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Enzymatic Hydrolysis of Biomass at High-Solids Loadings – A Review

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Abstract

Enzymatic hydrolysis is the unit operation in the lignocellulose conversion process that utilizes enzymes to depolymerize lignocellulosic biomass. The saccharide components released are the feedstock for fermentation. When performed at high-solids loadings ($\geq$15% solids, w/w), enzymatic hydrolysis potentially offers many advantages over conversions performed at low- or moderate-solids loadings, including increased sugar and ethanol concentrations and decreased capital and operating costs.

The goal of this review is to provide a consolidated source of information on studies using high-solids loadings in enzymatic hydrolysis. Included in this review is a brief discussion of the limitations, such as a lack of available water, difficulty with mixing and handling, insufficient mass and heat transfer, and increased concentration of inhibitors, associated with the use of high solids, as well as descriptions and findings of studies that performed enzymatic hydrolysis at high-solids loadings. Reactors designed and/or equipped for improved handling of high-solids slurries are also discussed. Lastly, this review includes a brief discussion of some of the operations that have successfully scaled-up and implemented high-solids enzymatic hydrolysis at pilot- and demonstration-scale facilities.

1. Introduction

Lignocellulose is the largest renewable source of carbon on the planet, as it is the main structural component of plants. Energy from lignocellulosic biomass has been tapped as one possible solution to decrease the United States' foreign dependence on petroleum, as well as serve as a more environmentally friendly source of energy. Lignocellulose can either be processed thermochemically or biochemically, depending on the desired product. The biorefinery concept is thought to be the desired model for biomass processing, where all of the biomass is exploited. The suite of products would be dictated by the market and selected to extract the greatest value possible out of lignocellulose (Figure 1).

Enzymatic hydrolysis of lignocellulose has long been studied as a method to depolymerize the biomass into fermentable sugars for conversion to biofuels and biochemicals, with a more recent focus on operating at high-solids loadings. It has been suggested that enzymatic hydrolysis conducted at high-solids loadings will be necessary to render the lignocellulosic conversion process more economically feasible. A process is considered "high solids" if the ratio of solids/liquid is such that very little to no free water is present in the slurry [1] or roughly a solids loadings ≥15% (w/w).

Enzymatic hydrolysis performed at high-solids loadings offers several advantages over low- and moderate-solids loadings, the main one being final sugar concentrations are higher [2, 3]. In theory, higher sugar concentrations translate into higher ethanol concentrations, which could reduce energy use and costs associated with the distillation process [4, 5]. For the purpose of this paper, the term "concentration" refers to the amount of a component dissolved in a given volume of liquid, while the terms "yield" and "conversion" refer to the quantity of a product obtained expressed as a percentage of the theoretical maximum. Distillation is most economical

55 when the ethanol concentration is ≥4% (w/w). In order to obtain this ethanol yield, glucose

56 yields must be at least 8% (w/w), which translated into a lignocellulose loading of ≥20% (w/w)

57 for enzymatic hydrolysis [6]. These estimates only account for conversion of cellulose;

58 however, as improvements are made to hemicellulose conversion (hydrolysis and fermentation)

59 technologies that work in combination with cellulose conversion, this initial solids loadings

60 estimate may decrease. Another potential advantage is the reduction of capital and production

61 costs. Smaller equipment and/or fewer reactors can be utilized to produce an equivalent output

62 [7, 8]. Fewer reactors also translate into reduced energy demands for heating, cooling and

63 mixing [3, 5], although the latter aspect may be a point of contention as increased solids makes

64 effective mixing more difficult. Additionally, less water is needed, which reduces the cost of

65 disposal or treatment of process water.

66   The goal for this review is to provide a consolidated source of information for the latest

67 technological advances for managing enzymatic hydrolysis at high-solids loadings. Following a

68 brief discussion of the factors limiting enzymatic hydrolysis at high solids, various aspects and

69 approaches pertaining to hydrolysis operating conditions are detailed. Additionally, reactors

70 designed to overcome some of the limitations associated with high-solids hydrolysis, as well as

71 pilot- and demonstration-scale plants operating at high-solids loadings are discussed. Lastly, the

72 authors comment on the envisioned direction for high-solids hydrolysis research, as well as the

73 necessary advances this technology must make to become commercially viable.

74

75 **2. Factors Limiting High-Solids Enzymatic Hydrolysis**

76   As solids loading increases, challenges that were negligible in low-solid systems become

77 more prominent, which has also been noted in high solids pretreatment [9]. One of the major

78   challenges for enzymatic hydrolysis at high solids loading is the lack of available water in the

79   reactor. Water is essential to effective hydrolysis for two reasons: mass transfer and lubricity.

80   Water increases the effectiveness of the enzymatic and chemical reactions, mainly by providing a

81   medium for solubilizing and aiding in the mass transfer of products. Water also reduces the

82   viscosity of the slurry by increasing the lubricity of the particles, which decreases the required

83   shear stress necessary to produce a given shear rate, allowing lower power input for mixing [1,

84   10]. The physical and chemical properties of the specific biomass affect the way biomass

85   absorbs water. As solids loadings approach 20% (w/w), the liquid fraction becomes fully

86   absorbed into the biomass leaving little free water [1]. With lower amounts of free water, the

87   apparent viscosity of the mixture increases, and consequently mixing and handling of material

88   become more difficult.

89   Gervais, Benoussan and Grajek [11] investigated the relationship between water content

90   and water activity on microorganisms in a high-solids cellulose environment. No free water

91   occurs when the matric potential of the substrate holds the water more tightly within its pores

92   than the gravitational force acts on it. The water potential (= osmotic potential + matric

93   potential) of the system is such that content affects mass transfer by limiting diffusion of

94   products away from enzyme [11]. Not only can the enzymes release compounds from the

95   biomass that are inhibitory to the organisms used in the fermentation step, but the sugar products

96   they produce are known inhibitors in the enzymatic feedback mechanism [2, 12, 13]. For

97   example, cellobiose inhibits the cellulase. Typically, cellulase is supplemented with β-

98   glucosidase to reduce the inhibition by cellobiose. However, it has recently been shown that

99   hydrolysis rates of cellulase and β-glucosidase are greatly impacted by hemicellulose-derived

100   products, like xylose, xylan and xylo-oligomers [14-16]. Pretreatment methods that do not

101   remove these products or enzyme cocktails that include xylanases may have detrimental effects

102   on glucose yields. While inhibition occurs at low solids, as well as at high solids, the increased

103   concentration of inhibitors, in addition to the reduced mass transfer rate away from the enzyme,

104   makes inhibition more apparent at high-solids loadings.

105   The challenges apparent at high solids are interrelated, so a less-than-ideal condition in

106   one property exacerbates the negative effects of another property. For example, the substrates'

107   physio/chemical properties affect the water retention value (WRV) of the biomass. A high WRV

108   (due to high-solids content and the specific properties of the substrate) reduces the diffusion of

109   inhibitors away from the enzymatic reaction, and increases the apparent viscosity of the mixture,

110   thereby increasing the difficulty of stirring the mixture to assist with diffusion. Zhang et al. [17]

111   found that the energy required to mix increased one order of magnitude when they increased the

112   solids loading of pretreated corn stover from 15% to 30% w/w (79.5 MJ/t slurry to 1009.2 MJ/t

113   slurry, respectively) to produce 854.9 and 1723.2 MJ/t slurry of ethanol respectively. The higher

114   solids loading did indeed achieve the goal of producing a higher concentration of ethanol in the

115   broth; however, over half of the energy produced in the ethanol was consumed in the mixing to

116   achieve the higher concentration of ethanol (compared to 9% of the energy needed to mix the

117   system producing the lower concentration of ethanol.

118   While it is widely recognized that increasing the solids content in a conversion process

119   increases product concentration [18], it is also widely recognized that the increase in yield is not

120   linear with increasing initial solids content because yield (percent conversion) decreases with

121   initial solids content (slope is a function of substrate type, pretreatment, and enzyme loading,

122   among other things) [10].   In fact, this well-recognized challenge was observed so often that

123   Kristensen et al. [10] coined the term *solids effect* to describe the persistence of a measured

124   reduction in conversion when solids loadings are increased. The scientific community has yet to

125   come to agreement as to the cause of the solids effect; however, theories include substrate

126   effects, product inhibition, water content and enzyme adsorption characteristics, just to name a

127   few [10].

128   Other challenges specific to high-solids enzymatic hydrolysis include long hydrolysis

129   times. Enzymatic hydrolysis is typically thought to be the bottleneck of the entire conversion

130   process in terms of both time and money, since the reaction time needed for most enzymes to

131   convert lignocellulose into sufficient glucose concentrations for fermentation is on the order of

132   days (usually ≥ 3 days). Long hydrolysis times can only be reduced so much by increasing

133   enzyme loading. Recent studies have suggested that enzymes can overcrowd accessible

134   cellulose sites, thus not reaching the full hydrolytic potential for the given enzyme loading [19,

135   20]. Adjacent cellulose chains are ~4-6 Å apart, whereas the diameter of the cellulases is about

136   10-fold larger at about 45 Å (Figure 2). Furthermore, as in low-solids hydrolysis, the cost of the

137   enzyme is also a limiting factor. Enzyme is typically added on a per weight of substrate basis.

138   As the solids loading increases so must the amount of enzyme. While the cost of enzymes has

139   decreased drastically over the years due to intense research developing cheaper production

140   schemes, the cost is still at a level that makes this step in the conversion process one of the most

141   expensive. Finding or developing enzymes with a high activity and inexpensive method of

142   production would greatly benefit the entire conversion process. Moreover, it is also important to

143   evaluate the economics when determining the balance between the loadings applied to the

144   lignocellulose and the amount of time needed to reach sufficient glucose concentrations.

145

146   **3.   Impacting Rheology of High-Solids Mixtures**

147   Rheology is the branch of physics that deals with the deformation and flow of matter. At

148   higher lignocellulose loadings, fundamental understanding of the rheology of these suspensions

149   becomes a powerful tool in designing conversion equipment and processes [21-24]. Factors

150   which contribute to the rheological properties of a suspension include particle size distribution,

151   particle aspect ratio, fiber flexibility [22, 25] and physio/chemical properties of the substrate.

152   Water retention value (WRV) of the substrate directly impacts the apparent viscosity of a

153   suspension, affecting mixing and handling of the slurries [26]. For example, pretreated corn

154   stover (PCS) slurries are considered "pourable" when yield stresses are at or below ~10 Pa or

155   ~10% insoluble solids [3, 23]. Dilute acid PCS at 20% insoluble solids is a thick, paste-like

156   substance that can be molded and formed into shapes that remain even after the applied forces

157   are removed [23]. At even higher solids loadings (>30%), particles are not as lubricated because

158   of the lack of free water, resulting in increased friction due to particles interacting with both

159   water and other particles. At this point, the mixture can no longer be called a slurry because it is

160   unsaturated and acts more like a wet, granular substance.   Substances with these varied

161   rheological properties present many unique challenges in materials handling throughout a

162   conversion process, particularly when continuous, industrial-scale processes are desired.

163   Several rheological models of interest, like the Bingham, Herschel-Buckley, Power Law,

164   Wildemuth-Williams and Casson models [3, 8, 21, 24, 27], have been developed to describe the

165   non-Newtonian behavior of these types of systems , but discussion of these models is beyond the

166   scope of this paper.

167   Um and Hanley [8] analyzed rheological properties of high-solids (10-20% w/v)

168   enzymatically hydrolyzed slurries of the model cellulose feedstock Solka Floc, a delignified

169   spruce pulp. Commercially-available *Trichoderma longibrachiatum*–sourced enzymes (30

170   FPU/g cellulose supplemented with β-glucosidase) were evaluated at 10, 15 and 20% solids

171   loadings. The enzymatic suspensions exhibited a pseudoplastic behavior overall, with viscosities

172   ranging from 0.04 to 0.01, 0.23 to 0.03, and 0.29 to 0.04 Pa·s for substrate concentrations of 10,

173   15 and 20% (respectively) initial solids measured at 50 °C. As the hydrolysis progressed, a

174   decrease in viscosity was observed for all solids loadings (dropping by approximately half in 3

175   hours). Zhang et al. [18] showed the same trend with high-solids steam exploded corn stover.

176   Several studies using dilute acid-pretreated corn stover also observed a reduction in yield stress

177   (and therefore viscosity) as solids loadings in enzymatic hydrolysis decreased (Figure 3) [3, 21,

178   22, 24, 27].

179   Additionally, Roche et al. [3] found that at 20% solids, >40% conversion was necessary

180   for the slurry to become pourable. They also reported a distinct difference between PCS that was

181   enzymatically hydrolyzed as compared to PCS that was just diluted. The yield stress for diluted

182   PCS is higher by a full order of magnitude than that of hydrolyzed PCS at corresponding particle

183   volume fractions. Although specific mechanisms for this difference were not investigated, one

184   theory is that the enzymes alter the particles during hydrolysis, converting them from complex

185   networks of material with distinct liquid and solid phases, to a homogeneous slurry as the liquid

186   and solid phases become indistinguishable.

187   Particle size affects the rheological properties of the suspensions, directly impacting

188   mixing and pumping costs [27]. Viamajala et al. [24] found that smaller particle sizes resulted in

189   smaller apparent viscosities under equivalent conditions. Mechanical pretreatment is often

190   utilized to reduce particle size to make the rheological properties more favorable for other steps

191   downstream in the process. However, temperature and acid concentration in dilute acid

192   pretreatment directly affect yield stress of a slurry, possibly as a result of a reduction in particle

193   size, as well as enhancing enzymatic hydrolysis due to the modification of the surface chemistry

194   of the particles [21, 27]. While a reduction in particle size lowers viscosity, as well as increases

195   conversion efficiency, the manner in which the size reduction occurs is also important. Size

196   reduction via pretreatment provides better digestibility and a reduced yield stress as compared to

197   mechanical size reduction, which did not significantly impact either property [27]. In some

198   cases, the pretreatment, like dilute acid pretreatment, hydrothermal pretreatment or SPORL

199   (sulfite pretreatment to overcome recalcitrance of lignocelluloses) performed prior to the

200   hydrolysis step alters the structure of the biomass significantly so that liquefaction occurs

201   quickly upon addition of the enzymes and mixing can resume [28, 29]. However, in most cases,

202   the solid fraction is still a complex network of fibrous material [21, 24, 30]. Sufficient mixing is

203   required for timely hydrolysis of the biomass, and traditional mixing methods like stirred-tank

204   reactors with impellers require excessive power and shaking does not provide adequate mixing.

205   Several mixing alternatives are discussed in a later section.

206   The pulp and paper industry has long used additives to modify rheological properties of

207   lignocellulosic slurries [25]. Knutsen and Liberatore [31] found that the most effective additive

208   groups (in descending order) to reduce yield stress were surfactants, additives with polar head

209   groups, additives with hydrophobic tails, unmodified protein and polymers. CTAB (cetyl

210   trimethylammonium bromide) and CPCl (cetylpyridinium chloride), both surfactants, were two

211   of the most effective additives for reducing yield stress. Samaniuk et al. [25] used water soluble

212   polymers (WSPs) like carboxymethyl cellulose (CMC), polyethylene oxide (PEO) and

213   polyacrylamide (PAM), to modify rheological properties of lignocellulosic slurries. Additives

214   like CMC reduced the friction between cellulose surfaces, making it easier to mix high-solids

215   suspensions. The addition of 2% CMC reduced the yield stress by ~67% from 55 kPa to ~18

216   kPa. A four-fold increase in CMC resulted in reducing by another 50%. They also found that a

217   lower degree of substitution for CMC had a positive impact on the yield stress; however, this

218   trend was more apparent at higher CMC loadings. Furthermore, a reduction in yield stress was

219   observed as the molecular weights of the WSPs increased up to a certain point. For example,

220   yield stress decreased with the addition of 600 kDa, as well as 2000 kDa, PEO, but no further

221   change in yield stress was observed with the addition of 7000 kDa PEO. Several other additives

222   were screened by monitoring the reduction in torque as measured by a torque rheometer to

223   determine whether they warranted further investigation. Fly ash and microcrystalline cellulose

224   were evaluated as possible additives, but their impact was limited. The surfactant Polysorbate 80

225   reduced the yield stress by 36% but required high concentrations (10%). Guar gum,

226   hydroxypropyl methyl cellulose (HPMC), a guar gum-xanthan gum mixture and a guar gum-

227   HPMC mixture were all more effective than CMC, where guar gum and the two mixtures

228   containing guar gum resulted in the highest reduction in torque (~80%). The addition of

229   additives may be costly, but like the pulp and paper industry, it may become economically

230   feasible to utilize such methods of modification for high-solids conversion processes. It is

231   important, however, that these additives be as inexpensive as possible and do not negatively

232   impact the conversion process by inhibiting the hydrolytic enzymes or fermentative organisms.

233

234   **4. Impacting Enzymatic Hydrolysis Rate and Extent**

235   The term "lignocellulosic biomass" refers to many different types of biomass, including

236   forestry and agricultural residues (woody biomass, straw, stover), fermentation by-products

237   (DDGS) and dedicated energy crops (grasses), just to name a few. Each type of lignocellulosic

238 material is slightly different in regards to composition, resulting in unique challenges in the

239 enzymatic hydrolysis step of the conversion process. The following sections are organized based

240 on various aspects in need of consideration during the conversion of lignocellulose and highlight

241 some of the challenges and breakthroughs associated with enzymatic hydrolysis performed at

242 high-solids loadings for different types of biomass. It is important to note that while each of

243 these processing approaches are discussed individually, it is often difficult to separate out the

244 combined effects of multiple process conditions.

245   Furthermore, when determining cellulose conversion, it is important to note that the

246 standard method of calculating conversions as described by [32] can grossly overestimate actual

247 conversion for high-solids systems. In some instances, conversions can be overestimated by up

248 to 36% [5]. Determining cellulose conversion in high-solids systems can become very

249 complicated, but several studies have proposed new methods for determining cellulose

250 conversion [5, 33, 34] under these high solids operating conditions. The standard method for

251 conversion calculations typically compares the amount of glucose measured in the hydrolyzate

252 (the liquid fraction) to the potential glucose found in the biomass (the solid fraction). This

253 method requires the assumption that all components have a consistent density throughout the

254 reaction and that it is approximately equal to that of water. As solids loadings increase, this

255 assumption no longer remains valid, resulting in overestimated conversions.

256

257 **4.1 Biomass Processing**

258   Enzymatic hydrolysis is an intermediate step in the conversion process, and while

259 producing high sugar yields is favorable, the resulting hydrolyzate must be subsequently capable

260 of supporting fermentative organisms while they produce biofuels. Some of the more expensive

261   steps in substrate preparation are washing the substrate following pretreatment and detoxifying

262   the hydrolyzate produced during enzymatic hydrolysis. It is likely that for industrial processes

263   unwashed, whole slurries (liquid + solids) from pretreatment will be used in enzymatic

264   hydrolysis [2], indicating a need for robust enzymes capable of maintaining their activity in the

265   presence of possible inhibitors and degradation products or developing pretreatments that do not

266   produce such products. Furthermore, the cost of hydrolyzate detoxification alone can be up to

267   22% of the total ethanol production cost [35].

268   Several studies have investigated the effects of eliminating washing and/or detoxifying

269   steps in the lignocellulose conversion process, with some promising results. Hodge et al. [2]

270   studied the effects of soluble and insoluble inhibitors on enzymatic hydrolysis by comparing the

271   glucose yields produced from a washed pretreated substrate (which introduces only potentially

272   insoluble inhibitors into the hydrolysis reaction since all soluble inhibitors are washed away) and

273   an unwashed whole slurry substrate (which introduces both potentially soluble and insoluble

274   inhibitors to the hydrolysis reaction). However, to maintain the high-solids loading and modify

275   the pH, the solid and liquid fractions were separated, the liquid fraction pH was adjusted, and the

276   two fractions were combined. Should the whole slurry be used at the industrial scale (as this

277   study states in its rationalization for this work), this method of pH modification may not be

278   feasible. This challenge is just one of many that must be solved prior to implementing a

279   complete conversion process. Regardless, this study utilized an insoluble solids loading of 5-

280   13% (~9-24% total solids loading) and relatively low enzyme loadings (<20 FPU/g cellulose).

281   Based on the glucose production from hydrolysis, the authors suggested that the limitations due

282   to mass diffusion are more prevalent than the sugar inhibition beyond a specific solid content.

283   For instance, sugar inhibition would result in a "leveling-off" of the hydrolysis rate, much like

284 what would be seen in a typical hydrolysis curve. However, a sharp decrease in the hydrolysis

285 rate was reported here. Using the washed substrate, this decrease is not prevalent until ~20%

286 insoluble solids loadings are reached, where convective mixing and available water are

287 negligible, likely indicating the point of mass transfer limitations. This decrease occurs at much

288 lower solids loadings (<10% insoluble solids) for unwashed substrate, indicating that the soluble

289 components contributed to a higher rate of enzyme inhibition or limited mass transfer by

290 reducing the amount of water available for reaction. (Further discussion on the restriction of

291 water can be found in Section 4.4 Solids Effects.)

292   Pristavka et al. [36] also conducted enzymatic hydrolysis studies with SO_2-catalyzed

293 steam exploded willow. These studies were concerned with simplifying the conversion process

294 by neglecting to wash the pretreated willow between the pretreatment and hydrolysis steps and

295 eliminating mechanical stirring of the biomass slurry. The reason for eliminating the washing

296 step was two-fold. First, less water would be used in the conversion process, making the process

297 more economical and more environmentally friendly. Secondly, washing usually leads to the

298 solubilization and removal of a significant portion of sugars. These sugars ultimately end up

299 accumulating in wastewater, resulting in an expensive processing step to recover them and/or

300 treating the water. The high-solids loadings (up to 25% ODM (organic dry matter)) used in this

301 study would make mechanical stirring of the slurry extremely energy intensive, so it was

302 removed. With these process modifications, a lower degree of conversion was observed as

303 compared to biomass that was washed prior to hydrolysis (53% vs. 74%). However, the degree

304 of cellulose conversion increased to >95% when the pH of the unwashed, pretreated willow was

305 adjusted with solid NaOH to the optimal pH of the enzymes. The significant increase in

306 conversion following pH adjustment highlights the importance of maintaining optimal hydrolysis

307 conditions for the enzymes, even if that means finding new, inexpensive and less resource-

308 intensive methods of doing so.

309   Lu et al. [37] investigated the effects (post-pretreatment) washed substrate had on

310 enzymatic hydrolysis and fermentation. Using steam-exploded corn stover, substantial

311 differences in conversion efficiencies were not observed for washed and unwashed substrates up

312 to a solids loading of 30% (w/w). However, closer examination of the conversion calculations

313 revealed differences between washed and unwashed substrates, since conversions were based on

314 water insoluble solids and not total solids content. (Essentially the denominators were different

315 for the two treatments.) Additionally, the pH of the unwashed corn stover was not adjusted prior

316 to addition of enzymes and buffer at pH 4.8. Cellulose conversion remained fairly consistent

317 (70-75%) for all solids loadings, although glucose content was higher for the washed substrate

318 than the unwashed substrate. Ethanol production was also independent of solids loading (up to

319 30% w/w) for the water-washed corn stover, reaching 92-94% of theoretical yield. However, the

320 results were quite different for the unwashed substrate. At the lower solids loadings studied (10-

321 15% w/w), ethanol production fell to 88% and 86%, respectively, and decreased as the solids

322 loading increased, until no ethanol could be measured (≥25% solids loading). The levels of

323 acetic acid and furfural measured at the higher solids loading reached inhibitory concentrations.

324 Inclusion of the water-washing step following pretreatment appears to eliminate the need for

325 another costly detoxification step following enzymatic hydrolysis for steam-exploded corn

326 stover.

327   In contrast to this study, others report contradicting results regarding the wash step [35,

328 38]. Lau et al. [35] reported that when AFEX-pretreated corn stover was fermented following

329 enzymatic hydrolysis at 18% (w/w) solids loading, the ethanol yield of ~93%, even though the

330   solids loading during hydrolysis and glucose concentration before fermentation were similar to

331   those reported in Lu et al. [37] who reported a 68% ethanol yield. While these results are so

332   different, it should be noted that different pretreatments, as well as fermentative organisms were

333   used (*E. coli* vs. *S. cerevisiae*, respectively), making it difficult to directly compare these

334   fermentation results. However, Lau and Dale [38] achieved higher ethanol production rates

335   fermenting unwashed substrates (~0.17 g/L/hr as compared to 0.12 g/L/hr for washed substrate)

336   with *S. cerevisiae* 424A (LNH-ST) (a genetically modified strain for improved xylose

337   fermentation), suggesting that the elimination of the washing step following pretreatment, and

338   with no adjustments made to the pH prior to hydrolysis, is beneficial for fermentation under the

339   conditions examined in this study. Ethanol concentration from unwashed substrate was 40 g/L

340   (no data given for washed substrate). Xylose metabolism from the genetically modified strain is

341   likely the largest contributing factor to the discrepancy in reported ethanol yields, but it was also

342   reported that the this strain of *S. cerevisiae* performed similarly on washed substrate as compared

343   to unwashed substrate. This study suggests that the washing step can be eliminated without any

344   loss in ethanol yield. Contradictory results indicate the need for further study of this issue, or at

345   the very least, optimization studies under specific process conditions.

346   In another study, LHW-pretreated sweet sorghum bagasse was hydrolyzed at 15-30%

347   solids (w/v) with either 20 or 30 FPU/g glucan cellulase [39]. Washing the substrate prior to

348   hydrolysis also did not improve the conversion rates. Washed substrate yielded 63.2 g/L of

349   sugar, whereas the unwashed substrate resulted in a sugar concentration of 66.1 g/L. It was

350   suggested, although not verified, that the washing step actually removed some of the smaller

351   cellulose particles that may have been easier to hydrolyze than larger cellulose particles.

352   The inconclusive results of these studies illustrate the complexity of defining appropriate

353   processing conditions that work in all situations. Operating conditions must be chosen carefully

354   in order to realize the full potential of using lignocellulose as a valuable energy source.   Table I

355   illustrates the wide variety of operating conditions that have been studied with regards to high-

356   solids loadings enzymatic hydrolysis. Depending on various factors, like substrate choice,

357   pretreatment conditions and hydrolysis conditions, it may be possible to eliminate certain steps

358   like washing pretreated substrate or detoxifying hydrolyzate prior to fermentation, thus

359   simplifying the overall conversion process. However, elimination of these steps may present

360   new problems that must be solved. For instance, should the washing step following pretreatment

361   be eliminated, it may be necessary to adjust the pH in another manner so the hydrolytic enzymes

362   can work most effectively.

363

364   **4.2 Feeding Strategies**

365   Fed-batch feeding schemes have been investigated as an alternative method of achieving

366   high-solids loadings in enzymatic hydrolysis [1, 26, 45, 46] because of some of the advantages it

367   offers over single feeding schemes. For instance, the initial viscosity is lower, so diffusion and

368   mixing limitations can be minimized or altogether avoided. A fed-batch feeding regime also

369   allows time for the slurry to liquefy before adding additional solids, which maintains a level of

370   free water that is available for the reaction process and for diffusion (away from the enzymes) of

371   potentially inhibitory products that result from the hydrolysis reaction. However, when a fed-

372   batch approach is selected, one must consider how and when to add substrate, as well as

373   enzymes, to the reaction in order to maintain high rates of conversion. Table II illustrates the

374   variety of substrate and enzyme application rates used in fed-batch studies.

375   Hodge et al. [1] conducted a study in which the fed-batch approach was utilized in order

376   to achieve a final insoluble solids content of 15% (w/w) (equivalent to a 25% (w/w) initial solids

377   loading). This solids loading was the upper limit of unhydrolyzed pretreated corn stover that

378   could be effectively mixed in the stirred tank reactors (STRs) available to the researchers. High

379   cellulose conversion (>80% cellulose conversion) was reported; however, the reaction time was

380   more than double the typical hydrolysis reaction time (168 hrs vs. 72 hrs). The extended time

381   problem may be overcome through the use of higher enzyme loadings or enzymes that can

382   tolerate higher sugar concentrations. The enzyme loading used in this study was 10.7 FPU/g

383   cellulose, a relatively low loading, and it was applied proportionally with each addition of

384   substrate. A study conducted by Yang et al. [46] obtained a similar cellulose conversion

385   (70.6%), with a higher solids loading (30%), an enzyme loading almost twice (20 FPU/g

386   cellulose) that used in the former study and with a much shorter reaction time (30 hrs). Both

387   studies attribute the high conversion rate, at least in part, to the fact that the substrates were

388   washed prior to hydrolysis, possibly eliminating any potential inhibitory products that resulted

389   from the pretreatments. The latter study also supplemented fresh enzyme with each addition of

390   new biomass, which increased the final enzyme loading from 10 to 15 FPU/g cellulose. The

391   fresh enzyme may have also enhanced the glucose yield, replacing the enzyme that may be non-

392   productively bound to the lignin or deactivated by extended hydrolysis times.

393   Zhang et al. [52] studied another fed-batch approach for the conversion of NaOH-

394   pretreated sugarcane bagasse and wheat straw. Pretreated biomass was fed into the reactor at

395   9%, 8%, 7%, and 6% solids over the course of 48 hrs to achieve a final solids loading of 30%

396   (w/v). All enzymes were added with the first addition of lignocellulose. Glucose conversion

397   from wheat straw reached a maximum (~60%) after the first feeding, but decreased with each

398   successive feeding. The higher rate of conversion was likely due to the low solids loading and

399   high enzyme loading at the beginning of the reaction. With each successive feeding, the

400   enzyme: substrate ratio decreased. After 72 hr of hydrolysis, the conversion began to level off,

401   resulting in a final glucose conversion of 39%. A slightly different conversion profile was

402   observed with the bagasse. The conversion continued to increase over the course of the

403   hydrolysis reaction, with the exception of the last feeding time (6% solids at 48 hr). The final

404   feeding resulted in a sharp decrease in conversion, but it recovered within 24 hr following the

405   feeding, leading to an increase in conversion over the batch. The final glucose conversion of the

406   sugarcane bagasse was 55%. Differences in the way the pretreatment affected the lignocellulose

407   may have led to the different glucose yields between the two substrates. It was reported that the

408   pretreatment caused the surface of the two substrates to become rough and fragmented as lignin

409   was removed, allowing for better access to the cellulose; however, the bagasse appeared to have

410   a rougher, more fragmented surface than the wheat straw. Following 144 hr of hydrolysis, the

411   surfaces were relatively smooth as compared to the start of the hydrolysis.

412   Wang et al. [39] considered the use of a fed-batch feeding scheme. Initially, the reactors

413   were charged with half of the final solids loading, followed by two additional feedings at 24 and

414   48 hr of one-fourth of the final solids loading. The system containing 30% solids achieved the

415   highest final sugar concentration with nearly 115 g/L. Even with the fed-batch system, the

416   conversion decreased with increasing solids loadings; however, the conversion of the 30% solids

417   reaction was only 5% less than the systems at 15% and 20% solids (55% vs. ~60%,

418   respectively).

419   Fed-batch was utilized by Ma et al. [55] to achieve a 25% (w/v) solids loading. Enzymes

420   were added either all at once at the beginning of the reaction or with each addition of the dilute

421   acid pretreated cassava bagasse. At this solids loading, the batch reaction reached ~50%

422   conversion, whereas the fed-batches with a single enzyme addition and multiple enzyme

423   additions achieved ~75% and 84% conversion, respectively. These results are similar to those

424   reported in other fed-batch studies [1, 46], indicating that under the right conditions fed-batch

425   systems may be a plausible solution for achieving higher conversion rates for hydrolysis

426   performed at high-solids loadings.

427   Rosgaard et al. [26] investigated several different regimes for batch and fed-batch

428   hydrolysis, including variations of sequential addition of substrate as well as substrate plus fresh

429   enzyme. The addition of fresh enzyme with each substrate addition maintained a constant

430   enzyme:substrate ratio throughout the whole reaction, as opposed to the other fed-batch feeding

431   schemes where all the enzyme was added in one application. In these cases, the effective

432   enzyme:substrate ratio decreased with each subsequent addition of substrate. Not surprisingly,

433   the fed-batch schemes that received the full enzyme application at the start of the reaction

434   produced higher glucose yields during the first few hours as compared to the fed-batch reactions

435   that received fresh enzyme with each substrate addition. However, the extent of the hydrolysis

436   reaction was not affected by the method of enzyme application as the final glucose

437   concentrations were not different for the fed-batch reactions with and without additional enzyme

438   applications (62-67 g/L). Furthermore, lower viscosity is often touted as an advantage of fed-

439   batch systems over batch systems because mixing becomes easier as viscosity decreases. The

440   viscosities of the fed-batch systems in this study were lower than in the batch systems, but no

441   benefits were observed in regards to glucose production as the batch system at 15% solids

442   resulted in higher glucose production (78 g/L) after 72 hr hydrolysis. Final glucose

443   concentrations of the fed-batch systems, though, were impacted by each addition of substrate.

444 Hydrolysis rates decreased and never fully recovered, resulting in lower final yields than the

445 batch systems.

446   Additionally, Chandra et al. [45] reported on a fed-batch approach at a moderate solids

447 loading that did not perform as well as a single stage feeding approach. The total solids loadings

448 achieved for both feeding schemes was 10%. Two enzyme loadings were tested (5 and 60

449 FPU/g cellulose), and at both loadings, the batch reaction produced the higher yields,

450 approximately 66% and 90% for steam-pretreated corn stover, respectively. However, when the

451 solids are fed at 24 hr intervals, the respective yields are lower (approximately 55% and 80%)

452 and the hydrolysis rates slower. The authors suggest these reductions in yields and rates are the

453 result of non-productive binding of enzyme to xylan or lignin fractions of the substrate or the

454 inability of the enzyme to desorb from partially hydrolyzed substrate and find accessible

455 cellulose sites in the fresh substrate. Free protein measurements taken at 72 hr indicate that 50-

456 70% of the cellulase was still adsorbed to the substrate for both enzyme loadings, while the

457 cellulose conversion ceased. The lower hydrolysis rate at the higher enzyme loading seems to

458 indicate that the enzymes are saturating the accessible cellulose sites, thus reaching a maximum

459 hydrolysis rate that is lower than that of the batch reaction when all the accessible cellulose sites

460 are available at once.

461   The results of fed-batch feeding schemes are currently still inconclusive, as indicated by

462 the preceding studies, making the decision to use a fed-batch approach unclear. Many

463 advantages are realized regarding the use of fed-batch systems, but questions persist. For

464 instance, at what point in the reaction should subsequent additions of substrate be applied to

465 maintain a high rate of conversion? Should enzymes be added in a single application, as a

466 supplement to the original application, or proportionally to the substrate? Does the benefit of

467 reduced viscosity make a difference in energy consumption during the conversion process to

468 overcome the potentially reduced sugar yield that may result from the fed-batch as compared to

469 the batch system?

470

471 **4.3 Effects of Enzyme Synergism**

472   Enzymatic hydrolysis, especially at high-solids loading, has been identified as the largest

473 impediment to achieving high yields in a timely manner in the lignocellulose to ethanol

474 conversion process, mainly because a significant portion of sugars produced are in oligomeric or

475 polymeric form, which cannot be used in the fermentation process. Several studies have

476 investigated this issue from the perspective of the enzyme (Table I), experimenting with enzyme

477 supplementation (in addition to cellulase) and alternative organism sources for cellulase [38, 47-

478 49]. Supplementing cellulase with β-glucosidase has long been used to minimize end-product

479 inhibition of the cellulase and achieve higher conversions. Lau et al. [48] investigated the use of

480 several different enzymes other than cellulase and β-glucosidase to enhance the conversion of

481 lignocellulose. Their enzyme cocktail included xylanase and pectinase to target the

482 hemicellulose that acts as a barrier to cellulose if not removed during pretreatment. The focus of

483 this work was on the fermentation step, so the details regarding the enzymatic hydrolysis are

484 limited. However, the hydrolyzates produced from AFEX-pretreated corn stover with these

485 enzyme cocktails were able to produce 40 g/L (5.1% v/v) of ethanol with *Saccharomyces*

486 *cerevisiae*.

487   Another study investigated the effects of supplementing the typical cellulase and β-

488 glucosidase enzyme cocktail with xylanase on the hydrolysis of steam-exploded barley straw

489 [50]. The addition of the xylanase to the enzyme mixture enhanced the conversion rate of the

22

490 cellulose, especially at low solids loading and early in the hydrolysis reaction. Conversion at

491 higher solids loadings may be reduced by the higher concentration of xylooligomers produced

492 with the addition of xylanases, as has recently been shown [15]. However, the xylanase used in

493 the supplementation study did contain some β-xylosidase activity, which, if present, might

494 counteract the inhibition caused by xylooligomers. The positive effects of the xylanase addition

495 reported in this study support the idea that overall enzyme loadings could be reduced if better

496 conversion is achieved by incorporating an array of different enzymes. However, a different

497 study conducted by Di Risio [44] also evaluated various enzyme cocktails made from

498 commercially-available enzyme solutions. All three cocktails assessed consisted of the same

499 base solution: cellulase and β-glucosidase. Each solution was supplemented with a third

500 commercial enzyme solution with different active components: cellulase + xylanase, cellulase +

501 xylanase + β-glucosidase, and xylanase. The highest glucose yields (44%) resulted from the

502 enzyme cocktail consisting of the base solution supplemented with the commercial solution

503 containing cellulase + xylanase + β-glucosidase activity. Surprisingly, the enzyme solution

504 supplemented with the enzyme promoted as a "xylanase" actually yielded significantly less

505 xylose than the other two enzyme solutions (39% as compared with 54% and 85%). However,

506 there is no indication that the xylanase activity of this commercial product was independently

507 verified prior to use. Glucose yields ranged from 32%-42%.

508   Taking it a step further, another group studied the effects of various addition schemes and

509 enzyme loadings using an enzyme cocktail containing cellulase, β-glucosidase and xylanase on

510 the hydrolysis of mixed hardwood chip pulps [42]. The enzyme cocktails consisted of fungal

511 cellulase (C), xylanases (X) and β-glucosidase (B) solutions mixed in the ratio of 10:3:3 (by

512 volume). The mixtures were added to the substrate in the following manners: (1) cellulase,

513   xylanases and β-glucosidase was mixed with substrate at the desired solids loading (CXB); (2)

514   cellulase was added to 5% solids, pressed or filtered to obtain the desired solids loading, and

515   hydrolyzed for a period of time before the xylanases and β-glucosidase mixture was added

516   (C+XB); and (3) half of the cellulase was added to 5% solids, pressed or filtered to obtain the

517   desired solids loading, and hydrolyzed for a period of time before the cellulase (half dose),

518   xylanases and β-glucosidase mixture was added (C+CXB). With the CXB mixture, a decrease in

519   conversion was observed with an increase in solids loading. Enzyme loading also plays an

520   important role in the optimization of biomass conversion. For example, with the CXB enzyme

521   mixture, the difference in sugar yields decreased with increased enzyme loadings. At 40 FPU/g

522   solids, conversion decreased from 70% to 68% for 5% and 20% solids loading, respectively,

523   which represents no significant difference in conversion. However, at 5 FPU/g solids,

524   conversion decreased from 40% to 19% for 5% and 20% solids loadings, respectively. The

525   authors hypothesized the decreased conversion was the result of ineffective mixing of the

526   enzyme mixture with the substrate as the solids loadings increased. Based on this hypothesis, the

527   authors added the enzyme to a low solids mixture, allowing time for the enzymes to adsorb to the

528   substrate, before filtering off 80% of the liquid to obtain 20% solids loadings. Enzyme activity

529   was tested following filtration to determine whether any enzyme was lost during this process.

530   Cellulase activity registered at 80% of the original activity, whereas only 20% of the xylanases

531   activity was retained. This observation resulted in the modified application of the enzyme

532   mixture (C+XB). At 20% solids and 20 FPU/g solids, sugar conversion increased from 44% for

533   the CXB mixture to 59% for the C+XB mixture. Sugar concentrations increased from 84 g/L to

534   114 g/L. This modified enzyme application process was also beneficial at low solids loadings

535   (5%), increasing conversion from 19% with CXB to 38% with C+XB. Taking this enzyme

536 application process one step further, additional cellulase was added with the xylanases and β-

537 glucosidase mixture (C+CXB). In this instance, although the sugar concentration increased to

538 121 g/L glucose (63% conversion), the conversion at 20% solids was similar to that at 5% solids

539 at all enzyme loadings tested. These experiments indicate the importance of determining enzyme

540 mixtures and application schemes that provide the optimal sugar yields and concentrations for

541 the conversion process.

542   Along with the feeding scheme and the enzyme loading, the type of enzyme used can

543 have a significant impact on the liquefaction of biomass. The term "cellulase" can refer to a

544 wide variety of enzymes, and commercially available enzymes can often be a crude mixture of

545 enzymes (i.e. *T. reesei* cellulase that is commonly used in hydrolysis studies). To be more

546 specific, for example, the *T. reesei* "cellulase" can refer to a mixture of cellobiohydrolases

547 (CBH), endoglucanases (EG), xylanases (XYLs), and β-glucosidase, among other enzyme

548 components. Using an array of CBHs, EGs, XYLs and a β-glucosidase, both individually and in

549 combination, Sjizarto et al. [30] assessed the enzymes on their ability to liquefy hydrothermally

550 pretreated wheat straw. For the *T. reesei* components, it was determined that the EGs (especially

551 Cel5A) were the most important in liquefying lignocellulose. This enzyme alone reduced the

552 viscosity of the slurry by nearly 90%. The CBHs and XYLs had little to no effect on the

553 viscosity, even though the sugar production was similar to that of some of the EGs.

554 Furthermore, a mixture of enzymes produced the highest sugar yields, even though the viscosity

555 was reduced by only about 82%, indicating that the amount of sugar hydrolyzed is not the main

556 factor in reducing viscosity, but that the sites at which the polysaccharides are cleaved is more

557 important.

558   Since enzymes play such a vital role in the conversion of lignocellulose, much of the

559   process integration depends on these biological catalysts. For instance, a balance must be struck

560   between the enzyme loading used and enzyme cost. High enzyme loadings not only increase the

561   total cost, but as discussed in the introduction, studies suggest that enzymes are overcrowding

562   accessible cellulose chains, thus reducing the rate at which cellulose is hydrolyzed. One such

563   study was conducted by Olsen et al. [58]. At a solids loading of 29% (w/w) pretreated corn

564   stover, a range of enzyme loadings (5-83 FPU/g cellulose) were evaluated for hydrolysis yields.

565   At enzyme loadings >66 FPU/g cellulose, the hydrolysis curves started to coincide. It was

566   suggested that the lack of improvement in hydrolysis rate and conversion was due to the

567   substrate being completely saturated with enzymes bound to all the accessible sites. High

568   enzyme loadings also do not make sense economically. Based on a techno-economic model of

569   the bioethanol conversion process, an optimum total solids loading of about 20% with an enzyme

570   loading of 20 mg/g solids (8.8 FPU/g solids) was determined to produce the minimum ethanol

571   selling price with currently available, commercial enzymes [4]. This model evaluated the cost of

572   production at 2007 enzyme production costs ($0.35/gal), as well as the enzyme production cost

573   projected by the Multi-Year Program Plan (MYPP) from the DOE's Office of Biomass Program

574   for 2012 ($0.12/gal) [59]. At the lower enzyme production cost, solids loadings could

575   potentially be increased up to 26% and remain economically viable. In the time since this study

576   was published, the MYPP re-evaluated the cost of enzyme production and the current projection

577   for 2012 was fairly consistent with the "high" cost of enzyme production reported in the study at

578    $0.34/gal of ethanol (2007$). Under the assumptions made constructing this model, 20% solids

579   loading remains the maximum that is economically feasible for the ethanol production process.

580   Zhang et al. [43] evaluated enzyme loading to determine the effect it had on glucose

581   concentration. A 50% reduction in enzyme loading decreased the glucose concentration by only

582   21%. The implication of this observation is that enzyme loading can be optimized to provide the

583   maximum concentration at the lowest unit cost. For example, it may not be worth converting an

584   extra 5% of glucose if it accounts for ~15% of the total enzyme cost unless the return on the

585   extra glucose recovers the cost of the additional enzyme.

586   While the cellulase system of *T. reesei* is one of the most commonly studied enzyme

587   systems, other organisms also produce cellulolytic enzymes that could potentially impart

588   superior activity under certain conditions. Ingram et al. [53] compared the conversion

589   efficiencies of enzymes from two different organisms, *T. reesei* and a genetically-modified (for

590   increased cellulase production) strain of *Penicillium janthinellum*. Enzyme mixtures from both

591   organisms contained cellulases, β-glucosidases and xylanase activity. With the cellulase from *T.*

592   *reesei*, an increase in glucose concentration as biomass loading increased was observed for the

593   organosolv and the LHW-pretreated rye straw. After 48 hrs of hydrolysis at 17.5% solids, the *P.*

594   *janthinellum* cellulase converted 72% of the soda-pretreated rye straw. Higher enzyme loadings

595   of *P. janthinellum* cellulase were necessary to achieve the same level of conversion produced by

596   the *T. reesei* cellulase (27 FPU/g cellulose vs. 13 FPU/g cellulose); however, the *P. janthinellum*

597   cellulase appeared to be more tolerant to changes in pH. This study highlights the fact that the

598   conversion process is dependent on many factors, including, but not limited to, the type of

599   biomass, the conditions of the pretreatment, and the source of enzymes.

600   In another study partially purified cellulase from the thermostable *Geobacillus* R7 was

601   evaluated as an alternative cellulase source [47]. For short hydrolysis times (36 hr), the

602   *Geobacillus* cellulase was comparable to a commercial enzyme preparation. However, for

603 hydrolysis of pretreated prairie cord grass using this cellulase, the glucose recovery at 96 hrs for

604 solids loadings ≥10% was between 46.2% and 48.7%. It does not appear that the solids loading

605 had much of an impact on conversion of the prairie cord grass; although the conversion of

606 cellulose into glucose utilizing the *Geobacillus* R7 cellulase was better than the conversion of the

607 pretreated corn stover at 27%-31%. *Geobacillus* R7 also has the added benefit of being

608 ethanologenic. During the hydrolysis, *Geobacillus* R7 produced a small amount of ethanol

609 (0.035 g/L) from the pretreated prairie cord grass, which has possible implications for

610 consolidated bioprocessing of lignocellulose materials. Subsequent fermentation of the

611 hydrolyzate with *S. cerevisiae* resulted in an ethanol production of 7.8 g/L (or 0.47 g ethanol/g

612 glucose) for the 20% solids loading of prairie cord grass.

613   Lastly, Matano et al. [60] engineered fermentative yeast to express three different types

614 of cellulase on its surface. This yeast was subsequently evaluated in SSF processes utilizing

615 25% (w/v) pretreated rice straw. Initially, a control yeast strain was supplemented with a

616 commercial cellulase (100 FPU/g biomass). This combination resulted in an ethanol yield of

617 80% and liquefaction after 72 hr. When combined with the modified yeast strain, the

618 commercial cellulase loading could be reduced to 10 FPU/g biomass and produce the same

619 ethanol yield (79%). Further study showed that a maximum ethanol concentration (43.1 g/L)

620 was obtained following a 2 hr liquefaction period prior to the addition of the modified yeast,

621 corresponding to an ethanol yield of 89%. Residual glucose was reduced by an order of

622 magnitude with the modified strain (16 g/L to 1.6 g/L). The authors hypothesized that the close

623 proximity of the cellulases on the surface of the yeast provided a synergistic effect that resulted

624 in an increased hydrolysis of cellulose. As commercial enzymes are still a relatively large

625 portion of the overall cost of the conversion process, the ability to reduce the commercial

626 enzyme loading and replace it with an organism capable of both the hydrolysis and fermentation

627 is very attractive.

628

629 **4.4 Solids Effect**

630   For conversion of lignocellulose into usable and valuable products, it makes economical

631 sense to utilize locally-available biomass, as shipping biomass over long distances greatly

632 reduces the beneficial impacts. Cara et al. [41] studied the conversion of olive tree pruning

633 biomass (consisting of leaves and thin branches) up to 30% (w/v) solids loadings. The final

634 glucose concentrations increased with increasing solids loading, achieving 61 g/L and 52 g/L

635 glucose at 30% solids loading of the liquid hot water (LWH) pretreated biomass and steam

636 exploded biomass, respectively. However, the conversions of the LHW-pretreated biomass

637 decreased nearly linearly from 76.2% at 2% solids to 49.9% at 30% solids. Conversions of the

638 SE-pretreated biomass held steady between 60% and 63% up to 10% solids loading before

639 decreasing to 39.6% at 30% solids. In a different study, the researchers also observed that the

640 glucose concentration decreased as the solids loading was increased beyond 10% solids for the

641 soda pretreated rye straw [53]. The overall conversion of cellulose decreased from ~65% to 40%

642 as solids loadings increased from ~10% to 17.5%. This result is not unusual, as most studies

643 performed at high-solids loadings sacrifice conversion for a more concentrated glucose product

644 [10, 29, 41].

645   Kristensen et al. [10] also studied four mechanisms that possibly contribute to the so-

646 called solids effect: (1) compositional and substrate effects, (2) product inhibition, (3) water

647 concentration, and (4) cellulase adsorption. These mechanisms were studied with filter paper,

648 which is essentially a pure cellulose substrate. The researchers observed the same decreasing

649 trend in conversion as solids increased using the filter paper, much like that observed with

650 lignocellulose. Therefore, it was concluded that lignin, which is absent in filter paper, is likely

651 not the reason for the solids effect. Study of the second mechanism, product inhibition, resulted

652 in significantly different conversions after 48 hours of hydrolysis for 5% DM and 20% DM

653 (64.5% vs. 38.6% or 30 g/L vs. 86 g/L, respectively). However, the final conversions for these

654 solids loadings with an additional 50 g/L glucose added resulted in fairly similar conversions

655 (29.7% and 26.3% or 64 g/L vs. 109 g/L for 5% DM + 50 g/L glucose and 20% DM + 50 g/L

656 glucose, respectively). This experiment did not elucidate the exact reason for the observed

657 similar conversions, but two hypotheses were offered. It was suggested that other components in

658 the hydrolysis mask the product inhibition or that enzymes are inhibited similarly once a certain

659 glucose concentration is reached.

660 Kristensen et al. [10] next attempted to quantify the effects of water on the hydrolysis

661 reaction. Water content was decreased by 25% and replaced by oleyl alcohol. The alcohol

662 allowed the viscosity of the slurry to remain constant, thus removing the effects of the viscosity,

663 while the water to solids (or enzyme) ratio was altered. With this decrease in water, a 5%

664 decrease in glucose yield was observed. However, increasing the solids content from 20% to

665 25% (which is essentially equivalent to a 25% reduction in water), typically decreases glucose

666 yields by ≥12%. The authors argue this discrepancy in glucose reduction indicates that lower

667 water content is apparently not the limiting factor responsible for the solids effect.

668 Lastly, cellulase adsorption was investigated as a possible source of the solids effect [10].

669 Cellulase adsorption to filter paper was determined by measuring the total nitrogen content of the

670 biomass after 24 hr of hydrolysis. The amount of adsorbed cellulase measured was halved (40%

671 to 17%) as solids loading increased from 5% to 25%. At the same time, conversion was reduced

672   from ~60% to <50%. A strong correlation between decreasing adsorption and conversion was

673   observed, indicating that cellulase is not effectively adsorbing onto cellulose causing a decrease

674   in yield. The authors hypothesize that increasing concentrations of glucose and cellobiose inhibit

675   the adsorption of enzymes. Knowledge of the mechanisms of high-solids product inhibition and

676   the mechanisms of high-solids enzyme adsorption inhibition can provide the key to improving

677   the overall conversion process, thus unlocking the full potential of high-solids conversions.

678   In contrast to the previous study, Roberts et al. [56] investigated the interactions of water

679   with biomass at high-solids loading without maintaining a constant viscosity. Time domain

680   NMR was used to measure the transverse (or spin-spin) relaxation times (T_2) of protons in water

681   molecules to indicate the extent of water constraint (or degree to which water is tightly bound to

682   biomass). Essentially, the nuclei of water molecules that are tightly bound have a shorter

683   relaxation time than nuclei that are less tightly bound. By measuring these relaxation times,

684   constraint can be determined. It was found that water was more tightly bound as solids loadings

685   increased, suggesting that an indirect relationship between water constraint and yield exists.

686   However, the relaxation time of the primary bound water (water that interacts directly with the

687   surface of the cellulose) was constant regardless of the solids loading. Interactions at the water-

688   solids interface appear to remain constant, suggesting the chemistry at the surface of the

689   cellulose does not change as water content changes. These results further suggest that the water

690   primarily interacts with the cellulose, and the impact of the solute is minimized. However, these

691   studies were conducted with bacterial cellulose, a substrate that is essentially pure cellulose. It is

692   unclear whether cellulose derived from pretreated lignocellulose would interact with water in a

693   similar manner or to what extent the type of pretreatment may affect these cellulose-water

694   interactions. With the addition of excess glucose or mannose to 5% solids, the hydrolysis rate

31

695   reduced to one similar to 15% solids loading. The authors hypothesize that the negative effects

696   on the hydrolysis rate are caused by water constraint as opposed to the monosaccharides

697   impacting the enzyme activity. It is also possible that the lack of available water limited the

698   uniform distribution of synergistic enzymes, thus hindering the hydrolysis rate. Also, in contrast

699   to the previous study, the results presented in this study indicate that water (or the lack of it) has

700   a great impact on the overall hydrolysis rate. Even though the addition of oleyl alcohol in the

701   former study reduced the water content in the reaction, the constant viscosity helped maintain

702   adequate mixing and therefore did not limit the diffusion of enzymes throughout the suspension.

703   While these studies draw conflicting conclusions on the effect of water on lignocellulose

704   conversion, they do highlight the need for effective mixing. Adequate mixing was provided in

705   the former study, even with a low water: substrate ratio because of the low viscosity afforded by

706   the addition of alcohol, whereas the latter study simply reduced the water: substrate ratio without

707   regard for the viscosity. These studies also highlight the difficulty of quantifying and assigning

708   the challenges of operating at high solids to any one factor (lack of water, high viscosity,

709   adequate mixing, etc.) when all these factors are so interrelated.

710

711   **4.5 Effect of Viscosity on Mixing**

712   High viscosity of high-solids slurries is another hurdle that must be overcome. Much of

713   the previous discussion (i.e. effects of enzymes on liquefaction and solids loadings) also affects

714   the rheology, but this section discusses specific viscosity modifiers and their effects on

715   enzymatic hydrolysis. Ineffective mixing increases the limitations associated with mass transfer,

716   including removal of local inhibitors and hydrolysis products and transfer of heat throughout the

717   reactor. The pulp and paper industry has long been using viscosity modifiers to enhance the

718 processability of fibrous slurries [31], much like the types of slurries produced by lignocellulose

719 materials prevalent in the conversion to biofuels and biochemicals. One study [31] investigated

720 the use of 18 different chemical additives and evaluated the effects on the slurry rheology and

721 hydrolysis rates. Several surfactants added to lignocellulosic slurries at 2% (w/w), including

722 CPCl, CTAB, sodium dodecylbenzene sulfonate (NaDBS) and sodium dodecyl sulfonate (SDS),

723 positively affected the rheological properties of the slurry by reducing the viscosity by nearly

724 four-fold as compared to the viscosity of the unmodified slurry. Although slight decreases in the

725 extent of the hydrolysis reactions were observed, only the CPCl and the CTAB did not reduce

726 hydrolysis rates. Additionally, Ma et al. [55] tested the surfactant Tween-80 and found that it did

727 not produce a significant increase in conversion at a 10% solids loading to warrant its use.

728 However, at 25% solids loading, the addition of the surfactant (2 g/L) increased cellulose

729 conversion by 30%. Contrary to what Kristensen et al. [10] said, the inhibition caused by non-

730 productive binding of the enzyme to lignin does not seem to have as large of an effect at low

731 solids as it does at high solids. These results show some promise in modifying viscosity

732 properties of lignocellulose slurries; however, more work is warranted to understand the

733 mechanism by which these surfactants work, as well as determining the economical value of the

734 use of such additives.

735   Another approach to reducing viscosity is to raise the temperature at which the hydrolysis

736 reaction takes place [61]. In order to work at higher temperatures, enzymes that can tolerate the

737 increased temperatures must be used. It has been shown that EGs from more thermotolerant

738 organisms worked better at reducing the viscosity of a lignocellulose slurry, while other types of

739 enzymes appeared to have little effect [61]. *T. aurantiacus* proved to be more thermotolerant

740 than *A. thermophilum*, as the *T. aurantiacus* EG continued to reduce the viscosity at temperatures

741   up to 75ºC. *A. thermophilum* enzymes were less active above 65ºC, resulting in a reduced effect

742   on the viscosity. The ability to use alternate sources of cellulase enzymes illustrates the number

743   of reaction condition variables (i.e. temperature, components in enzyme cocktail, and solids

744   content in slurry) open to modification.

745   The method of mixing the slurry can also have a substantial impact on the conversion of

746   lignocellulose. For example, Zhang et al. [43] observed a significantly reduced liquefaction time

747   when comparing hydrolysis at high solids (17-20% w/w) performed in shake flasks with a lab-

748   scale peg mixer. Peg mixers are commonly used in the pulp and paper industry, which routinely

749   utilizes solids loadings up to 35% [43]. (Readers are referred to the section entitled "Reactor

750   design for enzymatic hydrolysis at high solids" for more details on the peg mixer.) Liquefaction

751   occurred after 1 hr of hydrolysis in the peg mixer, whereas the shake flask required 40 hr. The

752   decrease in liquefaction time can most likely be attributed to the effective mixing provided by

753   the peg mixer and the breaking down of the large fiber network that tends to occur as solids

754   loadings surpass 8%. At 20% (w/w) solids loadings, hydrolysis performed in the peg mixer

755   resulted in 144 g/L and 158 g/L of glucose from unbleached hardwood and Organosolv

756   pretreated poplar, respectively. These concentrations are the highest glucose concentrations

757   achieved known to the authors at the time of writing this review.

758   One of the highest solids loadings in enzymatic hydrolysis reported to date is 40% (w/w)

759   [29, 51]. A horizontally-oriented rotating drum was utilized as the reactor in these studies in

760   order to effectively mix the solids. The studies found that cellulose and hemicellulose

761   conversion decreased from ~90% to ~33% and ~70% to 35%, respectively, with the increase in

762   solids loading from 2% to 40%, but the reactor was providing adequate mixing as evidenced by

763   the conversion of lignocellulose into fermentable saccharides (86 g glucose/kg at 40% solids)

764   [29]. At 40% solids, liquefaction occurred after only 4 hrs. The viscosity was still high, as the

765   slurry turned into a thick, clay-like paste and remained as a thick paste following 96 hrs of

766   hydrolysis. Additionally, the reactor was a very energy efficient solution to the mixing problem.

767   Mixing speed did not affect the liquefaction time, so relatively low speeds (6.6 rpm) could be

768   used. It was also shown that ethanol could be produced in the same rotating drum reactor from

769   the resulting slurries, where the highest ethanol yield (48 g/kg DM) reported was from the slurry

770   at 35% solids. Even at reduced enzyme loadings (5 FPU/g DM supplemented with β-glucosidase

771   at a 5:1 loading), ~40% conversion for both cellulose and hemicellulose can be achieved at 30%

772   solids loading [51]. These results suggest using one reactor for all processing steps in the

773   conversion of lignocellulose, with the implication that capital and equipment costs can

774   potentially be greatly reduced as both the number of reactors and amount of enzyme used

775   decreases. However, with the yield penalty for conversion at higher solids loadings being high, a

776   full techno-economic analysis would be needed to fully validate such a system operating under

777   the given conditions.

778

779   **4.6 Tools and Methods for Measuring the Progress of Enzymatic Hydrolysis at High-Solids**

780   **Loadings**

781   As more and more interest is expressed in the use of high-solids loadings in the

782   conversion of lignocellulose, it is also important that tools are available to properly measure and

783   study the progress of the hydrolysis reaction. Calorimetry has been studied as a new tool for

784   determining enzymatic kinetics of high-solids loadings in hydrolysis [58]. It provides higher

785   sensitivity than HPLC in the early stages of the hydrolysis, making calorimetry a useful tool to

786   evaluate initial rates of hydrolysis. Avicel showed that enzyme hydrolysis slowed when enzyme

787 loading of >30 FPU/g cellulose were used. It is believed that this reduction in rate is due to the

788 lack of available binding sites on the cellulose, as illustrated by the heat-flow curves converging

789 upon a single value, regardless of the enzyme loading.

790   Lavenson et al. [57] also implemented the use of new tools to monitor liquefaction and

791 the extent of hydrolysis of cellulose. Liquefaction and the spatial homogeneity of the enzyme

792 distribution in Solka-Floc suspensions (28% w/w) were monitored with magnetic resonance

793 imaging (MRI). The MRI signal is proportional to the amount of free water in the reaction,

794 which correlates to the degree of liquefaction in the system. Additionally, a penetrometer was

795 used to monitor the mechanical strength of the suspension. Measurements were taken on two

796 hydrolysis systems, where one contained a mixed Solka-Floc and enzyme suspension and the

797 other contained a Solka-Floc suspension that received an application of enzyme but no mixing.

798 Mechanical strength of the mixed suspension decreased by 20% of the initial strength after ~30

799 hrs, as compared to ~170 hrs for the unmixed suspension. Based on the MRI results, the mixed

800 samples did not show a spatial gradient, indicating uniform liquefaction when the enzyme and

801 substrate are initially well-mixed. The unmixed samples showed a slow change in spatial

802 gradients, which were attributed to ineffective diffusion of the enzyme to the substrate. Since

803 liquefaction occurs nearly six times faster for the mixed samples, it is not surprising that higher

804 final glucose concentrations are also obtained as compared to the unmixed samples and in much

805 less time. For example, the mixed suspension reached ~75 g/L glucose in only ~120 hrs,

806 whereas the unmixed suspension produced only ~50 g/L in 300 hrs. Furthermore, adequate

807 initial mixing of the enzyme and substrate resulted in an initial rate of hydrolysis an order of

808 magnitude higher (1.8 g/L/hr as compared to 0.21 g/L/hr).

809

5. Reactor Design for Enzymatic Hydrolysis at High Solids

810

811   Several groups studying the use of high-solids loadings for enzymatic hydrolysis have

812 embraced a horizontal orientation of the reactor [6, 29, 62, 63]. Gravitational or free-fall mixing

813 provides many advantages over typical vertical stirred tank reactors and are used in other

814 industrial processes that require mixing highly viscous slurries, like peanut butter, ketchup and

815 concrete [62, 63]. The horizontal orientation minimizes particle settling and local accumulation

816 of reaction products within the reactor, as well as ensuring better enzyme distribution. These

817 types of reactors are also easily scalable from bench-scale to pilot-scale and larger. Power

818 requirements are lower for horizontal reactors equipped with paddles over vertical stirred tank

819 reactors that provide the same level of effective mixing [62].

820   Roche et al. [63] employed free-fall mixing in their design for bench-scale reactors for

821 enzymatic hydrolysis. Polypropylene bottles (125 mL and 250 mL) were placed on a roller

822 apparatus in a horizontal orientation. The roller apparatus and bottles were placed in an

823 incubator for temperature control during enzymatic hydrolysis. This roller-bottle system

824 produced results comparable to shake flasks when utilizing intermittent hand mixing, especially

825 following enzyme addition and prior to sampling, for up to 30% solids (data not shown). At

826 20% solids loading, these two mixing schemes resulted in 80-85% cellulose conversion. The

827 roller-bottle reactors eliminated the human component of mixing, resulting in more consistent

828 mixing and better enzyme and reaction product distribution.

829   Hydrolysis studies conducted by Dasari et al. [62] utilized a horizontal reactor of

830 intermediate capacity (8 L). The reactor was constructed from a cylinder made of Pyrex glass

831 with aluminum lids fitted over the ends. An adjustable speed, rotating shaft with rubber-tipped,

832 stainless steel blades attached was inserted into the reactor. Three sampling ports were located

833   along the length of the reactor. Hydrolysis studies comparing the horizontal reactor to shake

834   flasks found, at 25% solids loading, approximately 10% more glucose was produced in the

835   horizontal reactor.

836   Jorgensen et al. [29] developed a reactor for use in pretreatment and enzymatic hydrolysis

837   processes with a total volume of 280 L. Several features have been implemented into the pilot-

838   scale drum reactor, as well as the smaller glass reactor, to address issues associated with high-

839   solids loadings. The horizontal orientation of the reactors takes advantage of free-fall mixing,

840   eliminating the need for mechanical mixing. Evaluation of a range of mixing speeds (3.3-11.5

841   rpm) by Jorgensen et al. [29] resulted in no significant differences in cellulose conversion over

842   the tested range, so energy input for mixing is significantly reduced as compared to vertically

843   oriented stirred tank reactors. In addition to free-fall mixing, a rotating shaft affixed with

844   paddles supplies additional mixing capabilities, as the shaft in the pilot-scale reactor can be

845   programmed to change rotational direction two times per minute. The paddles also provide a

846   scraping action that removes lignocellulosic material from the reactor walls, improving heat

847   transfer between the reactor and the biomass.

848   The Integrated Biomass Utilization System (IBUS) Project coordinated by DONG

849   Energy in Denmark also utilizes free-fall reactors. DONG Energy has free-fall reactors in a

850   variety of sizes for research and development purposes (400 L) and has successfully scaled one

851   up to a capacity of 11,000 L [6, 64]. These reactors routinely operate at approximately 40%

852   solids loading. Larger particle sizes can be used, since the mechanical work of the mixing helps

853   tear biomass fibers and particles apart [6]. This tearing action also increases the surface area of

854   the lignocellulose, resulting in increased enzyme accessibility to the cellulose and hemicellulose.

855   While most reactors implemented for high-solids enzymatic hydrolysis have employed

856   some form of free-fall mixing, Zhang et al. [18] investigated the effects of a helical impeller in a

857   vertical reactor on SSF at solids loadings up to 30% (w/w) and compared it to a typical Rushton

858   (paddle) impeller (Figure 4a-b). Helical impellers are suggested for use in highly viscous, non-

859   Newtonian fluid agitation, which describes high-solids biomass slurries. The helical impeller

860   performed better than the Rushton impeller with regard to every aspect tested. The feeding rate

861   of lignocellulose into the reactor was adjusted so that a liquefied slurry could be maintained

862   throughout the feeding period. The helical impeller provided better mixing, as the feeding period

863   was completed more than 2 hr sooner than that of the Rushton impeller. The helical impeller also

864   resulted in higher ethanol concentration (51.0 g/L vs. 43.9 g/L) and productivity, as well as

865   consuming less power. At 30% solids (prior to inoculation with the fermentative organism), the

866   Rushton impeller required nearly 40 W/kg corn stover (CS) before decreasing to ~29 W/kg CS

867   after 72 hr of saccharification and fermentation. The helical impeller required ~8 W/kg CS and

868   ~1 W/kg CS prior to inoculation and after 72 hr, respectively. (It should be noted that the

869   stirring rates for the two impellers were different; however, the power requirements were

870   normalized based on the "no-load" power consumption for each impeller.) Lastly, the mixing

871   efficiency of the helical impeller was superior to the Rushton impeller. The geometry of the

872   impeller can play a significant role in effectively mixing biomass slurries. Other geometries

873   tested by Wang et al. include a plate-and-frame impeller and a double-curved-blade impeller

874   (Figure 4c-d). The impellers were tested at various speeds and 100 rpm resulted in the best

875   conversion efficiencies for both geometries. However, the plate-and-frame impeller achieved a

876   higher conversion than the double-curved-blade impeller by nearly 18%, indicating that the

877   geometry of the impeller can have an effect on the hydrolysis. The authors suggested that the

878   plate-and-frame impeller provides a more consistent mixing regime at every depth in the reactor,

879   whereas the axial flow induced by the double-curved-blade impeller is a function of the distance

880   from the blades.

881   Another study investigated the use of a peg mixer (Figure 4e) for enzymatic hydrolysis at

882   high-solids loadings [43]. The mixer used in this study was a 9 L reactor fitted with a rotating

883   shaft with pegs extending out radially. The time for liquefaction of 20% (w/w) of unbleached

884   hardwood pulp was significantly reduced when comparing shake flasks to the peg mixer (40 hr

885   vs. 1 hr). The benefit of this mixer is that it has been proven effective with lignocellulosic

886   material. High-solids enzymatic hydrolysis is just another application for the peg mixer.

887   From the various aforementioned reactors utilized with high-solids enzymatic hydrolysis

888   reactions, there are several suggestions to improve the mixing of highly viscous slurries. Free-

889   fall mixing relies on gravity to effectively mix the slurry, which consumes less energy than a

890   stirred tank reactor providing a similar degree of mixing. An effective mixing regime can greatly

891   depend on the impeller geometry, as the shape of an impeller can cause large differences in

892   speed and shear effects at various impeller-slurry interfaces throughout the reactor. High shear

893   rates have been shown to disrupt the adsorption of cellulase onto biomass or to even cause the

894   denaturation of cellulase [65, 66]. Lastly, technology should be borrowed from other

895   applications, where possible. For instance, peg mixers are a "tried-and-true" technology that is

896   commonly used in the long-established pulp and paper industry. All of these ideas have shown

897   some promise but require more study and fine-tuning before being implemented into the

898   lignocellulose conversion process.

899

900   **6. Pilot and Demonstration-Scale Operations**

901   Several plants operating at pilot- and demonstration -scale level have recently come

902   online. These installations will help the industry gain valuable insights and improve upon the

903   challenges and limitations that are not recognized at the laboratory scale.

904   One such pilot plant constructed in Denmark is operated by Inbicon (a subsidiary of

905   DONG Energy), with a distillation capacity of ~1 ton fermentation broth/hr. Additionally, in

906   2010, Inbicon opened its demonstration-scale plant that is capable of producing 5.3 million liters

907   of ethanol each year. Enzymatic hydrolysis is performed here at 25-30% (w/w) solids content

908   with a relatively low enzyme loading of 3-6 FPU/g DM. However, the plant is capable of

909   handling up to 40% (w/w) solids in any of its process streams [6, 64]. Since this operation is

910   also used for developmental purposes, they have reactors that range from 400 L up to 11,000 L.

911   Additionally, pretreatment and fermentation are performed at high-solids loadings, 20-40% and

912   ~18% DM, respectively. At the end of the conversion process, the remaining lignin-rich material

913   (40-95% DM) is burned to produce heat and electricity that can be cycled back into the

914   conversion operation.

915   The National Renewable Energy Laboratory (Golden, CO, USA) recently expanded their

916   lignocellulose processing facilities to achieve a capacity of 4,000 L and to operate at solids

917   loading of ≥20% (w/w) [67]. The conversion process is designed as a semi-continuous operation

918   with pretreatment occurring in horizontal reactors with paddles, taking advantage of the reduced

919   energy inputs required with free-fall mixing of lignocellulose. Following liquefaction at ~24-30

920   hrs, the slurry is pumped into vertical, stirred tank reactors to complete the enzymatic hydrolysis

921   of the material. This operation is capable of processing about 0.5 to 1 ton dry biomass into

922   ethanol each day.

923

7. Direction of future work

In order to fully realize the benefits of operating enzymatic hydrolysis at high-solids, several issues must be addressed. There are many variables associated with enzymatic hydrolysis that can affect the efficiency of the conversion, including (but not limited to) biomass source, pretreatment method, enzyme source and enzyme mixture. Each of these components must be considered when designing a process for lignocellulose conversion, which makes optimal processing conditions difficult to devise. Further study for the optimization of glucose yields, especially in regards to the use of fed-batch systems, enzyme supplementation, washing and detoxification steps, and additives, both individually and in combination, is still very much needed. It is also important that a better understanding of some of the mechanisms that seem to have the greatest impacts on the conversion process is achieved. Robust reactors capable of effectively mixing biomass slurries to minimize end-product inhibition and heat and mass transfer limitations are needed. Additionally, the cost of enzymes, biomass and any necessary specialty equipment, as well as the best uses for any potential by-products produced in the conversion process, should be considered in the design stages.

939

8. Conclusions

Recent national and international focus on producing biofuels and chemicals from lignocellulose has led to significant research on the development and optimization of effective conversion processes. Several definitive conclusions regarding enzymatic hydrolysis performed at high-solids loadings can be made following a thorough review of the available literature on this topic:

946

- Free-fall mixing is effective. The advantages of this type of mixing system are numerous, and it has been employed successfully in other industrial processes.

- The solids effect is real. Although, the exact cause of this phenomenon has not been determined, there are several hypotheses that have been suggested, including

  - lower cellulase adsorption (increased concentrations of glucose and cellobiose have been shown to inhibit the adsorption of enzymes onto cellulose);

  - product inhibition of enzymes occurs earlier because of the higher concentration of products;

  - inadequate mixing, which can emphasize diffusional limitations exacerbating product inhibition and access of enzyme to substrate;

  - interaction of water with substrate (water has been shown to be more tightly bound to lignocellulose as the solids loadings increase, thus less water is available to the enzymes to perform the hydrolysis reaction).

- Contradictory evidence continues to raise questions regarding the lignocellulose conversion process. For example, some studies have shown that washing solids following pretreatment can enhance sugar production and fermentation, while others have found the opposite to be true. Additionally, arguments persist regarding the effects water has on the overall conversion process. Lastly, as long as enzyme cost remains a large portion of the overall conversion cost, enzymes also demand further attention, especially with regards to proper loadings and combinations.

967   • Fed-batch systems are worth investigating. While there have been some conflicting

968   results, many studies show overwhelming support for conducting high-solids operations

969   as a fed-batch system.

970   • The use of additives to reduce slurry viscosity has achieved some success at the lab-

971   scale. However, the economics of the use of additives on an industrial-scale should be

972   validated prior to implementation at that level.

973   The use of high-solids operations would make biofuels produced from the conversion of

974   lignocellulose more economical and more price-competitive with petroleum. Increasing sugar

975   and ethanol yields while reducing capital and production costs, lowering energy demands and

976   lowering water requirements will contribute to a more economically feasible process as

977   compared to one operated at low- or moderate-solids loadings. Despite all the benefits of

978   operating at high solids, the process remains restricted due primarily to the lack of available

979   water within the culture, high viscosities, which translate to difficulties with mixing and

980   handling, and increased concentration of inhibitors, which extends reaction times and increases

981   enzyme costs. Researchers are attacking these issues from many angles, experimenting with

982   different pretreatment methods and various enzyme sources and cocktails, while modifying

983   operating conditions and slurry properties. Although there has been some success at performing

984   enzymatic hydrolysis at high solids at the pilot and demonstration scale, many questions must be

985   resolved before the full potential of high-solids lignocellulose conversion will be realized.

986

44

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