Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse.

Geisa A. L. Gonçalves[a+], Yusaku Takasugi[a+], Lili Jia[a], Yutaro Mori[a], Shuhei Noda[b], Tsutomu Tanaka[c], Hirofumi Ichinose[d], Noriho Kamiya[a,e]*

[a] Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, Japan.

[b] Biomass Engineering Program, RIKEN, 1-7-22, Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan.

[c] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan.

[d] Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka, 812-8581, Japan.

[e] Center for Future Chemistry, Kyushu University, Japan.

[+] These authors contributed equally to this work.

*Corresponding Author

[a,e] 744, Motooka, Nishi-Ku, Fukuoka, Japan

*nori_kamiya@mail.cstm.kyushu-u.ac.jp

Phone: (+81) 092-802-2807, Fax: (+81) 092-802-2810

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Abstract

Recently, the new trend in the second-generation ethanol industry is to use mild pretreatments, in order to reduce costs and to keep higher content of hemicellulose in the biomass. Nevertheless, a high enzyme dosage is still required in the conversion of (hemi)cellulose. The interaction between cellulases and xylanases seems to be an effective alternative to reduce enzyme loading in the saccharification process. At first, to evaluate the synergism of xylanases on bagasse degradation, we have produced two xylanases from glycoside hydrolase family 10 (GH10) and three xylanases from glycoside hydrolase family 11 (GH11), from two thermophilic organisms, *Thermobifida fusca* and *Clostridium thermocellum*, and one mesophilic organism, *Streptomyces lividans*. Peracetic acid (PAA) pretreated bagasse was used as substrate. The combination of XynZ-C (GH10, from *C. thermocellum*), and XlnB (GH11, from *S. lividans*) presented the highest degree of synergy after 6 hours (3.62). However, the combination of XynZ-C and Xyn11A (GH11, from *T. fusca*) resulted in the highest total yield of reducing sugars. To evaluate the synergism between xylanases and cellulases, commercial cellulase preparation from *Trichoderma reesei* was combined with the selected xylanases, XynZ-C and Xyn11A. About 2-fold increase was observed in the concentration of reducing sugars, when both xylanases, XynZ-C and Xyn11A, were added together with *T. reesei* cellulases in the reaction mixture.

Keywords: Xylanase; Synergism; Lignocellulose; Bioconversion; Biofuel.

1. Introduction

Xylanases have been traditionally used in several industrial processes over the past decades, especially in the food, feed, detergents and pulp and paper segments. However, xylanases are becoming more and more attractive for the saccharification process of lignocellulosic biomass, once they can hydrolyze xylan and assist in the hydrolysis of cellulose, to obtain fermentable sugars with potential use in the production of biochemicals and biofuels [1]. Biofuel from cellulosic biomass is a major focus of different governments and industries around the world. One of the bottlenecks in the process of biofuel production is still the initial conversion of biomass into sugars, and new biotechnological solutions are needed to make this process cost-competitive [2].

Several biomass conversion processes rely on biomass pretreatment before enzymatic hydrolysis, to remove lignin and to reduce recalcitrance of the biopolymer [3], but a reduction in the hemicellulose content can also occur. Recently, less severe pretreatments of lignocellulosic biomass are gaining popularity, thus, hemicellulase characterization and studies regarding synergism of cellulases and xylanases, can contribute to reducing pretreatment severities and enhancing glucose and xylose release [4]. The use of xylanases, as accessory enzymes, has shown to improve the hydrolysis of xylan and cellulose, and it has contributed to the reduction of enzyme dosage, but it seems to be a substrate dependent reaction [5-6].

Hemicellulolytic microorganisms usually produce multiple enzymes with different mechanisms of action that can be combined to promote a unique synergism during the saccharification process. Furthermore, these microorganisms usually co-exist with other cellulolytic or xylanolytic organisms, creating an ideal ecosystem for plant

cell wall degradation [7]. Mimicking the nature, diverse enzymes are being combined in selected formulas of cocktails to be applied in industrial process. A recent study has shown that most of the xylanases used in the industry are from mesophilic and/or neutrophilic origin, but thermophilic and acidophilic enzymes would be of interest in many biotechnological processes [8]. In fact, highly stable enzymes, active under extreme conditions, could bring many advantages for several biotechnological processes, as contamination reduction and faster reaction rates, and it could lead to a breakthrough in the development of (hemi)cellulases for biomass conversion [9].

There is a growing interest in xylanases from thermophilic organisms at the industrial level, because of their ability to degrade xylan at high temperatures [10]. Thermophilic enzymes have also been identified in the deconstruction of lignocellulose, and the characterization of these enzymes could represent new opportunities in the renewable biofuel area [11]. As a result, thermophilic cellulolytic organisms and their enzymes are gaining notoriety in the bioconversion field [12]. Exploring the use of novel enzymes and the combination of enzymes from different organisms, with distinct characteristics, could contribute to the development of a highly efficient system for lignocellulose conversion.

To investigate the synergistic interaction of xylanases in the hydrolysis of pretreated bagasse, family 10 and 11 xylanases were produced. Overall, we observed that higher synergism occurred between the xylanases from different families (GH10 and GH11). Ultimately, the synergism between xylanases and cellulases were evaluated. The addition of the binary mixture of xylanases, XynZ-C and Xyn11A, has increased the activity of the commercial cellulase mixture in the hydrolysis of bagasse.

2. Materials and Methods

2.1. Biomass material and enzymes

The biomass, bagasse, particle size average of 200 μm, was purchased from Toyota Tsusho Corporation (Nagoya, Japan). Commercial cellulase from *Trichoderma reesei* was purchased from Sigma-Aldrich. Other enzymes used in this study are indicated in Table 1, and they were expressed and purified as follows.

2.1.1. Expression and purification of xylanases from *Clostridium thermocellum* Z (GH10)

Genomic DNA of *Clostridium thermocellum* (NBRC 103400) was obtained from NITE Biological Resource Center (Japan). A gene fragment encoding xylanase domain of XynZ (M22624) was amplified from the *C. thermocellum* genome using Phusion DNA polymerase (Thermo Fisher Scientific). The PCR mixture (50 μL) contained 1 U Phusion High-Fidelity DNA Polymerase, 1 × Phusion HF Buffer, 200 μM dNTPs, 10 ng genomic DNA, and 100 pmol each of primers (Forward primer; 5'-AAGGAGATATACATATGACTCCGACACAAACTCCTATCCCCA-3', Reverse primer; 5'-GGTGGTGGTGCTCGAGATAGCCCATAAGAGCTTCCTTTATTGC-3'). The gene fragment was amplified without the original stop codon to introduce a hexa-His-tag from an expression plasmid. PCR conditions were as follows: 94°C for 60 s followed by 94°C for 10 s, 60°C for 15 s, and 72°C for 60 s for 35 cycles, and then a final 2 min extension at 72°C. The amplified gene fragment was purified using a QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan) and cloned into NdeI/XhoI site of

pET22 vector (Novagen, Madison, WI, USA) using In-Fusion® HD Cloning Kit (Clontech Laboratories, Inc., Mountain View, CA, USA).

E. coli C41 (DE3) was transformed with the expression plasmid of xylanase. A fresh transformant was seeded into 50 mL of Luria-Bertani medium supplemented with carbenicillin (50 mg/L) and grown overnight with shaking (180 rpm) at 37°C. The overnight culture was transferred into 1 L of Terrific Broth medium supplemented with carbenicillin (50 mg/L). Bacterial cells were grown with shaking (180 rpm) at 37°C until the OD_{600} reached 0.8. The culture was then transferred to 27°C, supplemented with IPTG (0.5 mM), and incubated with shaking (180 rpm) for 48 h. After 48 h incubation, bacterial cells were harvested by centrifugation (7,000 *g*), washed with 100 mL of 50 mM potassium phosphate (pH 7.4), and then collected by centrifugation (7,000 *g*). The cells were re-supplemented in 40 mL lysis buffer consisting of 50 mM HEPES (pH7.4), 1mM EDTA, 1mM DTT, 1mM PMSF, and 20% glycerol, and then disrupted by sonication in an ice-cold water bath. After removing cell debris by centrifugation (10,000 *g*), the resultant cell-free extract was applied for His-tag affinity chromatography using an AKTA Prime plus system (GE Healthcare) equipped with HisTrap FF crude columns (GE Healthcare) at 4°C. After the affinity chromatography, recombinant protein was further purified by anion-exchange chromatography using HiTrap Q HP column (GE Healthcare). After the chromatographic purification, the recombinant protein was concentrated using a centrifugal filter device (Amicon Ultra-15, Millipore) and exchanged into 50 mM HEPES (pH 7.4) containing 20% glycerol, 0.5 mM DTT using a PD10 column (GE Healthcare).

2.1.2. Expression and purification of xylanases from *Thermobifida fusca* – Xyn10B or Tfu2791 (GH10) and Xyn11A or Tfu1213 (GH11), and *Streptomyces lividans* – XlnB and XlnC (GH11)

Each polymerase chain reaction (PCR) was carried out using PrimeSTAR HS (Takara, Shiga, Japan). The plasmids for expressing Tfu2791 (GH10) or Tfu1213 (GH11) were constructed as follows. The gene fragments encoding Tfu2791 (GH10) or Tfu1213 (GH11) were amplified by PCR using the *Thermobifida fusca* YX genome (ATCC number: BAA-629D-5) as a template with the following primers: 5'-TCGTTTAAGGATGCAATGAAACCGGTGCGTCTCATCACCG-3' and 5'-CGCTCAGTCGTCTCATCAGTGGTGGTGGTGGTGGTGGCAGTGATCGTGCTTGGGGC-3' or 5'-TCGTTTAAGGATGCAATGAACCATGCCCCCGCCAGTCTGA-3' and 5' CGCTCAGTCGTCTCATCAGTGGTGGTGGTGGTGGTGGTTGGCGCTGCAGGACACCG-3'. Each fragment was introduced into the *Sph*I and *Bgl*II sites of pUC702-pro-sig-term [13], using an In-Fusion HD Cloning Kit (Takara). The resultant plasmid was called pUC702-Tfu2791 or pUC702-Tfu1213.

The plasmids for expressing XlnB or XlnC (GH11) were constructed as follows. The gene fragments encoding XlnB or XlnC (GH11) were amplified by PCR using the *Streptomyces lividans* 1326 (NBRC15675) as a template with the following primers: 5'-TCGTTTAAGGATGCAATGAACCTGCTCGTCCAGCCGAGGC -3' and 5'-CGCTCAGTCGTCTCATCAGTGGTGGTGGTGGTGGTGGCCCGCGCTGCAGGACACGCTG -3' or 5' TCGTTTAAGGATGCAATGCAGCAGGACGGCACACAGCAGG-3' and 5'-CGCTCAGTCGTCTCATCAGTGGTGGTGGTGGTGGTGACCGCTGACCGTGATG

TTCGAG-3'. Each fragment was introduced into the *Sph*I and *Bgl*II sites of pUC702-pro-sig-term using an In-Fusion HD Cloning Kit. The resultant plasmid was called pUC702-XlnB or pUC702-XlnC.

Protoplasts of wild-type *S. lividans* 1326 was prepared according to the method of Hopwood et al. [14]. Briefly, the mycelium of each strain was treated with a solution of 1 mg/mL lysozyme (Wako, Osaka, Japan), and suspended mycelia were used as protoplasts. Each multi-copy plasmid (pUC702-Tfu2791, pUC702-Tfu1213, pUC702-XlnB or pUC702-XlnC) was introduced into wild-type *S. lividans* 1326 using the polyethylene glycol (PEG) method. Selection of transformants was carried out by overlaying soft agar containing 50 μg/ml of thiostrepton. After cultivation for 5 days, transformants were selected and named as *S. lividans*/Tfu2791, *S. lividans*/Tfu1213, *S. lividans*/XlnB, and *S. lividans*/XlnC.

Spore suspension of each transformant was inoculated in a test tube containing 5 mL of tryptic soy broth (TSB) medium (BD Diagnostic Systems, 199 Sparks, MD, USA) supplemented with 5 μg/ml of thiostrepton (MP Biomedicals, Illkirch-Graffenstaden, France), followed by cultivation at 28 °C for 3 days. Then, 5 ml of the preculture broth of each transformant was seeded into a shake flask with a baffle containing 100 ml of modified TSB medium with 5 μg/ml thiostrepton, 3 % glucose, and 5 % tryptone, followed by incubation at 28 °C for 4 days.

Each culture supernatant (300 ml) of *S. lividans*/Tfu2791, *S. lividans*/Tfu1213, *S. lividans*/XlnB, or *S. lividans*/XlnC was precipitated by ammonium sulfate. The precipitate of each was collected by centrifugation at 20,000g for 30 min, and dissolved with 50 mM phosphate buffer (pH 7.0) containing 300 mM NaCl. Tfu2791, Tfu1213, XlnB, and XlnC were separately purified using TALON metal affinity resins (Takara)

according to the manufacturer's protocol, and dialyzed with 50 mM phosphate buffer (pH 7.0) containing 300 mM NaCl.

2.2. Enzyme specific activity

Xylanase specific activity was assayed using beechwood xylan (Tokyo Chemical Industry Co., Ltd.). The beechwood xylan (0.25 w/v%) was dissolved in 50 mM phosphate buffer, pH 7.0, and xylanase was added to a final concentration of 2.5 μg/mL. The mixture was incubated at 50℃ for 10 min. The amount of reducing sugars released was determined by the dinitrosalicylic acid (DNS) method [15].

2.3. Peracetic acid (PAA) pretreatment

Bagasse sample (50 mg) was soaked in 1 mL of deionized water and 50 μL of PAA solution 40 % (w/w) (Mitsubishi Gas Chemical Company, Inc., Japan). The mixture was heated at 80 ºC with stirring (200 rpm) for 3 h. After that, 5 mL of deionized water was added to the mixture, and the sample was centrifuged at 5800 g for 20 min, supernatant was discharged after centrifugation. To remove residual acids, the solids were washed, by addition of 5 mL of water, followed by centrifugation (5800 g / 20 min), for 3 times. The regenerated bagasse was dried, overnight, by freeze-drying.

2.4. Chemical composition of the untreated and the PAA-treated bagasse

The chemical composition of the untreated and the PAA-treated bagasse was measured following the protocol (LAP TP-510-42619, 42618, 42622) from National Renewable Energy Laboratory, [16],[17], [18]. Briefly, the samples were treated with 72% sulfuric acid at 30 ºC for 1 h, then diluted to 4% and incubated in autoclave at

121ºC for 1 h. After cooling, calcium carbonate was used for neutralizing the pH to 7. Analysis of sugar content was performed using a high performance liquid chromatography (HPLC) system equipped with Shodex sugar KS-801 column (8.0×300 mm, Showa Denko Co., Tokyo, Japan) and RI detector at 80 ºC, with HPLC-grade water as the eluent (flow rate of 1 mL/min). The remaining acid insoluble lignin was measured after oven-dried at 45 ºC overnight, and the amount of acid soluble lignin was determined by measuring the absorbance at 240 nm, against a deionized water blank using a UV-Vis spectrophotometer (Jasco UV-Vis spectrophotometer V-550).

2.5. Enzymatic saccharification

The enzymatic saccharification was tested in a 2 mL final volume containing pretreated bagasse (0.25 wt% biomass), in 50 mM sodium phosphate buffer pH 7.0 for xylanase tests and pH 6.0 for cellulase and xylanase tests. The preliminary tests using xylanase only, enzyme loading was kept at 1 mg of protein / g of biomass, for total enzyme added in the reaction tube. A ratio of 50:50 was used, when xylanases were mixed. Further tests, with xylanase and cellulase, enzyme loading was kept at 2 mg of protein / g of biomass, for total enzyme added in the tube. A ratio of 50:50, cellulase:xylanase, or 50:25:25, cellulase:xylanase1:xylanase2, was used even when xylanases were combined to the cellulase commercial mix. All reactions were carried out at 50 °C with stirring (1000 rpm), for 72 hours. The DNS assay was performed to measure the reducing ends of (hemi)cellulose after the enzymatic reaction [15]. At the end of the enzyme reaction, 100 μL of DNS reagent containing 1.3 M DNS, 1 M potassium sodium tartrate, and 0.4 N NaOH was added to 100 μL of the reaction mixture containing substrate and incubated at 99 °C for 5 min to label the reducing ends

of the hydrolyzed cellulose. Samples were analyzed at hour 3, 6, 12, 24, 48 and 72.

2.6. Sugar analysis

Quantification of soluble sugars were conducted based on the derivatization of carbohydrates with ABEE (4-aminobenzoic acid ethyl ester) [19]. Briefly, to determine the concentration of xylose, xylobiose and xylotriose, enzymatic hydrolysis samples were centrifuged at 5800 g for 3 minutes, and the aqueous supernatant was filtered (0.20 µm), in order to completely remove bagasse content. After that, 10 µl of filtered samples were diluted in 40 µl of ABEE reagent containing 1.4 M sodium cyanoborohydride, 2.6 M benzocaine, 22.1 M methanol, and 1.8 M glacial acetic acid, and incubated at 83 ºC for 1 hour. After incubation, samples were cooled down to 15 ºC, 200 µl of water and 200 µl of chloroform were added to the mix. Samples were centrifuged at 5800 g for 3 minutes, and the final supernatant was used for HPLC analysis on a Shimadzu HPLC system equipped with a Honenpak C18 column (J-Oil Mills Inc., Japan, Tokyo, Japan), and an UV detector. HPLC method was run at a constant flow rate of 1.0 mL/min with, 10 % of acetonitrile and 90 % of 0.02 % TFA, as the mobile phase, at 40 ºC for 30 min and the elution was monitored at 305 nm. Standard curves of xylose, xylobiose and xylotriose, were prepared to determine the elution time and the peak areas as a function of the sugar concentration, in order to identify all sugars present in each sample as well as their concentrations. Samples were analyzed only in the end of the reaction, at hour 72.

2.7. Calculation method of degree of synergy

The calculation of degree of synergy (DS), between the xylanases, was determined as following equation [20] :

$$DS_{XYL} = Y_{1+2} / (\alpha Y_1 + \beta Y_2)$$

Where α and β correspond to the mass ratio of the enzyme in each reaction. In the case of the binary mixture of xylanases, α and β, correspond to 0.5, $Y_{1+2,}$ indicates the yield of reducing sugar achieved by the two enzymes working simultaneously, Y_1 and Y_2 indicate the yields of reducing sugar, achieved by each enzyme working separately.

When calculating DS of the combination of commercial *T. ressei* cellulase with the two selected xylanases, the following equation was applied:

$$DS_{XYL+CEL} = Y_{1+2+3} / (\alpha Y_1 + \beta Y_2 + \gamma Y_3)$$

Where α, β and γ correspond to the mass ratio of the enzyme in each reaction. In the case of the xylanases, Y_1 and Y_2, α and β, correspond to 0.25, and for the commercial cellulase Y_3, γ correspond to 0.5, Y_{1+2+3}, indicates the yield of reducing sugar achieved by all enzymes working simultaneously, Y_1, Y_2 and Y_3 indicate the yields of reducing sugar achieved by each kind of enzyme working separately.

As a general rule, DS indicates the cooperation between the involved enzymes:
If DS is higher than one, there is cooperation among the involved enzymes.
If DS is lower than one, there is competition of the involved enzymes.
High values of DS indicate high cooperation among the involved enzymes.

3. Results and Discussion

3.1. Purified xylanases and PAA-treated bagasse

The xylanases from *T. fusca* (Xyn10B and Xyn11A), *C. thermocellum* (XynZ-C), and *S. lividans* (XlnB and XlnC) were purified by affinity chromatography, as described in materials and methods section, enzyme purities were confirmed by SDS-PAGE (Figure 1). Specific activities were determined using beechwood xylan as substrate (Table 1). The mesophilic xylanase XlnC (GH11) showed the highest activity on beechwood xylan (119.5 U/mg), followed by the thermophilic, family 10, xylanase XynZ-C (93.0 U/mg). Family 11, xylanase XlnB (60 U/mg), and the thermophilic xylanases, family 11, Xyn11A (51 U/mg) and, family 10, Xyn10B (45.3 U/mg) presented lower hydrolytic activity on beechwood xylan. Therefore, it was not possible to conclude if xylanases from family 11 were more susceptible to have higher activity on soluble beechwood xylan substrate than xylanases from family 10, or vice versa. Previous studies have demonstrated distinct preferences between GH10 and GH11 xylanases to soluble xylan substrates [6], [12]. Thus, the affinity and hydrolytic activity of a certain enzyme to a specific substrate seems to vary according to the enzyme origin, type, structure, family, and other factors.

Pretreatment method is usually necessary, before saccharification process, to increase the accessibility of hemicellulase and cellulase enzymes to woody biomass. Aiming to remove maximum content of lignin, but at the same time, to keep maximum content of cellulose and hemicellulose, PAA method was chosen to pretreat the bagasse. The PAA treatment is a conventional method, well known and used in the pretreatment of lignocellulosic biomass, especially in softwoods and hardwoods. Nevertheless, the successful use of PAA on the pretreatment of agricultural residues, with high delignification and less severe impacts was demonstrated [21]. As expected, cellulose

content was not changed and xylan removal was minimum (4.5 %) in the PAA-treated bagasse. On the contrary, the removal of lignin was not as high as expected; only about 5.7 % of lignin content was removed. This could be likely due to the low PAA loading (0.4 g of PAA/ g of biomass) used in the bagasse pretreatment. Even thought pretreatment temperature was 80 ºC, reaction time was about 3 hours. Kumar and co-workers have demonstrated low lignin removal at low PAA loading (0.5 g of PAA/ g of biomass) at 25 ºC after 24 hours, but high lignin removal, at higher loading of PAA (5.5 g of PAA/ g of biomass) at 25 ºC after 48 hours [21]. Surprisingly, 68% of the arabinan content was removed from the original bagasse, likely favoring the action of the xylanases in the PAA-treated bagasse (Table 2). Arabinose is usually present in agricultural crops, combined with xylan. The inter- or intra-molecular crosslinks of arabinose and xylan could hinder the xylanase action during biomass degradation [22]. Previous study has demonstrated an inverse relationship between arabinose content and catalytic efficiency of a family 11 xylanase from *Thermobacillus xylanilyticus* [23].

3.2. Enzymatic saccharification of pretreated bagasse by xylanases from family 10 and family 11

The xylanolytic system of microbes to breakdown xylooligosaccharides is well known and described in the literature. Microorganisms usually produce different types of xylanases, which act in synergism to degrade plant cell wall [24]. Inspired by nature, enzyme cocktails are being formulated to hydrolyze lignocellulosic biomass in industrial processes. These cocktails of enzymes usually contain cellulases, and accessory enzymes, such as hemicellulases, from different families and/or microorganisms [25]. To investigate the synergism between distinct xylanases during

enzymatic hydrolysis of bagasse, we have mixed xylanases from family 10 and family 11 (Table 1), in three modes: xylanases from family 10 mixture, xylanases from family 11 mixture and xylanases from family 10 and family 11 mixture. Each individual enzyme was also evaluated, in order to analyze the synergism between the different combinations of enzymes. All enzyme mixtures were done in a ratio of 50:50 in this study. Other ratios (10:90, 20:80, 30:70, 40:60) were also analyzed, however the best synergism was observed when mixing the xylanases in a ratio of 50:50 (data not shown).

Among all the tested enzymes, XynZ-C, from *C. thermocellum*, and Xyn11A, from *T. fusca*, produced the highest concentration of reducing sugars, when tested sole (Figure 2). Normally, *Clostridium* species are specialized in crystalline cellulose degradation, but this organism produces several hemicellulases, especially xylanases [26]. Early studies, demonstrated that pure xylanase Z, from *C. thermocellum*, was shown to have high activity on xylan, but in nature XynZ is found to be associated with the cellulosome structure [27]. More recently, XynZ, purified from *C. thermocellum* cellulosome, was found to consist of a multidomain structure, with a N-terminus ferulolyl esterase domain, a family 6 carbohydrate binding domain, a dockerin and a glycoside hydrolase family 10 domain [28]. Sajjad and co-workers have previously demonstrated higher activity of XynZ without the native non-catalytic domains before in birchwood xylan [29]. The role of CBM was also analyzed by the attachment of CBM6 to the C-terminal or N-terminal, and CBM22 in the N-terminal, of the catalytic domain of XynZ, without the ferulolyl domain. The activity of XynZ was improved only by the addition of CBM22 in the N-terminal of the catalytic domain. Nonetheless, the specific activity of each enzyme form was demonstrated only on soluble xylan

substrate [30]. In this study, we have expressed and purified the catalytic domain of XynZ only, without ferulolyl esterase and CBM. Although, CBM is known to be essential for the proximity of the enzyme and the substrate, high saccharification efficiency was obtained by XynZ-C lacking CBM. Further work would be necessary to analyze the impact of CBM addition to XynZ-C on the hydrolysis of bagasse.

The xylanase Xyn11A, from *T. fusca*, was shown to have a special binding module that can bind strongly to both cellulose and xylan. Although Xyn11A has no activity in cellulose, this special feature can potentially increase hemicellulose degradation [31]. Recent study has demonstrated that a family 11 xylanase was more efficient in hydrolyzing xylan, than a family 10 xylanase, in different soluble substrates [6]. In addition, a GH11 xylanase from *Thermobacillus xylanilyticus*, had better hydrolytic activity in wheat bran than a GH10 xylanase from the same organism [23]. However, the enzyme action seems to be directly related to the substrate source. For example, the thermostable xylanase belonging to family 10 (Ta Xyn) was shown to be more efficient in solubilizing xylan, from birchwood glucuronoxylan and wheat straw, than a family 11 thermostable xylanase (Nf Xyn) [32]. Moreover, Kim and co-workers have demonstrated that the Xyn10B, from *T. fusca*, has specific activity on various substrates, and it produced more reducing sugars from corn fiber than the Xyn11A, for example [33]. In the case of PAA pretreated bagasse, the family 10 xylanase, from *C. thermocellum*, produced higher amounts of reducing sugars than both family 11 xylanases, from *S. lividans*, and similar amounts of reducing sugars when compared to the family 11 xylanase, from *T. fusca*. On the other hand, the family 10 xylanase, from *T. fusca*, yielded minimal amounts of reducing sugars during the hydrolysis of the

pretreated bagasse. In addition to the substrate, it is likely that enzymatic action during the degradation of hemicellulose is related to the enzyme-producing organism.

Interestingly, synergistic effect was observed when mixing the xylanases from family 10 and family 11, from different or same organisms, for all tested combinations. In contrast, minimum synergism was verified when mixing the xylanases from the same family, GH10 or GH11. Previous report has shown synergistic effect between Xyn11A and Xyn10B from *T. fusca*, during hydrolysis of corn fiber [33]. The synergistic effect between enzymes from two different families could be expected due to the complex structure of natural hemicellulose, such as bagasse, that would require a dynamic enzyme action to be hydrolyzed. Xylanases belonging to family 10 usually have small binding sites and are highly active on short xylo-oligossaccharides, and may also be active on cellulose substrate. On the other hand, family 11 xylanases are exclusively active on xylose containing substrates. In addition, it has been demonstrated that hydrolytic products from family 11 xylanases can be further hydrolyzed by family 10 xylanases [34]. Unlike our data, synergistic effect was not observed when mixing GH10 and GH11 xylanases from the thermophilic bacterium *Thermobacillus xylanilyticus*, on the hydrolysis of wheat bran arabinoxylans [23]. Despite the fact that minimum synergism was observed when mixing xylanases from the same family (10 or 11) during the hydrolysis of PAA-treated bagasse, other studies have shown synergism among xylanases from family 11 only, from distinct organisms, in the bleaching of bagasse pulp [35], or family 10 xylanases only, on hardwood and softwood paper pulps [36]. Taking into consideration the above-mentioned findings, it is clear that enzyme action is highly dependent on the nature of substrate, origin of enzyme-producing microorganism and enzymatic reaction conditions.

3.3. Soluble sugar analysis of bagasse after enzymatic hydrolysis using different combinations of GH10 and GH11

Commercial development of hemicellulases is less advanced than cellulases in the bioconversion field, especially because most of the biomass pretreatment still depends on acid preparation, removing the majority of the hemicellulose component. Nevertheless, studies involving hemicellulases have increased due to recent trends in reducing pretreatment severities to preserve hemicellulose content [4],[25]. Thus, there is a need to understand different aspects of plant biomass degradation. In order to evaluate and quantify the final content of soluble sugars on hydrolyzed bagasse, samples were analyzed by HPLC after 72 hours. Soluble sugar analysis has confirmed the results obtained in the quantification of reducing sugars, affirming that synergistic effect occurs when mixing xylanases from family 10 and family 11 (Figure 3). As expected, the xylanase Xyn10B, from *T. fusca*, generated shorter products than xylanases from family 11, basically only xylose and xylobiose. Although xylanase XynZ-C, from *C. thermocellum*, has produced the highest amount of xylose during hydrolysis of the bagasse, this enzyme has also generated small amounts of xylotriose. This could be explained by the differences in the active sites and/or substrate affinity, once XynZ-C and Xyn10B originated from different organisms. Interestingly, the xylanases from *S. lividans* (XlnB and XlnC), belonging to family 11, produced only xylobiose and xylotriose when tested sole. However, the same enzymes when mixed with Xyn10B, generated a mix of xylose and xylobiose, and no xylotriose was observed. Small amounts of xylose were also observed when mixing XlnB or XlnC with Xyn11A, although these enzymes belong to the same family (GH11). Nonetheless,

xylotriose was still produced in the reaction mix. In general, all reaction mixtures have increased the final amount of xylose and xylobiose, while reducing total xylotriose. Recent work has indicated that family 10 xylanases usually generate smaller products than family 11 xylanases, but this tendency can vary according to substrate type and enzyme-producing microorganism [8]. Amongst all the enzymes tested in this study, XynZ-C generated the highest amount of xylose and xylobiose, when tested individually. Xyn11A has produced similar amounts of reducing sugars compared to XynZ-C, but less soluble sugars. Nevertheless, long soluble xylooligomers, such as xylotetraose and xylopentose have not been measured here. In general, xylobiose and xylotriose are the main xylooligomers produced by xylanases during the hydrolysis of lignicellulosic biomass, however previous works have demonstrated the production of high chain xylooligomers (X6, X7, X8, X9, X10 and X11) by GH10 or GH11 xylanases, during the hydrolysis of birchwood and wheat arabinoxylan [8], [37].

3.4. Degree of synergy of xylanases from different families (GH10 and GH11) or from the same family (GH10 or GH11)

An effective method to quantitatively measure the extent of synergism between different enzymes is the calculation of the degree of synergy (DS). DS can be calculated based on the ratio of the yield of sugars released by enzymes working simultaneously to the sum of the yield of sugars released by each enzyme separately, once they were applied in the mixture in equal quantities [38]. There are several proposed models for enzymatic hydrolysis of cellulose, (for a review, see Zhang and Lynd, 2004 [39]). Here, we have used a type of semi-mechanistic method with respect to the enzyme to calculate the degree of synergy, using an equation already described and used in prior

studies [20], [40]. Enzymatic hydrolysis based on reducing sugar quantification was used for calculus purpose. Overall, higher degree of synergy was observed when mixing xylanases from different GH families, comparing to xylanase mixtures of the same family. At hour 6, DS was higher than one, for all the binary mixtures, between GH10 and GH11, indicating that the enzymes worked in cooperation to enhance hemicellulose hydrolysis. In contrast, the mixtures between xylanases of the same family, such as, XynZ-C and Xyn10B, and Xyn11A and XlnB, presented DS lower than one, indicating a competition of the involved enzymes (Figure 4). Nevertheless, DS was higher than one at hour 72, for all enzyme combinations (Table 3). The highest DS (3.62) was observed at hour 6, when mixing XynZ-C and XlnB, but the highest final DS obtained at hour 72, was observed when mixing Xyn10B and Xyn11A (2.16) or Xyn10B and XlnB (2.15). In this specific case, DS indicates the cooperation between the combined enzymes, to enhance the hydrolysis of hemicellulose. However, the degree of synergy between 2 hemicellulases is not always an indicative of the overall efficiency of hemicellulose degradation. The highest obtained DS did not correspond to the highest yield of reducing sugars in this work. A decrease of DS was observed with the increase of the reaction time, for most of the tested binary mixtures. Nevertheless, a contrasting difference was verified when changing the species of family 10 xylanase in the reaction mix. Less reduction in the final value of DS was observed when using Xyn10B from *T. fusca*, instead of XynZ-C from *C. thermocellum* (Table 3). Several experiments using xylanases to hydrolyze lignocellulose biomass have shown that DS value decrease with the extension of enzymatic hydrolysis. This decrease on DS appears to be associated with the substrate modification during hydrolysis, such as crystallinity index, and/or irreversible adsorption of enzymes by regions containing lignin [29-30].

3.5. Observation of continuing synergy between GH10 and GH11 xylanases and a commercial cellulase

After identifying a high synergism between the xylanases from family GH10 and GH11, we have tested the combination of the two top xylanases in this study, XynZ-C and Xyn11A, and the commercial cellulase from *T. reesei*. The optimum pH for *T. reesei* cellulase is known to be 5, while the optimum pH of both xylanases, XynZ-C and Xyn11A, is about 7, but here the combinations of these enzymes were analyzed at pH 6. Considering, that enzymes from different microorganisms can act synergistically, pH adjustment offers the possibility of developing enzyme mixtures with higher activity than each enzyme in the original crude extract. Previous studies have already used pH 6, as a compromise between the optimal pH for *T. reesei* and *T. fusca* enzymes [41], [42].

Family 10 xylanase, XynZ-C, showed higher synergism than family11, Xyn11A, when combined with *T. reesei* commercial cellulase. However, the use of GH10 and GH11 xylanases, simultaneasly, promoted the highest improvement in the hydrolytic activity of the commercial cellulase, at same enzyme loading (Figure 5). The synergistic interaction between XynZ-C and Xyn11A, proved to be efficient when combined to commercial cellulase mixture in the enhancement of pretreated bagasse hydrolysis. The development of new formulations of enzyme cocktails is being approached by different research groups, to reduce enzyme loading for biomass conversion. In addition, hemicellulase activity in commercial cellulase cocktails is usually minimum and not enough to convert all hemicellulose content, especially from biomass pretreated under milder conditions [43]. The addition of family 10 and 11

xylanases to commercial cellulase preparations seems to be of high relevance for industrial process. The interactions of xylan and cellulose in pretreated biomass reduce the accessibility of cellulase to celulose structure, and the synergy factor for glucose formation and reducing sugar release were shown to increase linearly with the xylan content in the substrate [44]. Recent study has demonstrated that the combination of xylanases (GH10 and GH11) and xyloglucanase (GH5), with commercial cellulase mixture improved the overall cellulose hydrolysis in pretreated corn fiber, sweet sorghum bagasse, corn stover, poplar and lodgepole pine, but the relative improvement seems to be substrate dependent [6]. Another recent study using sugarcane bagasse has shown that the interaction between cellulases and xylanases can also be related to the pretreatment method, and consequently chemical composition of the pretreated biomass [45] .

Here, pure xylanases, were used as accessory enzymes, and clear synergism was observed between pure xylanases and the commercial cellulase preparation. Interestingly, continuing synergy was observed between the commercial cellulase from *T. reesei* and the binary xylanase mixture (XynZ-C and Xyn11A) (Figure 4), with a DS reduction of 30 %, from hour 6 to hour 72, compared to a reduction of 50 % when the same binary mixture was applied sole to the pretreated bagasse (Table 4). These results demonstrate the potential use of xylanases as accessory enzymes to reduce total enzyme loading during the hydrolysis of pretreated bagasse. Further studies should be performed in order to optimize an ideal ratio between xylanases and cellulases for minimum enzyme dosage and maximum conversion of the pretreated biomass.

4. Conclusion

The majority of the xylanases characterized nowadays belong to GH families 10 and 11, thus a comprehension of the interaction between these xylanases and cellulases would be of the utmost importance for industrial application. In this study, we have first evaluated the interaction among xylanases from GH10 and GH11 from three different organisms, and we have identified that mixing the different GH families generates significant synergistic effect during pretreated bagasse hydrolysis. Furthermore, the synergism observed between GH10 and GH11 xylanases had positive effect on the hydrolytic activity of a commercial cellulase mixture from *T. reesei* during pretreated bagasse hydrolysis.

Acknowledgments

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The authors declare that they have no competing interests.

Author's contribution

Designed the experiments: GALG, YS, LJ, YM, SN, TT, HI and NK. Performed the experiments: GALG, YS, LJ, and HI. Analyzed the data: GALG, YS, LJ, YM, SN, TT, HI and NK. Contributed to reagents and materials: TT, HI and NK. Wrote the paper: GALG and NK. All authors read and approved the final manuscript.

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Figure Captions

Figure 1. SDS-PAGE of the purified enzymes used in this study: XynZ-C (lane1), Xyn11A (lane 2), Xyn10B (lane 3), XlnB (lane 4), XlnC (lane5) and marker (lane M).

Figure 2. Comparative hydrolysis of pretreated bagasse by xylanases from family GH10, sole or mixed, and GH11, sole or mixed, from *C. thermocellum*, *T. fusca* and *S. lividans*. Reactions were prepared by mixing enzymes from the same family (GH10 or GH11), or from both families (GH10 and GH11). Enzymatic activity was measured as mM of reducing sugars. Reactions were performed for 72 hours, and samples were taken at hour 3 (yellow bars), hour 6 (red bars), hour 12 (green bars), hour 24 (purple bars), hour 48 (blue bars) and hour 72 (orange bars). Error bars are shown from duplicates.

Figure 3. Determination of soluble sugars after hydrolysis of pretreated bagasse by xylanases from family GH10, sole or mixed, and GH11, sole or mixed, from *C. thermocellum*, *T. fusca* and *S. lividans*. Reactions were prepared by mixing enzymes from the same family (GH10 or GH11), or from both families (GH10 and GH11). The concentration of xylose (black bars), xylobiose (grey bars) and xylotriose (white bars) were determined as mM after 72 hours of reaction. Error bars are shown from duplicates.

Figure 4. Synergistic action of xylanases from family 10 and 11 during hydrolysis of pretreated bagasse. Synergies were calculated at hour 6, for mixing enzymes of different families, GH10 and GH11 (black), or same family, GH10 or GH11 (white).

Figure 5. Comparative hydrolysis of pretreated bagasse by commercial cellulase preparation from *T. reesei* (Cel), sole or mixed, with GH10 xylanase (XynZ-C) and/or GH11 xylanase (Xyn11A). Enzymatic activity was measured as mM of reducing sugars. Reactions were performed for 72 hours. Error bars are shown from duplicates.

Table 1. Enzymes used in this study

Protein name	**CBM**	**Microorganism**	**Xylanase activity (U/mg of enzyme)[d]**	**MW (kDa)**	**Family**
XynZ-C	_[a]	*Clostridium thermocellum*	93.0	38.0	GH10
Xyn10B (or Tfu2791)	_[b]	*Thermobifida fusca*	45.3	45.1	GH10
Xyn11A (or Tfu1213)	XBM[c]	*Thermobifida fusca*	51.0	36.4	GH11
XlnB	CBM	*Streptomyces lividans*	60.0	35.6	GH11
XlnC	_[b]	*Streptomyces lividans*	119.5	24.7	GH11

[a] XynZ consists of N-terminus ferulolyl esterase domain, a family 6 carbohydrate binding domain, a dockerin and a glycoside hydrolase family 10 domain. However, in this study, only the catalytic domain was expressed (XynZ-C).
[b] The specific enzymes do not contain CBM.
[c] XBM can bind to xylan or cellulose.
[d] 1 unit (U) = 1 μmol of reducing sugars / min

Table 2. Chemical composition of the untreated and PAA-treated bagasse

	Untreated	PAA pretreated
Glucan (%)	37.7 ± 1.1	38.6 ± 0.4
Xylan (%)	22.0 ± 5.6	21.0 ± 0.7
Arabinan (%)	2.8 ± 0.8	0.9 ± 0.3
Ash (%)	3.7 ± 0.4	2.7 ± 0.5
Extractives (%)	13.1 ± 1.6	11.5 ± 0.4
Acid insoluble lignin (%)	20.2 ± 0.8	14.9 ± 2.5
Acid soluble lignin (%)	2.3 ± 0.1	6.3 ± 0.3

Table 3 – The degree of synergy of xylanases from glycoside hydrolase (GH) families 10 and 11.

Reaction time (h)	Same GH family				Different GH families					
	XynZ-C + Xyn10B	**Xyn11A + XlnB**	**Xyn11A + XlnC**	**XlnB + XlnC**	**XynZ-C + Xyn11A**	**XynZ-C + XlnB**	**XynZ-C + XlnC**	**Xyn10B + Xyn11A**	**Xyn10B + XlnB**	**Xyn10B + XlnC**
6	0.27	0.95	1.14	1.19	2.83	3.62	3.30	3.25	1.41	3.25
12	0.98	0.99	0.85	1.51	1.89	3.58	2.28	2.44	2.93	2.14
24	1.38	1.22	0.99	1.46	1.80	2.72	2.14	2.58	2.59	2.75
48	1.47	1.22	1.08	1.30	1.46	2.22	1.73	2.23	2.30	2.02
72	1.43	1.31	1.12	1.35	1.50	1.86	1.72	2.16	2.15	1.97

Table 4. The degree of synergy of xylanases and cellulases

Reaction time (h)	**Cel + XynZ-C**	**Cel + Xyn11A**	**Cel + XynZ-C + Xyn11A**	**XynZ-C + Xyn11A**
6	2.39	2.19	3.67	2.83
12	2.41	1.91	3.19	1.89
24	2.34	1.89	3.01	1.80
48	2.26	1.81	2.73	1.46
72	2.20	1.92	2.55	1.50

Figure 1

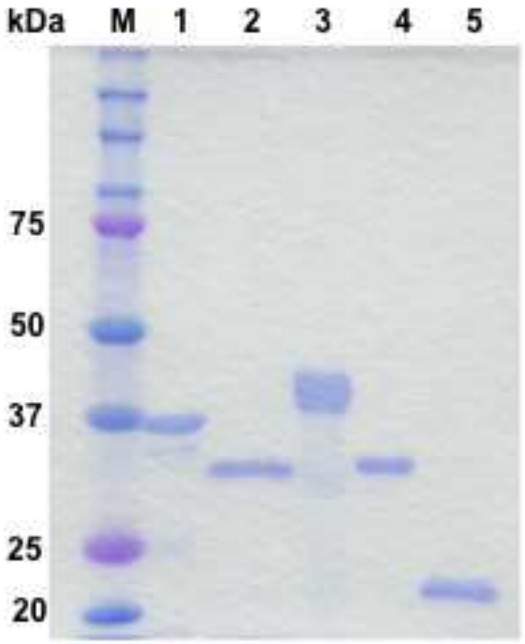
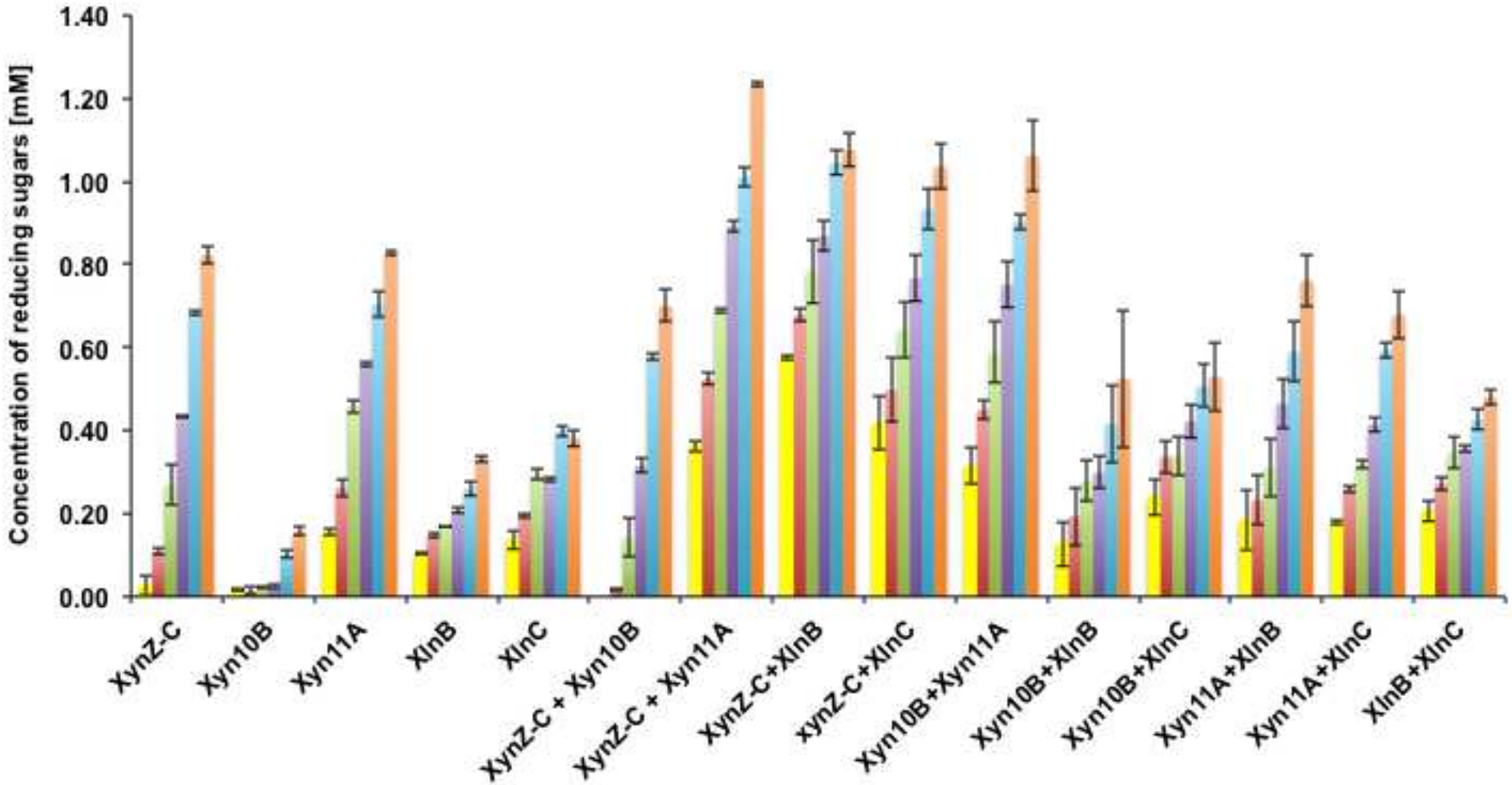


Figure 2


Concentration of reducing sugars [mM]
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
XynZ-C
Xyn10B
Xyn11A
XlnB
XlnC
XynZ-C + Xyn10B
XynZ-C + Xyn11A
XynZ-C+XlnB
xynZ-C+XlnC
Xyn10B+Xyn11A
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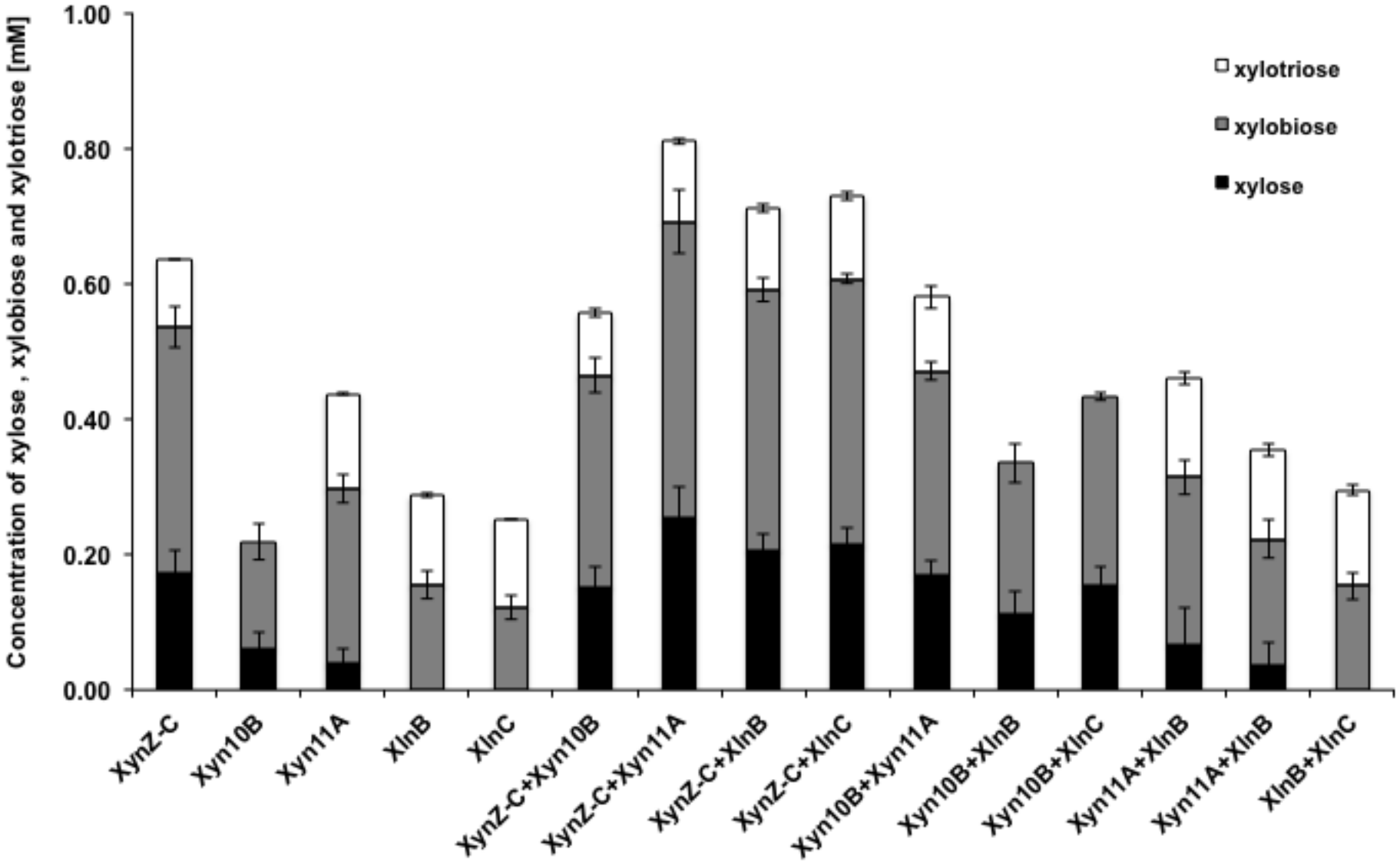
XlnB+XlnC

Figure 3


Concentration of xylose , xylobiose and xylotriose [mM]
1.00
0.80
0.60
0.40
0.20
0.00
xylotriose
xylobiose
xylose
XynZ-C
Xyn10B
Xyn11A
XlnB
XlnC
XynZ-C+Xyn10B
XynZ-C+Xyn11A
XynZ-C+XlnB
XynZ-C+XlnC
Xyn10B+Xyn11A
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Xyn11A+XlnB
Xyn11A+XlnB
XlnB+XlnC

Figure 4

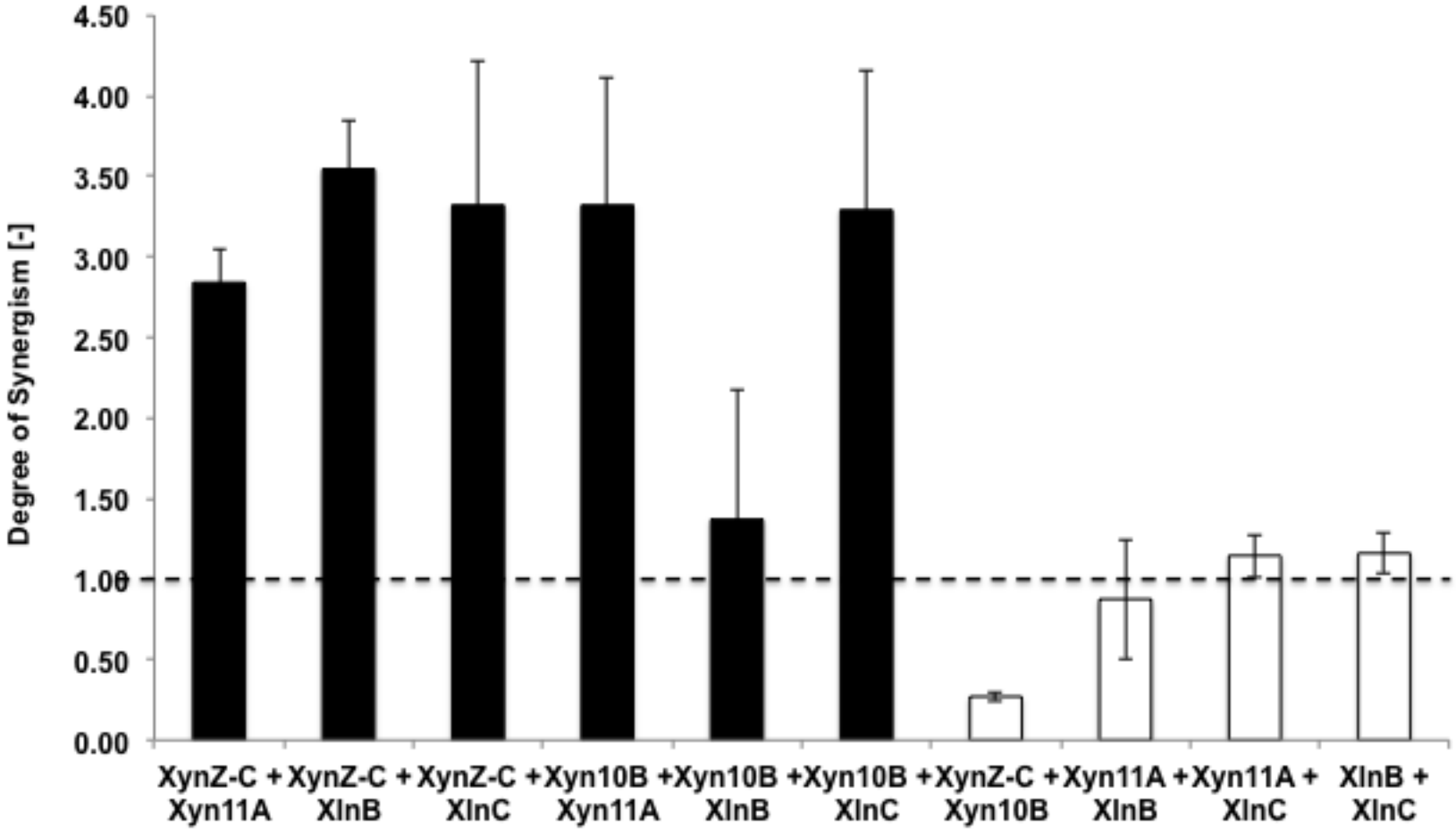
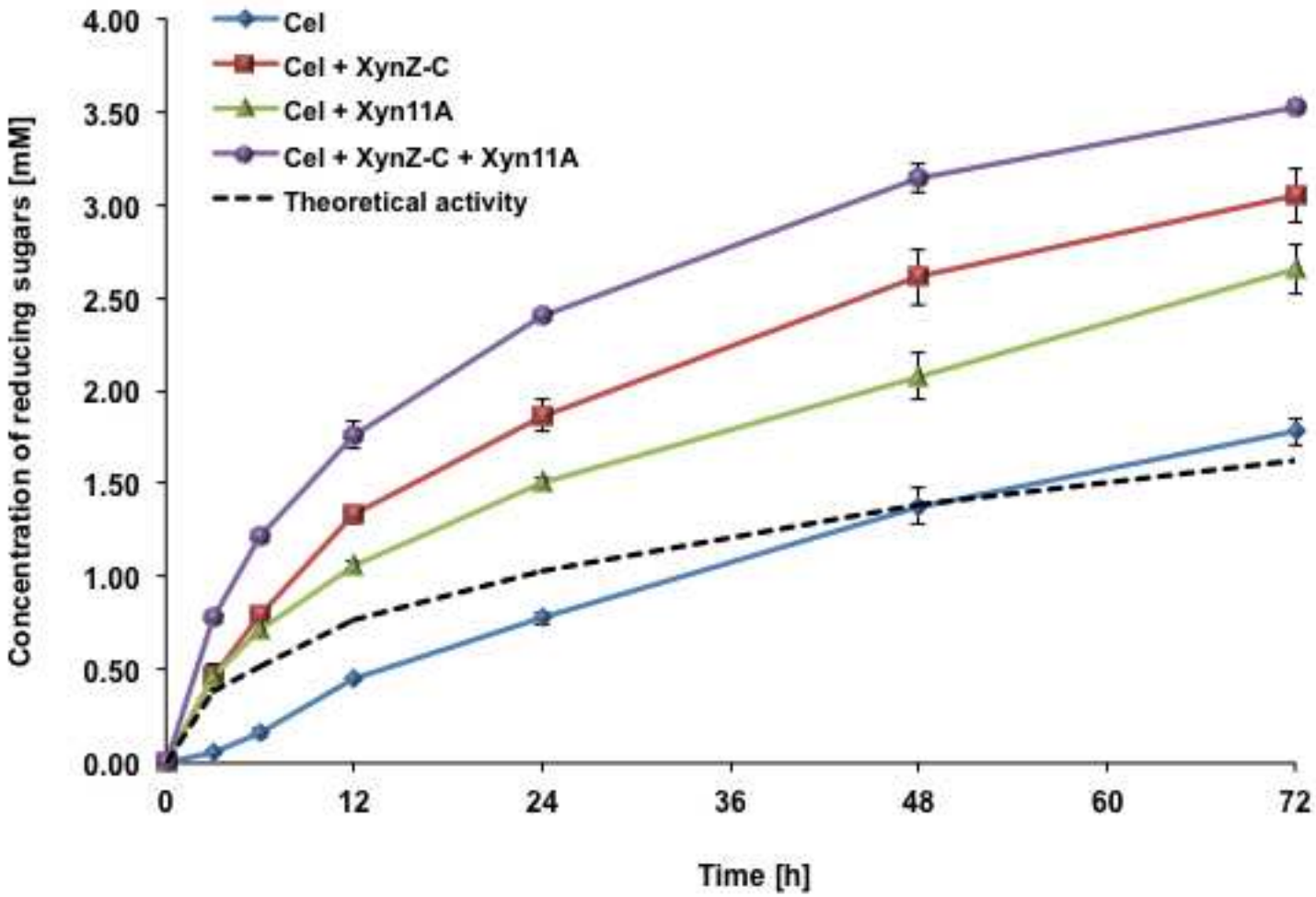

Degree of Synergism [-]
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
XynZ-C + Xyn11A
XynZ-C + XlnB
XynZ-C + XlnC
Xyn10B + Xyn11A
Xyn10B + XlnB
Xyn10B + XlnC
XynZ-C + Xyn10B
Xyn11A + XlnB
Xyn11A + XlnC
XlnB + XlnC

Figure 5


Cel
Cel + XynZ-C
Cel + Xyn11A
Cel + XynZ-C + Xyn11A
Theoretical activity
Concentration of reducing sugars [mM]
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
12
24
36
48
60
72
Time [h]