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Determining Yields in High Solids Enzymatic Hydrolysis of Biomass

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Abstract As technologies for utilizing biomass for fuel and chemical production continue to improve, enzymatic hydrolysis can be run at still higher solids concentrations. For hydrolyses that initially contain little or no free water (10–40% total solids, *w*/*w*), the saccharification of insoluble polymers into soluble sugars involves changes of volume, density, and proportion of insoluble solids. This poses a new challenge when determining the degree of hydrolysis (conversion yield). Experiments have shown that calculating the yield from the resulting sugar concentration in the supernatant of the slurry and using the assumed initial volume leads to significant overestimations of the yield. By measuring the proportion of insoluble solids in the slurry as well as the sugar concentration and specific gravity of the aqueous phase, it is possible to precisely calculate the degree of conversion. The discrepancies between the different ways of calculating yields are demonstrated along with a nonlaborious method for approximating yields in high solids hydrolysis.

Keywords Enzymatic hydrolysis · Biomass · Bioethanol · High solids · High dry matter · Yield

Introduction

The enzymatic saccharification of biomass to fermentable sugars is a well-known bottleneck in the production of bioethanol in an economically viable manner [1]. An important process parameter in enzymatic hydrolysis is the ability to work at high solids concentrations. A high substrate concentration allows for the production of a concentrated sugar solution, which in turn is beneficial for the subsequent fermentation and, in particular, distillation. The energy requirement for distillation is significantly reduced if the solution contains more than 4% (*w*/*w*) ethanol [2]. Furthermore, working at high solids concentrations lowers heating requirements and increases the volumetric productivity of the plant.

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To reach an ethanol concentration of more than 4% (*w*/*w*), a sugar level of at least 8% (*w*/*w*) is needed. For most types of lignocellulosic biomass, this requires an initial solids content above 20% [3]. Recently, much research has gone into being able to perform and handle enzymatic hydrolyses at high substrate concentrations [4–7]. High solids content could be defined as initial concentrations where little or no free water is present. One solution to increase solids levels has been to replace conventional stirred-tank reactors with so-called gravimetric mixing, enabling liquefaction, saccharification, and fermentation of pretreated biomass at up to 40% initial solids content [3, 7].

Lignocellulosic bioethanol is on the verge of commercial reality [8], and as processes improve and the solids content in enzymatic hydrolysis increase, so does the need to consider the most accurate way of determining the yield in order to compare various enzyme systems, processes, and technologies. Hydrolysis of biomass is a complex reaction where multiple, insoluble polymers are broken down and their constituents dissolved in the liquid phase. During the reaction, the content of insoluble solids decreases, the density of the liquid phase increases as does the volume of the liquid phase. When working at high solids concentrations, it is practical to measure the initial biomass or cellulose concentration in weight per weight (e.g., 25% *w*/*w*). However, usually, sugars are measured by high performance liquid chromatography (HPLC) and only in the aqueous phase, free of insoluble components, and are reported in weight per volume (e.g., 70 g/L).

Often, the above-mentioned factors (including the solids content) are not taken into consideration when calculating the percent-of-theoretical yield. In the following, it is shown how high solids hydrolysis yields are overestimated, when based on the initial volume. Also, we suggest a nonlaborious method for approximating the correct yield.

Materials and Methods

Compositional Analysis

The compositions of hydrothermally pretreated straw (pretreated at 195 °C for approximately 6 min as described in [3]) and filter paper (AGF 725, 140 g/m^2 from Frisenette ApS, Knebel, Denmark) were analyzed using two-step acid hydrolysis according to the procedure published by the National Renewable Energy Laboratory (NREL) [9]. Before hydrolysis, the samples were dried at 45 °C for 1 day. The dried samples were milled in a Braun coffee grinder. Dry matter was determined using a Sartorius MA 30 moisture analyzer at 105 °C. The released sugars were quantified with HPLC as described below.

Enzymatic Hydrolysis

Hydrolysis was performed using an enzyme mixture of Celluclast 1.5 L and Novozyme 188 (weight ratio 5:1, from Novozymes A/S, Bagsværd, Denmark) with a filter paper activity of 75 FPU g^{-1}, as measured by the filter paper assay [10].

The hydrolyses were performed in 100 mL plastic bottles (total reaction mass 50 g), at 5–30% solids content (*w*/*w*) in a 50-mM sodium citrate (pH 4.80) buffer and using an enzyme loading of 5–20 FPU g DM^{-1}. The bottles were placed in a heated (50 °C), horizontally placed drum, rotating at 60 rpm for 24 h. The 80-cm diameter drum was equipped with two inside paddles that lifted and dropped the plastic bottles during rotation, mimicking the gravimetric mixing described in [3, 7]. All experiments were performed in duplicate. Samples for sugar analysis were boiled for 10 min to terminate the reaction.

Samples were spun down in 50 mL falcon tubes (4,223×*g* for 10 min) and the density of the supernatant was measured. The remaining solids were washed with MilliQ water five times to remove any water-soluble material. The solids were then dried at 105 °C and weighed in order to calculate the amount of insoluble solids.

Sugar Analysis

The content of monosaccharides and disaccharides in the hydrolyzed samples (D-glucose, D-xylose, L-arabinose, and D-cellobiose) was quantified on a Dionex Summit HPLC system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80 °C with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL min^{-1}. Samples were filtered through a 0.45-μm filter and diluted with eluent before analysis on HPLC.

Results and Discussion

In the standard for enzymatic saccharification of lignocellulosic biomass proposed by the NREL [11], it is assumed that the specific gravity of all components of the hydrolysis is 1.000 g/mL. The equation for determining the yield can be written as:

$$\text{Percent hydrolysis} = \frac{[\text{Glc}] + 1.0526 \times [\text{Cel}]}{1.111 \times F_{\text{cellulose}} \times [\text{Ini. sol}]} \times 100\% \quad (1)$$

where [Glc] is the glucose concentration in the supernatant of the slurry (in grams per liter), [Cel] is the cellobiose concentration in the supernatant of the slurry (in grams per liter), $F_{\text{cellulose}}$ is the fraction of cellulose in the substrate, and [Ini. sol] is the initial solids concentration (in grams per liter) with the assumption that all solutions and biomass have a specific gravity of 1.000 g/mL. The volume of the reaction is assumed not to change during the hydrolysis and is thus omitted from the equation.

When working with a fixed mass reaction (e.g., 50 or 100 g assays) above a certain solids content, the assumption that all components are of the same specific gravity becomes invalid. Furthermore, calculating the yield by using the "initial" volume of the reaction (assuming that a 50-g assay equals 50 mL) to find the amount of cellulose digested (using the sugar concentration), usually leads to an overestimation of the yield. The reason is that the released sugar is dissolved in less than the "initial" volume, i.e., part of the fixed mass of the reaction is solid matter and thus, the liquid volume is significantly less than assumed.

Although the mass of the reaction is constant during the hydrolysis, there are significant changes to the volume of the reaction. As solids are hydrolyzed, the mass and density of the aqueous phase increases, although not at the same rate. This makes it difficult to calculate the precise amount of cellulose consumed, based purely on the resulting sugar concentrations. However, it is possible to measure the amount of insoluble solids remaining after hydrolysis as well as the specific gravity of the aqueous phase. As the mass is constant, the exact volume of the aqueous phase can be calculated, and based on the concentration of sugars, the exact amount of cellulose that has been converted. The equation for determining the yield then becomes:

$$\text{Percent hydrolysis} = \frac{\frac{m_{\text{reac}} - m_{\text{ins. sol}}}{\text{SG}_{\text{aq. phase}}} \times ([\text{Glc}] + 1.0526 \times [\text{Cel}])}{1.111 \times m_{\text{sub}} \times F_{\text{cellulose}} \times \text{DM}} \times 100\% \quad (2)$$

where m_{reac} is the mass of the whole reaction (in grams), $m_{ins.\ sol}$ is the mass of insoluble solids after hydrolysis (in grams), $SG_{aq.\ phase}$ is the specific gravity of the aqueous phase (in grams per liter), [Glc] is the glucose concentration in the supernatant of the slurry (in grams per liter), [Cel] is the cellobiose concentration in the supernatant of the slurry (in grams per liter), m_{sub} is the mass of the substrate (in grams), $F_{cellulose}$ is the fraction of cellulose in the substrate, and DM is the initial dry matter content (*w*/*w*).

We used this rather laborious method of measuring the fraction of insoluble solids and aqueous phase density to establish the exact yield at various time points/enzyme dosages for hydrothermally pretreated wheat straw at 5%, 10%, 15%, 20%, and 30% initial solids content. The yields were also calculated using Eq. 1 and compared with the exact yields. To illustrate the difference in yield between the two equations, the values for hydrolysis of hydrothermally pretreated straw at 30% initial solids content (50 g assay) are: [Glc]= 136.22 g/L, [Cel]=20.31 g/L, $F_{cellulose}$=0.532, and [Ini. sol]=300 g/L.

Using Eq. 1, this gives a yield (percent hydrolysis) of 88.9%. The additional values needed for Eq. 2 are: m_{reac}=50.00 g, $m_{ins.\ sol}$=10.32 g, $SG_{aq.\ phase}$=1085.7, m_{sub}=39.28 g, and DM=0.3819.

With Eq. 2, this equals a yield (percent hydrolysis) of 65.0%. This means that at 30% initial solids content, the proposed standard (Eq. 1) gives a yield that is more than 36% too high. This is a considerable and unwanted overestimation, in particular when comparing new technologies for bioethanol production and biorefineries.

As seen in Fig. 1, for every initial solids content, there is a near linear relationship between the actual yield and the yield based on initial volume/supernatant sugar concentration. This means that the error of the noncorrected yield (Eq. 1) is not dependent on the degree of hydrolysis but rather on the initial solids content. As expected, the degree of error increases with solids content. When the ratio between the actual (Eq. 2) and noncorrected (Eq. 1) yields is plotted as a function of initial solids content (Fig. 2), the relationship is also near linear. Thus, the slope of this graph can be used to calculate the actual yield from the noncorrected yield at each initial solids content.

The correction factor is substrate-dependent as it correlates to the composition of the biomass used, mainly the cellulose content. The experiment was repeated with filter paper containing a higher proportion of cellulose (for the composition of pretreated wheat straw

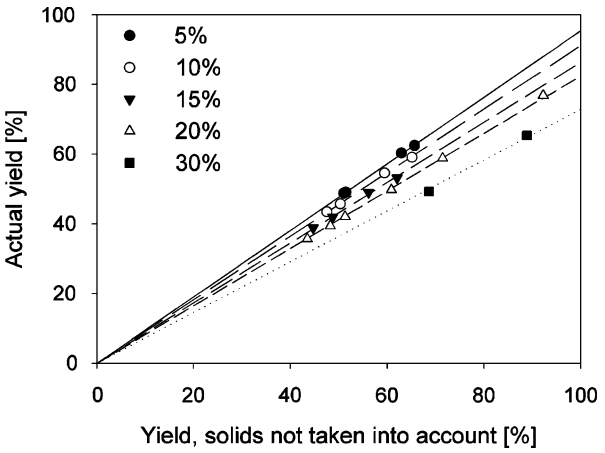

Fig. 1 Relationship between actual yield and yield calculated without taking solids content into account, when hydrolyzing pretreated straw at various initial solids contents (*w*/*w*). Each point is average of duplicates

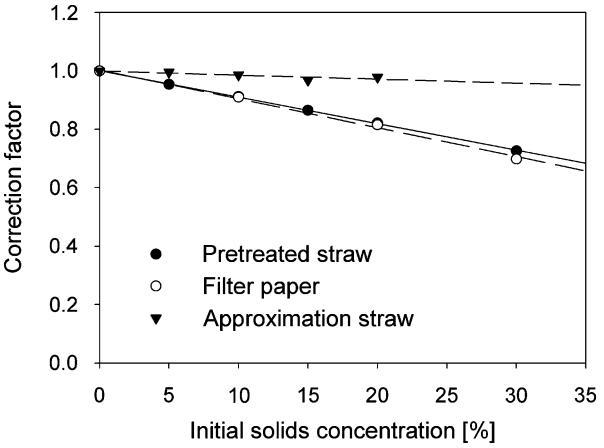

Fig. 2 Correction factor (for correcting yield overestimation) is the ratio between actual yield and yield calculated without taking solids content into account. The correction factor is shown as a function of initial solids content for pretreated straw and filter paper, respectively. The *upper graph* depicts the correction factor for pretreated straw when calculated from whole slurry sampling, thereby approximating the actual yield. Each point is the average of duplicates

and filter paper, see Table 1) and it was found that the correction curve for filter paper was slightly steeper. Thus, a correction factor must ideally be established for each substrate.

The errors seen for high solids enzymatic hydrolysis also apply for simultaneous saccharification and fermentation. Since the specific gravity of the aqueous phase starts decreasing when the released and dissolved sugars are converted to ethanol, the situation is more complex. The error of the yield is less than for enzymatic hydrolysis only, at least when a certain amount of the released sugar has been converted. Experiments with hydrothermally pretreated straw show that at 30% initial solids content, the yield was overestimated by 23% (results not shown).

Due to the inconsistency of pretreatments and natural substrates, calculating correction factors can be a laborious task. An alternative is to use the following approximation method: A representative slurry sample is weighed, e.g., 1.000 g, and diluted to 10.000 g (ten times dilution). It is then spun down and the amount of sugars in the supernatant is measured by HPLC. By knowing how large a fraction of the whole slurry is sampled, it can be calculated how much of the cellulose that has been hydrolyzed, as per Eq. 1. As the slurry was diluted ten times, the error caused by the solids content has been significantly reduced. Experiments have shown that the error (overestimate) for hydrolyses at up to 30% initial solids was reduced to a maximum of 3–5% (see Fig. 2).

Although it can be difficult to collect a representative slurry sample, especially early in the hydrolysis, we believe that this approximation method is more practical than measuring the fraction of insoluble solids and the density of the aqueous phase. Most importantly, it is better for calculating high solids yields rather than simply measuring the sugar concentration in the aqueous phase of the slurry and using the “assumed” initial volume, as prescribed by, e.g., NREL.

Table 1 Composition of pretreated straw and filter paper.

	Cellulose	Xylan	Arabinan	Klason lignin	Ash	Mannan
Pretreated straw	59.0	5.2	0.0	25.5	5.6	0.00
Filter paper	80.63	0.00	0.97	0.42	0.27	14.43

Contents expressed in percent, based on solids

Conclusions

It was found that, when working at high solids concentration (10–40% *w*/*w*), it is necessary to reconsider the way the yield is calculated in order to avoid significant overestimation in the order of up to 36%. As enzymatic saccharification of biomass is a complex and dynamic process, it is difficult to theoretically calculate the yield purely based on an assumed initial volume and sugar concentration in the aqueous phase.

By measuring the amount of insoluble solids, aqueous phase density, and sugar concentration, it is possible to precisely calculate the yield. This is, however, a laborious process and an alternative would be to dilute a representative portion of the slurry prior to measurement.

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