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The inhibitory effects of various substrate pre-treatment by-products and wash liquors on mannanolytic enzymes

Samkelo Malgas[a], J. Susan van Dyk[b], Sagaran Abboo[a], Brett I. Pletschke[a,*]

[a] Department of Biochemistry and Microbiology, Rhodes University, Grahamstown 6140, Eastern Cape, South Africa
[b] Forest Products Biotechnology Group, University of British Columbia, 2424 Main Mall, Vancouver, British Columbia V6T1Z4, Canada

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ABSTRACT

Biomass pre-treatment is essential for achieving high levels of bioconversion through increased accessibility of hydrolytic enzymes to hydrolysable carbohydrates. However, pre-treatment by-products, such as sugar and lignin degradation products, can negatively affect the performance of hydrolytic (mannanolytic) enzymes. In this study, two monomeric sugars, five sugar degradation products, five lignin derivatives and four liquors from biomass feedstocks pre-treated by different technologies, were evaluated for their inhibitory effects on mannanolytic enzymes (α-galactosidases, β-mannanases and β-mannosidases). Lignin derivatives elicited the greatest inhibitory effect on the mannanolytic enzymes, followed by organic acids and furan derivatives derived from sugar degradation. Lignin derivative inhibition appeared to be as a result of protein–phenolic complexation, leading to protein precipitating out of solution. The functional groups on the phenolic lignin derivatives appeared to be directly related to the ability of the phenolic to interfere with enzyme activity, with the phenolic containing the highest hydroxyl group content exhibiting the greatest inhibition. It was also demonstrated that various pre-treatment technologies render different pre-treatment soluble by-products which interact in various ways with the mannanolytic enzymes. The different types of biomass (i.e. different plant species) were also shown to release different by-products that interacted with the mannanolytic enzymes in a diverse manner even when the biomass was pre-treated using the same technology. Enzyme inhibition by pre-treatment by-products can be alleviated through the removal of these compounds prior to enzymatic hydrolysis to maximize enzyme activity.

1. Introduction

The use of lignocellulosic biomass as a low-cost feedstock for the production of renewable energy and value-added products (biochemicals) has gained a lot of interest. Lignocellulosic biomass is composed of polysaccharides such as cellulose, hemicellulose and pectin (which constitute approximately 75% of the dry mass of the biomass) and lignin [1]. Due to its high polysaccharide content, lignocellulosic biomass has been suggested as a model feedstock for cellulosic ethanol production [2,3]. However, lignocellulose has a very complex structure and is therefore very recalcitrant to enzymatic hydrolysis [4,5]. A biomass pre-treatment step is therefore necessary to increase the accessibility of the enzymes to the hydrolysable carbohydrates [2,5].

A number of pre-treatment approaches have been developed empirically with the combined aim of reducing particle size and changing the physical and chemical characteristics of lignocellulosic biomass, so that enzymatic hydrolyzability can be improved. However, most of these processes result in the generation of compounds that hamper enzymatic hydrolysis and microbial fermentation [6–8]. In addition, plants are reported to naturally release phenolic compounds that inhibit enzyme hydrolysis as a defence mechanism against pathogens that use hydrolases to gain entry into the plant cells [9]. The soluble inhibitors that can be found after biomass pre-treatment include sugars (monomers and oligomers), furan derivatives (hydroxymethyl furfural and furfural), organic acids (acetic, formic and levulinic acids), and lignin derivatives (poly- and mono-phenolic compounds such as p-coumaric acid, vanillin, vanillic acid and gallic acid) [6,10]. Fig. 1 illustrates the chemical structures of some of the common inhibitory pre-treatment by-products.

Although several studies on pre-treatment liquor effects on enzymatic hydrolysis have been reported, the effects of by-products (liberated through various pre-treatment methods) on enzymatic hydrolysis is not completely understood. The mechanisms of inactivation by these compounds remain unclear. The aim

* Corresponding author. Fax: +27 46 6037576.
E-mail address: b.pletschke@ru.ac.za (B.I. Pletschke).

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Table 1
Chemical characterisation of substrates pre-treated by various technologies as a percentage (%) of dry mass.

Substrate and pre-treatment method	Natural sugar composition				Lignin content		Ash/extractives	Reference
	Glu	Xyl	Man	Ara	AIL	ASL		
Douglas-fir (SE)	59.4	0.9	1.2	0.1	38.4	0.5	0.1	Nakagame et al. [24]
Sugarcane bagasse (L)	28.8	14.2	–	1.1	33.7	8.7	4.5	Beukes and Pletschke [33]
Sugarcane bagasse (SE)	43.2	7.2	5.8	2.3	40	1.2	–	Thoresen [34]
Sugarcane bagasse (SCD)	30.5	23.2	20.4	5.9	16.8	0.9	–	Malgas [35]

Glu: glucose, Xyl: xylose, Man: mannose, Ara: arabinose, AIL: acid insoluble lignin, ASL: acid soluble lignin, SE: steam explosion, L: lime treatment, SCD: sodium chlorite/acetic acid treatment. Results are shown as a percentage of dry mass and had a mass balance greater than 90%.

of this study was to elucidate the individual inhibitory properties of each of the known pre-treatment by-products and the effects of liquors obtained from substrates pre-treated by various technologies on mannanolytic enzymes.

2. Materials and methods

2.1. Materials

All substrates (*p*-nitrophenyl-β-D-mannopyranoside and *p*-nitrophenyl-α-D-galactopyranoside) were purchased from Megazyme™, except for locust bean gum, which was purchased from Sigma–Aldrich (South Africa). Two α-galactosidases; one from *C. tetragonolobus* (guar) seed (Aga27A, GH27) and the other from *A. niger* (AglC, GH36) were purchased from Megazyme™. A β-mannanase from *A. niger* (Man26A, GH26) was also purchased from Megazyme™. Another mannanase (ManA, GH5) from *C. cellulovorans* was expressed and purified according to Beukes et al. [11]. The two β-mannosidases were purchased from Prozomix™; *C. mixtus* mannosidase (Man5A, GH5) and *B. thetaiotaomicron* mannosidase (Man2A, GH2). The furans and phenolics were all purchased from Sigma–Aldrich.

2.2. Enzyme assays

The β-mannanase activity for the two enzymes, ManA and Man26A, was determined according to Malgas et al. [12]. The reducing sugar released was monitored with the modified dinitrosalicylic acid (DNS) method described by Malgas et al. [12]. Mannose was used as a suitable standard for the DNS assay.

The galactosidase activity for Aga27A and AglC was also determined according to Malgas et al. [12], with the released *p*-nitrophenyl product monitored at 405 nm. The β-mannosidase activity for Man2A and Man5A was performed using the same protocol for determining α-galactosidase activity, but with *p*-nitrophenyl-α-D-mannopyranoside (*p*NPM) as substrate.

2.3. Product inhibition profiles of the rate limiting mannanolytic enzymes

Inhibition of α-galactosidases (Aga27A and AglC) by D-galactose (2–100 mM) was determined using *p*NPG as the substrate (see Section 2.2). The inhibition of β-mannosidases (Man2A and Man5A) by D-mannose (2–100 mM) was also determined using *p*NPM as substrate (see Section 2.2).

2.4. Pre-treatment by-products inhibition assays

Enzyme activity assays were carried out with each individual inhibitor (1–2 g/L) and activity assays for the various mannanolytic enzymes were set up as described in Section 2.2. The inhibitors assessed were: acetic acid, formic acid, lignin, levulinic acid, furfural, hydroxymethylfurfural, *p*-coumaric acid, vanillin, gallic acid and vanillic acid. Rates of substrate hydrolysis in the presence and absence of added inhibitors were then compared.

2.5. Precipitation of proteins by phenolics

To directly measure the extent of protein precipitation by the phenolic compounds, the phenolics (gallic acid) at 0.05–0.1 mg

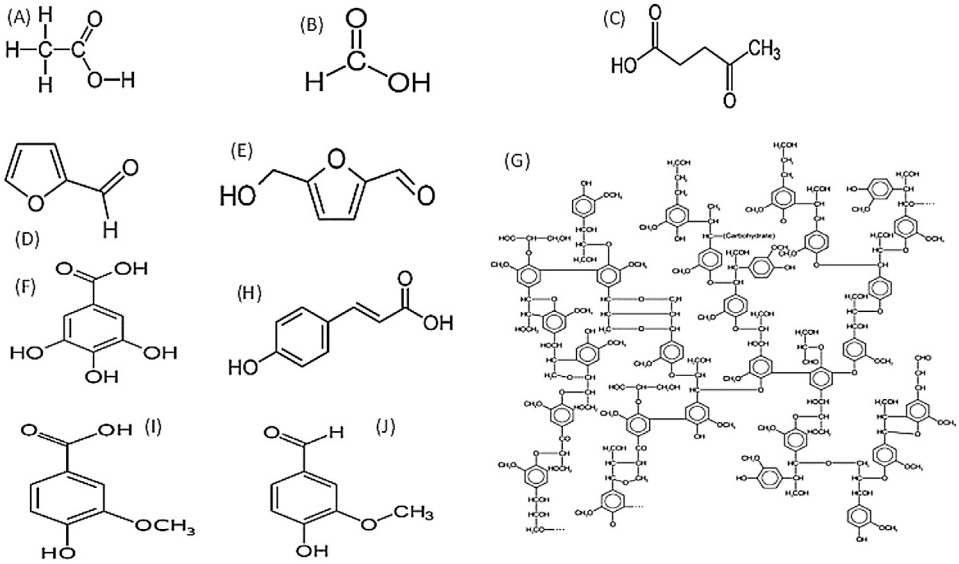

Fig. 1. Chemical structures of the various substrate pre-treatment by-products used in this study. Sugar degradation products are: (A) acetic acid, (B) formic acid, (C) levulinic acid, (D) furfural and (E) hydroxymethylfurfural, and lignin degradation products are: (F) gallic acid, (G) lignin, (H) *p*-coumaric acid, (I) vanillic acid and (J) vanillin [36].