Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars

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15

16   **Abstract**

17   Thermo-mechanical extrusion pretreatment for lignocellulosic biomass was

18   investigated using soybean hulls as the substrate. The enzyme cocktail used to

19   hydrolyze pretreated soybean hulls to fermentable sugars was optimized using response

20   surface methodology (RSM). Structural changes in substrate and sugar yields from

21   thermo-mechanical processing were compared with two traditional pretreatment

22   methods that utilized dilute acid (1% sulfuric acid) and alkali (1% sodium hydroxide).

23   Extrusion processing parameters (barrel temperature, in-barrel moisture, screw speed)

24   and processing aids (starch, ethylene glycol) were studied with respect to reducing sugar

25   and glucose yields. The conditions resulting in highest cellulose to glucose conversion

26   (95%) were screw speed 350 rpm, maximum barrel temperature 80°C and in-barrel

27   moisture content 40% wb. Compared with untreated soybean hulls, glucose yield from

28   enzymatic hydrolysis of soybean hulls increased by 69.6, 128.7 and 132.2%,

29   respectively, when pretreated with dilute acid, alkali and extrusion.

30

31   Keywords: soybean hulls; lignocellulosic; ethanol; pretreatment; extrusion

32   1. Introduction

33   The National Biofuels Action Plan released in October 2008 states that

34   expanding annual biofuels production to 36 billion gallons by 2022 would be a key

35   component in America's movement towards clean, affordable and secure energy

36   sources. Corn ethanol, which is categorized as a first generation biofuels, has grown

37   steadily over the last few years to 12.1 billion gallons in 2010 (Service, 2010). However,

38   corn ethanol alone cannot meet the increasing demand for biofuels because of

39   sustainability issues (Perlack et al., 2005). The Renewable Fuel Standard 2 (RFS2),

40   which was published in March 2010, introduced a 15 billion gallon cap for corn ethanol

41   by 2015 and set the goal of 21 billion gallons for advanced biofuels by 2022. The latter

42   include cellulosic ethanol, biodiesel and other nonconventional biofuels.

43   Lignocellulosic biomass is abundantly available, and has potential for better

44   energy balance than corn ethanol. Additionally, cellulosic ethanol provides the benefit

45   of reducing greenhouse gas emissions by up to 86% (Wang et al., 2007). Potential

46   feedstock includes perennial energy crops such as switch grass, forest residues such as

47   wood, crop residues such as sugarcane bagasse, corn cobs, corn stover and wheat straw,

48   and agricultural co-products such as soybean hulls and wheat bran. In the USA 10.8

49   billion pounds of soybean hulls are produced every year, as a result of processing 1.8

50   billion bushels of soybeans for oil, protein and flour (Corredor et al., 2008). This co-

51   product is used as a low-value protein/ fiber supplement in ruminant animal feed, but its

52   demand is expected to reduce due to availability of distiller's dry grains with solubles

53   (DDGS) as a substitute. Soybean hulls contain 46-51% cellulose, 16-18% hemicellulose

54   and 1.4-2% lignin (Corredor et al., 2008; Blasi et al., 2000), which makes them an

55   attractive source of fermentable sugars for ethanol production. In addition to their high

56 cellulosic content, soybean hulls do not require as extensive a grinding process prior to

57 pretreatment as some other feedstocks.

58   Cellulosic ethanol production is different from corn ethanol, due to the

59 'recalcitrance' of the substrate. An additional pretreatment step is required because the

60 lignocellulosic structure is very complex, highly crystalline, and resistant to enzymatic

61 degradation in its native state. Despite the ongoing research on pretreatment

62 technologies and other aspects of cellulosic ethanol production the current production

63 costs are still too high to compete in the market place (Galbe et al., 2007). In 2010,

64 production of cellulosic ethanol in the USA, all by demonstration facilities, was

65 expected to total 25.5 million gallons (Service, 2010). This is far less than original

66 expectations, due to the economic slump, uncertainty among policymakers, an

67 oversupply of first generation biofuels, transportation logistics for raw materials, and

68 technological gaps that make a cellulosic ethanol plant unviable. To overcome at least

69 part of these obstacles, transformational scientific breakthroughs are urgently needed in

70 pretreatment procedures, enzyme systems for conversion of pretreated substrate to

71 fermentable sugars and efficient microorganisms to ferment mixed sugars to ethanol

72 (Saha and Bothast, 1999).

73   In order to break down the structural integrity of lignocellulosic biomass and

74 enhance enzymatic action on cellulose, various chemical, mechanical, thermo-chemical

75 and biochemical pretreatment have been investigated, including acid hydrolysis, alkali

76 hydrolysis, the organosolv process, steam explosion, ammonia fiber explosion (AFEX),

77 pyrolysis, hot water treatment and microorganism treatment (Corredor et al., 2008;

78 Galbe et al., 2007; Galbe and Zacchi, 2007; Sun and Cheng, 2002). There have been

79 some successes in terms of increased ethanol yield, however no single method has yet

80 been found suitable for commercial application (Saha and Cotta, 2007). The challenge

81　lies in increasing the efficiency of cellulose conversion to the extent that the overall

82　ethanol production process becomes cost-effective.

83　　　　This article primarily focuses on pretreatment methods, and to a certain degree

84　on optimum enzyme systems. Most of the above mentioned pretreatment technologies

85　have inefficiencies related to batch processing, small capacity and low solids loading. A

86　recent publication by our laboratory (Lamsal et al., 2010) described the application of a

87　promising technology, extrusion processing, which can provide a unique and

88　continuously stirred thermo-chemical reactor environment in combination with

89　mechanical energy or shear. The shear forces applied by the extrusion screw serve to

90　continuously remove the softened surface regions of the substrate and expose the

91　interior to chemical and/or thermal action, thus improving the overall rate of cellulose

92　conversion. The high throughputs associated with extrusion further increase its

93　attraction as an effective pretreatment technology. In this study soybean hulls were used

94　as the model lignocellulosic substrate, with the objectives of development of a protocol

95　for thermo-mechanical extrusion pretreatment, optimization of the enzyme cocktail for

96　downstream hydrolysis to sugars, and comparison of conversion efficiency of extrusion

97　with two traditional pretreatments, acid hydrolysis and alkali hydrolysis.

98

99　**2. Materials and Methods**

100　*2.1 Lignocellulosic biomass*

101　　　　Soybean hulls (Archer Daniels Midland Company, Salina, KS) were ground to a

102　particle size less than 1041µm by using an experimental laboratory roller mill (Ross

103　Roller Mill, Ross Machine & Mill Supply Inc., Oklahoma City, OK). All experiments

104　described in this study utilized ground soybean hulls as the substrate. The

105　lignocellulosic composition of soybean hulls was determined using the ANKOM Fiber

106   Analyzer (ANKOM Technology, NY), and starch content was determined using the

107   standard glucoamylase method (920.40; AOAC 2010).

108   *2.2. Dilute acid and alkali pretreatment*

109   Ground soybean hulls were subjected to two traditional pretreatment methods,

110   dilute acid hydrolysis and alkali hydrolysis, which were adapted from previous studies

111   (Corredor et al., 2008; Saha and Bothast, 1999). The substrate (10%, w/w) was added to

112   1% (w/w) sulfuric acid or 1% (w/w) sodium hydroxide solutions (both chemicals were

113   obtained from Sigma Aldrich, St. Louis, MO), and autoclaved (SS-325E; Tomy Tech

114   USA, Inc., Fremont, CA) at a temperature of 121°C for 30 min. The remaining solids

115   were neutralized and washed three times with 500 ml of deionized water at 85°C. This

116   was followed by 10 min of centrifugation (10,000×g) and subsequent drying in an air

117   oven at 45°C for 24 h.

118   *2.3. Enzyme cocktail optimization*

119   A combination of cellulase (Celluclast 1.5L), β-glucosidase (Novozyme 188)

120   and a cell-wall degrading enzyme complex (Viscozyme® L) were used for enzymatic

121   saccharification of pretreated soybean hull. All enzymes were obtained from Novozyme,

122   Franklinton, NC. Cellulase was used to break down cellulose into glucose, cellobiose

123   and higher glucose polymers. The enzyme activity of Cellulcast 1.5L was 80 FPU (filter

124   paper unit)/mL with optimal pH range 4.5-6.0 and optimal temperature range of 50-

125   60°C. β-glucosidase with enzyme activity of 322 CBU (cellobiase unit)/mL was used to

126   hydrolyze cellobiose, which inhibits the initial stage of cellulose hydrolysis.

127   Viscozyme® L contained a wide range carbohrases, including arabinase, cellulase, β-

128   glucanase, hemicellulose and xylanase, which act on branched pectin-liked substances

129   found in plant cell walls. Its activity was 134 FBG (fungal beta-glucanase unit)/mL.

130   Response surface methodology was used to optimize the amount of each enzyme for

131 maximum reducing sugar yield, using soybean hulls pretreated with acid hydrolysis as

132 the substrate . The experimental design (Table 1) had fifteen combinations with 3 levels

133 (-1, 0, 1) of each enzyme and 3 replicates at the center point (Box and Draper, 1987).

134 Results were analyzed using SAS software (v.9.2, SAS Institute, Cary, NC) at $p < 0.05$.

135 Three dimensional plots of reducing sugar yield for various enzyme dosages were

136 generated, model coefficients were obtained using the response surface regression

137 (RSREG) procedure, and optimum enzyme dosage determined.

138 *2.4. Extrusion pretreatment - high starch*

139   Thermo-mechanical or extrusion pretreatment of soybean hulls, the primary

140 focus of this study, was conducted using a laboratory-scale twin-screw extruder (Micro-

141 18, American Leistritz, Somerville, NJ) with a six-head configuration, screw diameter

142 of 18 mm, L/D ratio of 30:1 and 2.4 mm circular die opening. Prior to extrusion,

143 soybean hulls were mixed with 10 and 20% corn starch (Archer Daniels Midland

144 Company, Salina, KS) as a processing aid to facilitate the flow of soybean hulls. The

145 soybean hull and corn starch blends were hydrated using a Hobart mixer (The Hobart

146 Mfg. Co., Troy, OH) and equilibrated for 24 h at room temperature before extrusion in

147 order to obtain 20 and 25% (wet basis or wb) moisture. This is also referred to as the

148 process moisture or in-barrel moisture. The extruder screw configuration and barrel

149 temperature profile are shown in Fig. 1. Three temperature profiles with maximum

150 barrel temperatures of 80, 110 and 140°C (at the discharge end) were used. Extruder

151 screw speed was fixed at 420 rpm. The feeder screw speed rate was set at 275 rpm,

152 which resulted in a feed rate ranging from 1.4 to 2.0 kg/h depending on the moisture and

153 starch content of the feed. Extruded soybean hull pellets were dried in a convection

154 oven at 45°C for 24 h. The dried pellets, having a moisture content of 9-10% (wb), were

155 stored at room temperature. Unlike acid or alkali hydrolyzed soybean hulls, the extruded

156 soybean hulls did not require neutralization or washing steps prior to enzymatic

157 hydrolysis.

158   Specific mechanical energy (SME) input during the extrusion process was

159 computed as follows:

160
$$SME\,(kJ/kg) = \frac{\frac{(\tau - \tau_0)}{100} \times (\frac{N}{N_r}) \times P_r}{\dot{m}}$$
(1)

161 where, τ is the % torque; τ_0 is the no load % torque; N is the screw speed; N_r is the

162 rated screw speed (500 rpm); P_r is the rated motor power (2.2 kW); and $\dot{m}$ is the mass

163 flow rate or feed rate (kg/s).

164 *2.5. Extrusion pretreatment – low starch*

165   Another set of thermo-mechanical pretreatments was carried out with low levels

166 of starch addition (0 and 5%). Results from the above experiment and also a previous

167 study (Lamsal et al., 2010) indicated that in the case of soybean hulls reduced amount or

168 absence of the processing aid led to surging, burning of the substrate and even blocking

169 of the die during extrusion. To avoid these problems, higher levels of in-barrel moisture

170 were required. Soybean hulls with 5% starch were hydrated to moisture levels of 20, 25,

171 30, 35 and 40% (wb), whereas the moisture levels for soybean hull without starch were

172 adjusted to 40, 45 and 50% (wb). All other process parameters were same as in the

173 previous experiment. At the same feeder screw speed of 275 rpm, the resultant feed rate

174 however was much lower, ranging from 0.48 to 0.84 kg/h, because of the higher

175 moisture and lower starch level. Only one barrel temperature profile was evaluated

176 (maximum 80°C). In an additional experiment, thermo-mechanical pretreatment of

177 soybean hulls without starch was evaluated at 40% (wb) in-barrel moisture content and

178 three different extruder screw speeds (280, 350 and 420 rpm). All other process

179 conditions were same as above.

180   *2.6. Extrusion pretreatment - ethylene glycol*

181   Ethylene glycol has been used for fibrillation of lignocellulosic materials such as

182   wood (Lee et al., 2009). One set of experiments was conducted to study its effectiveness

183   with soybean hulls in combination with extrusion pretreatment. Solutions were prepared

184   at ratios of ethylene glycol (Sigma-Aldrich, St. Louis, MO) to water of 1:1, 1:3 and 1:9.

185   Pure water and ethylene glycol were used as controls. Soybean hulls were blended with

186   solvent at a ratio of 2:1. The same extrusion processing conditions were used as

187   described above (Section 2.5)

188   *2.7. Enzymatic hydrolysis and sugar analysis*

189   Pretreated soybean hulls (10% w/w) were hydrolyzed by using the optimized

190   enzyme cocktail dosage in 0.05M sodium acetate buffer at pH 5, and incubation at 50°C

191   for 72 h. Soybean hulls without any pretreatment were also subjected to enzymatic

192   saccharification as a control. Total reducing sugar yield was determined by using the

193   3,5-dinitrosalicylic acid (DNS) reagent method (Miller, 1959), while glucose

194   concentration was analyzed by high performance liquid chromatography (Agilent 1100;

195   Agilent Technologies; Santa Clara, CA) using a Phenomenex Rezex ROA organic acid

196   column (130x7.8 mm, H+ (8%); Phenomenex, Torrance, CA). The mobile phase was

197   0.005N sulfuric acid at 60°C with flow rate of 0.6 ml/min.

198   Reducing sugar and glucose yields were expressed in terms of g/g extruded

199   pellet. The degree of conversion to reducing sugars (C_{RS}%) was calculated based on

200   total cellulose and hemicellulose content in soybean hulls and added starch, as follows.

201
$$C_{RS}\% = \frac{Y_{RS}}{(C+H)\times 1.1}\times 100$$
(2)

202   where, C = amount of cellulose in 1 g of pellet (g/g), H = hemicellulose in 1 g of pellet

203   (g/g), 1.1 = conversion factor for polymer to monomer sugar, Y_{RS} = reducing sugar

9

204   yield (g/g). The degree of conversion from cellulose to glucose conversion ($C_{CG}\%$) was

205   determined as the ratio of the glucose obtained to the theoretical yield based on the

206   amount of cellulose in soybean hulls.

207
$$C_{CG}\% = \frac{Y_G}{C \times 1.1} \times 100$$
(3)

208   where Y_G = glucose yield (g/g) and C = amount of cellulose in 1 g of pellet. Both

209   reducing sugar and glucose yields were corrected for the contribution of starch,

210   assuming 100% conversion of the latter.

211   *2.8. Structural characteristic by using X-ray diffraction*

212   X-ray diffraction (XRD) patterns of the lignocellulosic substrate before and after

213   pretreatment were analyzed using an X-ray diffractometer (D-8 Advance, Bruker AXS,

214   Karlsruhe, Germany) set at 40 kV and 40 mA. Samples were scanned in the range of

215   diffraction angle 2θ = 10-40° with a step size of 0.05° and a scan speed of 4°/min. The

216   crystallinity index (CrI) was the percentage of crystalline material in the biomass and

217   was used to compare the effects of various treatments on the structural modification of

218   lignocellulosic biomass. It was defined as follows.

219
$$CrI = \frac{(I_{002} - I_{am})}{I_{002}} \times 100$$
(4)

220   where I_{002} is the diffraction intensity at 2θ = 22.6°, which represents both the crystalline

221   and amorphous regions, and I_{am} is the diffraction intensity at 2θ = 18.7°, representing

222   the amorphous material (Yoshida et al., 2008; Zhao et al., 2008).

223

224   **3. Results and Discussion**

225   *3.1. Lignocellulosic structure of soybean hulls*

226   The soybean hulls contained 35.35, 17.21 and 2.33% (dry basis or db) of

227   cellulose, hemicellulose and lignin, respectively (Table 2), and 0.83% (db) starch. The

10

228 remaining portion is reported to comprise of protein, fat, pectin, sugars and ash

229 (Corredor et al., 2009; Schirmer-Michel et al., 2008). Cellulose is mainly comprised of

230 glucose monomers, while hemicellulose is composed of mannose and xylose (Huismann

231 et al., 1998; Stombaugh et al., 2000). Typically, cellulose exits as microfibrils, which

232 are sheathed with hemicellulose and aligned in the direction of cell walls. Lignin forms

233 the glue that holds the fibers together, and has a complex, cross linked structure whose

234 building units include monomers such as guaiacyl and syringyl. Pectin performs a

235 similar function and mainly consists of arabinose, galactose, rhamnose, fructose and

236 uronic acid. As cellulose is the only source of glucose, the theoretical yield of latter was

237 calculated as 0.39 g/ g soybean hull based on cellulose composition, and by multiplying

238 by 1.1 to account for the water of hydrolysis.

239 *3.2. Enzyme cocktail optimization*

240   Response surface analysis of enzymatic saccharification data resulted in a

241 statistical model for describing reducing sugar yield from dilute acid pretreated soybean

242 hulls. The model ($R^2 = 0.96$) equation is described below.

243 $$Y_{RS} = 5.18 + 699.51X + 75.72Y + 52.02Z - 3623.71X^2 - 365.15Y^2 - 375.27Z^2$$

244 $$- 824.76XY - 1.69XZ + 463.82YZ \qquad\qquad (5)$$

245 where Y_{RS} = reducing sugar yield (g/g), X= cellulase (mL/g cellulose), Y= β-

246 glucosidase (mL/g cellulose), and Z= cell wall degrading enzyme complex (mL/g

247 cellulose).

248   Based on the above, maximum reducing sugar release was predicted at 0.0873,

249 0.0806, and 0.1189 ml/g cellulose for cellulase (Celluclast 1.5L), β-glucosidase

250 (Novozyme 188) and cell-wall degrading enzyme (Viscozyme), respectively. This

251 combination of enzymes was used for all subsequent enzymatic saccharification

252 experiments in this study. Cellulose concentration and its second order term, i.e. *X* and

253   X^2, had the greatest contribution to the statistical model with p=0.0002 and 0.0037,

254   respectively. Cellulase acts directly on cellulose and degrades it to lower molecular

255   weight glucans and glucose. Previous studies on enzymatic saccharification of

256   lignocellulosic substrates have used cellulase loadings in the wide range of 7 to 33 FPU

257   /g substrate (Sun and Cheng, 2002). In this study, the optimum amount of 0.0873 ml/g

258   cellulose corresponded to 6.74 FPU/g cellulose, which is a lower cellulose dosage than

259   that used in most previous studies. The addition of β-glucosidase achieved better

260   saccharification by hydrolyzing cellobiose, which as mentioned before was an

261   intermediate product of cellulose hydrolysis and inhibited cellulase activity. Cellulase

262   has been supplemented with β-glucosidase in many other studies, and the application of

263   Novozyme 188 was recommended by the manufacturer at the ratio of 1:0.2 for

264   Celluclast 1.5L to Novozyme 188 for initial industrial trials (Note, 1990). Substantial

265   amount of hemicellulose was presumed to be dissolved and removed by the dilute acid

266   pretreatment. However, the presence of cell wall degrading enzyme complex did

267   contribute to the efficiency of enzymatic saccharification. The primary mechanism was

268   hydrolysis of the residual hemicellulose and increase in cellulase accessibility to the

269   cellulose (Fan et al., 1987). Cell wall degrading enzymes have been reported to act on

270   even untreated soybean hulls, resulting in more than 97% increase in sugar yield during

271   enzymatic hydrolysis with cellulase (Corredor et al., 2009).

272   It was clear from optimization results that enzyme concentration beyond a

273   certain level led to a decrease in the amount of reducing sugar liberated. Faster sugar

274   production with an enzyme overdose might lead to inhibition of the hydrolysis process

275   (Drissen et al., 2009). Enzyme dosage also significantly affects the cost of the overall

276   ethanol production process, which is another reason for optimizing the dosage.

277   *3.3. Crystallinity of pretreated soybean hull*

278   The crystallinity index (CrI) of soybean hulls prior and subsequent to various

279   pretreatments is shown in Fig. 2. Increase in cellulose crystallinity after pretreatment of

280   lignocellulosic substrates using sulfuric acid, sodium chlorite, peracetic acid or other

281   chemicals has been observed previously (Weimer et al., 1995; Corredor et al., 2008;

282   Yoshida et al., 2008; Zhao et al., 2008). In the current study also the average

283   crystallinity of the substrate increased due to chemical pretreatment with acid (AH) or

284   alkali (ALH). The two processes led to removal of hemicellulose and delignification,

285   respectively, and thus increased the concentration of cellulose and CrI. This was

286   confirmed by the lignocellulosic composition data (Table 2). After sulfuric acid and

287   sodium hydroxide pretreatments, the concentration of cellulose increased by 75 and

288   89%, respectively, due to removal of other components such as hemicellulose from the

289   substrate. This would lead to greater exposure of cellulose to enzymatic action and more

290   efficient sachharification. It was clear that the mechanism by which acid and alkali

291   pretreatments render the substrate more susceptible to enzymatic hydrolysis does not

292   rely on decreased crystallinity. Lee et al. (2009) suggested that opening of the cell wall

293   structure at the microscopic scale due to some pretreatment would be sufficient for

294   enzymatic saccharification regardless of cellulose crystallinity. Yoshida (2008) also

295   found that delignification increased the rate of enzymatic hydrolysis of cellulose and

296   hemicellulose, although delignified biomass showed higher crystallinity. They

297   concluded that lignin is the most significant factor interfering with the enzymatic

298   hydrolysis of lignocellulosic biomass. However, lowering of cellulose crystallinity and

299   increase in substrate surface area due to mechanical pretreatment processes such as ball

300   milling (Ouajai and Shanks, 2006; Yoshida et al., 2008) can also lead to improved

301   enzymatic saccharification.

13

302   In comparison, thermo-mechanical extrusion pretreatment appeared to have a unique

303   impact on substrate morphology. Cellulose crystallinity increased by 82% (Fig. 2), even

304   though there was no significant change in lignocellulosic composition (Table 2). This

305   indicated crystallization of the amorphous phase of cellulose during thermo-mechanical

306   processing. Other researchers have observed recrystallization of cellulose in the

307   presence of moisture and heat, during or subsequent to pretreatment processes such as

308   steam explosion and ball milling (Tanahashi et al., 1983; Bertran and Dale, 1985;

309   Ouajai and Shanks, 2006). Different degrees of recrystallization of ball milled hemp

310   cellulose, resulting from various water amounts, drying rates and agitation speed, were

311   observed by Ouajai and Shanks (2006). Bertran and Dale (1985) also reported that

312   amorphous cellulose from cotton linter and wood was recrystallized in aqueous media

313   subsequent to ball milling, and concluded that water promoted recrystallization.

314   Tanahashi et al. (1983) found that the crystallinity of cellulose in wood increased after

315   high pressure steam explosion due to the transformation of cellulose from amorphous to

316   crystalline phase. Similarly, extrusion is a high pressure and temperature process, and it

317   is reasonable to expect inducement of cellulose crystallization in the presence of

318   moisture as supported by data.

319   *3.4. Effect of on extrusion pretreatment on saccharification*

320   Extrusion is widely used in in the food and plastics industry and the

321   corresponding process dynamics have been extensively studied and understood. In

322   contrast, extrusion of lignocellulosic material is far more complex and challenging,

323   primarily because the material does not melt even at high temperatures and

324   consequently shows poor flow properties inside the extruder barrel and die (Prat et al.,

325   1999). This was observed in the current study also as described below.

326   *3.4.1. Barrel temperature*

327   Extrusion experiments were conducted at maximum barrel temperatures of 80,

328   110 and 140°C. At higher barrel temperature (110 and 140°C), processing conditions

329   were unstable. For example, large fluctuations in motor torque and die pressure were

330   observed, the flow occurred in surges, and burning of soybean hulls inside the barrel

331   and die led to frequent blockage and cessation of screw rotation. The little amount of

332   soybean hulls that could be extruded at 110 and 140°C appeared to be darker than those

333   extruded at 80°C, which again pointed towards burning. Reducing sugar yields are

334   summarized in Fig. 3. The contribution of starch, assuming complete hydrolysis, was

335   expressed in darker shade for each treatment. Y_{RS} ranged from 0.46-0.61 g/g pellet,

336   which corresponded to conversion percentage (C_{RS}%) of 79-104% after subtracting the

337   contribution of starch. Greater than 100% conversion was probably because of

338   naturally present sugars in untreated soybean hull. Significant trends in Y_{RS} were

339   however were not observed with respect to the barrel temperature. As soybean hulls

340   could be processed with relative ease at 80°C without any impact on sugar yield, that

341   barrel temperature was considered to be optimum and utilized in all subsequent

342   experiments and analyses.

343   *3.4.2. Starch and in-barrel moisture*

344   As mentioned earlier, starch was added to soybean hulls at levels of 5, 10 and 20%

345   as a processing aid that facilitated flow during extrusion. Lignocellulosic materials such

346   as soybean hulls are otherwise tough to extrude especially at low in-barrel moisture

347   (<35% wb), as determined previously by our research group (Lamsal et al., 2010).

348   Materials like wheat bran are an exception, as they naturally contain as much as 19%

349   starch, which allows extrusion at in-barrel moistures of 20-25% wb (Lamsal et al., 2008;

350 Lamsal et al., 2010). The starch is gelatinized during extrusion and forms a viscous melt,

351 which assists in pushing the lignocellulosic material through the extruder.

352 Glucose yields (Y_G) from experiments at barrel temperature of 80°C and

353 constant screw speed of 420 rpm are summarized in Fig. 4. As before the contribution

354 of starch, assuming complete hydrolysis, was expressed in darker shade. Without taking

355 the starch into account, the conversion percentage was as high as 130%. Thus it was

356 obvious that glucose and higher molecular weight dextrins were produced from

357 hydrolysis of starch during thermo-mechanical treatment and subsequent enzymatic

358 saccharification. The latter effect was probably due to side activities of Viscozyme,

359 Novozyme 188 and Celluclast 1.5L, which is typical of enzymes produced from natural

360 strains of fungi.

361 Overall glucose yields (Y_G) ranged from 0.32-0.38 g/g pellet, and did not exhibit

362 any trends with respect to starch level. It should be noted that soybean hulls without any

363 starch could also be extruded, provided in-barrel moisture was high enough (40-50%

364 wb), resulting in Y_G comparable to treatments with 5-20% starch addition. After

365 subtracting the contribution of starch, glucose yields ranged from 0.15-0.35 g/g and the

366 corresponding cellulose to glucose conversion percentage (C_{CG}%) ranged from 51-90%.

367 C_{CG}% had a clear decreasing trend with respect to starch level. For example, at 20% in-

368 barrel moisture C_{CG}% decreased from 78% to 51% as starch increased from 10 to 20%.

369 The corresponding decline was from 63% to 51% at 25% in-barrel moisture. Such a

370 trend was also observed for reducing sugar conversion percentage (C_{RS}%) (Fig. 3).

371 Specific mechanical energy (SME) ranged from 738-1905 kJ/kg, and increased starch

372 level resulted in higher SME due to greater viscosity development. However, it was

373 clear that both Y_G and C_{CG}% were not proportional to SME. One reason could be that

16

374 mechanical energy was distributed unevenly between soybean hulls and starch, in favor

375 of the latter.

376   The above reasoning also appeared to be valid for the relationship between in-

377 barrel moisture, SME and glucose yield at high starch levels (10-20%). Higher motor

378 torque was obtained as in-barrel moisture increased from 20 to 25% due to greater

379 viscosity development, which in turn led to increase in SME. However, Y_G and C_{CG}%

380 either decreased or remained the same. On the other hand at lower starch levels (0-5%),

381 torque exhibited the near universal trend of increasing with lower in-barrel moisture. In

382 the absence of adequate starch, mechanical energy was directed towards soybean hulls

383 and exposed the interior of the substrate to thermal energy and subsequent enzymatic

384 saccharifcation, thus leading to greater Y_G and C_{CG}%. This demonstrated the primary

385 mechanism involved in thermo-mechanical pretreatment of lignocellulosic substrates,

386 with higher torque breaking down the cell wall structure more efficiently and liberating

387 the cellulose microfibrils (Lee et al., 2009; Lamsal et al., 2010). The drop in glucose

388 yield observed as in-barrel moisture decreased from 45 to 40% was contrary to the

389 above mentioned trend and might be due to degradation of sugars. Factors other than

390 those discussed above might also be at work, including interactions between starch and

391 lignocellulosic components, competition between them for water, and also residence

392 time of material inside the extruder barrel.

393 *3.4.3. Screw speed*

394   Fig. 5 summarizes glucose yields (Y_G) from thermo-mechanical pretreatment

395 experiments at screw speeds ranging from 280 to 420 rpm, while barrel temperature and

396 in-barrel moisture were constant at 80°C and 40% wb, respectively. Specific mechanical

397 energy (1478-1528 kJ/kg) increased with screw speed, which is again a universal trend

398 observed during extrusion. Y_G ranged from 0.22 g/g pellet to 0.37g/g pellet, with the

17

399   maximum value obtained at the intermediate screw speed of 350 rpm. As discussed in

400   section 3.4.2, in the absence of starch mechanical energy was directed at soybean hulls,

401   thus improving glucose yield as screw speed increased from 280 to 350 rpm. The

402   excessive mechanical energy input at the highest screw speed of 420 rpm possibly

403   resulted in degradation of sugars and reduction in glucose yield. The low residence time

404   associated with high screw speeds might also be a factor, and could have led to reduced

405   thermal exposure of the substrate during extrusion and subsequently lower

406   saccharifcation.

407   *3.4.4. Ethylene glycol*

408   The use of ethylene glycol (EG) as a processing aid was based on a previous

409   study involving thermo-mechanical pretreatment of wood (Lee et al., 2009). EG was

410   used because of its cellulose affinity. Its addition was shown to accelerate the

411   fibrillation process during extrusion of lignocellulosic materials because it intercalates

412   between cellulose microfibrils, thus preventing reaggregation of liberated microfibrils

413   due to strong hydrogen bonds. While Lee et al. (2009) observed 62.4% cellulose to

414   glucose conversion by extruding Douglas fir with 200% EG, this approach was not

415   successful in the current study. Soybean hulls could not be processed with either 100%

416   EG or 1:1 solution of EG: water. Decreasing the EG: water ratio to 1:3, 1:9 and 100%

417   water allowed extrusion of the soybean hulls and led to successively higher glucose

418   yield at 0.19, 0.24 and 0.27 g/g pellets, respectively, which corresponded to 48-70%

419   cellulose to glucose conversion percentage. The low amount of solvent used (50%) and

420   differences in the extrusion process (counter rotating extrusion was used by Lee et al.,

421   2009) might be some reasons for the poor performance of EG.

422   *3.5. Comparison of thermo-mechanical pretreatment with other methods*

423   Fig. 6 shows data for cellulose to glucose conversion percentage (C_{CG}%) after

424   enzymatic hydrolysis of soybean hulls subjected to different pretreatments, including

425   acid (AH), alkali (ALH) and thermo-mechanical extrusion (EX). Enzymatic hydrolysis

426   of untreated soybean hulls (SH) was the control, which resulted in glucose yield and

427   conversion percentage of 0.16 g/g and 40.8%, respectively. The grinding process for

428   soybean hulls exposed enough of the cellulosic structure to allow substantial

429   saccharification even without any pretreatment. All three pretreatments led to

430   improvement in glucose yield as compared to the control (69.6, 128.7 and 132.2%

431   increase for AH, ALH and EX, respectively) although the mechanisms by which it was

432   brought about differed greatly.

433   After enzymatic hydrolysis of AH and ALH pretreated soybean hulls, glucose

434   yield was 0.27 and 0.36 g/g, respectively, which corresponded to 69.2 and 93.3%

435   cellulose conversion. Dilute sulfuric acid pretreatment improved the efficiency of

436   enzymatic hydrolysis by solubilization and removal of hemicellulose and increasing

437   cellulose accessibility (Torget et al., 1990). Alkaline pretreatment promotes cellulose

438   conversion by delignification due to the degradation of ester bonds and cleavage of

439   glycosidic linkages in the cell wall matrix, which lead to the alteration of the structure

440   of lignin and reduction of the lignin-hemicellulose complex (Cheng et al., 2010). This

441   might also be accompanied by swelling of cellulose and its partial decrystallization. The

442   cellulose conversion from AH and ALH treatments in this study were comparable with

443   results reported previously. Corredor et al. (2009) observed a maximum cellulose to

444   glucose conversion of 73% for soybean hulls by applying the combination of 2% H_2SO_4

445   at 140°C, followed by steam explosion and enzymatic hydrolysis for 36 h. Martin et al.

446   (2007) pretreated rice hulls with 2% of H_2SO_4 solution at a solid to liquid ratio of 1:10

447   at 121°C, resulting in 61.4% cellulose conversion after saccharifcation. Saha et al. (2005)

448 showed that fermentable sugar yields from rice hulls varied, depending on the acid

449 solution concentration, reaction time and temperature. Less fermentation inhibitors were

450 observed at lower reaction temperature, and the maximum monomeric sugar yield of 60%

451 was obtained by treatment with 1% H_2SO_4 at 121°C. Glucose yield from coastal

452 Bermuda grass after alkali hydrolysis using 0.75% NaOH at 121°C reached up to 90%

453 with 86% of lignin removal (Wang et al., 2010).

454 Thermo-mechanical pretreatment (EX) was comparable or better than the more

455 established chemical methods (AH and ALH), with regard to saccharification efficiency.

456 Enzymatic hydrolysis of EX pretreated soybean hulls (no starch, 40% moisture, 350

457 rpm) led to glucose yield of 0.37 g/g and conversion of 94.8%. The primary mechanism

458 was disruption of cell wall structure due to a combination of mechanical and thermal

459 energy. The conversion percentage for EX was even better than that reported by some

460 recent studies involving extrusion. Our lab group previously reported reducing sugar

461 yields of 60-73% and 25-36% for wheat bran and soybean hulls, respectively, after

462 pretreatment with twin screw extrusion followed by saccharification (Lamsal et al.,

463 2010). Karuppuchamy and Muthukumarappan (2009) and Karunanithy and

464 Muthukumarappan (2010a; 2010b) used a single screw extruder for pretreatment of

465 soybean hulls, corn stover, switchgrass and prairie cord grass followed by enzymatic

466 hydrolysis, resulting in 62, 61, 45 and 66% sugar recovery, respectively. These authors

467 also reported absence of fermention inhibitors such as hydroxymethylfurfural, which are

468 often produced during chemical pretreatment (Torget et al., 1990; Saha et al., 2005).

469

470 **4. Conclusions**

471 Thermo-mechanical extrusion was shown to be a feasible pretreatment method

472 for lignocellulosic ethanol production. The challenge of processing lignocellulosic

473   substrates with poor flow properties can be overcome by utilizing high in-barrel

474   moistures and/ or processing aids such as starch. Cellulose conversion from extrusion

475   pretreatment of soybean hulls was comparable or better than that obtained from

476   traditional chemical pretreatments utilizing acid and alkali. The enzyme loading used in

477   this study was much lower than in most previous studies, yet higher glucose yields were

478   obtained. This continuous pretreatment technology shows great promise, especially

479   since it can be scaled up easily to obtain high throughputs. Absence of fermentation

480   inhibitors is another advantage over traditional pretreatment methods.

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620
621
622

623 **Figure captions**

624 Fig. 1. Schematic of lab-scale twin extruder screw profile and barrel temperature

625   settings.

626 Fig. 2. Crystallinity of soybean hulls before and after pretreatment[a].

627 Fig. 3. Reducing sugar yield (g/g pellet) as determined by DNS assay[a,b].

628 Fig. 4. Effect of in-barrel moisture content and starch addition on glucose concentration
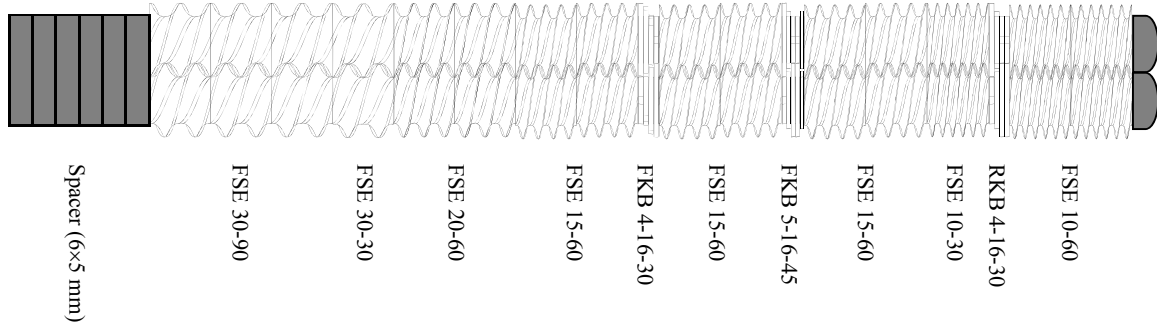
629   (g/g pellet)[a,b].

630 Fig. 5. The effect of screw speed on glucose concentration (g/g pellet)[a,b].

631 Fig. 6. Cellulose to glucose conversion (%) after saccharification of soybean hulls

632   subjected to different pretreatments[a].

Head No.	1	2	3	4	5	6
Barrel temperature						
Temp.(°C)	40	50	60	70	75	80
	40	50	65	80	90	110
	40	60	80	100	120	140

633

Spacer (6×5 mm)
FSE 30-90
FSE 30-30
FSE 20-60
FSE 15-60
FKB 4-16-30
FSE 15-60
FKB 5-16-45
FSE 15-60
FSE 10-30
RKB 4-16-30
FSE 10-60

FSE: forward conveying screw element (all double flight, intermeshing)
FKB: forward kneading block; RKB: reverse kneading block
Numbers on screw elements: pitch (mm)-element length (mm)
Numbers on kneading blocks: number of disks-total block length (mm)-staggering angle of disks
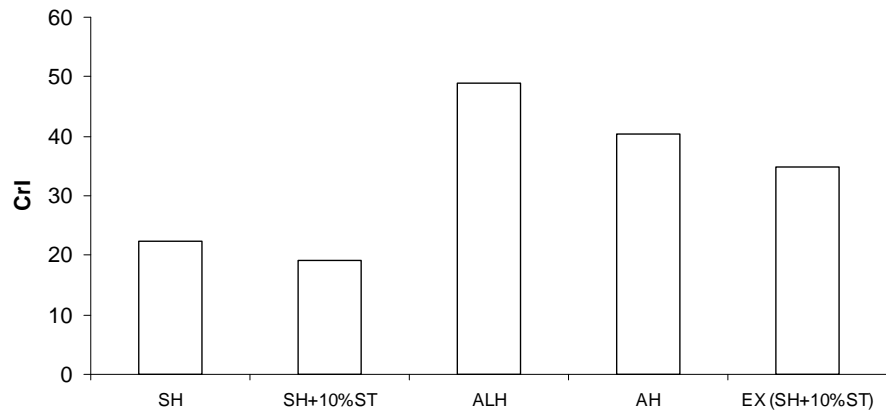
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640

641 ^aSH: untreated soybean hull; SH+10%ST:untreated soybean hull + 10% starch; ALH: alkali
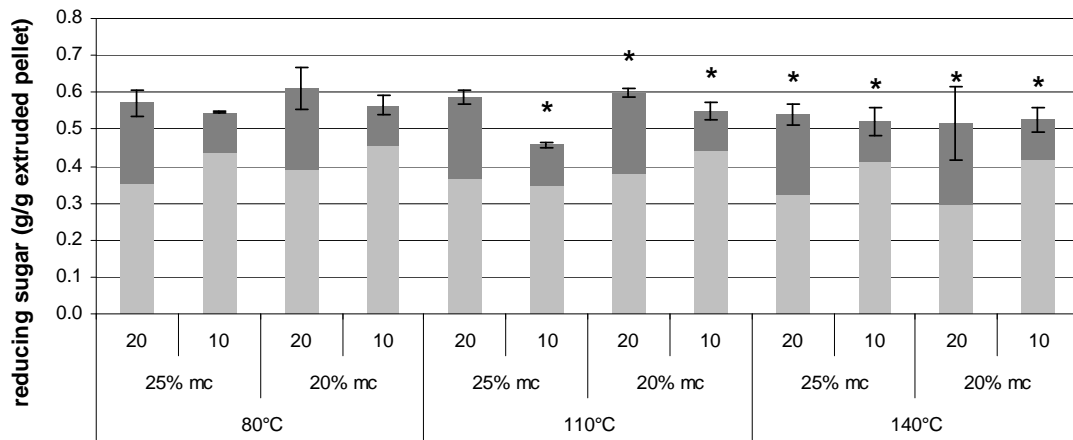
642 pretreated soybean hull; AH: dilute acid pretreated soybean hull; EX: extruded pellet from
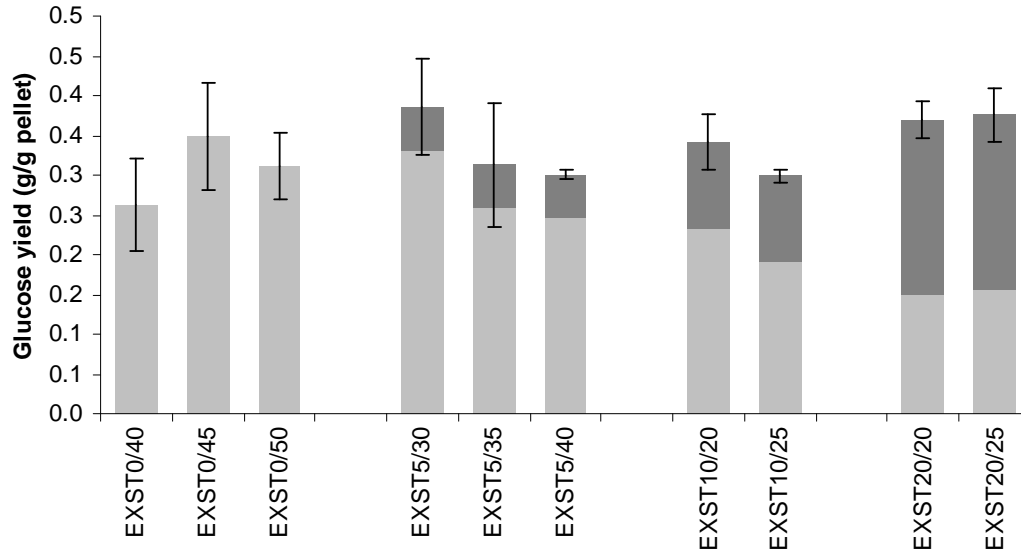
643 soybean hull + 10% starch.

644

645

646

647 [a]Dark shaded portion of each bar indicates starch contribution assuming 100% hydrolysis. Error bars

648 represent standard deviation.

649 [b]X-axis from top to bottom: starch addition %, in-barrel moisture content %, maximum barrel
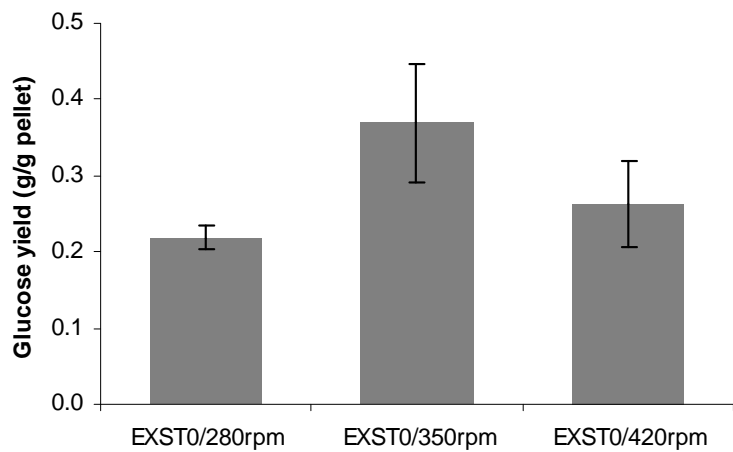
650 temperature.

651 * Surging and/ or burning observed.

652
653

654   [a]Screw speed 420 rpm and barrel temperature 80°C. Dark shaded portion of each bar indicates

655   starch contribution assuming 100% hydrolysis. Error bars represent standard deviation.

656   [b]X-axis: EXSTx/y; x = % starch addition, y = in-barrel moisture (%wb).
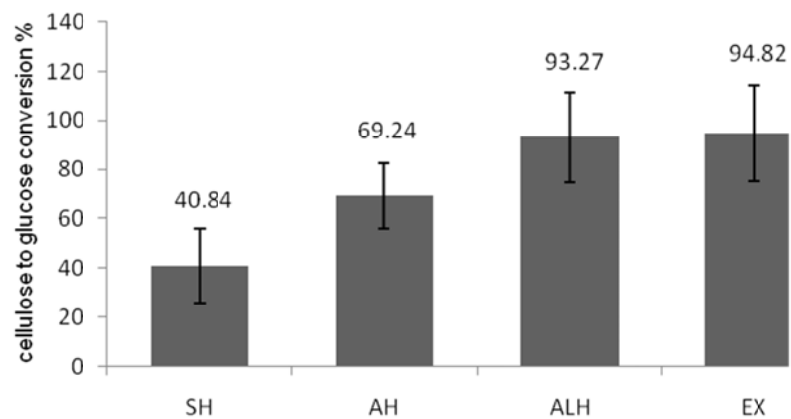
657

658   [a]No starch, barrel temperature 80°C and in-barrel moisture 40% wb. Error bars represent standard

659   deviation.

660   [b]X-axis: EXST0/screw speed.

661

663

666   [a]SH: untreated soybean hull; AH: dilute acid pretreated soybean hull; ALH: alkali pretreated

667   soybean hull; EX: extruded soybean hull (no starch, screw speed 350 rpm and in-barrel moisture

668   40% wb). Error bars represent standard deviation.

667

667　　　　　**Table 1. Experimental design for enzyme cocktail optimization (response**

668　　　　　**surface methodology).**

669

		mL/g cellulose		
		-1	0	1
X	Cellulase (Celluclast 1.5L)	0.0254	0.0609	0.1016
Y	β-glucosidase (Novozyme 188)	0.0254	0.0609	0.1016
Z	Cell wall degrading enzyme (Viscozyme® L)	0.0254	0.0609	0.1016

670

Test no.	X	Y	Z
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

671
672

Table 2 Lignocellulosic composition (% db) of soybean hull (SH) before and after pretreatment.

	Lignin	Hemicellulose	Cellulose
Untreated SH	2.33±0.05	17.21±0.06	35.35±0.20
Acid hydrolyzed SH	5.22±0.11	7.80±0.14	61.79±0.43
Akali treated SH	11.66±0.21	14.59±0.12	66.72±0.48
(SH + 0% starch) extrudate	1.52±0.01	20.42±0.22	36.88±0.05