THE EFFECT OF ALCOHOLS, LIGNIN AND PHENOLIC COMPOUNDS ON THE ENZYME ACTIVITY OF *CLOSTRIDIUM CELLULOVORANS* XynA

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In the production of biofuel from lignocellulose biomass, particularly in the case of consolidated bioprocessing where the saccharification and fermentation steps take place within the same bioreactor, many compounds may be present that could affect the enzymes within such a bioreactor. This study examined the effect of ethanol, butanol, propanol, lignin, ρ-coumaric acid, and gallic acid on the activity of XynA from *C. cellulovorans*. XynA from *C. cellulovorans* was purified, and the effects of various compounds on enzyme activity were assayed using the dinitrosalicylic acid method. In this study, it was found that XynA was very tolerant to ethanol and only lost 25% of activity even at high concentrations of ethanol. In the presence of lignin, XynA was inhibited at very low levels and retained ~85% of its activity. The highest degree of inhibition of XynA was experienced in the presence of ρ-coumaric acid (38%) and gallic acid (47%). The results indicate that the most problematic compounds within the bioreactor are likely to be soluble lignin degradation products resulting from pretreatment steps. Therefore, the removal of these compounds prior to saccharification should result in increased productivity within a bioreactor. This study indicates that XynA may be a suitable hemicellulase for use in bioethanol production, as it has very high tolerance for ethanol inhibition.

Keywords: Clostridium cellulovorans; Ethanol; Lignin; Phenolic compounds; Xylanase; XynA

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INTRODUCTION

Lignocellulose consists of approximately 75% polysaccharide sugars and can be a valuable feedstock for production of bioethanol (Lynd et al. 1991; Gomez et al. 2008). Lignocellulose consists of cellulose microfibrils embedded in a matrix of hemicellulose and pectin with lignin deposited in the cell walls to give rigidity and strength (Raven et al. 1999). The structural complexity of lignocellulose necessitates a consortium of enzymes in order to hydrolyse all the various bonds in the structure to release the sugars. These include enzymes such as cellobiohydrolases (EC 3.2.1.91), endo-1,4-β-glucanases (3.2.1.4), β-glucosidases (EC 3.2.1.21), endo-1,4-β-xylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterase (EC 3.1.1.6), α-glucuronidase (EC 3.2.1.131), pectate lyase (EC 4.2.2.2), and endo-β-1,4-D-mannanase (EC 3.2.1.78). Lignin represents a major obstacle to the access of all enzymes to the cellulose and hemicellulose. Allowing access to cellulose and hemicellulose requires removal of the lignin, which is generally achieved through pretreatment of biomass via mechanical or chemical means such as acid hydrolysis, steam treatment, or alkaline

treatment, which renders the biomass more susceptible to enzymatic degradation (Mielenz 2001; Lynd et al. 2002; Himmel et al. 2007). Pretreatments degrade the lignin, but lignin degradation products or undegraded lignin may remain present in the delignified substrate (Hendriks and Zeeman 2009).

In order to produce bioethanol from lignocellulose biomass, the cellulose and hemicellulose components must be hydrolysed (via a saccharification step) before fermentation to alcohol can take place. Consolidated bioprocessing is the term used when both the saccharification and fermentation steps take place within the same bioreactor, and this makes the process more economical (Elkins et al. 2010). Biological elements within such a reactor therefore include the enzymes responsible for saccharification as well as the yeast responsible for fermentation of sugars into ethanol. In order to achieve optimal productivity, the bioreactor environment and its effect on the biological elements must be understood. Elkins et al. (2010) identified tolerance to pretreatment inhibitors and solvent tolerance as two key elements in this process.

The impact of increased ethanol concentration on the saccharification and fermentation steps has to be considered. In this regard, much research has been conducted in understanding the effect of ethanol on the microorganisms involved in fermentation, but little is known about the impact of ethanol on the enzymes involved. Dien et al. (2003) reported that organisms producing bioethanol could achieve ethanol production of between 23 and 63 g/L. However, at such high levels of ethanol concentration, up to 50% inhibition of the organisms takes place (Demain et al. 2005). Some work has been done on the effect of ethanol on inhibition of cellulases (Holtzapple et al. 1990; Wu and Lee 1997; Taherzadeh and Karimi 2007), but very few researchers have investigated the impact of ethanol on other enzymes such as xylanases (Van Dyk et al. 2010). Nevertheless, the increased interest in expanding the fermentation to utilise hemicellulose sugars such as xylose, as well as glucose, necessitates investigating the effect of ethanol on other enzymes such as xylanases.

Although pretreatments attempt to remove the lignin component of lignocellulose biomass, some lignin may remain present in the substrate. The impact of this on the biological elements within the bioreactor must therefore be assessed. Upon degradation of the lignin during pretreatment, soluble degradation products such as phenolics may remain present on the substrate. Some studies have investigated the impact of lignin and phenolics on fermentation. Nishikawa et al. (1988) investigated the effects of lignin degradation products on xylose fermentation by *Klebsiella pneumonia* and found them to be inhibitory to growth and solvent production. They found that less substituted phenolics were more inhibitory. Delgenes (1996) also studied the impact of lignocellulose degradation products on ethanol fermentation. Research on the impact of phenolics from lignin degradation on enzymes has been limited to cellulases (Ximenes et al. 2011) and xylanases (Kaya et al. 2000). Kaya et al. (2000) investigated the impact of lignin and lignin degradation products on inactivation of enzymes and found that loss of activity is either caused by the binding of enzymes to the lignin or the inactivation by lignin degradation products. Further research in this regard is required to determine the impact of these compounds and whether all enzymes display the same response.

XynA is a cellulosomal endo-xylanase from *C. cellulovorans* and is a member of the family 11 glycoside hydrolases (Kosugi et al. 2002). As an important component of the cellulosome, it plays an important role in the synergistic degradation of complex biomass. Purified XynA was used in this study to investigate the impact of alcohols, lignin, ρ-coumaric and gallic acid on enzyme activity.

EXPERIMENTAL

Expression and Purification of XynA

Expression and purification were performed utilizing methods adapted from Kosugi et al. (2002).

Protein Concentration Determination

The protein concentration was determined using a modified method of Bradford (1976). The modified protocol consisted of 10 µL of protein sample to which 240 µL of Bradford reagent was added and left to stand for 5 minutes at room temperature. The samples were properly mixed and the readings were taken at 595 nm. Bovine serum albumin (BSA) was used as a standard.

Enzyme Assays

Xylanase activity was determined by measuring the amount of reducing sugars released according to a modified dinitrosalicylic acid assay (DNS) method as described by Miller (1959). Birchwood xylan was used as a substrate in all enzyme assays. The assay reaction mixture consisted of 50 µL enzyme solution, 100 µL substrate, and 200 µL 50 mM boric, acetic, and phosphoric (BAP) buffer (pH 5.5) (Britton and Robinson 1931). Enzyme reactions were conducted in triplicate at 45°C for 30 minutes. The reaction mixture was then centrifuged at 13,000 x g for 1 minute to pellet insoluble material. A volume of 150 µL of this reaction mixture was added to 300 µL DNS reagent and heated at 100 ºC for 5 minutes and then cooled on to ice for 5 minutes. Readings were then taken at 540 nm to determine reducing sugars present in the reaction mixture after deduction of appropriate controls and calculated using a standard curve. Enzyme activity was measured in units (U), with 1 unit defined as the quantity of enzyme releasing 1 µg of xylose, per ml, per minute, using xylose as a standard.

The Effect of Alcohols, Lignin and Soluble Phenolic Compounds on Enzyme Activity

To determine the effect of various compounds on XynA enzyme activity, the standard assay was conducted in the presence of ethanol, propanol, butanol, lignin, *p*-coumaric acid, and gallic acid, with appropriate controls. The effect of ethanol, propanol, and butanol were measured over the range 10-60 g L^{-1} of alcohol. The effect of lignin (soluble lignin with low sulfonate content) was investigated over a range of concentrations of 0 to 1.0 mg mL^{-1}, and the effect of ρ-coumaric and gallic acid was investigated using fixed levels of 0.1% and 0.2% (w/v). Lineweaver-Burk plots were prepared to identify the type of inhibition displayed by some of the compounds tested.

RESULTS AND DISCUSSION

Bacterial fermentation can result in the production of several alcohols, such as propanol, butanol, and ethanol. According to Dien et al. (2003), productivity in fermentation can result in concentrations of alcohols ranging between 23 and 63 g L^{-1}. Thus the effect of these compounds over this concentration range on XynA activity was determined. The percentage inhibition of XynA with ethanol, propanol and butanol at concentrations between 10 and 60 g L^{-1} was measured using birchwood xylan as a substrate is shown in Fig. 1.

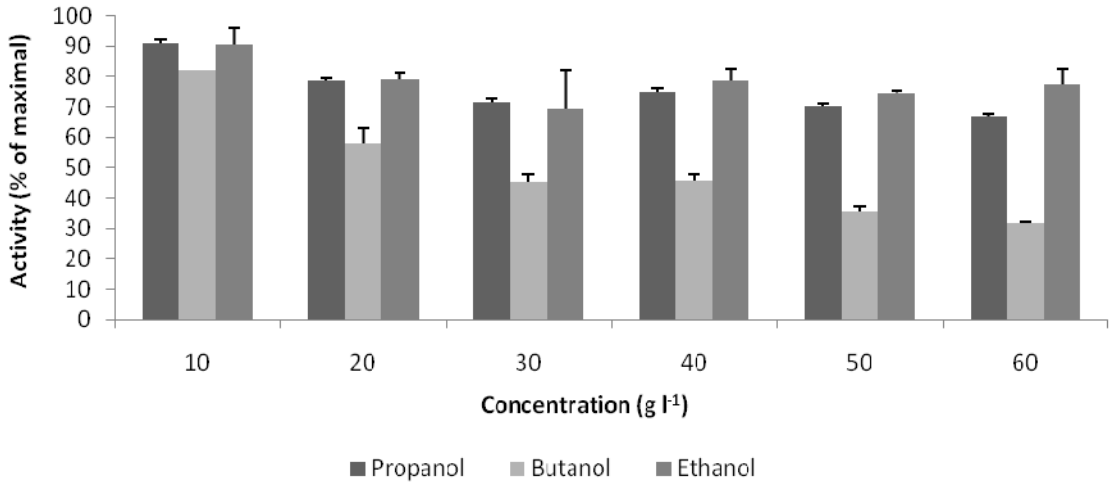

Fig. 1. XynA activity in the presence of ethanol, butanol and propanol at concentrations ranging from 0 to 60 g/l. Activity of XynA was measured using birchwood xylan as a substrate. Values are presented as mean values ±SD (n=3).

From Fig. 1 it was observed that butanol had the highest inhibitory effect on XynA activity. At a low concentration of 10 g L^{-1}, activity in the presence of butanol was reduced to 81%, while the activity continued to decrease with increased butanol concentration until only 31% of XynA activity remained at 60 g L^{-1}. Propanol and ethanol had less of an inhibitory effect on XynA activity. Even at 60 g L^{-1} propanol, 66% of XynA activity remained. Ethanol had the lowest impact on XynA activity and no more than 30% inhibition was recorded at 30 g L^{-1} of ethanol. Even though the concentration of ethanol was increased above this level, no further inhibition took place.

Since ethanol is generally considered to be the most prominent product in biofuel production, further experiments were conducted to determine the type of inhibition that XynA experienced in the presence of ethanol. For this purpose, XynA activity was measured using increasing substrate concentrations in the presence of two fixed levels of ethanol, namely 10 g/L and 20 g/L. This data was used to prepare a Lineweaver-Burk plot to identify the type of inhibition displayed by ethanol as shown in Fig. 2. From Fig. 2, it was concluded that the mode of inhibition displayed by ethanol is mixed in nature.

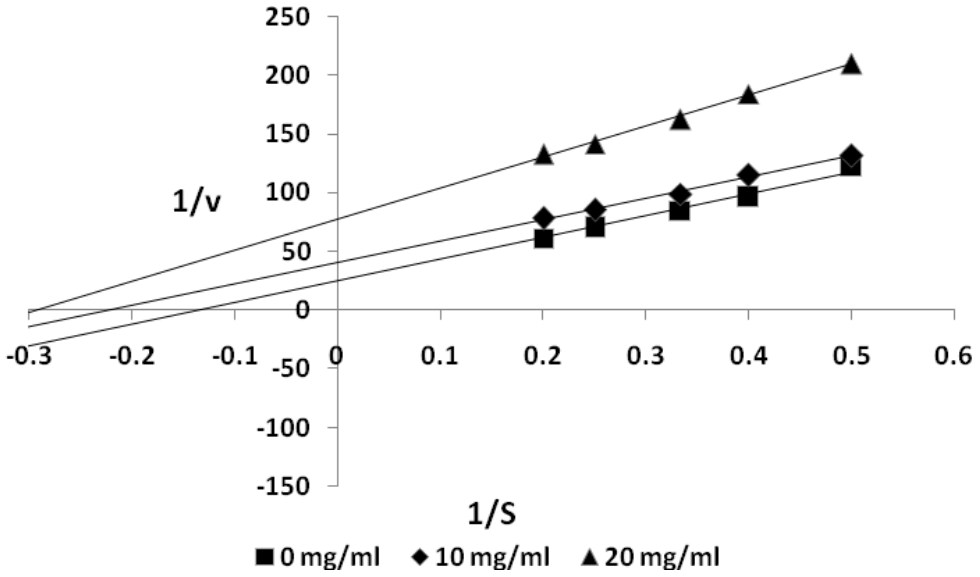

Fig. 2. A Lineweaver-Burk plot of the inhibition of XynA (using birchwood xylan as a substrate) at ethanol concentrations of 0, 10 g l^{-1} and 20 g l^{-1}. S = substrate concentration. V = velocity.

The impact of ethanol on enzymes as reported in the literature was reviewed for the sake of comparison. Van Dyk et al. (2010) investigated the inhibition of xylanase from *Bacillus licheniformis* SVD1 in the presence of ethanol. The xylanase from this organism was relatively tolerant to inhibition by ethanol and still retained 58% activity at 50 g L^{-1} of ethanol. Most studies on the inhibition of the hydrolytic enzymes have focused on cellulases. Holtzapple et al. (1990) reported that cellulases from *Trichoderma reesei* were non-competitively inhibited by ethanol. The result obtained with XynA in this study indicated mixed inhibition (non-competitive inhibition is a special form of mixed inhibition), although Van Dyk et al. (2010) found competitive inhibition for the xylanase from *B. licheniformis* SVD1. Wu and Lee (1997) also examined the inhibition of cellulases from *Trichoderma reesei* by ethanol. At 60 g l^{-1} ethanol, the cellulases were inhibited by 64%, thus displaying a far higher level of inhibition with XynA in this study. It is therefore clear that XynA would be a very good candidate for use in the saccharification step within a consolidated bioprocessing reactor, as it is very tolerant to high levels of ethanol.

The effect of soluble lignin on XynA activity was investigated at lignin concentrations ranging from 0 to 1.0 mg mL^{-1} and the results are shown in Fig. 3. At low lignin concentrations inhibition of XynA takes place with a 25% reduction in activity at 0.075 mg mL^{-1} of lignin. However, no further increases in inhibition were observed at higher concentrations of lignin. XynA activity reached a plateau at ~85% of the activity in the absence of any inhibitor.

Pretreatment of lignin may result in the production of different degradation products depending on the pretreatment method used. This is discussed in great detail in reviews by Palmqvist and Hahn-Hagerdal (2000) and Hendriks and Zeeman (2009). Two phenolic compounds, namely ρ-coumaric acid and gallic acid, were tested for their effect on XynA activity.

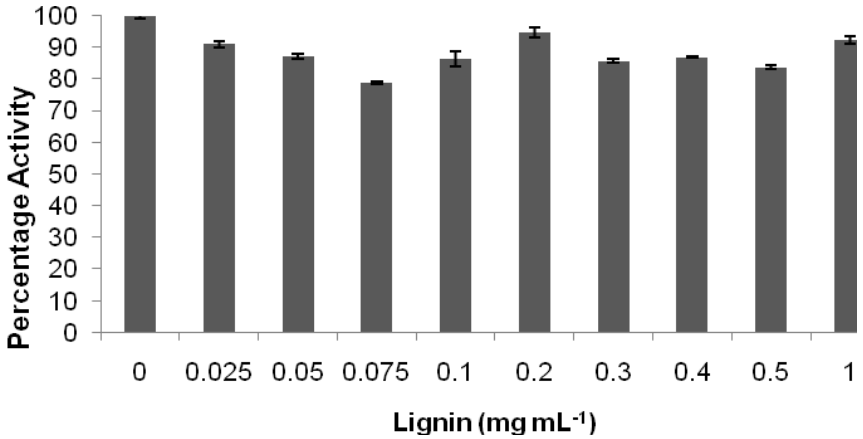

Fig. 3. The effect of lignin on XynA activity was determined using birchwood xylan as a substrate in the presence of soluble lignin concentrations from 0 to 1 mg ml^{-1}. Values are presented as mean values ±SD (n=3).

ρ-coumaric acid is a lignin degradation product that can be present in a bioreactor environment as a result of lignin degradation during a pretreatment step (Sharma et al. 1998). Gallic acid is a phenolic compound found in tannin that naturally occurs in plant biomass (Sharma et al. 1998). These compounds were tested at concentrations of 0.1% and 0.2 % (w/v). At 0.1% (w/v) of gallic acid, the activity of XynA was reduced to 84% compared to the activity in the absence of inhibitor. Upon increase of the gallic acid concentration to 0.2%, the activity of XynA was reduced to 52% of maximal. ρ-coumaric acid displayed less inhibitory effect on XynA activity. At 0.1% (w/v) concentration, the activity was reduced to 85% of maximal, while at 0.2% ρ-coumaric acid, activity was reduced to 63%. To determine the type of inhibition that took place in the presence of these compounds, Lineweaver-Burk plots were prepared at these two concentrations and compared with the activity in the absence of any inhibitor (see Fig. 4). As can be seen in Fig. 4, the type of inhibition displayed by gallic acid or ρ-coumaric acid was identified as non-competitive.

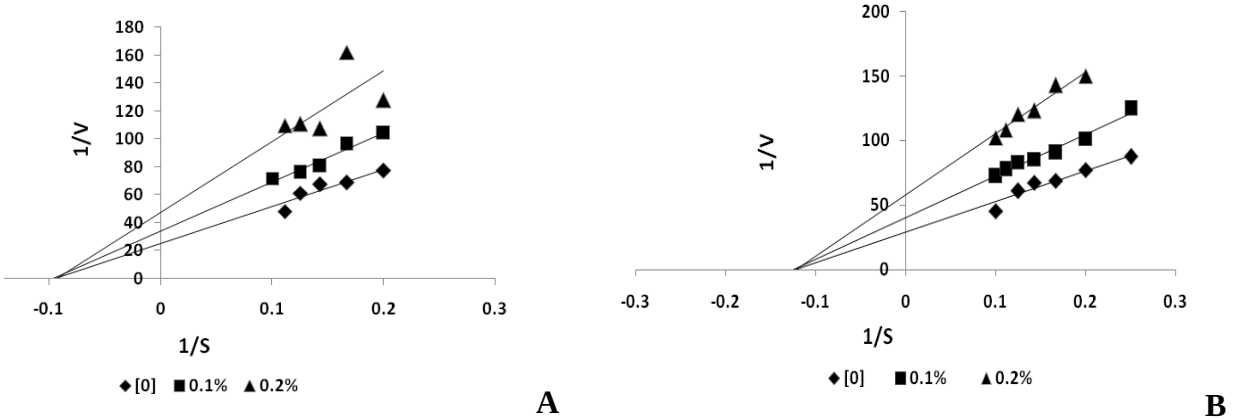

Fig 4. Lineweaver-Burk plots of the inhibition of XynA by gallic acid (A) and ρ-coumaric acid (B) using birchwood xylan as a substrate.

The substantial inhibition observed in the presence of these compounds could have a negative impact on enzyme activity within a bioreactor environment. In contrast to these results, Kaya et al. (2000) found that, at low concentrations (0.05%), lignin and lignin degradation products increased the activity of xylanases hydrolysing xylan. Soluble lignin increased xylanase activity in their study at all the concentrations tested (0-0.06%) (Kaya et al. 2000). However, in the case of the lignin degradation products, inhibition took place at higher concentrations although the behaviour of different compounds was inconsistent (Kaya et al. 2000). It should be noted, however, that they did not test ρ-coumaric acid or gallic acid for their effect on enzyme activity, but rather focused on compounds such as vanillic acid, acetovanillone, guaiacol, and protocatechuic acid. Based on circular dichroism, Kaya et al. (2000) were able to detect a change in the structure of the enzyme, which indicated that binding took place which accelerated the enzyme activity. They argued, however, that when the enzyme forms an insoluble complex with the compound present, a reduction in activity would be expected. According to Senior et al. (1991), the effect of lignin on the activities of xylanases was characterised by non-competitive inhibition.

Other authors have also investigated the effect of phenolic compounds from tannin and lignin degradation. Ximenes et al. (2011) reported that pre-incubation of the compound with enzyme led to a 20 to 80% deactivation of cellulases and β-glucosidases in the case of tannic, gallic, hydroxy-cinnamic, and 4-hydroxybenzoic acids, together with vanillin, with tannic acid leading to the greatest inhibition. The degree of inhibition varied depending on the enzyme, the microorganism it was derived from, and the phenolic compound tested (Ximenes et al. 2011).

Sewalt et al. (1997) studied the impact of lignin on cellulase activity and found that 14 to 60% inhibition occurred with increasing lignin concentration. They concluded that the lignin, in fact, precipitated protein out of the solution, which caused the reduction in activity. Berlin et al. (2006) examined the inhibition of cellulases, xylanases, and β-glucosidase by lignin preparations. Cellulases displayed varying levels of inhibition while xylanases displayed consistent inhibition and β-glucosidase was the least affected. According to Berlin et al. (2006), the actual lignin preparation and the functional groups present on the lignin could affect the molecular basis for enzyme inhibition. Palmqvist et al. (1996) investigated the effect of water-soluble inhibitors on enzymatic degradation and ethanol fermentation. They found that volatile compounds had no impact on these processes, but that non-volatile compounds displayed inhibitory effects (Palmqvist et al. 1996).

Various studies in the literature highlight the fact that more research is required using a wide range of compounds and a variety of enzymes in order to elucidate the behaviour of these enzymes in the presence of these degradation products. The relationship between the structure of the enzymes and the nature of the inhibition may also assist in understanding the interaction between these components within a bioreactor system. The results indicate that the most problematic compounds within the bioreactor are likely to be soluble lignin degradation products resulting from pretreatment steps. Therefore, the removal of these compounds prior to saccharification, should result in increased productivity within a bioreactor.

CONCLUSIONS

1. XynA was very tolerant to ethanol and only lost 25% of activity even at 60 g L^{-1} of ethanol.
2. In the presence of lignin, XynA was inhibited only slightly and retained approximately 85% of its activity at up to 1 mg mL^{-1} of lignin.
3. High levels of inhibition of XynA were experienced in the presence of 0.2% ρ-coumaric acid (37% inhibition) and 0.2% gallic acid (48% inhibition).

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