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Enzymatic degradation of Elephant grass (*Pennisetum purpureum*) stems: Influence of the pith and bark in the total hydrolysis

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HIGHLIGHTS

- The inner pith of Elephant grass stalks is more easily degraded than the outer cortex.
- Esterase supplementation increased deacetylation by reduced biomass solubilisation.
- Enzymatic deacetylation of Elephant grass was improved by the addition of DMSO.
- Low concentrations of DMSO can improve enzymatic removal of xylan and glucan.
- Acetyl esterases removed acetic acid from lignin-enriched materials.

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ABSTRACT

The internal pith of a high energy plant, Elephant grass (EG), was more extensively degraded (>50% dry matter) compared to the outer cortex (31%) or the whole stem (35%) by an enzyme preparation from *Humicola insolens*, Ultraflo. Reducing sugars and acetic acid release from the pith was also higher compared to the cortex. Supplementation of Ultraflo with a type-C feruloyl esterase increased the level of deacetylation but also led to reduced solubilisation. The addition of 20% dimethyl sulfoxide (DMSO) as a co-solvent also reduced the solubility of EG by Ultraflo, although acetic acid release was increased, complimenting previous results found on model substrates. The presence of DMSO was also shown to have a protective effect on xylanase activity but not acetyl esterase activity in Ultraflo. Xylan in the biomass was preferentially solubilised by DMSO, while Ultraflo removed more glucose than xylose.

1. Introduction

In addressing the demand for sustainable alternatives to fossil fuel as a source of energy and consumer products, low cost and renewable lignocellulosic residues from municipal wastes, forest residues, food side-streams and energy crops have been the recent focus of intensive research. Elephant grass (*Pennisetum purpureum*), also called Napier grass, is native to the tropical grasslands of Africa and has been introduced to most tropical and subtropical countries. Cultivation of Elephant grass can yield stems over 3 m in height, and can provide annual dry matter production levels of 88 metric tonnes per hectares per year (Somerville et al., 2010). This grass has been used as animal fodder for many years, but the high growth potential and the solid centre of the stems, similar to maize/corn stover (Zeng et al., 2012) and sugar cane, makes Elephant grass a potential source of precursors of fine chemicals and bioenergy. Recently the composition of the lipophilic extractives (Prinsen et al., 2012) and the lignin (del Rio et al., 2012) in the cortex and pith of Elephant grass stems have been determined. The cortex represents 84% (dry weight) of the whole material and the

Abbreviations: DMSO, dimethyl sulfoxide; AnFaeA, type-A feruloyl esterase from *Aspergillus niger*; MFA, methyl ferulate; MpCA, methyl *p*-coumarate; pNP, *p*-nitrophenyl; TsFaeC, type-C feruloyl esterase from *Talaromyces stipitatus*; MWL, milled wood lignin.

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pith the remaining constituent (del Rio et al., 2012). The cortex and pith have *p*-hydroxyphenyl (H)–guaiacyl (G)–syringyl (S) lignins (S/G ratios around 1.3–1.5), with associated *p*-coumarates and ferulates. Ferulates are mostly attached to the hemicellulosic arabinose while *p*-coumarates are primarily attached to the lignin polymer (and exclusively at the γ-carbon of the side-chain), as also occurs in other grasses (del Rio et al., 2012). Plant cell walls also contain abundant acetyl groups, mainly associated with the hemicellulose components, but also found associated with the lignin (del Rio et al., 2007; Martinez et al., 2008; Kim and Ralph, 2010). Such acetylation can inhibit both the saccharification process but also the subsequent fermentation of sugars to alcohol (Selig et al., 2009).

The utilisation of renewable crops depends on the availability of the cell wall polysaccharides for extraction, their hydrolysability into simple sugars and their fermentability, especially if considering bioalcohol production. However, the plant cell wall polysaccharides are enmeshed with the aromatic polymer, lignin, which hinders an enzyme-based degradation process through the structure and interaction between the wall polymers (Kumar et al., 2012; Zhang et al., 2012), non-specific adhesion of degradative enzymes to the lignin (Rahikainen et al., 2011; Kumar et al., 2012) or inhibition of enzyme activities through the release of small phenolic compounds, such as tannic acid (Li et al., 2010). The use of plant-degrading fungi and bacteria or commercially available multi-enzyme preparations requires the production of enzymes with different modes of activity working synergistically to open up the complex heterogeneous cell wall matrix for efficient and complete saccharification.

We have previously shown that the multi-enzyme preparation from *Humicola insolens*, Ultraflo L, contains high carbohydrate-acting esterase activity, both feruloyl and acetyl esterases (Faulds et al., 2011). This enzymatic cocktail achieved selective carbohydrate solubilisation of brewers' spent grain and wheat bran, two side-streams from the agro-food industry (30% solubilisation of the initial carbohydrate within 5 h; [Robertson et al., 2010]). Enzyme activity is maintained during treatment so the apparent limit of solubilisation is due to other factors, such as steric hindrance caused by substitution patterns and polymer–polymer and polymer–enzyme interactions (Rahikainen et al., 2011; Kumar et al., 2012). In the absence of structural constraints, lignification can decrease the rate and extent of hemicellulose and cellulose solubilisation (Grabber, 2005) and ferulate-induced cross-linking of the hemicellulose, lignin and protein components may account for nearly half of the inhibitory effects of lignin on cell wall fermentation (Grabber et al., 2009).

To exploit the components of the lignocellulosic biomass, the material needs to be pretreated with a variety of mechanical, chemical and/or biochemical methods. Hemicelluloses can be removed through mild alkali treatments (Mandalari et al., 2005) or through steam-explosion (Han et al., 2010). Lignin, however, is insoluble in aqueous environment and thus requires the use of strong alkali or organic solvents at high temperature in processes such as organosolv. Organic co-solvents can expand the use of enzymes in lignocellulose deconstruction by making substrates more soluble and thus more accessible. We recently showed that 10–30% dimethyl sulfoxide (DMSO) was an adequate co-solvent for esterase treatment of water-insoluble substrates, and that the de-acetylation activities of Ultraflo and of pure feruloyl esterases were activated in the presence of the co-solvent (Faulds et al., 2011).

In this paper, we have explored the use of Ultraflo in breaking down whole Elephant grass and the separate hydrolysis of the pith and cortex components of the grass stem, in particular the overall solubilisation, the reducing sugar release and the release of acetic acid. We have also studied how the addition of a co-solvent, such as DMSO, could be used to facilitate enzyme accessibility to the lignocellulosic fibres and how enzyme stability is affected by the presence of low concentrations of such organic solvents.

2. Methods

2.1. Methods

Elephant grass (*P. purpureum* Schumacher) stems, cultivar 'Paraiso', were kindly supplied by the University of Viçosa (Brazil). The stems were air-dried and subsequently separated into the cortex and the pith (Prinsen et al., 2012). Samples of *P. purpureum* pith and cortex were milled using a knife mill (Janke & Kunkel, Analysemühle) and subsequently ball-milled in an agate container in a Retsch S100 centrifugal ball mill in order to obtain a very fine flour. The material was used "as is" without any further pre-treatment steps, apart from the preparation of "Milled-wood lignins", described in the next section. The multienzyme preparation from *H. insolens* (Ultraflo) was kindly provided by Novozymes (Bagsvaerd, Denmark). This enzyme preparation contains xylanase, endo-glucanase, protease and esterase activities (Faulds et al., 2009). The A-type feruloyl esterase from *Aspergillus niger* (AnFaeA) was heterologously expressed in *Pichia pastoris* as previously described (Juge et al., 2001). The recombinant C-type feruloyl esterase from *Talaromyces stipitatus* (TsFaeC) (Crepin et al., 2003) was a kind gift from Biocatalysts Ltd (Cefn Coed, Wales, UK).

2.2. 'Milled-wood lignin' isolation

The milled-wood lignins (MWLs) were obtained according to the classical procedure (Björkman, 1956) as already previously described (Rencoret et al., 2009; del Rio et al., 2012). Extractive-free ground cortex and pith samples (prepared by successively extracting with acetone in a Soxhlet apparatus for 8 h, and with hot water at 100 °C for 3 h) were finely ball-milled in a Retsch PM100 planetary mill (40 h at 400 rpm for 25 g of wood) using a 500 mL agate jar and agate ball bearings (20 × 20 mm), and toluene as coolant. The milled samples were submitted to an extraction (3 × 12 h) with dioxane:water (9:1, v/v) (20 mL solvent/g milled sample). The suspension was centrifuged and the supernatant evaporated at 40 °C under reduced pressure. The residue obtained (raw MWL, 1.765 g) was redissolved in acetic acid/water 9:1 (v/v) (25 mL solvent/g raw MWL). The solution was then precipitated into stirred cold water and the residue was separated by centrifugation, milled in an agate mortar and dissolved in 1,2-dicloromethane:ethanol (2:1, v/v). The mixture was then centrifuged to eliminate the insoluble material. The resulting supernatant was precipitated into cold diethyl ether, centrifuged, and subsequently resuspended in 30 mL petroleum ether and centrifuged again to obtain the purified MWL, which was dried under a current of N_2. The final yields ranged from 15% to 20% based on the Klason lignin content.

2.3. Enzyme assays and biomass hydrolysis

Enzymatic time course digestions were performed in duplicate with 50 mg milled substrate or 50 mg MWL per mL enzyme preparation at 37 °C under agitation. Ultraflo was initially desalted through a PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) into 100 mM MOPS, pH 6, containing 0.05% sodium azide, prior to use. For the supplementation assays, additional feruloyl esterase activity was added as pure enzymes as described in the results section to the above Ultraflo preparation, and the reactions performed as described above. For the hydrolysis reactions performed in the presence of DMSO, MOPS buffer containing 20%

DMSO (v/v) was added to the dry substrate, vortexed to mix the material, then enzymes were added as described above for the non-DMSO reactions. Reactions were performed as described above. All reactions were terminated by centrifugation and immediate separation of residual biomass from supernatant. Feruloyl esterase activities were assayed using methyl ferulate (MFA; 60 μM) at 37 °C in 100 mM MOPS (pH 6.0) and the release of the corresponding free acid measured spectrophotometrically at 335 nm (Ralet et al., 1994). Acetyl esterase activities were assayed using *p*-nitrophenyl acetate (*p*NPA; 1 mM) and performed at 37 °C in 100 mM MOPS (pH 6.0) with the release of *p*-nitrophenol continuously monitored at 410 nm. Xylanase activity was determined with 1% (w/v) wheat arabinoxylan in 100 mM MOPS (pH 6.0), as previously described (Bailey et al., 1992). All assays were analysed in duplicate. For all enzymes investigated in this study, one unit of activity (1U) is defined as the amount of enzyme forming 1 μmol of product per min at pH 6.0 and 37 °C.

2.4. Analysis methods

Residual biomass was determined by drying the residues obtained after hydrolysis at 65 °C for 24 h. Reducing sugars in the supernatant were determined using the adapted DNS method of Miller (1959) and quantified against a xylose standard curve. The released acetic acid was determined using the Acetic Acid (Acetate Kinase Manual Format) kit (Megazyme Ltd, Brey, Ireland). The amount of acetic acid released was calculated as per the manufacturer's instructions. In order to determine the degree of acetylation of the initial biomass, 400 mg of material was treated with 2 M NaOH, covered in aluminium foil and left for 16 h at room temperature with constant agitation. Samples were then acidified with 1 M HCl, centrifuged at 3000*g* in a benchtop centrifuge (10 min) and the amount of acetic acid determined from the resultant supernatants. Klason lignin content was estimated as the residue after sulphuric acid hydrolysis of the pre-extracted material according to Tappi test method T222 om-88 (Tappi, 2004). Glucose and xylose were measured in the same hydrolysates after derivatization to their corresponding alditol acetates (Laine et al., 1972) and analysed by gas chromatography as previously reported (Bernabé et al., 2011). All experiments were performed in triplicate. The acid-soluble lignin was determined in the filtrates, after the insoluble lignin was filtered off, spectrophotometrically at 205 nm wavelength using 110 L cm^{-1} g^{-1} as the extinction coefficient as recommended in Tappi UM 250 (Tappi, 2004).

3. Results and discussion

3.1. Influence of incubation time and Ultraflo dosage on biomass solubilisation

Ball-milled Elephant grass was incubated over a 96 h period at 37 °C with Ultraflo (100 μL enzyme solution after PD-10 desalting, which corresponds to 908 xylanase-equivalent units or 0.56 acetyl esterase-equivalent units/g biomass) and samples analysed every 24 h for biomass solubilisation, reducing sugar release and acetic acid release (Fig. 1). The amount of alkali-extractable acetic acid in the material was determined to be: 143.3 mg/g (±0.24) whole Elephant grass (dry weight) (±5.7), 126.8 mg/g in the cortex and 121.3 mg/g (±0.36) in the pith. There was a small amount of easily aqueous extractable material in the cortex (8.8%) and much more in the pith (10.4%) (del Rio et al., 2012) hence the values for the 0 h solubilisation in Fig. 1A. The solubilisation of the whole material and the pith increased over the 96 h period, reaching 36% and 55% (w/w) solubilisation, respectively. In comparison to the pith, the cortex was poorly solubilised by Ultraflo, reaching 32.5% biomass reduction after 72 h. This lower degree of solubilisation was also reflected in the release of reducing groups or acetic acid from the cortex compared to the pith. 80% of the acetate bound to the pith was released by the esterases present in Ultraflo and a maximum of 56% from the cortex and 47% from the whole material. While we cannot yet say if the acetylated lignin in the cortex (39% of lignin units are γ-acylated, 21.8 μmol acetyl groups/g lignin) and pith (55% lignin units are γ-acylated, 10.1 μmol acetyl groups/g lignin) (del Rio et al., 2012) was a substrate for the esterases in Ultraflo, the high de-acetylation of the pith suggests that the enzymes are able to access some of the acetate groups in the lignin components of the inner stem as well as the acetylated hemicellulose, while the outer cortex and intact stem material are more inaccessible.

The cortex of Elephant grass is a relatively poor substrate for Ultraflo compared to the pith and the whole stem material, even after 96 h incubation. With increasing dosage of Ultraflo, solubilisation of the pith begins to level off with the equivalent of 908 U xylanase/g biomass, resulting in 54% solubilisation of the pith compared to 28% of the cortex and 32% of the whole material with the same dosage (Fig. 2A). The location of the material in the grass stem does not influence the release of reducing sugars with similar amounts being released from both pith and cortex at the end of the incubation period, although only 65% of the pith value was obtained with the milled whole stem (Fig. 2B). A significantly better glucose and xylose conversion was also measured after cellulase treatments of the pith compared to the cortex and leaves of corn stover (Zeng et al., 2012), although this material has also been initially pre-treated with liquid hot-water. Acetic acid release also reached a plateau with a dosage of Ultraflo of 0.56 acetyl esterase-equivalent units/g biomass, representing 52% of the alkali-extractable acetic acid in the whole stem, 69% of the acetic acid from the cortex and 81% of the acetic acid in the pith (Fig. 2C). From these results, it appears that acetylation of the Elephant grass polymers is not an insurmountable barrier to saccharification and the acetate is readily removed from the hemicellulose (and possibly lignin). However, in the intact stem, the degradation is more restricted as the presence of the pith would form a possible barrier to enzyme accessibility to the inner components of the cortex, even after plant tissue disruption through the mechanical milling pre-treatment step. One can suppose that the enzymatic degradation of the cortex layer proceeds from the innermost layer of this tissue type as the outermost layer is the cutin-coated epidermis and hence more recalcitrant to enzyme hydrolysis, and the extensive degradation of these cortex cells progresses until the lignified vascular bundles are reached. The presence of the pith and recalcitrant epidermis thus limits overall degradation of whole Elephant grass, even after particle size reduction. Zeng and co-workers suggested a physical fractionation of C4 grasses into soft and hard tissue types in order to reduce costs associated with enzyme hydrolysis, with the soft tissue being directed to saccharification and fermentation processes while the poorly digested material is used for other processes (Zeng et al., 2012). A similar fractionation process could also be applicable for Elephant grass.

3.2. Supplementation of Ultraflo with feruloyl esterases

Phenolic and acetic acid substitutions on the hemicellulose backbone are believed to impede biomass breakdown by glycoside hydrolases (Akin and Chesson, 1989), therefore the presence of carbohydrate-acting esterases in an enzyme cocktail should overcome this problem. Ultraflo itself has type-B feruloyl esterase and acetyl esterase activity (Faulds et al., 2002, 2011), while fungal feruloyl esterases display activity against model acetylated substrates, such

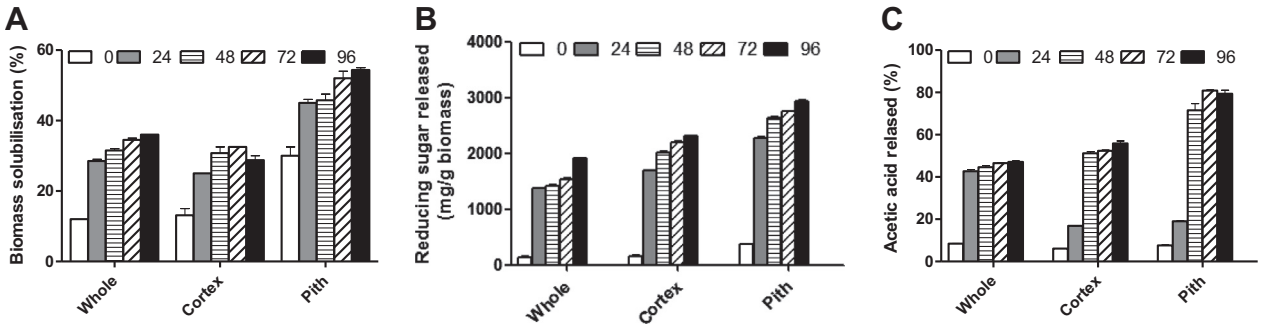

Fig. 1. The effect of Ultraflo on the (A) solubilisation, (B) release of reducing sugars, and (C) release of acetic acid from Elephant grass cortex and pith over a 96 h incubation at 37 °C.

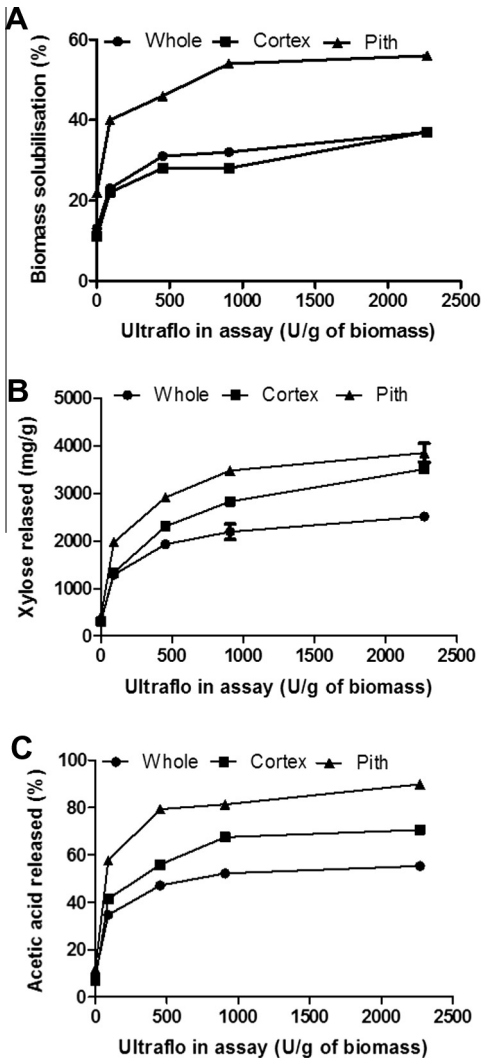

Fig. 2. The effect of Ultraflo dosage on the (A) solubilisation; (B) release of reducing sugar (expressed as xylose-equivalents); and (C) the release of acetate from whole Elephant grass (●), cortex (■) and pith (▲) after 96 h incubation at 37 °C.

as *p*-nitrophenyl acetate. Feruloyl esterases have been classed according to their sequence and biochemical activities (Crepin et al., 2004) and 12 families have been proposed based on esterase genes in *Aspergillus oryzae* and their activities on methyl hydroxycinnamates (Udatha et al., 2012). To Ultraflo (450 U xylanase-equivalent activity), was added the type-A feruloyl esterase, AnFaeA (FEF 12A of Udatha classification), or a type-C esterase, TsFaeC (FEF 4B). The addition of TsFaeC (0.1 U activity against *p*-nitrophenyl acetate) in the Ultraflo hydrolysis reaction was the only supplementation to result in an increase in the solubilisation of ball-milled whole stalk Elephant grass compared to Ultraflo (Fig. 3), although as expected, more acetic acid was released in the reactions supplemented with the esterases. However, when a higher concentration of TsFaeC was added ($\geqslant$0.5U), biomass solubilisation decreased. This suggests that the removal of acetic acid, and more probably ferulic acid, resulted in a more recalcitrant substrate for the hydrolases in Ultraflo. Increased arabinose and xylose release has also been noted when the feruloyl esterase-containing multienzyme cocktail Depol 740 was added to a triblend of pectinase, cellulase and β-glucosidase for the saccharification of AFEX and liquid hot-water pretreated dried distillers' grains with solubles (DDGS), with maximum effect recorded with the addition of 1–2U feruloyl esterase-equivalent activity, depending on the substrate (Dien et al., 2008). It is difficult to judge the individual effect of the feruloyl esterases in this paper, as Depol 740 also contains high levels of xylanase, β-glucanase and endo-glucanase activity (Faulds et al., 2009). The removal of acetyl- and feruloyl-groups will certainly enhance further carbohydrate release by arabinofuranosidases and xylanases, but the small percentages of these compounds in lignocellulosic biomass will not contribute to extensive improvement in solubilisation/saccharification is the polymeric interactions are not sufficiently disentangled.

3.3. Effect of DMSO on biomass hydrolysis by Ultraflo

Organic co-solvents can expand the use of enzymes in lignocellulose deconstruction through making substrates more soluble and thus more accessible (Quesada-Medina et al., 2010). In choosing the most adequate co-solvent for feruloyl esterases, the hydrolysis

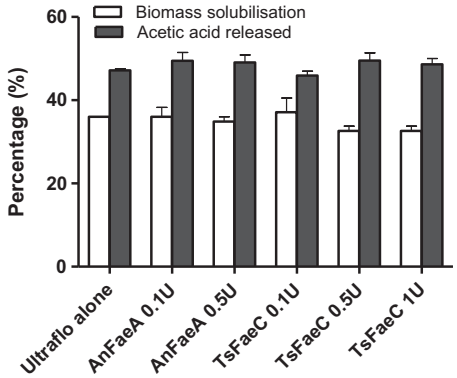

Fig. 3. Effect of feruloyl esterase supplementation to 450 U Ultraflo/g biomass on the solubilisation (white bar) and release of acetic acid (black bar) from whole Elephant grass after 96 h incubation at 37 °C.

of methyl *p*-hydroxycinnamates by Ultraflo and three pure enzymes was previously evaluated, and low concentrations of DMSO were found to enhance hydrolysis while at levels >20% DMSO, activity was reduced (Faulds et al., 2011). DMSO also enhanced acetyl esterase-type activity in these enzymes. To understand if this solvent effect is transferable to biomass degradation, comparative analysis regarding the levels of Klason lignin, acid-soluble lignin, and residual sugars was performed on the residues recovered after Ultraflo treatment on whole EG, pith or cortex in the absence and presence of 20% DMSO in the reaction buffer (Table 1), as well as determining the solubilisation, reducing sugar release and acetic acid release (Fig. 4). The presence of DMSO resulted in a decreased solubilisation in all cases. In fact, in the no-enzyme control, no actual change in the weight of the recovered whole stem Elephant grass occurred. An increase in solubilisation was observed only in the presence of TsFaeC ± DMSO. This decrease in solubilisation correlated to a decrease in the amount of reducing groups generated by the action of the enzymes (Fig. 4B). Only in the case of acetic acid release an increase in the presence of DMSO was measured in all reactions apart from the buffer control, indicating that the presence of 20% DMSO was activating the de-acetylating activity in a similar way to that reported previously on the model acetyl esterase substrates. It is also possible that ferulic acid and similar phenolic acid-derivatives are released by the action of the esterases. In theory, this would lead to an increase in hydrolysis by the main-chain and side-chain-acting hydrolases, and thus increased solubilisation and reducing sugar release. This was not observed in this study. Increased activity against methyl ferulate and methyl p-coumarate in the presence of DMSO was only observed previously for Ultraflo, not for the phenolic-acting activity of AnFaeA and TsFaeC esterases used in the supplementation studies (Faulds et al., 2011). This suggests that DMSO influences the accessibility of acetyl groups on the xylan backbone and lignin, but not necessarily improves deferuloyation.

When the residual enzyme activity in the reaction supernatants was measured, the presence of 20% DMSO protected the xylanase and esterase activity within the Ultraflo cocktail, especially with xylanase, where 78% of the initial activity was still present, in comparison to only 31% remaining in the absence of DMSO. 15% of the acetyl esterase activity remained in the supernatant without the addition of DMSO, compared to 23% when the co-solvent was added. The pure feruloyl esterases, AnFaeA and TsFaeC, do not appear to be as stable as the Ultraflo esterases in the presence of DMSO, as the recovered values were much lower when DMSO was present in the reaction medium. TsFaeC appears to be slightly more stable than AnFaeA.

The addition of DMSO to the hydrolysis reaction appeared to aid the solubilisation of the carbohydrate in preference to the lignin in the whole stalk material as illustrated with the same Klason lignin content with and without DMSO in all samples together with the reduction of glucose in the presence of the co-solvent. However the effect on the removal of glucose corresponded to the reduced solubility determined in the presence of DMSO (Fig. 4A), suggesting that the co-solvent is perhaps restricting more the removal of extractives during the hydrolysis treatment. This aspect and the nature of extractives being affected by the presence of organic co-solvents requires further studies. It is also interesting to point out that Ultraflo is removing material which normally is incorporated in the Klason lignin. It is probably unlikely that lignin is being broken down by this preparation and suggests that the cocktail acts on proteinaceous or lipid-type compounds which are entrapped within the matrix and hence not so easily extractable in an aqueous environment without the aid of enzymes.

Table 1
Solubilisation (%), lignin and sugar content (% of residual material) after treatment of Elephant grass (whole stem, pith and cortex) with Ultraflo in the absence or presence of 20% (v/v) DMSO at 37 °C and 72 h incubation, in comparison with treatment in a buffer.

Treatment	Solubilisation (%)	Lignin (%)	Acid-soluble lignin (%)	Glucose (%)	Xylose (%)	Arabinose (%)
Buffer (whole)	19	65	1.2	18.2	5.5	1.4
Buffer (whole) + DMSO	5	64	4	22.1	4.3	1.3
Ultraflo (whole)	38	70	3.9	7.3	16.0	2.5
Ultraflo (whole) + DMSO	27	63	3.9	4.7	8.0	1.5
Buffer (pith)	27	63	2.7	18.5	4.0	1.3
Buffer (pith) + DMSO	16	57	3.5	14.2	3.3	1.0
Ultraflo (pith)	51	65	3.4	8.1	10.5	2.4
Ultraflo (pith) + DMSO	43	60	3.9	7.3	9.5	2.6
Buffer (cortex)	11	68	2.8	19.0	6.0	1.4
Buffer (cortex) + DMSO	6	64	3.4	26.7	5.0	1.5
Ultraflo (cortex)	37	69	3.6	5.9	11.9	1.4
Ultraflo (cortex) + DMSO	29	66	3.8	1.2	3.0	0.6

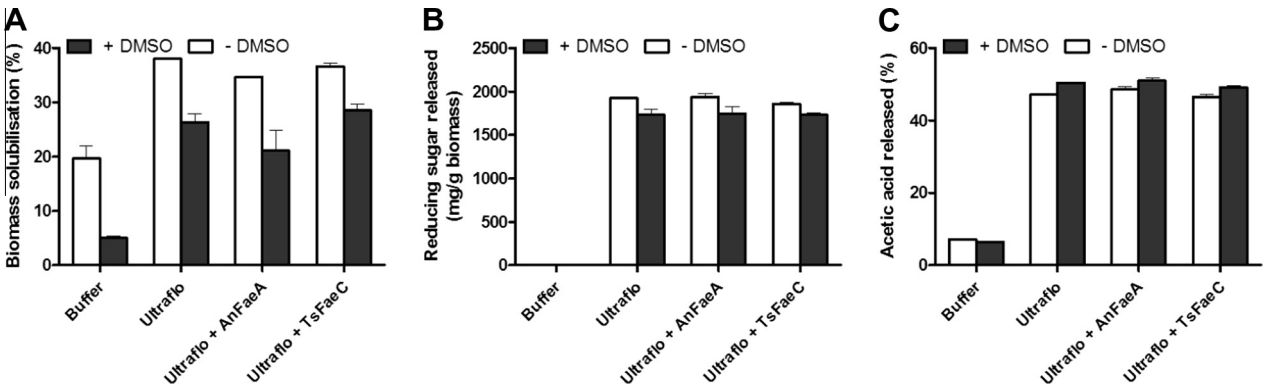

Fig. 4. The influence of DMSO on (A) solubilisation, (B) production of reducing groups and (C) acetic acid released from whole Elephant grass by Ultraflo with or without supplementation with feruloyl esterases, hydrolysis performed in the absence of DMSO (white bar) or in the presence of DMSO (black bar).

DMSO appears to be removing xylose and glucose from the walls of EG even in the absence of Ultraflo, while Klason lignin levels remain similar (90–95% similar ±DMSO), and the material recovered as acid-soluble lignin increases in the presence of DMSO (Table 1). This high recovery of acid-soluble lignin may reflect a precipitation of the extractives in the EG preparations. More glucose remains in the material after DMSO treatment compared to xylose although residual arabinose levels do not follow xylose levels. The co-solvent appears very efficient in aiding the enzymatic removal of sugars from the cortex-derived material, suggesting that the DMSO is indeed either solubilising the polysaccharides or at least swelling the matrix due to H-bond rupturing, allowing better enzyme accessibility to their substrates in this cortex matrix, while enzymatic sugar removal is more reduced in the pith. The higher extractives concentration in the pith (Prinsen et al., 2012) may cause the reduced sugar solubilisation due to their interaction with the DMSO, and hence cause the reduced solubilisation of the whole stem material as shown in Fig. 4A.

3.3.1. Can feruloyl esterases deacetylate isolated lignins?

The amount of ester-linked acetate in the Millable-wood lignins (MWL) of Elephant grass bark and pith was determined to be 44.0 mg/g dry matter (±2.9) in the cortex and 77.5 mg/g (±0.8) in the pith. Ultraflo, TsFaeC and AnFaeA were all able to release acetic acid from the pith MWL, with 30.5% of the total acetate removed by Ultraflo. TsFaeC (19%) was the better of the two feruloyl esterases in releasing acetic acid from this material, with AnFaeA only releasing 6% of the available acetic acid. The amount of acetic acid released from cortex MWL was in comparison lower. Ultraflo and TsFaeC released similar levels of acetic acid, 11% and 13.5%, respectively, although no significant hydrolysis was detected in the presence of AnFaeA (1.5%). It cannot be ascertained in this study if the acetate is being removed only from the small amount of hemicellulose remaining in the MWL sample (estimated to be approx. 10% w/w) or also from the lignin component comprising the majority of the MWL of Elephant grass pith and cortex. However, the high level obtained with Ultraflo on the pith suggests that the esterases present in this *H. insolens* cocktail as well as TsFaeC are capable of deacetylating lignin.

4. Conclusions

The inner pith of Elephant grass was more degradable by Ultraflo than either the whole stalk or the outer cortex. High acetic acid removal (80%) from the pith indicates that this substitution was not a barrier to enzyme hydrolysis. Supplementation of Ultraflo with an esterase (TsFaeC) led to higher acetic acid release but higher dosage led to decreased solubilisation, possibly due to increased polymeric interactions. TsFaeC was also able to remove acetyl groups from isolated lignin. Addition of an organic co-solvent decreased biomass solubilisation and reducing sugar release but activated the esterases in Ultraflo to release more acetic acid.

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