Universidade de Brasília - UnB

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DESENVOLVIMENTO DE COMPLEXOS ENZIMÁTICOS BASEADOS EM XILANASES DE *Clostridium thermocellum*

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Tese de doutorado apresentada ao Programa

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Desenvolvimento de complexos enzimáticos baseados em xilanases de *Clostridium thermocellum*

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Dedicatória

In memoriam

Wally Hamann

Elioteria Vieira das Virgens

Resumo

Clostridium thermocellum é uma bactéria anaeróbica termófila, especializada na degradação de compostos celulósicos por meio da secreção de um complexo enzimático chamado de celulossomas. Dentre as principais característica que chamam a atenção para celulossomas como maquinaria enzimática para degradação da parede celular vegetal, tem-se a presença de diversas atividades além das celulases, como xilanases e pectinases. Apesar do grande número de estudos em relação ao efeito da formação de complexos enzimáticos formados por celulases, pouco se sabe da vantagem da organização de xilanases em complexos enzimáticos, assim como o efeito de tal complexação em aplicações biotecnológicas. No presente trabalho, foi realizada a expressão heteróloga das xilanases XynZ, e XynA, assim como a porção n-terminal da proteína estrutural CipA, contendo dois domínios cohesin e um domínio de associação a celulose. Para a enzima XynA, foi realizado um primeiro trabalho experimental para avaliar o efeito de compostos fenólicos derivados da biomassa lignocelulósica em sua atividade xilanolítica. Dentre os compostos avaliados, ácido tânico causou maior perda na atividade quando a enzima foi exposta a temperatura de 60ºC. Quando a enzima foi exposta aos compostos fenólicos a baixa temperatura (40ºC), a perda da atividade tornou-se reversível. Ainda em relação a xilanase XynA, a mesma foi utilizada para formulação de complexo enzimático usando a região n-terminal da proteína estrutural CipA. Quando o complexo enzimático formado foi utilizado para hidrólise do bagaço da cana de açúcar tratado, apresentou maior desempenho na liberação de açúcares redutores, em relação a mesma carga enzimática utilizando a enzima em sua forma não associada ao complexo. Também foi observado que a proteína estrutural, quando utilizada para suplementar misturas enzimáticas de origem fúngica para hidrólise de biomassa lignocelulósica, foi capaz de melhorar a produção de D-glicose. A enzima XynZ também foi utilizada para produção de mini xilanossomas utilizando a proteína estrutural CipA com dois domínios cohesin e um domínio de associação a carboidratos, o complexo formado apresentou efeito de pH e temperatura similares ao da enzima não complexada, entretendo observou-se ganho na estabilidade térmica quando a enzima estava associada a um complexo. Quando o mini xilanossoma foi utilizado para hidrolisar fibras de sisal, foi observado geração de açúcares redutores até três vezes superiores em comparação quando utilizado a enzima em sua forma não complexada. Os resultados demonstrados no presente trabalho atestam que a complexação de xilanases em mini-xilanosomas podem contribuir para uma maior desconstrução da biomassa lignocelulósica.

Abstract

Clostridum thermocellum is an anaerobic thermophilic bacterium that is specialized in degrading cellulosic materials through the secretion of enzymatic complexes, namely cellulosomes. The main feature that calls attention for cellulosomes as machinery for plant cell wall degradation is several hydrolytic activities along with cellulases, including xylanases and pectinases. Despite the great number of studies regarding the cellulosome assembly using cellulases, little is known about the advantage of organizing xylanases in enzymatic complexes and the effect of complexed xylanases in biotechnological applications. In the present study, the heterologous expression of xylanases XynZ and XynA was done, and the N-terminus portion of the scaffolding proteins CipA, containing two cohesin domains and a cellulose-binding module. For the enzyme XynA, a first study was conducted to evaluate the effect of phenolic compounds derived from lignocellulose on XynA's xylanase activity. Among the evaluated compounds, tannic acid caused the highest activity decay when assays were conducted at 60°C. However, when XynA was exposed to phenolic compounds at a lower temperature (40ºC), activity loss could be reversible. Still, regarding XynA, this enzyme was used to assembly mini-xylanosomes with the N-terminus portion of scaffolding protein CipA. The formed enzymatic complex was used to hydrolyze chemically treated sugarcane bagasse and showed a better release of reducing sugar than the enzyme on its non-complexed state and same activity load. It was also observed that the scaffolding protein, when coupled with fungal enzymes in hydrolysis of lignocellulose, was capable of improving the D-glucose production. Regarding XynZ, this enzyme was also used to assemble mini-xylanosomes. The assembled complex displayed similar pH and temperature effect in xylanase activity compared to the free enzyme. However, was observed improvement in thermostability property for the mini-xylanosome. When applying the mini-xylansome to hydrolyze sisal fibers, was observed production of reducing sugar three times higher than those observed for non-complexed enzymes. Results shown in the present study attest that the assembly of xylanases into mini-xylanosomes can improve lignocellulose deconstruction.

Sumário

Apresentação da tese

A presente tese foi organizada em cinco capítulos, que foram publicados ou estão em avaliação por periódicos científicos internacionais. Antes da apresentação dos manuscritos, uma breve introdução é apresentada para facilitar o entendimento do contexto científico que cada capítulo se encontra.

O Capítulo I é uma ampla revisão bibliográfica contendo o histórico da descrição de cada enzima contendo atividade xilanolítica descrita para a bactéria *Clostridium thermocellum*, assim como as implicações biotecnológicas do uso desses biocatalisadores no contexto de biorefinaria.

O Capítulo II é uma revisão da literatura descrevendo um dos recentes tópicos em relação ao uso de enzimas em biorefinarias, a inibição e desativação gerados por compostos oriundos da biomassa lignocelulósica, tendo como enfoque as endo-β-1,4-xilanases, sendo esse capítulo base para entendimento dos experimentos realizados no capítulo seguinte.

O Capítulo III (publicado: Process Biochemistry, Elsevier) mostra o efeito de compostos aromáticos/fenólicos derivados da biomassa lignocelulósica na proteína recombinante XynA, sendo investigados o efeito de tais compostos em uma xilanase que apresenta elevada estabilidade térmica.

Os Capítulos IV e V (mostram a formação de complexos enzimático s tendo como base uma versão reduzida da proteína estrutural, mCipA, e aplicação desses mini-xilanossomas em aplicações biotecnológicas. O Capítulo IV mostrara a formação de complexos utilizando a xilanase XynA, e suas implicações na desconstrução do bagaço de cana-de-açúcar. O Capítulo V mostra a formação de complexos enzimáticos utilizando como base a xilanase XynZ, e sua aplicação na solubilização da hemicelulose presentes em fibras de sisal.

A última parte da tese consiste de um panorama geral dos achados científicos descritos no presente trabalho, assim como as perspectivas biotecnológicas, e os pontos que poderão ser abordados em trabalhos futuros a serem realizados no Laboratório de Enzimologia – UnB.

Pedro Ricardo Vieira Hamann

Brasília, junho de 2021

Objetivos

Devido a importância de *Clostridium thermocellum* como um organismo modelo na desconstrução da parede celular vegetal, a presente tese tem como objetivo central a montagem de complexos enzimáticos, tendo como base duas xilanases de *Clostridium thermocellum*, XynZ e XynA, tendo como principal questionamento saber se a associação de xilanases a uma proteína estrutural por meio da associação dos domínios dockerin:cohesin apresenta alguma vantagem em termos de desconstrução da hemicelulose, assim como suas implicações em aplicações biotecnológicas.

Além da formação dos complexos enzimáticos, a tese também tem como objetivo secundário a caracterização de xilanases termoestáveis de *Clostridium thermocellum*, levando em consideração temas de recente interesse industrial, como o efeito de inibidores derivados da lignocelulose, como os compostos fenólicos.

CAPÍTULO I – Revisão da literatura

XYLAN DECONSTRUCTION APPARATUS OF *Clostridium thermocellum*

Com as atuais demandas por desenvolvimento de energias renováveis e a mudança para uma matriz de produção com menor dependência de produtos de fontes não renováveis, existe uma vertente científica crescente para a utilização de rejeitos agroindustriais como fonte de carbono para uma nova matriz renovável. Dentre os principais problemas observados para o aproveitamento de resíduos agroindustriais como matriz para o desenvolvimento de novos produtos é a sua recalcitrância. Sendo a desconstrução da biomassa lignocelulósica um dos principais empecilhos para sua ampla utilização.

A recalcitrância dos resíduos agroindustriais faz com que várias metodologias venham sendo exploradas para que os diversos materiais presentes na constituição de tais resíduos possam ser disponibilizados como fonte de carbono para produção de compostos químicos de interesse industrial. No passado, a fração celulósica de tais compostos recebeu muita atenção, devido ao modelo de aproveitamento dos resíduos ser majoritariamente desenvolvido para a fermentação de hexoses. Entretanto, atualmente tem-se uma visão mais ampla em que os outros constituintes dos resíduos agroindústrias devem ser aproveitados ao máximo possível. Podendo assim serem gerados vários outros produtos de valor agregado.

Um dos componentes da biomassa lignocelulósica que vem recebendo interesse é a fração de carboidratos que estão ao redor das fibras celulósicas, a hemicelulose. Dentre os principais fatores que levaram a busca do aproveitamento da hemicelulose está o fato do desenvolvimento de linhagens de microrganismos que possam fermentar além de hexoses, as pentoses que estão presentes em sua maior parte na hemicelulose de resíduos industriais. Para a desconstrução por vias biológicas da hemicelulose composta por pentoses, dentre estes a xilana, um repertório de enzimas é necessário para que se obtenha a completa desconstrução dessa importante fração dos resíduos lignocelulósicos.

Enzimas com atividade contra a xilana recebem atenção por sua versatilidade em aplicações industriais. Classicamente enzimas que desempenham papel na degradação da parede celular vegetal recebem atenção para o setor de biocombustíveis. Entretanto, xilanases podem ser aplicadas em diversos outros setores industrias, como no preparado

de polpa celulósicas, adição para a nutrição de animais, produção de açúcares com função probiótica, remoção da hemicelulose para produção de fibras celulósicas, e na indústria da panificação.

Dentre os organismos estudados para desconstrução da hemicelulose, fungos filamentosos sempre foram a principal fonte a ser explorada. Entretanto a busca por diferentes biocatalisadores fez com que outras fontes menos tradicionais de microrganismos fossem exploradas para bioprospecção de hemicelulases. Na procura por organismos que possam de forma eficiente desconstruir os componentes da parede celular vegetal, a bactéria anaeróbica termófila *C. thermocellum* é muito estudada, por seu potencial em degradar a celulose. Sendo um dos organismos reportados com maior eficiência na desconstrução de compostos celulósicos.

O potencial de *C. thermocellum* em desconstruir a celulose ocorre pela forma com que suas enzimas são organizadas em complexos enzimáticos, o celulossoma. De forma geral, enzimas que constituem este complexo, celulossomas, produzidas por essa bactéria apresentam o domínio *dockerin* I que é capaz de se associar com os domínios *cohesin* I presentes em proteínas estruturais. Como exemplo de proteína estrutural tem-se a CipA, proteína que é dotada de nove domínios cohesin I, um módulo de associação a carboidratos (CBM3) e um domínio *dockerin* II para associação do complexo a membrana bacteriana por meio de interações *cohesin* II *dockerin* II. De forma geral, a composição mínima de um celulossoma é uma proteína estrutural (CipA) associadas a proteínas por meio da interação *dockerin*I:*cohesin*I.

Além da produção de complexos enzimáticos contendo celulases, outras atividades como hemicelulases e pectinases estão presentes na formulação do complexo enzimático. Apesar de *C. thermocellum* ser um organismo que apenas consome hexoses da fração de carboidratos da parede celular vegetal, suas xilanases vem recebendo atenção por seu potencial biotecnológico. Xilanases produzidas por *C. thermocellum* além de apresentarem domínios *dockerin* I para que sejam integradas ao celulosoma, apresentam outros domínios que auxiliam na desconstrução da hemicelulose, como domínios de associação a carboidratos específicos à hemicelulose, e também domínios catalíticos com atividades auxiliares, que serão detalhadas na revisão abaixo.

Juntamente com a capacidade em agregar xilanases em complexos enzimáticos, as propriedades catalíticas dessas enzimas produzidas por *C. thermocellum* apresentam características desejáveis em aplicações industriais. Dentre as propriedades que as

xilanases de *C. thermocellum* apresentam, tem-se a elevada estabilidade térmica e o efeito sinérgico/cooperativo entre glicosil-hidrolases e domínios acessórios presentes em uma única cadeia peptídica.

O presente capítulo mostrará um panorama histórico da descoberta e caracterização de xilanases de *C. thermocellum*, assim como uma abordagem relacionada a suas possíveis aplicações industriais. As informações contidas no presente capítulo servirão de base teórica para os capítulos subsequentes.

Xylan breakdown apparatus of *Clostridium thermocellum*

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Abstract

Clostridium thermocellum has been studied as a model organism highly specialized in cellulose deconstruction and secretion of cellulolytic enzymes organized into an enzymatic complex called the cellulosome. Lignocellulose deconstruction requires a varied group of enzymes; in general, hemicellulases first act to open access by removing the hemicellulose, making the cellulose core available for cellulases action. In addition to cellulase production, *C. thermocellum* is a producer of xylanases, which might be found as cellulosomes components. These enzymes work in synergy with cellulosomal cellulases for the complete hydrolysis of holocellulose. It is also worth mentioning that xylan-degrading enzymes are currently highly sought after for industrial applications, including biofuels and paper and pulp production. In this context, industrial processes could take advantage of *C. thermocellum*'s xylanases properties as thermal stability and specificity to xylans from different biomasses. In this review, we aimed at describing the potential of *C. thermocellum* in degrading xylan-based polymers by showing the main findings regarding xylan degrading enzymes synthesized by this organism. Features of these enzymes, including the modular architecture, specificity to different xylan polymers, and biochemical properties, are shown, and custom-tailored xylanosomes are proposed as enzymatic machinery for hemicellulose deconstruction.

Keywords: Xylan, Xylanase, *Clostridium thermocellum*, cellulosomes, biofuels

Clostridium thermocellum as a model organism in lignocellulose degradation: A brief introduction

The search for an organism with the natural ability to degrade cellulose has been gaining attention in the biorefinery scenario; many organisms were studied and investigated, including thermophiles (Turner et al. 2007). Despite most studies and commercial formulations of plant cell wall degrading enzymes rely on fungal enzymes, there is also interest in developing new enzyme blends with different working parameters, such as extreme temperatures and tolerance to hydrolyses end-products.

In this scenario of microorganisms bioprospection aiming at cellulose deconstruction, the Gram-positive thermophilic anaerobic bacterium *Clostridium thermocellum* has received attention due to its efficiency in consuming cellulosic materials. *C. thermocellum* isolates have been obtained from a diversity of environments, including cow dung (Blume et al. 2013), goat rumen (Hamann et al. 2015), cotton bale (Freier et al. 1988), horse manure, and soil (McBEE 1954). Isolates of *C. thermocellum* were reported as optimally growing at a temperature of 60 ºC and consume a variety of cellulosic materials such as cotton fibers, paper, steam-exploded hardwood, sugarcane bagasse, and cellulose (Freier et al. 1988; Blume et al. 2013; Hamann et al. 2015).

An early study regarding *C. thermocellum* was mainly focused on describing its potential in consuming cellulosic substrates and the general properties of its cellulosic system (Ng et al. 1977). The remarkable capacity of this bacterium for cellulosic material degradation was later revealed as a result of the unusual architecture of its cellulases into a well-organized enzymatic complex named cellulosome (Bayer et al. 1998, 2004). As a basic definition, cellulosomes are comprised of enzymes harboring domains called dockerin I that non-covalently interact with cohesin I modules present in the scaffoldin protein, resulting in a high molecular mass protein complex. The scaffoldin protein CipA is the major component of *C. thermocellum*'s cellulosome; this protein contains nine cohesins I modules, one carbohydrate-binding module family 3, and one dockerin II module (Demain et al. 2005).

Complementary to CipA, there is also the possibility of anchoring the cellulosome to membrane-bound proteins through dockerin II and cohesin II interactions. Besides the cohesin II module, these proteins also contain S-layer homology domains (SLH) responsible for attaching these anchoring structures to the bacterial cell surface. Examples of these proteins are SdbA, Orf2, and OlpB, respectively harboring one, two, and seven

cohesin II modules, and thus generating enzymatic complexes with up to 63 enzymes (Bayer et al. 2008; Fontes and Gilbert 2010). Other proteins harboring membrane anchoring proteins also possess cohesin I modules, allowing the unitary attachment of cellulosomal proteins to bacterial membrane; an example of those proteins are OlpC, OlpA, both carrying one cohesin I module (Fontes and Gilbert 2010).

Extracellular polycellulosomes are also generated by the dockerin II and cohesin II modules interaction present on scaffold proteins CipA and the protein ScaE (Cthe_0736). ScaE has seven cohesins II modules on its structure and no membrane associate domain, thus an extracellular polycellulosome. The possibility of assembling polycellulosomes brings a higher level of complexity for the plant cell wall degrading apparatus of *C. thermocellum*, and this novel level of complexity can be further explored for designing more efficient plant biomass deconstruction processes (Xu et al.). **Representation of cellulosomes is shown in figure 1**.

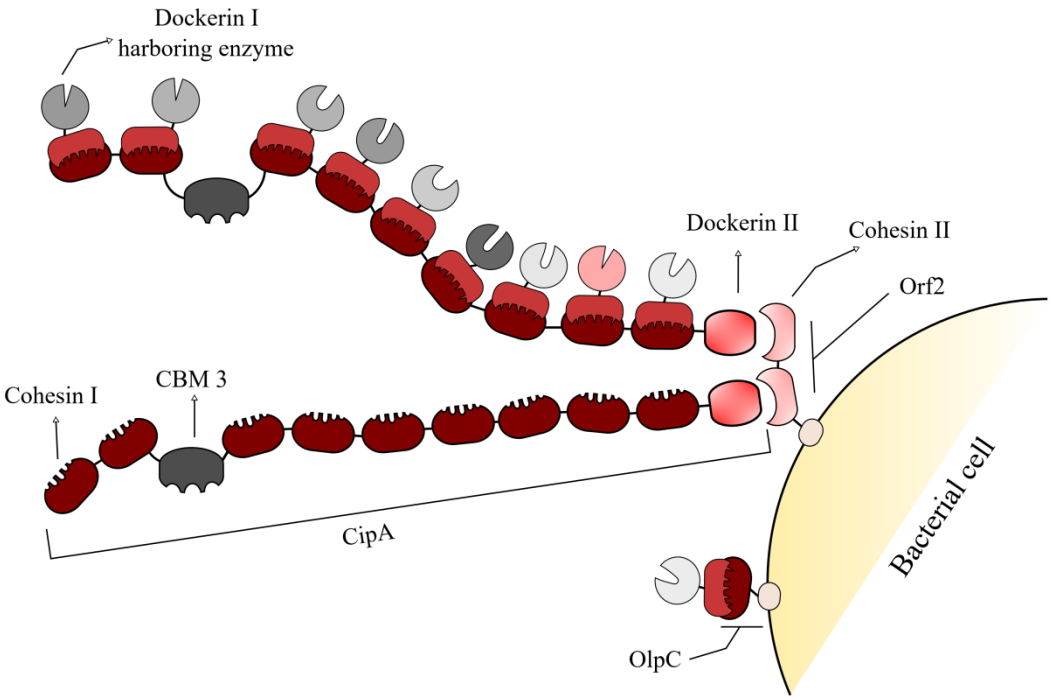

Figure 1: Schematic representation of *C.thermocellum*cellulosome. Cipa (Cthe_3077), OlpC (Cthe_0452), Orf2 (Cthe_3079). Dockerin II modules associates with membrane-bound anchoring proteins through DockerinII:Cohesin II interaction. Enzymes associate to scaffold protein (CipA), and membrane-bound proteins (OlpC), by dockerinI:cohesin I interaction. CBM3 (Carbohydrate binding module family 3).

Xylan structure and enzymes involved in xylan break down

Three major components constitute the plant cell wall: cellulose, hemicelluloses, and lignin, the hemicellulose formed by xylan is the second most abundant polysaccharide. Xylan is found in plant cell walls surrounding cellulose fibers and is covalently associated with lignin (Bastawde 1992). The xylan central backbone consist of D-xylose unities joined by β(1,4) glycosyl linkages, containing ramifications termed side chains. In a general representation, side chains included α(1,2) glucuronic acid, α(1,2)/α(1,3) arabinose or α(1,2) 4-O-methyl glucuronic acid, acetylation in xylan main structure is observed in O2/O3 positions (Bastawde 1992; Correia et al. 2011). In addition to sugars/acetyl groups, xylan may also have *p*-coumaric/ ferulic acids attached to O-5 arabinose residues through ester linkages (Smith and Hartley 1983).

The ratio of xylan's side chains and the acetylation level depend on the xylan source. In hardwoods, glucuronic acid is the main side chain; thus, they are named glucuronoxylans, and are usually irregularly distributed over the xylan backbone (Jacobs et al. 2001; Biely et al. 2016). Some of the lignin's phenylpropane unities are ester-linked to glucuronic acid groups; for instance, it was reported that one-third of glucuronic acid in the water-soluble lignin-carbohydrate complex from beech is ester-linked to lignin (Takahashi and Koshijima 1988). In addition to xylose and glucuronic acid, there is also evidence that hardwood xylan may also contain galactosyl residues attached to methyl glucuronic acid through α(1,2) linkage, for *Eucalyptus globulus*, the molar ratio of galactose, methylglucuronic acid, and xylose has been reported as 1:3:30 (Shatalov et al. 1999). The xylan rich in arabinose side chains, arabinoxylan, can be classified as neutral arabinoxylan, on which there is no glucuronic acid side chain attached to xylan's main backbone; this arabinoxylan is found in the cell wall of cereals (Biely et al. 2016). The second class of arabinoxylan, arabinoglucuronoxylan or acid arabinoxylan, has arabinose and methyl glucuronic ramifications, such class of xylan is present in several industrial relevant crops, including sugarcane and corn (Kim and Holtzapple 2006; Biely et al. 2016; Bartos et al. 2020).

For a complete enzymatic deconstruction of xylans, a diversified group of enzymes must remove side chains and hydrolyze the main backbone. Indeed, side groups and acetyl groups must be removed to make the xylan central core more accessible to other enzymes (Gilbert and Hazlewood 1993; Patel and Savanth 2015).

Acetyl groups present in the xylan backbone are removed by the action of acetyl xylan esterases (EC 3.1.1.72); this class of enzymes has been reported mainly for members of carbohydrate esterases (CE) families 1, 5, and 6 (Biely et al. 2016). These enzymes have been reported for a variety of microorganisms, and for *T. reesei*, it was described that acetyl xylan esterases could remove acetyl groups of high molecular weight xylan and acetylated monomeric xylose (Poutanen et al. 1990). Side chains such as glucuronic acid, and arabinose, are removed by α-glucuronidases (EC 3.2.1.131) and α-arabinofuranosidases (EC 3.2.1.55). α-glucuronidases are not in-deep investigated as seen for other classes of enzymes involved in xylan deconstruction. In general, this class of enzyme is found in glycoside hydrolase families 67 and 115. Those from GH67 are described as only capable of removing glucuronic acid side chains attached to a non-reducing end xylose, whereas those from GH115 can remove glucuronic acid from internal xylose residues (Biely et al. 2000). Ferulic acid that is covalently bound to hemicellulose is removed by the action of feruloyl esterases (EC 3.1.1.1). Representation of decorated xylan enzymatic deconstruction is present in **figure 2**.

The main backbone of xylan, in a simplified explanation, is depolymerized by two groups of enzymes, endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). Endo-β-1,4-xylanases (EC 3.2.1.8) catalyze the internal linkage β(1,4) in xylan's main backbone releasing saccharides, including xylobiose and xylotriose. Saccharides released by the action of endo-β-1,4 xylanases are further hydrolyzed by β-xylosidases, which catalyze the hydrolysis of β-1,4-bonds, releasing D-xylose.

Although endo-mode acting enzymes and β-xylosidases can simplify the classical mechanism of xylan deconstruction, currently, there is information that this deconstruction is also orchestrated by the presence of endo-β-1,4-xylanases from different families. The most-reported synergistic mechanism of endo-β-1,4-xylanases activity in xylan's primary backbone deconstruction is the action of GH10 and GH11 endo-β-1,4-xylanases. In summary, GH10 and GH11 xylanases catalyze the same reaction, cleaving β(1,4) linkages in the xylan backbone. However, they require different levels of side chains to having access to the xylan backbone. For instance, GH11 recognizes the main backbone when there are three sequential unramified xylose residues, and GH10 only needs two sequential unramified residues (Biely et al. 2016).

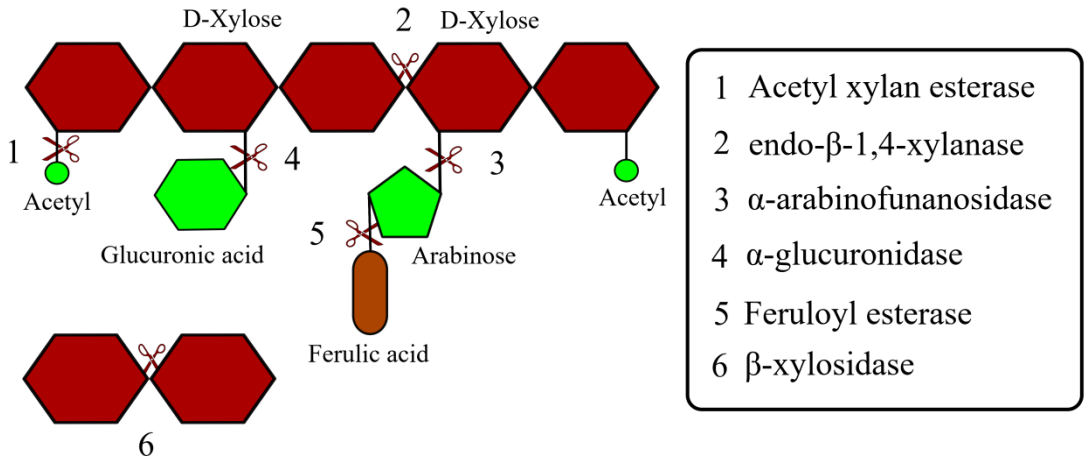

Figure 2: Representation of decorated xylan backbone with side chains (acetyl, glucuronic acid, arabinose), xylobiose, and enzymes related to xylan deconstruction: acetyl xylan esterase (EC 3.1.1.72), endo-β-1,4-xylanase (EC 3.2.1.8), α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.131), feruloyl esterase (EC 3.1.1.1), β-xylosidase (3.2.1.37). Enzymes and xylan architecture were based on the model proposed by Biely (1985) (Biely 1985).

For xylan containing glucuronic acid residues, GH10 and GH11 xylanases display different levels of substrate accessibility. GH10 can hydrolyze β(1,4) linkage of xylose residue containing the glucuronic acid, whereas GH11 requires an extra xylose residue to recognize the substrate and perform catalysis (Puchart et al. 2019). In addition to the GH11 and GH10 synergism, another group of endo-β-1,4-xylanases that have been receiving attention because of the mode they recognize xylan polymers containing glucuronic acid residues, GH30. This group of xylan degrading enzymes is specialized in hydrolyzing β(1,4) bonds in glucuronoxylans. It has been reported that GH30 endo-β-1,4-xylanases are specialized in recognizing the xylan's main backbone by identifying glucuronic acid side chains. Therefore GH30 xylanases are specialized in the deconstruction of glucuronoxylan (Šuchová et al. 2018)

More recently, in addition to endo-β-1,4-xylanases and β-xylosidases that are classically known for their role in xylan deconstruction, scientific evidence pointed out the existence of oxidative enzymes with activity against xylan (Couturier et al. 2018). Lytic polysaccharides monooxygenases (LPMOS) have recently been reported to be active in cellulose deconstruction. However, some oxidases may play a role in hemicellulose solubilization (Vidal-Melgosa et al. 2014; Vaaje-Kolstad et al. 2017).

C. thermocellum as a producer of xylanases, a brief historical panorama

Early evidence of *C. thermocellum* hemicellulolytic activity was reported by Gordon et al. (1978) after detection of production of xylose during growth in the presence of Solka-floc cellulose as a carbon source. Although Solka-floc is majorly formed by cellulose, the residual portion of hemicellulose preset was deconstructed during *C. thermocellum* growth; this study called attention to other authors. Later, Garcia-Martinez et al. (1980) showed that the supernatant of *C. thermocellum* ATCC 27405 grown in the presence of cellobiose could hydrolyze xylan. Ngt and Zeikus (1981) also detected the production of xylan degrading enzymes by *C. thermocellum* LQRI. This activity was detected against Larchwood xylan as well as 4-O-methylglucuronoxylan. Hydrolysis products showed that *C. thermocellum* xylanases could degrade Larchwood xylan and 4-O-methylglucurono xylan, the primary product released was xylobiose, and only small amounts of xylose were generated.

Briefly, Saddler and Chan (1982) described that the strains ATCC27450 and NRCC688 produced xylanases during growth utilizing Solka floc cellulose. Freier et al. (1988) demonstrated the capability in degrading xylan polymers when characterizing the strain JW20. Although the authors did not directly measure activity, they reported that *C. thermocellum* JW20 could hydrolyze xylan polymers. Kohring et al. (1990) studied the production of xylanases by *C. thermocellum* JW20. In their report, *C. thermocellum* was grown in the presence of ball-milled cellulose, Avicel, and cellobiose. The production of xylan degrading enzymes was detected on every tested condition, and they reported the presence of endo-β-1,4-xylanase activity against birchwood xylan on activity SDS-PAGE.

In addition to the previous description of *C. thermocellum* as a producer of xylanases, Morag et al. (1990) demonstrated endo-β-1,4-xylanases making part of cellulosomes. These enzymes also have been described in their free form, not associated with cellulosomes. Also, the authors reported that cellulosomes had minor β-xylosidase activity when assayed against synthetic substrates. However, they failed in converting β-xylanopiranoside into xylose. This report raised attention to the lack of β-xylosidase activity on *C. thermocellum*'s xylan degrading apparatus.

Despite most of the initial reports regarding the production of xylan-degrading enzymes by *C. thermocellum* employed carbohydrates in their purified form (cellulose,

Avicel, Solka Floc) as a carbon source, recent reports have focused on using more complexes carbon sources to the production of enzymes by *C. thermocellum* isolates.

As previously explained, more recent studies focused on the production of xylan degrading enzymes by *C. thermocellum* using industrial relevant carbon sources, as inexpensive lignocellulosic residues (Vieira et al. 2007; Blume et al. 2013; Hamann et al. 2015). Vieira et al. (2007) described the *C. thermocellum* ISOII as a producer of cellulosomes; when cultivating the bacterium using the banana stem as a carbon source, the principal hydrolytic activity associated with the complexes was xylanase. The enzymatic complex from ISOII also harbored residual β-xylosidase activity against the synthetic substrate *p*-nitrophenyl-β-D-xylopyranoside. Blume et al. (2013) characterize three isolates of *C. thermocellum*: ISOI, ISOII (same used by Vieira et al.), and JW20. They demonstrated the production of endo-β-1,4-xylanase when cultivating *C .themocellum* isolates in the presence of microcrystalline cellulose or sugarcane bagasse. Xylanase activities were found in the supernatant, as well as bound to the residual substrate.

As a result of the progress of omics techniques, the study of the production of plant cell wall degrading enzymes by *C. thermocellum* shifted to a broader analysis rather than directly detecting activities using traditional enzymatic assays. As an example of a proteomic study of cellulosomes, Zverlov et al. (2005) described the enzymatic profile of enzymatic complexes synthesized by the isolate *C. thermocellum* F7 (VKMB 2203) grown in the presence of microcrystalline cellulose. Among glycoside hydrolases identified, xylanases represented a significant portion of enzymes assembled in cellulosomes, as the xylanases XynC, XynZ, XynD. This kind of protein analysis permitted the identification of every component of *C. thermocellum's* enzyme repertoire secreted and actively working in xylan deconstruction.

Gold and Martin (2007) similarly detected xylanases as significant components of cellulosomes synthesized by strain ATCC27407. Protein identification was carried out using metallic isotope-labeling and nano-LC-ESI-MS, and the isolate was grown in the presence of Avicel or cellobiose. The following xylan-degrading enzymes were detected in cellulose-growth conditions: XynC, XynZ, XynA; additionally, it was described that different titers of xylanases and other glycoside hydrolases were seen as growth-condition dependent. For instance, the xylanase XynZ was more abundant in bacterial growth using cellobiose. Raman et al. (2009) performed quantitative proteomic analyses to compare

cellulosome composition of strain ATCC27405 grown in front of different carbohydrates and pretreated switchgrass. Similar to findings reported by Gold and Martin, xylanases were more abundant in bacterial growth in the presence of cellobiose in comparison to cellulose. Furthermore, it was noted that xylanases were less abundant in growth conditions in front of pretreated switchgrass than cellulose.

***C . thermocellum* xylan-degrading enzymes**

The advance on genome sequencing techniques dramatically improves the description of xylan-degrading enzymes, including those from *C. thermocellum*. For instance, nowadays, four genomes (AD2, ATCC27405, DSM1312, DSM2330, and LQRI) are assembled, and their glycosyl hydrolases annotation is available in the Cazy database (Terrapon et al. 2017). At first, the search for *C. thermocellum*'s enzymes involved in plant cell wall degrading relied on the screening of genomic libraries (Grepinet et al. 1988). Regarding cellulosomal enzymes, 81 genes are annotated as possessing at least one dockerin domain. Therefore cellulosomal enzymes, and from these proteins 21 as involved in hemicellulose deconstruction (Krauss et al. 2012; de Camargo et al. 2018)**.**

Grepinet et al. (1988) were the first authors to describe a *C. thermocellum*'s gene encoding for a xylan-active enzyme, xynZ [Cthe_1963: *5'*-CE1 (Feruloyl esterase)-CBM6-DockerinI-GH10 (endo-β-1,4-xylanase)-*3'*] (Grépinet et al. 1988). These authors also first suggested the presence of additional functional modules in xynZ. Indeed, the presence of a dockerin I domain was further reported, which is responsible for the anchorage of XynZ into the cellulosome and a feruloyl esterase domain in addition to the glycoside hydrolase catalytic domain (GH10) (Blum et al. 2000).

Although Grépinet et al. were assertive in discussing the nature of multidomain xylanases mined from *C. thermocellum*'s genome, other authors could not identify the full-length orfs coding for GH in *C. thermocellum*. For instance, Mackenzie et al. (1989) screened from a genomic library three different xylanases. However, just one form could efficiently hydrolyze xylan, and this form had only 25 kDa. Given the current knowledge in GH enzymes annotated for *C. thermocellum*, it is rational to assume that the 25 kDa protein obtained is not a full-length orf but a catalytic domain of a multidomain protein.

In 1995 Fontes et al. reported the gene xynY [Cthe_0912: *5'*-CBM9-GH10 (endo-β-1,4-xylanase)-CBM9-dockerin I-CE1 (Feruloyl esterase)-*3'*], with orf containing 3228 bp. Although the authors pointed out that some modules were unnecessary for the endo-

β-1,4-xylanase activity, they found that xynY had similarities to xynZ, an enzyme that harbors GH10 endo-xylanase and CE1 (Feruloyl esterase) domains.

In the late 90s the gene that encodes the XynC protein [Cthe_1838: *5'*-CBM22-GH10(endo-β-1,4-xylanase)-dockerin I-*3'*] was reported (Hayashi et al. 1997), and in 1999 Hayashi et al. described two contiguous xylanases genes with highly conserved domains, xynA [Cthe_2972: *5'*-GH11 (endo-β-1,4-xylanase)-CBM6-DockerinI-CE4 (acetyl xylan esterase)-*3'*], and xynB [Clo1313_0522: *5'*-GH11 (endo-β-1,4-xylanase)-CBM6-dockerin I-*3'*]. As described for xynZ, xynA (2049 bp) encoded a protein containing additional domains to the glycoside hydrolase domain, GH11, CBM6-*dockerin* I, and the extra catalytic domain of an acetyl xylan esterase. These modular structures seem to be conserved in genes encoding xylan active enzymes from *C. thermocellum*, regarding the protein XynB, encoded by 1371 bp gene that results in a protein very similar to XynA, the exception is the lack of the C-terminal acetylase domain (Hayashi et al. 1999). In the same year, Fernandes et al. (1999) reported xynU and xynV for the strain YS, being these genes homologous to xynA and xynB.

Despite the considerable number of genes encoding xylan-degrading enzymes be discovered and cloned employing screening of genomic libraries, the access of fully assembled *C. thermocellum* genomes and the improvement of annotated sequences facilitate the process of cloning and expressing these genes. Nowadays, there is a growing number of sequences that have never been validated, and their function is assumed by homology.

With the increment of annotated sequences, more enzymes started to be mined from the *C. thermocellum* genome, and in the early 2000s, the xynD [Cthe_2590: *5'*-CBM9-GH10 (endo-β-1,4-xylanase)-dockerin I-*3'*] was reported. In 2011, Correia et al. (2011) described the gene coding for CtXyl5A [Cthe_2193: 5'-GH5 (endo-β-1,4-xylanase)-CBM6-CBM13-CBM62-dockerin I-3'], one of the most complex modular enzymes reported to *C. thermocellum* because of the diversity of CBM domains, and the only GH5 reported with endo-β-1,4-xylanase activity reported to this bacterium. Ahmed et al. (2013) reported the gene coding to the first cellulosomal α-arabinofuranosidase, the Ct43Araf [Cthe_1271: *5'*-GH43 (α-arabinofuranosidase)-CBM6-CBM6-dockerin I-*3'*].

Recently, three enzymes of *C.thermocellum* involved in xylan deconstruction have been validated, the α-arabinofuranosidase AxB8 [Cthe_2196: GH43 (α-arabinofuranosidase)-CBM6-dockerin I] (de Camargo et al. 2018), the xylanase from

glycoside hydrolase family 141 XynE [Cthe_2195: GH141 (endo-β-1,4-xylanase)-CBM6-DockerinI] (Heinze et al. 2017), and the glucuronoxylan specific xylanases CtXyn30 [Cthe_2193: *5'*-GH30 (endo-β-1,4-xylanase)-CBM6-DockerinI-*3'*]. However, more sequences that encode proteins with a role in plant cell wall deconstruction will be further validated. A list of cellulosomal xylan-degrading enzymes that have been validated for *C. thermocellum* is shown in **table 1.**

Table 1: List of cellulosomal enzymes with a validated role in xylan degradation. Data were retrieved from the Cazy database (Terrapon et al., 2017). Locus tag is about strain *C. thermocellum* ATCC 27405, an exception to [a] which annotation was retrieved from *C. thermocellum* DSM1313.

Activity	Secondary activity	Enzyme	Locus tag	Modules
α-L-arabinofuranosidase	-	Ct43Araf	Cthe_1271	GH43, CBM6, CBM6, Dockerin I
	-	AxB8	Cthe_2196	GH43, CBM6, Dockerin I
endo-β-1,4-xylanase	-	XynD	Cthe_2590	CBM22, GH10, Dockerin I
	Acetyl xylan esterase	XynA	Cthe_2972	GH11, CBM6, CE4, Dockerin I
	-	XynB	Clo1313_0522 [a]	GH11, CBM6, Dockerin I
	-	CtXyn30	Cthe_3012	GH30, CBM6, Dockerin I
	-	CtXyl5	Cthe_2193	GH5, CBM6, CBM13, CBM62, Dockerin I
	-	XynC	Cthe_1838	CBM22, GH10, Dockerin I
	-	XynE	Cthe_2195	GH141, CBM6, Dockerin I
	Feruloyl esterase	XynY	Cthe_0912	CBM22, GH10, CBM22, CE1, Dockerin I
	Feruloyl esterase	XynZ	Cthe_1963	CE1, CBM6, GH10, Dockerin I

In addition to the significant number of sequences coding for GH enzymes that have been recently validated, a considerable number of orfs are assumed as putative *C. thermocellum*'s enzymes involved in xylan deconstruction. Although further functional validation may assign new biological roles to proteins encoded by putative genes, some are worth investigating. Regarding those putative genes, de Camargo et al. (2018) have discussed the diversity of GH 43 annotated to *C. thermocellum* that have not been functionally validated. For instance, Cthe_2138 encodes for a cellulosomal protein (contains a dockerin I domain and an extra CBM42) and possesses a GH43 module that can potentially be a novel α-arabinofuranosidase or even a cellulosomal β-xylosidase.

Thus far, to the best of our knowledge, this gene has not been validated, and in the case of it represents a β-xylosidase activity, it will be a fundamental finding since no β-xylosidase has been reported to *C. thermocellum*.

Still, regarding putative GH43, Cthe_2139 encodes for a cellulosomal GH43 containing an extra domain GH30 and a CBM42. None of these catalytic domains have been validated, and the GH30 domain calls attention because for *C. thermocellum*, glucuronoxylan-specific xylanase from this family was reported (Verma and Goyal 2016), and following the Cazy database (Terrapon et al. 2017) GH30 family comprises relevant xylan-degrading activities as endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37).

Another gene with particular interest is Cthe_2194, which contains a carbohydrate esterase family I domain and a CBM6 and dockerin I domain. The particular interest is that this annotation comes from its differential architecture, as the CE1 family, at least for *C. thermocellum*, represents feruloyl esterases, and they are present in modular enzymes, accompanied by the glucosyl-hydrolase module as seen for XynZ. Therefore, this could be the first cellulosomal feruloyl esterase that is not part of a bi-functional (two catalytic domains) enzyme, and the fact that a CBM6 (xylan-specific) is present highlights the potential of this gene be involved in hemicellulose deconstruction.

Regarding putative genes annotated with a role in hemicellulose deconstruction, there are the Cthe_2197 that encodes for a protein containing a GH2 domain, a carbohydrate-binding module 6, and a dockerin I domain, therefore a cellulosomal enzyme. Unlike other annotations, this gene is reported as possessing a GH2 domain that follows the Cazy database (Terrapon et al., 2017). This GH family represents several activities; however, some are unrelated to xylan depolymerization, including β-mannosidase (EC 3.2.1.31) and endo-β-mannosidase (EC 3.2.1.146). The presence of a xylan-specific carbohydrate-binding module highlights a possible role of this protein in xylan deconstruction, increasing the need to discover the role of this protein in cellulosomes and its role in xylan deconstruction.

Modulation of the expression of xylanases by *C. thermocellum*: a model still under development

Another point of extreme relevance is how these genes are regulated. Many early reports tried to conclude how these genes are regulated by employing traditional analyses of secreted proteins as zymograms (Bhat et al. 1993). After the public availability of *C.*

thermocellum's genomes, valuable insights were arisen into how these genes are organized, and thus further investigation on its regulation was possible.

Enzymes involved in plant cell degradation by *C. thermocellum* have been reported as scattered in the genome (Guglielmi and Béguin 1998). However, some functional operons associated with glycoside hydrolases and cellulosomal structural proteins were reported (Newcomb et al., 2011). The overall triggering mechanism to start transcription of genes encoding plant cell wall degrading enzymes involves an extracellular sensing mechanism. This mechanism relies on the presence of anti-σ^I factors in the external cell membrane; they display structures that resemble CBMs or even glycoside hydrolase modules.

Bahari et al. (2011) reported one anti-σ^I factor for *C. thermocellum* that harbors a GH10 module, had an affinity to arabinoxylan, and the GH10 module was functional. This result raised attention to specific extracellular sensors responsible for triggering a particular set of plant cell wall degrading enzymes. Kahel-Raifer et al. (2010) demonstrated that sigma factor I6 could trigger the expression of xylan encoding genes, as xynA, xynZ, xynD, xynY. Also, these authors proved that the presence of extracellular polysaccharides as xylan could boost the transcription of sigma factors responsible for triggering hemicellulases transcription. Cellobiose and cellulose could also trigger these hemicellulose-related σ factors, however, to a reduced extension. These results agree with proteomics, and early studies about *C. thermocellum*'s enzyme production, on which they have demonstrated that cellobiose and cellulose lead to a more diversified set of enzymes rather than just cellulases (Bhat et al. 1993; Gold and Martin 2007).

Analyses of transcription of *C. thermocellum*'s genes also brought essential insights into how these genes are expressed in response to polysaccharides. Wei et al. (2014) demonstrated the during *C. thermocellum* ATCC 27405 growth in the presence of dilute acid pretreated yellow poplar, genes as xynD, xynY, xynZ, and manA are highly expressed in comparison to cellobiose growth condition.

Although the knowledge of how extracellular polysaccharides modulates the secretion of glycoside hydrolases by *C. thermocellum,* it is expected that more operons and regulatory mechanisms are going to be discovered. The general mechanism of sensing extracellular carbohydrates seems to be a viable target for developing engineered strains to boost carbohydrate-active enzyme production.

Properties of xylan degrading enzymes from *C. thermocellum*

Despite the organization of enzymes into cellulosomes, *C. thermocellum* xylanases have also gained attention because of their enzymatic properties. In recent years, the search for industrial enzymes with properties aligned to the need in industrial processes has placed *C. thermocellum*'s enzymes into the spotlight.

One of the primary studies characterizing *C. thermocellum*'s hemicellulolytic enzymes was reported by Morag et al. (1990) when comparing cellulosomal and noncellulosomal xylanases. This study revealed endo-β-1,4-xylanases with maximum activity at 70 ºC, in pH range 6 to 7.5. The authors also showed xylanase thermal stability at 70 ºC for ten minutes. In addition, the endo-β-1,4-xylanases, loss of endo-β-1,4-xylanase activity was described in the presence of SDS and after boiling.

Vieira et al. (2007) described that endo-β-1,4-xylanases from *C. thermocellum* ISOII had optimum activity in a temperature range of 55 to 75 ºC, and the maximum activity was observed in pH 6. Thermal stability was evaluated at 65 ºC, the temperature on which enzymatic activity was stable for 12 hours, keeping 80% of the original activity. Osiro et al. (2017) reported the properties of cellulosomes assembled by isolate B8 in response to microcrystalline cellulose. Xylanase activity had its maximum activity in a range of 60 to 70 ºC, and pH 5 and 6. The stability of this enzymatic complex was evaluated either at 50 or 60 ºC, showing that the xylanase activity of cellulosomes could stand for 20 and 15 days without significant activity loss, respectively, at 50 and 60 ºC.

Xylan degrading enzymes secreted by isolate B8 were evaluated by Hamann et al. (2015). The authors showed that different carbon sources could lead to xylanases' production by isolated B8, and maximum activity was obtained at 70 ºC when assayed against birchwood or oat spelt xylan; regarding pH effect, uppermost activity was observed in a range from 5 to 7. Xylanases synthesized by isolate B8 showed no activity loss after 12 days at 50 ºC, and at 70 ºC, half-life activity was reached after 3 hours.

Hayashi et al. (1999) reported the enzyme XynA with maximum activity at 65 ºC in pH 6.5. In addition to the previous study, the glycoside hydrolase module of XynA (GH11) was cloned and expressed in rice plants as host cells (Kimura et al., 2003). This glycoside hydrolase module displayed minor activity loss after 24 hours incubation time at 60 ºC and had maximum activity at 80 ºC at pH 5.5; it was stable for 10 minutes at 70 ºC; however, at 80 ºC complete activity loss was observed.

Fernandes et al. (1999) demonstrated that the strain *C. thermocellum* YS had homologous genes for xynA and xynB (GH11, CBM6, dockerin I (Hayashi et al. 1999)), respectively named xylV and xylU. XylV (GH11, CBM6, dockerin I, acetyl xylan esterase) had maximum endo-β-1,4-xylanase activity in pH 5 to 8 and maximum activity at 75 °C. In that study, the function of the acetylase domain was investigated (previously known as NdoB domain). This domain performed acetylase activity against soluble and insoluble xylan. In addition to studying individual modules of XylV, the authors also evaluated the synergic effect of acetyl xylan esterase and the GH11 module activity. They reported that the deacetylation of xylan leads to enhancement in overall endo-β-1,4-xylanase activity.

The GH10 and feruloyl esterase modules of XynZ were separately expressed and characterized. XynZ-GH10 heterologously expressed in tobacco leaves displayed thermal stability at 60 ºC and had the maximum activity at 70 ºC (Herbers et al. 1995). XynZ-feruloyl esterase presented maximum activity in temperatures ranging from 50 to 60 ºC and in a broad pH range, from 4 to 7. Regarding thermal stability, the enzyme stability at 70 ºC was 6 hours, at 80 ºC, 50% of activity was lost within 3 hours (Blum et al. 2000).

Truncated forms of XynZ and XynC, containing the glycoside hydrolase module, were expressed to evaluate the role of non-catalytic domains as carbohydrate-binding modules (Sajjad et al. 2010). The presence of additional modules did not change the pH effect on GH10 activity, being observed maximum activity in a pH range from 5 to 9, at 60 ºC. However, the presence of a carbohydrate-binding module made the GH10 domain of XynC more heat stable, while the lack of CBM made the GH10 of XynZ less heat stable.

The GH10 domain from *C. thermocellum*'s XynY performed the maximum activity at 75 ºC in pH 6.8 (Fontes et al. 1995; Selvaraj et al. 2010). In addition to XynZ, XynC, and XynY, there is also the Xyn10D (XynD), this protein has similar architecture to XynC (CBM22, GH10, dockerin I), and the endo-β1,4-xylanase activity of XynC presented maximum activity at 80 ºC and pH 6.4 (Zverlov et al. 2005).

CtXynGH30, an endo-β-1,4-xylanase GH30 classified as a glucuronoxylan, has specificity in hydrolyzing xylan structures with glucuronic acid residues, as beechwood and birchwood xylans. However, this enzyme also displayed activity against arabinoxylans as oat spelt, and rye hemicellulose. CtXynGH30 displayed maximum

activity at 70 ºC in pH 6. By contrast, CtXyl5A harbors the GH5 (endo-β-1,4-xylanase) and three extra carbohydrate-binding modules of families 6, 13, 62, and dockerin I module (Brás et al. 2011). CtXyl5A is active against ramified arabinoxylans as rye and wheat arabinoxylan, has low activity against oat spelt xylan, and no activity against glucuronoxylans as beechwood xylan (Correia et al. 2011).

In a more recent study, Heinze et al. (2017) described a xylanase classified as GH141, designated XynE, that had higher activity against wheat and oat spelt xylan than glucuronoxylan. The activity was maximum in temperatures of 67 to 75 ºC in pH 6 to 6.5. This endo-β-1,4-xylanase displayed modular architecture with a GH141, one carbohydrate-binding module 6, and a dockerin I, being this protein one of the few GH141 fully characterized.

Also, concerning other enzymes with accessory activity in xylan deconstruction, the properties of α-arabinofuranosidases have been investigated. Ahmed et al. (2013) expressed and purified an α-arabinofuranosidase of GH43 with two tandem carbohydrate-binding modules at the C-terminus and a dockerin module I. This enzyme displayed activity against various xylan-decorated substrates as such arabinoxylan from rye, wheat and oat spelt being maximum activity detected at 50 ºC in pH 5.7. Still, regarding cellulosomal α-arabinofuranosidase, axb8, reported by de Camargo et al. (2018), displayed maximum activity at 50 ºC, pH 5 and 6. Axb8 displayed thermal stability for 14 hours at 50 ºC; however, at 60ºC, activity loss was observed after 2 hours of incubation time.

The third report of glycoside hydrolase belonging to family 43 from *C. thermocellum* was the Ctgh43, the truncated version of this protein possessing the catalytic domain was expressed and used for lignocellulosic hydrolysis by Das et al. (2012). This catalytic domain displayed activity against various xylan substrates as arabinoxylan from rye, wheat, and arabinogalactan, displaying maximum activity at 50 ºC in pH 5.4. Taylor et al. (2006) described the intracellular α-arabinofuranosidase araf51 from the glycoside hydrolase family 51. This enzyme displayed maximum activity at 82 ºC and low affinity to wheat arabinoxylan in caparison to the synthetic substrate.

As a general perception, accessory activities of cellulosomal enzymes involved in hemicellulose deconstruction display maximum activity in a more mesophilic temperature, as seen for arabinofuranosidases and feruloyl esterases. In contrast, endo-β-1,4-xylanase activities present maximum activity in higher temperatures 70 ~ 80 ºC. The

distribution of maximum activity of *C. thermocellum* xylan degrading enzymes is displayed in **figure 3**.

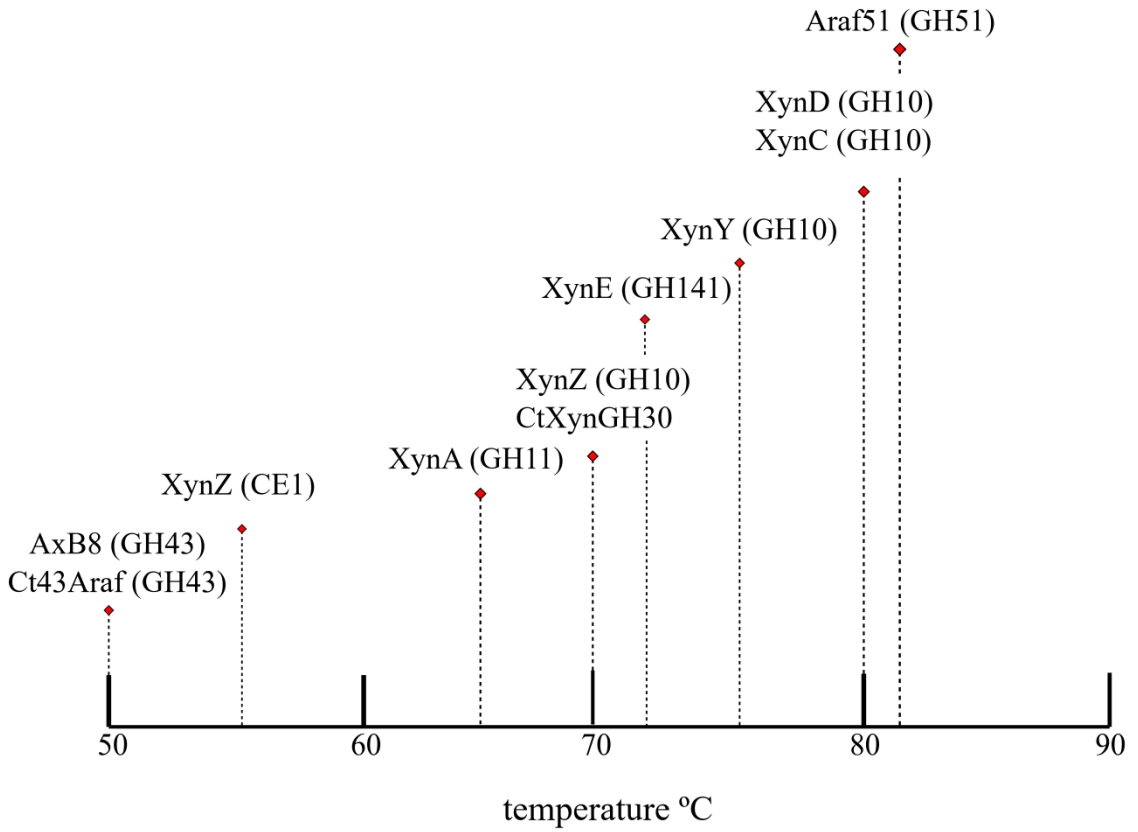

Figure 3: Temperature range on which maximum activity is observed for different xylan-degrading enzymes from *C.thermocellum.* XynZ is twice reported by characteristics of different modules, glycoside hydrolase family 10 (GH10) with endo-β-1,4-xylanase, and carbohydrate esterase family 1 (CE1) feruloyl esterase activity. Enzymes whose maximum activity was reported in a temperature range were placed in the middle point of the range.

Beyond the effect of temperature and pH on xylanase activity, different parameters are often assayed for this class of plant cell wall degrading enzymes. Amongst evaluated parameters, there is the effect of ions, surfactants, and the description of kinetics parameters. These properties are commonly evaluated for purified enzymes, and they help indicate a possible future application of these enzymes. For ions' effect on recombinant xylanase from *C. thermocellum*'s, XynE did not display significant activity changes in the presence of $CaCl_2$ and $MgCl_2$. However, EDTA, $CuSO_4$, ethanol, and SDS caused activity loss (Heinze et al., 2017). Significant activity loss was also reported for

the GH10 XynC when xylanase activity was assayed in the presence of $HgCl_2$, $FeCl_3$, $CuCl_2$, and partial activity was maintained in $MnCl_2$, $AlCl_3$, and *p*-chloromercuribenzoic acid. Although most of the assayed ions caused activity loss for xylanases, the GH30 CtXynGH30 displayed activity enhancement in the presence of Li^{2+} either at 1 or 10 mM. However, other compounds as SDS, β-mercaptoethanol caused activity loss, Hg^{2+} ions abolish the xylanase activity.

For accessory enzymes, GH43 α-arabinofuranosidase *Ct43Af* displayed activity enhancement in the presence of Na^{+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}. Nevertheless, ions such as Cu^{2+}, Hg^{2+}, and Al^{3+} caused significant activity loss (Ahmed et al. 2013). Still, regarding α-arabinofuranosidases from family GH43, the axb8 reported by de Camargo et al. (2018) displayed contrasting effects of ions, being reported activity enhancement in front of $FeCl_3$, and $CaCl_2$, and the ions $MnSO_4$, KCl, $NiSO_4$, and $MgSO_4$ caused slight inhibition. Similar to these results, the xylanase activity present on cellulosomes reported by Vieira et al. (2007) displayed slight activity enhancement in the presence of Fe^{3+} and Ca^{2+}, and as reported to other *C. thermocellum*'s xylanases, Zn^{2+}, Cu^{2+}, and Al^{3+} caused inhibition. In addition, Vieira et al. demonstrated that free amino acids as cysteine and tryptophan could drastically enhance xylanase activity present on cellulosomes.

Kinetic parameters of these enzymes have also been evaluated for purified enzymes and natural cellulosomes. Vieira et al. (2007) reported that natural cellulosomes synthesized by the strain ISOII displayed higher affinity to insoluble xylan than the soluble fraction, with k_M of 1.54 mg/mL for insoluble oat spelt xylan and 11.53 mg/mL for the soluble portion. However, the maximum velocity of reaction V_{max} was obtained of soluble xylan. The GH30 CtXynGH30 has k_M and *Vmax* of 2.2 mg/mL and 40.52 U/mL for purified enzymes, and catalytic efficiency of $5.5\times10^{4}min^{-1}.mg^{-1}$. Regarding accessory activities on xylan deconstruction, kinetic parameters of the carbohydrate esterase modules of XynZ were investigated. Blum et al. (2000) reported feruloyl esterase k_M of 5 mM and V_{max} of 12.5 µmol of ferulic acid per $min^{-1}.mg^{-1}$ when assayed against FAX3 (Xylose-β(1,4)-Xylose-(α1,3-Arabinose, Ferulic acid)-β(1,4)-Xylose).

The α-arabinofuranosidase reported by de Camargo et al. (2018) had its kinetics parameters reported and compared to other *C. thermocellum*'s accessory enzymes previously reported to have α-arabinofuranosidase activity. The AxB8 (GH43) reported by these authors showed lower affinity to the synthetic substrate pNP-α-L-

arabinofuranoside (23.04 mM) when comparing to previous reports, Araf51 GH43 (0.18) (Ahmed et al. 2013), and Araf51 GH51 (0.25) (Taylor et al. 2006).

Carbohydrate-binding modules

The presence/absence of carbohydrate-binding modules does not affect *C. thermocellum*'s xylan degrading enzymes capacity in hydrolyzing xylan β-1,4-linkages; however, they might help enzymes in anchoring to the substrate (Sajjad et al. 2010). XynZ, XynA, XynE, CtXyl5A, CtAraf, AxB8, and CtXyn30, have at least one carbohydrate-binding module 6 on their structure. CBM module and the recombinant form of CBM6 derived from xynA showed affinity to xylobiose, xylooligosaccharides, oat spelt, and birchwood xylan. However, it did not display affinity to cellulose-based polymers, chitin, or starch (Sakka et al., 2003).

Charnock et al. (2000) described the effect of CBM22 present on XynY (CBM22, GH10, CBM22, dockerin I) and reported that the CBM22 module caused enhancement on XynY GH10 activity when assayed in the presence of insoluble substrates. In addition, the CMB22 displayed higher binding constants against oat spelt xylan, wheat arabinoxylan, and rye arabinoxylan.

The CMB22 present on XynC was studied by Ali et al. (2005). The authors found that the GH10 module of XynC performed the maximum activity at 60 ºC; with a fusion of the CBM22, the maximum enzyme activity shifted to 80 °C. Others results as stability in different temperature and pH effects over xylanase activity were almost identical compared to the presence/absence of the CBM22. In addition, the CBM22 had an affinity to soluble and insoluble xylan and cellulose. Similar to the CBM22 module observed for XynY, the presence of CBM22 for XynC caused significant activity enhancement against an insoluble fraction of oat spelt xylan (Ali et al. 2005). Khan et al. (2013) reported that the change of XynZ native CBM6 module for a CBM22 resulted in enhancement of XynZ affinity to the substrate and improved thermal stability.

The multidomain CtXyl5A that harbors three distinguish CBMs (6, 13, and 62) had the binding properties of CBM6 evaluated in the truncated form GH5-CBM6. Different from the report for the CBM6 from XynZ, the CBM present on CtXyl5A had an affinity to cellobiose, cellotriose, cellohexaose, xylosaccharides, and wheat arabinoxylan (Correia et al. 2011; Brás et al. 2011; Labourel et al. 2016). In addition, Labourel et al. (2016) reported that the presence of CBM6 and 13 alongside the GH5

module of CtXyl5A leads to higher catalytic efficiency compared to the full-length protein (GH5, CBM6, CBM13, CBM62).

Overview of the application of thermostable xylanases and accessory enzymes: Applications of *C. thermocellum* thermostable xylan degrading enzymes and perspectives

The application of xylan degrading enzymes is diversified; however, nowadays, there is an increasing interest in bioprospecting new enzymes with more suitable industrial applications. In this scenario, thermostable enzymes are mainly studied for application at harsh temperatures, pH, or a combination of factors (Shukla and Kumar 2016). These enzymes are also desirable for industrial use because of their prolonged natural half-life, inhibition of microbial growth at higher temperatures, and enhancement of operation properties as the viscosity of the reactional mixture and mass transfer in elevated temperatures (Zamost et al. 1991). **Representantion of xylanases application is shown in figure 4**.

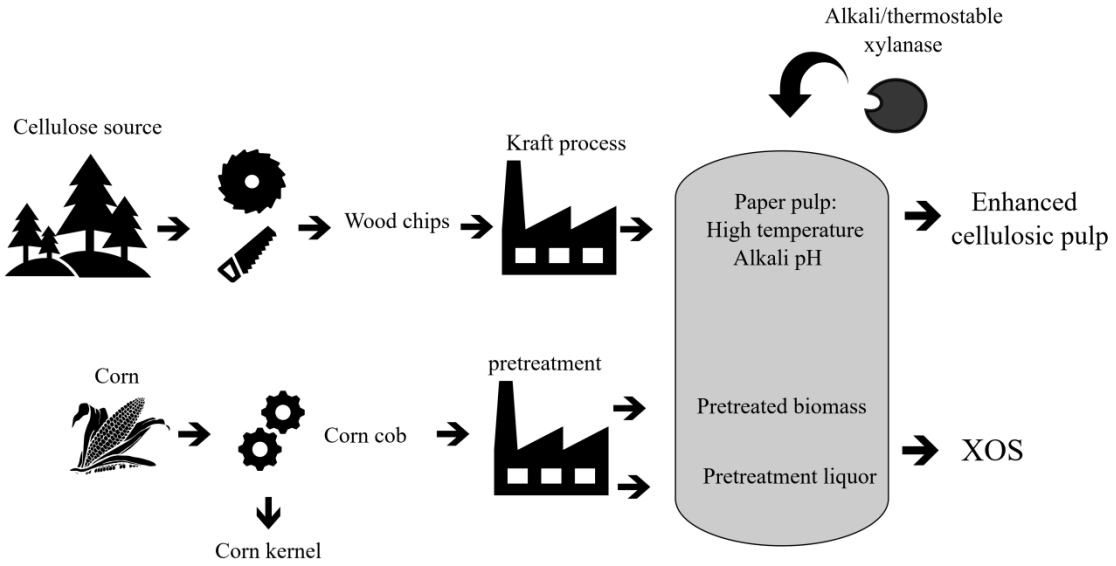

Figure 4: Representation of possible application of thermostable and alkali stable xylan degrading enzymes. XOS (Xylooligosaccharides).

Among the uses of thermostable xylanases, the paper and pulp industry still requires new enzymes with thermostable properties (Shukla and Kumar 2016). Xylanase application in this industrial sector is consolidated because these enzymes reduce bleaching chemicals and enhance paper quality. However, the harsh condition on which

the wood pulp is prepared delivers a final pulp in high temperature and alkali pH, being required a larger volume of enzymes and pH adjustments (Zamost et al. 1991).

In order to overcome these limitations, researchers have reported several xylan-degrading enzymes that could be used as biobleaching agents. Dhillon et al. (2000) described the production of thermostable xylanase by *Bacillus circulans* AB16, which could also resist alkali pH conditions. This xylanase activity produced by *Bacillus circulans* AB16 was mainly devoid of cellulolytic activity and thus assayed for eucalyptus pulp bleaching tests, resulting in enhanced pulp brightness and reduction in 20% of chlorine use. Also, using thermostable xylanase, Rättö et al. (1994) demonstrated that the xylanase produced by the anaerobic thermophilic bacterium *Dictyoglomu s*sp strain B1 displayed potential to be applied in birch and pine kraft pulp bleaching. This enzyme could hydrolyze part of the hemicellulose of kraft pulps in pH range from 6 to 9 when applied at 80 ºC, enhancing the brightness property of paper pulps.

Others thermostable alkali xylanases have been studied and proposed as biobleaching agents; Shrinivans et al. (2010) described cellulase-free xylanase produced by *Bacillus* sp. JB99, these enzymes displayed maximum activity in pH 8 and temperature of 70 ºC (Shrinivas et al. 2010). Ko et al. (2010) described a 41 kDa xylanase produced by *Panibacillus campinasensis* BL11, which was more stable at pH 9 than neutral pH, stable up to four hours at 55, or 60 ºC. This enzyme could hydrolyze hardwood kraft pulp, enhancing the brightness and viscosity of the final pulp.

Thermostable xylanases were also explored as tools for general lignocellulosic deconstruction and production of value-added chemicals. Teng et al. (2010) demonstrated that xylanase produced by *Paecilomyces thermophila* J18 with maximum activity at temperature 75-80 ºC could hydrolyze carbohydrates present on steam-exploded liquor from corn cob, and generate xylooligosaccharides at 70 ºC, mainly being produced xylobiose and xylotriose. Liu et al. (2018) used a recombinant multidomain thermostable xylanase (*PbXyn10A*) from *Paenibacillus barengoltzii* to hydrolyze pretreated corncob at 50 ºC, obtaining 75% of xylooligosaccharides.

Accessory thermostable enzymes have also been investigated for hydrolyses of oat spelt xylan; for instance, Huang et al. (2010) described adding a recombinant thermostable acetyl xylan esterase from *Thermobifida fusca* NTU22 with maximum activity at 60 ºC in pH 6.5 displayed synergism with xylanases during xylan hydrolyses.

However, the presence of these two enzymes did not display synergism during bagasse deconstruction.

Although cellulose-degrading enzymes mainly received attention for plant cell wall deconstruction purposes, proteins involved in hemicellulose deconstruction are also seen as a paramount player in plant material solubilization. The second group sought after aiming at lignocellulose deconstruction is hemicellulases. These hydrolytic enzymes solubilize the carbohydrate portion, shielding cellulose, thus reducing the physical barrier that protects cellulosic fibers from being hydrolyzed by cellulases.

Despite the natural ability of hemicellulases in making access to cellulosic fibers free for other hydrolyzing enzymes, these enzymes might be applied in their cellulase-free form or a consortium of proteins (Srinivasan and Rele 1999; Liu 2014). Specifically for xylan hydrolyzing enzymes, there is an interest in producing cellulase-free blends aiming at paper pulp industry application as a biobleaching agent.

Based on the industrial application on which other thermostable xylan-degrading enzymes have been utilized, it is possible to direct *C. thermocellum*'s xylanases to various industrial processes. Reports on regard to *C. thermocellum*'s xylan-degrading enzymes applications are not widespread. Das et al. (2012) described that a glycoside hydrolase from family 43 from *C. thermocellum* could be used to deconstruct pretreated agricultural residues and thus generated sugar be used to bioethanol production by *Candida shehatae.* In this study, authors have shown that simultaneous saccharification and fermentation were possible even in temperature bellows the optimum condition of *C. thermocellum*'s hemicellulases, making the process more efficient when using pretreated mango leaves.

A more recent study showed that the integration of XynY into designed cellulosomes enhanced the hydrolyzes of pretreated softwood (Leis et al. 2018). Gonçalves et al. (2015) demonstrated that the GH10 module of XynZ could be applied with other xylan-degrading enzymes from different organisms to achieve synergism in pretreated bagasse biomass solubilization. In addition, this enzyme could also perform synergism with commercial cellulases, and this catalytic module as a standalone enzyme could degrade bagasse, generating xylose, xylobiose, and xylotriose.

Native enzymes produced by *C. thermocellum* strains have also been investigated. Although the enzymes were assembled into cellulosomes, and not only xylan or hemicellulose degrading enzymes were present is clear that these enzymes play an essential role in lignocellulose deconstruction. For example, Osiro et al. (2017) showed

that during the deconstruction of pretreated sugarcane residues by cellulosomes, xylose and xylooligosaccharides were released.

Based on the diversity of xylanases found in the *C. thermocellum* genome, these proteins could be used to design custom xylanosomes aiming at specific applications on hemicellulose removal. Industrial applications are diversified, and hemicellulose is heterogeneous, contributing to the need for custom xylanase blends. Biomasses on which no pretreatment has been applied carry xylan backbone with all decorations as arabinose, glucuronic, and acetyl groups. This biomass class may require enzymes with the capacity to hydrolyze xylan and remove its side chains, enzymes as the GH30 CtXyn30 (activity against glucuronoxylan) and the GH5 CtXyl5 (active against highly decorate arabinoxylan) could be helpful tools in this scenario.

On the other hand, biomasses subjected to pretreatments are likely to have fewer side chains, thus a more available backbone. In this scenario, the synergism observed for GH10 and GH11 xylanases could boost its degradation. For instance, the GH10 XynD and the GH11 XynB could be valuable tools. Xylan deconstruction on which the aimed final product is xylose monomers may require a more diversified group of enzymes. In this scenario, additional activities might be incorporated into designed cellulosomes aiming at complete xylan deconstruction, hence by far, no β-xylosidase or α-glucuronidase have been reported as components on *C. thermocellum*'s cellulosome architecture. A model of tailored xylanosomes based on *C .thermocellum*'s xylanases is represented in **figure 5**.

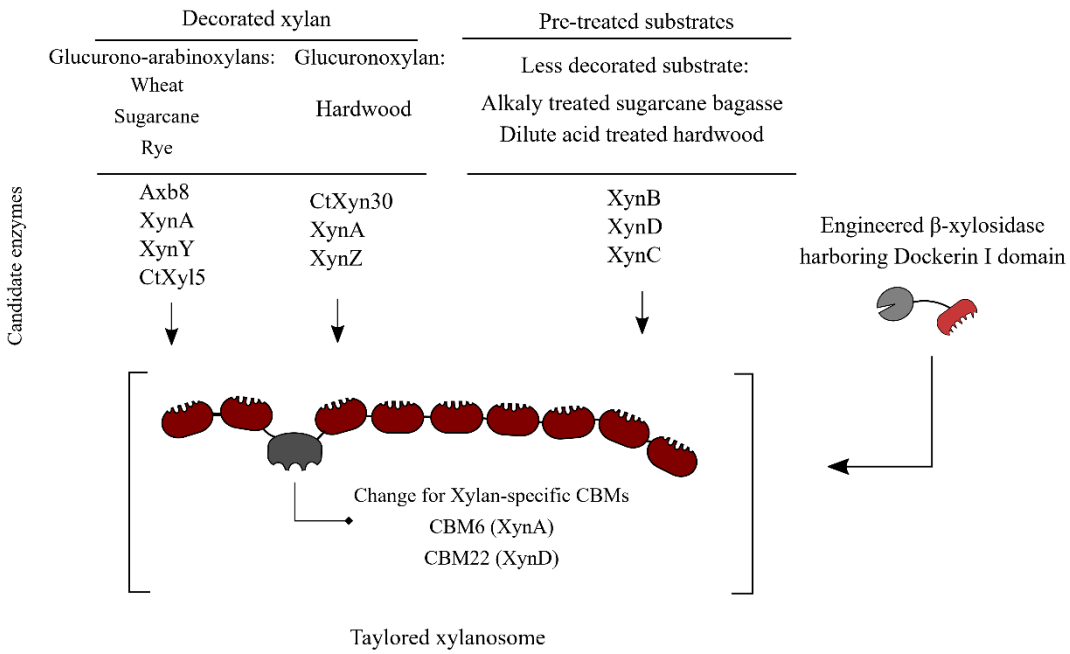

Figure 5: Representation of putative tailored cellulosomes aiming deconstruction of different decorated xylans. Enzymes were chosen based on their modules, and the data reported in regard of their affinity to decorated substrates. AxB8(Cthe_2196), XynA (Cthe_2972), XynY (Cthe_0912), CtXyl15 (Cthe_2193), XynB (Clo1313_0522 – *C.thermocellum* DSM 1313 as reference genome), XynD (Cthe_2590), XynC (Cthe_1838).

Hallmarks and future work

C. thermocellum has been proven to be versatile in producing cellulases, and it is also clear that this organism has a well-developed xylan degrading arsenal. These enzymes have operational parameters (pH, temperature, stability) aligned to industrial needs, and they must be further explored. The possibility of designing custom xylanosomes using *C.thermocellum* CipA and enzymes harboring *dockerin I*domain seems to be a promising strategy in developing more robust and efficient xylan degrading blends. Future work should also focus on the limitation present on *C. thermocellum*'s cellulosomes to fully achieve xylan depolymerization.

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CAPÍTULO II -Endoxilanases e inibidores derivados da lignocelulose: uma breve revisão

Endo-β-1,4-xylanases and lignocellulose-derived inhibitors: a brief review

Dentre um dos principais tópicos relacionados a utilização de enzimas em processos industriais, está a inibição e desativação de glicosil hidrolases por compostos derivados do substrato lignocelulósico. Dentre os compostos que são classicamente estudados como inibidores, estão os açúcares resultantes do processo de hidrólise, assim como os compostos aromáticos que podem advir de diversos processos, incluindo as atuais metodologias de pré-tratamento.

No presente capítulo, uma breve introdução será apresentada mostrando os principais relatos na literatura em relação a esses inibidores e sua influência na atividade de endoxilanases. Com base no que é descrito na literatura, pouco é estudado em relação a inibição de endoxilanases pelos açúcares resultantes da hidrólise da hemicelulose. A falta de estudos em relação a essa inibição pode ocorrer por diversos motivos, entretanto, uma das principais razões por essa escassez de estudos está relacionada à inadequação de metodologias que possam, de forma eficiente e prática, diferenciar os açúcares redutores gerados durante a hidrólise, e aqueles que foram utilizados como modelo de inibição. Um outro motivo para o baixo número de relatos em relação a açúcares redutores como inibidores de endoxilanases, encontra-se no enorme foco que a inibição de celulases recebe, sendo que endoxilanases e demais classes de enzimas não recebem a devida atenção.

Um tópico referente a desativação e inibição de endoxilanases que vem recebendo grande interesse é a relação entre os compostos fenólicos derivados da parede celular vegetal e a sua associação com as endoxilanases. Esse tópico vem recebendo atenção devido a enorme busca por endoxilanases que possam atuar na presença desses compostos, visto que em processos industriais, principalmente na indústria da polpa celulósica, técnicas de tratamento também geram uma enorme quantidade de compostos fenólicos, que são capazes de reduzir a atividade xilanolítica. Dentre os compostos fenólicos que apresentam maior desativação das endoxilanases, encontram-se os constituídos por diversas unidades fenólicas, entre eles o ácido tânico, e fragmentos de lignina

Em relação as medidas para evitar a inibição/desativação de endoxilanases por compostos fenólicos, encontra-se a suplementação das misturas enzimáticas com enzimas modificadoras de tais compostos, como tanases e lacases. Além da suplementação enzimática, ao menos para celulases, tem-se discutido a adição de compostos como polietilenoglicol e surfactantes como Tween 80 para desfazer a interação entre compostos fenólicos e enzimas. A adição de tais compostos ainda não foi validade para a ação de endoxilanases, entretanto, espera-se que assim como ocorrer para celulases, a interação proteína-fenol possa ser desfeita com adição de tais compostos.

Além da adição de compostos com a finalidade de reduzir a interação proteína-fenol, existe a possibilidade de desenvolver novos biocatalisadores. Para que isso seja possível, estudos ainda são necessários, para saber quais regiões das proteínas estão envolvidas no mecanismo de inibição/desativação e assim poder realizar a engenharia dos biocatalizadores.

Os dados mostrados no presente capítulo servirão de base teórica para o entendimento dos experimentos mostrados no capítulo III.

Endo-β-1,4-xylanases and lignocellulose-derived inhibitors: a brief review

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Bullet points

- Endoxylanases are inhibited by phenols derived from lignocellulose.
- Very little information is reported about the sugar inhibition of endoxylanases.
- Techniques used to reduce the detrimental effect of phenol in cellulases could be investigated for endoxylanases.

Endo-β-1,4-xylanases and lignocellulose-derived inhibitors: a brief review

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Abstract

Endo-β-1,4-xylanases represent a class of enzymes that have great potential for industrial application. They can be applied in industrial sectors as paper and cellulose pulp production and biofuels. However, one of the main challenges associated with the enzymatic biocatalysis of lignocellulosic biomass is the inhibition and deactivation by inhibitors derived from the plant cell wall. Endo-β-1,4-xylanases can be inhibited by a list of compounds found in lignocellulose, including sugars, phenols, aromatic compounds, and proteins synthesized by plants to avoid pathogens colonization. In the present review, we summarized the main findings regarding inhibition of endo-β-1,4-xylanases and their implication in the industrial application of this class of enzymes. It is also discussed how to avoid/reduce detrimental effects of lignocellulosic derived compounds.

Keywords

Xylanases, Trichoderma, lignocellulose, tannic acid, phenols

1-**Introduction: Endo-β-1,4-xylanases and their context**

For years, cellulose, the most abundant biopolymer, with annual biosynthesis of around 1.5×10^{12} tons (Klemm et al., 2005), has been targeted for studies aiming at its deconstruction. Much of this search for enzymes aiming at cellulose conversion into its monomeric unities was due to the direct use of industrial yeast to convert glucose into ethanol. Indeed still, there is an intense search for enzymes and proteins involved in cellulose bioconversion. However, with the advancement of new industrial green approaches, and the need to make use of as many as possible components from lignocellulosic biomass into the biorefinery context, more enzymes started to be explored, including hemicellulases (Cherubini, 2010; Dondelinger et al., 2016).

Regarding hemicellulose, xylan is the second most abundant polysaccharide in the plant kingdom. Although xylan is the most dominant form of hemicellulose, and its backbone is composed of xylose unities linked by β-(1,4) glycoside bounds, there are differences in its side chains. Xylan side chains can vary according to the plant source, which can be observed in different ratios of acetyl groups and D-arabinose, D-glucuronic acid, and methylated D-glucuronic acid (Bastawde, 1992).

The search for hemicellulases, more specific endo-β-1,4-xylanases (EC 3.2.1.8), is directly associated with the vast number of industrial applications this enzyme class has. Although endo-β-1,4-xylanases are enzymes that work in synergism with β-xylosidases (EC 3.2.1.37), and enzymes with catalytic activity against side chains to achieve the release of monomeric unities of D-xylose (Gilbert and Hazlewood, 1993; Gonçalves et al., 2015), there are industrial processes on which endo-β-1,4-xylanases can be applied as the unique hemicellulase, as paper pulp production, and animal feed additives (Prade, 1996; Srinivasan and Rele, 1999).

Endo-β-1,4-xylanases act in the hydrolysis of β-(1,4) in the xylan backbone, releasing xyloosaccharides and decorated sugars (Collins et al., 2005; Malgas et al., 2019). The potential of endo-β-1,4-xylanases in hydrolyzing isolate xylans or lignocellulosic biomass has extensively been reported, for instance, for xylanases from *Paenibacillus* sp. A59 was shown that both XynA (GH10) and XynB (GH11) could hydrolyze beechwood xylan and extruded wheat straw, generating xylotriose, xylobiose, and xylose from both substrates (Ghio et al., 2018). On the contrary, the recombinant purified PbXyn10A (GH10) from *Paenibacillus barengoltzii* could hydrolyze

commercial xylans liberating xylosaccharides with polymerization degree from two to five, but without the release of D-xylose (Liu et al., 2018). Regarding more complex biomasses, the liberation of small xyloosaccharides has also been reported for thermostable xylanase produced by *Paecilomyces themophila* J18 during enzymatic hydrolysis of liquor obtained from corn cob steam explosion (Teng et al., 2010).

In addition to the diversity of saccharides obtained from hemicellulose solubilization by xylanases, endo-β-1,4-xylanases are categorized in several glycoside hydrolase families. Enzymes with activity against the main backbone in xylan are typically classified as xylanases, and this group of enzymes is observed in glycoside hydrolase families 5, 8, 10, 11, and 30 (Paës et al., 2012). Aside from the protein architecture, these proteins display notable differences in their affinity to xylan substrates. A summary of endo-β-1,4-xylanases and their respective GH families is present in table 1.

Table 1: List of some endo-β-1,4-xylanase from different glycoside hydrolase families, and their general properties.

Organism	GH Family	Substrate	Properties	Reference
Bacillus sp. strain BP-7	5	Nonsubstituted xylooligosacharides	Temp. 55ºC pH 6	Scar Gallardo et al. (2010)
Penicillium purpurogenum	30	Glucuronoxylan, arabinoxylans	Temp. 45ºC pH 3-5	Espinoza and Eyzaguirre (2018)
Penicillium citrinum HZN_{13}	10	Birchwood and oat spelt xylan	Temp. 55 -75ºC pH 3.5 – 5	Bagewadi et al. (2016)
Streptomyces sp. SWU10	11	Birchwood, oat spelt xylan, wheat arabinoxylan	Temp. 40ºC pH 5	Deesukon et al. (2013)

In the industrial scenario, hemicellulases are traditionally obtained from microorganisms due to the fast growth rate and the scalability to industrial level production (Bastawde, 1992; Gilbert and Hazlewood, 1993). Microbial endo-β-1,4-xylanases are habitually obtained from filamentous fungi, and in some cases, bacteria are investigated as a source of these enzymes. Filamentous fungi specialized in biomass deconstruction are the prime target for hemicellulases prospection, including

Trichoderma and *Aspergillus* (Filho et al., 2015; Rezende et al., 2002). These organisms are specialized in plant-cell wall solubilization. Thus they also produce a set of cellulases (Borin et al., 2015; Gómez-Mendoza et al., 2014).

The secretion of cellulases and hemicellulases is fundamental for achieving a more effective plant cell wall solubilization. However, for a specific application in paper pulp enhancement, cellulase-free blends are sought after. In this respect, microorganisms secreting low to non-detectable cellulolytic activities are searched; for example, *Clostridium absonum* CFR-702 (Rani and Nand, 2000) and *Thermomyces lanuginosus* (Purkarthofer et al., 1993) have been reported with this particular trait. Also, heterologous expression of endo-β-1,4-xylanases in host cells lacking cellulolytic activity is a feasible strategy, with the potential for industrial scalability (Ahmed et al., 2009).

Even though xylanases (endo-β-1,4-xylanases) represent a versatile biotechnological instrument, many bottleneck points are originated during its action in lignocellulose deconstruction. Most of the detrimental effects caused in xylan-degrading enzymes are related to components associated with lignocellulose, as phenolic components and carbohydrates (Boukari et al., 2011). Still, other bottleneck points can be related explicitly to endo-β-1,4-xylanases as low thermal stability, low-affinity to the substrate, and promiscuity in bounds-cleavage (Polizeli et al., 2005).

Despite the numerous applications of endo-β-1,4-xylanases, most studies regarding inhibition and deactivation of plant-cell wall degrading enzymes are based on cellulases (Kim et al., 2011; Ximenes et al., 2011). It is rational that studies aiming to understand the effect of plant-cell wall inhibitors have as prime target cellulase since this group of enzymes has a central role in cellulosic bioethanol production. However, xylanases are receiving more attention not only because of their industrial relevance in the paper and pulp industry but also by the employment of pentoses into fermentative pathways to obtain chemicals of industrial relevance.

Given the already established importance of endo-β-1,4-xylanases, and the emerging biotechnological application of this class of enzyme, this review intends to summarize the main findings in the literature regarding the abatement of endo-β-1,4-xylanases activities caused by plant-derived components. Also, strategies aiming to reduce activity loss are discussed, considering operational changes (*e.g* additives) and enzyme engineering.

2- **Sugars inhibitors**

When discussing enzymes that act on carbohydrate deconstruction, it is logical to associate carbohydrate-active enzymes inhibition with their end-products. Most of this line of thought comes from the classical inhibition mechanism observed for β-glucosidases and cellobiohydrolases, wherein in several cases, their hydrolysis products can strongly inhibit their activity (Bezerra et al., 2011; Zhao et al., 2004). A correspondent inhibition pattern can be associate between β-glucosidases and β-xylosidases, regarding their inhibition by end products, D-glucose, and D-xylose, respectively. However, for endo-β-1,4-xylanases this process is much less studied, been few reports aiming to understand how this class of enzyme is inhibited by sugar.

Although inhibition by end products be expected for carbohydrate-active enzymes, Rahmani et al. (2019) reported two xylanases from family 10 and 11 from the bacterium *Kitaspora sp.*, in their recombinant form as not inhibited by xylose. The authors used concentrations ranging from 0.1 to 10 $mg.ml^{-1}$, making it possible to categorize these enzymes as tolerant to xylose. Similar results were found by Xiros et al. (2011) that described xylose concentration from 1 to 10 $g.l^{-1}$, as not inhibitory to xylanases produced by *Fusarium oxysporum* during alkali-treated brewers spent grain hydrolysis. On the other side, xylobiose has been shown as a potent inhibitor of *Fusarium oxysporum*'s endo-β-1,4-xylanases, displaying significant inhibition in concentrations of 0.05 to 0.6 $g.l^{-1}$.

In another study focusing on fungal enzymes, Ribeiro (2014) showed that *Penicillium griseofulvum* displayed approximately 20% endo-β-1,4-xylanases activity loss when tested in the presence of 20 or 40 mM of xylose. Contrary, in the same study, the authors discovered that *Malbranchea pulchella* xylanase activity was slightly activated in the presence of xylose. Xylose is an inhibitor that has also been reported to bacterial xylanases; Paul and Varma (1990) described that xylose at 1 $mg.ml^{-1}$ as a potent inhibitor of xylan-degrading enzyme produced by *Bacillus sp.*; contrary, D-glucose had no inhibitory effect.

Still, regarding bacterial xylanases, Lo Leggio and Pickersgill (1999) reported the inhibition constant for multiple xylosaccharides and cellosaccharides for the recombinant form of the endo-β-1,4-xylanase, xylanase A, from *Pseudomonas fluorescens sp. cellulosa*. In this study, the authors reported that xylobiose and xylosaccharides with five unities have inhibitory action, not being detected inhibitory effect for xylobiose. Besides, cellosaccharides also displayed inhibition, mainly those with a polymerization degree of three, four, and five.

For xylanases produced by *Trichoderma longibrachiatum*, Royer and Nakas, (1991) proved that for two different purified endo-β-1,4-xylanases the addition of xylotriose inhibited the hydrolysis of xylopentose. However, the authors also showed that xylobiose displays inhibition with a more negligible effect than xylotriose. Regarding *Trichoderma harzianum* xylanases, Tan et al. (1985) purified and characterized two, and neither displayed inhibition when assayed in concentration from 1 to 4% of xylose or xylobiose. Differently, it was shown that a higher concentration of sugars could enhance xylanase activity. The authors explain this enhanced activity by changes in the viscosity propriety of the reactional mixture, resulting in reduced sedimentation constant of xylan. Besides, it was shown that xylan concentration >1% could inhibit both enzymes.

Summarizing the main research findings, it is clear that few endoxylanases have their inhibition constants evaluated. Most of the studies focused on associating the inhibitor (xylosaccharides) with the decrease in hydrolysis products. This kind of approach is very significant when dealing with enzymes with the potential for industrial applications. Also, most of the colorimetric reactions commonly used to evaluated xylan hydrolysis are based on the detection of reducing ends generated upon carbohydrate hydrolysis as the method developed by Miller (1959), which can become more troublesome with the addition of inhibitors (xylobiose, and xylose) with higher reactivity to the colorimetric reaction. To contour this limitation, many authors have used xylan associated with azo dyes and directly analyzing changes in product formation in the presence of inhibitors.

At this point, is crucial that some considerations must be taken to correctly interpret inhibition studies from enzymes that are not in their purified form. Unpurified enzyme mixtures may contain alongside the assayed endo-β-1,4-xylanase, β-xylosidase, and other enzymes with accessory activity (Gómez-Mendoza et al., 2014; Yang et al., 2018). When assaying against natural substrates, both endo-β-1,4-xylanase and β-xylosidases will majorly contribute to the production of reducing ends. Thus the inhibitory profile observed for the mixture of enzymes may be interpreted with thoughtfulness. To circumvent this issue, purification and the production of recombinant endo-β-1,4-xylanase in host cells lacking such activity are the best alternatives so far. Moreover, in this respect, there are innumerous reports in the literature about the purification and production of recombinant xylanases (Basu et al., 2017; Gerber et al., 1997).

Based on the reports of sugar inhibition over xylanases, it can be inferred that xylobiose and small xylosaccharides with a low degree of polymerization pose as the primary sugar source of inhibition of this class of enzyme. However, xylose was constantly reported with low inhibition, the same observed for cellosaccharides. It is essential to highlight that in the context of plant cell wall deconstruction, many other sugars are present even in the xylan structure as arabinose, and the inhibitory effect of these other sugars has not been evaluated. In addition, acetylated sugars and decorated xylose residues may also be considered as inhibitors candidates. A general model of inhibition is shown in figure 1.

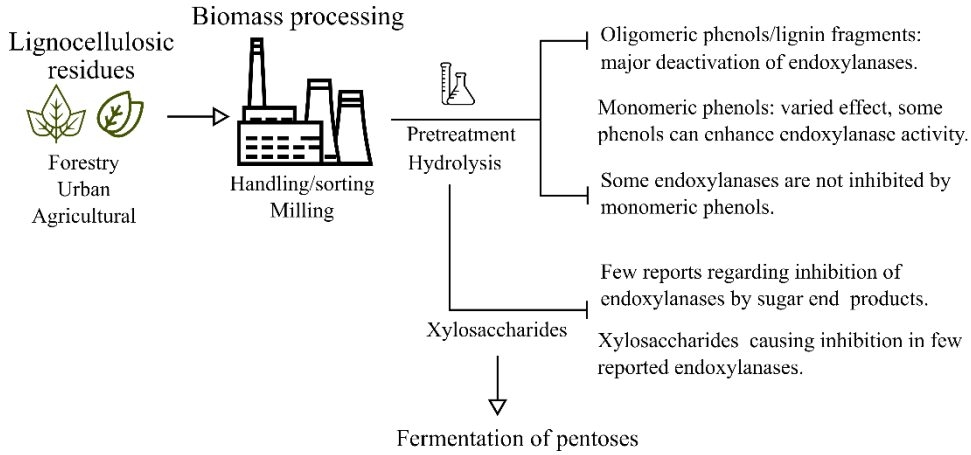

Figure 1: Schematic representation of endoxylanase inhibitors generated during lignocellulosic processing, and their effect over endoxylanase

3- **Aromatic and phenolic inhibitors**

Aromatic and lignin-related compounds are the main class of molecules that are investigated as a significant player in cellulases and hemicellulases deactivation and inhibition. The attention that this class of plant cell wall-derived components is receiving is because of the growing number of reports showing that not just the lignin but also free phenolic unities can decrease the performance of holocellulases (Ximenes et al., 2011).

Lignin is a macromolecule comprised of a covalently joined aromatic structure that conceals plants structural resistance and an efficient biochemical barrier against biological degradation of the carbohydrate core. Recent studies also addressed the formation of free phenolic unities upon lignocellulose pretreatment, and/or deconstruction; for example, Kim et al. (2011) reported that liquid hot water pretreatment of maple could generate free forms of phenols in the liquid fractions in values of 1.3 g/l.

García-Aparicio et al. (2006) reported forming many free aromatic compounds in stream-explosion barley straw liquid fraction, including ferulic, vanillic, and coumaric acid.

Although the current review focuses on the classical inhibition of endo-β-1,4-xylanases, many reports regarding aromatic compounds and lignin structure effects over this class of enzyme are not entirely associated with the inhibition mechanism. In many cases, lignin and related compounds can adsorb part of the enzyme, making them unavailable to the hydrolysis system (Rahikainen et al., 2013) or altering enzyme properties as thermal stability (De Souza Moreira et al., 2013). Other aromatic compounds as tannic acid are usually reported as a robust precipitating agent of enzymes, which can potentially make them not active in the deconstruction of lignocellulose (Goldstein and Swain, 1965; Marks et al., 1987). Regardless of the mechanism involved, reports about the abatement/inhibition of xylanase activity will be addressed.

In the industrial scenario, more precisely in the paper pulp brightening process mediated by enzymes, it is expected that xylanases will encounter lignin and/or aromatic compounds from the chemical disruption of hemicellulose and lignin. Thus, the prospection of endo-β-1,4-xylanases with tolerance to this class of macromolecule is well desired. In this regard, Morrison et al. (2011) reported the recombinant form of xylanase XynA from *Clostridium cellulovorans* with relative tolerance to lignin, showing only about ~85% activity loss when tested against lignin in concentration from 0.025 to 1 mg/ml. On the contrary, the authors described that some free forms of aromatic compounds had higher potential to decrease *C. cellulovorans*' XynA activity; for instance, coumaric acid at 0.2% concentration caused 37% inhibition.

Some other aromatic compounds associated with the plant cell wall are described as significant components in reducing the performance of hemicellulases. For instance, Ladeira Ázar et al. (2018) reported that enzymatic preparations containing the secretome of two fungi had the endo-β-1,4-xylanase activity strongly abated in the presence of tannic acid in the concentration of 50 mM. In higher concentrations, this effect was more prominent. Still, regarding tannic acid and fungi enzymes, Gomes et al. (2019) demonstrated that tannic acid in small concentrations of 0.2 mg/ml could reduce the xylanase activity present on *Penicillium chrysogenum* secretome.

The effect of tannic acid has also been investigated for purified enzymes synthesized by filamentous fungus. Silva et al. (2015) described the effect of tannic acid in a purified GH11 from *Emericella nidulans*; this compound assayed at 2 mg/ml (protein

ratio of 230 µg phenol/µg protein) caused a significant reduction in catalytic efficiency. The authors also explain that other aromatic compounds as vanillin and *p*-coumaric acid did not cause inhibition.

In addition to decreasing xylanase activity, some reports show that phenolic compounds can enhance xylanase activity, thus boosting hemicellulose hydrolysis. Kaya et al. (2000) reported that aromatic compounds as vanillic acid could enhance the hydrolytic activity of an enzyme preparation manufactured for biobleaching cellulosic pulps. The same effect was observed when adding small portions of black liquor from the kraft pulping of southern-yellow pine. Also, in addition to this report, Osiro et al. (2017) described that endo-β-1,4-xylanases assembled into the cellulosome of *Clostridium thermocellum* B8 are activated by ferulic acid, the same activation was also detected for the endo-β-1,4-xylanase activity present on the secretome of *Panibacillus barengoltzii* A1_50L2 when assayed in the presence of ferulic acid at 1 mg/ml.

While several reports have addressed the severity of phenolic compounds in hemicellulose solubilization, either by direct measuring activity change or performance in lignocellulose hydrolysis, very few studies have deeply investigated amino acid residues involved in protein/phenol interaction. Understanding how enzymes are modulated by phenols/lignin-related structures may require structural studies and engineered proteins to evaluate the reduction/enhancement of such interaction. In this regard, Boukari et al. (2011) studied the interaction of lignin-derived phenols and a GH11 endo-β-1,4-xylanase produced by *Thermobacillus xylanilyticus*. Using tryptophan fluorescence spectroscopy and classical enzyme kinetics, the authors proved that the hydroxyl radical of phenols is mainly responsible for protein/phenol interaction.

Despite the many recent reports describing how phenol/lignin structures modulate endo-β-1,4-xylanase activity, it is not possible to point out a general rule of which lignin-by products are more harmful xylan degrading enzymes. Taking the majority of reports as a point of view, structures containing polyphenols as tannic acid are the primary source of activity reduction. Since the abatement of xylanase activity appears to be caused by multiple factors arising from plant phenols, several possibilities of how to dimmish their detrimental effect will be further discussed in the following sections.

4- **Proteins specialized in inhibiting endoxylanases**

Although the present manuscript focuses on inhibitors/deactivators of endo-β-1,4-xylanases that are found in industrial applications, there is a growing number of studies

describing proteins involved in inhibiting xylanase activities in plant/pathogens interaction. These proteins are primarily found in plant tissues, and they are considered a biochemical mechanism developed by plants to avoid pathogens colonization of plant tissues.

The role of this class of xylanase inhibitor proteins was validated in various in vitro analyses and biological models. As an example of a study investigating the role of these proteins as a biochemical strategy of plant defense against pathogens, Moscetii et al. (2013) modified wheat plants to constitutively express TAXI-III protein (*Triticum aestivum* endoxylanase inhibitor) and noticed that the constative expression of such protein could delay the symptom caused by *Fusarium graminearum* plant colonization. Illustration of plant defense is shown in figure 2.

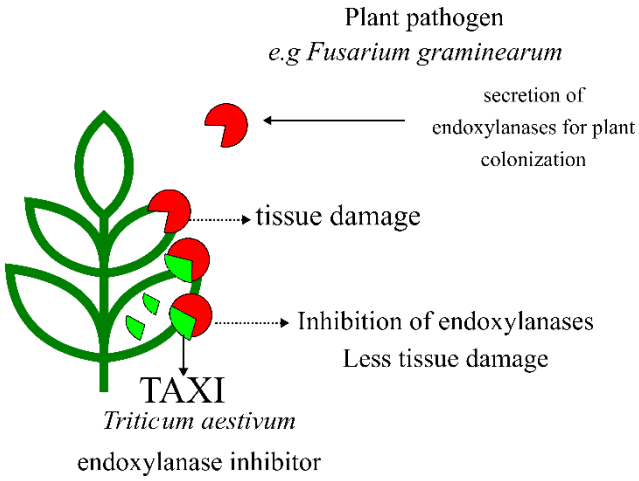

Figure 2: Plant tissue colonization mediated by secretion of endoxylanases, and the effect of TAXI (*Triticum aestivum* endoxylanase inhibitor) in inhibiting pathogens' endoxylanases to reduce plant cell wall damage.

Regarding proteins with the inhibitory role over xylanases and in vitro analyses, Juge et al. reported proteins of glycoside hydrolase family 18 produced by wheat (*Triticum aestivum*) that could inhibit fungal xylanases from family 10 and 11 produced by several different fungal species, including *Aspergillus niger*, *Trichoderma longibrachiatum*, and *Aspergillus oryzae*. The authors also evaluated the effect of these proteins during inhibition of bacterial xylanases from families 10 and 11; however, no inhibitory activity was observed (Juge et al., 2004).

Plants proteins that can inhibit endo-xylanases seem to be present in different other plants, possibly a conserved mechanism. Fierens et al. (2003) found a class of proteins for wheat that can inhibit xylanases. They also found similar genes for carrot, *Arabidopsis thaliana*, and rice. Still regard this study, they expressed the TAX-I (*Triticum aestivum* endoxylanase inhibitor) in *Escherichia coli* and evaluated the inhibitory role of the recombinant protein in xylanases of *A. niger* and *B. subtilis*.

Although the inhibition of endoxylanases by plant proteins be a topic centered on plant-pathogen interaction, in industrial conditions of lignocellulose deconstruction, these protein inhibitors are likely to not be present due to usual pretreatments/biomass handling. However, the study of the inhibitory mechanism of those proteins can be a valuable tool to understanding the role of these enzymes in lignocellulose deconstruction, and they can also be used as scientific tools to study the effect of inhibiting endoxylanases during biomass deconstruction; therefore, they are worth to mention in this review.

5- **Approaches to reduce the inhibition**

In addition to the significant number of reports describing potential inhibitors/deactivations of endoxylanases found in lignocellulose, there is also a growing number of reports describing new methodologies to surpass this inhibition. Many studies seeking new methodologies to surpass such inhibition are majorly based on scientific studies based on cellulases since this class of enzymes has been the primary scientific focus in plant cell wall deconstruction.

As an example of the prevalence of studies investigating the inhibition of cellulases during lignocellulose deconstruction, Qing and Wyman (2011) described integrating β-xylosidases and endoxylanases in enzymatic mixtures could lead to a reduction of the cellulase inhibition by xylan deconstruction end products.

Regarding aromatic/phenolic compounds that are present in lignocellulose and residual carbohydrate slurry after pretreatment, commonly referred to as black liquor (Bankeeree et al., 2018), there are studies on which endoxylanases possessing a certain level of tolerance for such compounds are described, including endoxylanases from *Emericella nidulans* (Silva et al., 2015) and *Aspergillus tamarii* (Av et al. 2016). These endoxylanases could be explored and used as a model to investigate further the reasons for their tolerance to some plant cell wall-derived inhibitors, and therefore the development of engineered enzymes.

Despite studies regarding aromatic/phenolic compounds as endoxylanases inhibitors, some reports describe such compounds as activators of this class of enzyme (Monclaro et al., 2019). Such reports analyzed single compounds effect over endoxylanases. Although they do not evaluate these enzymes in a broad context, this kind of study indicates that phenolic activation of endoxylanases can potentially balance the inhibition observed in the presence of different other phenolic inhibitors. Finding enzymes prone to activity enhancement in front of some phenolic compounds could be a viable strategy to formulate xylanases-blends that can withstand the enzymatic activity upon releasing such compounds in lignocellulose deconstruction.

In addition to engineering xylanases and screening enzymes that can tolerate the presence of a phenolic compound, there are also studies investigating the integration of laccases (phenol-active enzymes) to reduce the inhibitory effect observed during lignocellulose deconstruction in β-xylosidases and xylanases. In this regard, Ladeira Ázar et al. (2018) demonstrated that the laccase activity of fungal strains could potentially reduce the phenolic inhibition of xylan degrading enzymes, including β-xylosidase and endoxylanases. This report highlights that in addition to study endoxylanases and the inhibitory mechanism, the screening of enzymes that can modify phenolic compounds is also a valuable strategy to reduce such inhibition and consequently enhance hemicellulose deconstruction.

Another strategy to reduce enzyme (endoxylanases) negative interaction with lignin and phenolic compounds is adding proteins with no catalytic activity against lignocellulose. For instance, the possibility of using BSA protein to alleviate the inhibitory effect caused by lignocellulose-derived phenolic compounds has been described for cellulases (Yang and Wyman, 2006), and it is plausible that the same mechanism may aid endoxylanases to avoid deactivation/inhibition by such compounds.

Tejirian and Xu. (2011) reported that the addition of polyethylene glycol could reduce the detrimental effect caused by oligomeric phenols in cellulose conversion. Although few studies investigated the role of oligomeric phenolics in xylan deconstruction by endoxylanases, this class of compound (polyethylene glycol) can potentially disrupt the interaction between enzymes and phenolics, which could theoretically cause a positive effect in xylan solubilization.

In addition to polyethylene glycol, some other chemical compounds have been investigated to dimmish the unproductive interaction between cellulases and phenolic

compounds liberated during lignocellulose processing, including PVP and Tween 80 (Oh et al., 1980; Tejirian and Xu, 2011; Zanobini et al., 1967). These compounds, as reported to PEG, can disrupt protein interaction with phenolic compounds. As observed for PEG, these compounds were not investigated, aiming to understand the role of phenolic compounds in endoxylanases activity. Since such compounds aim at disrupting phenol/protein interaction, they could aid endoxylanases to avoid unproductive interaction during hemicellulose solubilization.

6- **Perspectives and future research**

The industrial importance of endoxylanases makes them a class of enzymes highly studied. The inhibition and deactivation of xylanases by lignocellulose compounds is a topic of great interest since understanding how enzymes are deactivated/inhibited may make possible the development of new methodologies and strategies to diminish such detrimental effects. Most of the studies aiming to alleviate inhibition by saccharification/ pretreatment end products are related to cellulases. Therefore there is a gap in the literature regarding methodologies to dimmish such inhibition in endoxylanases.

Among the alternatives that can readily be applied in hemicellulose deconstruction processes to alleviate the deactivation by polymeric phenols, enzymes such as laccases and tannases can modify phenolic compounds into less inhibitory molecules (Ladeira Ázar et al., 2018; Tejirian and Xu, 2011). Further alternatives may include the rational design of hemicellulases blends to incorporate endoxylanases with lower deactivation/inhibition by phenols and engineering of endoxylanases taking as a model those enzymes with relative tolerance to inhibitors.

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CAPÍTULO III - Influência de compostos fenólicos derivados da lignina na endo-β-1,4-xilanase XynA de *C. thermocellum*

1-Introdução

Com o avanço das técnicas de pré-tratamento de resíduos lignocelulósicos, muito é discutido em relação à melhora da acessibilidade à porção rica em carboidratos e a liberação de compostos fenólicos com elevado potencial em inibir/desativar enzimas envolvidas na desconstrução da fração composta por hemicelulose e celulose.

Historicamente, celulases de origem fúngica foram os principais alvos de estudos para avaliar o efeito desses compostos fenólicos que são gerados durante o processamento da biomassa lignocelulósica. Dentre os principais compostos que são liberados da biomassa encontram-se os monoméricos como ácido ferúlico, e os que são compostos por várias unidades, como o ácido tânico que é comumente encontrado em tecidos vegetais.

Apesar da literatura atual demonstrar que esses compostos são inibidores e desativadores de celulases fúngicas, pouco se sabe da relação da liberação de tais compostos e sua influência em outras enzimas envolvidas na desconstrução da parede celular vegetal, como xilanases. Em relação a xilanases e os compostos liberados durante o pré-tratamento, existe um crescente número de relatos investigando como tais compostos possam modular a atividade dessa classe de enzima, porém assim como ocorre com celulases, esses estudos são focados em enzimas fúngicas proveniente de mesófilos.

2-Objetivos experimentais do capítulo

Tendo como base a escassez de dados na literatura referente ao efeito de compostos fenólicos em xilanases, e mais específico xilanases resistentes a desativação térmica (termoestáveis). No presente estudo investigamos o efeito de tais compostos sobre a xilanase XynA de *C. thermocellum* que foi produzida de forma heteróloga em *Escherichia coli*. Dentre as perguntas que nortearam a execução desse estudo estava a questão se proteínas termoestáveis poderiam apresentar um menor índice de desativação/inibição por compostos fenólicos, assim como se tais compostos poderiam modificar a tolerância térmica de tais enzimas. A xilanase XynA foi utilizada como modelo devido a sua estabilidade térmica já relatada na literatura, assim como sua arquitetura que é clássica de enzimas que compõe o complexo enzimático de *C. thermocellum*, possuindo além do domínio dockerin I de associação ao celulossoma, um

domínio adicional de associação a carboidratos (CBM6, afinidade a xilana), e um outro contendo a atividade acessório de acetil-esterase.

Influence of lignin-derived phenolic compounds on the *Clostridium thermocellum* endo-β-1,4-xylanase XynA

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Abstract

Phenolic compounds released during pretreatment of lignocellulosic biomass influence its enzymatic hydrolysis. To understand the effects of these compounds on the kinetic properties of xylan-degrading enzymes, the present study employed the recombinant cellulosomal endo-β-1,4-xylanase, thermostable GH11 XynA protein from *Clostridium thermocellum*, as an enzyme model to evaluate the effects of 4-hydroxybenzoic acid, gallic acid, vanillin, tannic acid, *p*-coumaric acid, ferulic acid, syringaldehyde, and cinnamic acid. XynA was deactivated by the assayed phenols at 40 and 60ºC, presenting the strongest deactivation at 60ºC in the presence of tannic acid, with an activity reduction of about 80%. Thermal stability of XynA was influenced by ferulic acid, syringaldehyde, cinnamic acid, 4-hydroxybenzoic acid, and *p*-coumaric acid. The hydrolysis rate of oat-spelt xylan by XynA was influenced by temperature, being unable to hydrolyze at 40ºC in the presence of tannic acid. On hydrolysis at 60ºC, the presence of gallic and tannic acid caused a major reduction in reducing sugar production, generating 3.74 and 2.15 $mg.mL^{-1}$ of reducing sugar, respectively, whereas the reaction in the absence of phenols generated 4.41 $mg.mL^{-1}$. When XynA was pre-deactivated by phenols it could recover most of its activity at 40ºC; however, at 60ºC activity could not be reestablished.

Keywords

C. thermocellum, XynA, *p*-coumaric acid, ferulic acid, tannic acid

1.Introduction

Agricultural residues such as sugarcane bagasse, wheat bran, corn cob and straw, mainly constituted by lignocellulose, are low-cost carbon sources with the potential for use as raw materials in the production of value-added chemicals. Technologies based on the use of these residues are still under development, as an economically viable process requires the complete deconstruction of lignocelluloses, a process hampered by their natural recalcitrance and complex structure [1]. Currently, the most commonly used and best studied process involves an initial step of pre-treatment to remove hemicelluloses and lignin, followed by enzymatic hydrolysis of the carbohydrate portion using plant cell wall-degrading enzymes [2–4]. Pre-treatment approaches including chemical, physical and enzymatic treatments result in a less recalcitrant carbohydrate core, with a liquid fraction rich in xylosaccharides and soluble lignin components. Like the carbohydrate core, the liquid fraction, rich in carbohydrates, is an interesting source of molecules for further biorefinery use [5–7].

Lignin is the major plant cell wall component responsible for lignocellulose recalcitrance and may also act as an inhibitor of plant cell wall hydrolases during enzymatic hydrolysis steps [8]. This inhibition might be caused by larger fragments of lignin and/or by free forms of phenolic compounds released during pretreatment [9,10]. Liquid hot water and steam-explosion are examples of pretreatments that release free forms of lignin-derived phenols such as vanillin and coumaric acid from lignocellulose [11,12].

Xylan-active enzymes have the potential for application in different industrial processes such as paper bleaching, animal feed additives, and general lignocellulose deconstruction for biofuel production [12,13]. However, the residual slurry from lignocellulose pretreatments contains phenolic compounds and carbohydrates that may cause inhibition over glycoside hydrolase [14,15]. There is a growing interest in identifying organisms and enzymes that are tolerant to these compounds, allowing them to deconstruct residual slurry to its monomeric units that can be further applied as biorefinery building blocks [16].

Historically, endo-β-1,4-xylanases are prospected from fungal strains; however, there is currently a demand for identifying new enzymes that are thermostable [17]. In this search for enzymes with kinetic properties more appropriate for industrial processes (*e.g* paper and pulp bleaching), thermophilic organisms from natural environments are

commonly investigated with the goal of producing industrially relevant enzymes [18,19]. In addition, there is an interest in studying thermostable enzymes for engineering new biocatalysts [20].

With respect to microbial hemicellulases, endo-β-1,4-xylanases pertaining to family GH11 are commonly secreted by fungi and bacteria [21,22]. Family GH11 is usually reported as displaying synergism in working with other endo-β-1,4-xylanases from family 10, enhancing sugar yield from biomass deconstruction [23]. However, there is a number of reports showing the potential of this family as glycoside hydrolases with applications in industrial processes as an enhancer of paper pulp brightness [24], and saccharification of lignocellulosic residues [25].

Clostridium thermocellum is largely studied as an efficient cellulose-degrading microorganism; however, it also produces a considerable number of hemicellulases, including xylanases [29]. Among *C. thermocellum* xylanases, XynA is one of the most studied as it is mainly found in the cellulosome, a highly efficient cellulolytic enzyme complex [26,27,30–33]. XynA is a bifunctional enzyme characterized by a modular structure containing a carbohydrate-binding module family 6 (CBM6) domain, a dockerin domain, and GH11 and acetyl xylan esterase catalytic domains [26]. The presence of two catalytic domains provides the enzyme with the ability to simultaneously remove side chains and/or substituting groups, and to catalyze the hydrolysis of glycoside linkages of the xylan backbone. Indeed, enzymes presenting a substrate-binding domain together with more than one catalytic domain, and organized in a complex, present greater efficiency in solubilization of their substrates [30,34].

In previous studies, our research group showed the ability of *C. thermocellum* (isolate B8) to grow on agricultural residues such as sugarcane bagasse with minimal pre-treatment, and to produce a diversified set of xylanases [28]. In addition, we also demonstrated that xylanase-encoding genes are highly expressed (data not published) during *C. thermocellum* growth in the presence of sugarcane straw. Together, these data call attention to the importance of investigating the role of *C. thermocellum* xylan-degrading enzymes during degradation of more recalcitrant substrates.

Based on the relevance of thermostable enzymes in industrial processes, and the need to understand factors that negatively influence lignocellulose deconstruction, the present study was designed to evaluate the impact of plant phenolic compounds on the activity of recombinant XynA, in relation to deactivation and effects on thermal stability

and xylan hydrolysis. The data presented will further the understanding of the effects of phenolics, generated in pretreatments, on xylan solubilization.

2. Methodology

2.1 Amplification and cloning of *xynA*

C. thermocellum B8 was cultured in reducing liquid medium containing 1% (w/v) Avicel PH101 (Sigma Aldrich, USA) as a carbon source, as previously described [28]. After 48 hours of growth, the culture was vacuum-filtered (Whatman filter paper nº5). The filtrate was collected and centrifuged for 10 minutes at 10,000 g / 4°C, the supernatant was discarded, and the cell pellet collected and used for DNA extraction. DNA extraction was performed as previously described [35,36] and the DNA was stored in 10 mM tris-HCl buffer, pH 8, with 1 mM EDTA, at -20°C until further use.

The full-length sequence of the *xynA* gene encoding the domains: catalytic domain GH11, dockerin type I acetyl xylan esterase and carbohydrate-binding module 6 (*C.thermocellum* ATCC 27505 NCBI Cthe_2972), was retrieved from Genbank. The SignalP 4.1 online tool [37] was used to predict the signal peptide. Forward and reverse primers were designed using the primer3plus online tool [38] (*forward*: 5'-AATATTGAATTC GATGTAGTAATTACGTCAAACCAGACG; *reverse*: 5'-AATATTGCGGCCGCATTACGGTACAGAGT TATACATTCTTTTGA-3'). The primers were designed to remove the xynA native signal peptide and add restriction sites for *EcoRI* and *NotI* restriction enzymes to the *forward* and *reverse* primers, respectively (underlined sequences).

Amplification of *xynA* was carried using polymerase chain reaction containing 50 ng of genomic DNA, 0.50 μM forward and reverse primers, 200 μM dNTPs, 2.50 μL of 10x Taq DNA polymerase buffer (Cellco, Brazil), and one unit of high fidelity *Taq polymerase* (Cellco, Brazil), in a final volume of 25 μL. The cycling protocol was: 3 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 62°C, and 3 minutes at 72°C. The final extension was at 72°C for 5 minutes. PCR products were analyzed by electrophoresis on a 0.8% (w/v) agarose gel. The amplified product presenting the expected length (1994 bp) was extracted from the gel using QIAEX® II Gel Extraction Kit (Qiagen, Germany), and then digested with *EcoR1* and *Not1* (New England BioLabs® Inc., USA), following the manufacturer´s instructions. Digested xynA was then ligated to the pET-21a(+) vector (Novagen® Merck KGaA, Germany) previously digested with *EcoR1* and *Not1*. Ligation was carried out using T4 DNA ligase (New England BioLabs® Inc., USA) following the manufacturer's instructions.

Competent *E.coli* XL10-Gold® cells (Stratagene, USA) were transformed by heat-shock with the pET-21a-xynA construct; after transformation the cells were cultivated in one mL of LB-media for one hour at 37ºC in a shaker incubator at 200 rev.min^{-1}, and then spread on LB-agar plates. Positive colonies displaying ampicillin resistance were grown in five mL of LB broth for 16 h at 37 ºC in a rotary shaker at 200 rev.min^{-1}. An aliquot of 2 mL of each culture was removed, centrifuged for 10 minutes at 10,000 g at room temperature, and the cell pellet used for plasmid extraction using the GeneJET Plasmid Miniprep Kit (Thermo Fischer Scientific, USA). The presence of the *xynA* gene in pET-21a-xynA was confirmed by PCR using the protocol and primers described above, as well as, by analyzing the digestion pattern generated by *NotI* and *EcoRI*.

pET-21a-xynA was cloned in *E. coli* BL21-DE3 (Novagen® Merck KGaA, Germany). Competent cells were heat-shock transformed, spread on LB-agar plates, and then one out of 10 colonies displaying ampicillin resistance was selected for further protein expression (BL21-pET-21a-xynA). All *E. coli* screening procedures were carried out in agar-LB-medium supplemented with 100 μg.mL^{-1} ampicillin.

2.2 Protein expression

E. coli BL21-DE3 cells harboring pET-21a-xynA were inoculated in conical flasks containing 50 mL of LB-broth with ampicillin (100 μg.mL^{-1}). Inoculated flasks were incubated at 37 ºC in a shaker at 200 rev.min^{-1} until the growth reached an OD_{600} value of 0.6. Subsequently, induction was carried out using two concentrations of lactose (5 mM or 10 mM). After 30, 150 and 300 minutes of growth, 5 mL aliquots were collected. Induction experiments were performed in biological triplicates. Each aliquot was centrifuged at 10,000 g / 4°C for 20 minutes. The supernatant was discarded, and the cells pellet suspended in 5 mL of 10 mM tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF), and then sonicated at 40% amplitude for 3 minutes (30 seconds on/off cycles) (SONICS® Vibra-Cell™ VC 750, USA). Sonicated cells were centrifuged, and the collected supernatant was supplemented with 0.01% (*w/v*) sodium azide, and stored at 4°C until use as a source of enzymes for enzymatic characterization.

Alternatively, supernatant cultures were further incubated at 50°C for 12 hours, in order to precipitate *E. coli* native proteins, then centrifuged at 10,000 g / 4ºC for 20 minutes. The clarified supernatant was collected and used as a source of XynA for further

characterization (pH and thermal stability), and to assess the effects of phenolic compounds.

2.3 Enzymatic activity

The endo-β-1,4-xylanase activity of clarified supernatants was evaluated by measuring sugar released from oat-spelt xylan (Sigma Aldrich, USA) using the Miller method (1959) [39]. The enzymatic assay was carried out using 2% (*w/v*) oat-spelt xylan in 50 mM sodium acetate buffer (pH 5) as previously described [28]. One unit of enzymatic unit per mL ($IU.mL^{-1}$) was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per minute of reaction per volume of enzyme used. D-xylose was used as a standard reducing sugar.

2.4 Electrophoresis and protein quantification

Electrophoresis procedures were carried out under denaturing SDS-PAGE conditions, as previously described by Laemmli [40] using a 12% polyacrylamide gel and Mini-PROTEAN Tetra Cell apparatus (Bio-Rad USA). Zymograms to detect endo-β-1,4-xylanase activity were carried out as previously described [28]. Protein quantification was performed using the commercial kit Quick Start™ Bradford 1×Dye Reagent (Bio-Rad Laboratories Inc., USA), following the manufacturer´s instructions. The protein marker used was Pierce™ Unstained Protein MW Marker (Thermo Fischer Scientific, USA).

2.5 Temperature and pH effect on recombinant XynA

The effect of temperature on XynA activity was determined using the enzymatic assays as described above, at temperatures ranging from 30 to 80 °C. The effect of pH was evaluated by varying substrate buffering with 100 mM phosphate-citrate buffer in a pH range from 3.0 to 8.0.

2.6 Effect of lignin-derived phenolics on recombinant XynA

Deactivation of XynA was evaluated by incubating clarified supernatant (1 IU of activity) with tannic acid (used as a model for oligomeric phenols present in lignocellulose), vanillin, *p*-coumaric acid, trans-ferulic acid, 4-hydroxybenzoic acid, syringaldehyde, gallic acid, and trans-cinnamic acid (Sigma Aldrich, USA) at final concentrations of 1 $mg.ml^{-1}$, for 24 hours, buffered in 50 mM tris-HCl, pH 7.1, as previously reported [41,42]. Each assay was performed at 25, 40 or 60ºC, and after 24 hours of incubation residual activity was evaluated by using the standard enzymatic assay described above. Phenolic compounds were dissolved in water (tannic acid, vanillin) or in pure anhydrous ethanol (*p*-coumaric acid, trans-ferulic acid, 4-hydroxybenzoic acid,

syringaldehyde, gallic acid, and trans-cinnamic acid) prior to experiments. Chemicals were obtained from Sigma-Aldrich with the following purity values: Gallic Acid >97.5% Lot # SLBM8746V, Tannic acid ACS purity grade Lot # MKBV0516V, Vanillin 99% Lot # BCBR0420V, *p*-coumaric acid >98% Lot # BCBQ5364V, trans-Ferulic acid 99% Lot # BCBM6076V, 4-Hydroxybenzoic acid >99% Lot # STBD6654V, Syringaldehyde 98% Lot # S26323V, trans-Cinnamic acid >99% Lot # MKBP7752V. Controls were performed using deionized water or ethanol (10% *v/v* final concentration) as solvent.

Thermostability of XynA in the presence of plant phenols was determined using the same procedures as used for deactivation (phenols at a final concentration of 1 $mg.mL^{-1}$, reaction buffered with 50 mM tris-HCl, pH 7.1). Aliquots were taken at 24-hour intervals until 72 hours of incubation, and residual activity evaluated using the standard enzymatic assay described above. Controls containing ethanol and water were used, and activity was expressed as relative activity in relation to controls before incubation. Calculation of half-life ($t_{1/2}$) and deactivation constants (k_d) was performed as previously reported [43].

2.7 Influence of plant phenolic compounds on xylan hydrolysis by XynA

Xylan hydrolysis in the presence of phenolic compounds was evaluated in two different experiments. First, the phenolic compounds were added together with enzymes in xylan hydrolysis (inhibition), and second, the deactivation of these was evaluated by pre-incubating enzymes with phenols before proceeding to hydrolysis. Inhibition of xylan hydrolysis by lignin-derived phenolic compounds was carried out at 40 or 60°C for 72 hours with constant agitation at 150 $rev.min^{-1}$. Hydrolysis assays contained 10 $mg.mL^{-1}$ of oat-spelt xylan (Sigma Aldrich, USA) buffered with 100 mM tris-HCl, pH 7.1, 0.1 U of heterologous XynA, and 1 $mg.mL^{-1}$ of the lignin-derived phenols tannic acid, vanillin, *p*-coumaric acid, trans-ferulic acid, 4-hydroxybenzoic acid, syringaldehyde, gallic acid and trans-cinnamic acid (aromatic) at phenol/protein ratio of 100 wt/wt, in a final volume of 5 mL. Aliquots of 100 µL were withdrawn at 24-hour intervals, and used to quantify reducing sugars released [44].

To further evaluate the effects of the phenolic compounds on XynA deactivation, the enzyme was incubated in the presence of phenols (tannic acid, vanillin, *p*-coumaric acid, trans-ferulic acid, 4-hydroxybenzoic acid, syringaldehyde, gallic acid and trans-cinnamic acid (aromatic)) for 24 hours at 40ºC or 60ºC, as described previously for the initial deactivation assay. Hydrolysis was then carried out as described above (at 40 and

60ºC), using 100 µL of deactivated recombinant XynA (deactivation and hydrolysis at the same temperature). For this experiment, samples were withdrawn after 72 hours and reducing sugars represented as yield (%) in comparison to enzymes incubated in the absence of phenolics. Each hydrolysis experiment was carried out in triplicate.

2.8 Statistical analyses

Results were subjected to ANOVA and Tukey's test using SigmaPlot v.12.0 (Systat Software Inc, USA), and statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1 Enzyme production and characterization

E. coli BL21-DE3 clone 3, harboring pET-21a-xynA, displayed endo-β-1,4-xylanase activity after 30 minutes of induction with 5 and 10 mM lactose, reaching maximal activity after 150 minutes (**Figure 1**). No endo-β-1,4-xylanase activity was detected for control *E. coli* harboring only the empty pET-21a vector. A protein band with an estimated molecular mass of 74 kDa corresponding to XynA full-length peptide was the most prominent protein detected on SDS-PAGE after induction for 150 and 300 min with 5 and 10 mM of lactose, respectively (**Figure 2.A, 2.B**). Zymogram analysis showed different protein bands presenting endo-β-1,4-xylanase activity. Hayashi et al. [26] also detected production of multiple forms of *C. thermocellum*'s XynA activity expressed in *E. coli* M15, suggesting proteolytic cleavage as a major drawback.

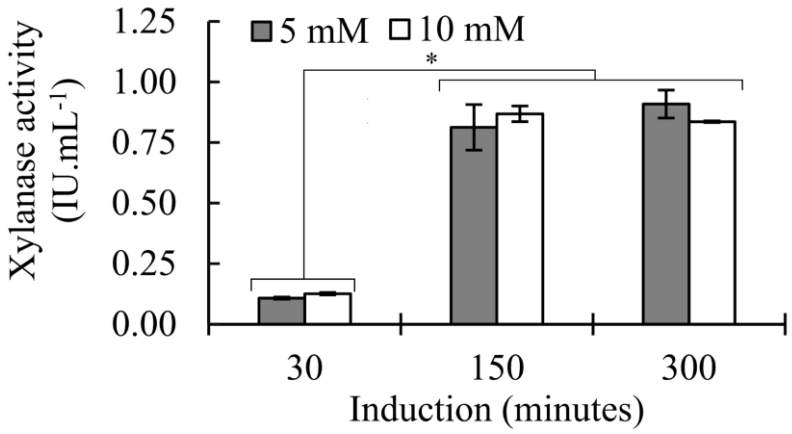

Figure (1): Time course of xylanase activity obtained after *E. coli* BL21(DE3) pET21a-xynA induction with 5 (grey bars), and 10 mM (white bars) lactose. Vertical bars represent standard deviation for biological triplicates. *E. coli* BL21(DE3) did not display xylanase activity. (*) $p<0.05$

The number of protein bands presenting endo-β-1,4-xylanase activity decreased to three after the supernatant heat treatment (Figure 3). The observed effect may be a consequence of a portion of proteins with xylanase activity being precipitated due to protein-protein interactions with native *E. coli* proteins. The isoforms detected correspond to the estimated molecular mass of full length XynA (74 kDa), a probable cleaved form without the acetyl xylan esterase module, and another cleaved form lacking both the acetyl xylan esterase and the dockerin domains (Figure 3). This hypothesis is based on the modular architecture of this protein and the molecular weight of each component. For instance, the GH11 domain (*n*-terminus portion) has a molecular weight of 21.93 kDa, the carbohydrate binding module corresponds to 12.97 kDa, the dockerin I domain to 7.5 kDa, and the acetylase domain to 19.64 kDa (Uniprot database accession code O87119_HUNTH). In addition, a construct of XynA lacking the *c*-terminus acetylase domain was produced in order to compare SDS-PAGE profiles (results not shown). Thermal treatment is a feasible strategy for obtaining heterologous thermophile proteins expressed in mesophiles without contamination by host proteins, due to their natural differences in heat tolerance [45].

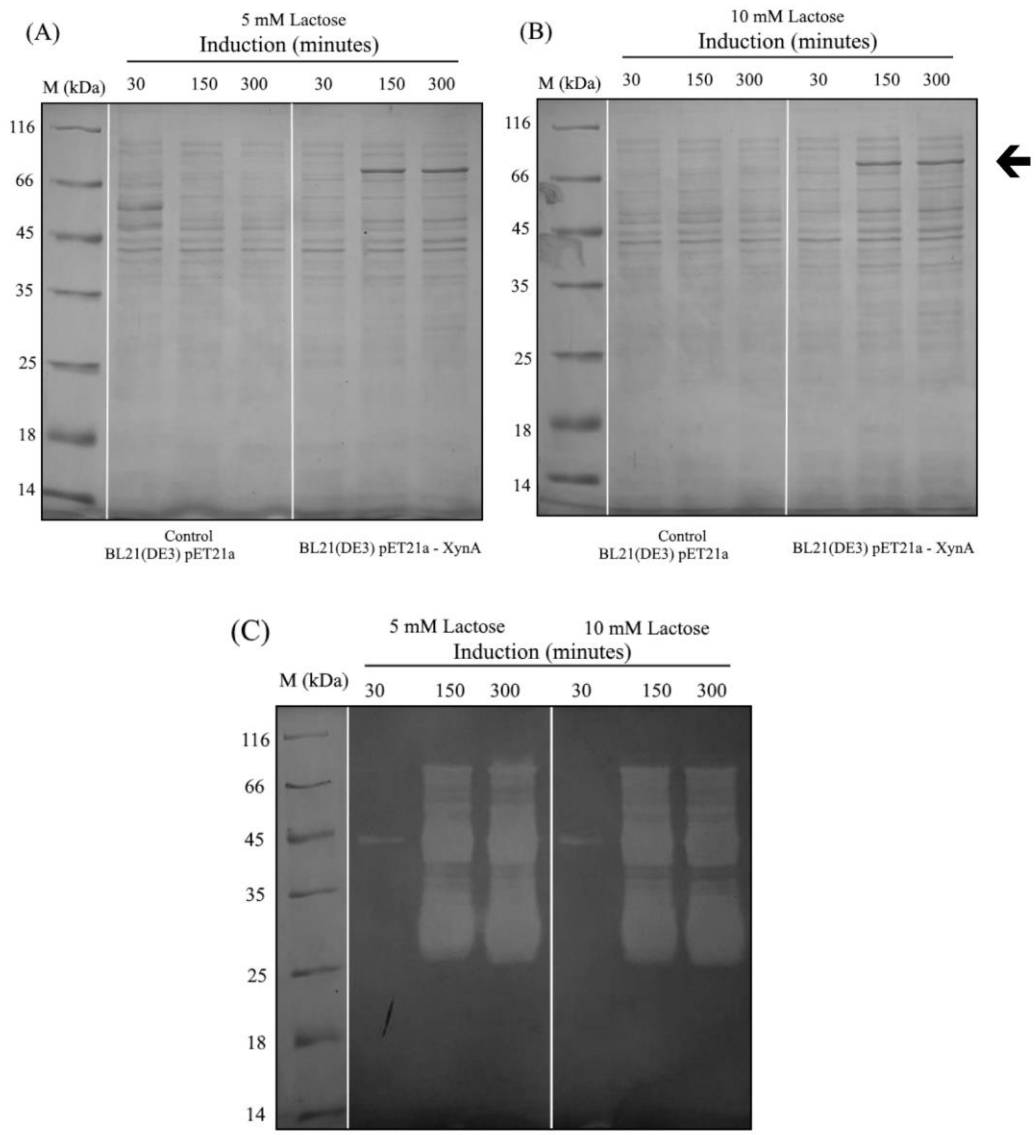

Figure (2): SDS-PAGE (12%) analyses of *E. coli* BL21(DE3) (control) and *E. coli* BL21(DE3) pET21a-XynA lysate showing expression of recombinant XynA, during induction with lactose at 5 (A) or 10 mM (B). Endo-β-1,4-xylanase zymogram (C). Black arrow represents the expression of the full-length XynA.

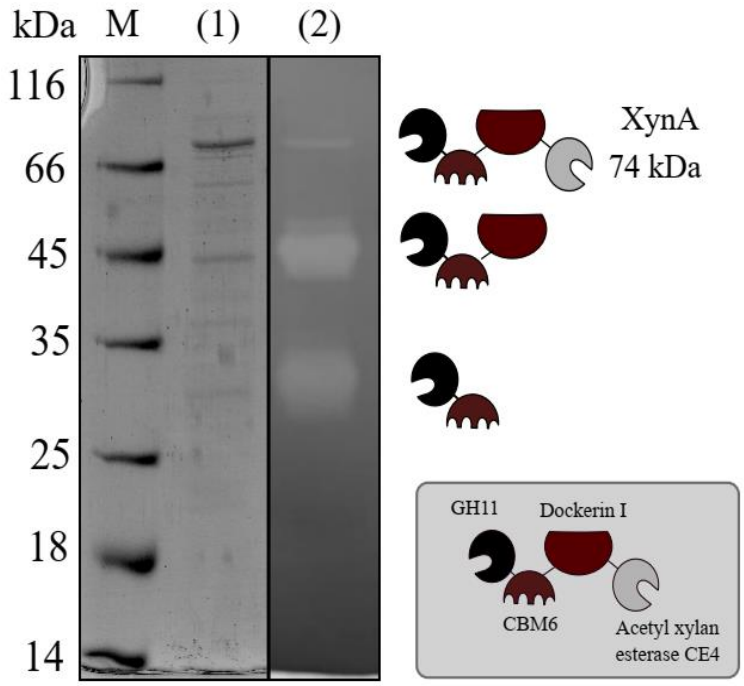

Figure (3): 12% SDS-PAGE of XynA (1), and endo-β-1,4-xylanase zymogram analyses after thermal treatment (2). Protein module identification was based on the protein sequence of *C. thermocellum* ATCC 27505 Cthe_2972. Glycosyl hydrolase family 11 (GH11), carbohydrate esterase family 4 (CE4), carbohydrate-binding module 6 (CBM6).

In the present work, for recombinant XynA, enzymatic activity was seen to increase for temperatures above 30ºC, reaching a maximal value at 60ºC. Regarding the effects of pH, XynA presented activity over a broad pH range from 5 to 8, with maximal activity at pH 6. These results are in agreement with the previously reported characterization of XynA [26], and activity at elevated temperatures and more alkali pH has also been reported for xylan-degrading enzymes produced by *Thermomyces lanuginosus* [25,46]. Therefore, despite the presence of truncated forms of XynA, the influence of temperature and pH was the same as that previously described for a purified full-length XynA [26].

3.2 Deactivation of XynA by phenolic compounds

Hydroxybenzoic acid and tannic acid caused deactivation of XynA for assays carried out at 25ºC, with retained activity values of 83.22 and 62.41%, respectively. On the other hand, trans-cinnamic acid, syringaldehyde, *p*-coumaric acid, and vanillin didn't affect XynA activity, while trans-ferulic acid caused around 15% activity enhancement (**Figure 4**). Osiro et al. 2017 [27] also reported increasing endo-β-1,4-xylanase activity

from purified *C. thermocellum* cellulosomes in the presence of trans-ferulic acid at 25ºC. The enhancement of endo-β-1,4-xylanase activity caused by trans-ferulic acid was also previously reported for a fungal endo-β-1,4-xylanase GH 11. Authors explain this activation based on the interaction between ferulic acid and the catalytic site from the GH11 domain [47]. These previous findings and the data in the present report indicate that trans-ferulic acid may have a positive effect on different endo-β-1,4-xylanases, especially those from family GH11.

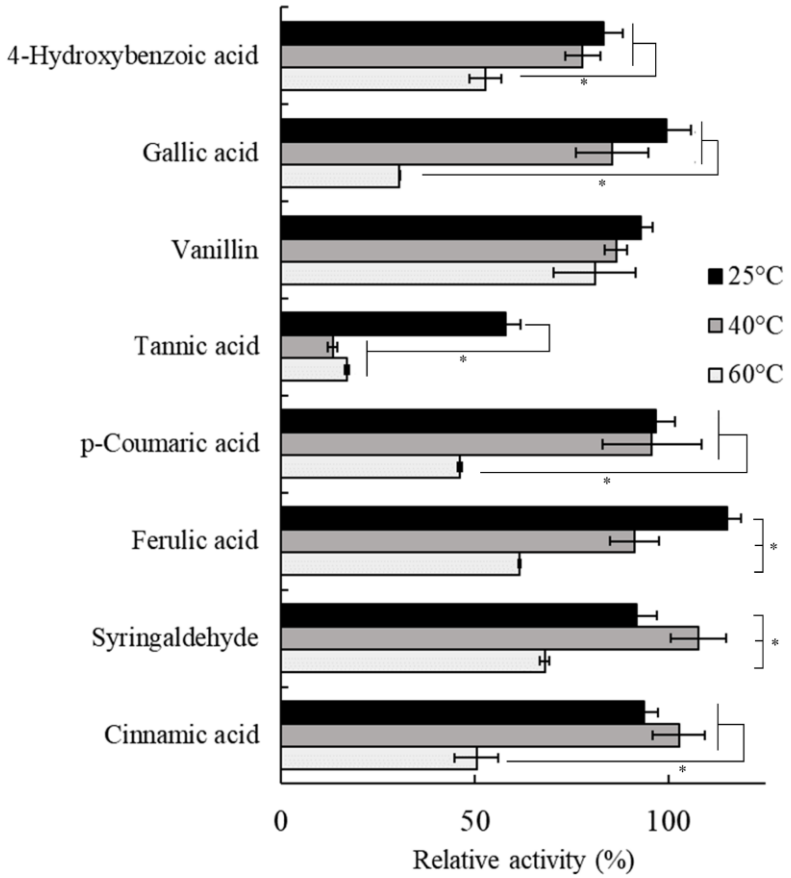

Figure (4): Effect of phenolic and aromatic (cinnamic acid) compounds on XynA-activity after 24 hours. Narrow bars represent the standard deviation from three separate experiments. (*) $p<0.05$

A higher deactivation level for XynA was observed for assays carried at 40ºC in the presence of tannic acid, with a retained activity value of 13.37%. The literature in respect to deactivation of hemicellulases by tannic acid is scarce [48]. Deactivation of endo-β-1,4-xylanases by tannic acid has been previously reported for fungal enzymes. Moreira et al. (2013) demonstrated this for an endo-β-1,4-xylanase produced by *Aspergillus terreus* [13]. The same kind of deactivation was also described for a GH11 endo-β-1,4-xylanase produced by *Emericella nidulans* [21]. Based on these reports and

the data shown in the present study, we suggest that the presence of oligomeric phenolic compounds might be one of the major drawbacks for application of endo-β-1,4-xylanases in lignocellulose waste hydrolysis.

As described above for incubation at 40ºC, 4-hydroxybenzoic acid, gallic acid, vanillin, *p*-coumaric acid, and cinnamic acid caused the same effect as that observed at 25ºC. The highest deactivation values were obtained at 60ºC, with the exception of vanillin. *p*-coumaric acid and 4-hydroxybenzoic acid presented the highest values with deactivation ranging from, 50-60% (**Figure 4**). These data suggest that, at 25 and 40ºC, XynA is resistant to deactivation by the lignin-derived phenols tested, and is sensitive at optimum temperature (60ºC), with the exception of response to tannic-acid. Based on these results, we propose that XynA deactivation by the phenolic compounds tested is temperature-dependent, with maximal values reached concomitant with temperature increase.

Our results suggest that temperature is a key component to be considered in order to fully understand tolerance/susceptibility of plant cell wall-degrading enzymes to lignin-derived phenolic compounds. As mentioned above, at low temperatures the recombinant XynA displays tolerance to 4-hydroxybenzoic acid, gallic acid vanillin, *p*-coumaric acid, and cinnamic acid, resulting in biased categorization of this protein as tolerant to lignin-derived components.

Mechanisms underlying the deactivation of xylanases by lignin structures have not yet been elucidated. For cellulases, Zanchetta et al. (2018) demonstrated adsorption to lignin as the main mechanism related to loss of activity; this appears to be a temperature-dependent process since the highest adsorption rate was detected at 45ºC [10]. It seems that this mechanism also can be the main way to deactivate XynA, at least for tannic acid, an oligomeric phenol.

The structural composition of phenolic compounds has also been described as critical to increasing levels of deactivation/inhibition. Boukari et al. (2010), demonstrated that for a GH11 endo-β-1,4-xylanase from *Thermobacillus xylanilyticus*, the hydroxyl component was the principal group causing enzyme inhibition by phenolics such as *p*-coumaric and caffeic acid, results aligned to findings in the present report [49]. As reported in the present study, lignin-derived phenolics such as vanillin, ferulic acid, and syringaldehyde, harboring methoxy groups, caused lower values of

inhibition/deactivation; the monomeric compound with the higher number of hydroxy groups, gallic acid, caused major activity loss at 60ºC (Figure 4).

Although phenols tested in the present study were used at a constant concentration, in lignocellulose processing it is expected that these values can differ in terms of quantity and species of phenolics originating from biomasses. The present study used values in the same range as for phenols previously employed in other studies [41,50]. Also, it is important to note that biomasses might contain predominant forms of free phenols after pre-treatment; for example, ferulic acid is consistently found in grass biomasses such as sugarcane bagasse and wheat straw [51].

3.3 Thermal stability in the presence of phenolic compounds

XynA retained 100% of its activity after incubation at 40ºC for 72 hours in the presence or absence of vanillin, *p*-coumaric acid, trans-ferulic acid, syringaldehyde, gallic acid, and trans-cinnamic acid. Tannic acid, on the other hand, caused a loss of activity of approximately 80% after 24 hours (**Figure 5**). In agreement with our results, Ximenes et al. [9] demonstrated that tannic acid deactivates fungal cellulases within 25 hours. The most accepted mechanism for deactivation by tannic acid is the precipitation of enzymes that might be a result of a stronger association between tannic acid and proteins, accelerating their deactivation when compared with other lignin-derived compounds.

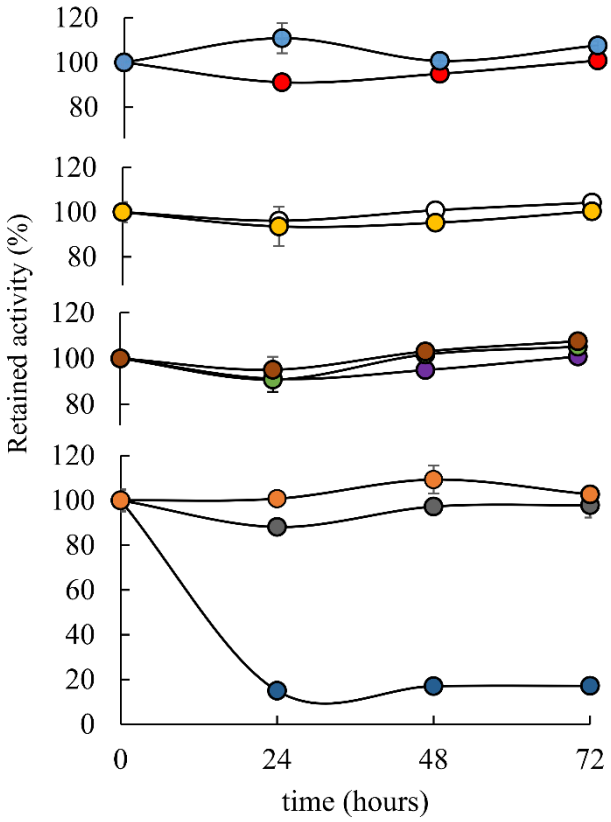

Figure (5): Thermal stability of XynA at 40°C in the presence or absence of lignin-derived phenolics and aromatic (cinnamic acid) compounds. Control water (–●–), control ethanol (–●–), cinnamic acid (–○–), syringaldehyde (–●–), ferulic acid (–●–), *p*-coumaric acid (–●–), tannic acid (–●–), vanillin (–●–), gallic acid (–●–), 4-hydroxybenzoic acid (–●–). Vertical bars represent the standard deviation from triplicates.

In addition, the deactivation constant observed at 60ºC for tannic acid is close to those values reported by Ximenes et al. [9] for total cellulase (FPase) and endoglucanase from fungal cellulase preparations. This report indicates that tannic acid may have an analogous effect on different enzymes involved in lignocellulose deconstruction, regardless of their source, bacterial or fungal.

Loss of XynA activity was detected at 60ºC after 24 hours. Ferulic acid, syringaldehyde, cinnamic acid, 4-hydroxybenzoic acid, and *p*-coumaric acid, enhanced the activity loss, reaching the highest value of approximately 60%, with respective half-lives of 43.8, 36.60, 36.0, 41.8 and 37.0 hours (**Figure 6**). By comparison, tannic acid and gallic acid displayed similar deactivation profiles with maximal deactivation in the first 24 hours, and after that time, constant activity was maintained at approximately 20%, resulting in the lowest activity half-life of 19.4 hours (**Table 1**).

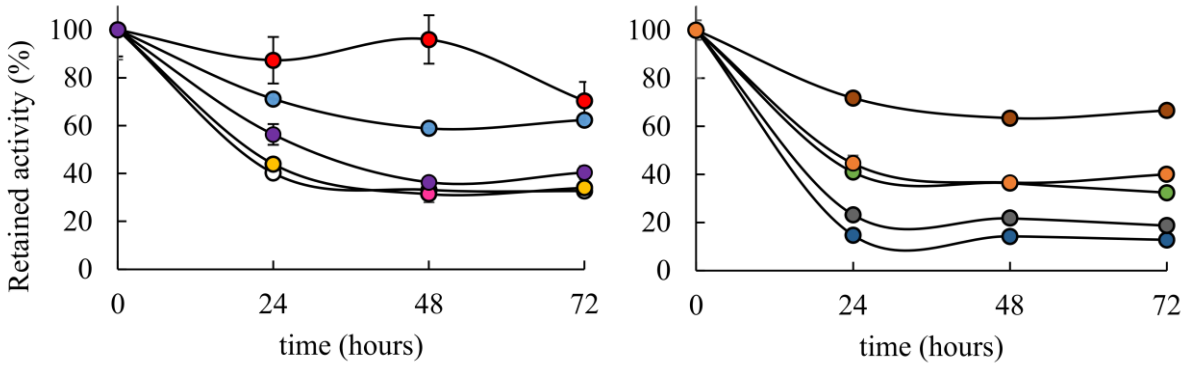

Figure (6): Thermal stability of XynA at 60°C in the presence or absence of ligin-derived phenolics and aromatic (cinnamic acid) compounds. Control water (–●–), control ethanol (–●–), cinnamic acid (–○–), syringaldehyde (–●–), ferulic acid (–●–), *p*-coumaric acid (–●–), tannic acid (–●–), vanillin (–●–), gallic acid (–●–), 4-hydroxybenzoic acid (–●–). Vertical bars represent the standard deviation from triplicates.

3.4 Oat spelt xylan deconstruction

The production of reducing sugars from oat spelt xylan by XynA at 40ºC increased in the presence of cinnamic and ferulic acid, producing 3.07 ± 0.25 and 3.37 ± 0.07 mg.mL^{-1} respectively, whereas water and ethanol controls resulted in 2.48 ± 0.004 and 2.57 ± 0.06 mg.mL^{-1}, respectively. Syringaldehyde, *p*-coumaric acid, vanillin, gallic acid, and 4-hydroxybenzoic acid didn't change the hydrolysis rate of xylan. Tannic acid abolished XynA hydrolysis yield and no reducing sugar was detected in its presence (**Figure 7**).

Table (1): XynA deactivation constants (k_d) and calculated half-life ($t_{1/2}$) of XynA in the presence of phenolic and aromatic (cinnamic acid) compounds at 60ºC.

	k_d x10^{-3} (h^{-1})	$t_{(1/2)}$ (hour)
H_2O	3.8	183.5
EtOH	8.4	82.7
trans-cinnamic acid	19.3	36.0
syringaldehyde	19.0	36.6
trans-ferulic acid	15.8	43.8
p-coumaric acid	18.7	37.0
tannic acid	35.7	19.4
vanillin	7.3	94.5
gallic acid	28.4	24.4
4-hydroxybenzoic acid	16.6	41.8

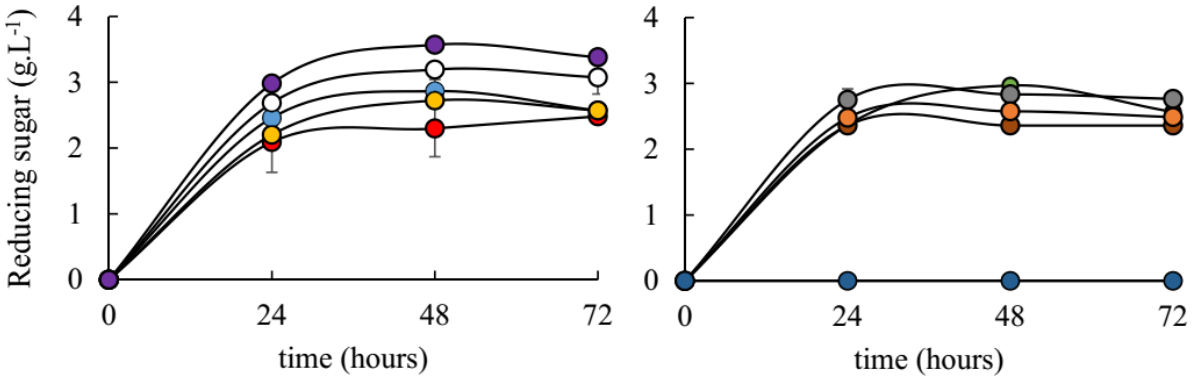

Figure (7): Reducing sugar generated during oat-spelt xylan hydrolysis by XynA in the presence of plant phenolic and aromatic (cinnamic acid) compounds at 40°C. Control water (–●–), control ethanol (–●–), cinnamic acid (–○–), syringaldehyde (–●–), ferulic acid (–●–), *p*-coumaric acid (–●–), tannic acid (–●–), vanillin (–●–), gallic acid (–●–), 4-hydroxybenzoic acid (–●–). Vertical bars represent the standard deviation from three experiments.

For hydrolysis carried out at 60ºC, ferulic acid displayed enhancement, generating 5.33 ± 0.51 mg.mL^{-1} of reducing sugars. The presence of syringaldehyde, cinnamic acid, *p*-coumaric acid, 4-hydroxybenzoic acid, and vanillin displayed the same levels of reducing sugars in comparison to controls with ethanol or water, generating 4.44 ± 0.55 and 4.56 ± 0.26 mg.mL^{-1} respectively. Hydrolysis in the presence of gallic acid and tannic acid generated 3.74 ± 0.10 and 2.15 ± 0.04 mg.mL^{-1}, respectively (**Figure 8**).

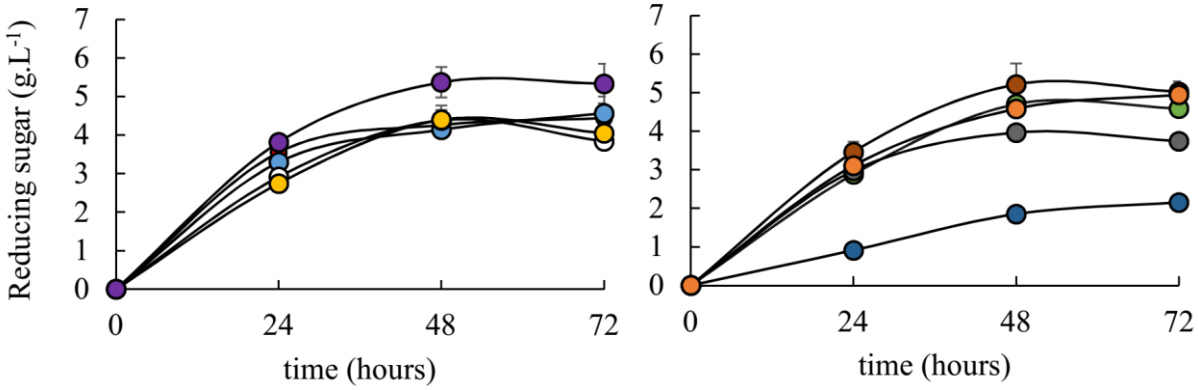

Figure (8): Reducing sugar generated during oat-spelt xylan hydrolysis by recombinant XynA in the presence of plant phenolic and aromatic (cinnamic acid) compounds at 60°C. Control water (–●–), control ethanol (–●–), cinnamic acid (–○–), syringaldehyde (–●–), ferulic acid (–●–), *p*-coumaric acid (–●–), tannic acid (–●–), vanillin (–●–), gallic acid (–●–), 4-hydroxybenzoic acid (–●–). Vertical bars represent the standard deviation from three experiments.

The presence of the substrate appears to prevent XynA deactivation, due to the enzyme/substrate interaction. XynA may be anchored to the substrate by the presence of carbohydrate-binding module 6, thus avoiding contact with monomeric compounds. Therefore, the substrate might be competing with the phenolic compounds to bind to the enzyme, consequently decreasing their negative effects.

Although the presence of CBM 6 can partially explain the apparent resistance to deactivation by monomeric phenols, previous studies demonstrated that carbohydrate-binding domains in fungal cellulases also interact with lignin by virtue of their natural hydrophobicity, contributing to cellulase interaction with lignin [52]. This phenomenon can bring insight into the present study, in which an oligomeric compound (tannic acid) caused major reduction in hydrolysis yield. The presence of a carbohydrate-binding module appears to be aiding the enzyme in surpassing the inhibition by monomeric phenolics in xylan deconstruction, as previously discussed. However, in the presence of high molecular weight compounds such as tannic acid that might show a higher hydrophobic interaction, this advantage is not observed.

As previously discussed, tannic acid is an oligomeric compound that is more likely to be present as a fraction of lignin, thus causing yield reduction [21]. The fact that at 40°C no reducing sugar was detected could be related to reduced activity of the XynA at lower temperatures.

Despite the deactivation profile of recombinant XynA not being correlated with hydrolase yields as previously observed, it is clear that compounds that most affected the thermal stability property caused lower reducing sugar yield at 60ºC. In addition to the reduction in hydrolase activity, a decline in thermal stability is also a drawback for the reuse of enzymes in industrial reactors; enzymes possessing prolonged stability are sought after for processes aiming to reuse biocatalysts.

Although the phenolics used in this study are in their free form (not aggregated to the lignin structure), it is expected that during lignocellulosic deconstruction a similar effect may be observed with respect to loss of thermostability at elevated temperatures, and activity loss by oligomeric phenolic compounds, as observed for tannic acid. In real lignocellulose waste processing, it is expected that the correlation between temperature and activity abatement by phenolics will be observed. For instance, Osiro et al. [27] reported that by employing the same protein load of cellulosomes produced by *C.*

thermocellum in deconstruction of alkali-treated sugarcane straw, lower temperature (50ºC) resulted in higher xylosaccharide yield in comparison to elevated temperature (60ºC), an effect not observed for cellosaccharides. At this point, it is relevant to highlight that *C. thermocellum* xylanases display their maximum activity at a temperature range of 60-70°C [27,28], a range in which XynA deactivation by oligomeric phenol is mostly irreversible (**Figure 9**), explaining the imbalance between lower activity and higher xylosaccharide yield observed in lignocellulose deconstruction.

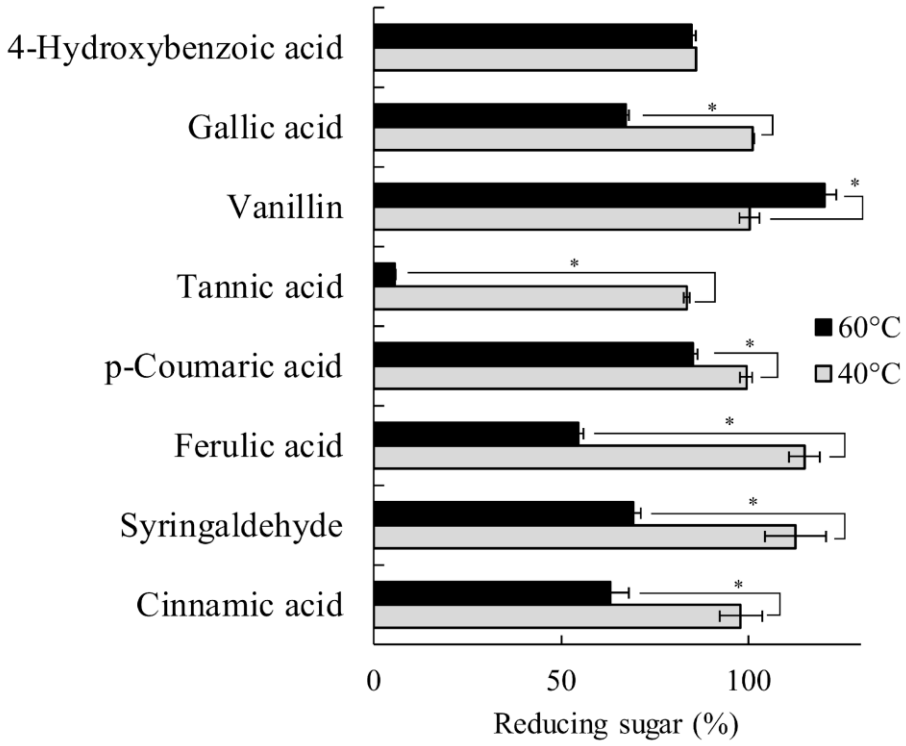

Figure (9): Oat-spelt xylan hydrolysis yield (%) by recombinant XynA pre-deactivated by phenolic and aromatic (cinnamic acid) compounds at 40ºC (grey bars) or 60ºC (black bars). Narrow bars represent the standard deviation from three independent experiments. (*) $p<0.05$.

Previously, Kaya et al. [53] reported that lignin components are xylanase activators, thus promoting hemicellulose hydrolysis enhancement. In the present study, ferulic acid was shown to be a hydrolysis enhancer, though not to as great as described by Kaya et al for vanillin and guaicol. Activation in the presence of ferulic acid was observed in the present study either after incubation in the presence of this compound at 25ºC, or during hydrolysis with ferulic acid at 40ºC and 60ºC. The addition of such compounds to the reactional mixture with the aim of enhancing xylan degradation may not be economically viable, and also may interfere in downstream processes. However,

understanding the changes caused by ferulic acid in XynA activity may be useful for designing more efficient biocatalysts.

With respect to hydrolysis after deactivation, tannic acid caused irreversible deactivation of recombinant XynA when incubated at 60ºC, whereas at 40ºC XynA was able to reestablish its activity and produce 83.51 ± 0.75% of the reducing sugar production level observed in the absence of phenolics. Also, cinnamic acid, 4-hydroxybenzoic acid, gallic acid, *p*-coumaric acid, syringaldehyde, at 40ºC, did not display irreversible deactivation, maintaining hydrolysis yields >85% in comparison to controls (Figure 9).

For pre-incubation at 60ºC, in addition to tannic acid, ferulic acid, syringaldehyde, cinnamic acid, and gallic acid caused major falls in hydrolysis yield. Under this condition, only in the presence of 4-hydroxybenzoic acid and vanillin was a hydrolysis yield above 85% observed (Figure 9). Particularly for vanillin, enhancement in reducing sugar yield was observed after pre-incubation (Figure 9). This result was not expected as adding vanillin during hydrolysis didn't result in enhancement in reducing sugar production (Figure 8). However, Kaya et al. [53] reported that small amounts of vanillin can enhance xylanase activity for commercial preparation, and similar results were obtained by Silva et al (2019) for *P. chrysogenum* endoglucanases [54]. These data lead us to believe that reduced amounts of vanillin may have a positive effect on some glycoside hydrolases, contributing to higher levels of released sugar.

Deactivation of XynA by the tested lignin-derived compounds is mostly reversible at 40ºC, indicating that their effects might be more related to transient inhibition than permanent damage to the enzyme. The reversible effect observed for tannic acid suggests that the possibility of recycling XynA in lignocellulose deconstruction processes in the presence of high molecular weight oligomeric phenols may only exist at mild temperatures (25 and 40ºC).

The fact that oligomeric phenols can cause temperature-dependent deactivation leads us to believe that application of these enzymes may require additional measures in order to avoid activity loss. For instance, Ázar et al. (2017) [48] demonstrated that the presence of lignin-modifying enzymes such as laccase can reduce deactivation of xylanases produced by fungal strains. Likewise, other authors have suggested that the addition of exogenous proteins may block the enzyme-lignin association, thus avoiding protein loss by lignin interaction [55].

In general, the mild deactivation observed for XynA at 40°C in the presence of monomeric phenols suggests that the use of this enzyme would be better suited to simultaneous saccharification and fermentation, processes that usually occur at temperatures below 40ºC. At such mild temperatures, enzymes can hydrolyze the carbohydrate core and also be reused in further lignocellulosic deconstruction processes. In order to reduce the detrimental effects caused by phenolics on XynA, further studies could be carried out for mapping the protein domain involved in the phenol-protein interaction, with a view to enzyme engineering to modify such regions, thus reducing the detrimental effects observed at elevated temperatures. Also, prospection of thermostable lignin-modifying enzymes to amend XynA in hemicellulose deconstruction could be a feasible strategy.

Conclusion

Deactivation/inhibition of thermostable recombinant XynA by lignin-derived compounds is temperature-dependent. These results indicate that at optimum temperature additional measures must be taken in order to reduce the negative effects of plant phenols, especially tannic acid, which caused major reduction in thermal stability. However, at lower temperatures XynA displayed resistance to phenolic compounds, making possible the reuse of this biocatalyst. In contrast to previous reports, the presence of monomeric phenols during xylan deconstruction didn't cause a decrease in reducing sugar release, indicating that the presence of substrate may modulate the influence of phenolics over XynA.

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CAPÍTULO IV - Montagem de mini-xilanossomas com a XynA de *Clostridum thermocellum*, e suas propriedades na desconstrução da biomassa lignocelulósica

Assembling mini-xylanosomes with *Clostridium thermocellum* XynA, and their properties in lignocellulose deconstruction

1-Introdução

A atual demanda por processos enzimáticos de desconstrução da biomassa lignocelulósica faz com que a procura pela melhora na eficiência desses catalisadores seja crescente. Dentre os principais problemas associados a catálise de rejeitos industriais, estão o elevado custo associado a aquisição de enzimas, assim como a perda de proteínas por diversos fatores como inibição por produtos finais da hidrólise e a desativação térmica. Em relação à melhoria da eficiência desses biocatalisadores, é demonstrado na literatura que, ao menos para celulases, a adição de celulases a complexos enzimáticos, tendo como base o complexo formado por *C. thermocellum* aumenta a eficiência na desconstrução de fibras celulósicas.

2-Objetivos experimentais do capítulo

Tendo em vista as propriedades atrativas para o setor industrial das xilanases de *C. thermocellum*, como elevada estabilidade térmica e presença de domínios adicionais como os de associação a carboidratos, e sítios catalíticos com atividades acessório, no presente trabalho foi investigado se a complexação da xilanase XynA, em um modelo de mini-celulossoma aumentaria a eficiência da hidrólise da porção de xilana do bagaço da cana-de-açúcar.

Assembling mini-xylanosomes with *Clostridium thermocellum* XynA, and their properties in lignocellulose deconstruction

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Highlights

- Mini-xylanosome had better performance at 60ºC when hydrolyzing pretreated bagasse.
- Mini-xylanosome and XynA generated the same level of reducing sugars at 60ºC for raw bagasse.
- At 70ºC mini-xylanosomes displayed better hydrolysis than XynA.
- mCipA works as a cellulose deconstruction enhancer when coupled with fungal enzymes.

Assembling mini-xylanosomes with *Clostridium thermocellum* XynA, and their properties in lignocellulose deconstruction

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Abstract

Lignocellulose is a prominent source of carbohydrates to be used in biorefineries. One of the main challenges associated with its use is the low yields obtained during enzymatic hydrolysis, as well as the high cost associate with enzyme acquisition. Despite the great attention in using the fraction composed by hexoses, nowadays, there is a growing interest in enzymatic blends to deconstruct the pentose-rich fraction. Among the organisms studied as a source of enzymes to lignocellulose deconstruction, the anaerobic bacterium *Clostridium thermocellum* stands out. Most of the remarkable performance of *C. thermocellum* in degrading cellulose is related to its capacity to assemble enzymes into well-organized enzymatic complexes, cellulosomes. A mini-version of a cellulosome was designed in the present study, using the xylanase XynA and the N-terminus portion of scaffolding protein, mCipA, harboring one CBM3 and two cohesin I domains. The formed mini-xylanosome displayed maximum activity between 60 and 70ºC in a pH range from 6 to 8. Although biochemical properties of complexed/non-complexed enzymes were similar, the formed xylanosome displayed higher hydrolysis at 60 and 70ºC for alkali-treated sugarcane bagasse. Lignocellulose deconstruction using fungal secretome and the mini-xylanosome resulted in higher D-glucose yield, and the addition of the mCipA scaffolding protein enhanced cellulose deconstruction when coupled with fungal enzymes. Results obtained in this study demonstrated that the assembling of xylanases into mini-xylanosomes could improve sugarcane deconstruction, and the mCipA protein can work as a cellulose degradation enhancer.

Keywords: Hemicellulose, *Clostridium thermocellum*, XynA, sugarcane bagasse, cellulose deconstruction

1-**Introduction**

Residues generated from agricultural activities are receiving attention because of their composition and feasibility to be used as a source of feedstock for biorefineries [1]. Lignocellulosic biomass is composed of three major components: lignin, hemicellulose, and cellulose. The ratio of each component may vary according to the biomass source, and pretreatments applied to the lignocellulosic biomass [2]. One of the major residues from agriculture is the sugarcane bagasse generated after the production of first-generation bioethanol. The global production of sugarcane bagasse can potentially reach 0.6 billion tons/year, representing a considerable amount of carbohydrates that can be employed in second-generation bioethanol production [3]. For instance, Mesa et al. (2011)[4] reported that from 1 ton of sugarcane bagasse could be produced 172 to 198 L of bioethanol.

Despite the great potential of agricultural residues as a carbon source to manufacture products of industrial relevance, the use of lignocellulosic biomass as feedstock for industrial processes is not extensively employed because of the natural recalcitrance of lignocellulosic residues. Such recalcitrance turns their deconstruction into a complex and expensive process [5,6]. Currently, there are plenty of commercially available enzymatic blends for lignocellulosic biomass deconstruction; however, there is a continuous demand to improve those enzymatic mixtures aiming to increase hydrolysis yields, consequently reducing industrial costs [6].

Historically, cellulose has been the main target for the development of enzymatic mixtures. Indeed, in the last decades, several new proteins have been reported as active in cellulose deconstruction and modification, as LPMOs [7] and swollenin [8]. In addition to cellulose, hemicellulose is a relevant source of sugars for fermentation and production of chemicals with industrial relevance [9].

Unlike cellulose, a homopolysaccharide composed of β-(1,4)-D- glucopyranose unities, hemicellulose is composed of a variety of sugars. The hemicellulose with β-(1,4)-D-xylopyranose backbone, xylan, can display ramification containing sugars such as arabinose, 4-*O*-methylglucuronic acid, glucuronic acid, and acetyl groups in the backbone [10]. To complete hydrolysis of xylan, a repertoire of carbohydrate-active enzymes must work in synergism, endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) hydrolyses the main backbone, ramifications and acetyl groups are respectively removed

by α-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.131), and acetyl xylan esterases (EC 3.1.1.72), [11].

Among the group of enzymes displaying activity against xylan, endo-β-1,4-xylanases receive attention due to their potential for industrial applications, including paper and pulp biobleaching, animal feed additives, and biofuels production [12]. Usually, endo-β-1,4-xylanases are obtained from filamentous fungi as *Aspergillus awamori* and *Aspergillus niger* [13,14].

In the context of obtaining new biocatalysts, thermophilic bacteria are an appealing source for the prospection of enzymes with thermal stability and activity over extreme pH conditions [15]. In the group of thermophiles, the anaerobic bacterium *Clostridium thermocellum* has been considered a model organism for the production of thermostable cellulases. One of the major features of *C. thermocellum* is its capacity to assemble high molecular weight enzymatic complexes, cellulosomes, through the interaction of cohesin modules present on an anchoring protein, CipA [UniProtKB - Q06851 (CIPA_HUNT2)], and dockerin modules present on enzymes [16,17].

The cellulosome of *C. thermocellum* has been studied as an enzymatic machine for cellulose hydrolysis [18]. Besides the cellulosome potential for degrading cellulose, this enzymatic complex also contains endo-β-1,4-xylanases. From the biotechnology perspective, the organization of hydrolytic enzymes into complexes using cellulosomes as a model has been investigated by several authors. In these studies, the complexing effect of cellulose-degrading enzymes has been the primary focus. For example, designed celluloses were assembled with β-glucosidases [19], and endoglucanases [20].

In addition to designed complexes containing cellulases, complexes based on xylanases were also proposed due to the importance of exploiting the hemicellulose fraction. For example, designed cellulosomes were built with activities from GH10 (*C. thermocellum*`s XynC), GH11 (*Bacillus halodurans)*, and feruloyl esterase (*C. thermocellum*`s XynZ) [21,22]. However, the advantage of assembling *C. thermocellum*'s xylanases, containing multiple activity domains and carbohydrate-binding modules, into complexes is not well understood, and its biotechnological potential has not been investigated. *C. thermocellum* is known for its great cellulolytic activity, and assembling enzymes into designed xylanosomes seems to be counterintuitive; however, there is evidence that complexed xylanases play an important role in *C. thermocellum*'s natural cellulosomes [23,24].

Given the relevant properties of *C.thermocellum*'s xylanases such as thermal stability, in the present study, a deep investigation was carried out, aiming at understanding the effect of complexing the cellulosomal, multidomain xylanase XynA [UniProtKB - O87119 (O87119_HUNTH)](glycoside hydrolase family 11, carbohydrate-binding module 6, dockerin I domain, acetyl xylan esterase family 4) in the N-terminus portion of the anchoring protein CipA that contains two cohesin I domains, and one carbohydrate-binding module 3. General biochemical properties (effect of pH and temperature) and hydrolysis of sugarcane bagasse were evaluated. Furthermore, the influence of the binding of proteins to the substrate was investigated to identify the key factors that allow the better performance of complexed xylanases. Findings reported in the present paper will help understand the advantages of complexing xylanases into mini-cellulosomes and enhance hemicellulose hydrolysis present in lignocellulosic residues.

2-**Material and methods**

2.1. Materials

Sugarcane bagasse was obtained in native form from local farms (Brasília, Brazil), autoclaved for one hour, and then rinsed with distilled water to remove water-soluble impurities. After drying at 60ºC to reach constant weight, the biomass was subject to milling using an industrial blender. The resulted powder named raw sugarcane bagasse was subject to alkali treatment. Raw sugarcane bagasse alkali treatment was performed incubating 20% (w/v) of the raw material in 200 mM sodium hydroxide solution at 30ºC. After one hour of incubation, the solids were filtered and then continuously washed with distilled water until reaching neutral pH, then the treated sugarcane bagasse was dried at 60ºC.

Avicel (Avicel® PH-101 Lot#0001440138, Fluka® Analytical, CH) and cellulose (Fibrous, medium Lot#106H1131, Sigma Aldrich, USA) were used as model cellulosic substrates. For hemicellulose activity detection, oat-spelt xylan was used as a substrate.

2.2. XynA and mCipA production

2.2.1.CipA cloning

The 5' portion of the scaffolding protein CipA gene (Cthe_3077, NCBI: Gene ID: 4809951) containing two cohesin I domains and one carbohydrate-binding module 3 was amplified. As the structural protein was amplified in an incomplete form, we called it mini-cipA (mCipA); therefore, we used an incomplete form of CipA to assembly mini complexes with XynA. Portion 5' of CipA was amplified using the following polymerase

chain reaction procedure: 50 ng of genomic DNA from *C. thermocellum* B8, 1 U of Taq. DNA polymerase (Taq High fidelity Pol, Cellco Biotec, BR), 0.50 µM of each primer (forward AACATATGGTATCGGCGGCCACAAT; reverse AAGTCGACTGAAGCAGACTTGAATGAGTAG; underlined sequences represent *NdeI* and *SalI* restriction sites, respectively), 2.50 µL of 10X buffer (High Fidelity Buffer, Cellco Biotec, BR), 200 µM of dNTPs, in a final volume of 25 µL. PCR cycling was performed as follows: 95ºC 1 minute, 35 cycles of 95ºC for 30 seconds and annealing and extension at 68ºC for 2 minutes, and a final extension at 68ºC for 5 minutes. Primers were designed using the online tool Primer3plus [25] without the native signal peptide, which was predicted using the toll SignalP [26].

Amplification was analyzed in 0.8% (w/v) agarose gel. The PCR product containing ~1416 bp was excised from the gel using a commercial kit (GeneJET Gel Extraction and DNA Cleanup Micro Kit, Thermo Scientific, US). Excised PCR product was digested with *NdeI* and *SalI* restriction enzymes using one enzyme unity at 37ºC for one hour in a thermocycler (New England Biolabs, US). The digested amplicon was ligated into pet-21a plasmid (digested with the same restriction enzymes). 17 ng of the insert, 20 ng vector, and one µL of T4 ligase prepared in reaction buffer provided by the manufacturer (New England Biolabs, US), the reaction proceeded at 4ºC for 16 hours.

The ligated construction was then used to heat-transform *Escherichia coli* DH5α [27]. Cells were spread in LB solid medium (10 $g.L^{-1}$ tryptone, 10 $g.L^{-1}$ NaCl, 5 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ agar, 100 $\mu g.mL^{-1}$ ampicillin, pH 7) and incubated at 37ºC. After 16 hours of growth, emerging colonies were collected and transferred to 5 mL of LB medium containing 100 $\mu g.mL^{-1}$ ampicillin and incubated at 37ºC in an orbital incubator set at 200 $rev.min^{-1}$, and grown for 12 hours. After growth time, the culture media was centrifuged, and plasmid extraction performed using alkaline-lysis. To confirm the presence of insert, a polymerase chain reaction was realized using specific primers, and analyses of digestion pattern on 0.8% (w/v) agarose gel. Clones that showed the correct insert size on digestion and polymerase chain reaction (~1416 bp) had their plasmid named pet-21a-mcipa and used in the next cloning steps.

The purified plasmid pet-21a-mcipa was used to transform *E. coli* BL21(DE3) by heat shock [27]. After transformation, growth was performed at 37ºC in an orbital incubator set at 200 $rev.min^{-1}$ in terrific broth (12 $g.L^{-1}$ tryptone, 24 $g.L^{-1}$ yeast extract, 0.4% (v/v) glycerol, 100 $\mu g.mL^{-1}$ ampicillin, buffered with 100 mM potassium phosphate

buffer, and pH adjusted to 7) until reaching OD (600 nm) 0.6. After growth time, 0.5 mM of isopropyl β-D-1-thiogalactopyranoside was added, and induction was performed for 16 hours at 28ºC 200 rev.min^{-1}. For soluble protein extraction, 100 mL of cells were centrifuged and sonicated. The cell-free soluble supernatant heat treatment and electrophoretic analysis were performed as henceforth described for XynA protein.

In addition to SDS-PAGE, to evaluate mCipA expression and the presence of C-terminus 6x histidine-tag, we applied 10 mL of the heat-treated supernatant containing mCipA into a 25 mL column packed with 1 mL agarose-nickel resin (Ni-NTA Agarose, Qiagen, DE) pre-equilibrated with 50 mM tris-HCl pH 7.5 supplemented with 10 mM Imidazole. The resin was washed with 100 mL of buffer containing 10 mM imidazole and then protein eluted with 5 mL of 50 mM tris-HCl pH 7.5 buffer containing 300 mM imidazole. Chromatography procedures were carried out at room temperature. The flow rate (3 mL.min^{-1}) was controlled by a peristaltic pump (Pharmacia Lkb-pump P-1, SE). 200 µL of the eluted protein was precipitated with trichloroacetic acid. Then protein homogeneity was analyzed in a silver-stained 12% SDS-PAGE.

2.2.2. XynA expression

Heterologous XynA production was performed using the strain *E. coli* BL21(DE3) harboring the plasmid pet-21a-xynA, *E. coli* BL21-XynA, as previously reported (Hamann, et al. 2020). *E. coli* BL21-XynA cells were grown at 37ºC using LB broth (10 g.L^{-1} tryptone, 10 g.L^{-1} NaCl, 5 g.L^{-1} yeast extract, pH 7) in an orbital incubator set at 200 rev.min^{-1} until reaching OD (600 nm) of 0.6, and then induction was initiated after addition of 10 mM lactose. After 5 hours of induction, 100 mL of cells were harvested by centrifuging at 12000×g for 20 minutes at 4ºC, and suspended in 10 mL of 50 mM tris-HCl pH 7.5 containing 5 mM of $CaCl_2$, 1 mM of phenylmethylsulfonyl fluoride (PMSF) and disrupted by sonication [40% amplitude for 5 minutes (30 seconds on/off cycles) (SONICS® Vibra-Cell™ VC 750, USA)]. The soluble fraction was subjected to heat treatment at 60ºC for 16 hours. The supernatant was centrifuged, and then the protein profile was evaluated in a 12% SDS-PAGE [29] stained with Coomassie Blue (Brilliant Blue G250, USB Corporation, USA).

2.3 Mini-xylanosomes assembling

For assembling mini-xylanosomes, the quantification of mCipa and XynA was performed by analyzing the SDS-PAGE profile by band intensity using IMAGE J [30]. For XynA protein quantification, the band density of the full-length XynA (~74 kDa) was

used as a reference (SDS-PAGE is shown in **Fig. 1.**). Proteins, mCipA and XynA, were mixed in the molar basis of 1:2 (mCipA [30 nM]:XynA [60 nM]), and incubated at 60 ºC with the addition of 2 mM β-mercaptoethanol. After 1 hour of incubation, we evaluated the formation of the mini-xylanosome in three different methodologies: association to cellulose, purification by immobilized metal affinity chromatography (IMAC), and analyses in a 5 % NATIVE-PAGE [31]. Briefly, 1 mL of the protein mixture was added to 100 mg of cellulose, incubated at 60ºC, after 1-hour cellulose suspension was centrifuged at 12000×g at room temperature for 20 minutes, the supernatant was discarded and residual cellulose washed with 50 mM tris-HCl pH 7.5 buffer with 5 mM $CaCl_2$, this procedure was performed twice, and then was checked if the cellulose had xylanase activity associated to it.

To evaluate the association between mCipA and XynA by immobilized metal affinity chromatography (IMAC), a total of 10 mL of the formed complex was applied into an agarose-nickel column, and protein purification performed as described for the mCipA. We verified the presence of the eluted mini-xylanosome from IMAC purification by precipitating 200 µL of eluted proteins with trichloroacetic acid and then applying precipitated proteins into a silver-stained 12% SDS-PAGE. This procedure was carried out because the XynA enzyme had no fusion histag, and thus, elution of the formed complex could only happen in virtue of the dockerin:cohesin interaction between proteins, as mCipA had a 6x histag.

2.4 Enzymatic activity quantification and pH/temperature effect.

Xylanase activity assay was performed by mixing 10 µL of the enzyme to 30 µL of 1 % (w/v) oat-spelt xylan prepared in 50 mM sodium acetate buffer pH 5, and the reaction mixture incubated at 60ºC. After 15 min, 60 µL of DNS was added, and the reaction was boiled for 10 minutes, according to Miller (1959) methodology. The final colorimetric reaction was read in a spectrophotometer at 540 nm. One enzymatic unity was defined as 1 µmol of product formed per minute. D-xylose was used as the sugar standard. pH effect over xylanase activity was performed in enzymatic assays buffered at different pHs, using 100 mM sodium citrate-phosphate buffer from pH 3 to 8. The temperature effect was evaluated by performing enzymatic assays in a temperature range of 30 to 80ºC. All enzymatic assays were performed in triplicates. Protein quantification was carried out using the methodology reported by Bradford (1976) [32], using Quick

Start™ Bradford Protein Assay Kit (Bio-Rad Laboratories, USA), with bovine serum albumin (BSA) protein as standard.

2.5 Binding of enzymes in cellulosic and lignocellulosic materials

Assessment of the binding capacity of individual proteins, mCipA and XynA, was performed by incubating 1 mL of a solution of each protein, prepared in 50 mM tris-HCl pH 7.5 with 5 mM $CaCl_2$, in a water bath at 60ºC for one hour in the presence of substrate. For this experiment, 100 mg of each substrate (raw sugarcane bagasse, treated sugarcane bagasse, cellulose, or Avicel) were used as binding substrates. After incubation time, the reaction was centrifuged at 12000×g for 10 minutes at room temperature, and 15 µL of unbound proteins were pooled and applied into a 12 % SDS-PAGE and stained with Coomassie brilliant blue G250. For this experiment, SDS-PAGE analyses of the proteins, mCipA or XynA, incubated without the substrate, were used as a control. In other words, no association with carbohydrates. The difference between unbound proteins, present in the supernatant from interaction with the substrate, and the controls, was assumed as bound proteins.

2.6 Sugarcane bagasse hydrolysis by mini-xylanosomes or free-enzyme.

Initial hydrolysis experiments were conducted to observe differences between the complex (mCipA:XynA) and free-XynA using sugarcane bagasse as substrate. Hydrolysis experiments were carried out with 5% (w/v) of pretreated/raw sugarcane bagasse in 5 mL reaction, buffered with 50 mM tris-HCl pH 7.5 with 5 mM $CaCl_2$, and 2 IU of xylanase/g substrate. This set of experiments was performed at 50 and 60ºC in an orbital shaker set at 150 $rev.min^{-1}$. Samples were retrieved every 24 hours until 72 hours and centrifuged at 12000×g for 20 minutes at room temperature. Total reducing sugar released was quantified by DNS methodology. Hydrolysis was also carried out at 70ºC (4 IU of xylanase/g substrate); however, performed in a water bath and the released reducing sugar was measured after 72 hours of incubation. Hydrolysis experiments were carried out in duplicates; reaction controls containing only the enzyme or the substrate were also performed in duplicates. Every hydrolysis experiment was carried out with a buffer containing 0.01% (w/v) sodium azide to avoid microbial growth.

Sugarcane hydrolysis was also performed using *Trichoderma harzianum* TR_{274} supernatant as a source of carbohydrate-active enzymes to supplement mini-xylanosomes, or XynA. For *Trichoderma harzianum* TR_{274} enzyme production, the fungus was grown in MYG plate (2.5 $g.L^{-1}$ yeast extract, 5 $g.L^{-1}$ malt extract, 10 $g.L^{-1}$

glucose, and 20 $g.L^{-1}$ agar) at room temperature for seven days, and after that period one mycelia disc (Ø 1cm) was inoculated into 50 mL of minimal media supplemented with 1% (w/v) raw sugarcane bagasse [33]. The fungus was grown in an orbital shaker set at 28ºC 120 $rev.min^{-1}$ for seven days. After growth time, the supernatant was filtered, centrifuged at 12000×g 4ºC for 10 minutes, and stored at 4ºC until the subsequent experiments. Experiments using *Trichoderma harzianum* supernatant were performed at 50ºC as previously described: 2 IU of mini-xylanosome, or XynA, per gram of substrate, with the addition of 2 mL of fungal enzymes. To evaluate enhancement in cellulose hydrolysis, final D-glucose formation was also quantified using a glucose-oxidase assay.

2.7 Cellulosic substrates hydrolysis by *T. harzianum*'s TR_{274} hydrolases supplemented with mCipA

To evaluate the binding effect of mCipA on cellulosic substrates, hydrolysis experiments were carried with 3% (w/v) Avicel or cellulose in 5 mL reactions buffered with 50 mM tris-HCl pH 7.5 with 5 mM $CaCl_2$, 2 mL of *T. harzianum*'s hydrolases, and 40 µg of mCipA. Hydrolysis were carried out at 50ºC in an orbital incubator set at 150 $rev.min^{-1}$. After 72 hours of hydrolysis, total reducing sugars and D-glucose were quantified as previously described. In addition, we performed a comparative analysis for cellulose hydrolysis by adding BSA, or mCipA. BSA was used as a positive control because it has previously been identified as a model protein to improve cellulose hydrolysis [34]. Experiments were carried out as above described, and protein load, BSA or mCipA, was: 40 µg (8 $\mu g.mL^{-1}$), 80 µg (16 $\mu g.mL^{-1}$), 160 µg (32 $\mu g.mL^{-1}$).

2.8 Statistical analyses

Statistical analyses were performed using SigmaPlot v.12.0 (Systat Software Inc, USA). Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1 mCipA heterologous production and mini-xylanosomes assembling

The N-terminus portion of *C. thermocellum*'s CipA was expressed and obtained as a soluble protein with the correct size of 50 kDa (**Fig. 1.**). Although many studies focused on expressing the full-length CipA that encodes a 196 kDa protein containing nine cohesin I domains, one carbohydrate-binding module 3, and a dockerin II domain [UniProtKB - Q06851 (CIPA_HUNT2)]. The expression of the entire CipA protein can be troublesome due to many repetitive domains and proteolytic cleavage that usually occurs when expressing multidomain proteins from *C. thermocellum* in *E. coli* as the host

cell [35]. Therefore, to explore the advantage of the enzyme proximity effect granted by the two adjacent cohesin I modules and the additional carbohydrate-binding module (CBM3), the N-terminus portion was chosen as the model of mini-cellulosome.

The heat-treated mCipA was purified by using the affinity to immobilized metal in an agarose-nickel resin. The primary purpose of this procedure was not to obtain a highly pure form of mCipA protein to further structural characterization but to certify that the 6x histidine-tag was available for protein purification by IMAC methodology (**Supplementary Fig. 1**). Interaction of mCipA:XynA was also verified by IMAC methodology. This strategy was used because the XynA employed in the present study has a native stop codon. Consequently, co-elution of both proteins from IMAC could only happen in the case of cohesin:dockerin interaction, as seen in SDS-PAGE and Native-PAGE of purified mini-xylanosome (**Fig. 2**).

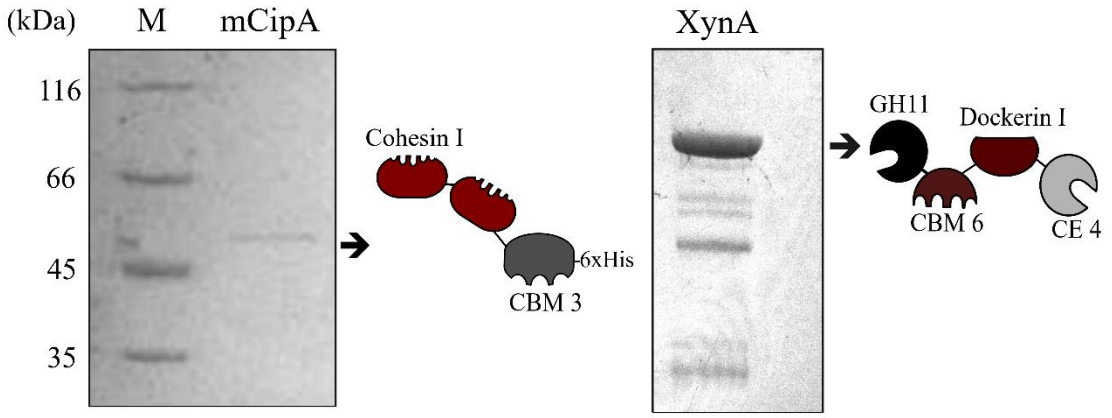

Fig. 1. SDS-PAGE (12%) profile of partially purified mCipA, and XynA after heat-treatment at 60º for 16 hours. Gels were stained with Coomassie blue G-250, proteins modules were based on proteins sequence of XynA [UniProtKB - O87119 (O87119_HUNTH)], and CipA [UniProtKB - Q06851 (CIPA_HUNT2)]. Protein marker (M): 116 kDa (β-galactosidase); 66 kDa bovine serum albumin); 45 kDa (ovalbumin); 35 kDa (lactate dehydrogenase).

The formation of mini-xylanosomes was also confirmed by the interaction of the formed complex with cellulose. Xylanase activity from the mini-xylanosome was found associate with cellulose after successive washing steps with buffer. Usually, *C. thermocellum's* CBM3 present on scaffolding proteins possess a sturdy association to cellulose, and this association is classically used to obtain natural cellulosomes with an

elevated purity level [23]. Extra carbohydrate-binding modules are also seen as a feasible strategy to purify and immobilize heterologously expressed proteins. For instance, the carbohydrate-binding module 3 of *C. thermocellum* has previously been employed as an affinity to cellulose-tag to purify the human cathelicidin-derived peptide LL37 [36].

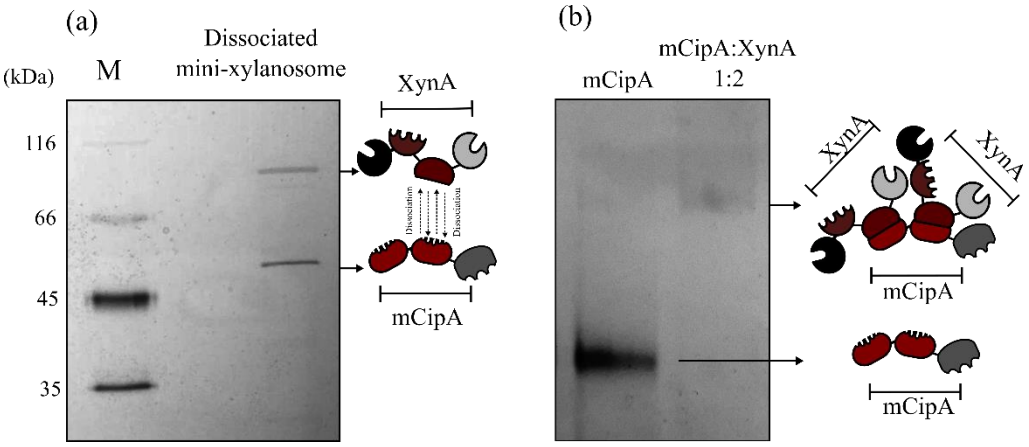

Fig. 2. (a) SDS-PAGE (12%) profile of partially purified mini-xylanosome after IMAC purification. (b) Native-PAGE (5%) of mCipA, and the steichometric 1:2 (CipA:XynA) mini-xylanosome. Gels were silver stained. Proteins modules were based on proteins sequence of XynA [UniProtKB - O87119 (O87119_HUNTH)], and CipA [UniProtKB - Q06851 (CIPA_HUNT2)]. Protein marker (M): 116 kDa (β-galactosidase); 66 kDa bovine serum albumin); 45 kDa (ovalbumin); 35 kDa (lactate dehydrogenase).

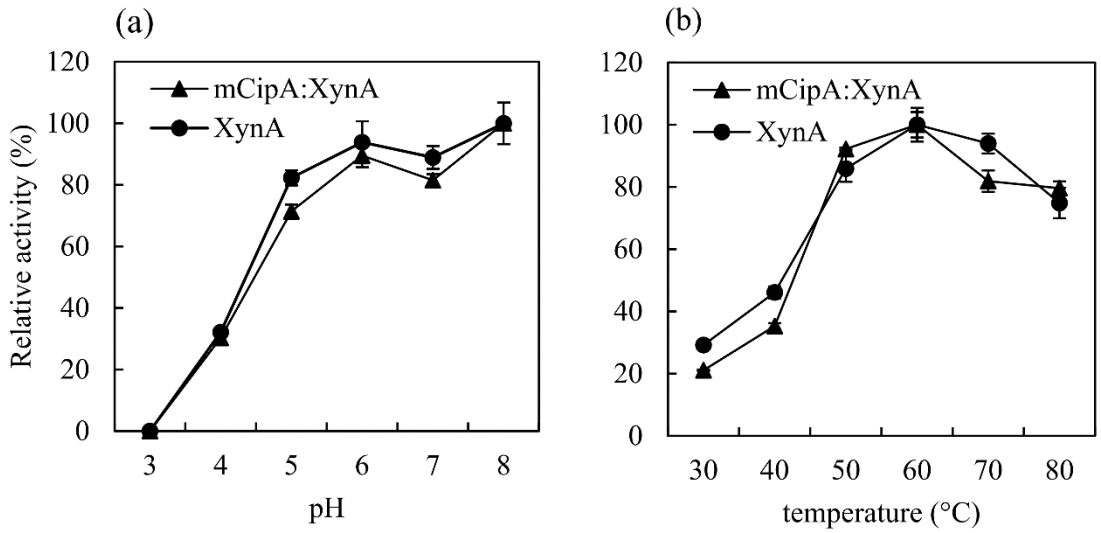

Fig. 3. (a) pH and (b) temperature effect on endo-β-1,4-xylanase activity of recombinant XynA (circle) and mini-xylanosome [mCipA:XynA] (triangle). Vertical bars represent the standard deviation from triplicates. Maximum activity corresponds to 1.19 IU.mL^{-1}.

3.2 Enzymatic properties of mini-xylanosome and XynA

Free and complexed XynA (mini-xylanosome) presented the same optimum pH and temperature (**Fig. 3**). Maximum activity was observed in a relatively broad pH range, from 5 to 8, and in temperatures between 60 and 70ºC. Our results agree with findings previously described by Morag et al. (1990) [37] and with other xylan-active enzymes from *C. thermocellum*, as the GH 141 XynE [38].

Despite the alignment of the results obtained for mini-xylanosome and XynA to other enzymes previously obtained from *C. thermocellum*, biochemical-properties described in this study diverge from endo-β-1,4-xylanases found to other anaerobic bacteria as *Clostridium beijerinckii* G117, that a GH11 xylanase was reported with maximum activity between 40 and 50ºC [39]. *C. thermocellum*'s xylanases display maximum activity at elevated temperatures, making these proteins attractive to industrial processes as lignocellulose hydrolysis for bioethanol production.

Regarding proteins association to carbohydrates, mCipA displayed binding to the cellulosic substrates, Avicel and cellulose, while XynA displayed reduced binding to this class of substrate (**Fig. 4**). This distinct binding capacity of XynA (CBM6) and mCipA (CBM3) to cellulosic substrates agrees with the role of each carbohydrate-binding module present on these proteins. The CBM3 present on mCipA is widely reported as a cellulose-specific module [40], and the CBM6 present on XynA is a xylan-binding domain [41].

Although a different binding pattern was observed for mCipA and XynA concerning cellulosic substrates, this diverging binding capacity is an interesting strategy for developing more efficient glycoside-hydrolase blends. For instance, Pinheiro et al. (2021) [42] demonstrated that the insertion of a CBM3 into a GH11 xylanase enhances its initial performance in lignocellulosic deconstruction. The fact that mCipA harbors a carbohydrate-binding module with affinity to cellulose may aid complexed enzymes in reaching more regions of lignocellulosic biomass. Also, complexing enzymes with a scaffolding protein (harboring CBM3) might be an interesting strategy to incorporate additional carbohydrate-binding modules into holocellulases.

The association of XynA and mCipA to raw sugarcane bagasse might result from the specific association of CBM module to carbohydrates in sugarcane bagasse surface (**Fig. 4**). However, the non-specific associations between protein-lignin cannot be neglected. Oliveira et al. (2018) [43] reported that *C. thermocellum*'s CBM3 does not

undergo unproductive interaction with lignin extracted from *Eucalyptus globulus*. Still, in the present study, in addition to the CBM3 module, there are two cohesins I domains that can potentially interact with lignin. The fact that non-pretreated substrates have higher lignin content may pose a relevant drawback to using designed xylanosomes/cellulosomes. Since the scaffolding protein by itself seems to interact with lignin, it can make complexes unavailable to perform carbohydrate hydrolysis, thus increasing hydrolysis costs.

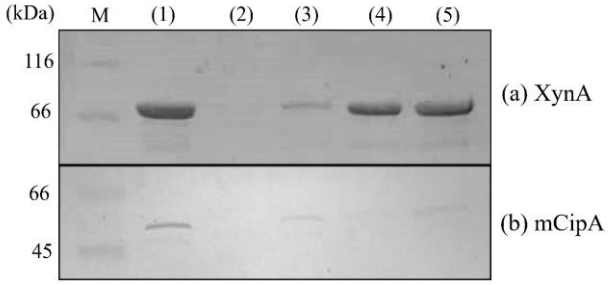

Fig. 4. SDS-PAGE 12% profile of (a) XynA, and (b) mCipA binding assay at 60°C against raw sugarcane bagasse (2), alkali-treated sugarcane bagasse (3), microcrystalline cellulose (4), Avicel PH101® (5), control sample incubated without substrate (1). Protein marker (M): 116 kDa (β-galactosidase); 66 kDa bovine serum albumin); 45 kDa (ovalbumin).

3.3 Hydrolysis experiments

In the hydrolysis of sugarcane bagasse by the formed mini-xylanosome or the enzyme XynA, it was observed that either raw or pretreated sugarcane bagasse could partially be deconstructed (**Fig. 5**). The amount of released sugar from raw sugarcane bagasse hydrolysis using either, XynA or mini-xylanosome, was similar, reaching values of reducing sugar around 0.40 $mg.mL^{-1}$ after 72 hours. A significant increase in released reducing sugar was observed for hydrolysis experiments using pretreated sugarcane bagasse, obtaining values twice as much as the obtained for raw sugarcane bagasse. In addition to the increase in reducing sugar, at 60ºC, the assembled complex had higher performance, reaching 0.83 $mg.mL^{-1}$ of released sugars. In contrast, XynA displayed a maximum of 0.67 $mg.mL^{-1}$ after 72 hours of hydrolysis time.

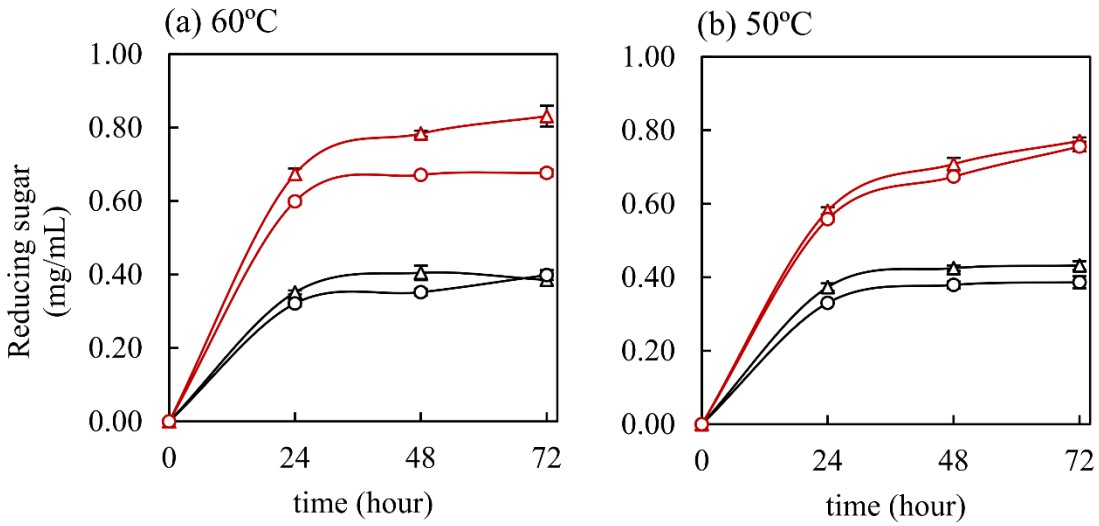

Fig. 5. Hydrolysis of sugarcane bagasse (black lines) and alkali-treated sugarcane bagasse (red lines) by XynA (circle), and mini-xylanosomes (triangles) at (a) 60ºC and (b) 50ºC. Vertical bars represent standard deviation from two individual hydrolysis experiments. The activity was normalized in 2 IU.g^{-1} of substrate.

The fact that alkali treatment of sugarcane bagasse drastically enhanced the hydrolysis yield is expected since this kind of pretreatment is usually employed before lignocellulose saccharification [2]. Alkali treatment has previously been reported as a methodology to decrease the lignocellulosic crystallinity and improve the specific surface area of corn cob, thus improving the accessibility of cellulases [44]. An increase in biomass surface area is a plausible explanation for the higher performance observed for mini-xylanosomes samples. Consequently, different structures will be available for enzyme interaction. The duality in CBMs, 3 and 6 may help anchor the mini-xylanosome in more regions of lignocellulosic biomass than XynA, which is mainly anchored to hemicellulose.

In terms of composition, the raw sugarcane employed in the present study has earlier been determined: ~26% lignin, ~72% carbohydrate, on which ~45% correspond to cellulose and ~25% of xylan [45], similar values to another report regarding raw sugarcane bagasse composition [46]. In addition to improvement of the surface area of lignocellulose [44], alkali treatment of sugarcane bagasse has been shown to promote great lignin removal; Gao et al. (2013) reported 77% [46], Chang et al. (2017) 41% [47];

the reduction of lignin content can also contribute to higher values for alkali-treated sugarcane bagasse hydrolysis observed in the present study.

Compared to hydrolysis experiments carried out at 50 and 60ºC, raw sugarcane bagasse hydrolysis at 70ºC displayed low sugar release, with the maximum value obtained for the mini-xylanosome sample, 0.16 mg.mL^{-1} (**Fig. 6**). Reducing sugar values obtained from hydrolysis at 70ºC of treated sugarcane bagasse were close to thrice of those obtained for raw sugarcane bagasse. As seen for raw sugarcane bagasse, maximum values were obtained for the mini-xylanosome sample, 0.60 mg.mL^{-1}. The reduced performance observed for hydrolysis of raw sugarcane bagasse at the higher temperature can directly be supported by the unproductive association of enzymes to lignin. Many studies have demonstrated that elevated temperatures lead to an increment in such interaction, reducing the number of enzymes available for hydrolysis [48].

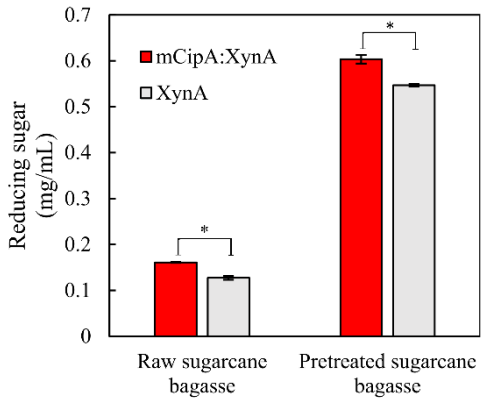

Fig. 6. Hydrolysis of raw sugarcane bagasse and alkali-treated sugarcane bagasse by XynA (grey bars) and mini-xylanosomes (red bars) at 70ºC. Vertical bars represent standard deviation from two separate hydrolysis experiments. The activity was normalized in 4 IU.g^{-1} of substrate. * p<0.05

Changes caused by alkali-treatment in sugarcane residues were investigated by Bartos et al. (2020) [49]. Their findings indicate that alkali treatment of this class of biomass can result in a reduction of hemicellulose, as well as lignin, and increment in cellulose content. The lignin content reduction can partially explain the higher amount of sugars released from pretreated sugarcane bagasse hydrolysis. The increment in cellulose content reported for alkali-treated sugarcane bagasse can corroborate making more sites

available to mini-xylanosome anchoring using the CBM3. Therefore making the mini-xylanosomes a viable tool for hydrolyzing the hemicellulose from alkali-treated substrates.

Fungal enzymes also hydrolyzed alkali-treated sugarcane bagasse as an addition to mini-xylanosomes or XynA (**Fig. 7**). Results obtained indicate that the addition of extra xylanases to *T. harzianum*'s enzymes can boost the substrate deconstruction, increasing in about 30% total reducing sugar released, reaching a maximum of around 2 $mg.mL^{-1}$. The total D-glucose generated was maximum when the *T. harzianum*'s enzymes were combined with mini-xylanosome (**Fig. 7**), resulting in 0.79 $mg.mL^{-1}$ of D-glucose released after 72 hours hydrolysis. This result is 8.86% higher than the total obtained when supplementing the fungal enzymatic mixture with XynA, indicating that the assembled complex augmented the cellulose deconstruction.

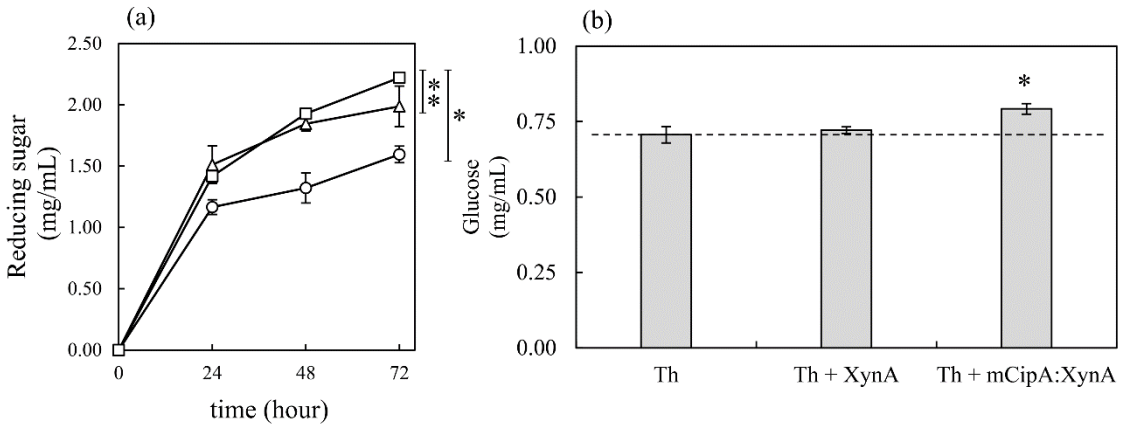

Fig. 7. (a) Hydrolysis of pretreated sugarcane bagasse by enzymatic mixtures at 50ºC, *Trichoderma harzianum*'s holocellulases (circle), *Trichoderma harzianum*'s holocellulases + XynA (triangle), *Trichoderma harzianum*'s holocellulases + mini-xylanosome (square). (b) D-glucose released after 72 hours hydrolysis of pretreated sugarcane bagasse by enzymatic mixtures. Vertical bars represent standard deviation from two separate hydrolysis experiments $**p>0.05$, $* p<0.05$.

The increment observed in released glucose can be related to the role of the CBM3 present on mCipA. Previous studies investigated the role of carbohydrate-binding modules, and although they do not have catalytic activity against polysaccharides, they can increase cellulose degradation [50]. The mechanism on which CBMs cause

augmentation in cellulose biodegradation is not fully understood. However, there is evidence that they may avoid the unproductive interaction of enzymes during hydrolysis [43].

Different results were observed when *T. harzianum*'s cellulases were employed for cellulose or Avicel degradation with the addition of mCipA (**Fig. 8**). For hydrolysis conditions supplementing the fungal enzymes with mCipA using Avicel as substrate, a more recalcitrant form of cellulose, values of 0.78 and 0.38 mg.mL^{-1} of reducing sugars and D-glucose were respectively obtained, and not different from values obtained using only fungal enzymes. On the other hand, while supplementing fungal enzymes with mCipA in cellulose hydrolysis, there was an increase of 37 and 25% in total reducing sugar and released D-glucose, respectively.

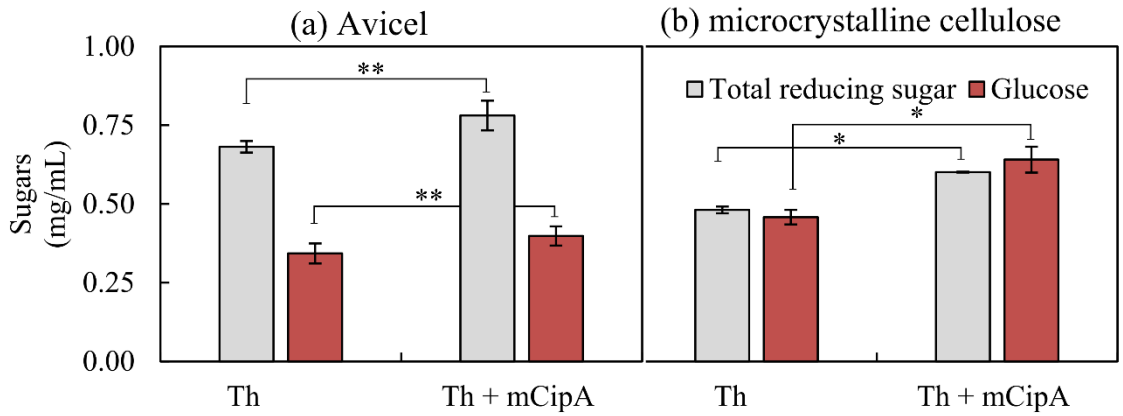

Fig. 8. (a) Hydrolysis of Avicel PH101® and (b) microcrystalline cellulose by *Trichoderma harzianum*'s cellulases [Th], and *Trichoderma harzianum*'s cellulases [Th] supplemented with mCipA, hydrolysis was carried out for 72 hours at 50ºC. Vertical bars represent standard deviation from two separate hydrolysis experiments $^{**}p > 0.05$, $^{*}p < 0.05$.

The results obtained in this study are aligned to findings described by Oliveira et al. (2018) [43], on which the addition of *C. thermocellum*'s CBM3 to enzymatic hydrolysis of *Eucalyptus globulus*'s whole slurry enhanced the D-glucose yield. The fact that only for microcrystalline cellulose the addition of mCipA had the D-glucose yield improved led us to believe that the fungal enzymes would require a higher titer of cellobiohydrolases because of the crystallinity of Avicel.

In addition to the end-point experiments of cellulose hydrolysis by fungal enzymes supplemented with mCipA, a time-course comparative analysis was made to evaluate different titers of mCipA and compare it to a well-characterized cellulase enhancer, the protein BSA. Results obtained in this experiment attested that mCipA is a cellulase enhancer, as seen for BSA (**Fig. 9**). Hydrolysis in the absence of enhancers generates a maximum of 0.34 $mg.mL^{-1}$ of reducing sugars. In the presence of 40 µg of BSA or mCpA, BSA could not improve sugar yield; however, at this protein load, mCipA improved cellulose deconstruction and enhanced in 17% final reducing sugar. At 80 µg, both samples showed the same enhancement in about 20%. At the highest load, 180 µg BSA caused no improvement, and mCipA at this load displayed the greatest improvement in hydrolysis yield, 26%. mCipA at highest titer also caused a change in the kinetic production of reducing sugars, displaying maximum release after 24 hours, 18.86 $µg.mL^{-1}.min^{-1}$, whereas the 80 µg of BSA had 13.96 $µg.mL^{-1}.min^{-1}$ (**Supplementary Fig. 2**).

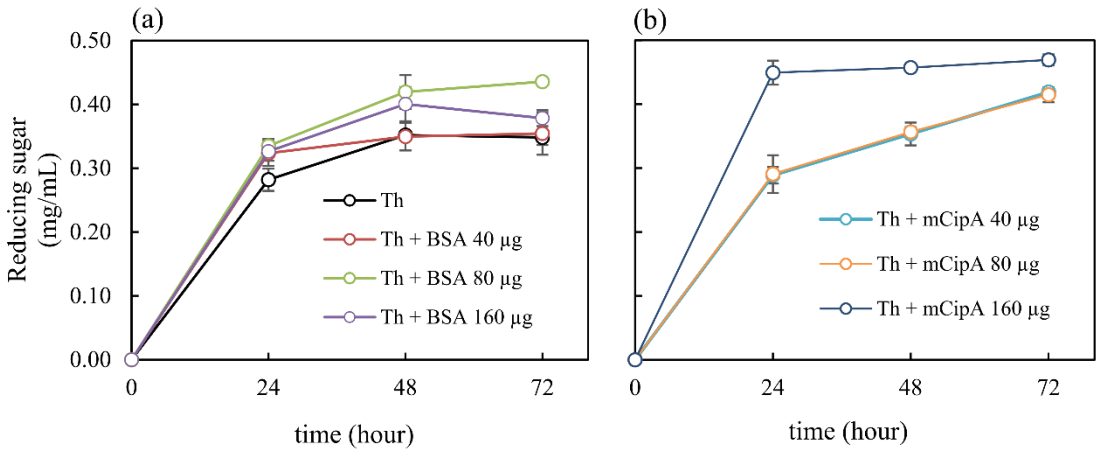

Fig. 9. (a) Hydrolysis of microcrystalline cellulose by *Trichoderma harzianum*'s cellulases [Th] supplemented with BSA at 40 µg (red line), 80 µg (green line), 160 µg (purple line); or (b) mCipA at 40 µg (light blue), 80 µg (orange line), 160 µg (dark blue). Control, without additive, only *Trichoderma harzianum*'s cellulases [Th] (black line). Vertical bars represent standard deviation from two separate hydrolysis experiments.

Although BSA could enhance cellulose deconstruction, based on the present study, the scaffolding protein harboring the CBM3 displayed better results than the classical enhancer, BSA. In current literature, the addition of BSA has been widely investigated [34,43], and one of the most discussed reasons for this augment in lignocellulose hydrolysis might be related to helping enzymes not to undergo

unproductive biding to lignin structures [34]. However, in the time-course hydrolysis experiment, we have used the purified cellulose. In other words, unproductive lignin interaction is not a possible explanation. A reasonable explanation is that BSA may function as a surfactant, facilitating cellulases to be released from cellulosic fibers. Indeed, Oliveira et al. (2018) [43] have demonstrated that the addition of CBM3 is beneficial for the enzymatic hydrolysis of a partially soluble substrate. Our study proves that proteins harboring *C .thermocellum*'s CBM3 can also enhance the deconstruction of completely insoluble substrates as cellulose and sugarcane bagasse as a model of lignocellulose.

4. **Conclusions**

The formation of a mini-xylanosome using as protein chassis *C. thermocellum*'s XynA and the N-terminus portion of the scaffolding protein mCipA was possible. Both samples displayed similar kinetic parameters. In hydrolysis experiments, alkali-treated sugarcane bagasse was the best substrate, and the tailored xylanosome displayed the best deconstruction performance. When combined with fungal holocellulases, the mini-xylanosome resulted in a higher D-glucose yield. Further investigation showed that the presence of the mCipA protein could improve cellulose deconstruction when applied together with fungal cellulases. The results present in this study attests that the duality of carbohydrate-binding modules may be a feasible strategy to enhance enzyme-based lignocellulose hydrolysis and that the addition of proteins harboring the CBM3 can be a strategy to improve cellulose and sugarcane bagasse deconstruction.

Author contribution

Pedro R.V. Hamann conceived and designed the research and wrote the manuscript. Tainah C. Gomes, and Luísa de M.B. Silva, conducted experiments. Eliane F. Noronha, wrote and revised the manuscript.

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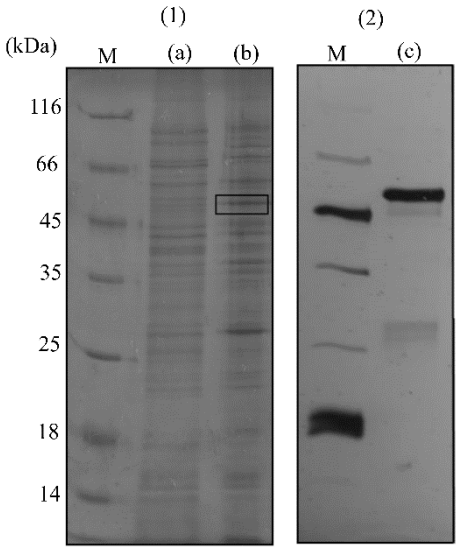
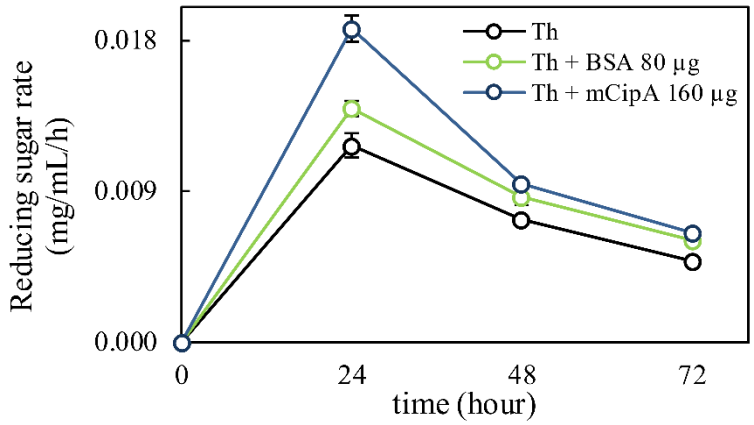


Supplementary Fig. 1. (1) 12% SDS-PAGE of (a) intracellular protein profile *E. coli* BL21(DE3) harboring empty pet21a vector, and (b) intracellular protein profile of *E. coli* BL21(DE3) harboring pet21a-mCipa after protein expression, area delimited by the square represents the expression of mCipA; the gel was stained with Coomassie blue G-250. (2) 12% SDS-PAGE of (c) purification of mCipA by IMAC, the gel was stained with silver nitrate. Protein marker (M): 116 kDa (β-galactosidase); 66 kDa bovine serum albumin); 45 kDa (ovalbumin); 35 kDa (lactate dehydrogenase); 25 kDa (REase Bsp98L); 18 kDa (β-lactoglobulin); 14 kDa (Lysozyme).



Supplementary Fig. 2. Time-course reducing sugar release ($mg.mL^{-1}.h^{-1}$) from microcrystalline cellulose (3% w/v) hydrolysis by *Trichorderma harzianum*'s cellulases [Th] (black lines), or *Trichoderma harzianum*'s cellulases supplemented with 80 µg of BSA (green lines), or 160 µg of mCipA (purple lines). Vertical bars represent standard deviation from two separate hydrolysis experiments.

CAPÍTULO V - Mini-xilanossomas como ferramenta biotecnológica para desconstrução de fibras de sisal, e imobilização enzimática

Using mini-xylanosomes as a biotechnological tool for sisal fibers deconstruction, and enzyme immobilization

1-Introdução

Dentre os principais desafios para a mudança da matriz energética baseada em fontes não renováveis encontra-se a substituição de polímeros derivados do petróleo por polímeros provenientes de uma matriz biológica e renovável. Uma das opções discutidas é a incorporação de fibras vegetais a materiais compostos, podendo assim esses serem utilizados como material para construção de materiais a serem aplicados como função estrutural. Um dos desafios associados à incorporação dessas fibras a materiais compostos é a remoção da hemicelulose, que atualmente é realizada por tratamentos químicos, o que faz difícil o aproveitamento dos carboidratos removidos para futura aplicação em processos microbiológicos.

2-Objetivos experimentais do capítulo

Tendo em vista a necessidade do desenvolvimento de metodologias que visam a remoção da hemicelulose de fibras naturais, no presente trabalho foi investigado a possibilidade da utilização de mini-xilanossomas como ferramenta biotecnológica para despolimerização da hemicelulose presente nas fibras de sisal. O mini-complexo enzimático formado no presente estudo teve como base a xilanase XynZ de *Clostridium thermocellum*, uma enzima que contém um domínio GH10 possuindo atividade de endo-β-1,4-xilanase, assim como domínios adicionais sendo eles: um de associação à hemicelulose (CBM6), um de associação da enzima à proteína estrutural do complexo, dockerin I, e um domínio catalítico adicional, CE4 possuindo atividade de feruloil esterase. A enzima XynZ foi expressa em *Pichia pastoris* e secretada ao meio de cultura por meio do uso do sinal de secreção α-factor presente no vetor pPIC9. Como modelo de proteína estrutural foi utilizada a proteína mCipA (proteína estrutural demostrada no capítulo IV).

Using mini-xylanosomes as a biotechnological tool for sisal fibers deconstruction and enzyme immobilization

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Highlights

- Hydrolysis of sisal fibers using mini-xylanosomes generates three times more reducing sugar in comparison to non-complexed xylanase.
- Cellulose-immobilized mini-xylanosomes could be subject to 72 hours of reuse cycles at 50 and 60 ^{o} C.
- At 70ºC, xylanases assembled into mini-xylanosomes display better thermal stability than non-complexed.
- Complexing the xylanase into mini-xylanosomes does not change the hydrolysis yield of soluble oat spelt xylan.

Abstract

Currently, there is an increasing demand for developing enzymatic blends that can efficiently deconstruct the hemicellulose present in plant fibers, since these fibers can be further employed to build new engineered materials. Sisal fibers are investigated as a source of cellulosic material to be used in the manufacture of new materials, as reinforced fiber with plastic additives. A critical step to obtain the cellulosic matrix is the removal of hemicellulose present on these fibers. In the present study, a mini-xylanosome was tailored using a miniature version of *C. thermocellum*'s scaffolding protein harboring two cohesins I domains and one carbohydrate-binding module family 3, and the xylanase XynZ. The designed xylanosome displayed higher thermal stability at 70 ºC in comparison to the free form XynZ, 134 and 90 minutes of half-lives, respectively. Complexing xylanases into mini-xylanosomes or immobilizing complexes into cellulose did not reduce activity loss by tannic acid. Regarding sisal fiber deconstruction, the mini-xylanosome displayed better hydrolyzing capacity than the non-complexed xylanase, reaching a result three times fold higher. Immobilized mini-xylanosome could be subjected to 72 hours of reuse cycles at 50 and 60 ºC, and at 70 ºC for 48 hours.

Keywords

Hemicellulase, *Clostridium thermocellum*, fibers, cellulosomes, ferulic acid.

Introduction

The demand for bio-based material is constantly growing; one of the great reasons is the mitigation of greenhouse gas emissions and change from a petroleum-based matrix to a renewable and greener production process. In this scenario, plant-based materials have a fundamental role, as they are renewable, cheaper, and abundant. Lignocellulosic biomass is generated from many industrial activities, such as food processing, paper, pulp production, and urban waste, making them widely available for further use in biorefineries [1, 2].

In the biorefinery context, natural fibers can be employed as a carbon source to produce a vast array of chemicals of industrial relevance, as biofuels [3, 4]. Besides chemicals of industrial relevance, compounds derived from lignocellulose are also sought after for the production of biomaterial with many application in industrial processes, as micro fibrillated cellulose, and reinforced fibers. For cellulose-based polymers production, usually, wood crops are exploited, but reports indicate that other biomasses can be used as a source of cellulosic material, like sugarcane and bamboo crops.

In respect to fiber production, several crops are usually dedicatedly cultivated to obtain fibers of industrial relevance, as *Agave sisalana*, which is primarily produced in countries as Brazil (82,923 tonnes/2018), the United Republic of Tanzania (32,460 tonnes/2018), Kenya (21,486 tonnes/2018) (Food and Agriculture Organization of the United Nations, http://www.fao.org). *A. sisalana* fibers, sisal fibers, are usually employed to produce ropes and twines [5]. Also, this fiber is studied as feasible to be incorporated into other materials, therefore obtaining biocomposites with the structural application [6].

For sisal fiber use as bio-matrix in composed material, usually, the fibers are subjected to alkali treatment aiming to remove hemicellulose and preserve the cellulosic core, which can be further impregnated with additives as epoxy [6], polyester [7], or waste polypropylene [8]. Resulting in a composed material with desirable physical properties for industrial production of structural components, as reinforced fibers, and cement-fiber composites. The composition of raw sisal fibers was reported as 73% (w/w) cellulose, 10.1% (w/w) hemicellulose, 7.3% (w/w) lignin, 6.2 % (w/w) extractives and 3.1 (w/w) ashes [9].

Chemical modification of *A. sisalana* fibers by alkali treatment is a common procedure for obtaining cellulosic fibers. However, there is an option to use glycoside hydrolases enzymes to deconstruct the amorphous part, including the hemicellulose,

reducing the use o chemicals and making possible the solubilized fraction of hemicellulose as sugars for further fermentation [10, 11]. Hemicellulose from *A. sisalana* is majorly composed of xylose residues 68.5 % (w/w), galactose 16.6 % (w/w), and arabinose 2.5 (w/w) [12].

Biological/enzymatic deconstruction or modification of sisal fibers have been investigated in some studies. Zwane *et al*. investigated changes in *Agave sisalana*'s fiber by hemicellulases produced by *Aspergillus niger*, on which 0.91% of total fiber weight could be hydrolyzed [13]. González *et al*. demonstrated that sisal fibers could be hydrolyzed by *Penicillium echinulatum*'s cellulases, resulting in reducing the amorphous region, thus enhancing fiber crystallinity [14]. These fibers have also been employed as a carbon source for methane generation in an anaerobic bioreactor. Mshandete *et al*. reported that during the aerobic pre-digestion of sisal fiber, glycoside-hydrolases such as cellulases and xylanases were secreted by a bacterial community [15].

Although some reports describe sisal fibers as feedstock to the production of biomolecules of industrial relevance, there is also a growing interest in investigating different biocatalysts that can improve hemicellulose removal and improve cellulose yield obtained from natural fibers. In this context, other enzymes instead of classical fungal enzymes could be investigated as a green approach in hemicellulose solubilization. In this respect, enzymes produced by the thermophilic anaerobic bacteria *Clostridium thermocellum* call attention due to their thermal stability and modular architecture, allowing the removal of side chains associated with hemicellulose backbone, and hydrolyzing the hemicellulose core structure [16, 17]. This bacteria is known for its natural capacity to assemble glycoside hydrolase enzymes into a well-organized complex, cellulosome, throughout dockerin and cohesin interaction [18, 19]. Although these complexes are reported as well specialized in cellulose solubilization, there are also hemicellulases in this complex that are worth to be investigated to further industrial applications [20–22].

Research focusing on sisal fibers deconstruction has been chiefly focused on mesophiles' enzymes, and few investigated the detailed role of xylanases in *A. sisalana*'s hemicellulose deconstruction. In this regard, in the present research, the N-terminus portion of CipA [UniProtKB - Q06851 (CIPA_HUNT2)] cellulosome anchoring protein, and the xylanase XynZ [UniProtKB - P10478 (XYNZ_HUNT2)], were used to assemble a mini-xylanosome, and evaluate its enzymatic properties, including pH and temperature

effect, thermal stability, association to cellulose, and finally its performance in hydrolyzing the hemicellulose present in *A. sisalana* fibers. XynZ was chosen as model xylanase because of its modular architecture containing two catalytic domains, a feruloyl esterase, and glycosyl hydrolase family 10, yielding higher xylan hydrolysis. The mini-xylanosome was designed aiming to take advantage of the proximity effect given by adjacent cohesin modules and the presence of an additional carbohydrate-binding module. The results shown in the present research will be further used in bioprocesses aiming to valorize sisal fibers and their integration in biorefineries.

Materials and methods

Cloning and expression of *C. thermocellum*'s XynZ in *Pichia pastoris*

XynZ encoding gene, Cthe_1963 [5'- feruloyl esterase module, carbohydrate-binding module family 6 (xylan specific carbohydrate-binding module 6), dockerin I domain, glycoside hydrolase family 10 -3'], was amplified from *C. thermocellum* B8 DNA using the following polymerase chain reaction: 50 ng of genomic DNA, 0.50 µL of Taq DNA polymerase (corresponding to one unity, Platinum Taq DNA polymerase High Fidelity, Invitrogen – US), 0.50 µL of forward (AATATT GCG GCC GCA GCATCCTTGCCAACCA), and reverse (AATATT GCG GCC GCA TCAATAGCCCATAAGAGCTTCC) primers, 200 µM of dNTPs, 2.50 µL of 10× High fidelity buffer (Invitrogen, US), in a final volume adjusted of 25 µL. PCR cycling was performed as follows: 1 minute at 95 ºC, 35 cycles (30 seconds at 95 ºC, 30 seconds at 62 ºC, extension at 72 ºC for three minutes), final extension at 72 ºC for 5 minutes, and hold-temperature of 15 ºC. Primers were designed using the gene Cthe_1963 *C. thermocellum* ATCC 27405 as a template, the online tool Primer3plus [23], and the predicted signal peptide was removed [24].

Amplified sequence containing the estimated size of XynZ (~2430 bp) was excised from agarose gel 0.8% (w/v) using the commercial kit QIAEX® II Gel Extraction Kit (Qiagen, DE). Then the purified sequence was digested with the restriction enzyme *NotI* following the manufacturer's instruction (New England Biolabs, US), 1 enzyme unity with the reaction for 1 hour at 37ºC, finally, the digested amplicon was applied into an 0.8% agarose gel and purified using the commercial kit QIAEX® II Gel Extraction Kit (Qiagen, DE). The vector pPIC9 was digested with *NotI* , 1 enzyme unity for 1 hour at 37ºC, and then treated with alkaline-phosphatase following the manufacture's instructions (New England Biolabs, US). The digested amplicon was ligated to linearized

pPIC9 vector using T4 DNA ligase (1 µL of T4 DNA ligase and reaction proceeded overnight at 10ºC) and then used to transform in *Escherichia coli* XL-10 Gold. Transformed cells were grown on LB-agar medium containing 100 µg/mL of ampicillin; emergent colonies were collected and cultivated in liquid LB for plasmid extraction using alkaline lysis [25]. To certify the correct orientation of Cthe_1963 in the plasmid pPIC9, the construct was digested with the restriction enzyme *NdeI* (1 enzyme unity and the reaction proceeded for 1 hour at 37ºC), generating fragments of 5163, 4479, and 811 bp.

The constructed plasmid pPIC9-XynZ was linearized (5 µg) using the restriction enzyme *SalI* (5 enzyme unities, and the reaction proceeded at 37ºC for 16 hours). Linearized construction was used to transform *Pichia pastoris* GS115 cells by electroporation method following the manufacturer's instructions. Then *P. pastoris* cells were selected by growth on solid MD medium (1.34 % (w/v) Yeast Nitrogen Based medium without amino acids (Difico), 2 % (w/v) glucose, $4x10^{-5}$ % biotin, and 2 % (w/v) agar) for five days at 30 ºC. Fifty colonies were collected and initially screened for their xylanase activity. Briefly, these colonies were grown in 5 mL of liquid medium BMMY (1 % (w/v) yeast extract, 2 % (w/v) peptone, 1.34 % YNB – without amino acids, $4x10^{-5}$ % biotin, buffered with 100 mM sodium phosphate pH 6.0) for 72 hours, at 30 º C and 200 rev/min, with daily addition of 0.5 % (v/v) methanol. After this initial induction, the supernatant was collected by centrifugation at 10000×*g* for 10 minutes at 4ºC, and the cell-free supernatant was used to detect xylanase activity. Finally, clones were chosen based on the secreted xylanase activity. All clones were preserved at -80ºC in 50 % (v/v) glycerol.

For enzyme production, selected clones, namely clone 5 and 9, were cultivated in 50 mL of BMGY medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 1.34 % YNB – without amino acids, $4x10^{-5}$ % biotin, buffered with 100 mM sodium phosphate pH 6, 1% (v/v) glycerol) in 400 mL Erlenmeyer flasks at 30 ºC and 200 rev/min. After 48 hours of growth, cells were centrifuged and then transferred to 50 mL of BMMY medium (initial OD_{600} = 1); each clone culture was daily added with 0.5 % (v/v) of methanol. After every 24 hours, samples of 1 mL were retrieved from cultures and used to measure optical density at 600 nm (OD_{600}), centrifuged 10000×*g* for 10 minutes at 4 ºC, and cell-free supernatant was used for determination of xylanase activity. A total of 10 µL of the clarified supernatant was applied into a silver-stained 12 % SDS-PAGE. Alternatively, induction was also performed in minimal liquid medium (1.34 % (w/v) YNB, buffered

with 100 mM sodium phosphate pH 6), with daily addition of methanol 0.5 % (v/v). Clones cultivation was performed in biological duplicates, *P. pastoris* GS115 transformed with *SalI* linearized pPIC9 vector without insert was used as a control.

XynZ production to further experiments (characterization, formation of mini-xylanosomes, and hydrolysis) was carried using clone 5, which was cultivated in 50 mL of liquid BMMY medium with daily 0.5% (v/v) methanol induction. After 120 hours of growth, the culture was centrifuged at 10000×g for 10 minutes at 4 ºC, cells were discarded, the supernatant was subjected to overnight dialyzes (10 kDa cut-off) against 10 mM tris-HCl pH 7.1 with 5 mM $CaCl_2$, and finally, the supernatant was added with 1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.01% (w/v) sodium azide. This sample used as XynZ source was stored at 4 ºC until further use.

CipA expression

In this study, the 5' portion of the *C. thermocellum*'s scaffolding protein, CipA (Cthe_3077, NCBI: Gene ID: 4809951) coding for the N-terminus fraction of CipA containing two cohesins I domains, and a carbohydrate-binding module 3 (CBM3). For DNA amplification, the genomic DNA from *C. thermocellum* isolate B8 was used as a template. The polymerase chain reaction was formed by 50 ng of genomic DNA, 1 U of Taq. DNA polymerase (Taq High fidelity Pol, Cellco Biotec, BR), 200 µM of dNTPs, 0.50 µM of each primer (forward AACATATGGTATCGGCGGCCACAAT; reverse AAGTCGACTGAAGCAGACTTGAATGAGTAG; underlined sequences represent *NdeI* and *SalI* restriction sites, respectively), 2.50 µL of 10× buffer (Hight Fidelity Buffer, Cellco Biotec, BR), in a final volume of 25 µL. PCR cycling was performed as follows: 95ºC 1 minute, 35 cycles of 95ºC for 30 seconds, annealing and extension at 68ºC for 2 minutes, and a final extension at 68ºC for 5 minutes.

After PCR reaction, the amplicon was run in a 0.8% agarose gel, purified, digested with *NdeI*, *SalI* restriction enzymes (1 hour at 37ºC), and then ligated into a pET21a vector predigested with same restriction enzymes. The ligated vector was cloned in *E. coli* DH5α for plasmid propagation, and then the plasmid pET21-mcipA was used to transform *E. coli* BL21(DE3) and used as an expression host. Protein expression was conducted by cultivating *E. coli* BL21(DE3) harboring the plasmid pET21-mcipA in 100 mL autoinduction broth [26] in shaker-incubator set at 28 ºC 200 rev/min for 16 hours. After growth time, cells were collected by centrifugation at 10000×g for 20 min at 4 ºC, resuspended in 10 mL of 50 mM tris-HCl buffer pH 7.1, sonicated [40% amplitude for 5

minutes (30 seconds on/off cycles) (SONICS® Vibra-Cell™ VC 750, USA)] and the supernatant containing the soluble protein collected by centrifugation and heat-treated at 60 ºC for 16 hours in a water bath. After heat-treatment, the supernatant was cleared by centrifugation at 10000×g for 20 min at 4 ºC, and then stored at 4ºC until further use.

Formation of enzymatic complexes

Indirect protein relative abundance for stoichiometric complex assemble was calculated using the software ImageJ [27], analyzing protein band density on a silver-stained 12 % SDS-PAGE. Mini-xylanosomes (mCipA:XynZ) were assembled by mixing XynZ with mCipA on a molar basis of 1:2. Briefly, the protein mixture containing mCipA and XynZ was incubated at 60 ºC for 1 hour with 2 mM β-mercaptoethanol. After mCipA and XynZ incubation time, samples were cooled at room temperature and stored at 4ºC until further use.

In order to evaluate the formation of complexes, 7.5 % Native-PAGE [28] was performed, and then silver-stained. The presence of the complex was also evaluated by comparing the elution profile in gel filtration chromatography of XynZ to the elution profile of the formed mini-xylanosome. For gel filtration chromatography procedures, a column containing 480 mL of Sephacryl S-100 resin (GE Life Sciences, US) was equilibrated with 50 mM tris-HCl pH 7.1 added with 150 mM $NaCl_2$ and 5 mM $CaCl_2$, and then 5 mL of samples (XynZ, or mini-xylanosomes) were applied and separated in 5 mL fractions at a flow rate of 18 mL/hour at room temperature (FRAC-920 Fraction collector, GE Life Sciences, US). Protein detection was performed by reading at A_{280}, and xylanase activity was assessed in every fraction. The void volume was estimated by applying 500 mg of Blue Dextran protein into the column and fractioning it as previously described.

Xylanase activity detection

Endo-β-1,4-xylanase activity, from now on referred to as xylanase activity, was determined using standard enzymatic assays at 60 ºC using 1 % (w/v) oat spelt xylan (Sigma Aldrich, US) prepared in 50 mM sodium acetate buffer pH 5.0 for 30 minutes as previously described [29]. One enzymatic unit was defined as µmol of reducing sugar released per minute (IU), and xylose was used as a sugar standard. Protein quantification was carried out using the commercial kit Quick Start™ Bradford 1x Dye Reagent (BioRad Laboratories, US), following the Bradford methodology [30], and bovine serum albumin was used as the protein standard.

Xylanase activity characterization

The effect of temperature on XynZ free-form and assembled in a mini-xylanosome was carried out by conducting standard enzymatic assays in different temperatures from 30 to 80 ºC. The pH effect was carried out using standard enzymatic assays varying the buffer, 100 mM sodium citrate (pH 3 and 4), 100 mM sodium acetate (pH 5), 100 mM sodium phosphate (pH 6), and 100 mM Tris-HCl (pH 7.1 and 8). Thermal stability was evaluated by incubating 5 mL of enzymatic samples (XynZ free-form or mini-xylanosome) at 70 or 80 ºC in a water bath, samples were periodically removed, and then residual activity was evaluated using standard enzymatic assays. The enzymatic assay was performed in triplicates, and values are represented with their corresponding standard deviation.

Mini-xylanosomes immobilization on cellulose and reuse in hemicellulose hydrolysis

Immobilization of mini-xylanosomes in cellulosic fibers was carried out to evaluate the activity loss during xylan hydrolyses of the mini-xylanosome, and its performance during reuse cycles. Binding of mini-xylanosomes on cellulosic fiber was carried out by incubating 200 µg of protein (mini-xylanosome) with 100 mg of cellulose (Fibrous, medium Lot#106H1131, Sigma Aldrich, USA) at 60º C. After one hour of incubation; samples were centrifuged at 12000×*g* for 20 minutes at room temperature. The cellulose pellet containing the immobilized enzyme was washed three times with 1 mL of 50 mM tris-HCl pH 7.1 containing 5 mM of $CaCl_2$. Then cellulose-containing immobilized mini-xylanosome was used as a source of hydrolytic activity to degrade oat spelt xylan. For xylanase activity quantification of cellulose-immobilized mini-xylanosomes, the enzymatic assay was carried out as previously shown, replacing the 50 µL of enzyme for 50 mg of cellulose-containing the bound complex.

Oat spelt xylan hydrolysis by immobilized mini-cellulosome was carried out by adding 6 mg of soluble oat spelt xylan to 100 mg of cellulose-containing xylanolytic activity. The volume was adjusted to one mL, and the hydrolysis pH adjusted to 7.1 using 50 mM tris-HCl buffer added with 5 mM $CaCl_2$. The hydrolysis experiment was carried out at three temperatures, 50, 60, or 70 ºC.

In order to assess cellulose-immobilized mini-xylanosome reusability in hydrolysis condition, the whole reaction was centrifuged after every 24 hours hydrolysis time, and the cellulose pallet containing the immobilized mini-xylanosome was thrice washed with 50 mM tris-HCl buffer. After the washing step, the recovered cellulose-

containing immobilized mini-xylanosome was again used as the source of enzymes for a new hydrolysis cycle. This procedure was carried out every 24 hours for 72 hours; the total reducing sugar generated was quantified every 24 hours during the hydrolysis course [31]. Experiments were performed in two separate hydrolysis experiments, and results are shown with corresponding standard deviations from them. Controls containing the immobilized mini-xylanosome or the substrate were also performed.

Effect of lignocellulosic derived phenolic inhibitors

For assessment of the inhibition or activation of xylanase activity by lignocellulosic derived phenolic and aromatic compounds, an initial screening was performed using 4-hydroxybenzoic acid, gallic acid, vanillin, tannic acid *p*-coumaric acid, ferulic acid, syringaldehyde, and cinnamic acid as previously described [32]. Briefly, XynZ was incubated with each compound in a final concentration of 1 mg/mL at room temperature; after one hour, enzymatic assays were performed at 60ºC using the standard methodology. After this initial screening (results not shown), tannic acid and ferulic acid were chosen as inhibitor and activator models, respectively. The effect of tannic acid and ferulic acid was further evaluated for the mini-xylanosome sample using the same procedure previously described for XynZ. Tannic acid inhibition was also performed using the immobilized mini-xylanosome on cellulose (obtained as described above).

For this set of experiments, controls using ultrapure distilled water or ethanol 10% (v/v) were performed since tannic was solubilized in water and ferulic acid in ethanol. Every test was carried out in triplicates, and results are given with standard deviation from triplicates. Phenolic and aromatic compounds used in the present study were purchased from Sigma Aldrich (USA), and their purity grade is above 98 %.

Sisal fiber and soluble oat-spelt xylan hydrolysis

Sisal fiber was obtained from the local market. Briefly, untreated sisal fiber was manually cut into fine pieces 1 cm wide and then stored at room temperature for further experiments. For hydrolysis of sisal fibers by XynZ or the mini-xylanosome, experiments were carried out in 1 mL reactions volume containing 5 % (w/v) of substrate load, buffered with 100 mM tris-HCl pH 7.1 added of 5 mM $CaCl_2$, and then incubated in a water bath at 60 ºC for 24 and 72 hours. For this experiment, the enzymatic load of 0.1 IU (2 IU/g), 0.2 IU (4 IU/g), 0.3 (6 IU/g) (xylanase activity/ grams of the substrate) were employed. After hydrolysis time, samples were retrieved from the water bath, centrifuged at 12000×*g* at 4 ºC for 20 minutes, and reducing sugar quantified using the DNS method

[31]. Controls containing only the enzyme solution or the biomass were performed. Soluble oat spelt xylan hydrolysis was performed as described for sisal fiber, replacing the lignocellulosic substrate with 6 mg of soluble oat spelt xylan, and enzymatic loads were 0.05 IU, 0.1 IU, and 0.15 IU. Controls containing only the soluble substrate or the enzyme solution were performed. Hydrolysis experiments and control samples were performed in duplicates, and results are given with standard deviation from duplicates.

Results and discussion

XynZ heterologous expression in *Pichia pastoris* GS115

A total of 50 clones of *Pichia pastoris* GS115 transformed with pPIC9-XynZ in the correct orientation were obtained in the first screen. Clones named 5 and 9 were capable of secreting xylanase activity (Fig. 1). The activity was detected for growth in both medium, BMMY, and minimal; however, values found for BMMY were higher. Maximal values were obtained after 120 hours of growth time, reaching 2.07 IU/mL. Values of secreted xylanase and growth curve were similar to clones 5 and 9; the stationary growth phase was achieved after 72 hours. In SDS-PAGE analyses, for the expressing XynZ *Pichia pastoris* clones, a prominent protein band close to 90 kDa was observed, indicating the expression of full-length XynZ (Fig. 1).

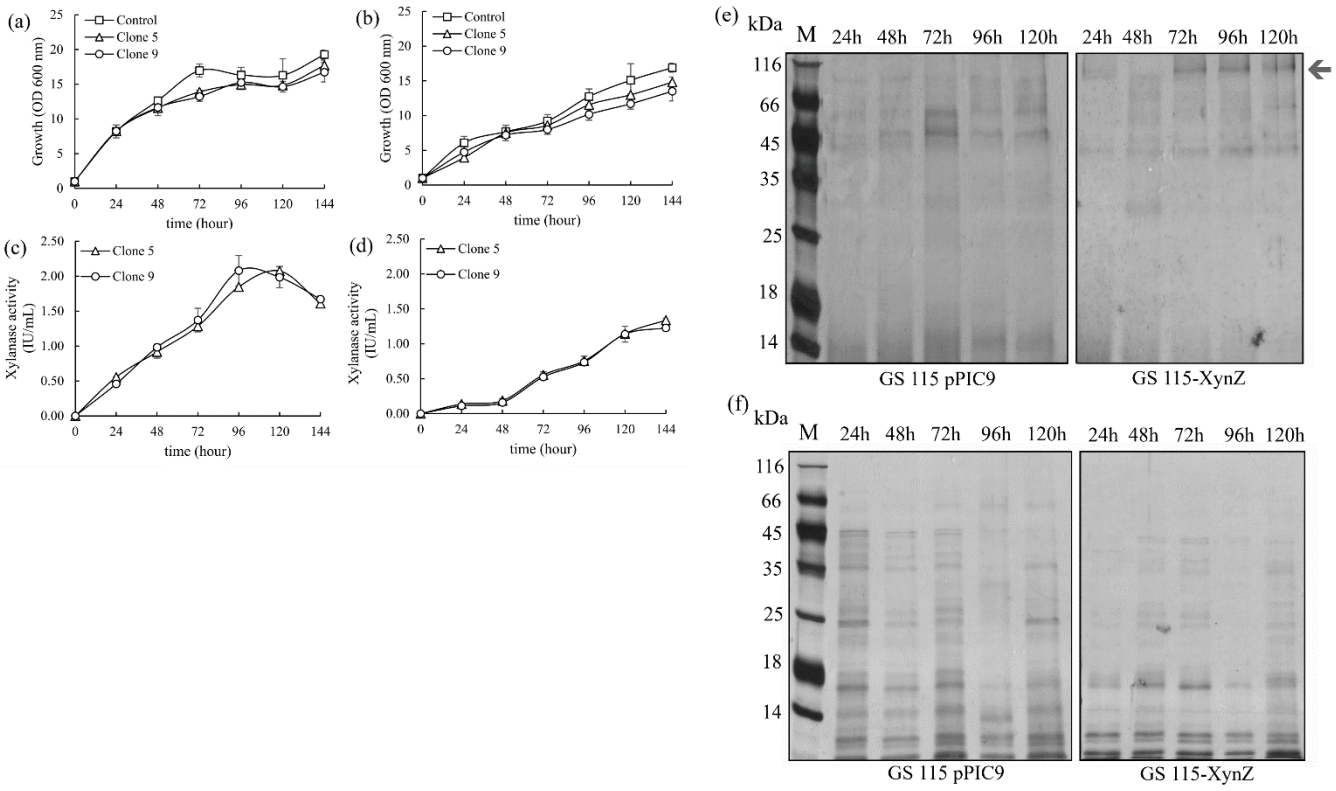

Fig 1: Growth profile of *P. pastoris* GS115 (square), and transformants clones 5 (triangle) and 9 (circle) in BMMY (a) and minimal medium (b). Vertical bars correspond to the standard deviation of reads from biological duplicates. Xylanase activity was obtained in the supernatant after *P. pastoris* transformants growth in BMMY (c) and minimal medium (d). Vertical bars correspond to the standard deviation from biological duplicates. SDS-PAGE profile of secreted proteins of *P. pastoris* GS115, and the transformant clone 9 in BMMY (e), and minimal medium (f). Induction was performed with the addition of 0.5 % (v/v) methanol every 24 hours. Protein marker (M): β-galactosidase (116 kDa), bovine serum albumin (66 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β-lactoglobulin (18 kDa), Lysozyme (14 kDa)

Results obtained for induction of the recombinant protein in *P. pastoris* indicate that the fusion with the α-factor for protein secretion was functional and differ from previous studies of heterologous expression of *C. thermocellum*'s genes. Previously, a *C. thermocellum* cellulase was expressed in yeast cells using the native bacterial signal peptide and resulted in intracellular protein accumulation [33]. In general, the expression of bacterial xylanases in yeast is seen as a common strategy to increase enzyme production; and yeast's secretion signal to obtain a soluble protein in the culture media supernatant. Expression of bacterial xylanase was also previously investigated; for instance, Cheng et al. reported the heterologous production of a 36 kDa xylanase of *Thermobifida fusca* in *P. pastoris* using the α-factor secretion signal in reactor scale, reaching values above 300 IU/mL activity [34]. A 29 kDa xylanase from *Bacillus pumilus* HBP8 was expressed in *P. pastoris* and secreted using the α-factor, and values above 500 IU/mL were obtained in bench-scale production [35].

The reports above described higher enzymatic activity values than our results; this can arise from many reasons, including the complexity of the xynZ gene, and multimodular proteins can go through proteolytic cleavage after expression [36]. Growth conditions also can contribute to this difference since stirred tanks/bioreactors can ensure better conditions for yeast growth than bench-scale growth, resulting in higher optical density and, consequently, higher heterologous enzyme production.

mini-xylanosome formation using XynZ and mCipA

In native page analysis, mini-xylanosome assembly was observed (Fig. 2-b), and a slight dissociation from dockerin and cohesin modules from the formed complex (Fig. 2). The formation of the mini-xylanosome could also be observed by analyzing the molecular-exclusion chromatography profile (Fig. 2-b), on which the addition of the mCipA to XynZ displaced the protein to an early elution (fractions 10 to 38), differing from the XynZ standalone profile (fractions 38 to 48). This displacement of proteins and xylanase activity indicates the mini-xylanosome formation. The mini-xylanosome's activity showed a similar elution profile compared to blue-dextran protein (void-volume), indicating the formation of proteins with molecular mass above 100 kDa (Sephacryl S-100 cut-off). The activity released in the void volume from chromatography procedures had more than 70 % of total xylanase activity.

The dissociation observed in native page analysis (Fig. 2) for the formed complex was not expected, and further addition of mCipA to XynZ to ensure a complete formation of mini-xylanosomes did not cause a reduction in dissociation (results not shown). Previous studies have partially elucidated this phenomenon, on which the yeast glycosylation pattern on dockerin domains can reduce the recognition of cohesin modules [37]. This information corroborates the results obtained in the present study; since no deglycosylation was performed before assembling mini-complexes.

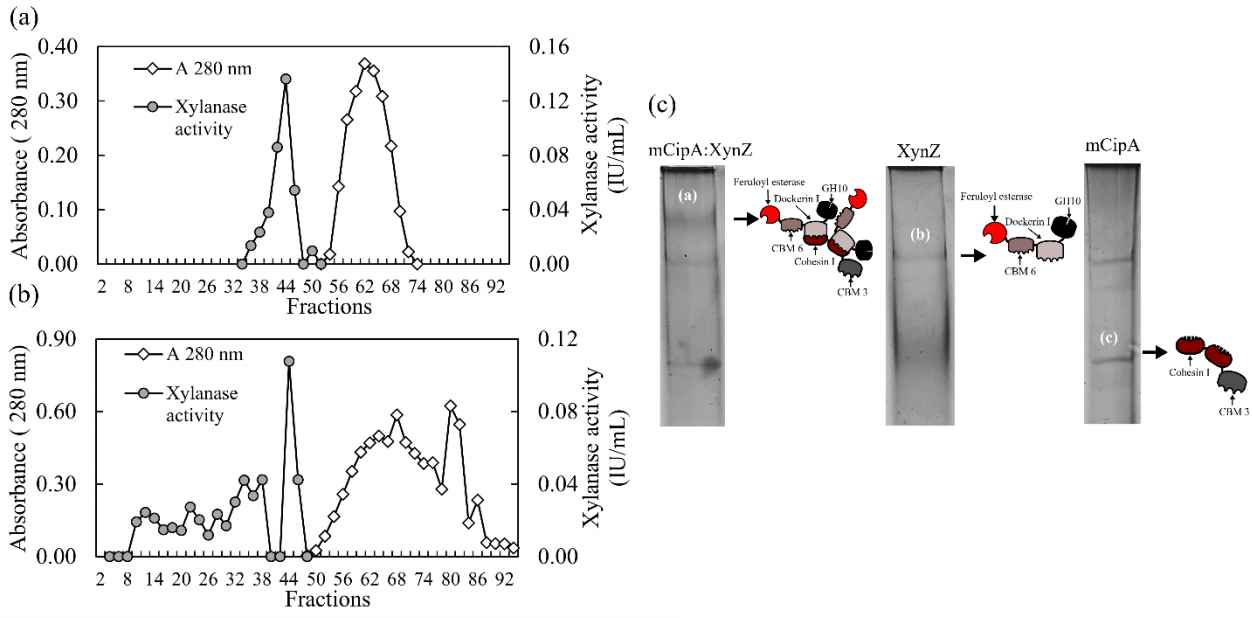

Fig. 2: Gel filtration chromatography profile of recombinant XynZ (a), and mini-xylanosome (b) separation in a Sephacryl-100 column. White diamonds represent protein elution (absorbance at 280 nm), and the grey circles represent the xylanase activity elution profile. 7.5 % Native-PAGE profile (c) of assembled mini-xylanosome (mCipA:XynZ), XynZ, and the recombinant scaffolding protein mCipA. Proteins modules are based on XynZ [UniProtKB - P10478 (XYNZ_HUNT2)], and mCipA [UniProtKB - Q06851 (CIPA_HUNT2)].

Biochemical properties of XynZ and mini-xylanosome

XynZ assembled in mini-xylanosome or non-complexed displayed maximum activity in a pH range from 6 to 8, presenting a residual activity at pH 3 (Fig. 3). Regarding the temperature effect, both enzymatic samples displayed maximum activity between 60 and 70 °C, activity values below 20 % were detected at 30 and 40 ºC. The effect of pH is in agreement with other *C. thermocellum*'s enzymes, including the expressed GH10 domain of XynZ [36] and the modular GH30 CtXynGH30 [38]. In our study, both

samples presented more activity above 60 ºC, whereas other xylanases from *C.thermocellum* as the CtXynGH30, show around 80 % activity at 50 ºC, and the GH43 α-arabinofuranosidase axb8 which shows peak activity between 40 and 50 ºC [39].

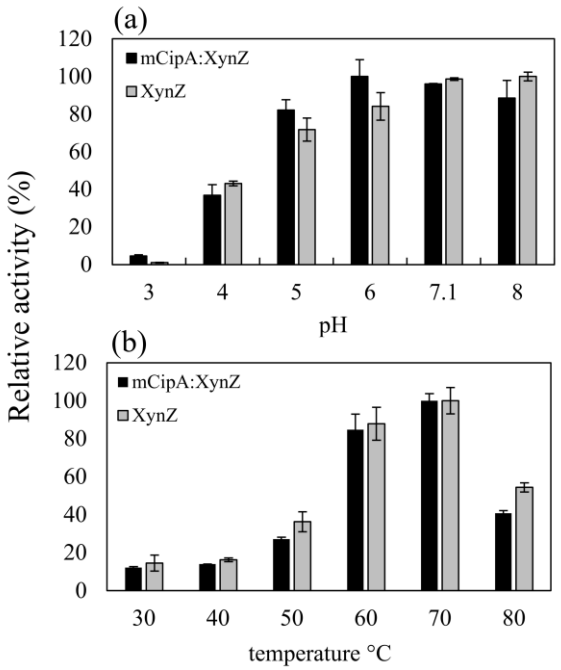

Fig. 3: pH (a) and temperature effect (b) on XynZ (grey bars), and on the mini-xylanosome (mCipA:XynZ, black bars). Vertical lines represent standard deviation from triplicates. Maximum activity corresponds to 1.19 IU/mL.

Regarding thermal stability, similar activity decay at 80 ºC was observed, with half-lives for the mini-xylanosome and XynZ of 28 and 22 minutes, respectively. At 70 ºC, a difference was observed between samples, the assembled xylanosome showed relatively higher thermal stability, with an estimated half-life of 134 minutes, and the enzyme XynZ showed an estimated half-life of 90 minutes (Fig. 4). The lower thermal stability of the enzymes (XynZ, or mini-xylanosome) observed in the present study for the temperature of 80 ºC was expected, since *C.thermocellum*'s glycoside-hydrolases are commonly reported to be more stable in a temperate range from 50 to 70 ºC, above this range prominent activity decay is expected to happen [29, 40].

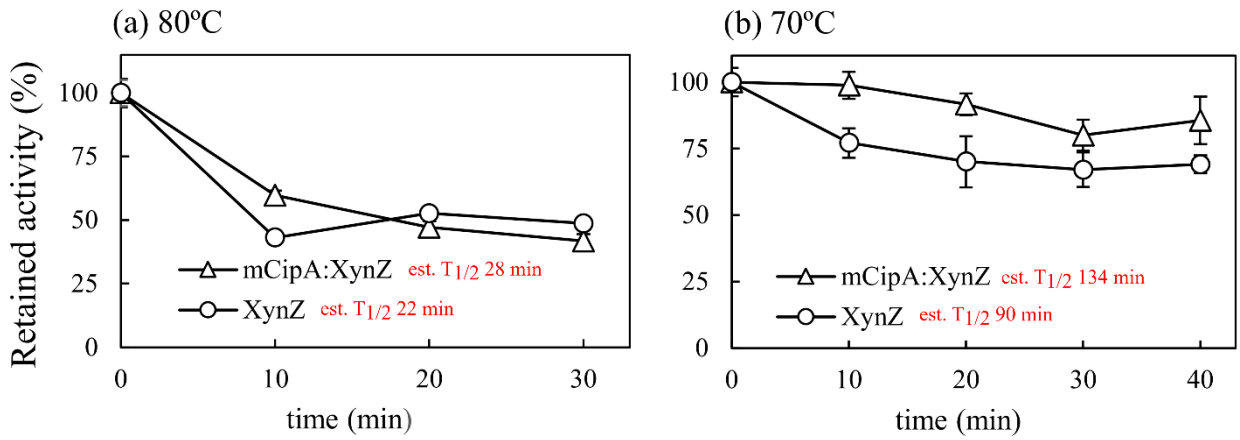

Fig. 4: Thermal stability profile of mini-xylanosomes (mCipA:XynZ, triangles), and XynZ (circles) at 80 ºC (a) and 70 ºC (b). Vertical bars represent the standard deviation from triplicates. Maximum activity corresponds to 1.19 IU/mL.

The mini-xylanosome sample showed higher thermal stability than the free form of XynZ, which might be a direct result of the protein-protein interaction granted by the dockerin:cohesin interaction with the scaffolding protein mCipA. The increment in thermal stability of proteins associated with cellulosomes using dockerin:cohesin interaction was proven to the cellulase Cel8A of *C. thermocellum* associated with a scaffolding protein harboring one cohesin and one carbohydrate-binding module [41]. Based on the previous reports and the data shown for thermal stability in the present study, it is reasonable to assume that the assembly of cellulosomal enzymes into scaffolding proteins harboring cohesin modules may enhance thermal stability for cellulases and cellulosomal hemicellulases as the XynZ.

Immobilization of the mini-xylanosome in cellulosic fibers

Cellulose-immobilized mini-xylanosome displayed activity against soluble xylan in temperatures of 50, 60, and 70 ºC, with maximum hydrolysis at 50 °C, resulting in reducing sugars above 0.40 mg/mL after 24 hours of hydrolysis (Fig. 5). For experiments of mini-xylanosome reuse carried out at temperatures of 50 and 60 °C, better reusability of the formed mini-xylanosomes was observed at the third cycle of reuse (72 hours), reaching 57.53 and 51.62 % of hydrolytic activity, respectively for 50 and 60 °C. For the temperature of 70 °C, at the third cycle (72 hours), no hydrolytic activity was obtained against soluble xylan, indicating that the formed mini-xylanosome had lost most of its activity.

A previous study demonstrated the immobilization of xylanases into different other matrices, such as Q-sepharose resin, gelatin, and HP-20 beads [42]. The immobilization process, shown in the present study, works as proof that the xylanase activity was associated with the scaffolding protein harboring the CBM3, i.e., assembled into a xylanosome.

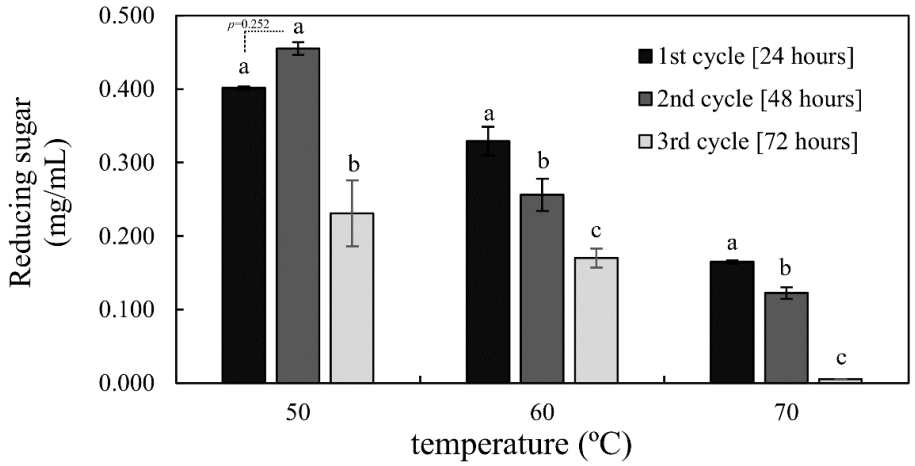

Fig. 5: Reducing sugar released after reuse cycles of immobilized mini-xylanosome on cellulose fibers during hydrolysis of oat spelt xylan. Black bars (first cycle, 24 hours), grey bars (second cycle, 48 hours), light grey bars (third cycle, 72 hours). Vertical lines represent standard deviation from two individual hydrolysis experiments. Bars containing the same letter represent values with $p>0.05$ in each temperature group analyzed by ANOVA followed by Tukey's test.

Furthermore, the immobilization of xylanases on cellulose also works as a strategy to evaluate xylanase thermal stability in real conditions of hemicellulose deconstruction, on which the presence of substrate and immobilization may modulate the thermal deactivation. For instance, the thermal deactivation of the formed complex indicates that at 70 ºC, the half-life activity is 134 minutes (Fig. 4). However, at the second cycle of reuse (48 hours), 74 % of initial hydrolysis was obtained, indicating the immobilization and/or the presence of substrate can positively module the thermal deactivation of mini-xylanosomes.

Effect of lignocellulose derived inhibitors in XynZ and mini-xylanosomes

An initial screening was performed to evaluate which class of phenolic/aromatic compound derived from lignocellulose could inhibit or enhance the xylanase activity of

XynZ, indicating that ferulic acid can enhance xylanase activity by about 50% and tannic acid significantly decrease hydrolytic activity (data are now shown).

Regarding the tannic acid effect, both mini-xylanosome and XynZ were strongly inhibited, resulting in 20 and 18.95 % of residual activity, respectively (Fig. 6). For ferulic acid, mini-xylanosome and XynZ displayed activity enhancement above 30 %. Tannic acid also caused a great activity reduction in cellulose-immobilized mini-xylanosome, resulting in 11.11 % of residual activity (Fig. 6).

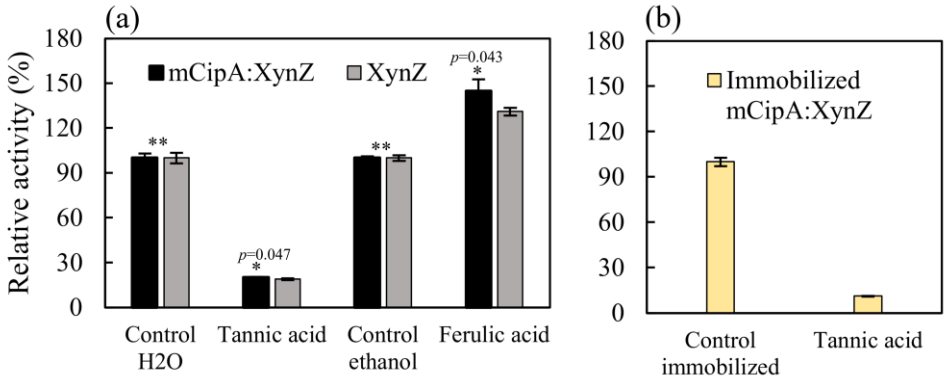

Fig. 6: Effect of lignocellulosic derived phenolics on xylanase activity of mini-xylanosome (mCipA:XynZ, black bars), and XynZ (grey bars) (a). Effect of tannic acid on cellulose-immobilized mini-xylanosome xylanase activity (mCipA:XynZ, yellow bars) (b). Vertical lines represent standard deviation from triplicates. *p<0.05, **p>0.05 determined after means comparison by Student's t-test.

In literature, there are plenty of reports demonstrating that tannic acid, and oligomeric phenols, as lignin, are some of the major players in the inhibition of cellulases and hemicellulases [32, 43, 44]. Our results are in agreement with previous reports regarding xylanases and tannic acid. Results from the cellulose-immobilized mini-xylanosome also attest that further immobilization will not prevent activity loss in the presence of tannic acid.

The activation of xylanases by ferulic acid has been previously reported to an enzyme belonging to the GH11 family [45] and the enzymatic complex produced by *C. thermocellum* B8 [40], containing two GH10 xylanases XynC and XynY. The results described above, along with the data shown in the present study, highlights that among phenolic compounds present on lignocellulosic residues, some can have positive modulation of xylanase activity. The activation by ferulic acid seems to be a common fact for xylanases from GH10 and GH11 families, as seen for the XynZ (GH10); this

activation could be advantageous during the hydrolysis of grasses, which have higher ferulic acid content on its structure [46].

Hydrolysis of sisal fibers and oat spelt xylan

Hydrolysis results were diverging for complexed to non-complexed XynZ, being observed higher releasing of reducing sugars for the mini-xylanosome sample at an enzymatic load of 0.2 IU/mL, 0.62 mg/mL in the first 24 hours. Whereas at the same enzyme load, hydrolysis using XynZ released 0.18 mg/mL. Indeed, reducing sugar released in 24 hours for the XynZ in all enzymatic loads generated comparable levels of sugars around ~0.2 mg/mL (Fig. 7 a,b).

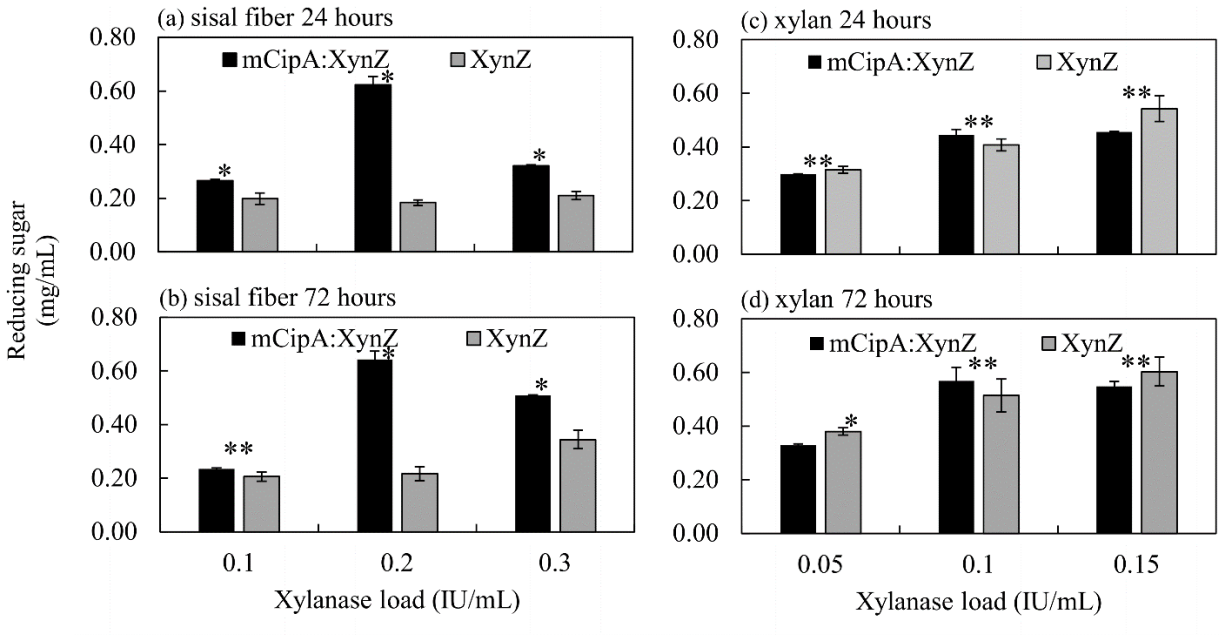

Fig. 7: Reducing sugar released after 24 (a,c) and 72 hours (b,d) hydrolysis time of sisal fibers (a,b), and oat spelt xylan (c,d), by mini-xylanosome (mCipA:XynZ, black bars), and XynZ (grey bars). Vertical lines correspond to the standard deviation from two separate hydrolysis experiments. *p<0.05, **p>0.05 determined after means comparison by Student's t-test.

As observed for 24 hours results, the mini-xylanosome sample also showed the best hydrolysis at 0.2 IU/mL after 72 hours, releasing 0.64 mg/mL of reducing sugar. Meanwhile, XynZ generates 0.21 mg/mL of reducing sugars. Time increment of reducing sugars from 24 to 72 hours was majorly observed for enzymatic loads of 0.3 IU/mL, on

which the reducing sugar generated by the complex shifted from 0.32 to 0.50 mg/mL, and the XynZ from 0.21 to 0.34 mg/mL.

In general, hydrolysis of sisal fibers was more efficient when employing the assembled mini-xylanosome. This result was obtained for several reasons, including the higher thermal stability of the complex sample and the addition of an extra carbohydrate-binding module from scaffolding protein mCipA. The higher thermal stability of the complexed sample, as previously discussed, may arise from two factors: the protein/ protein interaction granted by the dockerin:cohesin interactions, as well as the biding of the complex to the cellulosic substrate, can prolong its stability. As previously demonstrated, the complexing of cellulosomal enzymes to scaffolding proteins can enhance thermal stability [41].

Despite the enhancement in thermal stability, the presence of an extra carbohydrate-binding module from family 3 (cellulose-specific CBM) can be the main reason to explain the higher efficiency of complexed samples. There are reports regarding the addition of cellulose-specific carbohydrate-binding modules into xylanases that may enhance hemicellulose solubilization. The presence of CBMs specific to cellulose may enhance the accessibility to a fraction of hemicellulose that is close to cellulosic fibers [47, 48]. This interface cellulose-hemicellulose may be more present in sisal fibers, with relatively low hemicellulose content. Thus the mini-xylanosome sample possessing a CBM3 can have higher efficiency on its deconstruction. This effect has previously been confirmed to GH10 xylanase. The addition of cellulose-specific CBM could enhance hydrolytic performance [48], resulting in agreement with the present study.

The CBM3 as one of the primary reasons for the higher performance of the complexed sample is also supported by the results observed for oat spelt xylan deconstruction (Fig. 7 c,d). In the hydrolysis of a pure hemicellulose matrix, the CBM3 cannot give the enzyme the binding properties; since this class of carbohydrate-binding module is specific for cellulose. Thus, hydrolysis of a soluble hemicellulose component resulted in close values when comparing the XynZ to the mini-xylanosome sample. The targeting effect given by the CBM3 has also been described as the main reason for enhancing the hydrolysis of soluble cellulosic substrates by engineered cellulosomes [49]. In addition to the findings described in the previous report, in our study, we suggest that the addition of this carbohydrate-binding module can also improve the deconstruction of insoluble lignocellulosic substrates.

Conclusion

In this study, the *C. thermocellum*'s cellulosomal xylanase, XynZ, was assembled into a mini complex, and its properties were evaluated. The remarkable difference between the non-complexed XynZ and the mini-xylanosome was observed for the thermal stability, on which complexed samples displayed better heat tolerance. The assembled complex also showed higher performance for hydrolyzing sisal fibers. This result highlights that complexing xylanases into mini-complexes can be further used to improve sisal fiber hydrolysis, working as an alternative to chemical treatments to hemicellulose removal. The results obtained in the present study can be further extended to other biomasses as a model of thermostable xylanase to hemicellulose deconstruction. Further studies are required to identify changes in fibers' physical properties and their applicability in the production of reinforced materials.

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Author's contribution

Pedro R.V Hamann wrote the manuscript and conceived experiments; Tainah C. Gomes conceived experiments; Luísa de M.B Silva conceived experiments; Eliane F. Noronha revised the manuscript, conceived experiments, and was responsible for funding acquisition.

Ethical statement

The manuscript was read and approved by all authors as it is; the authors would like to declare that there is no conflict of interest, and credit has appropriately been given to everyone who contributed to the present study.

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Principais pontos abordados e perspectivas

XynA e a os compostos fenólicos

Dentre os principais achados em relação a enzima XynA e os compostos fenólicos, observa-se a tendência temperatura-dependente em que a desativação da XynA ocorre na presença dos compostos fenólicos, temperaturas mais elevadas corroboram para que a atividade da xilanase seja reduzida na presença de compostos como ácido gálico, ácido *p*-cumárico e ácido ferúlico. Em relação a modulação da estabilidade térmica, quando a enzima termoestável está na presença dos compostos fenólicos a baixa temperatura (40ºC) apenas ácido tânico é capaz de reduzir a estabilidade térmica, entretanto a 60ºC os compostos conseguem reduzir drasticamente a estabilidade térmica.

No presente estudo também foi observado que nos experimentos de hidrólise em que a enzima além do composto fenólico tinha acesso ao substrato, o efeito da desativação foi reduzido, indicando que a interação enzima-substrato pode levar a uma menor perda de atividade na presença de compostos fenólicos. Além da interação por meio do sítio catalítico (GH10), o módulo de associação a carboidratos específico a xilana (CBM6) deve contribuir para facilitar a associação proteína-substrato.

Experimentos de pré-desativação por compostos fenólicos da XynA, mostrou que quando a enzima é exposta a tais compostos a baixa temperatura (40ºC), ela é capaz de sofrer reativação, e ainda continuar capaz de realizar a hidrólise da xilana. Entretanto, quando a pré-desativação ocorre em temperaturas elevadas (60ºC) a enzima não é capaz de recuperar sua atividade catalítica.

Os resultados atestam que compostos fenólicos podem apresentar efeitos negativos na XynA, e que esses efeitos são de certa forma dependentes da temperatura. Para contornar tal problema, enzimas termoestáveis podem ser aplicadas em processes simultâneos de sacarificação e fermentação que usualmente ocorrem a baixas temperaturas, de tal forma as propriedades como estabilidade térmica seriam preservadas, possibilitando a enzima ser reutilizada em processos industriais.

Formação de complexos enzimáticos utilizando mCipA e XynA

Após realizar a complexação da xilanase XynA, com uma versão reduzida da proteína estrutural, mCipa, foi observado que propriedades gerais como efeito da temperatura e pH apresentaram valores similares para a proteína complexada e a não complexada, tendo atividade máxima em uma faixa de 60 a 70ºC em pH 6 a 8. Apesar do efeito da pH e da temperatura apresentarem valores similares, quando as duas amostras foram utilizadas para realizar a hidrólise a 60ºC do bagaço de cana tratado quimicamente, a amostra complexada demonstrou maior liberação de açúcares redutores, 0.83 $mg.mL^{-1}$, enquanto a amostra não complexada gerou 0.67 $mg.mL^{-1}$.

Além da melhor performance na hidrólise da biomassa lignocelulósica, foi observado que a suplementação dos mini-xilanosomas formados a misturas enzimáticas fúngicas produzidas por *Trichoderma harzianum* elevam a liberação de D-glicose. O fato de os mini-complexos formados melhorarem a performance das enzimas fúngicas na hidrólise do bagaço de cana tratado quimicamente pode ser relacionado a presença do domínio de associação a celulose presente na proteína estrutural que foi empregada no presente estudo. Apesar do domínio de associação a carboidrato (CBM3) não desempenhar atividade catalítica na desconstrução da celulose, existem relatos que a associação de tais domínios as fibras celulósicas podem causar uma menor associação inespecífica entre o substrato e a enzimas, dessa forma facilitando a catálise.

Para avaliar o efeito aditivo da proteína estrutural possuindo o CBM3 e a hidrólise da celulose, experimentos adicionais foram realizados. Para tais experimentos, as enzimas fúngicas foram adicionadas da proteína estrutural, mCipA, e então a hidrólise enzimática de celulose e Avicel foi realizada. Na hidrólise de fibras celulósicas a adição da proteína estrutural contendo o CBM3 foi capaz de melhorar a hidrólise e a produção de D-glicose final, entretanto para a condição de hidrólise utilizando Avicel como substrato não foi observado incremento na liberação de açúcares, resultado que possivelmente ocorreu pela maior recalcitrância de tal material celulósico.

De forma geral, como inicialmente proposto, as condições de hidrólise em que os mini-xilanosomas foram utilizados resultaram em uma maior liberação de açúcares redutores quando a biomassa lignocelulósica, bagaço de cana quimicamente tratado, foi utilizado como substrato. Os resultados obtidos para a hidrólise de materiais celulósicos indicam que a adição da proteína estrutural é capaz de facilitar a desconstrução dessa

classe de material, o que pode ser utilizada como uma ferramenta para o incremento na hidrólise de resíduos agroindustriais.

Formação de complexos enzimáticos utilizando mCipA e XynZ

De forma resumida, propriedades relacionadas ao efeito do pH e temperatura foram os mesmos para a proteína XynZ complexada ou não complexada. Entretanto, quando avaliada a propriedade de estabilidade térmica foi observada que nas temperaturas testadas (70 e 80ºC) a amostra que estava em seu estado complexado apresentou maior estabilidade térmica, mantendo a capacidade catalítica por maior tempo.

Tendo como base a associação do complexo contendo um domínio de associação a celulose (CBM3) foi realizado a associação dos complexos a fibras celulósicas, e então a amostra foi utilizada como modelo de imobilização enzimática. Os resultados obtidos indicam que o mini-xilanossoma montado é capaz de ser utilizado em ciclos de reuso por até três dias quando utilizadas temperaturas de 50 e 60ºC, entretanto quando temperatura mais elevada é empregada, 70ºC, o mini-xylanossoma é apenas capaz de ser reutilizados por dois turnos de 24 horas. Esses resultados servem como prova de conceito para: demonstrar a associação/ formação do complexo por meio da presença da associação da XynZ as fibras celulósicas, e demonstrar de forma funcional a estabilidade térmica do mini-xylanassoma, uma vez que a estabilidade térmica foi avaliada em condições reais de uso.

Os experimentos da hidrólise das fibras de sisal pelo mini complexo formado e pela enzima XynZ em seu estado livre mostram que ambas as amostras são capazes de liberar açúcares redutores das fibras vegetais, porém é observado que a amostra complexada apresenta valores de liberação de açúcar redutor três vezes superior em comparação com a XynZ em sua forma não complexada. Dentre os fatores que podem corroborar para a maior eficiência dos mini-xilanossomas em relação a enzima livre no que diz a respeito das hidrólises das fibras vegetais, pode-se citar: a presença de um domínio de associação a celulose, que pode fazer com que o mini complexo formado tenha acesso a demais áreas da lignocelulose, e como demonstrado anteriormente, os mini complexos formados apresentam maior estabilidade térmica.

Tendo como base os resultados mostrados, é possível propor a utilização de mini complexos enzimáticos contendo xilanases termoestáveis como uma opção para despolimerização da hemicelulose presentes em fibras celulósicas. A hidrólise enzimática de tal componente presente nas fibras vegetal pode facilitar o aproveitamento das fibras

celulósicas como materiais para formulação de novos compostos, assim como aproveitamento dos açúcares que foram solubilizados para futura fermentação por microrganismos

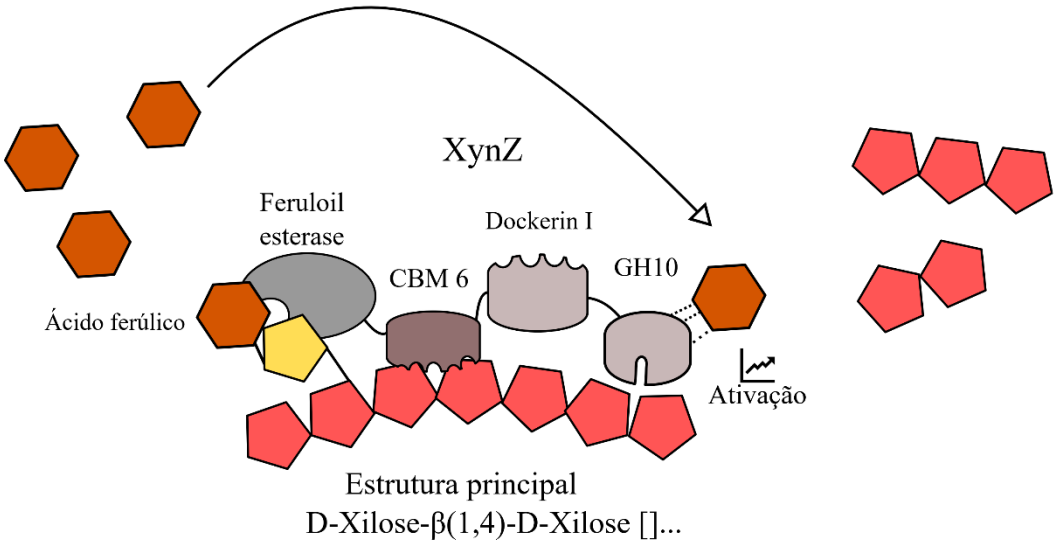
Perspectivas

Dar continuidade a montagem de mini xilanossomas, porém incorporando as duas atividades xilanolíticas, XynA (GH11) e XynZ (GH10). De tal forma, a avaliar o efeito sinérgico entre as atividades de GH11 e GH10 no mini xilanossoma.

Avaliar a modificação do domínio de associação a carboidratos (CBM3) presente na mCipA por um domínio com associação específica a hemicelulose, para assim tentar potencializar o efeito da complexação de xilanases em um xilanossoma

Investigar outras xilanases de *C. thermocellum* que tenham em sua estrutura mais de uma atividade contra a hemicelulose, e assim avaliar o efeito sinérgico entre os módulos catalíticos com função nas ramificações, e na estrutura principal da xilana.

Investigar a ativação do ácido ferúlico na xilanase XynZ, uma vez que essa enzima apresenta um sítio catalítico com função de feruloil esterase, e um domínio GH10, é possível que exista uma relação de ativação/cooperação, em que o produto da reação do domínio de feruloil esterase funcione como um ativador do módulo GH10.



Produção científica durante o doutorado

Artigo da tese

Hamann, P. R.V, Gomes, T. C., de MB Silva, L., & Noronha, E. F. (2020). Influence of lignin derived phenolic compounds on the *Clostridium thermocellum* endo-β-1, 4-xylanase XynA. Process Biochemistry. https://doi.org/10.1016/j.procbio.2020.02.034

Artigo publicado com o grupo de pesquisa como autor correspondente

de MB Silva, L., Gomes, T. C., Ullah, S. F., Ticona, A. R., **Hamann, P. R.V**, & Noronha, E. F.(2019). Biochemical properties of carbohydrate-active enzymes synthesized by *Penicillium chrysogenum* using corn straw as carbon source. Waste and Biomass Valorization, 1-12. https://doi.org/10.1007/s12649-019-00589-x

Artigos publicado com o grupo de pesquisa

Silva, J. P., Ticona, A. R., **Hamann, P. R**., Quirino, B. F., & Noronha, E. F. (2021). Deconstruction of Lignin: From Enzymes to Microorganisms. Molecules, 26(8), 2299.

Ticona, A. R., Ullah, S. F., Hamann, P. R.V, Lopes, F. A., & Noronha, E. F. (2020). *Paenibacillus barengoltzii* A1_50L2 as a Source of Plant Cell Wall Degrading Enzymes and Its Use on Lignocellulosic Biomass Hydrolysis. Waste and Biomass Valorization, 1-13. https://doi.org/10.1007/s12649-020-00975-w

Ullah, S. F., Souza, A. A., **Hamann, P. R. V**., Ticona, A. R. P., Oliveira, G. M., Barbosa, J. A.R., ... & Noronha, E. F. (2019). Structural and functional characterization of xylanase purified from *Penicillium chrysogenum* produced in response to raw agricultural waste. International journal of biological macromolecules, 127, 385-395. https://doi.org/10.1016/j.ijbiomac.2019.01.057

Artigo publicado com outro grupo de pesquisa

Souto BdM,de Araujo ACB, **Hamann PRV**, Bastos AdR, Cunha IdS, Peixoto J, et al.(2021) Functional screening of a Caatinga goat (Caprahircus) rumen metagenomic library reveals a novel GH3β-xylosidase. PLoSONE. https://doi.org/10.1371/journal.pone.0245118

Patente depositada junto ao grupo do professor Dr. Tatsuya Nagata

VETORES VIRAIS DERIVADOS DO MATERIAL GENÉTICO DEPEPPER RINGSPOT VIRUS E SEU USO PARA PRODUÇÃO DE PROTEÍNAS RECOMBINANTES EM PLANTAS VIA SISTEMA HETERÓLOGO DE EXPRESSÃO Número do Processo: BR 10 2021 011096 1

Função desempenhada: Seleção, validação, e purificação de proteínas com potencial biotecnológico utilizando o sistema de expressão desenvolvido.

Orientações durante o doutorado

Luisa de M.B Silva: Prospecção e caracterização de β-galactosidases microbianas. Aluna de graduação em Biotecnologia. Iniciação Científica (2017-2018).

Tainah. C. Gomes: Purificação e caracterização de β-glicosidases produzidas por *Clostridium thermocellum* B8. Aluna de graduação em Biotecnologia. Iniciação Científica (2017-2018).

Artigos submetidos em revisão

Assembling mini-xylanosomes with *Clostridium thermocellum* XynA, and their properties in lignocellulose deconstruction. Periódico: **Enzyme and Microbial Technology**. Autor.

Xylan breakdown apparatus of *Clostridium thermocellum*. Periódico: **Cellulose**. Autor.

Analysis of *Trichoderma harzianum* TR 274 secretome to assign candidate proteins involved in symbiotic interactions with Phaseolus vulgaris. Periódico: **Fungal Biology**. Co-autor

Anexo dos artigos publicados durante o doutorado

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Influence of lignin-derived phenolic compounds on the *Clostridium thermocellum* endo-β-1,4-xylanase XynA

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ABSTRACT

Phenolic compounds released during pretreatment of lignocellulosic biomass influence its enzymatic hydrolysis. To understand the effects of these compounds on the kinetic properties of xylan-degrading enzymes, the present study employed the recombinant cellulosomal endo-β-1,4-xylanase, thermostable GH11 XynA protein from *Clostridium thermocellum*, as an enzyme model to evaluate the effects of 4-hydroxybenzoic acid, gallic acid, vanillin, tannic acid, *p*-coumaric acid, ferulic acid, syringaldehyde, and cinnamic acid. XynA was deactivated by the assayed phenols at 60 °C, presenting the strongest deactivation in the presence of tannic acid, with an activity reduction of about 80 %. Thermal stability of XynA was influenced by ferulic acid, syringaldehyde, cinnamic acid, 4-hydroxybenzoic acid, and *p*-coumaric acid. The hydrolysis rate of oat-spelt xylan by XynA was influenced by temperature, being unable to hydrolyze at 40 °C in the presence of tannic acid. On hydrolysis at 60 °C, the presence of gallic and tannic acid caused a major reduction in reducing sugar production, generating 3.74 and 2.15 $g.L^{-1}$ of reducing sugar, respectively, whereas the reaction in the absence of phenols generated 4.41 $g.L^{-1}$. When XynA was pre-deactivated by phenols it could recover most of its activity at 40 °C, however, at 60 °C activity could not be reestablished.

1. Introduction

Agricultural residues such as sugarcane bagasse, wheat bran, corn cob and straw, mainly constituted by lignocellulose, are low-cost carbon sources with the potential for use as raw materials in the production of value-added chemicals. Technologies based on the use of these residues are still under development, as an economically viable process requires the complete deconstruction of lignocelluloses, a process hampered by their natural recalcitrance and complex structure [1]. Currently, the most commonly used and best studied process involves an initial step of pre-treatment to remove hemicelluloses and lignin, followed by enzymatic hydrolysis of the carbohydrate portion using plant cell wall-degrading enzymes [2–4]. Pre-treatment approaches including chemical, physical and enzymatic treatments result in a less recalcitrant carbohydrate core, with a liquid fraction rich in xylosaccharides and soluble lignin components. Like the carbohydrate core, the liquid fraction, rich in carbohydrates, is an interesting source of molecules for further biorefinery use [5–7].

Lignin is the major plant cell wall component responsible for lignocellulose recalcitrance and may also act as an inhibitor of plant cell wall hydrolases during enzymatic hydrolysis steps [8]. This inhibition might be caused by larger fragments of lignin and/or by free forms of phenolic compounds released during pretreatment [9,10]. Liquid hot water and steam-explosion are examples of pretreatments that release free forms of lignin-derived phenols such as vanillin and coumaric acid from lignocellulose [11,12].

Xylan-active enzymes have the potential for application in different industrial processes such as paper bleaching, animal feed additives, and general lignocellulose deconstruction for biofuel production [12,13]. However, the residual slurry from lignocellulose pretreatments contains phenolic compounds and carbohydrates that may cause inhibition over glycoside hydrolase [14,15]. There is a growing interest in identifying organisms and enzymes that are tolerant to these compounds, allowing them to deconstruct residual slurry to its monomeric units that can be further applied as biorefinery building blocks [16].

Historically, endo-β-1,4-xylanases are prospected from fungal strains; however, there is currently a demand for identifying new enzymes that are thermostable [17]. In this search for enzymes with kinetic properties more appropriate for industrial processes (*e.g* paper and pulp bleaching), thermophilic organisms from natural environments are commonly investigated with the goal of producing industrially relevant enzymes [18,19]. In addition, there is an interest in studying thermostable enzymes for engineering new biocatalysts [20].

With respect to microbial hemicellulases, endo-β-1,4-xylanases

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ORIGINAL PAPER

Biochemical Properties of Carbohydrate-Active Enzymes Synthesized by *Penicillium chrysogenum* Using Corn Straw as Carbon Source

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Abstract
Lignocellulosic material is an alternative, renewable and cheaper source of molecules to be applied in greener industrial processes. Its utilization for this purpose requests steps of pre-treatment and hydrolysis. Filamentous fungi are receiving attention as source of plant cell wall degrading enzymes to apply in lignocellulosic biomass hydrolysis. In the present study, a strain of *Penicillium chrysogenum* CCDCA10756 isolated from Brazilian Cerrado soil (Savannah like biome) was evaluated as a producer of plant cell wall degrading enzymes aiming industrial application. The fungus cultivated in the presence of corn straw as sole carbon source secreted cellulases (endo-β-1,4-glucanases, cellobiohydrolases, β-glucosidases), endo-β-1,4-xylanases, and pectinases. Endo-β-1,4-xylanases and pectinases presented earlier production reaching maximum values after 3 days of growth in comparison to cellulolytic activities mostly produced after 5 days. Cellobiohydrolases and endo-β-1,4-glucanases present maximal activity in acid pH (3 and 4) and at 50 °C, whereas β-glucosidase presents maximal activity at pH 5.0 and 60 °C. Pectinases showed maximum activity in pH 8 at 50 °C. Furthermore, endo-β-1,4-glucanases and cellobiohydrolases displayed remarkable thermostability at 40 °C. Lignin-derived compounds, trans-ferulic acid, 4-hydroxybenzoic and syringaldehyde inhibited cellobiohydrolases. Pectinolytic activity, instead, was improved in the presence of *p*-coumaric acid, trans-ferulic acid, and syringaldehyde.

Keywords Cellulases · Biorefinery · Lignocellulosic biomass · Bioethanol

Statement of Novelty

In the present work, we showed the potential use of a strain of *Penicillium chrysogenum* from Savannah-like biome in Brazil as model fungi to produce plant cell wall degrading enzymes using corn straw as a carbon source. In addition to previous works which showed *P. chrysogenum* as a producer of hemicellulases, we described a natural blend containing all the enzymes related to the complete hydrolysis of holocellulose. We provided also a detailed enzymatic characterization of produced enzymes and novel information concerning pectinases tolerance to lignin derived phenolics and activity on alkaline pH, as well as, cellulases' thermal stability. Data presented here will support the use of *P. chrysogenum*'s hollocellulases in industrial lignocellulose deconstruction processes.

Introduction

Lignocellulose is an abundant and renewable source of molecules with biotechnological interest, daily produced and discarded as waste worldwide in result of agro-industrial activities [1]. In Brazil, agriculture is a relevant economic sector corresponding to 21.6% of Brazilian Gross Domestic Product (Base year 2017 CEPEA/Esalq http://www.cepea.esalq.usp.br). Corn is the fourth largest crop of Brazil, in 2010 its production generated about 46 million tons of agricultural residues, which would be converted to 4.4 billion liters of cellulosic ethanol [2].

Lignocellulose mainly found in plant cell wall is constituted by cellulose (45–60%), hemicellulose (20–40%), pectin

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Structural and functional characterisation of xylanase purified from *Penicillium chrysogenum* produced in response to raw agricultural waste

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ABSTRACT

Commercial interest in plant cell wall degrading enzymes (PCWDE) is motivated by their potential for energy or bioproduct generation that reduced dependency on non-renewable (fossil-derived) feedstock. Therefore, underlying work analysed the *Penicillium chrysogenum* isolate for PCWDE production by employing different biomass as a carbon source. Among the produced enzymes, three xylanase isoforms were observed in the culture filtrate containing sugarcane bagasse. Xylanase (*PcX1*) presenting 35 kDa molecular mass was purified by gel filtration and anion exchange chromatography. Unfolding was probed and analysed using fluorescence, circular dichroism and enzyme assay methods. Secondary structure contents were estimated by circular dichroism 45% α-helix and 10% β-sheet, consistent with the 3D structure predicted by homology. *PcX1* optimally active at pH 5.0 and 30 °C, presenting $t_{1/2}$ 19 h at 30 °C and 6 h at 40 °C. Thermodynamic parameters/melting temperature 51.4 °C confirmed the *PcX1* stability at pH 5.0. *PcX1* have a higher affinity for oat spelt xylan, K_M 1.2 mg·mL^{-1}, in comparison to birchwood xylan K_M 29.86 mg·mL^{-1}, activity was inhibited by Cu^{+2} and activated by Zn^{+2}. *PcX1* exhibited significant tolerance for vanillin, *trans*-ferulic acid, *ρ*-coumaric acid, syringaldehyde and 4-hydroxybenzoic acid, activity slightly inhibited (17%) by gallic and tannic acid.

1. Introduction

Plant cell wall degrading enzymes (PCWDE) can be applied to different industrial processes. Therefore, there is a crescent demand of these enzymes, especially PCWDE presenting kinetic properties compatible with industrial processes. The alternative use of lignocellulosic biomass as a source of fermentable sugars to produce second generation ethanol and chemicals derived from lignocellulose and hemicelluloses also boost the search for PCDWE to deconstruct the feedstock [1]. Lignocellulosic biomass is daily produced and accumulated as a result of agro-industrial activities around the world and might be used as raw material to obtain a set of products with commercial value. Lignocellulose is composed of cellulose (45–60%), hemicellulose (20–40%), pectin (5–10%) and lignin (10–40%) [2]. Cellulose filaments are tightly linked and microfibrils are covered by hemicelluloses, pectin and lignin. It hampers their degradation and contributes to plant cell wall recalcitrance, one of the major limitation in the conversion of lignocellulosic biomass to value-added products [3]. Microorganisms are able to deconstruct lignocellulosic biomass by employing a consortium of enzymes, acts in synergism to depolymerise this complex material. At first, considered as accessory activities, hemicelluloses degrading enzymes including xylanases are essential to complete the deconstruction of lignocellulose [4].

Hemicelluloses hydrolysing enzymes are classified into *endo*-β-1,4-xylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37), and debranching enzymes, including α-glucuronidase (EC 3.2.1.139), L-arabinase, feruloyl esterase (EC 3.1.1.73) and *ρ*-coumaric esterase (EC 3.1.1.1). Endo-β-1,4-xylanases catalyse the hydrolysis of β-1,4 linkages of xylan backbone. This cleavage is not random and depends on the nature of substrate, size ramification of the chain, as well as the degree of branching [5]. These enzymes are classified into GH 10 and GH11 glycoside hydrolases families. In general, GH10 xylanases are characterised as a higher molecular mass (Mm > 30 kDa), lower *p*I values with a three-dimensional folding in barrel (β/α), whereas GH 11 presents a lower molecular mass (Mm < 30 kDa), higher *p*I value of xylanases and three-dimensional folding in β-jelly-roll [6].

Previous literature has largely debated the *Trichoderma* and *Aspergillus* species particularly efficient in the production of cellulases and their ability to deconstruct the lignocellulose [8,9]. However, other species are also focused of the research due to their potential to produce in addition to cellulases, hemicellulases and lignin active enzymes [7–10].

Penicillium species, such as *Penicillium chrysogenum*, studied previously as a source of antimicrobial activities, nowadays are gaining

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ORIGINAL PAPER

Paenibacillus barengoltzii A1_50L2 as a Source of Plant Cell Wall Degrading Enzymes and Its Use on Lignocellulosic Biomass Hydrolysis

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Abstract
Lignocellulosic residues daily generated as a by-product of economic activities worldwide, stand out as potential feedstock to biorefineries. Industrial processes based on their use still face a challenge in the development of low-cost enzymatic mixtures resistant to inhibitors produced during pre-treatment step and efficient in the complete deconstruction of different lignocellulosic wastes. In the present work, *Paenibacillus barengoltzii* A1_50L2 isolated from bovine rumen was studied as a source of plant cell wall degrading enzymes. In addition, the natural produced enzymatic cocktail was characterized and applied to the hydrolysis of lignocellulosic substrates. *P. barengoltzii* grew in liquid media containing cellulose, sugarcane bagasse and wheat bran, as carbon source producing endo-1,4-β-xylanases, pectinases, mannanases, endo-1,4-β-glucanases, exo-1,4-β-glucanases and β-glucosidases, with highest values of endo-1,4-β-xylanase activity. Six isoforms of endo-1,4-β-xylanase (45 to 116 kDa), three of endo-1,4-β-glucanase (40 to 66 kDa) and a single band of mannanase (62 kDa) were secreted by the bacterium during growth on wheat bran, all enzymes presented maximal activity in pH ranging from 4 up to 8 and temperature from 45 up to 60 °C. Endo-1,4-β-xylanases, endo-1,4-β-glucanases, exo-1,4-β-glucanases, pectinases, mannanases and β-glucosidases produced by *P. barengoltzii* are tolerant to lignin-derived compounds showing a minimal residual activity of 61%. Ferulic, gallic, tannic and cinnamic acids enhanced activities of endo-1,4-β-glucanases/endo-1,4-β-xylanases, mannanase and pectinase, respectively. *P. barengoltzii* enzymatic cocktail (PbEC) hydrolyzes sugarcane bagasse, banana stem and corncob with hydrolysis values comparable to those obtained for the commercial blend Viscozyme®. The addition of PbEC to Viscozyme® increases the hydrolysis rate of sugarcane bagasse, banana stem and corncob in three to four times.

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Review

Deconstruction of Lignin: From Enzymes to Microorganisms

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Abstract: Lignocellulosic residues are low-cost abundant feedstocks that can be used for industrial applications. However, their recalcitrance currently makes lignocellulose use limited. In natural environments, microbial communities can completely deconstruct lignocellulose by synergistic action of a set of enzymes and proteins. Microbial degradation of lignin by fungi, important lignin degraders in nature, has been intensively studied. More recently, bacteria have also been described as able to break down lignin, and to have a central role in recycling this plant polymer. Nevertheless, bacterial deconstruction of lignin has not been fully elucidated yet. Direct analysis of environmental samples using metagenomics, metatranscriptomics, and metaproteomics approaches is a powerful strategy to describe/discover enzymes, metabolic pathways, and microorganisms involved in lignin breakdown. Indeed, the use of these complementary techniques leads to a better understanding of the composition, function, and dynamics of microbial communities involved in lignin deconstruction. We focus on omics approaches and their contribution to the discovery of new enzymes and reactions that impact the development of lignin-based bioprocesses.

Keywords: lignin; bacteria; biodegradation; auxiliary activities; metagenomics; metaproteomics; metatranscriptomics

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1. Introduction

The conversion of lignocellulosic biomass into biofuels and chemicals has gained interest because of its potential application in biorefineries as a green platform. Lignocellulosic biomass is mainly composed of lignin and polysaccharides (i.e., cellulose, hemicellulose, and pectin), arranged in plant cell walls. Cellulose is a homopolymer, composed of D-glucose monomers joined by linear β (1–4) linkages. Hemicellulose is also a sugar polymer, but it is composed of different monosaccharide molecules mainly joined by β-1,4 glycosidic linkages. Among the sugars that compose hemicellulose, D-xylose, D-mannose, and arabinose are present. Hemicellulose polymers are strongly interlinked through covalent and non-covalent bonds, and also linked to lignin, which together with cellulose will form the recalcitrant lignocellulosic matrix [1,2]. In the plant cell wall, lignin is linked to the carbohydrate moiety via the ester linkage. This association gives the plant cell wall greater strength and impermeability [3]. Lignin is formed by radical coupling reactions involving the three main phenylpropane units: p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), linked by C–C and C–O linkages [4,5].

Lignin is the main plant cell wall component responsible for recalcitrance [6]. Thus, pretreatment is an essential step for removing lignin in the process of lignocellulosic biomass conversion into biofuels [7,8]. The high carbon/oxygen ratio and the natural abundance of lignin make it a promising feedstock material for biological conversion into value-added products [9]. In addition to the biofuel industry, lignin may also be found as a by-product from wood-biomass in industrial processes for paper and pulp production.

PLOS ONE

RESEARCH ARTICLE

Functional screening of a Caatinga goat (*Capra hircus*) rumen metagenomic library reveals a novel GH3 β-xylosidase

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Abstract

Functional screening of metagenomic libraries is an effective approach for identification of novel enzymes. A Caatinga biome goat rumen metagenomic library was screened using esculin as a substrate, and a gene from an unknown bacterium encoding a novel GH3 enzyme, BGL11, was identified. None of the BGL11 closely related genes have been previously characterized. Recombinant BGL11 was obtained and kinetically characterized. Substrate specificity of the purified protein was assessed using seven synthetic aryl substrates. Activity towards nitrophenyl-β-D-glucopyranoside (pNPG), 4-nitrophenyl-β-D-xylopyranoside (pNPX) and 4-nitrophenyl-β-D-cellobioside (pNPC) suggested that BGL11 is a multifunctional enzyme with β-glucosidase, β-xylosidase, and cellobiohydrolase activities. However, further testing with five natural substrates revealed that, although BGL11 has multiple substrate specificity, it is most active towards xylobiose. Thus, in its native goat rumen environment, BGL11 most likely functions as an extracellular β-xylosidase acting on hemicellulose. Biochemical characterization of BGL11 showed an optimal pH of 5.6, and an optimal temperature of 50˚C. Enzyme stability, an important parameter for industrial application, was also investigated. At 40˚C purified BGL11 remained active for more than 15 hours without reduction in activity, and at 50˚C, after 7 hours of incubation, BGL11 remained 60% active. The enzyme kinetic parameters of K_m and V_{max} using xylobiose were determined to be 3.88 mM and 38.53 μmol.min^{-1}.mg^{-1}, respectively, and the K_{cat} was 57.79 s^{-1}. In contrast to BLG11, most β-xylosidases kinetically studied belong to the GH43 family and have been characterized only using synthetic substrates. In industry, β-xylosidases can be used for plant biomass deconstruction, and the released sugars can be fermented into valuable bioproducts, ranging from the biofuel ethanol to the sugar substitute xylitol.