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Evaluation of a *Hypocrea jecorina* Enzyme Preparation for Hydrolysis of Tifton 85 Bermudagrass

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Abstract Tifton 85 bermudagrass, developed at the ARS-USDA in Tifton, GA, is grown on over ten million acres in the USA for hay and forage. Of the bermudagrass cultivars, Tifton 85 exhibits improved digestibility because the ratio of ether- to ester-linked phenolic acids has been lowered using traditional plant breeding techniques. A previously developed pressurized batch hot water (PBHW) method was used to treat Tifton 85 bermudagrass for enzymatic hydrolysis. Native grass (untreated) and PBHW-pretreated material were compared as substrates for fungal cultivation to produce enzymes. Cellulase activity, measured via the filter paper assay, was higher for fungi cultivated on PBHW-pretreated grass, whereas the other nine enzyme assays produced higher activities for the untreated grass. Ferulic acid and vanillin levels increased significantly for the enzyme preparations produced using PBHW-pretreated grass and the release of these phenolic compounds may have contributed to the observed reduction in enzyme activities. Culture supernatant from Tifton 85 bermudagrass-grown fungi were combined with two commercial enzyme preparations and the enzyme activity profiles are reported. The amount of reducing sugar liberated by the enzyme mixture from *Hypocrea jecorina* (after 192 h incubation with untreated bermudagrass) individually or in combination with feruloyl esterase was 72.1 and 84.8%, respectively, of the commercial cellulase preparation analyzed under the same conditions.

Keywords Bermudagrass · *Trichoderma* · *Hypocrea* · Cellulases · Hemicellulases

Introduction

Global demand for food is expected to double within the next 50 years, and global demand for transportation fuels is expected to increase even more rapidly [1]. Diversion of ethanol production from food and feed can be reduced if other agricultural and forestry products are considered for this purpose [2]. There is also a great need for renewable energy supplies

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that do not cause significant environmental harm. Biofuels such as cellulosic ethanol have potential to provide fuel supplies with greater environmental benefits than either petroleum or current food-based biofuels [3].

Crops such as bermudagrass (*Cynodon dactylon* L.) have the capacity to produce large quantities of lignocellulose as substrates for biofuels production. Bermudagrass is already grown on 10–15 million acres throughout the southern United States for hay and forage. Genetic improvements in digestibility obtained by traditional plant breeding [4, 5] are directly related to the ability to digest the biomass with enzymes [6]. Tifton 85 is a variety of bermudagrass with improved animal digestibility developed at the ARS-USDA (Tifton, GA). Phenolic acids occurring within grass cell walls are associated with lignin and are recalcitrant to biodegradation [7, 8], thus inhibiting sugar release from the biomass [6]. Prior studies indicate a negative relationship between ether-linked ferulic acid concentrations and extent of digestibility among bermudagrass cultivars [9]. Tifton 85 bermudagrass exhibits improved digestibility because the ratio of ether- to ester-linked phenolic acids has been lowered using traditional plant breeding techniques [4, 5].

Three steps are involved in bioconversion of grasses to ethanol: (1) a pretreatment process to reduce substrate recalcitrance, (2) enzymatic hydrolysis of cellulose and hemicellulose components to simple sugars, and (3) fermentation of the sugars to ethanol. The first two steps are considered to be economic hurdles for full-scale process commercialization [10, 11]. Pretreatments help to increase the rate and extent of hydrolysis by chemicals, enzymes, or microorganisms, and improve the action of enzymes to increase sugar yields. An ideal pretreatment is cost-effective and avoids the loss of potential sugars and formation of inhibitory by-products [2]. Pressurized batch hot water pretreatment (PBHW) [12] of plant materials provides an effective way to pretreat cellulosic material by disrupting hemicellulose before enzymatic hydrolysis without using chemicals such as sulfuric acid, lime, or ammonia. Liquid water at 220°C has a pH of approximately 5.5 as a result of an ion product of 10^{-11} [13], and exposure of biomass causes liberation of acetyl groups from hemicellulose and increases depolymerization. These reactions decrease the pH of the solution further, mimicking very dilute acid hydrolysis, a common technique which uses low concentrations of acid in hot water to break down hemicellulose [14]. PBHW-pretreated grass was investigated as a substrate for enzyme production using *Hypocrea jecorina*.

The ascomycete *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) is currently used for commercial manufacturing of cellulase and hemicellulase products and has been proposed as the most promising organism for production of enzymes for lignocellulose conversion to fermentable sugars [15]. In addition to a long history of safe commercial use, this fungus secretes significant quantities of enzymes and has well-developed genetic systems [16, 17]. *H. jecorina* strains have been cultivated on different lignocellulosic substrates to generate an enzyme cocktail suited for a particular substrate. Certain natural substrates may induce secreted enzymes suited to degrade particular combinations of polysaccharides and chemical bonds found in the carbon source [18].

The goal of this study was to determine the enzyme profile of *H. jecorina* when grown either on untreated or PBHW-pretreated Tifton 85 bermudagrass and to compare enzyme cocktails produced in this fashion to commercially available preparations. Commercial enzyme preparations are very effective at hydrolyzing biomass; however, they are expensive, although this cost may decrease in the near future. If an enzyme cocktail produced by growing *H. jecorina* on Tifton 85 bermudagrass showed enhanced activity, it could translate into cost savings for the overall grass-to-ethanol process. Enzyme preparations thus obtained were also evaluated for release of sugars and phenolic

compounds from PBHW-pretreated Tifton 85 bermudagrass, either alone or in combination with commercially available feruloyl esterase. These results were compared to those obtained from commercial enzyme preparations. In addition, the important inhibitory effects of phenolic compounds, such as ferulic acid, on cellulase activity of preparations were explored. Economically viable enzyme production and amelioration of enzyme inhibition are of crucial importance when designing grass-to-ethanol processes.

Materials and Methods

Materials, Microbial Strains, and Medium

H. jecorina (anamorph *T. reesei*) NRRL 11460 (Rut C-30) was provided by Dr. Xin-Liang Li [from the ARS Culture Collection (NCAUR, Peoria, IL)]. The fungus was routinely propagated in potato dextrose agar (PD) containing 0.4% (*w/v*) potato and 2.0% (*w/v*) dextrose supplemented with 2% (*w/v*) agar for solid medium. Tifton 85 bermudagrass was provided by Dr. William F. Anderson (ARS-USDA, Tifton, GA). All chemicals and media ingredients were of research quality. The experiments were performed in triplicate. Values obtained were very similar; therefore, to facilitate reading, standard deviations are not presented in the tables. In the graphs, standard deviations are presented as error bars.

PBHW Pretreatment of Tifton 85 Bermudagrass

Pressurized batch hot water (PBHW) pretreatment was conducted in a 2-l pressure vessel (Model 4600 Parr Instrument Co., Moline, IL), surrounded by retractable ceramic heaters [12]. Tifton 85 bermudagrass (15 g) was placed in a 500-μm (35 mesh) stainless steel basket, which was immersed in 1,450 ml of deionized water in the vessel. The sample was treated at 230°C for 2 min essentially as previously described [12]. Hydrolyzed solids were dried at 40°C for 90 min using a fluidized bed drier (Endecott, FBD2000, London, UK), and stored at 4°C until further use.

H. Jecorina Flask Cultures

Conidia spores (approximately 1×10^7) were transferred to a shake flask (250 ml) containing 50 ml PD medium. The flask was shaken at 250 rpm at 28°C for 48 h. A 5% (*v/v*) inoculum was transferred to 50 ml of production medium in a 250-ml Erlenmeyer flask. Production media for *H. jecorina* contained per liter 15.0 g KH_2PO_4, 20.0 g corn steep liquor (Sigma-Aldrich, St. Louis, MO), 5 g NH_4SO_4, 0.5 g $Mg(SO_4)_2{\cdot}7H_2O$, 1.0 ml Tween 80, and 30 ml solution of untreated or PBHW-pretreated Tifton 85 bermudagrass containing a 2.0 g of grass. Basal medium was adjusted to pH 4.8 with 1 M NaOH. The fungus was grown at 28°C with agitation (250 rpm) for 8 days. Flasks were sampled daily (2 ml). For evaluation of the inhibitory effect of ferulic acid on cellulase activities, *H. jecorina* was grown as described above on untreated bermudagrass with different concentrations of ferulic acid (0, 0.02, 0.06, and 0.10% *w/v*).

Bermudagrass Digestion Assay

Tifton 85 bermudagrass solids recovered after PBHW treatment at 230°C at a solids loading of 5% (*w/v*) were hydrolyzed by commercial (Spezyme CP, Danisco, Genencor Division,

Rochester, NY; and DEPOL 740LL, Biocatalysts Ltd., Cardiff, Wales, UK) and *H. jecorina* preparations (supernatant of samples collected after 8 days of growth on 4% *w/v* solids untreated or PBHW-pretreated Tifton 85 bermudagrass). The performance of the enzyme preparations were compared by standardizing either xylanase (400 IU/ g grass) or FPAase (8 FPU/ g grass) units. Assays were conducted at 50°C, pH 4.8, with an agitation of 100 rpm for 24 h, under sterile conditions. Samples were boiled, centrifuged (10,000 rpm/ 4°C), and supernatant was collected and kept at −20°C until further analysis. Reducing sugars, sugar composition and phenolic compounds were analyzed after the enzymatic hydrolysis.

Enzyme Assays

Enzyme activities in the presence of 1% *w/v* substrates (Sigma-Aldrich) including oat-spelt xylan, polygalacturonic acid, carboxymethylcellulose (CMC, low viscosity), 15 mmol/ l cellobiose, and 2.5 mmol/l *p*-nitrophenyl (*p*-NP) conjugated substrates were determined at 50°C in the presence of 50 mmol/l sodium acetate buffer, pH 4.8 using published methods [19, 20]. Filter paper activity (FPAase) was assayed as described by Mandels et al. [21]. Release of reducing sugars was determined according to Miller [22]. Glucose determination for the cellobiase assay used a D-glucose (GOPOD Format) assay kit from Megazyme (Megazyme International Ireland Ltd., Co. Wicklow, Ireland). One unit of cellulase (for CMC as substrate), cellobiase, xylanase and polygalacturonase activities was defined as the release of one μmol of glucose, xylose or galacturonic acid, respectively, per min. For *p*-NP conjugated substrates, one unit of activity was defined as one μmol of *p*-NP released per min.

Protein Content

The protein content of the commercial enzyme preparations and the fungal supernatant samples were determined using the Bradford assay [23].

Analysis of Soluble Carbohydrates

A 25-μl filtered liquid sample was blown to dryness by nitrogen after adding 50 μl of MeOH containing 91 μg of phenyl glucose as the internal standard. Two drops of acetonitrile were also added to dried samples and then blown to dryness again. Silylation was performed by adding 50 μl of both trimethylsilane (TMS) and N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) to dried samples by incubation at 75°C for 30 min. Arabinose, xylose, and glucose, both α and β conformations, and sucrose concentrations were determined for 1 μl aliquots of silylated sugar derivatives by gas chromatography (model 5890, Hewlett Packard Inc., Atlanta, GA) using J&W (Agilent, Wilmington, DE) DB-5 capillary column (30 M×0.25 mm I.D.). The temperature program started at 100°C, and increased to 320°C at a rate of 6°C/min. Injector temperature was 250°C and detector temperature was 350°C.

Ferulic Acid Determination

The procedure for ferulic acid determination was adapted from a chlorogenic acid quantification protocol [24]. A 100-μl sample was diluted with 100 μl dH_2O. 50 μl of MeOH containing 0.0041 mg of chrysin was added as an internal standard. Ferulic and p-coumaric acid concentrations were determined for 20 μl aliquots of the solution by reverse-phase HPLC (model 1050, Hewlett Packard) using an H_2O/MeOH linear gradient from 10% (*v/v*) to 100%

MeOH in 35 min and a flow rate of 1 ml/min. The column was a 250×4.6 mm i.d., 5 μm Ultrasphere C18 (Beckmann Instruments, Norcross, GA). The detector was a diode array system, and 340 nm was used for further analysis. Each solvent contained 0.1% (*v*/*v*) H_3PO_4. Response factors were determined with pure authentic compounds (Sigma-Aldrich). Quantification of ferulic acid was based on the internal standard (chrysin) and peak identification was based on co-chromatography (spiking) and spectral analysis.

Results and Discussion

Hydrolytic Enzyme Production by *H. Jecorina* Grown on Untreated or PBHW-pretreated Tifton 85 Bermudagrass

Tifton 85 bermudagrass has been used for fermentations using both yeast and engineered bacteria with commercially available cellulase mixtures generating the sugars that are subsequently fermented to ethanol [9, 12, 19]. This is the first report of Tifton 85 bermudagrass as a substrate for production of different enzyme activities. The goal of this study was to determine the enzyme profile of *H. jecorina* when grown either on untreated or PBHW-pretreated Tifton 85 bermudagrass. All activities were similar or higher for the fungus grown on untreated bermudagrass (Table 1). These results agree with those of Acebal et al. [25] where ammonia treated wheat was used as the substrate. Using *T. reesei* (*H. jecorina*) QM9414, growth was maximal with alkali-pretreated straw, however cellulase yields expressed as activity units were higher when the alkali pretreatment was omitted [31]. These results are in contrast to those obtained during cultivation of the *H. jecorina* on liquid hot water (LHW) treated DDGS, where the enzyme activities were equal to or better than those using untreated DDGS [19]. The results obtained for xylanase production using untreated bermudagrass as substrate are similar to those obtained for *T. reesei* (*H. jecorina*) Rut C-30 cultured in LHW-treated DDGS and corn fiber, and *T. reesei* (*H. jecorina*) QM9414 cultured in corn fiber (Table 2). On the other hand, considerably higher enzyme production results were reported for these two *Hypocrea* strains when grown in corn fiber arabinoxylan (Table 2) [26]. Efficient inducers of xylanase production in *H. jecorina* include xylan, xylan hydrolysis intermediates (xylobiose, D-xylose), lactose, L-sorbose, L-arabinose and sophorose [27, 28]. In addition, cellulose is known to induce xylanases [28, 29]. However, the mechanisms by which the hydrolytic enzymes are induced are still not well understood [18, 30].

When grown on cellulose (50 g/l), the production of α-arabinosidase activity by *H. jecorina* Rut C-30 reached its highest value of 0.009 IU/ml after 106 h [18]. Higher α-arabinosidase activity (0.56 IU/ml and 0.28 IU/ml, respectively) was produced by *H. jecorina* when grown on untreated and PBHW-pretreated bermudagrass after a longer incubation (192 h; Table 1). When cultivated in LHW-treated DDGS, even greater amounts of α-arabinosidase activity was produced (1.86 IU/ml) [19].

Maximum polygalacturonase (PG) activity produced by *H. jecorina* Rut C-30 when grown on cellulose (50 g/l) was 0.10 U/ml measured after 128 h of incubation. Total PG activity on sugar beet as substrate was 0.82 U/ml, while 0.12 U/ml was produced by another strain (QM9414) when grown on the same substrate [31]. Our results show that when cultivated in untreated bermudagrass, *H. jecorina* produces similar levels of PG activity (0.60 IU/ml; Table 1) after 8 days of incubation. Under conditions tested, no PG activity was detected when the fungus was cultivated in PBHW-pretreated bermudagrass (Table 1).

Similar results were obtained for FPAase activity whether the fungus was grown on PBHW-pretreated bermudagrass (Tables 1 and 2) or LHW-treated DDGS, although the two

Table 1 Comparison of *H. jecorina* enzyme profile after 8 days growth on untreated or 230°C PBHW-pretreated Tifton 85 bermudagrass as substrate with that of commercial preparations.

	FPAase (FPU)	CMCase (IU/ml)	β-glucosidase (ρ-NPG) (IU/ml)	Cellobiase (CBU)	Xylanase (IU/ml)	β-xylosidase (IU/ml)	α-arabinofuranosidase (IU/ml)	α-galactosidase (IU/ml)	Polygalacturonase (IU/ml)	Amylase (IU/ml)	Protein (mg/ml)
H. jecorina (untreated bermudagrass)	0.53	14.8	0.21	0.25	85.0	0.53	0.56	0.60	0.60	0.31	0.058
H. jecorina (230°C PBHW-pretreated bermudagrass)	1.16	11.5	0.12	0.17	32.2	0.70	0.28	0.15	0.19	0.00	0.051
Spezyme CP[a]	48.1	3,086.0	65.1	79.2	2,411.1	4.30	Not determined	1.03	5.90	31.8	37.5
Depol 740L[b]	0.50	876.5	32.6	26.8	2,100.0	2.86	Not determined	0.20	1.12	1.63	8.60

[a] Commercial enzyme preparation (Danisco, Genencor Division, Rochester, NY)

[b] Commercial enzyme preparation (Biocatalysts Ltd., Cardiff, Wales, UK)

Table 2 The effect of various carbon sources on production of xylan-degrading enzymes by different *H. jecorina* (*T. reesei*) strains.

Organism	Substrate	Cultivation conditions	Substrate for determination of enzyme activity	Xylanase (IU/ml)	Reference
H. jecorina Rut C-30	Untreated Tifton 85 bermudagrass	Shake flask 28°C, 192 h	1% oat spelt xylan	85.0	This work
H. jecorina Rut C-30	PBHW-pretreated Tifton 85 bermudagrass	Shake flask 28°C, 192 h	1% oat spelt xylan	32.0	This work
H. jecorina Rut C-30	LHW- DDGS	Shake flask 28°C, 132 h	1% oat spelt xylan	93.4	18
H. jecorina QM 9414	corn fiber	Shake flask 28°C, 192 h	1% oat spelt xylan	98.5	24
H. jecorina Rut C-30	corn fiber	Shake flask 28°C, 192 h	1% oat spelt xylan	86.1	24
H. jecorina QM 9414	Corn fiber Xylan	Shake flask 28°C, 192 h	1% oat spelt xylan	221	24
H. jecorina Rut C-30	Corn fiber Xylan	Shake flask 28°C, 192 h	1% oat spelt xylan	621	24

substrates are very different. The tendency to produce higher FPAase activity when grown in PBHW- or LHW-treated substrate was observed in both cases (Fig. 1a; Table 1). On the other hand, LHW-treated DDGS was a better substrate for the production of β-glucosidase and β-xylosidase [19]. Xylanase activity was higher in the fungal culture grown on untreated bermudagrass than when the fungus was cultivated on PBHW-pretreated bermudagrass (Fig. 1b; Table 1). Starch is not present in grass [32], which could explain the very low levels of amylase activity found (Table 1). The same pattern of reducing sugars release was observed for the fungus grown in both conditions tested (Fig. 2). There was not a significant difference in terms of protein production after 8 days growth on either of the substrates evaluated (Table 1).

PBHW-pretreated Tifton 85 Bermudagrass Digestion Assay: Production of Soluble Carbohydrates and Inhibition by Phenolic Compounds

After we determined the enzyme profile of *H. jecorina* grown on either untreated or PBHW-pretreated Tifton 85 bermudagrass, we compared our enzyme preparations to the commercial cellulase preparation, Spezyme CP (Genencor International, Rochester, NY). *H. jecorina* is the source for this commercial preparation; however, Spezyme CP was not made specifically for the degradation of grass. Kabel et al. [33] have pointed out that the choice of an efficient enzyme preparation is dependent rather on substrate characteristics than on standard enzyme activities measured. Therefore, we compared the commercial preparation results with the enzyme mixture produced by growing the fungus in untreated or PBHW-pretreated bermudagrass. These enzyme preparations were evaluated individually or in combination with a commercial feruloyl esterase (Depol 740L). Studies were conducted by standardizing xylanase activity for Spezyme CP and the *H. jecorina* preparations for 400 IU/g grass. Cellulase activity was standardized for 8 FPU/g grass.

The amount of reducing sugars released by the enzyme mixture from *H. jecorina* (after 8 days incubation with untreated bermudagrass) individually or in combination with feruloyl esterase was 72.1 and 84.8%, respectively, of the Spezyme CP preparation

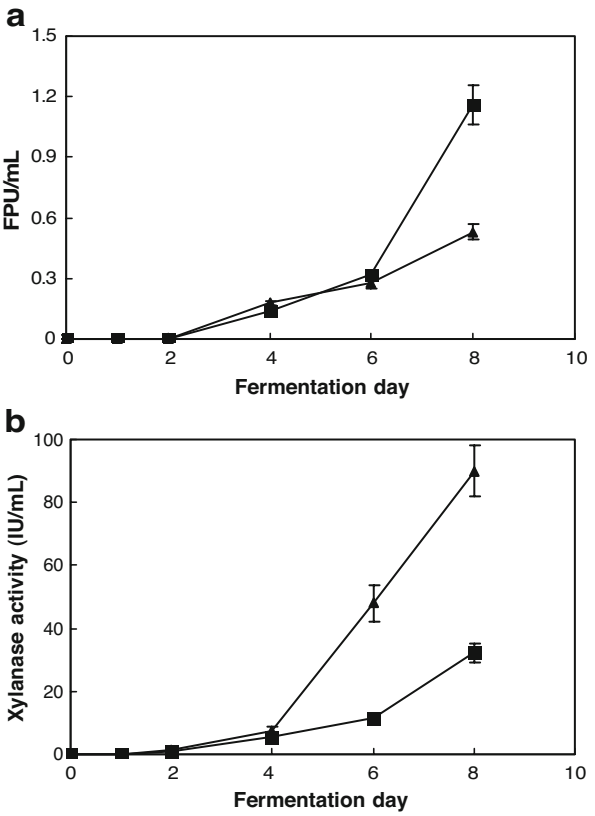

Fig. 1 Cellulase (Filter Paper Unit=FPU) (**a**) and Xylanase (**b**) and activities produced by *H. jecorina* after growth on 230°C PBHW-pretreated (*filled square*) and untreated (*filled triangle*) Tifton 85 bermudagras. Standard deviations are presented by error bars

analyzed under the same conditions (Table 3). It is important to note that although Depol 740L is mainly a feruloyl esterase commercial preparation there are other enzyme activities present, most notably xylanase activity. The addition of Depol 740L to Spezyme CP and to the *H. jecorina* enzyme preparation increased reducing sugars by 11.4 and 24.2%, respectively (Table 3). It is unclear if the increase in reducing sugars liberated was due to synergism between enzymes from our *H. jecorina* preparation with the feruloyl esterase or the action of xylanases and/or other enzymes present in this commercial preparation. The

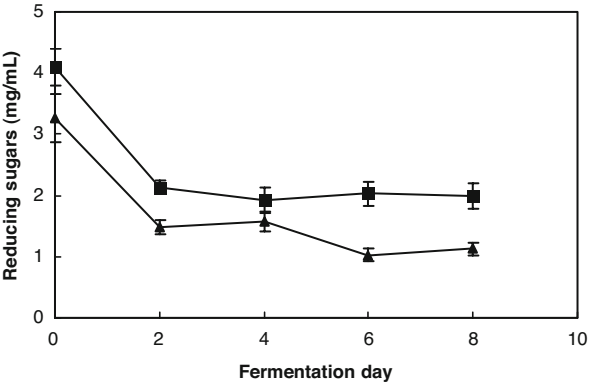

Fig. 2 Reducing sugars released from grass by *H. jecorina* after grown on 230°C PBHW-pretreated (*filled square*) or untreated (*filled triangle*)Tifton 85 bermudagrass. Standard deviations are presented by error bars

Table 3 Reducing sugar and monomeric sugar composition for *H. jecorina* enzyme preparations and commercial enzymes for the hydrolysis of 230°C PBHW-pretreated bermudagrass[a].

	Reducing sugars (mg/ml)	Reducing sugars (mg/g grass)	Glucose	Xylose	Arabinose	Ferulic acid (mg/g grass)
Spezyme CP	19.5	391.2	221.4	72.0	0.78	0.25
H. jecorina preparation[b]	14.1	282	132.8	64.0	0.77	0.26
Depol 740L (Feruloyl esterase)	10.9	218	94.8	63.3	7.48	0.88
Spezyme+ Depol 740L	22.0	440	123.3	85.9	3.73	0.36
H. Jecorina +Depol 740L	18.6	373	92.6	78.4	3.7	0.54

[a] Xylanase activity standardized for 400 IU/g grass for Spezyme CP and *H. jecorina* enzyme preparations; Feruloyl activity used: 7.8 IU/g grass; Cellobiase activity used: 78.2 CBU/ g grass

[b] Using a preparation taken from the fungus grown on untreated bermudagrass after 8 days of cultivation

dinitrosalycilic acid (DNS) colorimetric assay has been commonly used for cellulose/cellulase studies for the quantification of the number of reducing sugars associated with the soluble face, such as glucose, cellobiose, as well as low-DP cellooligosaccharides, although the DNS assay tends to overestimate absolute numbers of reducing ends per unit mass cellulose [34].

When cellulase activity was standardized for 8 FPU/g d wt grass for both cellulase preparations, there was no significant difference in reducing sugars released (Table 4). For the 230°C PBHW-pretreated bermudagrass with 7.8 IU Depol 740L/g d wt grass added, doubling the cellulase did not increase reducing sugar (data not shown). There was not a significant increase in reducing sugar when commercial feruloyl esterase was added to the *H. jecorina* preparation as observed in Table 3 (with higher xylanase units of activity). Overall, the fungus grown on the untreated grass gave a better balanced enzyme mixture for the degradation of the PBHW-pretreated substrate. Vanillin and ferulic acid levels were higher in the PBHW-pretreated grass samples and increased during incubation with *H. jecorina* enzymes (data not shown). The phenolic compounds appear to be more easily

Table 4 Comparison of *H. jecorina* enzyme preparations to commercial enzymes for the hydrolysis of 230°C PBHW-pretreated bermudagrass[a].

	Reducing sugars (mg/ml)	Reducing sugars (mg/g grass)	Glucose	Xylose	Arabinose	Ferulic acid (mg/g grass)
Spezyme CP	16.05	321	218.1	60.5	0.45	0.17
H. jecorina preparation[b]	15.8	316	180.9	51.2	1.27	0.21
Spezyme CP+Depol 740L	19.5	390	122.8	71.2	8.04	0.36
H. jecorina+Depol 740L[b]	16.9	338	115.9	89.2	1.85	0.39
H. jecorina preparation+Cellobiase[b]	17.6	352	230.1	47.9	0.13	0.27
Spezyme CP+Cellobiase	18.2	364	306.8	53.0	0.15	0.12
Spezyme CP+Depol 740L+ Cellobiase	23.0	460	230.9	54.5	1.04	0.42

[a] Cellulase (FPAase) activity standardized for 8 FPU/g grass for Spezyme CP and *H. jecorina* enzyme preparations; Feruloyl activity used: 7.8 IU/g grass; Cellobiase activity used: 78.2 CBU/ g grass

[b] Using a preparation taken from the fungus grown on untreated bermudagrass after 8 days of cultivation

liberated from the PBHW-pretreated grass and may be inhibiting certain enzyme components, although further studies are needed to confirm this hypothesis.

Phenolic compounds, freed from lignin, are known to inhibit extracellular cellulases and hemicellulases produced by fungi, although cellulase preparations can differ significantly in their sensitivity to lignin [35–37]. The mechanism of enzyme inhibition may involve both the adsorption of enzyme to the major particulate lignin component and the interactions with the minor low-molecular lignin component [5]. Vohra et al. [35] reported that Avicelase, CMCase and β-glucosidase activities were reduced when *H. jecorina* was grown in Avicel in the presence of different concentrations of lignin, vanillin and ferulic acid. In the case of ferulic acid, the β-glucosidase activity was the most affected, although the ferulic acid had little effect on the growth of the fungus itself. DNS reducing sugar estimation was not affected by the phenolic concentration used in their investigations. We also observed this effect in the *H. jecorina* cellulase activity, when the fungus was grown on untreated bermudagrass for 8 days in the presence of different ferulic acid concentrations. All the activities tested (FPAase, CMCase and β-glucosidase) were drastically reduced in the presence of 0.02% (*w/v*) ferulic acid (Fig. 3). In addition, when our *H. jecorina* cellulase preparations and the commercial feruloyl esterase were combined, the sugar compositional analysis showed that glucose production from PBHW-pretreated bermudagrass decreased in comparison to the amount liberated by each enzyme mixture alone (Tables 3 and 4). Even when a third enzyme, a commercial β-glucosidase to supplement the insufficient cellobiase activity from *H. jecorina* preparations, was added to the Spezyme CE and feruloyl esterase treatment, the inhibitory effect was also observed (Table 4). For both cellulolytic enzyme preparations acting only in the presence of the feruloyl esterase compared to their action individually, the amount of xylose and arabinose released was increased. It is unclear if this increase is related to some synergism between the two enzyme preparations or due to the hemicellulolytic activity present in the Depol 740L preparation.

Considering that the microorganism source for the Spezyme CP enzyme preparation is also *H. jecorina*, our results using a different approach confirm previous work showing that low molecular weight phenolic compounds are inhibitory to the action of cellulases from *H. jecorina.* Vohra et al. [35] demonstrate that at least some of *H. jecorina* cellulases are inhibited in the presence of different concentrations of ferulic acid. *H. jecorina* itself does not produce feruloyl esterase activity [12], so it may not be surprising that the cellulases do not seem to act in synergism with feruloyl esterases and are inhibited by the esterase

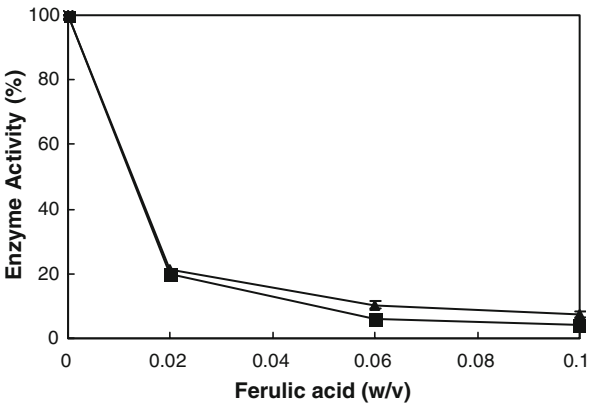

Fig. 3 Effect of ferulic acid on the cellulase activities of *H. jecorina* after grown on untreated bermudagrass. The activities tested were: FPAase (*filled triangle*) and β-glucosidase (*filled square*). The CMCase activity was reduced to almost 0% in the presence of 0.02% (*w/v*) ferulic acid. Standard deviations are presented by error bars

products. Berlin et al. [20, 38] have discussed the engineering or selection of enzymes with reduced affinity for lignin as a potential strategy to improve enzymes for hydrolysis of lignocellulosic substrates. Perhaps this approach would be helpful when cellulase activity is needed in the presence of substantial amounts of phenolic compounds. *H. jecorina* has been successfully engineered for the over-expression of homologous and heterologous genes coding for different hydrolytic enzymes [17, 39], and future work will explore the engineering of *H. jecorina* enzymes for resistance to phenolic compounds.

Ferulic acid, ρ-coumaric acid, and vanillin are low-molecular-weight phenolic compounds frequently isolated from forages [37]. Ferulic acid is a precursor to natural vanillin, and may be used as a UV absorber and antioxidant in skin care formulations. Phenolic compounds, especially ρ-coumaric acid, act as an inhibitor of microbial growth and enzyme activities and may have potential value as natural compounds for pest control [9]. Ferulic acid, in particular, seems to have an inhibitory effect on the cellulase action from the *H. jecorina* and Spezyme CP preparations. Interestingly, when feruloyl esterase was used in combination with Spezyme CP and the *H. jecorina* preparations, less ferulic acid was released from the PBHW-pretreated bermudagrass, compared to the amount of ferulic acid liberated by Depol 740L alone (Table 3). In developing a methodology for using bermudagrass for ethanol and co-products production, incubating with the feruloyl esterase, removing the phenolics, then treating with cellulases has worked well [6], although this maybe costly. Alternatively, *H. jecorina* may be engineered for the expression of cellulases less susceptible to inhibition by phenolic compounds, or other sources of non-phenolic acid inhibited cellulolytic enzymes may be used.

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