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# Quantitative and visual analysis of enzymatic lignocellulose degradation

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ABSTRACT

Lignocellulosic ethanol is a promising second-generation biofuel. However, production still faces huge challenges, especially in the field of pretreatment. Mechanical and enzymatic methods have been widely investigated, still little is known about the process of decomposition. Therefore, in this work three substrates: Miscanthus, switchgrass and wheat straw have been enzymatically hydrolyzed, after steam explosion treatment. Additionally, three additives Tween 80, peptone and ethanol have been tested for their influence on the enzymatic hydrolysis. The degree of decomposition has been evaluated by the amount of released sugars and by examination of the fiber structure by scanning electron microscopy. The images gave highly interesting insights into the degradation of the lignocellulosic scaffold and the weakening of the cellular cohesion. Hereby, the smaller fragments with epidermal structures showed a much higher degree of destruction than the stem parts. Therefore mechanical disintegration and use of certain additives is highly recommended as a pretreatment method.

## 1. Introduction

For a very long time, fossil fuels have been the world's major source of energy. However, the supplies are limited, the energy demand is growing with every year and environmental issues are closing in. Particularly in the transportation sector, alternatives are desperately needed, because private, as well as public and industrial transport still relies on fuels, derived from crude oil. On this matter, lignocellulosic ethanol constitutes a promising alternative, as the variety of potential substrates, which do not compete with food or feed production is very large. These so called energy crops include agricultural residues, such as corn husks, sugarcane bagasse or wheat straw. However, also unpretentious plants, which produce a lot of biomass, such as Miscanthus, switchgrass or millet are widely investigated (Bai et al., 2008; Claassen et al., 1999; Mussatto et al., 2010; Sanchez and Cardona, 2008).

In contrast to sugar or starch based substrates, lignocellulosic biomass requires costly and time-consuming pretreatments. The lignin scaffold, which under natural conditions coats and therefore protects the cellulose fibrils has to be destroyed. In addition, not only cellulose but also hemicellulose, which connects lignin and cellulose need to be converted to monosaccharides, in order to be of further use. Therefore, the main production steps are the breaking of the lignocellulosic structure, hydrolysis of cellulose and hemicellulose, fermentation of the resulting sugars and separation of ethanol from the mash (Alvira et al., 2010; Behera et al., 1996).

A large variety of pretreatment techniques have been under research, from which some are biological or chemical, others are physical or even physico-chemical (Alvira et al., 2010). They include for example soft, brown or white rot fungi (Sánchez, 2009); ammonia fiber expansion (AFEX), dilute acid, lime, sulfur dioxide (Kumar and Wyman, 2009); organosolv, e.g. ethanol (Papatheofanous et al., 1995) and finally steam explosion, which is one of the most commonly used pretreatment.

During this latter hydrothermal process, the biomass is heated (typically to temperatures of 160–260 °C) under elevated pressure (0.69–4.83 MPa) for a certain time until the pressure is suddenly reduced. This causes the fibers to break, due to the almost explosive expansion of water steam in the material. In addition to the mechanical separation of lignin, cellulose and hemicellulose, there are also chemical effects, such as autohydrolysis, which result in partial degradation of hemicellulose (Alvira et al., 2010; Sun and Cheng, 2002).

The consequent enzymatic hydrolysis is mostly carried out by cellulase, as well as hemicellulase complexes, which are highly specific and work under mild conditions, including temperatures of 40–50 °C and a pH of 4.5–5.0 (Converse et al., 1988; Taherzadeh and Karimi, 2007).

However, one of the major problems still is the substrate handling. In order to obtain an efficient process including recovery, fermentable

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sugar concentrations should be at a relatively high level of above 10% (Alvira et al., 2010). This in turn requires high amounts of enzymes, which makes the whole process very expensive, but lowers mash viscosity severely. The initial ratio of solids in lignocellulosic mashes is very high and makes mixing and mass transfer rather difficult (Taherzadeh and Karimi, 2007). Several additives have been tested, which all had the goal to reduce the amount of enzyme utilization during hydrolysis. They include surfactants, such as Tween 80 (Jin et al., 2016) or non-enzymatic proteins, such as BSA or peptone (Wang et al., 2015).

Unfortunately, so far no ideal method has been found yet and there is still a lot of research carried out on the improvement of technologies and the understanding of lignocellulose degradation. The project, in whose course this research was carried out, aims to develop a continuously working bioethanol reactor, based on the model of a biogas reactor. Therefore, only limited process control can be employed and all substrates, microorganisms or other additives should be included from the beginning. Hereby, the process duration time, does not play an important role, as it can be counteracted by the number or volume of bioethanol reactors. For this reason and also in order to keep production costs and effort low, the extent of pretreatment can be minimalized. In accordance with these requirements, in this work only steam explosion and enzymatic hydrolysis were employed. The effects of these pretreatment techniques were investigated for three different substrates: Miscanthus, switchgrass and wheat straw. Furthermore, the effect of low-cost additives such as Tween 80, peptone and ethanol on enzymatic hydrolysis were tested for switchgrass. The degree of degradation was evaluated by scanning electron microscopy (SEM) and measurement of resulting sugar concentration by high-performance liquid chromatography (HPLC).

## 2. Materials and methods

### 2.1. Substrate preparation

Wheat straw (*Triticum aestivum*) was supplied by Meiereihof (University of Hohenheim) in 2015, Miscanthus (*Miscanthus × giganteus*) samples and switchgrass (*Panicum virgatum*) were both grown at Ihinger Hof (University of Hohenheim) for field trial purposes. Miscanthus was harvested in 2013 and switchgrass in 2015.

All plants were harvested, chopped, dried and milled with a hammer mill, containing a 2.5 mm sieve. They were stored dry until further use in sealed plastic barrels at a temperature of approximately 15 °C.

### 2.2. Steam explosion

First of all, the substrates were soaked in cold water for 24 h (ratio: 1 kg dry substrate per 9 kg water). The suspension was then poured into a double-walled 20 L steam reactor (H & K GmbH Behälter und Edelstahltechnik, Kehl, Germany) and heated to 160 °C and an inside pressure of approximately 6 bar by indirect steam injection. The temperature was kept for 45 min, while stirring at a constant speed of 22 rpm with an anchor stirrer. Finally the pressure was abruptly reduced to ambient pressure by opening a valve at the reactor bottom. The moist substrates and supernatant were transferred to 1 L plastic bottles and stored at −18 °C until further processing.

### 2.3. Substrate analysis

In order to analyze the substrate composition the Laboratory Analytical Procedures by the National Renewable Energy Laboratory have been used (Sluiter et al., 2008b, 2008a). Initially, the substrates were dried for determination of the oven dry weight (ODW) and the total solid content. The oven dry substrates were then used for determination of acid insoluble residues (AIR) and –lignin (AIL), as well as the acid soluble lignin (ASL), the ash content and cellulose-, hemicellulose- and total lignin content. Therefore, the substrates first needed to be degraded completely with 72% sulfuric acid and after dilution, by heating to 121 °C for one hour. Subsequently, the suspension was filtered through filtering crucibles and the liquor used for UV and HPLC analysis. The solids were dried and finally ashed. From the concentrations of monomeric sugars, the cellulose- and hemicellulose contents were calculated. All samples were analyzed in triplicate and the average values were calculated.

**Table 1**
Total solid content and structural carbohydrate composition of miscanthus, straw and switchgrass.

| | Miscanthus | Switchgrass | Wheat Straw |
|---|---|---|---|
| Total solids [%] | 12.64 | 13.42 | 12.58 |
| AIR [%TS] | 27.66 | 29.79 | 35.25 |
| AIL [%TS] | 26.45 | 28.85 | 30.76 |
| ASL [%TS] | 1.28 | 1.14 | 1.57 |
| Ash [%TS] | 1.21 | 0.94 | 4.50 |
| Total lignin [%TS] | 27.73 | 29.99 | 32.31 |
| Cellulose [%TS] | 49.37 | 42.20 | 44.03 |
| Hemicellulose [%TS] | 19.86 | 22.70 | 19.77 |

### 2.4. Enzymatic hydrolysis

The steamed and moist substrates were transferred to steal cups, together with a certain amount of supernatant. For the switchgrass experiments with the additives also 4 $g/L_{liquid}$ peptone (Bacto; Sparks, USA), 4 $g/g_{TS}$ Tween 80 (Sigma; Darmstadt, Germany) and 10 $g/L_{liquid}$ ethanol was mixed into the suspension. The cups were then placed in a water bath at a temperature of 50 °C. Cellic CTec2 enzyme preparation (Novozymes; Bagsværd, Denmark) was added at concentrations of 3% and 30% of the absolute cellulose content, respectively. This enzyme preparation is a special blend and consists of aggressive cellulases, a high level of β-glucosidases and hemicellulase for use in degradation of cellulose to fermentable sugars. Stirrer lids, consisting of lids with silicone fittings to seal the cups and crossed blade impellers, were placed on top. Samples were taken after 0, 24, 48, 72 and 96 h and examined for their cellobiose, glucose, xylose and arabinose content by HPLC (Rezex ROA-Orgabic Acid $H^+$, Phenomenex, Torrance, USA). Acetic acid and ethanol concentrations were additionally checked, in order to see, if any contamination had occurred. Cellobiose and glucose and respectively xylose and arabinose concentrations were then used to calculate the ratio of converted cellulose and hemicellulose. This conversion ratio was defined as the amount of sugars (in g) released from cellulose or hemicellulose, divided by the initial amount of the specific polysaccharide (in g).

### 2.5. SEM imaging

A scanning electron microscope (Zeiss, EVO MA10; Oberkochen, Germany) was used to evaluate the changes in fiber structure by the enzymatic hydrolysis. The SEM was fitted with a tungsten filament and the accelerating voltage used was 8 kV.

The samples for SEM imaging were centrifuged, the supernatant was discarded and the fibers were washed three times with double distilled water. Subsequently the substrates were dried at a temperature of 50 °C for four days. To increase the electric conductivity, the samples were sputter covered (SC7620 Mini Sputter Coater, Quorum Technologies; Laughton, UK). The samples were sputtered for 120 s with gold/ palladium using argon gas to provide the ionization medium, resulting in approximately 11 nm thick cover.