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Research paper

Interactive effect of enzymes and surfactant on the cellulose digestibility of un-washed and washed dilute ammonia pretreated energy cane bagasse

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ABSTRACT

The interaction effect of cellulase (Cellic® CTec2), xylanase (Cellic® HTec2) and laccase along with a non-ionic surfactant (Tween® 80) on cellulose digestibility of unwashed and washed liquid ammonia pretreated energy cane bagasse was investigated. A polynomial quadratic model was fitted and solved for the optimum value of each variable for the desired response using response surface methodology (RSM). Statistical analysis of the results showed that the interactive effect of all variables with cellulose were significant ($p < .05$) and resulted in 75.85% and 12.74% improvement in cellulose digestibility of unwashed and washed biomass, respectively. Highest cellulose digestibilities observed were 84.30% and 97.10% for values set within the design range for the unwashed and washed biomass, respectively. Optimum enzymatic hydrolysis conditions for unwashed substrate were 19.39% (mass ratio of glucan) CTec2, 12.04% (mass ratio of glucan) HTec2, a laccase loading on dry biomass of 46.32 IU g^{-1}, and 10.15% (mass ratio of biomass) Tween® 80. Optimum enzymatic hydrolysis conditions for washed substrate were 16.90% (mass ratio of glucan) CTec2, 14.17% (mass ratio of glucan) HTec2 (w/w), a laccase loading on dry biomass of 34.64 IU g^{-1}, and 14.86% (mass ratio of biomass) Tween® 80.

1. Introduction

Sugars released during enzymatic hydrolysis of pretreated lignocellulosic material can be used in the production of fuels and chemicals. The challenge is to find the optimum combination of enzymes (i.e., cellulase), accessory enzymes (i.e., xylanase, laccase) and enzyme stabilizers (i.e., surfactants) for maximum sugar yield based on the unique chemical composition of the substrate (i.e., cellulose, hemicellulose, lignin), pretreatment technology (i.e., acid, alkaline) and by-products generated or released during pretreatment (i.e., furan derivatives, carboxylic acids, phenolic compounds) as result of the degradation of lignin, cellulose and hemicellulose.

Xylanase, as an accessory enzyme, can increase cellulose accessibility by hydrolyzing the hemicellulose and eliminating its physical barrier [1]. Moreover, the addition of xylanase can address some of the issues associated with the presence of solubilized hemicellulose, hemicellulose-derived products and xylo-oligomers [2,3]. These compounds are known to have inhibitory effects on cellulase activity by blocking its accessibility to cellulose [4,5]. Xylanase, increases fiber porosity and biomass swelling which improves glucose yields [6]. Xylanase has shown interactive effects with cellulase [7–9]; however, the effect was dependent on the chemical composition of the substrate, and type and severity of pretreatment [10]. Cellulose crystallinity and the molecular structure of enzymes can also affect the degree of interaction [11].

The addition of laccase (a multicopper-containing phenoloxidase) as an accessory enzyme during enzymatic hydrolysis can potentially remove the lignin by oxidizing phenols, anilines and aromatic thiols [12–14]. This would create microspores in the biomass where enzymes can go through and hydrolyze the substrate [12,15]. Al-Zuhair et al. [16] reported that the combined application of laccase, xylanase and cellulase during the enzymatic hydrolysis of date palm biomass resulted in a 60% cellulose conversion as compared to 45.60% conversion under the sequential application of these enzymes and a 5.60% conversion by using just cellulase. However, some studies have reported a negative effect of laccase on enzymatic hydrolysis yield due to its inhibitory effect on β-glucosidase activity [17,18]. Oliva-Taravilla et al. [18] reported that the addition of a laccase loading on dry biomass of 10 IU g^{-1} to the enzymatic hydrolysis of acid pretreated wheat straw resulted in a 10% decrease in enzymatic digestibility.

Non-ionic surfactants such as Tween® 80 have been reported to increase enzymatic digestibility of biomass in favor of reducing enzyme loadings [19–23]. However, the activity of surfactants depends on the type of pretreatment used. Acid pretreated wheat straw showed a better response to the addition of a non-ionic surfactant (poly ethylene glycol) when compared to ammonia pretreated straw [24]. Jin et al. [25]

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reported that a 0.50% loading of Tween® 80 enhanced enzymatic digestibility of steam exploded reed by 1.7 fold as compared to samples without added surfactant. Yang et al. [23] showed that Tween® 80 increased the activity of endoglucanase and exoglucanase by preventing deactivation of the adsorbed cellulase during their interaction with the substrate. Tween® 80 has also been reported to increase sugar yields of larger size biomass particles thus reducing the energy required for fine grinding of the material [22].

Washing is considered the simplest method for the removal of non-sugar compounds (i.e., furan derivatives, carboxylic acids, phenolic compounds) that are generated or released during the pretreatment of biomass materials [26–28]. These non-sugar compounds can have a negative effect on downstream processes (i.e., enzymatic hydrolysis, fermentation) [29–31].

In this study, the interactive effect of enzymes (cellulase, xylanase and laccase) with or without the addition of a surfactant (Tween® 80) on the cellulose digestibility of unwashed and washed dilute ammonia pretreated energy cane bagasse was assessed using response surface methodology (RSM).

2. Material and methods

2.1. Biomass

Energy cane is a cross breed between sugarcane (*Saccharum officinarum*) and its wild ancestors (*S. spontaneum*) and has interesting features as a potential energy crop. Compared to sugarcane, energy cane has higher fiber content (26.4% versus 13.5% (dry basis)), higher cane yield (88.90 t ha^{-1} versus 69.20 t ha^{-1} (dry basis)) and it is more resistant to cold, disease and requires less water input [32,33]. Energy cane non-commercial variety Ho 02-113 was bred in Houma, LA through collaboration between the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) and the Sugar Research Station at Louisiana State University Agricultural Center in St. Gabriel, LA. Energy cane was harvested at the Sugar Research Station (30° 16′ 7.77″ N, 91° 6′ 15.75″ W) and was milled three times using a roller press (Farrel Company, Ansonia, CT) to remove the juice. The milled solid fraction is called bagasse. Bagasse was air dried (10% final moisture content), milled (Wiley Mill, Arthur Thomas Co, PA), sieved (2 mm mesh sieve), and stored at −20 °C until further use.

2.2. Dilute ammonia pretreatment

Energy cane bagasse was pretreated with liquid ammonium hydroxide (28% mass fraction NH_4OH, Fisher Scientific (catalog number: A669S-500), Pittsburgh, PA) in a 4 L stirrer reactor (316SS and HASTELLOY® C-276 materials) (Autoclave Engineers, Erie, PA) at 208 °C for 36 min to a final mass ratio of ammonium hydroxide to bagasse to water of 0.4:1:20. The pretreatment conditions used in this study had been previously optimized for maximum sugar yields using RSM [34]. RSM is a statistical modeling technique that uses quantitative data generated from an appropriately designed experiment to determine a multivariate equation. Solving this equation would give the optimal values of variables for the desired response. RSM can reduce the number of experimental trials, while evaluating the interaction effect of multiple variables on the response [35]. The pretreated slurry was pressed to recover the solid fraction. The solid fraction was divided in two parts, half was washed with deionized water (6 vol) and the other half was kept unwashed. The biomass was dried at 45 °C in an oven to a final moisture content below 10%. Composition analysis of untreated and pretreated energy cane bagasse was performed following NREL's Laboratory Analytical Procedures [36–39]. NREL reference material 8491 (for sugarcane bagasse) was analyzed as an internal sample to ensure the accuracy of the procedures.

2.3. Experimental design and data analysis

Central Composite Design (CCD) and RSM were run using software Design-Expert 9.0.3 (State Ease Inc., Minneapolis, MN) to design the experiment, to analyze the interaction effect of variables (cellulase, xylanase, laccase, and surfactant) on the response (glucose yield) and to find the optimum combination that would result in the highest glucose yield. CCD consists of 2^k factorial points, 2 k axial points ($\pm \alpha$), and six center points for replications, where k is the number of variables. A total of 30 experiments were performed in duplicate for washed an unwashed dilute ammonia pretreated energy cane bagasse (Table A.1). A quadratic polynomial equation (Equation A.1) was assumed to approximate the data. Center points of the design were replicated six times to estimate the error sum of squares. Significance of each coefficient was evaluated with analysis of variance (ANOVA).

2.4. Enzymatic hydrolysis and sample analysis

Cellulase (Cellic® CTec2) and xylanase (Cellic® HTec2) were obtained from Novozymes (Novozymes A/S, Bagasvaerd, Denmark). Laccase from *Rhus vernicifera* and Tween® 80 were purchased from Sigma (Sigma–Aldrich, Inc., St. Luis, MO, USA). Enzyme assays were performed to measure cellulase, xylanase and β-glucosidase activities of CTec2 and HTec2. Cellulase activity for CTec2 (132 FPU cm^{-3}) and HTec2 (90.75 FPU cm^{-3}), and β-glucosidase activity for CTec2 (3229 IU cm^{-3}) and HTec2 (12.61 IU cm^{-3}) were measured following the Ghose method [40]. Xylanase activity of Ctec2 (12100 IU cm^{-3}) and Htec2 (56000 IU cm^{-3}) were determined according to Bailey et al. [41]. Laccase activity (50 IU cm^{-3}) was measured using syringaldazine as substrate based on the Ride method [42]. Bagasse and a sodium citrate solution (2.58 g L^{-1}, Fisher Scientific, Pittsburgh, PA) at 8:100 mass ratio were mixed to a final pH of 4.8. Corresponding amounts of enzymes and Tween® 80 were then added to each flask. Flasks were incubated at 50 °C in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) at 3 Hz for 72 h. Samples were taken at 0 h (before the addition of enzymes), 24 h, 48 h, and 72 h and kept at −20 °C until further analysis. Experiments were done in duplicate. All collected samples were centrifuged at 130 Hz (Spectrafuge 24D, Labnet International Inc., Woodbridge, NJ) for 5 min and filtered (0.2 μm nylon Syringe Filters, Environmental Express Inc., Mt. Pleasant, SC). Samples were diluted accordingly and analyzed for sugars by high performance liquid chromatography (HPLC) (Agilent 1200 Series) with a BioRad Aminex HPX-87P (P), Pb form, 3000 × 7.8 mm (ID), 9 μm column and a differential refractive index detector (G1362A Agilent). Theoretical yields for cellulose were calculated using Equation (1) [43].

$$\%\text{Theoretical Cellulose Yield} = \frac{m_{Glucose} + 1.053\, m_{Cellobiose}}{1.111\, w_{Cellulose}\, m_{Biomass}} \times 100\% \quad \text{(Eq. 1)}$$

where $m_{Glucose}$, is the residual glucose mass; 1.053, is the multiplication factor that converts cellobiose to equivalent glucose; $m_{Cellobiose}$, is the residual cellobiose mass; 1.111 is the factor that converts cellulose to equivalent glucose; $w_{cellulose}$, is the cellulose mass fraction; $m_{Biomass}$, is the biomass mass at the beginning of the hydrolysis.

2.5. Mass balance

Mass balances were calculated as described below for washed and unwashed dilute ammonia pretreated energy cane bagasse. Liquid and solid streams of pretreated and enzymatically digested biomass were analyzed for lignin, cellulose, hemicellulose, monomeric sugars, and total solids by NREL procedures [36–39]. Oligomeric sugars present in the liquid streams were further hydrolyzed to their monomeric form [36]. Glucan and xylan values in the solid fraction were reported as glucose and xylose using the conversion factor of 1.111 for glucan to glucose and 1.135 for xylan to xylose conversion [43].