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Effect of lime pre-treatment on the synergistic hydrolysis of sugarcane bagasse by hemicellulases

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ABSTRACT

Agricultural crop wastes are typically lignocellulosic in composition and thus partially recalcitrant to enzymatic degradation. The recalcitrant nature of plant biomass and the inability to obtain complete enzymatic hydrolysis has led to the establishment of various pre-treatment strategies. Alkaline pre-treatments increase the accessibility of the exposed surface to enzymatic hydrolysis through the removal of acetyl and uronic acid substituents on hemicelluloses. Unlike the use of steam and acid pre-treatments, alkaline pre-treatments (e.g. lime) solubilise lignin and a small percentage of the hemicelluloses. The most common alkaline pre-treatments that are employed make use of sodium hydroxide and lime. This study compared the synergistic degradation of un-treated and lime pre-treated sugarcane bagasse using cellulosomal and non-cellulosomal hemicellulases as free enzymes. The enzyme combination of 37.5% ArfA and 62.5% ManA produced the highest amount of reducing sugar of 91.834 μmol/min for the degradation of un-treated bagasse. This enzyme combination produced a degree of synergy of 1.87. The free enzymes displayed an approximately 6-fold increase in the enzyme activity, i.e. the total amount of reducing sugar released (593.65 μmol/min) with the enzyme combination of 37.5% ArfA, 25% ManA and 37.5% XynA for the lime pre-treated substrate and a degree of synergy of 2.14. To conclude, this study indicated that pre-treating the sugarcane bagasse is essential, in order to increase the efficiency of lignocellulose enzymatic hydrolysis by disruption of the lignin sheath, that the lime pre-treatment did not have any dramatic effect on the synergistic relationship between the free enzymes, and that time may play an important role in the establishment of synergistic relationships between enzymes.

1. Introduction

The possible depletion of fossil fuel resources has sparked a renewed interest in the potential use of renewable sources such as agricultural waste products for the production of a variety of biofuels. Sugarcane bagasse (SCB) is well established as an important agricultural waste product. Sugarcane bagasse is the solid lignocellulosic residue that remains after the sugarcane liquor has been extracted and is commonly burnt as a fuel source; however, SCB may serve as a valuable renewable source of biomass for the production of biofuel, as SCB is produced in large quantities in sugar mills and ethanol plants (Frollini et al., 2004; Monterio et al., 1998; Paiva and Frollini, 2002; Simkovic et al., 1990; Zarate et al., 2002). Aside from being used as an energy source in sugar mills, SCB has been used as the raw material to generate electricity, and the hydrolysed SCB products have also been used in different fermentation processes (Pandey et al., 2000).

Synergism with complex lignocellulose biomass has been observed in several enzyme systems, and facilitates an enhanced hydrolytic activity which is greater than the collective sum of the activities obtained by individual enzymes (Din et al., 1994; Teeri, 1997). Unfortunately, the synergistic relationships between cellulosomal enzymes and other cellulosomal and/or non-cellulosomal enzymes are not well established. These synergy studies occurred between a variety of proteins/enzymes such as: scaffoldin proteins, xylanasases, acetyl xylan esterase, α-arabinofuranosidase, β-galactosidase and mannanases (Beukes et al., 2008; Blum et al., 2000; Ciruela et al., 1998; Kosugi et al., 2002a; Koukiekolo et al., 2005; Murashima et al., 2003). Here, synergy was observed between cellulosomal cellulases and xylanase from *Clostridium cellulovorans* when a substrate, such as corn stem powder, was used. Minicellulosomes containing either XynA, EngE, EngH or ExgS were used to degrade corn cell walls. Besides the synergistic action between cellulosomal enzymes, synergy was found to occur between specific cellulosomal enzymes and non-cellulosomal enzymes. This was exemplified by the action of cellulosomal hemicellulase XynA and non-cellulosomal hemicellulases ArfA and BgaA.

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Despite the enhanced enzymatic hydrolysis of lignocellulose resulting from synergistic relationships between the different enzymes in non-complexed and complex systems, the complete hydrolysis of lignocellulose is never achieved due to the recalcitrant nature of plant biomass. The recalcitrant nature of biomass is due to the complexity of the biomass structure. The recalcitrant nature of plant biomass and the inability to obtain complete enzymatic hydrolysis led to the establishment of various pre-treatment strategies. Alkali pre-treatments proceed at ambient temperatures and at low pressures, which is advantageous as it eliminates the cost of maintaining the high temperatures and pressures that are usually required in other pre-treatments. Alkali pre-treatments increase the accessibility of the surface exposed to enzymatic hydrolysis through the removal of acetyl and uronic acid substituents on hemicellulose (Chang and Holtzapple, 2000). Unlike the use of steam and acid pre-treatments, alkali pre-treatments solubilise lignin and a small percentage of the hemicellulose (Chang, 2007; Kaar and Holtzapple, 2000).

Previously, the synergistic associations between two hemicellulases and EngE from *C. cellulovorans* on various substrates were investigated (Beukes et al., 2008). Novel synergistic associations were established between the three recombinant cellulosomal enzymes XynA, EngE and ManA, in the degradation of SCB, xylan, carboxymethylcellulose and locust bean gum. The combination with the molar ratio of 75% XynA to 25% EngE produced the largest degree of synergy (4.65) with SCB.

The aim of this study was to extend the previous study and to determine if synergistic relationships exist between the recombinant *C. cellulovorans* cellulosomal enzymes (XynA and ManA), and the non-cellulosomal enzyme (ArfA) with SCB as substrate, in the presence or absence of a lime pre-treatment step; and to establish the ideal molar ratio of the three recombinant enzymes (as free enzymes) to effectively degrade SCB under these conditions. Arabinofuranosidases are important enzymes and act in synergy with other glycosyl hydrolases to degrade arabinose containing polysaccharides. They also exhibit wide substrate specificity. SCB was chosen as a substrate due to the importance of the sugarcane industry to the South African agricultural sector, and the fact that this feedstock is also important for a number of other countries. Once the ideal molar ratio of cellulases and hemicellulases has been established, it will allow for the improvement of existing cellulase and hemicellulase producing strains and also allow for the design of an enzyme cocktail for the optimal hydrolysis of SCB.

2. Methods

2.1. Substrate

The chemical characterisation of the SCB was performed according to established National Renewable Energy Laboratory (NREL) protocols (Hames et al., 2005). The SCB was finely ground, autoclaved for 20 min at 121 °C, then washed to remove residual sugar from the milling process and to remove any potential bacterial and fungal spores that were present on the bagasse, and then finally air dried.

2.2. Lime pre-treatment

The SCB was milled with a bench top Waring blender to increase the surface area of the bagasse. The bagasse was treated to a solution of calcium hydroxide (lime) (reagent grade lime-Merck, cat. No. 1020470500) in a ratio of 0.4 g lime per gram dry bagasse and incubated at 70 °C for 36 h, shaking at 100 rpm. The pre-treated bagasse was filtered, and washed with distilled water until a neutral pH of 7.0 was reached. The pre-treated bagasse was air dried and stored in an air tight container.

2.3. Expression and purification of recombinant enzymes

Escherichia coli BL21 (DE3) was grown in 5 ml 2 × YT broth (Yeast, Tryptone and sodium chloride – see Bergey, 1974) for 12 h at 37 °C, shaking on a Labcon bench shaker at 200 rpm. Three cultures of the *E. coli* BL21 (DE3) cells that had been transformed with one of the recombinant plasmids *pET29-arfA, pET 29b-manA* and *pET 29b-xynA* were used as pre-inocula. The pre-inocula were incubated at 37 °C for 14 h. The pre-inocula were used to inoculate 500 ml 2 × YT broth, which was incubated at 37 °C, shaking at 200 rpm, until an optical density of 0.8 at 600 nm for ArfA and 0.6 at 600 nm was obtained for ManA and XynA. The expression of the recombinant enzymes was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) for ArfA, ManA and XynA. The cultures were incubated at 18 °C for 16 h (Murashima et al., 2002). *E. coli* BL21 (DE3) cells harbouring the recombinant plasmids were harvested by centrifugation at 8000*g* for 20 min at 4 °C and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mg lysozyme/ml, pH 8). The solution was incubated on ice for 30 min, and the soluble recombinant proteins were subsequently extracted from the cells by sonication (3 times 10 s with 10 s intervals) and centrifugation at 10,000*g* for 20 min at 4 °C. The clear lysates were applied to Protino® Ni-TED (Macherey–Nagel) columns. The flow-through was collected and the unbound proteins were removed with two 10 ml washing steps using the washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7), prior to the elution of the bound proteins with 10 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 7). The purified enzymes were desalted with 50 mM Tris–HCl buffer (pH 7.5) and concentrated with PEG 20,000 (Murashima et al., 2002). The samples were resolved on a 12% reducing SDS–PAGE gel (Laemmli, 1970).

2.4. Protein determination

The quantity of protein present was measured using Bradford's reagent (Bradford, 1976). Bovine serum albumin was used as a standard. The molar quantity of each enzyme was subsequently calculated using the theoretical molecular weight.

2.5. Enzyme assays

The enzyme concentrations in the reaction mixture were 0.35 mM of ArfA, 0.42 mM for ManA and 0.33 mM for XynA; these concentrations being the lowest identical protein concentration in mg/ml of the individual enzymes that were active with the SCB. A wide range of different enzyme combinations, ranging from 0% to 100% of the individual enzymes were tested with the SCB as previously described (Beukes et al., 2008). Once the optimal enzyme combination was established, the effect of time on the degree of synergy and the production of reducing sugar was determined for the optimal enzyme combinations obtained with both the untreated and lime pre-treated bagasse. The amount of sugar released into solution from the pre-treatment was also determined. The liberated reducing sugars were quantified using a dinitrosalicylic acid (DNS) reagent (Miller, 1959). A single standard curve for the DNS assay was prepared using xylose as a suitable standard and expressing the activity in terms of reducing sugars released as D-xylose equivalents. This standard curve was used to measure individual activities as well as the combined activity. The enzyme assays were performed in triplicate, and the activities were expressed in units (U), where 1 unit was defined as the quantity of enzyme required to release 1 μmol of reducing sugar per min.