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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 (≥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.

Keywords: High-solids loadings; enzymatic hydrolysis; lignocellulose conversion; reactor design; corn stover; straw; woody biomass
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
when the ethanol concentration is ≥4% (w/w). In order to obtain this ethanol yield, glucose yields must be at least 8% (w/w), which translated into a lignocellulose loading of ≥20% (w/w) for enzymatic hydrolysis [6]. These estimates only account for conversion of cellulose; however, as improvements are made to hemicellulose conversion (hydrolysis and fermentation) technologies that work in combination with cellulose conversion, this initial solids loadings estimate may decrease. Another potential advantage is the reduction of capital and production costs. Smaller equipment and/or fewer reactors can be utilized to produce an equivalent output [7, 8]. Fewer reactors also translate into reduced energy demands for heating, cooling and mixing [3, 5], although the latter aspect may be a point of contention as increased solids makes effective mixing more difficult. Additionally, less water is needed, which reduces the cost of disposal or treatment of process water.

The goal for this review is to provide a consolidated source of information for the latest technological advances for managing enzymatic hydrolysis at high-solids loadings. Following a brief discussion of the factors limiting enzymatic hydrolysis at high solids, various aspects and approaches pertaining to hydrolysis operating conditions are detailed. Additionally, reactors designed to overcome some of the limitations associated with high-solids hydrolysis, as well as pilot- and demonstration-scale plants operating at high-solids loadings are discussed. Lastly, the authors comment on the envisioned direction for high-solids hydrolysis research, as well as the necessary advances this technology must make to become commercially viable.

2. Factors Limiting High-Solids Enzymatic Hydrolysis

As solids loading increases, challenges that were negligible in low-solid systems become more prominent, which has also been noted in high solids pretreatment [9]. One of the major
challenges for enzymatic hydrolysis at high solids loading is the lack of available water in the reactor. Water is essential to effective hydrolysis for two reasons: mass transfer and lubricity. Water increases the effectiveness of the enzymatic and chemical reactions, mainly by providing a medium for solubilizing and aiding in the mass transfer of products. Water also reduces the viscosity of the slurry by increasing the lubricity of the particles, which decreases the required shear stress necessary to produce a given shear rate, allowing lower power input for mixing [1, 10]. The physical and chemical properties of the specific biomass affect the way biomass absorbs water. As solids loadings approach 20% (w/w), the liquid fraction becomes fully absorbed into the biomass leaving little free water [1]. With lower amounts of free water, the apparent viscosity of the mixture increases, and consequently mixing and handling of material become more difficult.

Gervais, Benoussan and Grajek [11] investigated the relationship between water content and water activity on microorganisms in a high-solids cellulose environment. No free water occurs when the matric potential of the substrate holds the water more tightly within its pores than the gravitational force acts on it. The water potential (= osmotic potential + matric potential) of the system is such that content affects mass transfer by limiting diffusion of products away from enzyme [11]. Not only can the enzymes release compounds from the biomass that are inhibitory to the organisms used in the fermentation step, but the sugar products they produce are known inhibitors in the enzymatic feedback mechanism [2, 12, 13]. For example, cellobiose inhibits the cellulase. Typically, cellulase is supplemented with β-glucosidase to reduce the inhibition by cellobiose. However, it has recently been shown that hydrolysis rates of cellulase and β-glucosidase are greatly impacted by hemicellulose-derived products, like xylose, xylan and xylo-oligomers [14-16]. Pretreatment methods that do not
remove these products or enzyme cocktails that include xylanases may have detrimental effects on glucose yields. While inhibition occurs at low solids, as well as at high solids, the increased concentration of inhibitors, in addition to the reduced mass transfer rate away from the enzyme, makes inhibition more apparent at high-solids loadings.

The challenges apparent at high solids are interrelated, so a less-than-ideal condition in one property exacerbates the negative effects of another property. For example, the substrates’ physio/chemical properties affect the water retention value (WRV) of the biomass. A high WRV (due to high-solids content and the specific properties of the substrate) reduces the diffusion of inhibitors away from the enzymatic reaction, and increases the apparent viscosity of the mixture, thereby increasing the difficulty of stirring the mixture to assist with diffusion. Zhang et al. [17] found that the energy required to mix increased one order of magnitude when they increased the solids loading of pretreated corn stover from 15% to 30% w/w (79.5 MJ/t slurry to 1009.2 MJ/t slurry, respectively) to produce 854.9 and 1723.2 MJ/t slurry of ethanol respectively. The higher solids loading did indeed achieve the goal of producing a higher concentration of ethanol in the broth; however, over half of the energy produced in the ethanol was consumed in the mixing to achieve the higher concentration of ethanol (compared to 9% of the energy needed to mix the system producing the lower concentration of ethanol.

While it is widely recognized that increasing the solids content in a conversion process increases product concentration [18], it is also widely recognized that the increase in yield is not linear with increasing initial solids content because yield (percent conversion) decreases with initial solids content (slope is a function of substrate type, pretreatment, and enzyme loading, among other things) [10]. In fact, this well-recognized challenge was observed so often that Kristensen et al. [10] coined the term solids effect to describe the persistence of a measured
reduction in conversion when solids loadings are increased. The scientific community has yet to come to agreement as to the cause of the solids effect; however, theories include substrate effects, product inhibition, water content and enzyme adsorption characteristics, just to name a few [10].

Other challenges specific to high-solids enzymatic hydrolysis include long hydrolysis times. Enzymatic hydrolysis is typically thought to be the bottleneck of the entire conversion process in terms of both time and money, since the reaction time needed for most enzymes to convert lignocellulose into sufficient glucose concentrations for fermentation is on the order of days (usually ≥3 days). Long hydrolysis times can only be reduced so much by increasing enzyme loading. Recent studies have suggested that enzymes can overcrowd accessible cellulose sites, thus not reaching the full hydrolytic potential for the given enzyme loading [19, 20]. Adjacent cellulose chains are ~4-6 Å apart, whereas the diameter of the cellulases is about 10-fold larger at about 45 Å (Figure 2). Furthermore, as in low-solids hydrolysis, the cost of the enzyme is also a limiting factor. Enzyme is typically added on a per weight of substrate basis. As the solids loading increases so must the amount of enzyme. While the cost of enzymes has decreased drastically over the years due to intense research developing cheaper production schemes, the cost is still at a level that makes this step in the conversion process one of the most expensive. Finding or developing enzymes with a high activity and inexpensive method of production would greatly benefit the entire conversion process. Moreover, it is also important to evaluate the economics when determining the balance between the loadings applied to the lignocellulose and the amount of time needed to reach sufficient glucose concentrations.
3. Impacting Rheology of High-Solids Mixtures

Rheology is the branch of physics that deals with the deformation and flow of matter. At higher lignocellulose loadings, fundamental understanding of the rheology of these suspensions becomes a powerful tool in designing conversion equipment and processes [21-24]. Factors which contribute to the rheological properties of a suspension include particle size distribution, particle aspect ratio, fiber flexibility [22, 25] and physio/chemical properties of the substrate. Water retention value (WRV) of the substrate directly impacts the apparent viscosity of a suspension, affecting mixing and handling of the slurries [26]. For example, pretreated corn stover (PCS) slurries are considered “pourable” when yield stresses are at or below ~10 Pa or ~10% insoluble solids [3, 23]. Dilute acid PCS at 20% insoluble solids is a thick, paste-like substance that can be molded and formed into shapes that remain even after the applied forces are removed [23]. At even higher solids loadings (>30%), particles are not as lubricated because of the lack of free water, resulting in increased friction due to particles interacting with both water and other particles. At this point, the mixture can no longer be called a slurry because it is unsaturated and acts more like a wet, granular substance. Substances with these varied rheological properties present many unique challenges in materials handling throughout a conversion process, particularly when continuous, industrial-scale processes are desired.

Several rheological models of interest, like the Bingham, Herschel-Buckley, Power Law, Wildemuth-Williams and Casson models [3, 8, 21, 24, 27], have been developed to describe the non-Newtonian behavior of these types of systems, but discussion of these models is beyond the scope of this paper.

Um and Hanley [8] analyzed rheological properties of high-solids (10-20% w/v) enzymatically hydrolyzed slurries of the model cellulose feedstock Solka Floc, a delignified
spruce pulp. Commercially-available *Trichoderma longibrachiatum*-sourced enzymes (30 FPU/g cellulose supplemented with β-glucosidase) were evaluated at 10, 15 and 20% solids loadings. The enzymatic suspensions exhibited a pseudoplastic behavior overall, with viscosities 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, 15 and 20% (respectively) initial solids measured at 50 °C. As the hydrolysis progressed, a decrease in viscosity was observed for all solids loadings (dropping by approximately half in 3 hours). Zhang et al. [18] showed the same trend with high-solids steam exploded corn stover.

Several studies using dilute acid-pretreated corn stover also observed a reduction in yield stress (and therefore viscosity) as solids loadings in enzymatic hydrolysis decreased (Figure 3) [3, 21, 22, 24, 27].

Additionally, Roche et al. [3] found that at 20% solids, >40% conversion was necessary for the slurry to become pourable. They also reported a distinct difference between PCS that was enzymatically hydrolyzed as compared to PCS that was just diluted. The yield stress for diluted PCS is higher by a full order of magnitude than that of hydrolyzed PCS at corresponding particle volume fractions. Although specific mechanisms for this difference were not investigated, one theory is that the enzymes alter the particles during hydrolysis, converting them from complex networks of material with distinct liquid and solid phases, to a homogeneous slurry as the liquid and solid phases become indistinguishable.

Particle size affects the rheological properties of the suspensions, directly impacting mixing and pumping costs [27]. Viamajala et al. [24] found that smaller particle sizes resulted in smaller apparent viscosities under equivalent conditions. Mechanical pretreatment is often utilized to reduce particle size to make the rheological properties more favorable for other steps downstream in the process. However, temperature and acid concentration in dilute acid
pretreatment directly affect yield stress of a slurry, possibly as a result of a reduction in particle size, as well as enhancing enzymatic hydrolysis due to the modification of the surface chemistry of the particles [21, 27]. While a reduction in particle size lowers viscosity, as well as increases conversion efficiency, the manner in which the size reduction occurs is also important. Size reduction via pretreatment provides better digestibility and a reduced yield stress as compared to mechanical size reduction, which did not significantly impact either property [27]. In some cases, the pretreatment, like dilute acid pretreatment, hydrothermal pretreatment or SPORL (sulfite pretreatment to overcome recalcitrance of lignocelluloses) performed prior to the hydrolysis step alters the structure of the biomass significantly so that liquefaction occurs quickly upon addition of the enzymes and mixing can resume [28, 29]. However, in most cases, the solid fraction is still a complex network of fibrous material [21, 24, 30]. Sufficient mixing is required for timely hydrolysis of the biomass, and traditional mixing methods like stirred-tank reactors with impellers require excessive power and shaking does not provide adequate mixing. Several mixing alternatives are discussed in a later section.

The pulp and paper industry has long used additives to modify rheological properties of lignocellulosic slurries [25]. Knutsen and Liberatore [31] found that the most effective additive groups (in descending order) to reduce yield stress were surfactants, additives with polar head groups, additives with hydrophobic tails, unmodified protein and polymers. CTAB (cetyl trimethylammonium bromide) and CPCl (cetylpyridinium chloride), both surfactants, were two of the most effective additives for reducing yield stress. Samaniuk et al. [25] used water soluble polymers (WSPs) like carboxymethyl cellulose (CMC), polyethylene oxide (PEO) and polyacrylamide (PAM), to modify rheological properties of lignocellulosic slurries. Additives like CMC reduced the friction between cellulose surfaces, making it easier to mix high-solids
suspensions. The addition of 2% CMC reduced the yield stress by ~67% from 55 kPa to ~18 kPa. A four-fold increase in CMC resulted in reducing by another 50%. They also found that a lower degree of substitution for CMC had a positive impact on the yield stress; however, this trend was more apparent at higher CMC loadings. Furthermore, a reduction in yield stress was observed as the molecular weights of the WSPs increased up to a certain point. For example, yield stress decreased with the addition of 600 kDa, as well as 2000 kDa, PEO, but no further change in yield stress was observed with the addition of 7000 kDa PEO. Several other additives were screened by monitoring the reduction in torque as measured by a torque rheometer to determine whether they warranted further investigation. Fly ash and microcrystalline cellulose were evaluated as possible additives, but their impact was limited. The surfactant Polysorbate 80 reduced the yield stress by 36% but required high concentrations (10%). Guar gum, hydroxypropyl methyl cellulose (HPMC), a guar gum-xanthan gum mixture and a guar gum-HPMC mixture were all more effective than CMC, where guar gum and the two mixtures containing guar gum resulted in the highest reduction in torque (~80%). The addition of additives may be costly, but like the pulp and paper industry, it may become economically feasible to utilize such methods of modification for high-solids conversion processes. It is important, however, that these additives be as inexpensive as possible and do not negatively impact the conversion process by inhibiting the hydrolytic enzymes or fermentative organisms.

4. Impacting Enzymatic Hydrolysis Rate and Extent

The term “lignocellulosic biomass” refers to many different types of biomass, including forestry and agricultural residues (woody biomass, straw, stover), fermentation by-products (DDGS) and dedicated energy crops (grasses), just to name a few. Each type of lignocellulosic
material is slightly different in regards to composition, resulting in unique challenges in the enzymatic hydrolysis step of the conversion process. The following sections are organized based on various aspects in need of consideration during the conversion of lignocellulose and highlight some of the challenges and breakthroughs associated with enzymatic hydrolysis performed at high-solids loadings for different types of biomass. It is important to note that while each of these processing approaches are discussed individually, it is often difficult to separate out the combined effects of multiple process conditions.

Furthermore, when determining cellulose conversion, it is important to note that the standard method of calculating conversions as described by [32] can grossly overestimate actual conversion for high-solids systems. In some instances, conversions can be overestimated by up to 36% [5]. Determining cellulose conversion in high-solids systems can become very complicated, but several studies have proposed new methods for determining cellulose conversion [5, 33, 34] under these high solids operating conditions. The standard method for conversion calculations typically compares the amount of glucose measured in the hydrolyzate (the liquid fraction) to the potential glucose found in the biomass (the solid fraction). This method requires the assumption that all components have a consistent density throughout the reaction and that it is approximately equal to that of water. As solids loadings increase, this assumption no longer remains valid, resulting in overestimated conversions.

4.1 Biomass Processing

Enzymatic hydrolysis is an intermediate step in the conversion process, and while producing high sugar yields is favorable, the resulting hydrolyzate must be subsequently capable of supporting fermentative organisms while they produce biofuels. Some of the more expensive
steps in substrate preparation are washing the substrate following pretreatment and detoxifying the hydrolyzate produced during enzymatic hydrolysis. It is likely that for industrial processes unwashed, whole slurries (liquid + solids) from pretreatment will be used in enzymatic hydrolysis [2], indicating a need for robust enzymes capable of maintaining their activity in the presence of possible inhibitors and degradation products or developing pretreatments that do not produce such products. Furthermore, the cost of hydrolyzate detoxification alone can be up to 22% of the total ethanol production cost [35].

Several studies have investigated the effects of eliminating washing and/or detoxifying steps in the lignocellulose conversion process, with some promising results. Hodge et al. [2] studied the effects of soluble and insoluble inhibitors on enzymatic hydrolysis by comparing the glucose yields produced from a washed pretreated substrate (which introduces only potentially insoluble inhibitors into the hydrolysis reaction since all soluble inhibitors are washed away) and an unwashed whole slurry substrate (which introduces both potentially soluble and insoluble inhibitors to the hydrolysis reaction). However, to maintain the high-solids loading and modify the pH, the solid and liquid fractions were separated, the liquid fraction pH was adjusted, and the two fractions were combined. Should the whole slurry be used at the industrial scale (as this study states in its rationalization for this work), this method of pH modification may not be feasible. This challenge is just one of many that must be solved prior to implementing a complete conversion process. Regardless, this study utilized an insoluble solids loading of 5-13% (~9-24% total solids loading) and relatively low enzyme loadings (<20 FPU/g cellulose).

Based on the glucose production from hydrolysis, the authors suggested that the limitations due to mass diffusion are more prevalent than the sugar inhibition beyond a specific solid content. For instance, sugar inhibition would result in a “leveling-off” of the hydrolysis rate, much like
what would be seen in a typical hydrolysis curve. However, a sharp decrease in the hydrolysis rate was reported here. Using the washed substrate, this decrease is not prevalent until ~20% insoluble solids loadings are reached, where convective mixing and available water are negligible, likely indicating the point of mass transfer limitations. This decrease occurs at much lower solids loadings (<10% insoluble solids) for unwashed substrate, indicating that the soluble components contributed to a higher rate of enzyme inhibition or limited mass transfer by reducing the amount of water available for reaction. (Further discussion on the restriction of water can be found in Section 4.4 Solids Effects.)

Pristavka et al. [36] also conducted enzymatic hydrolysis studies with SO₂-catalyzed steam exploded willow. These studies were concerned with simplifying the conversion process by neglecting to wash the pretreated willow between the pretreatment and hydrolysis steps and eliminating mechanical stirring of the biomass slurry. The reason for eliminating the washing step was two-fold. First, less water would be used in the conversion process, making the process more economical and more environmentally friendly. Secondly, washing usually leads to the solubilization and removal of a significant portion of sugars. These sugars ultimately end up accumulating in wastewater, resulting in an expensive processing step to recover them and/or treating the water. The high-solids loadings (up to 25% ODM (organic dry matter)) used in this study would make mechanical stirring of the slurry extremely energy intensive, so it was removed. With these process modifications, a lower degree of conversion was observed as compared to biomass that was washed prior to hydrolysis (53% vs. 74%). However, the degree of cellulose conversion increased to >95% when the pH of the unwashed, pretreated willow was adjusted with solid NaOH to the optimal pH of the enzymes. The significant increase in conversion following pH adjustment highlights the importance of maintaining optimal hydrolysis
conditions for the enzymes, even if that means finding new, inexpensive and less resource-intensive methods of doing so.

Lu et al. [37] investigated the effects (post-pretreatment) washed substrate had on enzymatic hydrolysis and fermentation. Using steam-exploded corn stover, substantial differences in conversion efficiencies were not observed for washed and unwashed substrates up to a solids loading of 30% (w/w). However, closer examination of the conversion calculations revealed differences between washed and unwashed substrates, since conversions were based on water insoluble solids and not total solids content. (Essentially the denominators were different for the two treatments.) Additionally, the pH of the unwashed corn stover was not adjusted prior to addition of enzymes and buffer at pH 4.8. Cellulose conversion remained fairly consistent (70-75%) for all solids loadings, although glucose content was higher for the washed substrate than the unwashed substrate. Ethanol production was also independent of solids loading (up to 30% w/w) for the water-washed corn stover, reaching 92-94% of theoretical yield. However, the results were quite different for the unwashed substrate. At the lower solids loadings studied (10-15% w/w), ethanol production fell to 88% and 86%, respectively, and decreased as the solids loading increased, until no ethanol could be measured (≥25% solids loading). The levels of acetic acid and furfural measured at the higher solids loading reached inhibitory concentrations. Inclusion of the water-washing step following pretreatment appears to eliminate the need for another costly detoxification step following enzymatic hydrolysis for steam-exploded corn stover.

In contrast to this study, others report contradicting results regarding the wash step [35, 38]. Lau et al. [35] reported that when AFEX-pretreated corn stover was fermented following enzymatic hydrolysis at 18% (w/w) solids loading, the ethanol yield of ~93%, even though the
solids loading during hydrolysis and glucose concentration before fermentation were similar to those reported in Lu et al. [37] who reported a 68% ethanol yield. While these results are so different, it should be noted that different pretreatments, as well as fermentative organisms were used (E. coli vs. S. cerevisiae, respectively), making it difficult to directly compare these fermentation results. However, Lau and Dale [38] achieved higher ethanol production rates fermenting unwashed substrates (~0.17 g/L/hr as compared to 0.12 g/L/hr for washed substrate) with S. cerevisiae 424A (LNH-ST) (a genetically modified strain for improved xylose fermentation), suggesting that the elimination of the washing step following pretreatment, and with no adjustments made to the pH prior to hydrolysis, is beneficial for fermentation under the conditions examined in this study. Ethanol concentration from unwashed substrate was 40 g/L (no data given for washed substrate). Xylose metabolism from the genetically modified strain is likely the largest contributing factor to the discrepancy in reported ethanol yields, but it was also reported that the this strain of S. cerevisiae performed similarly on washed substrate as compared to unwashed substrate. This study suggests that the washing step can be eliminated without any loss in ethanol yield. Contradictory results indicate the need for further study of this issue, or at the very least, optimization studies under specific process conditions.

In another study, LHW-pretreated sweet sorghum bagasse was hydrolyzed at 15-30% solids (w/v) with either 20 or 30 FPU/g glucan cellulase [39]. Washing the substrate prior to hydrolysis also did not improve the conversion rates. Washed substrate yielded 63.2 g/L of sugar, whereas the unwashed substrate resulted in a sugar concentration of 66.1 g/L. It was suggested, although not verified, that the washing step actually removed some of the smaller cellulose particles that may have been easier to hydrolyze than larger cellulose particles.
The inconclusive results of these studies illustrate the complexity of defining appropriate processing conditions that work in all situations. Operating conditions must be chosen carefully in order to realize the full potential of using lignocellulose as a valuable energy source. Table I illustrates the wide variety of operating conditions that have been studied with regards to high-solids loadings enzymatic hydrolysis. Depending on various factors, like substrate choice, pretreatment conditions and hydrolysis conditions, it may be possible to eliminate certain steps like washing pretreated substrate or detoxifying hydrolyzate prior to fermentation, thus simplifying the overall conversion process. However, elimination of these steps may present new problems that must be solved. For instance, should the washing step following pretreatment be eliminated, it may be necessary to adjust the pH in another manner so the hydrolytic enzymes can work most effectively.

4.2 Feeding Strategies

Fed-batch feeding schemes have been investigated as an alternative method of achieving high-solids loadings in enzymatic hydrolysis [1, 26, 45, 46] because of some of the advantages it offers over single feeding schemes. For instance, the initial viscosity is lower, so diffusion and mixing limitations can be minimized or altogether avoided. A fed-batch feeding regime also allows time for the slurry to liquefy before adding additional solids, which maintains a level of free water that is available for the reaction process and for diffusion (away from the enzymes) of potentially inhibitory products that result from the hydrolysis reaction. However, when a fed-batch approach is selected, one must consider how and when to add substrate, as well as enzymes, to the reaction in order to maintain high rates of conversion. Table II illustrates the variety of substrate and enzyme application rates used in fed-batch studies.
Hodge et al. [1] conducted a study in which the fed-batch approach was utilized in order to achieve a final insoluble solids content of 15% (w/w) (equivalent to a 25% (w/w) initial solids loading). This solids loading was the upper limit of unhydrolyzed pretreated corn stover that could be effectively mixed in the stirred tank reactors (STRs) available to the researchers. High cellulose conversion (>80% cellulose conversion) was reported; however, the reaction time was more than double the typical hydrolysis reaction time (168 hrs vs. 72 hrs). The extended time problem may be overcome through the use of higher enzyme loadings or enzymes that can tolerate higher sugar concentrations. The enzyme loading used in this study was 10.7 FPU/g cellulose, a relatively low loading, and it was applied proportionally with each addition of substrate. A study conducted by Yang et al. [46] obtained a similar cellulose conversion (70.6%), with a higher solids loading (30%), an enzyme loading almost twice (20 FPU/g cellulose) that used in the former study and with a much shorter reaction time (30 hrs). Both studies attribute the high conversion rate, at least in part, to the fact that the substrates were washed prior to hydrolysis, possibly eliminating any potential inhibitory products that resulted from the pretreatments. The latter study also supplemented fresh enzyme with each addition of new biomass, which increased the final enzyme loading from 10 to 15 FPU/g cellulose. The fresh enzyme may have also enhanced the glucose yield, replacing the enzyme that may be non-productively bound to the lignin or deactivated by extended hydrolysis times.

Zhang et al. [52] studied another fed-batch approach for the conversion of NaOH-pretreated sugarcane bagasse and wheat straw. Pretreated biomass was fed into the reactor at 9%, 8%, 7%, and 6% solids over the course of 48 hrs to achieve a final solids loading of 30% (w/v). All enzymes were added with the first addition of lignocellulose. Glucose conversion from wheat straw reached a maximum (~60%) after the first feeding, but decreased with each
successive feeding. The higher rate of conversion was likely due to the low solids loading and high enzyme loading at the beginning of the reaction. With each successive feeding, the enzyme: substrate ratio decreased. After 72 hr of hydrolysis, the conversion began to level off, resulting in a final glucose conversion of 39%. A slightly different conversion profile was observed with the bagasse. The conversion continued to increase over the course of the hydrolysis reaction, with the exception of the last feeding time (6% solids at 48 hr). The final feeding resulted in a sharp decrease in conversion, but it recovered within 24 hr following the feeding, leading to an increase in conversion over the batch. The final glucose conversion of the sugarcane bagasse was 55%. Differences in the way the pretreatment affected the lignocellulose may have led to the different glucose yields between the two substrates. It was reported that the pretreatment caused the surface of the two substrates to become rough and fragmented as lignin was removed, allowing for better access to the cellulose; however, the bagasse appeared to have a rougher, more fragmented surface than the wheat straw. Following 144 hr of hydrolysis, the surfaces were relatively smooth as compared to the start of the hydrolysis.

Wang et al. [39] considered the use of a fed-batch feeding scheme. Initially, the reactors were charged with half of the final solids loading, followed by two additional feedings at 24 and 48 hr of one-fourth of the final solids loading. The system containing 30% solids achieved the highest final sugar concentration with nearly 115 g/L. Even with the fed-batch system, the conversion decreased with increasing solids loadings; however, the conversion of the 30% solids reaction was only 5% less than the systems at 15% and 20% solids (55% vs. ~60%)

Fed-batch was utilized by Ma et al. [55] to achieve a 25% (w/v) solids loading. Enzymes were added either all at once at the beginning of the reaction or with each addition of the dilute
acid pretreated cassava bagasse. At this solids loading, the batch reaction reached ~50% conversion, whereas the fed-batches with a single enzyme addition and multiple enzyme additions achieved ~75% and 84% conversion, respectively. These results are similar to those reported in other fed-batch studies [1, 46], indicating that under the right conditions fed-batch systems may be a plausible solution for achieving higher conversion rates for hydrolysis performed at high-solids loadings.

Rosgaard et al. [26] investigated several different regimes for batch and fed-batch hydrolysis, including variations of sequential addition of substrate as well as substrate plus fresh enzyme. The addition of fresh enzyme with each substrate addition maintained a constant enzyme:substrate ratio throughout the whole reaction, as opposed to the other fed-batch feeding schemes where all the enzyme was added in one application. In these cases, the effective enzyme:substrate ratio decreased with each subsequent addition of substrate. Not surprisingly, the fed-batch schemes that received the full enzyme application at the start of the reaction produced higher glucose yields during the first few hours as compared to the fed-batch reactions that received fresh enzyme with each substrate addition. However, the extent of the hydrolysis reaction was not affected by the method of enzyme application as the final glucose concentrations were not different for the fed-batch reactions with and without additional enzyme applications (62-67 g/L). Furthermore, lower viscosity is often touted as an advantage of fed-batch systems over batch systems because mixing becomes easier as viscosity decreases. The viscosities of the fed-batch systems in this study were lower than in the batch systems, but no benefits were observed in regards to glucose production as the batch system at 15% solids resulted in higher glucose production (78 g/L) after 72 hr hydrolysis. Final glucose concentrations of the fed-batch systems, though, were impacted by each addition of substrate.
Hydrolysis rates decreased and never fully recovered, resulting in lower final yields than the batch systems.

Additionally, Chandra et al. [45] reported on a fed-batch approach at a moderate solids loading that did not perform as well as a single stage feeding approach. The total solids loadings achieved for both feeding schemes was 10%. Two enzyme loadings were tested (5 and 60 FPU/g cellulose), and at both loadings, the batch reaction produced the higher yields, approximately 66% and 90% for steam-pretreated corn stover, respectively. However, when the solids are fed at 24 hr intervals, the respective yields are lower (approximately 55% and 80%) and the hydrolysis rates slower. The authors suggest these reductions in yields and rates are the result of non-productive binding of enzyme to xylan or lignin fractions of the substrate or the inability of the enzyme to desorb from partially hydrolyzed substrate and find accessible cellulose sites in the fresh substrate. Free protein measurements taken at 72 hr indicate that 50-70% of the cellulase was still adsorbed to the substrate for both enzyme loadings, while the cellulose conversion ceased. The lower hydrolysis rate at the higher enzyme loading seems to indicate that the enzymes are saturating the accessible cellulose sites, thus reaching a maximum hydrolysis rate that is lower than that of the batch reaction when all the accessible cellulose sites are available at once.

The results of fed-batch feeding schemes are currently still inconclusive, as indicated by the preceding studies, making the decision to use a fed-batch approach unclear. Many advantages are realized regarding the use of fed-batch systems, but questions persist. For instance, at what point in the reaction should subsequent additions of substrate be applied to maintain a high rate of conversion? Should enzymes be added in a single application, as a supplement to the original application, or proportionally to the substrate? Does the benefit of
reduced viscosity make a difference in energy consumption during the conversion process to overcome the potentially reduced sugar yield that may result from the fed-batch as compared to the batch system?

4.3 Effects of Enzyme Synergism

Enzymatic hydrolysis, especially at high-solids loading, has been identified as the largest impediment to achieving high yields in a timely manner in the lignocellulose to ethanol conversion process, mainly because a significant portion of sugars produced are in oligomeric or polymeric form, which cannot be used in the fermentation process. Several studies have investigated this issue from the perspective of the enzyme (Table I), experimenting with enzyme supplementation (in addition to cellulase) and alternative organism sources for cellulase [38, 47-49]. Supplementing cellulase with β-glucosidase has long been used to minimize end-product inhibition of the cellulase and achieve higher conversions. Lau et al. [48] investigated the use of several different enzymes other than cellulase and β-glucosidase to enhance the conversion of lignocellulose. Their enzyme cocktail included xylanase and pectinase to target the hemicellulose that acts as a barrier to cellulose if not removed during pretreatment. The focus of this work was on the fermentation step, so the details regarding the enzymatic hydrolysis are limited. However, the hydrolyzates produced from AFEX-pretreated corn stover with these enzyme cocktails were able to produce 40 g/L (5.1% v/v) of ethanol with *Saccharomyces cerevisiae*.

Another study investigated the effects of supplementing the typical cellulase and β-glucosidase enzyme cocktail with xylanase on the hydrolysis of steam-exploded barley straw [50]. The addition of the xylanase to the enzyme mixture enhanced the conversion rate of the
cellulose, especially at low solids loading and early in the hydrolysis reaction. Conversion at higher solids loadings may be reduced by the higher concentration of xylooligomers produced with the addition of xylanases, as has recently been shown [15]. However, the xylanase used in the supplementation study did contain some β-xylosidase activity, which, if present, might counteract the inhibition caused by xylooligomers. The positive effects of the xylanase addition reported in this study support the idea that overall enzyme loadings could be reduced if better conversion is achieved by incorporating an array of different enzymes. However, a different study conducted by Di Risio [44] also evaluated various enzyme cocktails made from commercially-available enzyme solutions. All three cocktails assessed consisted of the same base solution: cellulase and β-glucosidase. Each solution was supplemented with a third commercial enzyme solution with different active components: cellulase + xylanase, cellulase + xylanase + β-glucosidase, and xylanase. The highest glucose yields (44%) resulted from the enzyme cocktail consisting of the base solution supplemented with the commercial solution containing cellulase + xylanase + β-glucosidase activity. Surprisingly, the enzyme solution supplemented with the enzyme promoted as a “xylanase” actually yielded significantly less xylose than the other two enzyme solutions (39% as compared with 54% and 85%). However, there is no indication that the xylanase activity of this commercial product was independently verified prior to use. Glucose yields ranged from 32%-42%.

Taking it a step further, another group studied the effects of various addition schemes and enzyme loadings using an enzyme cocktail containing cellulase, β-glucosidase and xylanase on the hydrolysis of mixed hardwood chip pulps [42]. The enzyme cocktails consisted of fungal cellulase (C), xylanases (X) and β-glucosidase (B) solutions mixed in the ratio of 10:3:3 (by volume). The mixtures were added to the substrate in the following manners: (1) cellulase,
xylanases and β-glucosidase was mixed with substrate at the desired solids loading (CXB); (2) cellulase was added to 5% solids, pressed or filtered to obtain the desired solids loading, and hydrolyzed for a period of time before the xylanases and β-glucosidase mixture was added (C+XB); and (3) half of the cellulase was added to 5% solids, pressed or filtered to obtain the desired solids loading, and hydrolyzed for a period of time before the cellulase (half dose), xylanases and β-glucosidase mixture was added (C+XB). With the CXB mixture, a decrease in conversion was observed with an increase in solids loading. Enzyme loading also plays an important role in the optimization of biomass conversion. For example, with the CXB enzyme mixture, the difference in sugar yields decreased with increased enzyme loadings. At 40 FPU/g solids, conversion decreased from 70% to 68% for 5% and 20% solids loading, respectively, which represents no significant difference in conversion. However, at 5 FPU/g solids, conversion decreased from 40% to 19% for 5% and 20% solids loadings, respectively. The authors hypothesized the decreased conversion was the result of ineffective mixing of the enzyme mixture with the substrate as the solids loadings increased. Based on this hypothesis, the authors added the enzyme to a low solids mixture, allowing time for the enzymes to adsorb to the substrate, before filtering off 80% of the liquid to obtain 20% solids loadings. Enzyme activity was tested following filtration to determine whether any enzyme was lost during this process. Cellulase activity registered at 80% of the original activity, whereas only 20% of the xylanases activity was retained. This observation resulted in the modified application of the enzyme mixture (C+XB). At 20% solids and 20 FPU/g solids, sugar conversion increased from 44% for the CXB mixture to 59% for the C+XB mixture. Sugar concentrations increased from 84 g/L to 114 g/L. This modified enzyme application process was also beneficial at low solids loadings (5%), increasing conversion from 19% with CXB to 38% with C+XB. Taking this enzyme
application process one step further, additional cellulase was added with the xylanases and β-glucosidase mixture (C+CXB). In this instance, although the sugar concentration increased to 121 g/L glucose (63% conversion), the conversion at 20% solids was similar to that at 5% solids at all enzyme loadings tested. These experiments indicate the importance of determining enzyme mixtures and application schemes that provide the optimal sugar yields and concentrations for the conversion process.

Along with the feeding scheme and the enzyme loading, the type of enzyme used can have a significant impact on the liquefaction of biomass. The term “cellulase” can refer to a wide variety of enzymes, and commercially available enzymes can often be a crude mixture of enzymes (i.e. *T. reesei* cellulase that is commonly used in hydrolysis studies). To be more specific, for example, the *T. reesei* “cellulase” can refer to a mixture of cellobiohydrolases (CBH), endoglucanases (EG), xylanases (XYLs), and β-glucosidase, among other enzyme components. Using an array of CBHs, EGs, XYLs and a β-glucosidase, both individually and in combination, Sjizarto et al. [30] assessed the enzymes on their ability to liquefy hydrothermally pretreated wheat straw. For the *T. reesei* components, it was determined that the EGs (especially Cel5A) were the most important in liquefying lignocellulose. This enzyme alone reduced the viscosity of the slurry by nearly 90%. The CBHs and XYLs had little to no effect on the viscosity, even though the sugar production was similar to that of some of the EGs. Furthermore, a mixture of enzymes produced the highest sugar yields, even though the viscosity was reduced by only about 82%, indicating that the amount of sugar hydrolyzed is not the main factor in reducing viscosity, but that the sites at which the polysaccharides are cleaved is more important.
Since enzymes play such a vital role in the conversion of lignocellulose, much of the process integration depends on these biological catalysts. For instance, a balance must be struck between the enzyme loading used and enzyme cost. High enzyme loadings not only increase the total cost, but as discussed in the introduction, studies suggest that enzymes are overcrowding accessible cellulose chains, thus reducing the rate at which cellulose is hydrolyzed. One such study was conducted by Olsen et al. [58]. At a solids loading of 29% (w/w) pretreated corn stover, a range of enzyme loadings (5-83 FPU/g cellulose) were evaluated for hydrolysis yields. At enzyme loadings >66 FPU/g cellulose, the hydrolysis curves started to coincide. It was suggested that the lack of improvement in hydrolysis rate and conversion was due to the substrate being completely saturated with enzymes bound to all the accessible sites. High enzyme loadings also do not make sense economically. Based on a techno-economic model of the bioethanol conversion process, an optimum total solids loading of about 20% with an enzyme loading of 20 mg/g solids (8.8 FPU/g solids) was determined to produce the minimum ethanol selling price with currently available, commercial enzymes [4]. This model evaluated the cost of production at 2007 enzyme production costs ($0.35/gal), as well as the enzyme production cost projected by the Multi-Year Program Plan (MYPP) from the DOE’s Office of Biomass Program for 2012 ($0.12/gal) [59]. At the lower enzyme production cost, solids loadings could potentially be increased up to 26% and remain economically viable. In the time since this study was published, the MYPP re-evaluated the cost of enzyme production and the current projection for 2012 was fairly consistent with the “high” cost of enzyme production reported in the study at $0.34/gal of ethanol (2007$). Under the assumptions made constructing this model, 20% solids loading remains the maximum that is economically feasible for the ethanol production process.
Zhang et al. [43] evaluated enzyme loading to determine the effect it had on glucose concentration. A 50% reduction in enzyme loading decreased the glucose concentration by only 21%. The implication of this observation is that enzyme loading can be optimized to provide the maximum concentration at the lowest unit cost. For example, it may not be worth converting an extra 5% of glucose if it accounts for ~15% of the total enzyme cost unless the return on the extra glucose recovers the cost of the additional enzyme.

While the cellulase system of T. reesei is one of the most commonly studied enzyme systems, other organisms also produce cellulolytic enzymes that could potentially impart superior activity under certain conditions. Ingram et al. [53] compared the conversion efficiencies of enzymes from two different organisms, T. reesei and a genetically-modified (for increased cellulase production) strain of Penicillium janthinellum. Enzyme mixtures from both organisms contained cellulases, β-glucosidases and xylanase activity. With the cellulase from T. reesei, an increase in glucose concentration as biomass loading increased was observed for the organosolv and the LHW-pretreated rye straw. After 48 hrs of hydrolysis at 17.5% solids, the P. janthinellum cellulase converted 72% of the soda-pretreated rye straw. Higher enzyme loadings of P. janthinellum cellulase were necessary to achieve the same level of conversion produced by the T. reesei cellulase (27 FPU/g cellulose vs. 13 FPU/g cellulose); however, the P. janthinellum cellulase appeared to be more tolerant to changes in pH. This study highlights the fact that the conversion process is dependent on many factors, including, but not limited to, the type of biomass, the conditions of the pretreatment, and the source of enzymes.

In another study partially purified cellulase from the thermostable Geobacillus R7 was evaluated as an alternative cellulase source [47]. For short hydrolysis times (36 hr), the Geobacillus cellulase was comparable to a commercial enzyme preparation. However, for
hydrolysis of pretreated prairie cord grass using this cellulase, the glucose recovery at 96 hrs for solids loadings ≥10% was between 46.2% and 48.7%. It does not appear that the solids loading had much of an impact on conversion of the prairie cord grass; although the conversion of cellulose into glucose utilizing the Geobacillus R7 cellulase was better than the conversion of the pretreated corn stover at 27%-31%. Geobacillus R7 also has the added benefit of being ethanologenic. During the hydrolysis, Geobacillus R7 produced a small amount of ethanol (0.035 g/L) from the pretreated prairie cord grass, which has possible implications for consolidated bioprocessing of lignocellulose materials. Subsequent fermentation of the hydrolyzate with S. cerevisiae resulted in an ethanol production of 7.8 g/L (or 0.47 g ethanol/g glucose) for the 20% solids loading of prairie cord grass.

Lastly, Matano et al. [60] engineered fermentative yeast to express three different types of cellulase on its surface. This yeast was subsequently evaluated in SSF processes utilizing 25% (w/v) pretreated rice straw. Initially, a control yeast strain was supplemented with a commercial cellulase (100 FPU/g biomass). This combination resulted in an ethanol yield of 80% and liquefaction after 72 hr. When combined with the modified yeast strain, the commercial cellulase loading could be reduced to 10 FPU/g biomass and produce the same ethanol yield (79%). Further study showed that a maximum ethanol concentration (43.1 g/L) was obtained following a 2 hr liquefaction period prior to the addition of the modified yeast, corresponding to an ethanol yield of 89%. Residual glucose was reduced by an order of magnitude with the modified strain (16 g/L to 1.6 g/L). The authors hypothesized that the close proximity of the cellulases on the surface of the yeast provided a synergistic effect that resulted in an increased hydrolysis of cellulose. As commercial enzymes are still a relatively large portion of the overall cost of the conversion process, the ability to reduce the commercial
enzyme loading and replace it with an organism capable of both the hydrolysis and fermentation is very attractive.

4.4 Solids Effect

For conversion of lignocellulose into usable and valuable products, it makes economical sense to utilize locally-available biomass, as shipping biomass over long distances greatly reduces the beneficial impacts. Cara et al. [41] studied the conversion of olive tree pruning biomass (consisting of leaves and thin branches) up to 30% (w/v) solids loadings. The final glucose concentrations increased with increasing solids loading, achieving 61 g/L and 52 g/L glucose at 30% solids loading of the liquid hot water (LWH) pretreated biomass and steam exploded biomass, respectively. However, the conversions of the LHW-pretreated biomass decreased nearly linearly from 76.2% at 2% solids to 49.9% at 30% solids. Conversions of the SE-pretreated biomass held steady between 60% and 63% up to 10% solids loading before decreasing to 39.6% at 30% solids. In a different study, the researchers also observed that the glucose concentration decreased as the solids loading was increased beyond 10% solids for the soda pretreated rye straw [53]. The overall conversion of cellulose decreased from ~65% to 40% as solids loadings increased from ~10% to 17.5%. This result is not unusual, as most studies performed at high-solids loadings sacrifice conversion for a more concentrated glucose product [10, 29, 41].

Kristensen et al. [10] also studied four mechanisms that possibly contribute to the so-called solids effect: (1) compositional and substrate effects, (2) product inhibition, (3) water concentration, and (4) cellulase adsorption. These mechanisms were studied with filter paper, which is essentially a pure cellulose substrate. The researchers observed the same decreasing
trend in conversion as solids increased using the filter paper, much like that observed with lignocellulose. Therefore, it was concluded that lignin, which is absent in filter paper, is likely not the reason for the solids effect. Study of the second mechanism, product inhibition, resulted in significantly different conversions after 48 hours of hydrolysis for 5% DM and 20% DM (64.5% vs. 38.6% or 30 g/L vs. 86 g/L, respectively). However, the final conversions for these solids loadings with an additional 50 g/L glucose added resulted in fairly similar conversions (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 glucose, respectively). This experiment did not elucidate the exact reason for the observed similar conversions, but two hypotheses were offered. It was suggested that other components in the hydrolysis mask the product inhibition or that enzymes are inhibited similarly once a certain glucose concentration is reached.

Kristensen et al. [10] next attempted to quantify the effects of water on the hydrolysis reaction. Water content was decreased by 25% and replaced by oleyl alcohol. The alcohol allowed the viscosity of the slurry to remain constant, thus removing the effects of the viscosity, while the water to solids (or enzyme) ratio was altered. With this decrease in water, a 5% decrease in glucose yield was observed. However, increasing the solids content from 20% to 25% (which is essentially equivalent to a 25% reduction in water), typically decreases glucose yields by ≥12%. The authors argue this discrepancy in glucose reduction indicates that lower water content is apparently not the limiting factor responsible for the solids effect.

Lastly, cellulase adsorption was investigated as a possible source of the solids effect [10]. Cellulase adsorption to filter paper was determined by measuring the total nitrogen content of the biomass after 24 hr of hydrolysis. The amount of adsorbed cellulase measured was halved (40% to 17%) as solids loading increased from 5% to 25%. At the same time, conversion was reduced
from ~60% to <50%. A strong correlation between decreasing adsorption and conversion was observed, indicating that cellulase is not effectively adsorbing onto cellulose causing a decrease in yield. The authors hypothesize that increasing concentrations of glucose and cellobiose inhibit the adsorption of enzymes. Knowledge of the mechanisms of high-solids product inhibition and the mechanisms of high-solids enzyme adsorption inhibition can provide the key to improving the overall conversion process, thus unlocking the full potential of high-solids conversions.

In contrast to the previous study, Roberts et al. [56] investigated the interactions of water with biomass at high-solids loading without maintaining a constant viscosity. Time domain NMR was used to measure the transverse (or spin-spin) relaxation times ($T_2$) of protons in water molecules to indicate the extent of water constraint (or degree to which water is tightly bound to biomass). Essentially, the nuclei of water molecules that are tightly bound have a shorter relaxation time than nuclei that are less tightly bound. By measuring these relaxation times, constraint can be determined. It was found that water was more tightly bound as solids loadings increased, suggesting that an indirect relationship between water constraint and yield exists. However, the relaxation time of the primary bound water (water that interacts directly with the surface of the cellulose) was constant regardless of the solids loading. Interactions at the water-solids interface appear to remain constant, suggesting the chemistry at the surface of the cellulose does not change as water content changes. These results further suggest that the water primarily interacts with the cellulose, and the impact of the solute is minimized. However, these studies were conducted with bacterial cellulose, a substrate that is essentially pure cellulose. It is unclear whether cellulose derived from pretreated lignocellulose would interact with water in a similar manner or to what extent the type of pretreatment may affect these cellulose-water interactions. With the addition of excess glucose or mannose to 5% solids, the hydrolysis rate
reduced to one similar to 15% solids loading. The authors hypothesize that the negative effects on the hydrolysis rate are caused by water constraint as opposed to the monosaccharides impacting the enzyme activity. It is also possible that the lack of available water limited the uniform distribution of synergistic enzymes, thus hindering the hydrolysis rate. Also, in contrast to the previous study, the results presented in this study indicate that water (or the lack of it) has a great impact on the overall hydrolysis rate. Even though the addition of oleyl alcohol in the former study reduced the water content in the reaction, the constant viscosity helped maintain adequate mixing and therefore did not limit the diffusion of enzymes throughout the suspension. While these studies draw conflicting conclusions on the effect of water on lignocellulose conversion, they do highlight the need for effective mixing. Adequate mixing was provided in the former study, even with a low water: substrate ratio because of the low viscosity afforded by the addition of alcohol, whereas the latter study simply reduced the water: substrate ratio without regard for the viscosity. These studies also highlight the difficulty of quantifying and assigning the challenges of operating at high solids to any one factor (lack of water, high viscosity, adequate mixing, etc.) when all these factors are so interrelated.

4.5 Effect of Viscosity on Mixing

High viscosity of high-solids slurries is another hurdle that must be overcome. Much of the previous discussion (i.e. effects of enzymes on liquefaction and solids loadings) also affects the rheology, but this section discusses specific viscosity modifiers and their effects on enzymatic hydrolysis. Ineffective mixing increases the limitations associated with mass transfer, including removal of local inhibitors and hydrolysis products and transfer of heat throughout the reactor. The pulp and paper industry has long been using viscosity modifiers to enhance the
processability of fibrous slurries [31], much like the types of slurries produced by lignocellulose materials prevalent in the conversion to biofuels and biochemistries. One study [31] investigated the use of 18 different chemical additives and evaluated the effects on the slurry rheology and hydrolysis rates. Several surfactants added to lignocellulosic slurries at 2% (w/w), including CPCl, CTAB, sodium dodecylbenzene sulfonate (NaDBS) and sodium dodecyl sulfonate (SDS), positively affected the rheological properties of the slurry by reducing the viscosity by nearly four-fold as compared to the viscosity of the unmodified slurry. Although slight decreases in the extent of the hydrolysis reactions were observed, only the CPCl and the CTAB did not reduce hydrolysis rates. Additionally, Ma et al. [55] tested the surfactant Tween-80 and found that it did not produce a significant increase in conversion at a 10% solids loading to warrant its use. However, at 25% solids loading, the addition of the surfactant (2 g/L) increased cellulose conversion by 30%. Contrary to what Kristensen et al. [10] said, the inhibition caused by non-productive binding of the enzyme to lignin does not seem to have as large of an effect at low solids as it does at high solids. These results show some promise in modifying viscosity properties of lignocellulose slurries; however, more work is warranted to understand the mechanism by which these surfactants work, as well as determining the economical value of the use of such additives.

Another approach to reducing viscosity is to raise the temperature at which the hydrolysis reaction takes place [61]. In order to work at higher temperatures, enzymes that can tolerate the increased temperatures must be used. It has been shown that EGs from more thermotolerant organisms worked better at reducing the viscosity of a lignocellulose slurry, while other types of enzymes appeared to have little effect [61]. *T. aurantiacus* proved to be more thermotolerant than *A. thermophilum*, as the *T. aurantiacus* EG continued to reduce the viscosity at temperatures
up to 75°C. *A. thermophilum* enzymes were less active above 65°C, resulting in a reduced effect on the viscosity. The ability to use alternate sources of cellulase enzymes illustrates the number of reaction condition variables (i.e. temperature, components in enzyme cocktail, and solids content in slurry) open to modification.

The method of mixing the slurry can also have a substantial impact on the conversion of lignocellulose. For example, Zhang et al. [43] observed a significantly reduced liquefaction time when comparing hydrolysis at high solids (17-20% w/w) performed in shake flasks with a lab-scale peg mixer. Peg mixers are commonly used in the pulp and paper industry, which routinely utilizes solids loadings up to 35% [43]. (Readers are referred to the section entitled “Reactor design for enzymatic hydrolysis at high solids” for more details on the peg mixer.) Liquefaction occurred after 1 hr of hydrolysis in the peg mixer, whereas the shake flask required 40 hr. The decrease in liquefaction time can most likely be attributed to the effective mixing provided by the peg mixer and the breaking down of the large fiber network that tends to occur as solids loadings surpass 8%. At 20% (w/w) solids loadings, hydrolysis performed in the peg mixer resulted in 144 g/L and 158 g/L of glucose from unbleached hardwood and Organosolv pretreated poplar, respectively. These concentrations are the highest glucose concentrations achieved known to the authors at the time of writing this review.

One of the highest solids loadings in enzymatic hydrolysis reported to date is 40% (w/w) [29, 51]. A horizontally-oriented rotating drum was utilized as the reactor in these studies in order to effectively mix the solids. The studies found that cellulose and hemicellulose conversion decreased from ~90% to ~33% and ~70% to 35%, respectively, with the increase in solids loading from 2% to 40%, but the reactor was providing adequate mixing as evidenced by the conversion of lignocellulose into fermentable saccharides (86 g glucose/kg at 40% solids).
At 40% solids, liquefaction occurred after only 4 hrs. The viscosity was still high, as the slurry turned into a thick, clay-like paste and remained as a thick paste following 96 hrs of hydrolysis. Additionally, the reactor was a very energy efficient solution to the mixing problem. Mixing speed did not affect the liquefaction time, so relatively low speeds (6.6 rpm) could be used. It was also shown that ethanol could be produced in the same rotating drum reactor from the resulting slurries, where the highest ethanol yield (48 g/kg DM) reported was from the slurry at 35% solids. Even at reduced enzyme loadings (5 FPU/g DM supplemented with β-glucosidase at a 5:1 loading), ~40% conversion for both cellulose and hemicellulose can be achieved at 30% solids loading [51]. These results suggest using one reactor for all processing steps in the conversion of lignocellulose, with the implication that capital and equipment costs can potentially be greatly reduced as both the number of reactors and amount of enzyme used decreases. However, with the yield penalty for conversion at higher solids loadings being high, a full techno-economic analysis would be needed to fully validate such a system operating under the given conditions.

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

Calorimetry has been studied as a new tool for determining enzymatic kinetics of high-solids loadings in hydrolysis [58]. It provides higher sensitivity than HPLC in the early stages of the hydrolysis, making calorimetry a useful tool to evaluate initial rates of hydrolysis. Avicel showed that enzyme hydrolysis slowed when enzyme
loading of >30 FPU/g cellulose were used. It is believed that this reduction in rate is due to the lack of available binding sites on the cellulose, as illustrated by the heat-flow curves converging upon a single value, regardless of the enzyme loading.

Lavenson et al. [57] also implemented the use of new tools to monitor liquefaction and the extent of hydrolysis of cellulose. Liquefaction and the spatial homogeneity of the enzyme distribution in Solka-Floc suspensions (28% w/w) were monitored with magnetic resonance imaging (MRI). The MRI signal is proportional to the amount of free water in the reaction, which correlates to the degree of liquefaction in the system. Additionally, a penetrometer was used to monitor the mechanical strength of the suspension. Measurements were taken on two hydrolysis systems, where one contained a mixed Solka-Floc and enzyme suspension and the other contained a Solka-Floc suspension that received an application of enzyme but no mixing. Mechanical strength of the mixed suspension decreased by 20% of the initial strength after ~30 hrs, as compared to ~170 hrs for the unmixed suspension. Based on the MRI results, the mixed samples did not show a spatial gradient, indicating uniform liquefaction when the enzyme and substrate are initially well-mixed. The unmixed samples showed a slow change in spatial gradients, which were attributed to ineffective diffusion of the enzyme to the substrate. Since liquefaction occurs nearly six times faster for the mixed samples, it is not surprising that higher final glucose concentrations are also obtained as compared to the unmixed samples and in much less time. For example, the mixed suspension reached ~75 g/L glucose in only ~120 hrs, whereas the unmixed suspension produced only ~50 g/L in 300 hrs. Furthermore, adequate initial mixing of the enzyme and substrate resulted in an initial rate of hydrolysis an order of magnitude higher (1.8 g/L/hr as compared to 0.21 g/L/hr).
5. Reactor Design for Enzymatic Hydrolysis at High Solids

Several groups studying the use of high-solids loadings for enzymatic hydrolysis have embraced a horizontal orientation of the reactor [6, 29, 62, 63]. Gravitational or free-fall mixing provides many advantages over typical vertical stirred tank reactors and are used in other industrial processes that require mixing highly viscous slurries, like peanut butter, ketchup and concrete [62, 63]. The horizontal orientation minimizes particle settling and local accumulation of reaction products within the reactor, as well as ensuring better enzyme distribution. These types of reactors are also easily scalable from bench-scale to pilot-scale and larger. Power requirements are lower for horizontal reactors equipped with paddles over vertical stirred tank reactors that provide the same level of effective mixing [62].

Roche et al. [63] employed free-fall mixing in their design for bench-scale reactors for enzymatic hydrolysis. Polypropylene bottles (125 mL and 250 mL) were placed on a roller apparatus in a horizontal orientation. The roller apparatus and bottles were placed in an incubator for temperature control during enzymatic hydrolysis. This roller-bottle system produced results comparable to shake flasks when utilizing intermittent hand mixing, especially following enzyme addition and prior to sampling, for up to 30% solids (data not shown). At 20% solids loading, these two mixing schemes resulted in 80-85% cellulose conversion. The roller-bottle reactors eliminated the human component of mixing, resulting in more consistent mixing and better enzyme and reaction product distribution.

Hydrolysis studies conducted by Dasari et al. [62] utilized a horizontal reactor of intermediate capacity (8 L). The reactor was constructed from a cylinder made of Pyrex glass with aluminum lids fitted over the ends. An adjustable speed, rotating shaft with rubber-tipped, stainless steel blades attached was inserted into the reactor. Three sampling ports were located
along the length of the reactor. Hydrolysis studies comparing the horizontal reactor to shake flasks found, at 25% solids loading, approximately 10% more glucose was produced in the horizontal reactor.

Jorgensen et al. [29] developed a reactor for use in pretreatment and enzymatic hydrolysis processes with a total volume of 280 L. Several features have been implemented into the pilot-scale drum reactor, as well as the smaller glass reactor, to address issues associated with high-solids loadings. The horizontal orientation of the reactors takes advantage of free-fall mixing, eliminating the need for mechanical mixing. Evaluation of a range of mixing speeds (3.3-11.5 rpm) by Jorgensen et al. [29] resulted in no significant differences in cellulose conversion over the tested range, so energy input for mixing is significantly reduced as compared to vertically oriented stirred tank reactors. In addition to free-fall mixing, a rotating shaft affixed with paddles supplies additional mixing capabilities, as the shaft in the pilot-scale reactor can be programmed to change rotational direction two times per minute. The paddles also provide a scraping action that removes lignocellulosic material from the reactor walls, improving heat transfer between the reactor and the biomass.

The Integrated Biomass Utilization System (IBUS) Project coordinated by DONG Energy in Denmark also utilizes free-fall reactors. DONG Energy has free-fall reactors in a variety of sizes for research and development purposes (400 L) and has successfully scaled one up to a capacity of 11,000 L [6, 64]. These reactors routinely operate at approximately 40% solids loading. Larger particle sizes can be used, since the mechanical work of the mixing helps tear biomass fibers and particles apart [6]. This tearing action also increases the surface area of the lignocellulose, resulting in increased enzyme accessibility to the cellulose and hemicellulose.
While most reactors implemented for high-solids enzymatic hydrolysis have employed some form of free-fall mixing, Zhang et al. [18] investigated the effects of a helical impeller in a vertical reactor on SSF at solids loadings up to 30% (w/w) and compared it to a typical Rushton (paddle) impeller (Figure 4a-b). Helical impellers are suggested for use in highly viscous, non-Newtonian fluid agitation, which describes high-solids biomass slurries. The helical impeller performed better than the Rushton impeller with regard to every aspect tested. The feeding rate of lignocellulose into the reactor was adjusted so that a liquefied slurry could be maintained throughout the feeding period. The helical impeller provided better mixing, as the feeding period was completed more than 2 hr sooner than that of the Rushton impeller. The helical impeller also resulted in higher ethanol concentration (51.0 g/L vs. 43.9 g/L) and productivity, as well as consuming less power. At 30% solids (prior to inoculation with the fermentative organism), the Rushton impeller required nearly 40 W/kg corn stover (CS) before decreasing to ~29 W/kg CS after 72 hr of saccharification and fermentation. The helical impeller required ~8 W/kg CS and ~1 W/kg CS prior to inoculation and after 72 hr, respectively. (It should be noted that the stirring rates for the two impellers were different; however, the power requirements were normalized based on the “no-load” power consumption for each impeller.) Lastly, the mixing efficiency of the helical impeller was superior to the Rushton impeller. The geometry of the impeller can play a significant role in effectively mixing biomass slurries. Other geometries tested by Wang et al. include a plate-and-frame impeller and a double-curved-blade impeller (Figure 4c-d). The impellers were tested at various speeds and 100 rpm resulted in the best conversion efficiencies for both geometries. However, the plate-and-frame impeller achieved a higher conversion than the double-curved-blade impeller by nearly 18%, indicating that the geometry of the impeller can have an effect on the hydrolysis. The authors suggested that the

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plate-and-frame impeller provides a more consistent mixing regime at every depth in the reactor, whereas the axial flow induced by the double-curved-blade impeller is a function of the distance from the blades.

Another study investigated the use of a peg mixer (Figure 4e) for enzymatic hydrolysis at high-solids loadings [43]. The mixer used in this study was a 9 L reactor fitted with a rotating shaft with pegs extending out radially. The time for liquefaction of 20% (w/w) of unbleached hardwood pulp was significantly reduced when comparing shake flasks to the peg mixer (40 hr vs. 1 hr). The benefit of this mixer is that it has been proven effective with lignocellulosic material. High-solids enzymatic hydrolysis is just another application for the peg mixer.

From the various aforementioned reactors utilized with high-solids enzymatic hydrolysis reactions, there are several suggestions to improve the mixing of highly viscous slurries. Free-fall mixing relies on gravity to effectively mix the slurry, which consumes less energy than a stirred tank reactor providing a similar degree of mixing. An effective mixing regime can greatly depend on the impeller geometry, as the shape of an impeller can cause large differences in speed and shear effects at various impeller-slurry interfaces throughout the reactor. High shear rates have been shown to disrupt the adsorption of cellulase onto biomass or to even cause the denaturation of cellulase [65, 66]. Lastly, technology should be borrowed from other applications, where possible. For instance, peg mixers are a “tried-and-true” technology that is commonly used in the long-established pulp and paper industry. All of these ideas have shown some promise but require more study and fine-tuning before being implemented into the lignocellulose conversion process.
6. Pilot and Demonstration-Scale Operations

Several plants operating at pilot- and demonstration-scale level have recently come online. These installations will help the industry gain valuable insights and improve upon the challenges and limitations that are not recognized at the laboratory scale.

One such pilot plant constructed in Denmark is operated by Inbicon (a subsidiary of DONG Energy), with a distillation capacity of ~1 ton fermentation broth/hr. Additionally, in 2010, Inbicon opened its demonstration-scale plant that is capable of producing 5.3 million liters of ethanol each year. Enzymatic hydrolysis is performed here at 25-30% (w/w) solids content with a relatively low enzyme loading of 3-6 FPU/g DM. However, the plant is capable of handling up to 40% (w/w) solids in any of its process streams [6, 64]. Since this operation is also used for developmental purposes, they have reactors that range from 400 L up to 11,000 L. Additionally, pretreatment and fermentation are performed at high-solids loadings, 20-40% and ~18% DM, respectively. At the end of the conversion process, the remaining lignin-rich material (40-95% DM) is burned to produce heat and electricity that can be cycled back into the conversion operation.

The National Renewable Energy Laboratory (Golden, CO, USA) recently expanded their lignocellulose processing facilities to achieve a capacity of 4,000 L and to operate at solids loading of ≥20% (w/w) [67]. The conversion process is designed as a semi-continuous operation with pretreatment occurring in horizontal reactors with paddles, taking advantage of the reduced energy inputs required with free-fall mixing of lignocellulose. Following liquefaction at ~24-30 hrs, the slurry is pumped into vertical, stirred tank reactors to complete the enzymatic hydrolysis of the material. This operation is capable of processing about 0.5 to 1 ton dry biomass into ethanol each day.
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.

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:
• 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
  o lower cellulase adsorption (increased concentrations of glucose and cellobiose have been shown to inhibit the adsorption of enzymes onto cellulose);
  o product inhibition of enzymes occurs earlier because of the higher concentration of products;
  o inadequate mixing, which can emphasize diffusional limitations exacerbating product inhibition and access of enzyme to substrate;
  o 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.
Fed-batch systems are worth investigating. While there have been some conflicting results, many studies show overwhelming support for conducting high-solids operations as a fed-batch system.

The use of additives to reduce slurry viscosity has achieved some success at the lab-scale. However, the economics of the use of additives on an industrial-scale should be validated prior to implementation at that level.

The use of high-solids operations would make biofuels produced from the conversion of lignocellulose more economical and more price-competitive with petroleum. Increasing sugar and ethanol yields while reducing capital and production costs, lowering energy demands and lowering water requirements will contribute to a more economically feasible process as compared to one operated at low- or moderate-solids loadings. Despite all the benefits of operating at high solids, the process remains restricted due primarily to the lack of available water within the culture, high viscosities, which translate to difficulties with mixing and handling, and increased concentration of inhibitors, which extend reaction times and increases enzyme costs. Researchers are attacking these issues from many angles, experimenting with different pretreatment methods and various enzyme sources and cocktails, while modifying operating conditions and slurry properties. Although there has been some success at performing enzymatic hydrolysis at high solids at the pilot and demonstration scale, many questions must be resolved before the full potential of high-solids lignocellulose conversion will be realized.

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