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Pretreatment of lignocellulosic biomass using Fenton chemistry

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HIGHLIGHTS

- We investigated the viability of Fenton chemistry for biomass pretreatment.
- We assessed pretreatment using enzymatic and microbial fermentation methods.
- Fenton chemistry made cellulose more bioavailable to enzymes and microbial species.

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ABSTRACT

In an attempt to mimic white-rot fungi lignin degradation via *in vivo* Fenton chemistry, solution phase Fenton chemistry (10 g biomass, 176 mmol hydrogen peroxide and 1.25 mmol Fe^{2+} in 200 mL of water) was applied to four different biomass feedstocks. An enzymatic saccharification of Fenton pretreated biomass showed an average 212% increase relative to untreated control across all four feedstocks ($P < 0.05$, statistically significant). A microbial fermentation of the same Fenton pretreated biomass showed a threefold increase in gas production upon a sequential co-culture with *Clostridium thermocellum* and *Clostridium beijerinckii*. These results demonstrate the use of solution phase Fenton chemistry as a viable pretreatment method to make cellulose more bioavailable for microbial biofuel conversion.

1. Introduction

Worldwide energy demand is increasing while the availability of petroleum based resources continues to dwindle. This situation has led to renewed interest in sustainable sources of energy such as lignocellulosic plant materials or biomass. Biomass feedstocks like miscanthus (*Miscanthus giganteus*), switchgrass (*Pancium virgatum*), (*Triticum aestivum*) wheat straw, and (*Zea mays*) corn stover are either dedicated energy crops or agricultural waste products, both of which are renewable and can be utilized for biofuel production (http://energy.gov/eere/energybasics/articles/biomass-resources). Biomass to biofuel conversion into two and four carbon alcohols occurs via microbial fermentation. However, the fermentation process is hindered by the recalcitrant nature of lignocellulosic biomass to chemicals and enzymes (Himmel et al., 2007).

Lignocellulosic biomass is composed of three main polymeric constituents: lignin, cellulose and hemicellulose; other polymers such as proteins and nucleic acids are present, but are not the focus of this study. Cellulose is highly sought after because it can be microbially converted to biofuels such as acetone, ethanol and butanol, and is also highly utilized in the paper industry. However, lignin negatively affects cellulose availability and must be removed or modified by pretreatment. An ideal pretreatment protocol removes the need to reduce biomass particle size, prevents cellulose degradation and limits formation of microbial inhibitory compounds (Arantes and Goodell, 2012; Mosier et al., 2005).

Lignocellulolytic *Basidiomycetes* have the ability to degrade the polymeric components of biomass and are split into two categories: white-rot fungi and brown-rot fungi (Arantes et al., 2012). Exposure to white-rot fungi, like *Phanerochaete chrysosporium*, or brown-rot fungi, like *Gloeophyllum trabeum*, are examples of an *in vivo* chemical pretreatment using Fenton (iron/peroxide) chemistry (Arantes et al., 2011; Palmer and Evans, 1983; Watanabe et al., 2009). These fungi have enzymes, such as peroxidases, some of which have iron at the catalytic center, which slowly degrade lignin via the catalytic decomposition of hydrogen peroxide by the ferrous ion, generating hydroxyl radicals, also known as Fenton chemistry (Villa et al., 2008).

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In attempt to mimic white-rot and brown-rot fungi enzymes, solution phase Fenton chemistry provides a non-selective oxidation of organic compounds (Balddrian and Valaskova, 2008; Flournoy, 1994; Kirk et al., 2009; Watanabe et al., 2009). The hypothesis and goal of this study is that solution based Fenton chemistry will enhance cellulose bioavailability. Solution phase Fenton chemistry holds the potential to provide a straight forward and efficient pretreatment for biofuel production (Michalska et al., 2012).

This study will show the effectiveness of solution phase Fenton chemistry as a pretreatment on various biomass feedstocks. Utilization of lignin and enzymatic saccharification assays will evaluate the ability of solution phase Fenton pretreatment to make cellulose more bioavailable for microbial fermentation leading to biofuel production from various biomass feedstocks.

2. Methods

2.1. Materials

All chemicals of reagent grade were obtained and used without further purification. Ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), potassium iodide, ammonium molybdate, ammonium hydroxide, sulfuric acid, starch, sodium thiosulfate and hydrogen peroxide (H_2O_2, 50%) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). *Trichoderma reesei* cellulase enzyme was obtained from Alltech (Nicholasville, KY, USA). Distilled water (18 MΩ) was used to make appropriate solutions/dilutions. Miscanthus, switchgrass, corn stover and wheat straw samples (all ~2 mm particle size) were obtained from collaborators in the Michael Montross Laboratory, University of Kentucky, Lexington, KY, USA.

2.2. Solution phase Fenton pretreatment

In a typical Fenton pretreatment experiment, 10 grams of dry biomass (~2 mm) was further ground using a commercial coffee grinder (Hamilton Beach Fresh-Grind coffee grinder model #80335 to ⩽2 mm particle size). The biomass was added to an Erlenmeyer flask (1000 mL) followed by addition of 100 mL of a 12.5 M solution of Fe^{2+} (1.25 mmol Fe^{2+}, or 250 mg $FeCl_2{\cdot}4H_2O$) and 100 mL of a 1.76 M solution of hydrogen peroxide (176 mmol H_2O_2, or 6% H_2O_2). This stirred mixture was allowed to react for a specified amount of time according to individual experiments. Upon filtration, the solid was dried (105 °C) overnight and the filtrate was kept for future experimentation (Jain and Vigneshwaran, 2012; Michalska et al., 2012; Kang et al., 2002). Control experiments were conducted by adding ground, dry biomass (10 g) to an Erlenmeyer flask (1000 mL) with distilled water (18 MΩ, 200 mL); the suspension was left to react for a specified amount of time according to individual experiments. Upon filtration, the solid was dried (105 °C) overnight and the filtrate was kept for future experimentation.

2.3. Hydrogen peroxide consumption

Hydrogen peroxide consumption assays were conducted using an iodometric titration according to the published procedure from US peroxide (US Peroxide). At various time points, an aliquot of supernatant (1 mL) was taken from the suspension and weighed. Distilled water (50 mL), 4% sulfuric acid solution (10 mL), 1% (w/v) KI solution (10 mL), and ammonium molybdate solution (0.4 M, 2 drops) were added to the procured aliquot followed by a titration with sodium thiosulfate (0.1 N) to a faint yellow color. Upon the addition of a 1% (w/v) starch solution (2 mL), titration proceeded until completion, indicated by a flash of blue color (US Peroxide).

2.4. Enzymatic saccharification

Enzymatic saccharification assays were conducted according to the published National Renewable Energy Laboratory (NREL) protocols (Selig et al., 2008) with slight modifications. Biomass feedstocks were added to a glass test tube with cellulase provided by Alltech, Lexington, KY, USA, (150 mg, activity: 56 FPU/mL), distilled water (5 mL) and 0.1 M sodium citrate buffer pH 4.8 (5 mL). The reaction tubes were heated in a sand bath (50 °C) and rocked overnight. Glucose was measured using an over the counter Abbott Precision Xtra glucometer and Abbott Precision Xtra blood glucose test-strips purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.5. Lignin determination

Acid-soluble (ASL) and acid-insoluble (AISL) lignin assays were conducted according to the National Renewable Energy Laboratory (NREL) procedures (Sluiter et al., 2008, 2012). Briefly, biomass (300 mg) was weighed out into a glass culture tube and dried (105 °C) overnight to determine the total solids. A 72% sulfuric acid solution (2 mL) was added, mixed and incubated in a water bath (30 °C, 1 h, stirring every 10 min). The suspension was diluted to a 4% sulfuric acid solution with distilled water (18 MΩ), transferred to an autoclavable bottle, and autoclaved on a 40 min, 121 °C, liquid cycle. It was then vacuum filtered using ashless quantitative filter paper in order to prevent material loss during transfer. The filtrate was analyzed with a single beam UV–Vis spectrophotometer (Cary 50 scan, Varian) at 205 nm to determine ASL, and the solid remained on the ashless filter paper and was transferred to a constant weight crucible to dry overnight (105 °C). The sample was reduced to ash in a muffle furnace for 4 h (575 °C) (Sluiter et al., 2008, 2012) to determine AISL.

2.6. Total organic carbon (TOC) analysis

Total organic carbon (TOC) analyses were conducted using a Shimadzu TOC 5000A. Control and Fenton pretreated filtrates were diluted 1:3 with distilled water (18 MΩ). Total organic carbon was determined based on external calibration curves conducted using the instrument software by subtracting the inorganic carbon from the total carbon.

2.7. Strain and media composition

Clostridium thermocellum ATCC 27405 was derived from the culture collection of Herbert J. Strobel, University of Kentucky, Lexington, KY, USA. The basal medium composition was recently described by Dharmagadda et al. (2010). The pH was adjusted to 6.7 with NaOH. The medium was autoclaved (121 °C, 104 kPa, 20 min) and cooled under O_2-free CO_2 sparge. The buffer, Na_2CO_3 (4 mg ml^{-1}) was added when broth was approximately 30 °C. The broth was anaerobically dispensed into serum bottles and sealed with butyl rubber stoppers, and autoclaved for sterility. *C. thermocellum* was maintained (63 °C) in the basal medium with cellulose (Whatman #1 filter paper; 4 mg ml^{-1}).

Clostridium beijerinckii ATCC 51743 was ordered from the American Type Culture Collection (Manassas, VA, USA). Reinforced Clostridial Media (RCM; Difco Laboratories, Detroit, MI, USA) was composed of (per liter) 10.0 g peptone, 10.0 g beef extract, 3.0 g yeast extract, 5.0 g NaCl, 5.0 g dextrose, 1.0 g soluble starch, 0.5 g cysteine, 3.0 g $C_2H_3NaO_2$, and 0.5 g agar. RCM was autoclaved and cooled under O_2-free N_2. It was anaerobically dispensed into