SUNLIBB

Sustainable Liquid Biofuels from Biomass Biorefining

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Deliverable 5.8

"Four new cellulases trialled for biomass saccharification"

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Workpackage: **5**
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SUNLIBB deliverables

Del No: 5.8	Deliverable Name: Four new cellulases trialled for biomass saccharification			
WP: 5	**Lead partner:** P1	**Dissemination level:*** PU	**Delivery date (proj month):40**	**Actual delivery date:** 40

Objective:

The objective of this deliverable is to test new enzymes originated during the course of the project from different partners in SUNLIBB, but above all from Projeto 3 in different Brazilian laboratories involved in CeProBio. The development of new enzyme cocktails or the improvement of existing ones are major foci of research in the biorefinery area. P1 has lead an enzyme discovery program in the UK and several Brazilian collaborators lead similar programs in Brazil. These programs provided enzymes to P1, where an automated platform tests the cellulases either in combination with commercial cocktails or isolated against a range of substrates.

Results:

a) *Limnoria quatripunctata* GH7

P1 has successfully led an enzyme discovery program funded by the BBSRC, where a marine wood borer organism, *Limnoria quatripunctata*, is used as a source of lignocellulose degrading mechanisms. A glycosyl hydrolase family 7 (GH7) with unique characteristics has recently been described in this organism (Kern et al., PNAS, 2013). This novel enzyme has high halotolerance and represents a promising cellulotic element to improve biomass hydrolysis. Within the present project, we tested the addition of LqGH7 into a standard commercial cocktail from Novozymes. The automated platform in York for the determination of enzyme activities (Whitehead, Methods in enzymology, 2012) allows a fast screen of the effects of the addition of enzymes into a cocktail at increasing concentrations and different incubation times. Figure 1 shows that the addition of LqGH7 produces a significant increase in the sugar release from filter paper co-digested with a Novozyme cocktail. Figure 2 shows that when biomass (Miscanthus giganteus) is used as a substrate, the synergic effect remains, increasing by approximately 30% the amount of reducing sugars obtained from the biomass. These experiments show that LqGH7 can have a significant impact on the improvement of biomass hydrolysis.

b) Laccase/hemocyanin pretreatment system

P1's work in revealing the mechanism present in *Limnoria* for the digestion of lignocellulosic biomass has shown that free radical chemistry takes part in the deconstruction of wood to extract sugars. In this free radical loosening of the cell wall, hemocyanins seem to mediate Fenton reactions. In a recent paper (Rico et al, Biotechnology for Biofuels, 2014) it has been shown that laccase treatment in combination with a phenolic mediator, enhances the saccharification of Eucalyptus biomass. We decided to test a similar mechanism *in vitro* to assess the effects of this free radical pretreatment in the saccharification of plant biomass.

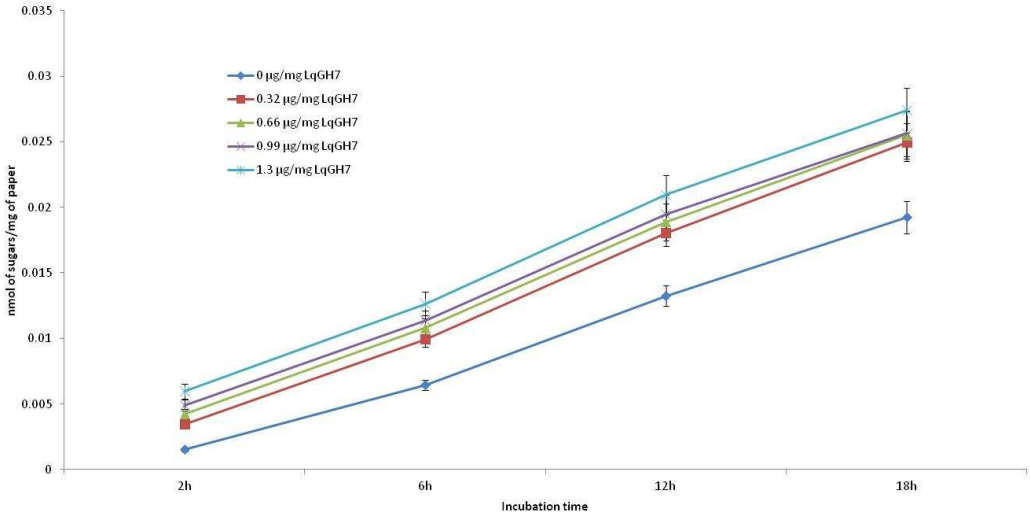


Figure 1: Addition of increasing LqGH7 amounts into Celluclast/Novozyme 188 cocktail against filter paper.

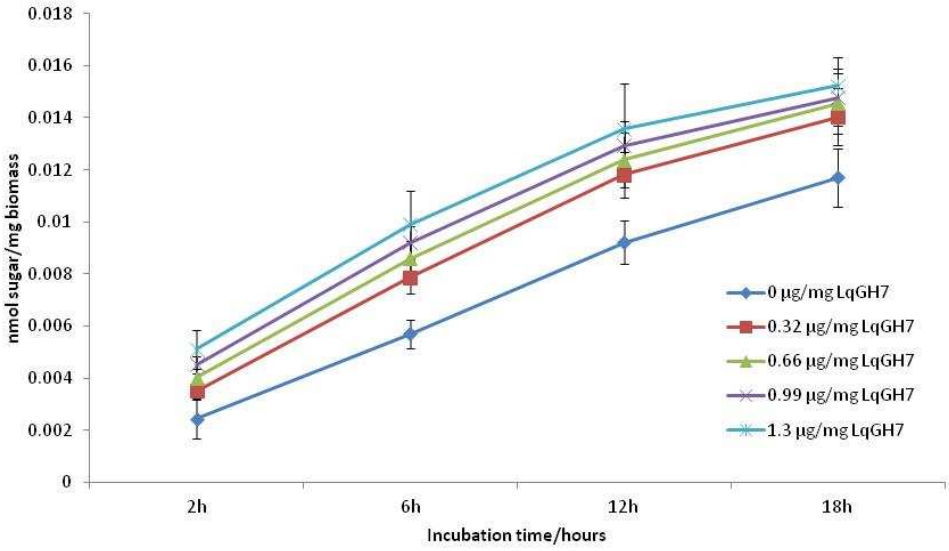


Figure 2: Addition of increasing LqGH7 amounts into Celluclast/Novozyme 188 cocktail against powdered miscanthus.

We tested the effect of hemocyanin pretreatment with and without the addition of methyl syringate (MeSy) as a phenolic mediator, on the subsequent saccharification. As a positive control of this mechanism we used laccase and MeSy, as indicated in the literature.

In spite of following the published protocol as closely as possible, we did not see an enhancer effect of these free radical pretreatments on any of the biomass or substrate trialled. Figure 3 shows the results corresponding to miscanthus after 8 hours of incubation.

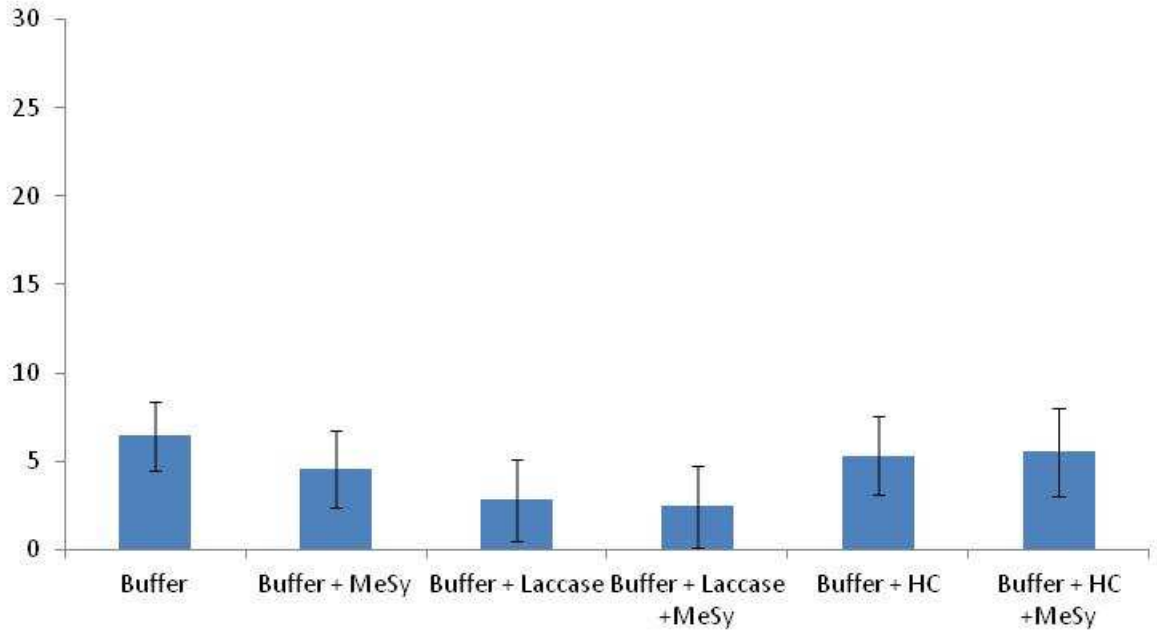
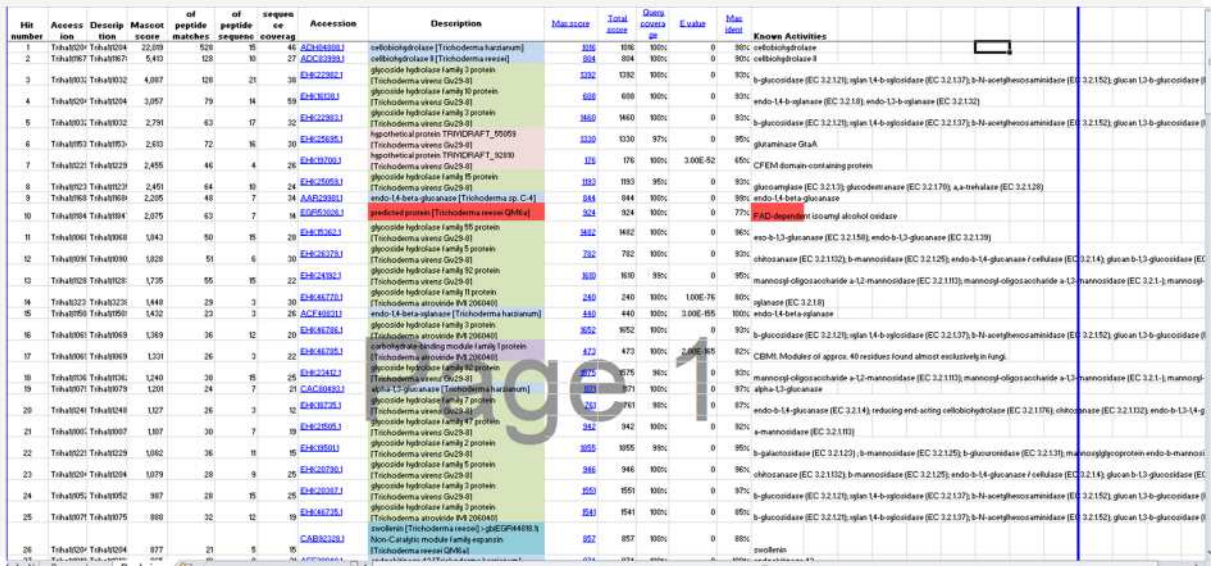


Figure 3: Effect of free radical mediated pretreatments on the saccharification of powdered miscanthus biomass.

c) Accessory proteins from *Trichoderma hartzianum* secretome

As part of the exchange between SUNLIBB and CeProBio, a cooperation was established between P1 and Prof. Nei Pereira Jr. from the Federal University of Rio de Janeiro. A Brazilian PhD student spent one year in York in order to explore the function of accessory proteins present in the secretome of the fungus *Trichoderma hartzianum*. Previous work carried out in Brazil established the optimal conditions for the production of lignocellulolytic enzymes in Trichoderma, and the objective of the present work was to obtain the proteomic profile of the secreted enzymes, select components from such secretome, express the proteins, and evaluate their effect on the saccharification of plant biomass. Figure 4 shows the list of the top 26 protein hits in the secretome.



From these proteins we chose two for further work: a swollenin abundantly produced by Trichoderma and vanillin oxydase.
These proteins were expressed in *Aspergillus niger* and the purified proteins were used in the automated platform to evaluate their effect on saccharification. None of the proteins had effect on the saccharification when used as a pretreatment.

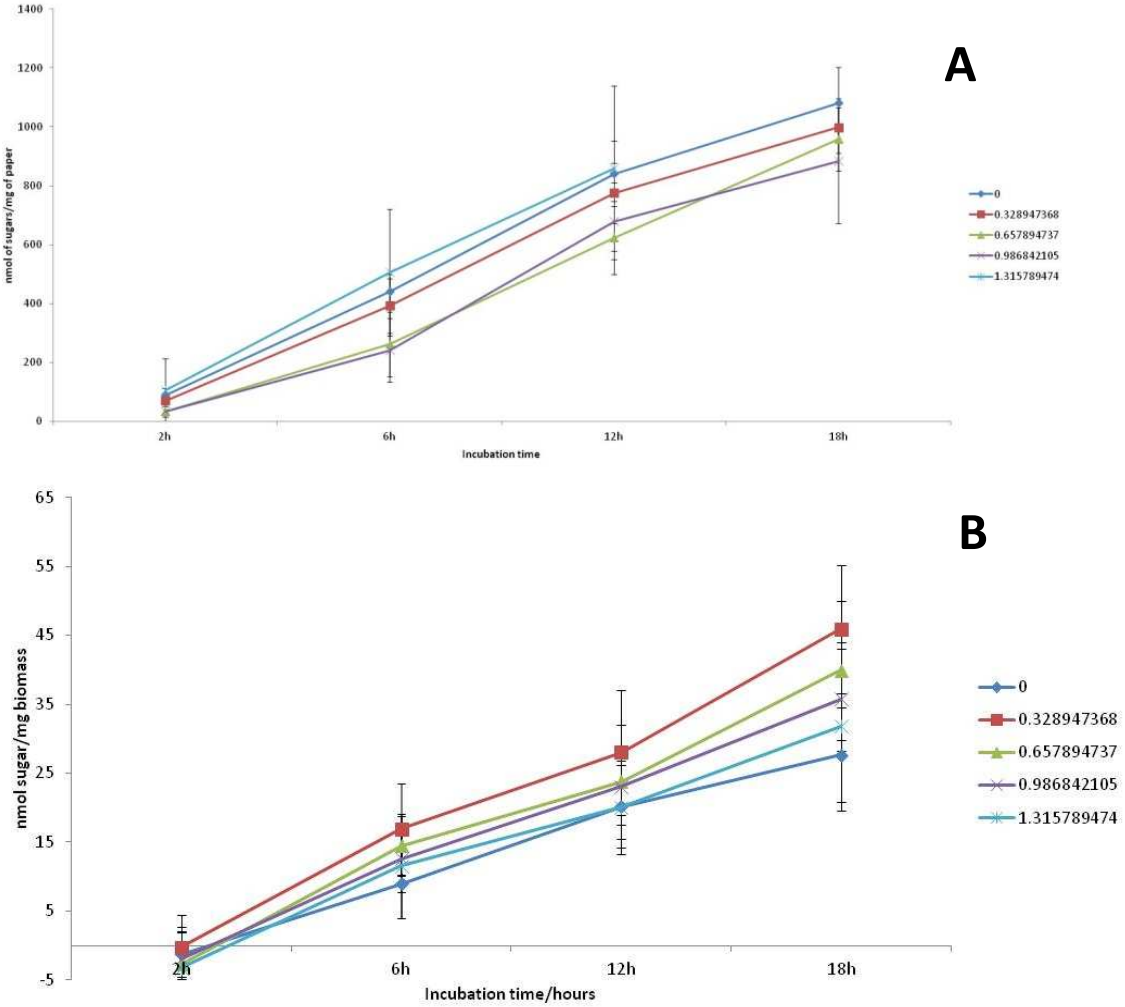


Figure 5: Time course of saccharification using Novozymes commercial cocktail with the addition of VOX in increasing concentrations. A: filter paper as substrate; B: miscanthus as substrate.

Figure 5 shows the time course of saccharification when vanillin oxydase (VOX) was added in the commercial cellulose cocktail. No significant enhancement of the saccharification was observed on the saccharification of filter paper (A) or miscanthus (B).

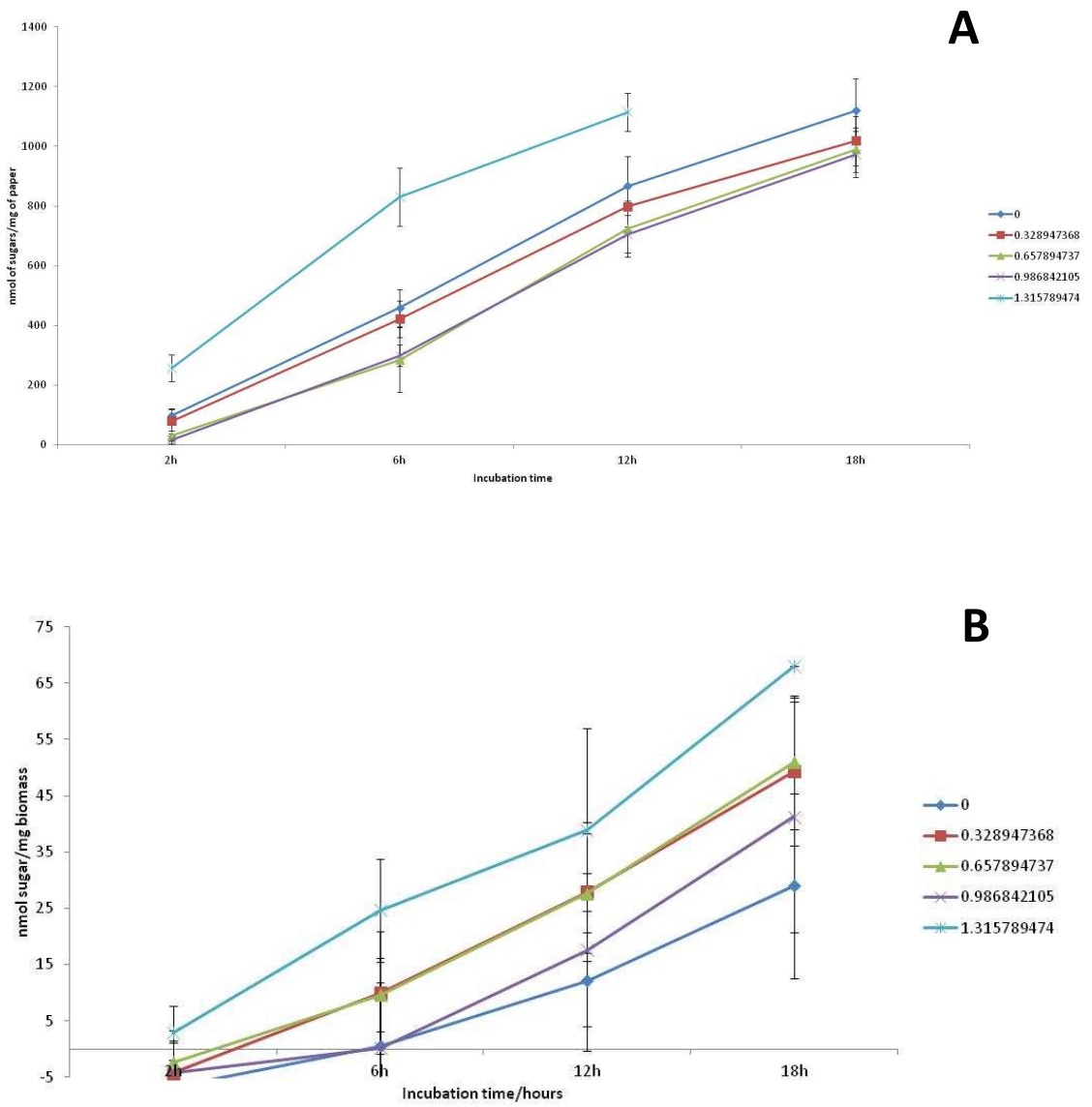


Figure 6: Time course of saccharification using Novozymes commercial cocktail with the addition of *Th* swollenin in increasing concentrations. A: filter paper as substrate; B: miscanthus as substrate.

When *Trichoderma hartziamun* swollenin was added to the enzyme cocktail (Figure 6) there was a small, but significant increase in the saccharification of both filter paper and biomass with increasing amounts of swollenin added.

d) Xylanase chimaeras

A collaboration with Professor Richard Ward, from Sao Paulo University, brought on board of SUNLIBB a set of chimeric enzymes designed to combine different functions to improve biomass hydrolysis. The original approach taken in Brazil to design enzymes involves the use of product stimulated enzymes. In this particular case, a microbial XylA was subjected to mutagenesis and the clones were evaluated to determine the degree of xylose stimulation (Figure 7)

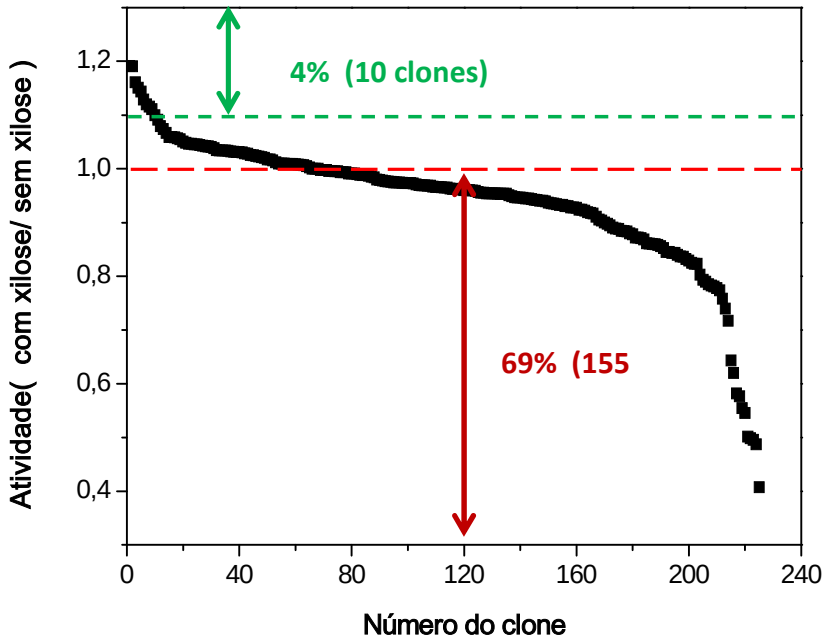


Figure 7: Selection of product-stimulated clones. Insertion at position 262 gives the chimera showing the highest stimulation with xylose.

These product-stimulated clones were linked to a Xylose binding protein (XBP) to increase the enzyme-substrate affinity. These clones were sent to York and the automated platform was programmed to measure the effect of these chimaeras on the hydrolysis of plant biomass (miscanthus), with or without the addition of commercial enzymatic cocktails, without pretreatment, after water pretreatment, and after 0.4M NaOH pretreatment.

Although we could see clear differences between pretreatments, the addition or not of the chimeric enzymes had no effect on the amount of reducing sugars released from the biomass. The chimeric enzymes had been tested against purified substrate (xylan) but not against biomass. At present we are trying to establish the reason for the lack of effect under our test conditions. Figure 8 shows the results for the 4 constructs tested at York.

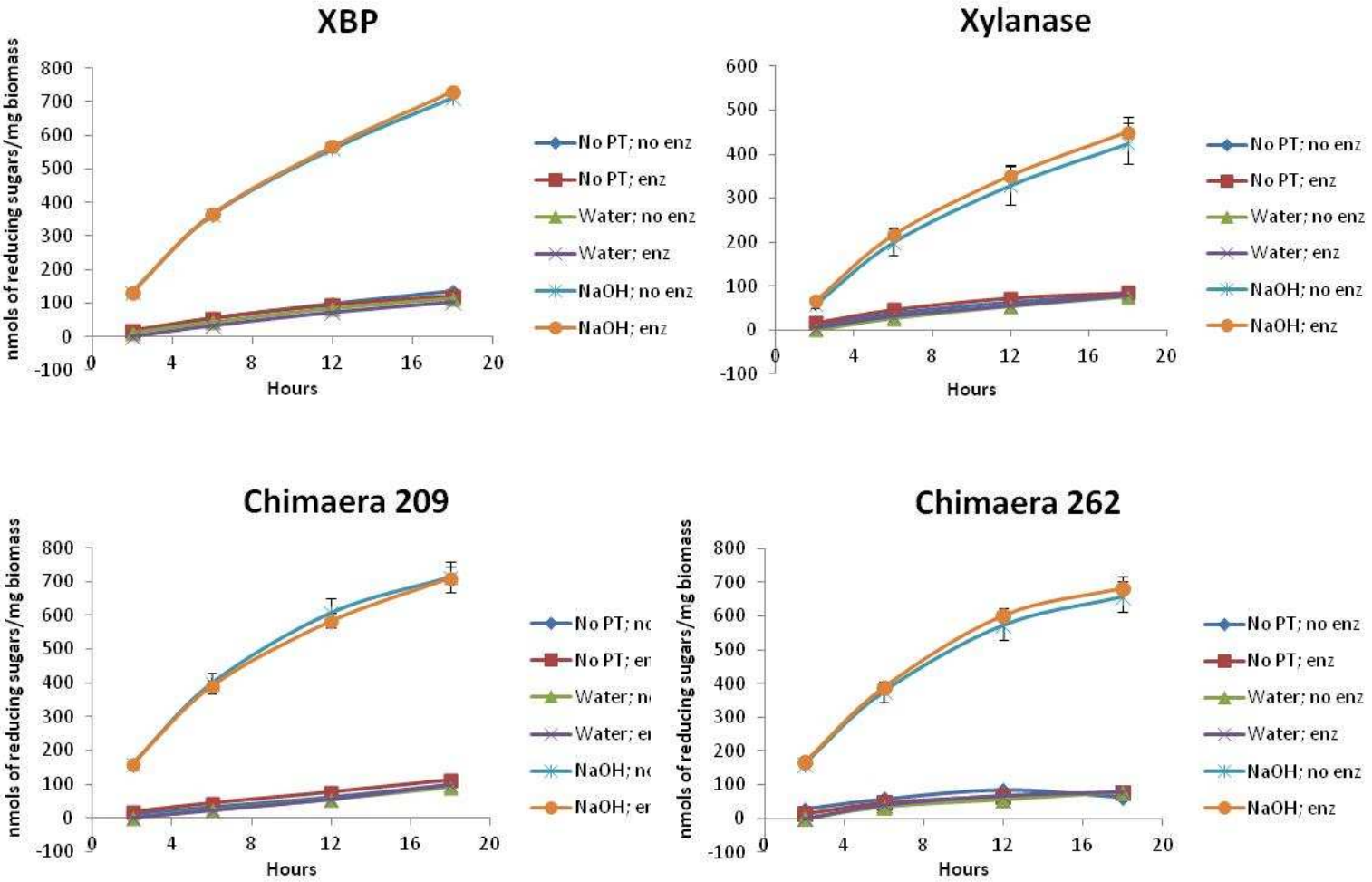


Figure 8: Saccharification of miscanthus after no pretreatment, water pretreatment, and NaOH pretreatment followed by 4 hours incubation in the presence of one of the chimeric xylanases, followed by 8 hours of hydrolysis with commercial enzymes.