Journal of Industrial and Engineering Chemistry 18 (2012) 707–714

Contents lists available at SciVerse ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

# Split addition of enzymes in enzymatic hydrolysis at high solids concentration to increase sugar concentration for bioethanol production

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ARTICLE INFO

*Article history:*
Received 16 April 2011
Accepted 11 July 2011
Available online 11 November 2011

*Keywords:*
High total solids
Enzymatic hydrolysis
Split addition
Sugar concentration
Sugar conversion

ABSTRACT

One challenge in making bioethanol production economical is to increase total solids in hydrolysis system while maintaining sugar conversion efficiency. Because the removal of excess water from hydrolysate requires enormous amounts of heat, large volume of reaction towers and high capital expenditure (CAPEX) for equipment, a lengthy operating time, and high operating costs. When solids loading in hydrolysis system increased from 5% to 20% with no mixing strategies, final sugar conversion decreased markedly. If cellulase is mixed with pulp at 5% solids and pressed to 20% solids, then 80% of the cellulase retained in the pulp thinned down the pulp mixture in 2 h. This thinning effect enabled additional cellulase, xylanase, and β-glucosidase to be mixed into the slurry. Sugar concentration was significantly improved; from 26 g/L to 121 g/L, while sugar conversion was remained as enzymatic hydrolysis with 5% total solids enzymatic hydrolysis. A US patent has been granted to NCSU for this concept and licenses have been granted to various companies.



## 1. Introduction

Biomass has been considered as the only sustainable source of organic carbon. Biofuels, or fuels derived from plant biomass, are the only current sustainable source of liquid fuels [1–4]. Global biofuel production has been increased sharply, but still accounts less than 3% of the global transformational fuel supply [5]. The expanding demand of starch as energy source has driven food price to near record level [6]. Fuel from lignocellulosic waste, could be one of the solution to alleviate the impact of food price of biofuel production, and also relief fossil fuel crises and climate change [7]. Fuel ethanol from lignocellulosic biomass can be produced in three integrated stages: pretreatment, hydrolysis, and fermentation. The purpose of the pretreatment stage is to reduce the recalcitrance of lignocellulose for more effective hydrolysis. Pretreatment can include alkalis, acids, water, gases, enzymes, organic solvents, ionic solvents at a specific temperature, pressure, or physical forces [8]. The advantage of alkaline pretreatment is its ability to recover most of the chemicals used in process. Alkaline pretreatment in the presence of sodium sulfide ($Na_2S$) can help alkali interact and dissolve lignin in the pretreatment liquor. The chemicals and dissolved lignin can be recovered through burning and causticization, which can tremendously decrease the cost of pretreatment [9]. Recycling the chemicals not only decreases the cost of pretreatment, but also decreases the concentration of inhibitors present in enzymatic hydrolysis and fermentation, created by the pretreatment chemicals and byproducts. Therefore, it presented an opportunity for the study of enzymatic hydrolysis with high total solids. Therefore, alkaline pretreatment was selected for this study.

The second stage of this study involves hydrolysis to convert the polysaccharides in biomass to fermentable mono sugars. To efficiently convert pretreated lignocellulosic biomass to fermentable sugars employing enzymatic hydrolysis, three main types of hydrolytic enzymes are involved: cellulase, hemicellulase (xylanase), and β-glucosidase.

Cellulose, one of the major components of biomass, is a β-1,4 linked cellobiose chain. Cellulase is comprised of a family of different enzymes, including exo-1, 4-β-D-glucanase (cellobiohydrolase, also abbreviated as CBH) (EC 3.2.1.91), which cuts cellobiose units off from the ends. Two types of CBHs work for this function: one works from the reducing end (CBH I), and the other works from the non-reducing end (CBH II). Endo-1, 4-β-D-glucanase (EG) (EC 3.2.1.4) randomly cleaves β-1,4-glucosidic bonds inside the cellulose chain. 1,4-β-D-Glucosidase (cellobiase) (EC 3.2.1.21) hydrolyzes cellobiose into glucose and also cleaves off glucose units from cello-oligosaccharide chains. These enzymes work synergistically to hydrolyze cellulose by creating new accessible sites for each other, removing obstacles, and relieving product inhibition [10,11]. For example, as CBHs increase the concentration of cellobiose in the hydrolytic system, the activity of

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1226-086X/$ – see front matter © 2011 Published by Elsevier B.V. on behalf of The Korean Society of Industrial and Engineering Chemistry.
doi:10.1016/j.jiec.2011.11.132

CBH could be inhibited by cellobiose, its end product. Cellobiase can further digest cellobiose into glucose, and therefore reduce the end product inhibition of CBHs by cellobiose.

Hemicellulose in biomass can hinder the reaction of cellulase with the cellulose chain. The function of hemicellulase is to depolymerize the heterogeneous hemicellulose into mono sugars. Hence, hemicellulase also works synergistically with cellulase as it removes hemicellulose from the biomass structure. Hemicellulose removal releases cellulose bundles and provides easier access to its reaction sites [12–14].

Typical enzymatic hydrolysis of woody biomass was conducted at 2–5% total solids substrate (w/w) to ensure proper contact between enzyme and substrate [15]. For enzymatic hydrolysis with 5% total solids, the sugar concentration can never exceed 50 g/L. Inefficient mixing would occur above 10% total solids, during which no obvious whirling of liquid can be observed [16]. With high total solids, cellulose can swell to create a thick mixture, creating difficulty, for instance, in transfer between reactors. Moreover, the removal of excess water from hydrolysate requires an enormous amount of heat, large volume reaction tanks, and a high capital cost for equipment [17]. Further, distillation energy consumption decreases sharply when ethanol concentration increases to 4–5 wt.%, which is equivalent to about 100 g/L of sugar concentration [18,19]. Such a high sugar concentration requires an enzymatic hydrolysis process with high total solids, and high total solids in enzymatic hydrolysis, and it is therefore one of the key issues in decreasing bioethanol production costs. There are limited studies that discuss a enzymatic hydrolysis process with high total solids industry application, such as Cara et al. [20]. However, the hydrolysis efficiency at 20% total solids is 13–25% lower than observing 5% total solids at 48 h. Therefore, a novel enzymatic hydrolysis process for real biomass is needed for better economical feasibility of bioethanol production.

This work is based on the concept of an enzyme binding onto its substrate. Most studies of adsorption were performed with cellulase mono components or domains under 2–8 °C, with pure cellulose as a substrate, while some of the studies were performed at room temperature [21–24]. Data related to adsorption of enzymes onto lignocellulose feedstock at higher temperatures is limited [15,25]. A higher temperature is preferable for industrial processes, given the fact that the substrate is usually at a high temperature from pretreatment before enzymatic hydrolysis [26]. Moreover, 40–50 °C is the range that most lignocellulytic enzymes digest substrates at the highest efficiency [27]. The adsorption of enzymes was investigated on pretreated biomass at 50 °C in this work.

In this study, the adsorption behavior of enzymes onto pretreated biomass was measured, the feasibility of decreasing the amount of water used in the process as much as possible without hurting the hydrolysis performance of enzymes was assessed, the process development was presented, and the best-case scenario was reported. The results suggested that it is beneficial to dose enzymes according to their adsorption characteristics, in order to develop a better process for an industrial application.

## 2. Materials and methods

### 2.1. Green liquor-pretreated pulps

Mixed hardwood chips from a mill in the southeastern United States were used in this study. The chips were screened and the accepts from 3/8 in. and 5/8 in. holes were used for pretreatment. The pretreatments were done in a 7-L M&K Digester with 800 OD grams of chips. The green liquor (comprised of $Na_2CO_3$ and $Na_2S$, Fisherbrand Fair Lawn, NJ) was charged, based on percent Total Titratable Alkaline as $Na_2O$ and the contents pulped to the target H Factor. Table 1 shows the pulping conditions that were used. The pulp yield was 78.4% based on wood. After pulping, the samples were washed overnight. The yield was measured by centrifuging the chip mass and measuring the total solids and total weight. The chips were then disintegrated using a refiner with a 0.005 in. gap and screened using a 0.008 in. screen plate. The rejects were refined with a disk gap of 0.001 in. and added back to the accepts. The pulp was then centrifuged and fluffed for further processing.

**Table 1**
Pretreatment condition of wood chips for green liquor pretreated pulp.

| | |
|---|---|
| TTA[a] | 16% |
| Sulfidity[b] | 25% |
| H Factor[c] | 800 |
| Liquor to wood ratio | 4:1 |
| Cooking temp | 160 °C |

[a] Total of all viable sodium alkali compounds. Calculated as $Na_2O$ based on bone dry wood chips.
[b] Sulfidity was defined as the ratio of $Na_2S$ to TTA.
[c] When relative reaction rate was plotted against cooking time in hours, the area under the curve was defined as H factor.

### 2.2. Raw material analysis

Small scale TAPPI method was conducted for raw materials analysis [28]. Air-dried 0.1 OD gram pulp with 92–95% total solids was added with 1.5 mL of 72% sulfuric acid (Fisherbrand, Fair Lawn, NJ) in a room temperature water bath. The pulp was stirred every 15 min for the duration of the 2-h reaction and transferred into a serum bottle with 56 mL of deionized water. Sealed with an aluminum cap, the serum bottle with the sample solution was heated up to 120 °C at 1.25 atm pressure for 90 min in an autoclave. The autoclaved suspension was then filtered with a fine-sized crucible after being cooled down with running tap water. The filtrate was collected for acid-soluble lignin content analysis at 205 nm wavelength with a UV-Vis spectroscope (Perkin Elmer, Model Lambda XLS, Waltham, MA), and the residue in the crucible was oven dried for Klason lignin content analysis. The filtrate was also used for sugar analysis, which was performed on an Ion Chromatography with Carbo Pac PA 10 (Dionex, Sunnydale, CA). The component of the pulps are shown in Table 2.

### 2.3. Enzyme activity assays

Enzyme activity for cellulase and xylanase test was tested according to the IUPAC standard method by Ghose [29]. A straight standard line was built with a Whatman No. 1 filter paper (Whatman, England). The 1 cm × 6 cm strip reacted with diluted cellulase solution for 60 min in the working buffer (NaAc, $NaN_3$, HAc were from Fisherbrand, Fair Lawn, NJ). The reducing sugar was then tested with 3,5-dinitrosalysilic acid solution (3,5-dinitrosalysilic acid, potassium sodium tartarate, and sodium metasulfite, were from Sigma Aldrich, St. Louis, MO and phenol were from Fluka, Switzerland) for color development by reading UV absorbance at 540 nm (PerkinElmer UV spectroscope, model Lambda XLS) and back-calculated for enzyme stock solution activity. When the cellulase activity was too low for the Ghose method (lower than 0.37 FPU/mL), a standard curve was created by reacting

**Table 2**
Raw materials analysis.

| Pulp | Glucan | Xylan | AISL[a] | ASL[b] | Balance | Error |
|---|---|---|---|---|---|---|
| GL16 | 61.1% | 15.0% | 20.0% | 2.9% | 99.0% | 1.1% |

[a] Acid insoluble lignin content.
[b] Acid soluble lignin content.