Biomass and Bioenergy 109 (2018) 284–290

Contents lists available at ScienceDirect

Biomass and Bioenergy

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

Research paper

Unravelling the effect of pretreatment severity on the balance of cellulose accessibility and substrate composition on enzymatic digestibility of steam-pretreated softwood

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ARTICLE INFO

Keywords:
Cellulose accessibility
Enzyme hydrolysis
Digestibility
Enzyme inhibition
Pretreatment
Softwood
Radiata pine

ABSTRACT

Pretreatment is essential for effective enzymatic digestion of lignocellulosic biomass. Steam pretreatments increase the digestibility by increasing the accessibility of the carbohydrates to the enzymes. However, they can also cause yield loss and lowered digestibility *via* increased non-productive binding of enzymes to lignin. The relative importance of these effects is not well defined, especially for softwoods which require more severe pretreatments than other types of biomass. *Pinus radiata* wood was steam pretreated at 180 °C and 215 °C to Combined Severity Factors of −3.31 or −2.61 and the digestibilities of the washed insoluble fractions examined before and after ball-milling to a common accessibility, as determined by Simons' stain measurements. Pretreatments at 215 °C for 2 min with citric and sulfuric acid catalysts were also investigated. Results showed that the digestibility of the pretreated substrates increased with pretreatment severity, rising from ~5% with no pretreatment to ~20% after the most severe pretreatment. However, when the substrates were ball-milled to a common accessibility, the digestibility decreased on increasing pretreatment severity. At a common accessibility and low enzyme dose the digestibility dropped six-fold from ~30% for the original wood to ~5% for the most severely pretreated substrate. This showed that while increasing pretreatment severity does lead to greater enzyme inhibition, this was being overridden by increases in the accessibility.

1. Introduction

Pretreatment to modify the structure and/or the chemical composition of a substrate is essential for effective digestibility of lignocellulosic biomass [1]. Short high-temperature (180–230 °C) steam treatments are generally effective for pretreating agricultural and hardwood biomass, whereas softwoods require harsher conditions such as the presence of an acid catalyst during the steam pretreatment [2–4].

Several substrate properties have been reported to influence the rate and/or extent of enzymatic digestion of pretreated substrates [5,6]. These include: the area of cellulose accessible to the cellulase enzymes; the size of the particles; the cellulose crystallinity; and inhibition of the enzymes by non-productive binding of the enzymes to the lignin or carbohydrate degradation products. In spite of being the subject of many studies, the relative importance of these different factors is still not clearly understood. What has however become clear is that the response of a given pretreated biomass to enzyme treatment is the result of a balance between two or more of these factors.

Pretreatments that increase the surface area of the substrate, leaving the cellulose more accessible to the enzymes, frequently lead to higher sugar yields [6]. Accessibility can be increased by either removing hemicelluloses or lignin from a lignocellulosic substrate, or by mechanically opening up the fibre to increase the exposed carbohydrates [7–9].

Components in the original biomass, or formed during biomass pretreatment can inhibit the enzymes by non-productively binding to the enzymes and therefore reducing their effectiveness. These inhibitors may be components of the fibre that remain in the water-insoluble fraction, particularly lignin [10–13], or water-soluble components from the pretreated substrate such as phenolics, organic acids or sugars [14]. Lignin is known to inhibit enzyme digestibility by binding to cellulases [15,16]. Non-productive binding of enzymes to lignin is a widely studied and accepted phenomenon, but is still poorly understood [10,12,13,16–19].

A number of studies have shown that particle size is a good predictor of digestibility [20,21]. This is due to the fact that smaller

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https://doi.org/10.1016/j.biombioe.2017.12.018
Received 1 June 2017; Received in revised form 19 December 2017; Accepted 21 December 2017

particles result in increased surface area, which gives enzymes easier access to the polysaccharides. It has been shown that increasing external surface area by reducing particle size is particularly important when a substrate is not well pretreated and has a low accessibility.

Cellulose crystallinity has also been suggested to influence the digestibility of lignocellulosic substrates, but the effects are inconsistent [22]. Many studies have shown that accessibility and lignin inhibition are the dominant factors and crystallinity is only a major contributor to digestibility for pure cellulosic substrates [6].

All of the above factors are affected by the pretreatment conditions. The pretreatment conditions of temperature, time and pH used to maximise overall sugar yields are a balancing act. On one hand, the pretreatment conditions should aim to maximise the accessibility of the enzymes to the cellulose and/or reduce cellulose crystallinity. At the same time yield losses due to dissolution or degradation of the biomass and inhibitor formation that occurs from the degradation and modification of sugars and/or lignin should be minimised. The relative importance of accessibility and inhibition at any given pretreatment severity ultimately should determine the sugar yield. A better understanding of this balance could lead to lower enzyme usage, as well as less expensive polysaccharide conversion and lower biofuel production costs.

This study evaluates the relative importance of factors controlling the digestibility of steam-pretreated radiata pine and in particular, the effect of pretreatment severity on the balance between the insoluble inhibitors present in pretreated wood and increasing cellulose accessibility. To do this, the digestibility of the insoluble fractions of steam-pretreated *P. radiata* wood derived from various pretreatment conditions was examined both before and after wet ball-milling to a common cellulose accessibility, as determined by Simons' stain measurements [23]. Comparisons of the digestibility of substrates at a common cellulose accessibility allowed for the impact of enzyme inhibitors to be evaluated independent of changes in the cellulose accessibility. The effect of particle size on digestibility was also evaluated.

2. Experimental

2.1. Materials

Fresh radiata pine (*P. radiata*) sawdust was collected from McAlpines Sawmill, Rotorua, New Zealand. This mill processes logs from plantation-grown radiata pine forests having a typical rotation time of 25–30 years. This material had a composition typical of that expected for radiata pine sapwood [24]. The sawdust was screened on a Williams vibratory screen (round holes) to collect the fraction of 1.29–3 mm in size and any bark particles removed. Citric acid, sulfuric acid, sodium azide and direct blue (Chicago Sky Blue 6B) were obtained from Sigma Aldrich. Direct orange dye (Pontamine Fast Orange 6RN) was obtained from Pylam Products Co. Inc (Garden City, NY). The commercial cellulose enzyme cocktail Cellic CTec2® (88.5 FPU g^{-1} enzyme) was sourced from Novozymes (Franklinton, NC, USA).

2.2. Sample preparation-steam pretreatment

Fresh sawdust (300 g oven dried (O.D.) equivalent of 1.29–3 mm fraction) was soaked overnight in either water, or a solution of citric acid or sulfuric acid of required concentration, to deliver a loading of 20 kg m^{-3} acid on sawdust (oven-dry basis) after filtration and removal of excess liquid. Duplicate samples were steam exploded after heating for the temperature and times shown in Table 1 following the procedure of Clarkand Mackie [25]. The Severity Factor (SF) and Combined Severity Factor (CSF) of each treatment was calculated using Equations (1) and (2), where t is time in minutes, T is temperature in degrees Celsius, and pH is of the soaking liquid [26,27].

$$\text{Severity Factor (SF)} = \text{Log}\,(R_0) = \text{Log}\left[t \times \exp\left(\frac{T-100}{14.75}\right)\right] \quad (1)$$

$$\text{Combined Severity Factor(CSF)} = \text{Log(CSF)} = \text{Log}\,(R_0) - \text{pH} \quad (2)$$

The slurry produced from steam explosion was filtered using 100 μm nylon cloth on a Büchner funnel to give a solid and a liquid fraction (filtrate). The solid fraction was pressed to raise solids content and improve recovery of the filtrate, it was then thoroughly washed with hot water (60 °C) to remove any remaining soluble components. The wash-water was discarded. The filtrates were preserved using 0.1 g of sodium azide per litre of filtrate and both the filtrates and solids were stored at 4 °C.

2.3. Chemical composition

Solid fractions (air-dried) were ground in a Wiley mill to pass a 40 mesh screen and extracted with dichloromethane in a Soxtec apparatus. The extracted substrate was analysed in duplicate for acid insoluble lignin by TAPPI Standard Method T 222 om-88 (scaled down to 250 mg), and for acid-soluble lignin by TAPPI UM 250. Ash contents were measured using TAPPI Standard Method T 211 om-93.

Fucose was added to the hydrolysate from the lignin analysis as an internal standard and the carbohydrates (L-arabinose, D-glucose, D-galactose, D-mannose and D-xylose) were analysed by anion chromatography [28]. Carbohydrates (total sugars) in the filtrates were analysed after hydrolysis with 4% sulfuric acid at 121 °C for 60 min. All sugar results are the average of duplicates that agree within ± 5% and are reported on anhydro sugar-basis by using conversion factors of 0.90 and 0.88 for hexoses and pentoses, respectively.

The cellulose and hemicellulose mass fractions of the original wood were calculated by means of equations (3) and (4) [29].

$$\text{Cellulose}(\%) = (\text{Glucose content} - 0.27 \times \text{Mannose content}) \times \text{yield}/100 \quad (3)$$

Table 1
Steam-pretreatment conditions and yield recoveries after pretreatment.

Sample #[a]	Catalyst	Temperature (°C)	Time (min)	CSF[b]	Yield (%)[c]		
					Insoluble	Soluble	Total
2	–	215	2	−3.31	83.3, 81.7	9.5, 9.6	92.8, 91.9
3	–	180	21.5	−3.31	77.3, 79.9	8.5, 5.9	85.8, 85.8
4	–	215	10	−2.61	71.1, 75.8	8.1, 9.1	79.3, 84.9
5	–	180	108	−2.61	69.9, 70.4	3.2, 4.2	73.1, 74.7
6	Citric acid	215	2	1.57	72.7, 74.9	18.0, 17.2	90.7, 92.1
7	Sulfuric acid	215	2	2.67	64.4, 63.3	21.5, 20.9	86.0, 84.2

[a] Sample 1 was untreated sawdust.
[b] Using initial pH values of soaking solution: Water 7.00, Citric acid 2.12, Sulfuric acid 1.02.
[c] Yields are a mass fraction of the original wood and duplicates are represented as individual values.