% solids, which contained 6.5% (db) oil, 16.5% (db) protein, 6% (db) ash and 71% (db) carbohydrates. Ethanol concentration in liquid part was determined by HPLC method described in HPLC analysis section.

**Effect of pretreatment method on ethanol production in IF fermentation**

Three treatment methods were tested to maximize saccharification and hence ethanol production in IF fermentation: (1) soaking in aqueous ammonia (SAA) (Figure 1a) (2) liquid hot water (LHW) (Figure 1b), and (3) enzymatic hydrolysis using cocktail of pectinase, xylanase, and cellulase. Process conditions for each pretreatment method are listed in Table 1. SAA pretreatment was performed by soaking IF (30 g db) with 15% (wt.) aqueous ammonia at solid-to-liquid ratio of 1:10 (w/w, db) in a 1 L screw-capped Pyrex laboratory bottle. The bottle was placed in a preheated oven at 80°C for 12 h. After 12 h, treated slurry was centrifuged at 3000×g for 15 min; the recovered solids were washed with distilled water until neutral pH was achieved (6-15 washing cycles). The washed solids were weighed and stored in sealed plastic cups at 4°C. The pretreated IF was then saccharified with cellulase and fermented with *S. cerevisiae*. Solid recovery was calculated by drying preweighed samples in forced air oven at 130°C for 3 h.

Treatment of IF with LHW was conducted in a 1 L PARR reactor (4522M PARR Instrument Company, Moline, IL). IF (1 kg; 14.4% solids) was added to the stainless steel reactor. The reactor was heated to a target temperature of 160°C at 0.68 MPa. After being held at the target temperature for 20 min, the reactor was cooled down to room temperature by turning on the cooling water. The pretreated slurry was then saccharified with cellulase and fermented with *S. cerevisiae*.

Enzymatic hydrolysis was conducted by adding PCX cocktail at the SSF step in the IF fermentation slurry. Slurry of IF (2% glucan loading) was prepared as described in SSCF of IF section. Four concentrations of PCX enzymes were tested (5%, 10%, 15%, or 20% of soy solids db). SSF with *S. cerevisiae* was conducted only when 5% of PCX was added to the slurry. SSCF with *S. cerevisiae* (at 37°C for 48 h) followed by *E. coli* KO11 (37°C for 48 h) was carried out for all four PCX concentrations. Two replications of each pretreatment were performed and change in ethanol concentration (g/L) and carbohydrate composition (g/L) was estimated.
Figure 1. Schematic flow diagram of (a) soaking aqueous ammonia (SAA) and (b) liquid hot water (LHW) pretreatment methods for IF.

Table 1. Experimental parameters for pretreatment and saccharification and fermentation of IF.

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Enzymatic saccharification/fermentation variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA</td>
<td>80°C, 12 h</td>
</tr>
<tr>
<td>LHW</td>
<td>160°C, 20 min</td>
</tr>
<tr>
<td>Enzyme cocktail</td>
<td>NA</td>
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</tr>
</thead>
<tbody>
<tr>
<td>SAA</td>
<td>80°C (Sacc), 35°C (SSF), Cellulase, S. Cerevisiae</td>
</tr>
<tr>
<td>LHW</td>
<td>160°C (Sacc), 35°C (SSF), Cellulase, S. Cerevisiae</td>
</tr>
<tr>
<td>Enzyme cocktail</td>
<td>35°C (SSF), Pectinase, Cellulase, Xylanase, S. Cerevisiae</td>
</tr>
</tbody>
</table>

Sacc. Saccharification, SSF: simultaneous saccharification and fermentation

Scale-up of saccharification and fermentation of IF

The effect of scaling up saccharification and fermentation of IF from laboratory scale (0.9 kg; 150 mL) to intermediate scale (6 kg; 10 L) and pilot plant scale (35 kg; 60 L) on ethanol production was determined. At the laboratory scale, saccharification and fermentation was performed in Tornado IS6 Overhead Stirring System (Radleys Discovery Technologies, Shire Hill, Saffron Walden, UK) equipped with an anchored stirring shaft and six 250 mL round bottom flasks. At the intermediate scale saccharification was performed in a 20 L bioreactor vessel (17.5-in x 12-in; internal diameter 10 in). The 34.6-in stirrer shaft consisted of a flake cup, 5-in agitator, and a 7.5-in high viscosity agitator with an 8-in diameter. The flake cup was 15.5-in from top to shaft, 5-in agitator was located 10.5-in from flake cup, and high viscosity agitator was placed 1.6-in below the 5-in agitator. Pilot scale bioreactor vessel consisted of bottom-drive agitator with two Rushton impellers (5-in diameter; 6-in width and 12-in from bottom) and one marine
impeller (8-in diameter, down-flow; 2-in from bottom). Interior vessel diameter was 12.4-in and contained one 1-in wide baffle. Effect of scale up of SAA pretreatment method (from lab scale-150 mL to pilot scale-10 L) on ethanol production was also evaluated. Treatment conditions are listed in Table 1.

Integrated corn-soy fermentation

Integrated corn-soy fermentation was performed according to Sekhon et al. (2015). Briefly, soy IF was mixed with coarsely ground corn and cold deionized (DI) water in a 250 mL flasks of the Tornado IS6 Overhead Stirring System. The ratio of corn-to-IF and water-to-solids were set at 1:0.16 on db and 2.5:1 (by weight), respectively. The amount of corn was kept constant in all fermentations. α-Amylase (0.167 mL) was added when temperature of the slurry reached 85°C. After 3 h, the flasks were cooled to 30°C in an ice bath and the pH of the cooled slurry was adjusted to 4.5 with 3 M sulfuric acid. Chlorine dioxide (0.028 mL; antimicrobial), ammonium sulfate (0.077 mL of 0.2 g/g solution; nitrogen source), glucoamylase (0.167 mL), soy fiber-hydrolyzing enzymes PCX and dry yeast (0.167 g) were then added. After 48 h of fermentation, the pH was adjusted to 7 with sodium hydroxide and the fermentation slurry was inoculated with 10 mL of *E. coli* KO11 inoculum. The slurry was then fermented at 37°C for 24 h. At the end of a 72 h fermentation period, a 3 mL aliquot of slurry was pipetted into a microfuge tube, heated in water bath at 75°C for 10 min, cooled in an ice bath for 10 min and centrifuged at 9000×g. Supernatant was transferred to an HPLC vial and analyzed for ethanol concentration (g/L) by using HPLC.

Effect of ethanol and sugar concentration on *E. coli* KO11

LB broth (10 mL) were prepared with different sugar types (glucose or xylose), different sugar concentrations (xylose 50 or 150 g/L), and different ethanol concentrations (0, 20, 50 or 100 g/L) in Erlenmeyer flasks. The broths were sterilized by autoclaving at 121°C/60 min. *E. coli* KO11 inocula (1 mL) were prepared (as described in the IF treatment section) and added to each flask. Flasks were covered with aluminum foil and allowed to incubate (with agitation) at 37°C for 24 h in a Shaker incubator (Lab-line Orbit Shaker Bath model 3540). Change in absorbance (DU 640 Spectrophotometer, Beckman Coulter, Brea, CA) of the broths was measured at 550 nm at 1 h intervals for 8 h and then measured at 22 h and 24 h. Absorbance of broths inoculated with *E. coli* KO11 at the start of fermentation was ~ 0.6-0.7. Control samples were incubated along with fermentation broth to account for turbidity caused by factors other than *E. coli* KO11 growth: LB broth only (no additional sugars), LB broth with glucose or xylose only (with no *E. coli* KO11 inoculum), and LB broth with sugars and ethanol (but no inoculum). Flasks containing LB broth were agitated at 37°C before adding the inoculum. Sugar and ethanol concentrations in broths were measured by HPLC analysis before the start of fermentation (0 h) and at the end of fermentation (24 h).

Prediction of *E. coli* KO11 kinetics using Michaelis-Menten equation

If *E. coli* KO11 growth follows Michaelis-Menten kinetics, a plot of initial absorbance of broth containing *E. coli* cells against time can be expressed as:

\[ Abs = \frac{V_{\text{max}} \times t}{K_m + t} \]  

(1)
Where, \( \text{Abs} \) = absorbance at 550 nm, \( V_{\text{max}} \) = maximum initial velocity, \( K_m \) = Michaelis constant, and \( t \) = time (h). The equation (1) was linearized using Hanes-Woolf model and \( V_{\text{max}} \) and \( K_m \) were estimated by plotting experimental data, \( \text{Abs}/t \) versus \( t \):

\[
\frac{\text{Abs}}{t} = \frac{K_m}{V_{\text{max}}} + \frac{1}{V_{\text{max}}} \cdot t
\]  

(2)

Straight line was fitted to the points with slope \( 1/V_{\text{max}} \) and y-intercept \( K_m/V_{\text{max}} \). The two parameters, \( V_{\text{max}} \) and \( K_m \) were then calculated: \( V_{\text{max}} = 1/\text{slope} \); \( K_m = \text{y-intercept} \times V_{\text{max}} \). The \( V_{\text{max}} \) and \( K_m \) values were then used to predict \( E. \text{coli} \) KO11 growth kinetics at any time, \( t \), using equation (1).

**HPLC analysis**

Samples were analyzed for carbohydrates using HPLC (Varian Inc., Victoria, Australia) equipped with an auto sampler, an isocratic pump, a refractive index detector, and an Aminex HPX-87P carbohydrate column (Bio-Rad Laboratories, Hercules, CA, USA) set at 85°C. The sample injection volume was 20 µL and elution was conducted using 18 mX NANO pure water at a flow rate of 0.6 mL/min. The glucose peaks were quantified using a 6-point external standard curve.

Ethanol was quantified using the same HPLC with an Aminex HPX-87H organic acid column (Bio-Rad Laboratories, Hercules, CA) at 65°C. The sample injection volume was 20 µL and 0.005 M sulfuric acid at 0.6 mL/min flow rate was used for the mobile phase.

**Statistical Analysis**

The data was analyzed using Analysis of Variance (ANOVA) in SAS (SAS Inc., Cary, NC, ver. 9.3). The means were compared using t-test and Duncan’s test at \( p < 0.05 \) level of significance.

**Results and Discussion**

**Pretreatment of IF**

The effect of pretreatment method, SAA, LHW, and enzymatic hydrolysis, on IF was evaluated by determining change in composition of IF, and production of ethanol after saccharification and fermentation of treated IF. Among the three pretreatment methods, maximum ethanol production was obtained when enzyme hydrolyzed IF was used for fermentation (Table 2). Although SAA increased glucan content in the treated IF, solid content was significantly reduced by washing IF to remove chemicals and neutralizing pH before enzymatic saccharification. This resulted in increased water consumption and loss of solids including proteins. No significant increase in ethanol production was observed when IF was pretreated with SAA or LHW compared to UIF. Soybeans for EAEP were prepared by cracking (using corrugated roller mill), aspirating (to remove hulls), conditioning (60°C), flaking (to 0.25 mm thickness using a smooth-surface roller mill), and extruding (using twin-screw extruder at 100°C) before extracting with water and protease (de Moura et al., 2011). The mechanical pretreatment ruptures the soybean cell wall comprising of cellulose, hemicellulose, pectin and lignin. Adding PCX during SSF further facilitated breaking down pectin and hemicellulose to release cellulose, arabinose and xylose.
Ouhida, Perez, and Gasa (2002) investigated individual effect of pectinase, cellulase, and xylanase on cellulosic soybean fraction. They reported significant degradation of cellulose after complete extraction of protein, pectin and hemicellulose from the fiber. Cellulase contains all three enzymatic activities (endo and exo-cellulase and β-glucosidase activities) for complete hydrolysis of cellulose; however, its activity is restricted by the presence of other polysaccharides and lignin. Therefore, other enzymes, such as pectinase, are required to synergistically breakdown the cell wall matrix (Beldman, Rombouts, Voragen, & Pilnik, 1984; Sun & Cheng, 2002). Marsman, Gruppen, Mul, and Voragen (1997) found that in defatted soybean water extractable fractions incubated with polysaccharide–degrading enzymes (Bio-Feed Plus, SP-249, Energex, and Driselase), 85% of the released monomers were galactose and arabinose with limited amounts of glucose, mannose, and xylose.

Further, addition of PCX to the IF fermentation slurry led to a decrease in viscosity of the slurry and hence allowed higher glucan loading. It should be noted that in this experiment E. coli KO11 fermentation step was not performed, therefore ethanol was produced primarily from glucose and galactose. It can be concluded from these results that enzymatic hydrolysis (by PCX) is the most efficient method for maximizing ethanol yield and fiber hydrolyzing enzymes and PCX can be added at the SSF step along with S. cerevisiae.

Tests were also performed to evaluate the effect of adding different concentrations of PCX enzymes (5%, 10%, 15% and 20% of soy solids) at the SSF step on ethanol production in IF fermentation. Addition of fiber-hydrolyzing enzymes significantly increased ethanol yield (~2 g/L to 11 g/L; Table 2) compared to no PCX treatment. Cellulase and pectinase have been reported to increase ethanol yields in ethanol fermentations (Beldman et al., 1984; Sun & Cheng, 2002). However, increasing PCX concentration from 5% to 20% (of soy solids) did not have a significant effect on ethanol production from IF slurries. Therefore, 5% concentration of PCX was used in all future fermentations. SSCF of IF by adding E. coli KO11 at the fermentation step in addition to S. cerevisiae fermentation further increased ethanol production (~ 11 g/L to 21-30 g/L; Figure 2). This increase in ethanol production can be attributed to E. coli's ability to convert both hexoses and pentoses (arabinan and xylan) into ethanol as compared to S. cerevisiae, which can only utilize hexoses (glucan and galactan). Figure 2 shows evolution of ethanol production and carbohydrate concentration in IF slurry with time. It can be seen that the increase in ethanol concentration after addition of E. coli KO11 was due to conversion of arabinose and xylose to ethanol.
Table 2. Composition of pretreated IF subjected to SSF with S. cerevisiae.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Total solids (%)</th>
<th>Glucan (%)</th>
<th>Xylan (%)</th>
<th>Total Lignin (%)</th>
<th>Glucan loading (%)</th>
<th>Ethanol concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14.4 ± 0.6a</td>
<td>26.9 ± 6.6b</td>
<td>23.2 ± 13.9a</td>
<td>4.7 ± 0.4a</td>
<td>1%</td>
<td>2.3 ± 0.1b</td>
</tr>
<tr>
<td>SAA</td>
<td>5.1 ± 1.3b</td>
<td>45.8 ± 3.9a</td>
<td>18.7 ± 6.2a</td>
<td>6.0 ± 2.8a</td>
<td>1%</td>
<td>5.2 ± 0.2ab</td>
</tr>
<tr>
<td>LHW</td>
<td>14.8 ± 1.1a</td>
<td>28.4 ± 6.4b</td>
<td>27.0 ± 14.8a</td>
<td>7.3 ± 1.7a</td>
<td>1%</td>
<td>3.8 ± 0.3b</td>
</tr>
<tr>
<td>Enzyme cocktail (PCX)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2%</td>
<td>11.8 ± 2.3a</td>
</tr>
</tbody>
</table>

*SAA: soaking in aqueous ammonia, LHW: liquid hot water, PCX: pectinase, cellulase, xylanase added at 5% of soy solids.

Figure 2. Effect of adding PCX at different concentrations (a) 5% (b) 10% (c) 15%, and (d) 20% of soy solids on ethanol and sugar concentration in IF fermentation.
Scale-up of SSCF of IF

The effect of scaling up of IF fermentation from laboratory scale to pilot plant scale on ethanol production was investigated with two pretreatment methods: SAA and enzymatic hydrolysis (cocktail of enzymes, PCX, added at 5% of soy solids). Scale-up of SAA pretreatment from laboratory (150 mL) to intermediate scale (10 L) significantly decreased ethanol concentration (0.20 kg/kg of dry biomass from 150 mL scale to 0.12 kg/kg of dry biomass from 10 L scale). In addition to safety issues and challenges in washing the fiber after the treatment, SAA pretreatment was difficult to scale up compared to enzymatic treatment. Scaling up enzymatic treatment from 250 mL to 10 L and 60 L using PCX enzyme cocktail resulted in similar ethanol concentrations (Table 3). SSF combined with enzymatic treatment at the fermentation step was more efficient at a 60 L scale compared to 10 L scale. SSCF of IF by the addition of *E. coli KO11* fermentation step in addition to *S. cerevisiae* fermentation resulted in a significant increase in ethanol concentration (Figure 3). Glucan and galactan are major components of IF and can be potentially fermented by *S. cerevisiae* and *E. coli* KO11. Xylan and Arabinan could only be fermented by *E. coli KO11*, which justifies its use in addition to *S. cerevisiae*. Further, utilization of sugars during fermentation increased protein content of the treated IF under all conditions, which will be beneficial for treated IF’s use in animal feed.

Table 3. Composition of initial starting material and effect of scale-up from 10 L to 60 L of enzymatic treatment and fermentation with *S. cerevisiae* and *E. coli KO11* on IF composition (% db) and ethanol production (kg/kg).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (%</th>
<th>Oil (%</th>
<th>Glucan (%)</th>
<th>Galactan (%)</th>
<th>Arabinan (%)</th>
<th>Xylan (%)</th>
<th>Lignin (%)</th>
<th>Solid conversion (%)</th>
<th>Ethanol (kg/kg dry solids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF</td>
<td>11.5 ± 0.5b</td>
<td>2.5 ± 1.7a</td>
<td>27.1 ± 2.7a</td>
<td>31.5 ± 7.8a</td>
<td>15.2 ± 1.0a</td>
<td>13.0 ± 2.2a</td>
<td>7.5 ± 2.0b</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SSCF-10 L</td>
<td>16.5 ± 0.0a</td>
<td>6.5 ± 0.4a</td>
<td>21.7 ± 0.4a</td>
<td>22.3 ± 0.7a</td>
<td>NA</td>
<td>NA</td>
<td>13.8 ±</td>
<td>6.0</td>
<td>0.16</td>
</tr>
<tr>
<td>SSCF-60 L</td>
<td>15.3 ± 0.6a</td>
<td>2.8 ± 0.5a</td>
<td>12.4 ± 0.5b</td>
<td>16.8 ± 0.2a</td>
<td>7.5 ± 0.2a</td>
<td>8.3 ± 0.0a</td>
<td>9.1 ±</td>
<td>5.1</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Figure 3. Evolution of carbohydrate composition and ethanol concentration during (a) lab-scale (150 mL) and (b) pilot-scale (60 L) SSCF with 5% enzyme cocktail (for 10 L only ethanol concentration is shown).
Integrated corn-soy fermentation

Integrated corn-soy fermentation was performed using untreated IF (UIF) and coarsely ground corn. Fermentation of [corn + UIF] slurry compared to [corn only] slurry lead to a significant increase in ethanol production rate (5.5 ± 0.1 g/L in [corn + UIF] from 4.2 ± 0.4 g/L in [corn only]) and ethanol yield (119.5 ± 0.1 g/L in [corn + UIF] from 113.4 ± 1.4 g/L in [corn only]). SSCF of corn-soy slurry with *E. coli KO11* for 24 h after 48 h of yeast-only fermentation did not significantly increase ethanol yield. This additional *E. coli KO11* step was investigated because yeast can only ferment hexoses, while *E. coli KO11* can ferment both hexoses and pentoses (Lau, Christa Gunawan, Venkatesh Balan, & Dale, 2010). As pentoses were present in the slurry, an increase in ethanol concentration was expected. This was true for SSCF of [IF only]. When IF was fermented with *E. coli KO11* after yeast fermentation, an increase in ethanol concentration was observed. However, in corn-soy integrated slurry, the ethanol concentration was greater than 100 g/L, which was not favorable for *E. coli KO11* growth and the conversion of pentoses to ethanol in the integrated [corn + UIF] slurry was not achieved. This was also evident from higher xylan and arabinan concentrations in the whole beers (fermented slurry) (Table 4).

![Figure 4. Ethanol production (g/L) from corn alone and corn + UIF slurries with and without an additional *E. coli KO11* fermentation step. UIF: untreated soy insoluble fiber.](image)

**Table 4. Proximate analyses (db) of the various fractions tested.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Solids (%)</th>
<th>Protein (%)</th>
<th>Oil (%)</th>
<th>Ash (%)</th>
<th>Glucan (%)</th>
<th>Xylan (%)</th>
<th>Galactan (%)</th>
<th>Arabinan (%)</th>
<th>Total Lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UIF</td>
<td>14.4 ± 0.30</td>
<td>11.5 ± 0.50</td>
<td>2.5 ± 0.20</td>
<td>4.0 ± 1.00</td>
<td>27.1 ± 2.70</td>
<td>13.0 ± 2.20</td>
<td>31.5 ± 7.80</td>
<td>15.2 ± 1.70</td>
<td>7.5 ± 0.40</td>
</tr>
<tr>
<td>TIF</td>
<td>14.3 ± 0.10</td>
<td>16.5 ± 0.20</td>
<td>6.5 ± 0.10</td>
<td>6.6 ± 0.20</td>
<td>21.7 ± 0.40</td>
<td>-</td>
<td>22.3 ± 0.07</td>
<td>-</td>
<td>13.8 ± 0.30</td>
</tr>
<tr>
<td>[corn + UIF] a/ferm</td>
<td>11.2 ± 0.20</td>
<td>26.5 ± 0.40</td>
<td>12.0 ± 0.10</td>
<td>4.5 ± 0.20</td>
<td>16.0 ± 0.70</td>
<td>29.4 ± 0.20</td>
<td>9.6 ± 1.70</td>
<td>8.2 ± 0.20</td>
<td>11.8 ± 0.40</td>
</tr>
</tbody>
</table>

*UIF: untreated soy insoluble fiber; TIF: soy insoluble fiber after fermentation, a/ferm: after fermentation. The results (mean ± standard deviation) reported for [corn + UIF] are after fermentation with *S. cerevisiae* only.
Effect of ethanol and sugar concentration on *E. coli KO11*

In a controlled experiment the effect of ethanol and sugar concentration on *E. coli KO11* growth in LB broth was investigated by measuring absorbance values (at 550 nm) over a period of 24 h. Absorbance of LB broth inoculated with *E. coli KO11* at the start of fermentation was ~0.6-0.7.

As ethanol concentration was gradually increased in the LB broth from 10 g/L to 20 g/L, no delay in *E. coli KO11* growth was noticed as indicated by the absorbance values (marked scatter in Figure 5). However, no change in absorbance values was observed when ethanol concentration was increased to 50 g/L or 100 g/L. Ethanol produced in dry-grind corn fermentation is ~110 g/L. Increasing xylose concentration in the LB broth from 50 g/L to 150 g/L did not increase ethanol yield (scatter plot in Figure 5). Hence, ethanol resistance of *E. coli KO11* was independent of the type of fermentable sugars, i.e. glucose (hexose) or xylose (pentose). This was in agreement with the results of Yomano, York, and Ingram (1998). Based on this finding xylose was used as sugar source in the experiments. However, it has been reported that if a mixture of glucose and xylose is present in the media, *E. coli KO11* would utilize glucose first and then xylose Jin et al. (2012).

To test LB broth for production of any other interfering compounds during fermentation, aliquots (1.5 mL) of the fermentation broth were taken at the start and the end of fermentation and analyzed by HPLC (as described in HPLC analysis section). No such interfering compounds were observed. HPLC results revealed consumption of xylose in fermentation broths containing no or low ethanol concentration (0 g/L or 20 g/L) and no xylose consumption in broths containing high ethanol concentrations (50 g/L or 100 g/L) (Table 5). These results validate *E. coli KO11*’s intolerance to higher ethanol concentrations. Intolerance of *E. coli KO11* to ethanol concentration could be a limiting factor in its use in enhancing ethanol production by utilizing pentoses (xylose and arabinose) in corn-soy fermentation. Therefore, to effectively utilize pentoses in corn-soy fermentation either *E. coli KO11* should be added in a step before yeast fermentation when ethanol concentration in the slurry is low (< 50 g/L); or after distillation when ethanol has been removed from whole beer (Taylor, Marquez, Johnston, Goldberg, & Hicks, 2010). A more robust strain of *E. coli* can also be used that can withstand higher ethanol concentrations (Jin et al., 2012; Wen, Bond-Watts, & Chang, 2013; Yomano et al., 1998).
Table 5. Change in Xylose (g/L) and ethanol (g/L) concentration in LB broth (containing 50 g/L or 150 g/L of xylose) during E. coli KO11 fermentation.

<table>
<thead>
<tr>
<th>Ethanol added (g/L)</th>
<th>Time 0 h</th>
<th>Time 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylose (g/L)</td>
<td>Ethanol (g/L)</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>0 (no E.coli)</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>22</td>
</tr>
<tr>
<td>50</td>
<td>49</td>
<td>46</td>
</tr>
<tr>
<td>100</td>
<td>46</td>
<td>87</td>
</tr>
<tr>
<td>50 g/L*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (no E.coli)</td>
<td>87</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>86</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>86</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>84</td>
<td>49</td>
</tr>
<tr>
<td>100</td>
<td>82</td>
<td>88</td>
</tr>
<tr>
<td>150 g/L*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Xylose and ethanol concentrations were determined by HPLC analysis. Ethanol produced (g/L) = E_{24}-(E_0-0.15*E_0); where E_{24}: ethanol concentration (g/L) in broth after 24 h, E_0: ethanol concentration (g/L) in broth at 0 h, 0.15 is average ethanol lost during fermentation.

Prediction of E.coli KO11 kinetics

E. coli KO11 growth in LB broths (at lower ethanol concentrations; 0-20 mg/mL) followed Michaelis-Menten kinetics. To determine kinetics parameters, the data was linearized by Hanes-Woolf plot. The parameters, V\text{max} and K_m with R^2 values are listed in Table 6. V\text{max} values were equivalent when two different sugar sources were used: glucose or xylose, but were higher for fermentation broths containing lower concentrations of ethanol and smaller for broths containing higher concentrations of ethanol. Same was true for K_m values. Lower V\text{max} values indicate slower reaction rate or slower growth of E.coli depicted by lower absorbance values. These parameters were then successfully used to accurately predict the effect of ethanol concentration on E. coli KO11 kinetics (smooth lined scatter in Figure 5). From these results it can be concluded that E. coli KO11 growth prediction is possible using Michaelis-Menten equation and Hanes-Woolf linearization. One disadvantage of using Hanes-Woolf plot is that ordinate and abscissa are not independent variables; both are dependent on fermentation time. However, Lineweaver-Burk plot and Eadie-Hofstee diagram were not able to predict E. coli KO11 growth kinetics as accurately as the Hanes-Woolf plot.
Figure 5. Effect of sugar type (a) glucose or (b) xylose; sugar concentration (c) xylose-50 g/L or (d) xylose-150 g/L; and ethanol concentration on *E. coli* KO11 growth as measured by absorbance values (550 nm). Plots 1 to 3 and 5 to 7 in (a) and (b) are 0, 10, and 20 g/L ethanol concentration in LB broths. Plots 1 to 4 and 5 to 8 in (c) and (d) are 0, 20, 50, and 100-g/L ethanol concentration in LB broths.

Table 6. Michaelis-Menten parameters as predicted by Hanes-Woolf linearization.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Ethanol conc. in LB broth (g/L)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0</td>
<td>2.84</td>
<td>2.11</td>
<td>0.9962</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.84</td>
<td>2.47</td>
<td>0.9989</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.12</td>
<td>3.06</td>
<td>0.9901</td>
</tr>
<tr>
<td>Xylose</td>
<td>0</td>
<td>2.67</td>
<td>1.96</td>
<td>0.9951</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.98</td>
<td>2.99</td>
<td>0.9968</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.97</td>
<td>2.93</td>
<td>0.9961</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Xylose (g/L)</th>
<th>Ethanol conc. in LB broth (g/L)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0</td>
<td>2.48</td>
<td>2.04</td>
<td>0.9842</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.61</td>
<td>5.01</td>
<td>0.9842</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.2</td>
<td>1.09</td>
<td>0.9945</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.11</td>
<td>-0.9</td>
<td>0.9943</td>
</tr>
<tr>
<td>150</td>
<td>0</td>
<td>2.85</td>
<td>7.37</td>
<td>0.8826</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.35</td>
<td>19.91</td>
<td>0.8826</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.2</td>
<td>0.58</td>
<td>0.8954</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.1</td>
<td>-0.99</td>
<td>0.9992</td>
</tr>
</tbody>
</table>
CONCLUSIONS

It was demonstrated that enzymatic hydrolysis is the best pretreatment method, compared to SAA and LHW pretreatment methods, to enhance ethanol production in IF fermentation. SSCF of IF with *S. cerevisiae* and *E. coli* KO11 with the addition of fiber hydrolyzing enzymes, pectinase, cellulase and xylanase, led to maximum ethanol production. Co-fermentation of IF with *E. coli* KO11 enhanced ethanol production by conversion of arabinose and xylose into ethanol. Addition of soy IF to dry-grind corn fermentation increased ethanol production rate, but low ethanol tolerance of *E. coli* KO11 was a limiting factor in achieving SSCF of corn-soy slurry with *S. cerevisiae* and *E. coli* KO11. Michaelis-Menten equation was successfully used to predict *E. coli* KO11 growth kinetics by Hanes-Woolf linearization.

ACKNOWLEDGEMENTS

This research was funded by USDA NIFA grant # 416-45-45-13-5362. We acknowledge the assistance of Shannon Box, Dr. Bishnu Karki, and Dr. Linxing Yao.
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