Enzyme and Microbial Technology 47 (2010) 363–371

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Carbohydrate-hydrolyzing enzyme ratios during fungal degradation of woody and non-woody lignocellulose substrates

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ARTICLE INFO

Article history:
Received 26 May 2010
Received in revised form 5 July 2010
Accepted 6 July 2010

Keywords:
Rot fungi
Cellulase
Hemicellulase
Ratios
Lignicellulose

ABSTRACT

Understanding the order that enzymes are secreted during lignocellulosic degradation is relevant both to better understanding basic fungal degradation mechanisms and to industrial attempts to control reactions for biofuels production and other bioprocessing technology. Much is known about the enzymes that are produced and their effect on individual substrates, but little is known about temporal variation and relative enzyme activity on different lignocellulosics substrates. Wood decay fungi *Trametes versicolor* and *Postia placenta* were grown in liquid culture with different substrates (aspen, pine, corn stover, prairie grass and alfalfa) over a 16-week period. Samples of liquid media were taken every 2 weeks for endoglucanase, β-glucosidase and xylanase activity measurement. Endoglucanase:β-glucosidase:xylanase ratios varied for both fungi over the sampling period. *T. versicolor* showed large differences in cellulase enzyme (total cellulase:endoglucanase:β-glucosidase) composition when grown on woody substrates compared with non-woody substrates; there were also difference between the two wood types. This research presents evidence that the ratio of carbohydrate-hydrolyzing enzymes secreted by fungi is not influenced solely by lignin:carbohydrate content of the substrate and other factors including cell anatomy and constituent composition have some control on enzyme production. This provides a useful and broad survey of natural adaptations to various plant tissues relevant to bioenergy and general bioprospecting.

1. Introduction

Lignocellulose is a complex matrix consisting of three major chemical components: lignin, cellulose and hemicellulose. The amount of each component varies among and within different plant species. In perennial plants, these three chemical components are bound together in different ways to create barriers to decomposition. Grasses, which include corn stover, are high in phenolic acid esters [1], which pose an additional barrier [2]. These barriers are overcome in nature, largely enzymatically, and this natural success represents a current goal in biomass processing.

Along with wood chemistry, anatomical characteristics of the wood and cell wall ultrastructure influence biodegradation [3]. Gymnosperms (softwoods) like southern yellow pine tend to have a simpler structure consisting of tracheids and non-lignified parenchyma cells contained in rays or epithelial cells surrounding resin canals [4]. Gymnosperms contain a relatively higher lignin content than other woods and only guaiacyl lignin monomers. Angiosperms (hardwood) including aspen have a more complex structure, containing a variety of cell types including vessels, fibres, parenchyma cells, fibre tracheids, libriforms fibres and others [5]. Angiosperms contain less lignin but higher hemicellulose content when compared with softwoods and contain both syringyl and guaiacyl lignin monomers. The non-woody plants like grasses and forage crops such as corn, prairie grass and alfalfa are all angiosperms. Grasses are monocotyledons containing all three lignin monomers. Alfalfa for example, is a perennial dicotyledon and is rich in protein, minerals, and vitamins [6] and the lignin polymer is predominately guaiacyl and syringyl units with low levels of *p*-hydroxyphenyl units. All three contain ferulic and *p*-coumaric acids that are esterified to hemicellulosic sugars [2,7].

Hydrolysis of the β-1,4-glycosidic bonds in cellulose is achieved with a suite of cellulases including endo-cleaving (endoglucanases), exo-cleaving (cellobiohydrolases) and β-glucosidases. Endoglucanases (EG) (Endo-1,4-β-glucanases (EC 3.2.1.4, endocellulase)) initiate cellulose breakdown by hydrolyzing glycosidic bonds internally, particularly in amorphous regions of cellulose, making it more accessible for cellobiohydrolases by providing new free chain ends [8]. Cellobiohydrolases (CBH) (Cellobiohydrolases (EC 3.3.1.91, exocellulase)) preferentially hydrolyze β-1,4-glycosidic bonds from chain ends, producing cellobiose as the main product. Both EG and CBH produce mostly a disaccharide cellobiose which is hydrolyzed to glucose by a third group of enzymes called β-glucosidases (EC 3.2.1.21) [9]. The degradation of hemicellulose is more complicated than cellulose due to hemicellulose being heterogenic. Xylanases are able to hydrolyze β-1,4 linkages in

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0141-0229/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2010.07.007

xylan, the most abundant component of hemicellulose and produce oligomers which can be further hydrolyzed into xylose by β-xylosidase. Additional enzymes such as β-mannanases, arabinofuranosidases or α-L-arabinanases are needed depending on the hemicellulose composition which can be mannan-based or arabinofuranosyl-containing [10].

Lignocellulolytic fungi are able to overcome the barriers of lignocellulose chemistry and anatomy to degrade lignocellulosic substrates. They use enzymatic methods or a combination of non-enzymatic and cellulolytic mechanisms to achieve decomposition. The two main groups of fungi that degrade woody materials are basidiomycetous white-rot and brown-rot fungi, and these are the best studied lignocellulose-degrading organisms due to prevalence in forest and wood in service. The distinguishing feature of brown rot is the generation of hydroxyl free radical via Fenton chemistry ($H_2O_2 + Fe_2^+ + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\bullet}OH$), which has long been implicated as one of the small oxidants that contributes to rapid polysaccharide depolymerisation [11]. Along with depolymerising cellulose, brown-rot fungi modify lignin, but the majority of this modified lignin remains in situ as a polymeric residue [12,13]. Brown-rot fungi typically do not produce exocellulase. Martinez et al. [14] confirmed this in *Postia placenta* when the genome was sequenced and genes encoding exocellobiohydrolases and cellulose-binding domains, typical of cellulolytic microbes, were absent. Further only one β-1–4 endoglucanase gene was found. Many hemicellulases (including endoxylanases, β-xylosidases, L-β-arabinofuranosidases, endo-β-mannanases, and β-mannosidases), and the single putative β-1–4 endoglucanase have been measured at high activity when *P. placenta* is grown in media containing cellulose [14]. White-rot fungi degrade all components of plant cell walls, including lignin. In addition to free hydroxyl radical production [15,16], white-rot fungi produce lignin-olytic enzymes [17]. The removal of lignin exposes the structural polysaccharides, making them susceptible to hydrolysis by synergistic cellulases.

The differences in lignocellulosic material chemistry and plant cell anatomy leads to the question of how rot fungi adjust carbohydrate-hydrolyzing enzymes to degrade the distinct plant materials. By culturing both a white-rot fungus and a brown-rot fungus on a variety of substrates with varying lignin, cellulose and hemicellulose contents and from different wood groups, we have shown that fungi adapt to different feedstocks using different ratios of cellulolytic and hemicellulolytic enzymes. In particular, there are broad trends that maybe useful towards adapting enzyme ratios commercially.

2. Methods

2.1. Substrates

Non-chemically treated aspen (*Populus tremuloides* Michx.) (hardwood) and southern yellow pine (*Pinus* spp.) (softwood) boards were collected from a local lumber supplier. Corn stover (*Zea mays* L.) (monocot) and alfalfa (*Medicago sativa* L.) (dicot) were gifted by a local farmer, and the mixed prairie grass (37 species, Table 1) was gifted by Professor David Tilman from the Cedar Creek Ecosystem Science Reserve, East Bethel, Minnesota. All non-woody substrates were a mixture of leaves and stalks. Corn stover contained corn cobs. Prairie grass contained seed heads. All non-woody plants were harvested at maturity. Kiln dried sapwood of aspen and southern yellow pine (pine) was chipped, prior to milling. All substrates were milled to pass a 40-mesh screen in a Wiley mill to homogenize the substrate. Milled substrate (10 g wet weight) was added to a 500 ml flask and autoclaved at 121 °C and 16 psi for 30 min.

2.2. Substrate characterization

Lignin, cellulose, and hemicellulose fractions (% oven dry weight) were determined for each treatment. Milled tissues were analyzed following the TAPPI standard protocol for acid-insoluble lignin (TAPPI T 222 om-23). The acid-insoluble lignin from the corn stover, prairie grass and alfalfa was determined by ashing the lignin at 575 °C in a muffle furnace for 5 h in order to remove the weight contribution of silica. Carbohydrate content was determined by analyzing the filtrate after acid-insoluble lignin removal. Filtrate was analyzed using high-performance liquid chromatography (HPLC) according to the conditions described by Schilling et al. [18]. Glucose, xylose, galactose, arabinose, and mannose were separated, along with a cellobiose internal standard.

2.3. Fungal cultures

The brown-rot fungus *P. placenta* strain MAD-698-R (USDA, Forest Mycology Center, Madison, WI)) and white-rot fungus *Trametes versicolor* isolate ATCC 12679 were used as fungal inocula for the enzyme production studies. *T. versicolor* was chosen because it is a test organism for the ATSM D 1413–07 Standard Test Method for Wood Preservatives by Laboratory Soil-Block Cultures. *P. placenta* was chosen because its genome has been sequenced [14] and only one endoglucanase gene was found. A 1 cm^2 plug of the stock cultures was aseptically cut from an agar plate and put into 50 ml sterilized yeast malt (YM) broth in a 250 ml flask (composition per liter: glucose, 10.0 g; peptone, 5.0 g; yeast extract, 3.0 g; and malt extract, 3.0 g). The liquid culture was incubated without agitation at room temperature in the dark for 2 weeks until the mycelia had grown as a dense mat on top of the liquid.

2.4. Fungal culture preparation

A similar method used by Shrestha et al. [19] was used to inoculate the basal media containing substrate. The media with mycelial mats were aseptically transferred into sterile 50 ml centrifuge tubes. The tubes were centrifuged at 2000 × *g* for 20 min. The supernatant was decanted and 50 ml of basal medium which contained 0.25 g of KH_2PO_4, 0.063 g of $MgSO_4{\cdot}7H_2O$, 0.013 g of $CaCl_2{\cdot}2H_2O$, and 1.25 ml of trace element solutions in 1 l of deionised water [20]. The trace element solution (in 1 l deionised water) contained 3.0 g of $MgSO_4{\cdot}7H_2O$, 0.5 g of $MnSO_4{\cdot}H_2O$, 1.0 g of NaCl, 0.1 g of $FeSO_4{\cdot}7H_2O$, 0.181 g of $CoSO_4{\cdot}7H_2O$, 0.082 g of $CaCl_2{\cdot}2H_2O$, 0.1 g of $ZnSO_4$, 0.01 g of $CuSO_4{\cdot}5H_2O$, 0.01 g of $Al_2(SO_4)_3{\cdot}2H_2O$, 0.01 of H_3BO_3, and 0.01 g of $NaMoO_4$ [20] was added to the centrifuge tube. The pellets were resuspended in the basal medium and the centrifugation and supernatant decantation procedure was repeated. After final resuspension, the 50 ml of basal media with fungal inoculums (approximately 110 mg mycelium dry weight for *T. versicolor* and 140 mg mycelium dry weight for *P. placenta*) was added to the flask containing 10 g of substrate. An additional 200 ml of basal media was added. Controls consisting of 250 ml basal media and 10 g substrate were set up to measure changes in enzyme supernatant in the absence of fungal inoculum. Each substrate was replicated five times ($n = 5$). Flasks were incubated at room temperature, in the dark, unshaken. Flasks were swirled every 2 weeks when 10 ml samples of the basal salts medium (no substrate was removed) were taken aseptically for 16 weeks. Samples were centrifuged at 2000 × *g* for 20 min and 6 ml was removed and frozen at −20 °C while the remaining 4 ml was frozen at −70 °C for future studies.

Table 1
Species contained in our mixed prairie grass substrate. (*Note*: Not all are grasses, but instead represent a native perennial consortium.).

Species	Species	Species	Species
Achillea millefolium (*lanulosa*)	*Bromus inermis*	*Lupinus perennis*	*Rudbeckia serotina*
Agropyron smithii	*Buchloe dactyloides*	*Monarda fistulosa*	*Schizachyrium scoparium*
Amorpha canescens	*Calamagrostis canadensis*	*Panicum virgatum*	*Solidago nemoralis*
Andropogon gerardi	*Coreopsis palmata*	*Petalostemum candidum*	*Solidago rigida*
Asclepias tuberosa	*Elymus canadensis*	*Petalostemum purpureum*	*Sorghastrum nutans*
Astragalus canadensis	*Koeleria cristata*	*Petalostemum villosum*	*Sporobolus cryptandrus*
Baptisia leucantha	*Leersia oryzoides*	*Poa pratensis*	*Stipa spartea*
Bouteloua curtipendula	*Lespedeza capitata*	*Quercus ellipsoidalis*	*Stipa spicata*
Bouteloua gracilis	*Liatris aspera*	*Quercus macrocarpa*	*Vicia villosa*
			Zizia aurea

2.5. Enzyme activity

Enzyme activity assays for endoglucanase, β-glucosidase and xylanase were performed on enzyme supernatant for both fungi (*T. versicolor* and *P. placenta*). Total cellulase activity assay was performed on the enzyme supernatant for the white-rot fungus (*T. versicolor*). Exoglucanase activity is included in total cellulase activity following the DOE-NREL standard for measurement of cellulase activities [21] using filter paper as a crystalline cellulose source and glucose as the assay standard. Endoglucanase activity was measured following the method of Duncan et al. [22] using hydroxyethyl cellulose as the substrate and glucose as the assay standard. β-Glucosidase activity was determined by the method described by Wood and Bhat [23] using *p*-nitophenyl β-D-glucopyranoside as the substrate and *p*-nitrophenol as the assay standard. Xylanase activity was measured using the methods described by Bailey et al. [24] using birchwood xylan as the substrate and xylose as the assay standard. Each enzyme supernatant replicate was assayed in duplicate with one blank. Enzyme activity was expressed as enzyme units (1 U is defined as the amount of the enzyme that catalyzes the conversion of 1 micro-mole (μM) of substrate per minute) and specific activity for each enzyme was calculated as μmol product min^{-1} mg^{-1} protein.

2.6. Protein content

Soluble protein was assayed with the Bio-Rad Protein Assay Kit 1 (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard. The concentration is expressed in mg ml^{-1}. This was used for standardising the specific enzyme activity and to monitor protein levels during the decay process.

3. Results

3.1. Substrate characterization

Chemical compositions of untreated aspen, pine, corn stover, prairie grass and alfalfa varied among the substrates (Table 2). Lignin content in non-degraded substrates ranged from 21% in aspen and alfalfa to 30% in pine. The total fermentable sugar content of the 5 substrates ranged from 56% in aspen to 43% in alfalfa (Table 2). The changes in composition after 16 weeks of degradation with the white-rot fungus *T. versicolor* were generally a reduction in lignin content and a slight reduction in hemicellulose content, while glucan content slightly increased relative to other fractions, typical of simultaneous white rot. The brown-rot fungus *P. placenta* reduced the glucan and hemicellulose content for aspen, pine and prairie grass while lignin content increased relative to other chemical components. *P. placenta* did not make significant changes to the composition of the corn stover or alfalfa.

3.2. Specific activity of carbohydrate-degrading enzymes

Endo-1,4-β-glucanase activity in *P. placenta* enzyme supernatant ranged from 1 to 30 μmol of glucose min^{-1} mg^{-1} protein for substrates aspen and pine (Fig. 1A). The specific activity peaks for corn stover, prairie grass and alfalfa substrate supernatant ranged from 0.01 to 14 μmol of glucose min^{-1} mg^{-1} protein (Fig. 1B). With *P. placenta* degraded substrates, the profiles were similar, with activity peaks for endo-1,4-β-glucanase at 8 weeks and 14 weeks for all five substrates (Fig. 1A and B). The activity was lower for corn stover and alfalfa. Both aspen and alfalfa produced an additional peak at 4 weeks. The *T. versicolor* enzyme supernatants which ranged from 0.03 to 2.4 μmol of glucose min^{-1} mg^{-1} protein for substrates aspen and pine (Fig. 1C) and 0.02 to 2 μmol of glucose min^{-1} mg^{-1} protein for the non-woody substrates (Fig. 1D) were lower than the *P. placenta* enzyme supernatant. There were multiple peaks in the specific activity profile for endo-1,4-β-glucanase from *T. versicolor* enzyme supernatant over the 16-week decay process but the major peak for each substrate was anytime from 4 weeks to 12 weeks (Aspen 8 weeks, pine 4 weeks, corn stover 12 weeks, prairie grass 12 weeks and alfalfa 10 weeks) (Fig. 1C and D) and profiles were not similar among the substrates.

Both *P. placenta* and *T. versicolor* enzyme supernatants contained similar β-glucosidase activity (0.002–0.27 μmol of *p*-nitrophenol min^{-1} mg^{-1} protein and range 0.003–0.45 μmol of *p*-nitrophenol min^{-1} mg^{-1} protein respectively). The β-glucosidase activity peak profiles for *P. placenta* and *T. versicolor* enzyme supernatant had varying peaks which were not similar among the substrates. The specific activity peaks for *P. placenta* enzyme supernatant from corn stover and alfalfa were a factor of 10 lower when compared with the other substrates.

The endo-1,4-β-xylanase activity produced by *P. placenta* ranged from 2.9 to 50 μmol of xylose min^{-1} mg^{-1} protein for woody substrates and ranged from 0.4 to 23 μmol of xylose min^{-1} mg^{-1} protein for non-woody substrates. *T. versicolor* endo-1,4-β-xylanase activity range was 0.8–25 μmol of xylose min^{-1} mg^{-1} protein for woody substrates and 0.5–4.4 μmol of xylose min^{-1} mg^{-1} protein. The endo-1,4-β-xylanase activity peak profile for *P. placenta* enzyme supernatants had varying numbers of peaks and the profiles were not similar among the five substrates. Multiple activity peaks for endo-1,4-β-xylanase were seen from *T. versicolor* enzyme supernatant and the profile of these peaks were not similar among the substrates.

Total cellulase activity (exoglucanase, endoglucanase and β-glucosidase) was only measured for the white-rot fungus *T. versicolor* because *P. placenta* does not produce exoglucanases and FPU activity remains zero [25]. For *T. versicolor*, the range of specific activity was from 0.05 to 2.3 μmol of glucose min^{-1} mg^{-1} protein for woody substrates (Fig. 2A) and 0.01 to 5.2 μmol of glucose min^{-1} mg^{-1} protein for non-woody substrates (Fig. 2B). The activity profiles for total cellulase from *T. versicolor* enzyme supernatants contained one broad peak for aspen (weeks 8–14) and pine (weeks

Table 2
Characterization of chemical constituents (wt.%) in non-decayed aspen, pine, corn stover, prairie grass and alfalfa degraded for 16 weeks by white-rot fungus *Trametes versicolor* (Tv) or brown-rot fungus *Postia placenta* (Pp).

Substrate	Decay type	Lignin (%)	Glucan (%)	Mannan (%)	Xylan (%)	Galactan (%)	Arabinan (%)
Aspen	None	20.8 (0.8)	41.8 (1.3)	2.2 (0.2)	11.1 (0.7)	0.4 (0.3)	0.4 (0.2)
Aspen	Tv	19.3 (1.2)	44.8 (0.3)	2.3 (0.2)	13.3 (0.2)	1.4 (0.2)	0.2 (0.0)
Aspen	Pp	21.9 (0.7)	36.1 (0.6)	1.7 (0.4)	9.2 (0.6)	0.0 (0.0)	0.0 (0.0)
Pine	None	30.3 (0.8)	37.6 (0.4)	8.2 (0.8)	5.6 (0.3)	2.3 (0.3)	0.8 (0.3)
Pine	Tv	29.1 (0.7)	40.1 (0.7)	8.9 (0.4)	5.9 (0.1)	2.5 (0.3)	0.5 (0.1)
Pine	Pp	32.1 (1.7)	31.9 (0.2)	5.6 (0.5)	3.5 (0.2)	1.7 (0.2)	0.4 (0.1)
Corn stover	None	22.6 (0.8)	35.8 (0.7)	0.9 (0.0)	14.7 (0.4)	0.5 (0.1)	2.4 (0.6)
Corn stover	Tv	21.1 (0.5)	37.2 (0.8)	0.9 (0.1)	17.0 (0.5)	0.9 (0.2)	2.1 (0.1)
Corn stover	Pp	23.2 (0.8)	35.1 (1.1)	0.8 (0.1)	15.0 (0.9)	1.3 (0.6)	2.2 (0.4)
Prairie grass	None	25.7 (1.0)	29.4 (1.2)	1.3 (0.3)	11.3 (0.3)	2.6 (0.4)	3.6 (0.3)
Prairie grass	Tv	23.7 (0.6)	33.3 (0.8)	0.8 (0.2)	15.2 (0.5)	1.2 (0.4)	2.6 (0.2)
Prairie grass	Pp	26.6 (0.7)	24.3 (1.6)	0.4 (0.1)	10.2 (0.3)	1.0 (0.3)	3.2 (0.2)
Alfalfa	None	20.7 (0.7)	28.0 (0.2)	1.4 (0.2)	9.6 (0.2)	1.6 (0.8)	2.7 (0.5)
Alfalfa	Tv	19.4 (0.9)	33.5 (0.9)	1.2 (0.5)	13.6 (0.8)	1.9 (0.3)	2.2 (0.3)
Alfalfa	Pp	22.0 (0.8)	26.5 (3.0)	1.3 (0.3)	13.4 (0.8)	0.6 (0.4)	2.9 (0.3)

Data are expressed as a percentage of total biomass (±standard error) ($n = 5$).

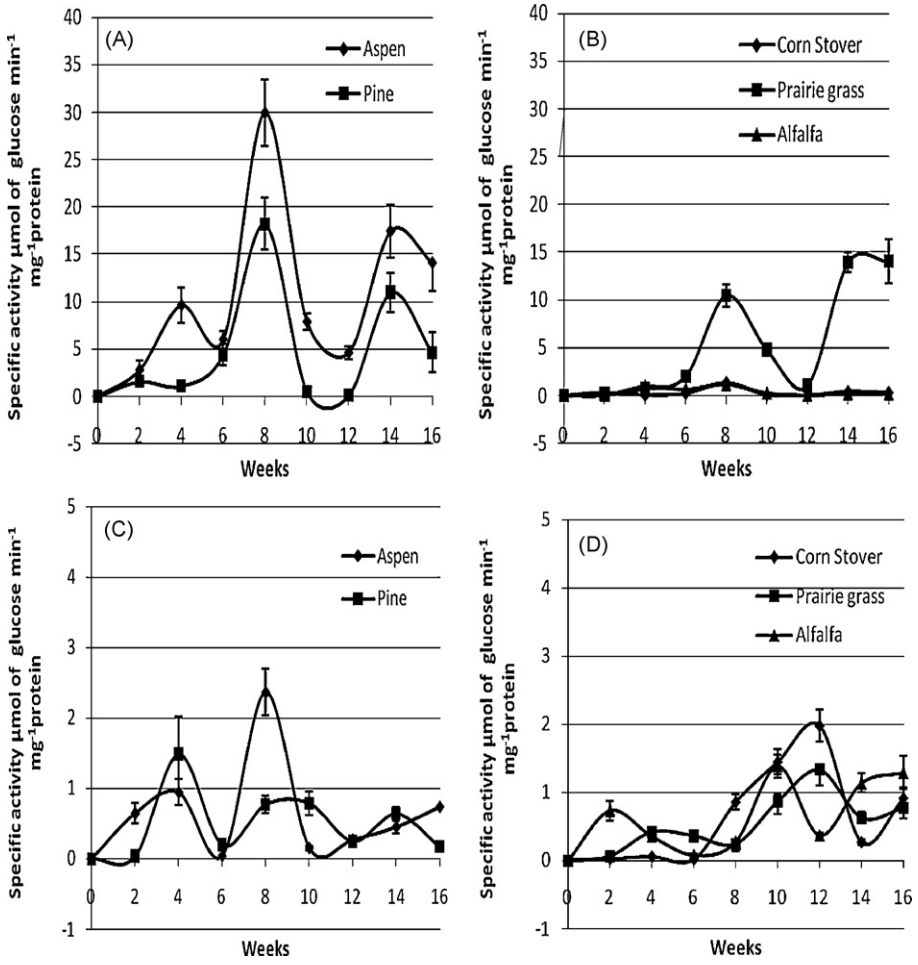

Fig. 1. Endo-1,4-β-glucanase specific activity of degraded substrate for aspen, pine, corn stover, mixed prairie grass and alfalfa powder degraded 16 weeks by (A) and (B) brown-rot fungus *Postia placenta* (C) and (D) white-rot fungus *Trametes versicolor* (±standard error) ($n = 2$).

2–8) and very little activity for the non-woody substrates until week 16 when the highest concentration recorded for all substrates 5.2 μmol of glucose min^{-1} mg^{-1} protein in corn stover enzyme supernatant.

3.3. Ratio of carbohydrate-degrading enzymes

Endo-1,4-β-glucanase and endo-1,4-β-xylanase enzyme unit activities (1 U is defined as the amount of the enzyme that catalyzes the conversion of 1 micro-mole (μM) of substrate per minute) were adjusted in proportion to the β-glycosidase enzyme unit activity, which allowed the enzyme composition of the enzyme supernatants to be compared. The enzyme units of β-glucosidase in the enzyme supernatant rarely exceeded 10% of the endo-1,4-β-glucanase activity for either of the test fungi. Both *T. versicolor* and *P. placenta* produced similar β-glucosidase activity when comparing specific activity. Overall, endo-1,4-β-glucanase:β-glycosidase:endo-1,4-β-xylanase activity in the

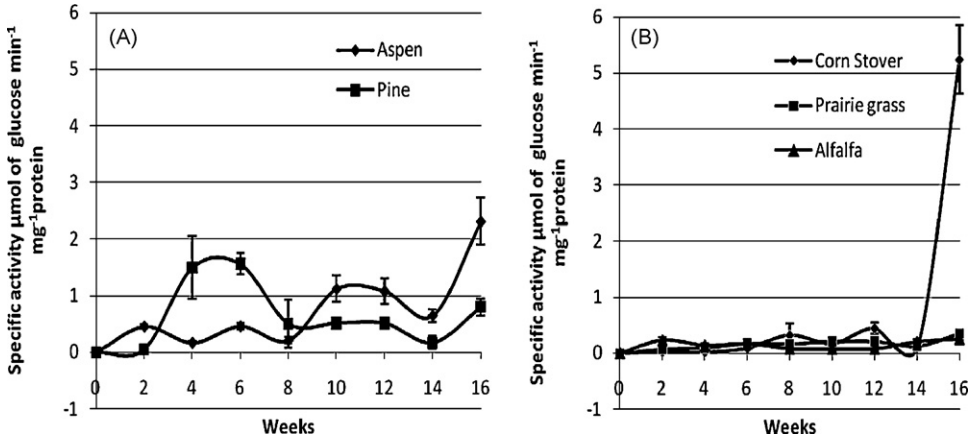

Fig. 2. Total cellulase specific activity of degraded substrate for aspen, pine, (A) corn stover, mixed prairie grass and alfalfa (B) powder degraded 16 weeks by *Trametes versicolor* (±standard error) ($n = 2$).

Table 3
Endoglucanase (EG):β-glucosidase (βG):xylanase (X) ratios, in enzyme supernatants using enzyme units with β-glucosidase activity standardized to 1 when aspen, pine, corn stover, prairie grass and alfalfa were degraded by *Postia placenta* over a 16-week period. Glucose and xylose content of enzyme supernatant when aspen, pine, corn stover, prairie grass and alfalfa were degraded by *Postia placenta* over a 16-week period.

Substrate	Sugar content in supernatant (μmol/ml)	Enzyme	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
	Glucose		0.11	0.10	0.10	0.09	0.12	0.12	0.66	0.48
		EG	157	162	107	671	1298	195	9908	5530
Aspen		βG	1	1	1	1	1	1	1	1
		X	420	80	73	113	421	104	1028	1440
	Xylose		0.9	0.9	0.9	0.9	0.7	0.7	0.7	0.6
	Glucose		0.21	0.06	0.10	0.06	0.05	0.07	0.67	0.54
		EG	165	103	53	1508	87	34	1087	586
Pine		βG	1	1	1	1	1	1	1	1
		X	550	344	101	189	379	165	186	484
	Xylose		0.9	0.7	0.6	0.6	0.6	0.7	0.2	0.2
	Glucose		0.15	0.44	0.33	0.79	0.33	0.27	0.91	0.71
		EG	40	8	79	99	13	1	393	35
Corn stover		βG	1	1	1	1	1	1	1	1
		X	33	54	67	13	20	13	65	5
	Xylose		0.9	1.0	0.9	1.3	1.7	0.9	0.2	1.0
	Glucose		2.13	2.22	1.09	1.35	1.02	1.45	0.97	0.69
		EG	6	17	43	77	54	23	69	61
Prairie grass		βG	1	1	1	1	1	1	1	1
		X	20	36	19	28	36	33	4	30
	Xylose		2.0	2.1	4.1	2.9	1.9	1.4	2.0	1.4
	Glucose		3.54	3.64	3.68	4.39	4.10	4.28	3.83	2.98
		EG	4	110	246	304	49	17	99	46
Alfalfa		βG	1	1	1	1	1	1	1	1
		X	45	29	108	44	26	35	31	24
	Xylose		3.6	5.7	4.6	6.3	5.8	5.6	4.6	4.0

enzyme supernatants when degraded by *P. placenta* was higher than when degraded by *T. versicolor* (Tables 3 and 4). Of the enzyme supernatants assayed for each fungi, the number of assays where the proportion of endo-1,4-β-glucanase was greater than endo-1,4-β-xylanase was 19 of 40 for *T. versicolor* and 26 of 40 for *P. placenta*. Wood substrates degraded by *P. placenta* always had a higher proportion of endo-1,4-β-xylanase in the enzyme supernatant than non-woody substrates and peaks of endo-1,4-β-xylanase activity

Table 4
Endoglucanase (EG):β-glucosidase (βG):xylanase (X) ratios, in enzyme supernatants using enzyme units with β-glucosidase activity standardized to 1 when aspen, pine, corn stover, prairie grass and alfalfa were degraded by *Trametes versicolor* over a 16-week period. Glucose and xylose content of enzyme supernatant when aspen, pine, corn stover, prairie grass and alfalfa were degraded by *Trametes versicolor* over a 16-week period.

Substrate	Sugar content in supernatant (μmol/ml)	Enzyme	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16
	Glucose		0.14	0.16	0.07	0.18	0.02	0.01	0.09	0.00
		EG	20	37	19	30	20	17	38	20
Aspen		βG	1	1	1	1	1	1	1	1
		X	105	48	70	287	190	94	109	9
	Xylose		1.1	1.1	0.9	0.8	0.8	0.6	0.7	0.7
	Glucose		0.20	0.18	0.15	0.21	0.55	0.09	0.08	0.06
		EG	1	24	7	55	6	3	92	2
Pine		βG	1	1	1	1	1	1	1	1
		X	13	50	28	56	30	45	163	4
	Xylose		1.0	0.8	1.0	1.0	0.8	0.6	0.7	0.3
	Glucose		0.35	0.37	0.55	0.87	2.13	0.65	0.43	0.54
		EG	5	4	1	29	20	51	8	7
Corn stover		βG	1	1	1	1	1	1	1	1
		X	19	19	10	16	8	14	10	5
	Xylose		1.6	1.4	1.9	3.4	2.6	1.9	1.9	1.4
	Glucose		0.80	0.53	0.76	2.70	1.73	1.16	1.01	0.80
		EG	4	21	19	8	15	30	15	9
Prairie grass		βG	1	1	1	1	1	1	1	1
		X	11	13	15	14	8	11	9	6
	Xylose		2.3	2.1	1.8	2.2	1.6	1.5	2.6	0.8
	Glucose		3.41	4.79	4.31	1.96	1.39	1.53	0.73	1.28
		EG	138	51	6	21	44	30	46	22
Alfalfa		βG	1	1	1	1	1	1	1	1
		X	25	31	19	17	15	13	15	4
	Xylose		5.2	5.2	4.0	2.4	2.3	2.5	2.3	2.4

Table 5
Total cellulase (exoglucanase, endoglucanase and β-glucosidase activity)(TC): endoglucanase (EG):β-glucosidase (βG) ratios, in enzyme supernatants using enzyme units with β-glucosidase activity standardized to 1 when aspen, pine, corn stover, prairie grass and alfalfa were degraded by *Trametes versicolor* over a 16-week period.

Substrate	Enzyme	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14	Week 16	TC:EG ratio average
Aspen	TC	22	11	59	128	110	62	12	10	1
	EG	20	37	19	30	20	17	38	20	0.5
	βG	1	1	1	1	1	1	1	1	
SYP Pine	TC	0.5	7.2	9.4	31.4	6.4	10.6	14.4	1.3	1
	EG	1	24	7	55	6	3	92	2	2.3
	βG	1	1	1	1	1	1	1	1	
Corn stover	TC	1.6	2.0	0.9	2.3	1.1	1.6	0.6	0.4	1
	EG	5	4	1	29	20	51	8	7	12
	βG	1	1	1	1	1	1	1	1	
Prairie grass	TC	1.8	1.6	1.6	1.5	0.6	0.8	1.1	0.5	1
	EG	4	21	19	8	15	30	15	9	13
	βG	1	1	1	1	1	1	1	1	
Alfalfa	TC	14	10	4.7	1.6	2.3	2.2	2.4	1	1
	EG	138	51	6	21	44	30	46	22	9
	βG	1	1	1	1	1	1	1	1	

in the enzyme mixtures were more dramatic (Table 3). The proportion of endo-1,4-β-glucanase in the enzyme supernatant peaked for all 5 substrates later in the decay process at weeks 8–10 and again at weeks 14–16. The endo-1,4-β-glucanase activity in the *P. placenta* degraded aspen enzyme supernatant is very high during weeks 10, 14 and 16 which are also weeks when the endo-1,4-β-xylanase activity was high.

Table 4 shows the proportion of endo-1,4-β-xylanase in the enzyme supernatant when *T. versicolor* degraded aspen was highest of the 5 substrates. There was also a noticeable higher proportion of endo-1,4-β-xylanase in the pine enzyme mixture compared with the three non-woody substrates. Of the 19 samples where the proportion of endo-1,4-β-glucanase in the enzyme supernatant is higher than endo-1,4-β-xylanase, 2 were in the enzyme supernatant of woody substrates and 17 in non-woody substrates.

The ratios of the three cellulase hydrolyzing enzymes using the white-rot fungus *T. versicolor* are presented in Table 5. Enzyme unit of total cellulase (endoglucanase, exoglucanase and β-glucosidase) and endo-1,4-β-glucanase were adjusted in proportion to the β-glycosidase enzyme unit activity. Generally the ratios fluctuated over the 16-week decay process. The enzyme supernatant that contained the highest proportion of total cellulase, also contained the highest proportion of endo-1,4-β-glucanase, except for aspen where when the highest proportion of total cellulase was report the endo-1,4-β-glucanase proportion was not the highest recorded. Only the wood substrates produced enzyme supernatants where the proportion of total cellulase in the enzyme supernatant was higher than the endo-1,4-β-glucanase proportion. The enzyme supernatants with the highest proportion of total cellulase activity were in aspen enzyme supernatants. The lowest proportions of total cellulase activity were seen in the enzyme supernatants of corn stover and prairie grass. The highest endo-1,4-β-glucanase activity in the enzyme supernatant were in alfalfa enzyme supernatants, while enzyme supernatants with the lowest proportion of endo-1,4-β-glucanase activity were corn stover and prairie grass. Differences were seen in the average ratio of total cellulase:endo-1,4-β-glucanase (Table 5) between the two wood species and also between wood and non-woody substrates. The proportion of endo-1,4-β-glucanase activity in wood substrates enzyme supernatants using enzyme units (aspen 1:0.5, pine 1:2.3) was less than non-woody substrates (corn stover 1:12, prairie grass 1:13 and alfalfa 1:9). There was also a noticeable difference between the two wood substrates. The proportion of endo-1,4-β-glucanase was slightly less when the SI unit for enzymes activity (nanokatal) was used for the calculation (aspen 1:0.4, pine 1:2, corn stover 1:9, prairie grass 1:11 and alfalfa 1:8) but the trends were the same.

3.4. Protein content

The amount of protein in the enzyme supernatant at week 0 was highest in corn stover, prairie grass and alfalfa at 91, 88 and 70 $\mu g\,ml^{-1}$ (respectively) compared with 3.2 and 9 $\mu g\,ml^{-1}$ for aspen and pine. The protein content stayed fairly constant during the experiment for all enzyme supernatant for the *T. versicolor* decayed substrate and the *P. placenta* degraded aspen, pine and prairie grass but in *P. placenta*-decayed corn stover and alfalfa the protein climbed steadily to highs of 200 and 550 $\mu g\,ml^{-1}$ (respectively).

3.5. Amount of sugars in the enzyme supernatant

The glucose content of the enzyme supernatant of the five substrates aspen, pine, corn stover, prairie grass and alfalfa at the start of the experiment were 0.1, 0.13, 0.6, 1.9 and 5.4 μmol/ml respectively. During the course of the experiment, the amount of glucose fluctuated up and down in the enzyme supernatant from the *T. versicolor* and *P. placenta* degraded substrates but all had dropped by week 16 for *T. versicolor* (Table 4) but had risen in 3 of the five *P. placenta* substrates and dropped in two, prairie grass and alfalfa enzyme supernatant (Table 3). The xylose content in the enzyme supernatant of the five substrates aspen, pine, corn stover, prairie grass and alfalfa at the start of the experiment were 0.9, 1.0, 0.7, 3.2 and 5.6 μmol/ml respectively. After fluctuating during the experiment period for both the *T. versicolor* (Table 4) and *P. placenta* (Table 3) degraded substrates, by week 16 the amount had dropped.

4. Discussion

The five substrates used in this research varied, in chemical composition, wood types, cell anatomy, microstructure and cell wall ultrastructure. Compositions of substrate used in this research were similar to other published results [18,26–28]. The only slight difference was the xylan content of aspen and corn stover which was lower than reported in the NREL Biomass Feedstock Composition and Property Database (www1.eere.energy.gov/biomass/feedstock_databases.html), 11.1% and 14.7% in this research compared with 17.6% and 18.3% by NREL. Large fungal mycelium mats formed in the liquid media for all substrates after 2 weeks and were present at harvest after 16 weeks. Weight loss could not be measured for the substrates as the mycelium mats were a mixture of milled substrate and fungus. The substrate characterization showed that both fungi degraded the lignocellulosic material less than when grown in

ASTM microcosms [26], slowing the 16-week study to equivalent to early decay of a wood block in a microcosm. As reported by Curling et al. [29], the hemicellulose components of the substrates were removed before the cellulose components with two sugars (galactan and arabinan) found on the hemicellulose side chains being used before the two main chain sugars (mannan and xylan). Although *P. placenta* mycelial mats formed in the liquid media for corn stover and alfalfa, the fungus did not substantially degrade the substrate as there was no change in substrate composition and the enzyme activity were lower than other substrates indicating that *P. placenta* was utilizing other more readily usable nutrients in the substrate. These enzyme supernatants had the highest protein contents.

Apart from minerals and trace elements found in the basal media, no nitrogen or carbon was added apart from that within the lignocellulosis substrates. The carbon and nitrogen source are two important factors affecting growth of microorganisms [30]. Non-wood substrates contained far more nitrogen (corn stover 0.67% mass) than the wood substrates (aspen 0.21%mass) according to the NREL Biomass Feedstock Composition and Property Database (www1.eere.energy.gov/biomass/feedstock_databases.html). Possible sources of carbon are the natural proteins in the substrate. The non-woody enzyme supernatants contained more protein 70–91 $\mu g\,ml^{-1}$ for alfalfa, corn stover and prairie grass compared to 9–32 $\mu g\,ml^{-1}$ for aspen and pine.

Two striking differences were observed in the specific activity of endo-1,4-β-glucanase in the enzyme supernatant between substrates degraded by *P. placenta* and *T. versicolor*. The first was that *P. placenta* produced endo-1,4-β-glucanase activity 10 times higher than *T. versicolor*. Martinez et al. [14] reported that *P. placenta* does not produce a cellulose-binding domain which would lead to the endoglucanase not being bound to the substrate and higher activity being present in the supernatant compared to *T. versicolor* where at least a third of the endoglucanases are associated with the fungal mycelium and undecayed substrate as reported by Valášková and Baldrian [31]. The second difference is the cyclic nature of the endo-1,4-β-glucanase specific activity when substrates were degraded by *P. placenta*. All five substrates had peaks at 8 and 14 weeks with two substrates aspen and prairie grass having a smaller additional peak at 4 weeks, which can be contributed to the one endoglucanse being constitutively secreted and not influenced by free glucose concentrations. There was no peak pattern in endo-1,4-β-glucanase specific activity when *T. versicolor* degraded the different substrates. This could be due to *T. versicolor* producing a mixture of endo-1,4-β-glucanase which are induced or repressed at different rates by different levels of accumulating hydrolysis products during the decay process. The β-glucosidase specific activity showed no peak pattern for both fungi. The endo-1,4-β-xylanase specific activity were similar for both fungi and showed no peak pattern possibly due to a situation similar to the *T. versicolor* endo-1,4-β-glucanase where multiple enzymes are secreted to degrade the hemicellulose in the plant material. Commercially available cellulase mixtures also contain a variety of exo, endo and β-glucosidase enzymes which all work synergistically to degrade the pretreated substrates in biofuels production. The total cellulase specific activity for the two woody substrates when degraded by *T. versicolor* had activity peaks at the same time as there were endo-1,4-β-glucanase activity peaks but the total cellulase peaks spanned a 4-week period rather than 2 weeks for endo-1,4-β-glucanase suggesting exoglucanase activity in response to endoglucanase activity. Two of the five substrates (especially corn stover) showed large increases in total cellulase activity in the 16-week samples suggesting increased decay rates after the 16-week period of this study.

Both the carbohydrate and cellulose degrading enzyme ratios changed at every sampling and were different between fungi and between the five different substrates. This suggests the fungi were able to adapt their cellulase profiles and ratios over time depending on substrate. Both the carbohydrate and cellulose degrading enzyme ratios were based on β-glucosidase because β-glucosidase is the enzyme which controls the accumulation of cellulase enzyme inhibitors and inducers, it was considered the most appropriate to base the activity of the other carbohydrate-degrading enzymes. The ratio of each carbohydrate degrading enzyme, endo-1,4-β-glucanase:β-glucosidase:endo-1,4-β-xylanase was generally higher in the enzyme mixtures produced by *P. placenta*. *P. placenta* produces only one endoglucanase and no exoglucanase suggesting that non-enzymatic degradation, increasing concentration of endo-1,4-β-glucanase and changing hemicellulase composition are the best option to adapt to different substrates. The larger number of enzyme mixtures (17 of 24) from non-woody substrates degraded by *T. versicolor* that contain more endo-1,4-β-glucanase than endo-1,4-β-xylanase compared with 2 of 16 for woody substrates suggests that *T. versicolor* has different approaches to degrading woody and non-woody substrates. *T. versicolor* seemed to need the most endo-1,4-β-xylanase in the enzyme mixture to degrade aspen, with pine requiring less than aspen but more than the non-woody substrates.

Differences in wood anatomy or lignocellulose chemistry could be factors in these differences. It is probable that the distribution and composition of lignin is as important as the concentration of lignin in terms of enzyme accessibility and lignocellulose digestibility [32]. Ramos et al. [33] suggested that guaiacyl lignin restricts fibre swelling and thus the enzymatic accessibility more than syringyl lignin. Gymnosperms contain only guaicyl lignin while angiosperm also contain syringyl lignin. Although gymnosperms have higher overall lignin content, they also have non-lignified parchenchyma cells which give rot fungi an opportunity to establish and an easier and quicker route to decay wood. In all wood species cell wall thickness, lignin content, and cellulose microfiber orientation all contribute to the carbohydrate degrading enzymes ability to get to available cellulose and hemicellulose [3]. Lignin content, amount of syringyl lignin and the syringyl/guaicyl ratio increase with maturity of grasses [34]. High lignin content correlates with high *p*-coumaric:ferulic acid ratios [35] indicating higher crosslinking of lignin to hemicellulose to cellulose.

The most noticeable differences were changes in enzyme ratios between substrates. When comparing the cellulose degrading enzymes of *T. versicolor*, there were differences in the ratio of total cellulase:endo-1,4-β-glucanase between wood species and between wood substrates and non-woody substrates. The average of the total cellulase:endo-1,4-β-glucanase showed that aspen had double the total cellulase to endo-1,4-β-glucanase (ratio 1:0.5) while the pine had 2.3 times the endo-1,4-β-glucanase than total cellulase (ratio 1:2.3) and the non-woody had similar ratios of total cellulase:endo-1,4-β-glucanase, approximately 10 times more endo-1,4-β-glucanase to total cellulase (corn stover 1:11.9, praire grass 1:12.7 and alfalfa 1:9.3). These differences could be attributed to aspen being a hardwood, having a more complicated structure and containing both syringyl and guaiacyl lignin monomers. Southern yellow pine being a softwood, has a uniform cell structure and contains only guaiacyl lignin monomers. Two (corn stover and prairie grass) of the three non-woody plants contain *p*-hydroxyphenyl lignin monomers and all three have additional phenolic acid esters which form ether linkages to other aromatic constituents. This comparison could not be done for *P. placenta* due to *P. placenta* not producing exoglucanases and cannot degrade crystalline cellulose.

Other research investigating enzymatic degradation of lignocellulosic substrates focused on enzyme activity rather than ratios of one enzyme to another. Rasmussen et al. [36] investigated the use of brown-rot fungus *Gloeophyllum trabeum* to degrade wet milled corn fibre to produce reducing sugars for future conversion to ethanol.

They used suspended-culture, solid-state fermentation in combination with addition of acetate buffer, anaerobic conditions and addition of *Saccharomyces cerevisiae*. Enzyme activity was studied from solid-state experiments and amylase and cellulase were detected but no xylanase activity was observed. Shrestha et al. [19] reported on research into the degradation of wet milled corn fibre by the white-rot fungus *Phanerochaete chrysosporium*, brown-rot fungus *G. trabeum* and soft-rot fungus *Trichoderma reesei* by aerobic submerged culture. α-Amylase, glucoamylase, xylanase, endocellulase, and exocellulase specific enzyme activity was determined. The corn fibre induced enzyme secretion in all three fungal cultures. They concluded that both starch and hemi/cellulose fractions contributed significantly to enzyme induction and that residual starch and hemi/cellulose fractions resulted in higher enzyme induction for the white-rot fungus as compared to the brown-rot and soft-rot fungi tested. α-Amylase and glucoamylase activities for all three fungal cultures were similar but xylanase, endo-, and exocellulase activities were significantly different between the fungal species. Non-enzymatic oxidative degradation was suggested as the reason for the low specific enzyme activity but higher ethanol yields in the case of the brown-rot fungus *G. trabeum*.

Designing more efficient mixtures of cellulases will help reduce the cost for processes that utilize cellulases [37]. In the early 1990s, NREL researchers determined that a 90:9:1 mixture of a cellobiohydrolase from *T. reesei* (CBH I), a thermal-tolerant endoglucanase from *Acidothermus cellulolyticus* (EI), and a β-D-glucosidase was capable of nearing the performance observed for a leading commercial *T.reesei* preparation at comparable protein loading http://www1.eere.energy.gov/biomass/printable_versions/cellulase_enzyme.html. None of the ratios in this study were as high as in these previous investigations but the fungi in this study are degrading the substrate on an 'as needed basis' rather than total release of all sugars in the shortest possible time.

Our research suggests that decay fungi appear to use enzymes at different activities during the lignocellulosic decay process of different substrates. Both the white-rot fungus *T. versicolor* and the brown-rot fungus *P. placenta* used in this research utilized decay enzymes at different activities and in different combinations to degrade the varied lignocellulosic substrates. We suggest that the concentration of lignin, cellulose and hemicellulose are not the only factor that affects enzyme ratios but that cell wall anatomy and microstructure all contribute. While factors affecting enzyme ratios are complex, we observed broad patterns among wood types and non-woody substrates that may guide future attempts to plan ratios to suit feedstock types more broadly.

Acknowledgments

This work was supported with funding by the U.S. Department of Energy (DOE), project no. GO18088. The authors wish to acknowledge the experimental assistance by Jacob Inda and Brook Jacobson. Prof. Robert Blanchette, Department of Plant Pathology, University of Minnesota, is warmly thanked for his critical reading of the manuscript.

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