GABRIELA PICCOLO MAITAN-ALFENAS

ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS FOR SECOND GENERATION ETHANOL PRODUCTION

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Agrícola, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2014

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
M232e
2014

Maitan-Alfenas, Gabriela Piccolo, 1986-
Enzymatic hydrolysis of lignocellulosic biomass for second generation ethanol production / Gabriela Piccolo Maitan-Alfenas. – Viçosa, MG, 2014.
x, 123f. : il. (algumas color.) ; 29 cm.

Orientador: Valéria Monteze Guimarães.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Etanol. 2. Enzimas. 3. Bagaço de cana. 4. Biomassa. I. Universidade Federal de Viçosa. Departamento de Bioquímica e Biologia Molecular. Programa de Pós-graduação em Bioquímica Agrícola. II. Título.

CDD 22. ed. 662.88

GABRIELA PICCOLO MAITAN-ALFENAS

ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS FOR SECOND GENERATION ETHANOL PRODUCTION

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Agrícola para obtenção do título de *Doctor Scientiae*.

APROVADA: 18 de novembro de 2014

Prof. Ronald Peter de Vries	Dr. Evan Michael Visser
Prof. Luciano Gomes Fietto	Prof. Wendel Batista da Silveira

Profa. Valéria Monteze Guimarães
(Orientadora)

Ao meu marido Rafael Ferreira Alfenas

Aos meus pais, meu irmão e minha irmã

Dedico

AGRADECIMENTOS

Primeiramene eu gostaria de agradecer a Deus pela oportunidade de chegar até aqui.

Gostaria de agradecer à minha orientadora, Profa. Valéria Guimarães, pela sua amizade, entusiasmo, paciência, e por me ajudar no desenvolvimento deste trabalho. Obrigada por estar sempre presente em inúmeras ocasiões.

Um agradecimento especial ao meu coorientador Prof. Ronald de Vries por sua amizade, otimismo, paciência e pelas incontáveis oportunidades. Obrigada por ter me aceitado em seu renomado grupo de pesquisa e por ter me ensinado a ver a ciência com uma nova perspectiva.

Gostaria de agradecer ao Dr. Evan Visser por sua amizade e apoio técnico sempre que precisei. E ao Prof. Sebastião Tavares por sua amizade e ensinamentos científicos.

Agradeço ao Prof. Luciano Fietto e ao Prof. Wendel Silveira pela enorme contribuição que deram a este trabalho.

Sou muito grata ao meu marido Rafael por seu apoio incondicional, pelo seu amor e pelo companheirismo, inclusive na ciência.

Gostaria de agradecer aos meus pais Denizart e Angelina, ao meu irmão, Júlio, e especialmente à minha irmã, Paula, pelo amor, incentivo e apoio constante. Também gostaria de agradecer aos meu sogros pelos válidos conselhos.

Sou grata ao Prof. Pedro Crous e à Karina Crous pelo apoio imensurável que nos deram no nosso ano de Holanda.

Gostaria de agradecer à Universidade Federal de Viçosa e particularmente ao Programa de Pós-Graduação em Bioquímica Agrícola pela oportunidade.

Agradeço à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) pela minha bolsa de doutorado e ao CBS Fungal Biodversity Center pelo apoio financeiro e logístico durante meu Doutorado Sanduíche.

Sou muito grata aos amigos do LABQ pelos momentos inesquecíveis. Obrigada ao Bráulio e à Mariana B. por toda ajuda e apoio técnico. Obrigada Rafaela L., Rafaela V., Lorena, Larissa, Kamila, Amanda, Tiago, Maíra, Daniel, Thiago D. e Lilian por tornarem meus dias mais divertidos.

Agradeço especialmente aos Dr. Joost van den Brink, Dr. Isabelle Benoit e Dr. Ourdia Bouzid pela amizade e por estarem sempre disponíveis para discutir ideias e resultados.

Agradeço aos amigos do CBS/Fungal Physiology Group: Ad , Eline e Henriek por serem os melhores técnicos do mundo; Alexa, Sylvia, Sebnem e Alexandra pela companhia diária e pela amizade; Robson pela importante ajuda; Miaomiao e Diogo pelos momentos inesquecíveis.

Agradeço ao Eduardo Monteiro e à Marcia Brandão pela amizade e total disponibilidade em todos os momentos que eu precisei.

Finalmente eu agradeço a todas as pessoas que passaram pela minha vida e me ajudaram em diferentes sentidos para que eu pudesse tentar me tornar uma pessoa melhor!

BIOGRAFIA

Gabriela Piccolo Maitan-Alfenas, filha de Denizart Ribeiro Maitan e Maria Angelina Piccolo Maitan, nasceu em Viçosa, Minas Gerais, em 18 de janeiro de 1986.

Em março de 2004 iniciou seus estudos em Engenharia de Alimentos, pela Universidade Federal de Viçosa (UFV), Minas Gerais.

De setembro de 2006 a agosto de 2007 participou de um programa sanduíche em que cursou um ano de sua graduação na *École Nationale Supérieure d'Ágronomie et des Industries Alimentaires*, em Nancy, na França.

Em janeiro de 2009, graduou-se em Engenharia de Alimentos, pelaUFV.

Em julho de 2009, iniciou o Curso de Mestrado em Bioquímica Agrícola, na UFV, sob orientação da Profa. Valéria Monteze Guimarães.

Em dezembro de 2010, foi aprovada a sua mudança de nível passando do mestrado para o doutorado com defesa de dissertação, no dia 22 de fevereiro de 2011, quando obteve o título de *Magister Scientiae* com a dissertação intitulada: "Produção, purificação e caracterização de β-glucosidases livre e imobilizada de *Debaryomyces hansenii* UFV-1".

Em dezembro de 2010 ingressou no Programa de Pós-Graduação, em nível de Doutorado em Bioquímica Agrícola na UFV, sob a orientação da Profa. Valéria Monteze Guimarães.

Em maio de 2012 foi realizar o doutorado sanduíde por um ano no instituto de pesquisa *CBS-KNAW Fungal Biodiversity Centre*, em Utrecht, na Holanda, sob orientação do Prof. Ronald P. de Vries.

Em novembro de 2014 concluiu os requisitos necessários para obter o título de *Doctor Scientiae*, no dia 18 de novembro, com defesa de tese intitulada: "Enzymatic hydrolysis of lignocellulosic biomass for second generation ethanol production".

SUMÁRIO

RESUMO

MAITAN-ALFENAS, Gabriela Piccolo, D. Sc., Universidade Federal de Viçosa, Novembro de 2014. **Hidrólise enzimática de biomassa lignocelulósica para a produção de etanol de segunda geração.** Orientadora: Valéria Monteze Guimarães. Coorientador: Ronald Peter de Vries.

A produção de etanol de segunda geração apresenta grande potencial para ser uma realidade sustentável, especialmente no Brasil que prossui grandes quantidades de bagaço de cana-de-açúcar. Os maiores obstáculos deste processo são os pré-tratamentos e a hidrólise da biomassa, principalmente esta última etapa visto que as enzimas ainda apresentam custos muito elevados. Assim, esforços têm se concentrado em tornar o processo mais econômico com a descoberta de enzimas mais efetivas. Novas fontes de enzimas são continuamente encontradas e várias estratégias de prospecção e produção enzimática têm sido estudadas. Uma estratégia bastante utilizada na busca por novas enzimas e/ou enzimas mais eficientes é a análise de genômica comparativa de diferentes micro-organismos que permite a seleção de vários candidatos de interesse num curto período de tempo. Além disso, as enzimas podem ser produzidas por fungos quando estes são crescidos em biomassas que apresentam baixo custo e alta disponibilidade. Este trabalho foi dividido em cinco capítulos sendo que o primeiro consiste de uma revisão atual sobre a produção de etanol de segunda geração focada na etapa de sacarificação enzimática. Várias estratégias de prospecção e produção enzimáticas foram discutidas e detalhadas. No segundo capítulo, a sacarificação de bagaço de cana-de-açúcar após pré-tratamentos ácido e alcalino foi comparada usando o extrato enzimático do fungo fitopatógeno *Chrysoporthe cubensis* e três coquetéis comerciais. Para o bagaço de cana utilizado neste estudo, o pré-tratamento alcalino promoveu os melhores rendimentos de sacarificação sendo o extrato do fungo *C. Cubensis* o responsável pela maior liberação de glicose e xilose quando comparado às misturas enzimáticas comerciais. Além disso, o extrato de *C. cubensis* produziu maiores valores de atividade específica comparados aos dos coquetéis comerciais. No terceiro capítulo, o potencial genômico de fungos candidatos foi avaliado e as enzimas mais interessantes para a hidrólise de bagaço de cana-de-açúcar foram expressas em *Aspergillus vadensis.* Nove enzimas de três fungos diferentes, *Aspergillus terreus*, *Nectria haematoccoca* e *Phaeosphaeria nodorum*, foram

clonadas e expressas por sistema heterólogo e representam uma nova possiblidade para a melhor degradação do bagaço de cana. Dentre estas enzimas, quatro β-xilosidases foram bioquimicamente caracterizadas e apresentaram atividade máxima em valores de pH 4,5-5,0 e em temperaturas 55-60°C. No quarto capítulo, duas xilanases de *Aspergillus nidulans* previamente clonadas em *Pichia pastoris*, aqui denominadas Xyn1818 e Xyn3613, foram expressas, purificadas e caracterizadas. Xyn1818 apresentou ótima atividade em pH 7.5 e à 60°C enquanto Xyn3613 alcançou máxima atividade em pH 6.0 e à 50°C. Xyn1818 apresentou-se bastante termoestável à 50°C mantendo 50% de sua atividade original após 49 horas de incubação nesta temperatura. Xyn1818 apresentou maior atividade contra arabinoxilana de trigo enquanto o melhor substrato para Xyn3613 foi xilana beechwood. Testes de sacarificação mostraram que os coquetéis comerciais liberaram mais açúcares (glicose e xilose) quando suplementados com as xilanases Xyn1818 e Xyn3613 de *A. nidulans*. Finalmente, no quinto capítulo, os fungos *Aspergillus niger* e *Trichoderma reesei* foram avaliados quanto à produção de enzimas após crescimento em dois substratos de "segunda geração": palha de trigo e bagaço de cana-de-açúcar. Os fungos produziram diferentes tipos de enzimas (hemi)celulolíticas, o que foi refletido pelo forte efeito sinergístico na liberação de açúcares durante a sacarificação dos substratos utilizando o conjunto de enzimas dos dois microorganismos. Foi constatado que a remoção de monossacarídeos do meio de produção de enzimas é muito importante quando combinações de enzimas de *T. reesei* and *A. niger* são utilizadas para aprimorar a hidrólise de biomassas.

ABSTRACT

MAITAN-ALFENAS, Gabriela Piccolo, D. Sc., Universidade Federal de Viçosa, November, 2014. **Enzymatic hydrolysis of lignocellulosic biomass for second generation ethanol production.** Advisor: Valéria Monteze Guimarães. Co-advisor: Ronald Peter de Vries.

Second generation ethanol production has great potential to be a sustainable reality, especially in Brazil due to the large amount of available sugarcane bagasse. Pretreatment methods and biomass hydrolysis continue to be the bottlenecks of the overall process, mainly this second step since the enzymes present high costs. Therefore, efforts have been taken to make the process more cost-effective with regards to the discovery of more effective enzymes. New sources of enzymes are continuously encountered and several strategies of enzyme prospection and production have been studied. One strategy used in the search for new and/or more efficient enzymes is comparative genomic analysis of different microorganisms which allows for the screening of several candidates of interest in a short period of time. Moreover, plant-degrading enzymes can be produced by fungi grown on abundantly available low-cost plant biomass. This work was divided in five chapters being the first chapter a current review about second generation ethanol production focused mainly on the saccharification step. Several strategies of enzyme prospection and production were discussed and detailed. In the second chapter, saccharification of acid- and alkali-pretreated sugarcane bagasse was compared using the enzymatic extract from the pathogen fungus *Chrysoporthe cubensis* and three commercial enzymatic mixtures. For the sugarcane bagasse studied in this work, the alkaline pretreatment promoted the best saccharification yields, where the *C. cubensis* extract was responsible for the higher release of glucose and xylose when compared to the commercial enzymatic mixtures Furthermore, the *C. cubensis* extract was able to produce high specific enzyme activities when compared to the commercial cocktails. In the third chapter, the genomic potential of the candidate fungi was evaluated and the most interesting enzymes for sugarcane bagasse hydrolysis were expressed in *Aspergillus vadensis*. Nine enzymes from three different fungi, *Aspergillus terreus*, *Nectria haematoccoca* and *Phaeosphaeria nodorum*, were successfully cloned and expressed by heterologous system and these enzymes represent a possibility for a better degradation of sugarcane bagasse. Four β-xylosidases were biochemically

characterized and showed maxima activity in the pH range 4.5-5.0 and at temperatures of 55-60°C. In the fourth chapter, two xylanases from *Aspergillus nidulans* previously cloned in *Pichia pastoris*, here nominated as Xyn1818 and Xyn3613, were expressed, purified and characterized. The optima pH and temperature for Xyn1818 were 7.5 and 60°C while Xyn3613 achieved maximal activity at pH 6.0 and 50°C. Xyn1818 showed to be very thermostable, maintaining 50% of its original activity after 49 hours when incubated at 50°C. Xyn1818 presented higher activity against wheat arabinoxylan while Xyn3613 had the best activity against xylan from beechwood. Saccharification results showed that the commercial enzymatic cocktails were able to release more sugars (glucose and xylose) after supplementation with the xylanases Xyn1818 and Xyn3613 from *A. nidulans*. Finally, in the fifth chapter, *Aspergillus niger* and *Trichoderma reesei* were evaluated for the production of enzyme sets after growth on two 'second generation' substrates: wheat straw and sugarcane bagasse. The fungi produced different sets of (hemi-)cellulolytic enzymes which was reflected in an overall strong synergistic effect in releasing sugars during saccharification using the enzyme blends from both fungi. It was observed that removing monosaccharides from the enzyme production media is very important when *T. reesei* and *A. niger* enzyme blends are combined to improve plant biomass saccharification.

CHAPTER 1

Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products

Authors: Gabriela Piccolo Maitan-Alfenas, Evan Michael Visser, Valéria Monteze Guimarães

Published in: Current Opinion in Food Science, DOI: 10.1016/j.cofs.2014.10.001

Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products

Gabriela Piccolo Maitan-Alfenas, Evan Michael Visser, Valéria Monteze Guimarães*
Department of Biochemistry and Molecular Biology, Federal University of Viçosa, Brazil

* Corresponding Author
Email: vmonteze@ufv.br
Telephone: (+55 31) 3899 2374
Fax: (+55 31) 3899 2373

Abstract

Second generation ethanol production has great potential to be a sustainable reality, especially in Brazil due to the large quantity of sugarcane bagasse encountered as industrial waste. Moreover, this process does not require that food crops are converted into biofuels. Biomass hydrolysis continues to be a bottleneck of the overall process due to the high costs of enzyme production. Therefore, efforts have been taken to make the process more cost-effective with regards to the discover of more effective enzymes. New sources of enzymes are continuously encountered and several strategies of enzyme prospection and production have been studied. The fermentation step is also an area of interest for biochemical alteration since pentoses are not effectively fermented to ethanol via traditional methods.

Highlights

- Second generation ethanol production does not compete with food production.
- The high-cost of enzymes is a challenge to be overcome for biomass hydrolysis.
- New technologies are being studied to find more effective enzymes for saccharification.
- Fermentation of pentoses in high yields has yet to become viable.

1- Introduction

Lignocellulosic biomass consists of forestry, agricultural, agro industrial and food wastes that are abundant, renewable and inexpensive energy sources. These lignocellulose wastes accumulate in large quantities and can cause environmental problems. Since the chemical composition of these materials consists mainly of polymer sugars (cellulose and hemicellulose) and lignin, these chemical components can be recycled and used for the production of a number of value added products, such as ethanol, food additives, organic acids, enzymes, and others.

The production of biofuels and alternative chemical products from agricultural residues is considered one of the most promising strategies to replace non-renewable fossil fuels. Most biofuels are produced from first generation substrates including sugarcane, corn, sugar beet, etc. that directly compete with food production. For this reason, more attention has been given to the development of biofuels from agricultural residues such as corn stover, wheat straw, rice straw and sugarcane bagasse. First generation biofuels are produced from simple vegetal components including sucrose and starch, while second generation biofuel production requires the conversion of lignocellulosic biomass into simple sugars. This sustainable method requires complexes enzyme mixtures due to the different compositions of lignocellulose from different agricultural residues.

This process of second generation ethanol production has great potential because cellulose is the most abundant renewable resource in the world. Moreover, this process contributes to improve energy security and to decrease air pollution by reducing CO_2 accumulation in the atmosphere [1].

Brazil is the largest producer of sugarcane in the world and the 2013/2014 sugarcane harvest was 653.32 million tons [2]. Sugarcane is used in the food industry for production of brown, raw and refined sugars, syrup and “cachaça”. As a general rule, in Brazil one ton of raw sugarcane generates 260 kg of bagasse [1]. About 50% of this residue is used in distilleries as a source of energy and the remainder is stockpiled [2]. Due to the large quantity of this biomass as an industrial waste, it presents potential for application of the biorefinery concept which permits the production of fuels and chemicals that offer economic, environmental, and social advantages (Figure 1).

2- Biomass Processing to Second Generation Ethanol Production

The process of ethanol production from lignocellulosic biomass includes three major steps: pretreatment, hydrolysis and fermentation. Pretreatment is required to alter the biomass structure as well as its overall chemical composition to facilitate rapid and efficient enzyme access and hydrolysis of carbohydrates to fermentable sugars [3]. Pretreatment is responsible for a substantial percentage of process cost, and as a result, a wide variety of pretreatment methods have been studied; however these methods are typically specific to the biomass and enzymes employed [4].

Hydrolysis refers to the processes that convert polysaccharides into monomeric sugars. The fermentable sugars obtained from hydrolysis can be fermented into ethanol and other products by microorganisms, which can be either naturally obtained or genetically modified [5].

2.1- Biomass Hydrolysis

Lignocellulose can be hydrolytically broken down into simple sugars either enzymatically by (hemi)cellulolytic enzymes or chemically by sulfuric or other acids [6]. However, enzymatic hydrolysis is becoming a suitable way because it requires less energy and mild environment conditions, while fewer fermentation inhibitor products are generated [7]. Enzymatic deconstruction of lignocellulose is complex because numerous structural features make it very recalcitrant. In addition to the complex network formed by cellulose, hemicellulose and lignin, some enzymes can be absorbed by condensed lignin which decrease the hydrolysis yield by non-specific linkages of these enzymes [8].

Optimal conditions for cellulases have been reported as temperature of 40–50°C and pH 4–5, while optimal assay conditions for xylanase are often similar. For complete cellulose degradation the synergistic action of four cellulase enzymes is necessary: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.176), exoglucohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21). Endoglucanases act randomly on internal glucosidic linkages, in the amorphous portion of cellulose, releasing oligosaccharides with several polymerization degrees. Cellobiohydrolases degrade cellulose by removing cellobiose molecules; they can act on the crystalline portion of cellulose and attack from the reducing and non-reducing ends of the glucose chain [9]. Exoglucohydrolases are responsible for removal of

glucose units from the non-reducing ends of cyclodextrins. Finally, β-glucosidases hydrolyze cellobiose into glucose and also remove glucose units from non-reducing ends of small cyclodextrins.

Hydrolysis of the hemicellulose fraction requires a more complex group of enzymes, referred to as hemicellulases. Complete enzymatic hydrolysis of xylan, the major polymer founded in hemicelluloses, requires endo-β-1,4-xylanase (EC 3.2.1.8), which acts randomly on the internal bond of xylan to release xylo-oligosaccharides, β-xylosidase (EC 3.2.1.37) which hydrolyzes the non-reducing ends of xylose chains to release xylose, and several accessory enzymes including α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetylxylan esterase (EC 3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) [10,11].

The concept of accessory enzymes has evolved over time since most are considered essential in enzymatic cocktails to increase sugar yields during biomass saccharification [12]. Moreover, studies have shown that supplementation of cellulase mixtures with hemicellulases can improve the rate and yield of glucan conversion since the removal of hemicellulose exposes the cellulose fibrils and increases substrate accessibility [13]. Synergism between the enzymes is a widely observed phenomena in biomass hydrolysis and it depends on several factors including the nature of the substrate and the source of enzymes [13].

Design of glycoside hydrolase mixtures with small amounts of synergistic proteins to release sugars from biomass presents to be an effective strategy. Recently, combined utilization of synergistic proteins lacking glycoside hydrolase activity (non-GH), such as carbohydrate-binding modules, plant expansins, expansin-like proteins, and Auxiliary Activity family 9 (formerly GH61) proteins, have been suggested as an effective option to facilitate the release of sugars from lignocellulosic biomass since they act by inducing structural modifications in cellulose without causing significant hydrolysis [14].

2.2- Enzyme Production

Microorganisms play an essential role on production of enzymes for biomass saccharification. Therefore, different strategies are used for the prospection of novel and/or more efficient enzymes that hydrolyze lignocellulose. One example consists of bioprospecting of microorganisms in specific environmental niches with posterior

investigation of their ability to hydrolyze crude substrates, followed by screening of the best candidates that possess interesting enzymes. Another strategy is the metagenomic tool which is extensively utilized for the genetic composition analysis of microorganism mixtures using probes or group-specific primers for seeking new (hemi)cellulases [15]. Unfortunately, this technique is unable to detect the metabolic potential of the microorganisms since the isolation and cultivation steps are not performed. The comparative genomics of different microorganisms is also commonly used for the screening of several candidates of interest in a short period of time. This strategy permits the genome analysis of a determined microorganism for evaluation of its proteome [16,17]. Recombinant technology may always be used to improve enzymatic production, in homologous and heterologous systems, and several methods have been developed to increase recombinant protein production in fungi [18] (Figure 2).

Numerous microorganisms are involved in the production of cellulases and hemicellulases, and most are filamentous fungi including *Trichoderma* spp. and *Aspergillus* spp., native or genetically modified [19,20]. However, *Trichoderma* generally lacks β-glucosidase activity and *Aspergillus* is one of the fungi genera most studied for production of this enzyme [21]. Thus, many studies have reported blending enzymes from these two microorganisms as a method to maximize conversion of lignocellulose to monosaccharide sugars.

Recently, some studies have concentrated efforts on isolation of cellulases and hemicellulases from plant pathogenic fungi. These microorganisms produce hydrolases for plant cell wall degradation and fast invasion [22]. Therefore, some works have reported the utilization of these fungi in production of enzymes for biomass saccharification. Fungi such as *Pycnoporus sanguineu*s [23], *Chrysoporthe cubensis* [24,25] and *Fusarium verticillioides* [20,26], presented great potential for plant biomass saccharification, specially alkali pretreated sugarcane bagasse.

It is already known that enzyme extracts obtained from a single microorganism are not so efficient in biomass hydrolysis, mainly because of the misbalance of enzymes. Normally cocktails have different enzymes in an adequate proportion so they are specific to individual pretreated biomass compositions. Furthermore, enzymes need to present stability for temperature and pH ranges, resistance to product inhibition, synergism in actuation and high catalytic activity. Blending of

individual enzymes and complementing crude enzyme extracts shows promise, since it can result in synergistic effects to improve biomass saccharification efficiency [25].

Co-cultivation has often been performed to obtain improved lignocellulose hydrolysis. This technique consists in the cultivation of more than one compatible fungal species that secret hydrolytic enzymes and results in better degradation of the substrate [27]. Another alternative is enzymatic production on-site [28,29]. In this case the enzymes do not need to be highly concentrated, and furthermore no accessory enzyme activity is lost in intense concentration/purification processes, which contributes to reduce the process costs.

The industrial use of (hemi)cellulases is still very limited because these enzymes are often used as purified enzyme extracts marketed by enzyme distributers, which significantly elevates costs. A more cost-effective option may therefore be the application of crude enzyme extracts obtained from fungi, in which case an array of enzyme activities is maintained and enzyme concentration costs are minimized.

Furthermore, few studies have been performed on recycling of enzymes [30]; where most focus on separating the enzyme from the solid or liquid phase or recycling of the solid and/or liquid phase directly. However, a more straightforward approach to enzyme recycling is direct recycle of the solid fraction to which most of the enzymes are likely bound. Studies have shown that enzyme productivity (product yield per quantity of enzyme applied) can be significantly increased in this type of recycle process [30].

2.3- Fermentation of monomeric sugars

Hexoses such as glucose, galactose, and mannose are readily fermented to ethanol by many naturally occurring organisms, but pentoses, including xylose and arabinose, are fermented to ethanol by few native strains, and usually at relatively low yields. Commercial utilization of xylose-fermenting microorganisms is often limited due to slow fermentation rates, carefully regulated oxygen requirements, sensitivity to inhibitors and low ethanol tolerance. However, because xylose and arabinose generally comprises a significant fraction of lignocellulosic biomass, its utilization makes the economics of biomass to ethanol conversion more feasible. The development of recombinant ethanogenic strains has resulted in bacteria and yeasts

capable of co-fermenting pentoses and hexoses into ethanol and other value-added products at high yields [31].

On the other hand, the hemicellulose hydrolysate can be used for xylitol and butanol production by xylose fermentation. Xylitol, which is a sugar substitute, has been widely used in food, medicine and other fields. Additionally, xylitol is identified as one of added-value chemicals that can be produced from biomass. Several yeasts are suitable for xylose fermentation to xylitol. Because the traditional xylitol production process involves the chemical synthesis of xylose crystal using a toxic catalyst, which imposes a high environmental burden and is unfavorable for large-scale production, bioprocessing could potentially be applied for industrial xylitol production [32]. A butanol production system from xylose fermentation was established using the high-density *Clostridium saccharoperbutylacetonicum* N1-4 generated by cell recycling [33].

The yeast *Saccharomyces cerevisiae* is that most studied and is known for its inherent resistance to low pH, high temperature, and various inhibitors [34]. Other wild-type microorganisms used in the fermentation process include *Escherichia coli*, *Zymomonas mobilis*, *Kluyveromyces marxianus*, *Pichia stipitis* and *Candida brassicae*, where some are capable of fermenting pentose sugars, but often at rates significantly lower than that of *S. cerevisiae* [35-37]. Temperature resistance of fermentation microorganisms is of great importance due to the high temperature of enzymatic hydrolysis, where for an effective application of simultaneous processes, the fermentation temperature should be as close to the optimal enzymatic hydrolysis temperature as possible.

Fermentation can be sequential to hydrolysis or it can occur simultaneously. Some methods have been developed to maximize efficiency while reducing costs and simplifying the overall process. These methods include: Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), Hybrid Hydrolysis and Fermentation (HHF), Separate or Simultaneous Co-Fermentation (SSCF) and Consolidated Bioprocessing (CPB), and these processes can also be broken down into batch, fed-batch and continuous processes. Integration of the different processes (enzyme production, saccharification and fermentation) reduces costs, but also complicates the process since optimal operating conditions are typically different [34,38]. This is further complicated in consolidated bioprocessing

where a single microorganism is utilized for enzyme synthesis as well as monosaccharide fermentation. However, cellulases are inhibited by glucose, and if saccharification is consolidated with fermentation, conversion of glucose to ethanol reduces this inhibitory effect.

3- Conclusions

The process of second generation ethanol production from different agricultural residues and food wastes is a strategy that decreases environmental impacts. However, further advances to this process must to be achieved to make it more cost-effective and a sustainable reality. Future strategies focus on advances in biotechnological tools which are necessary to discover new and/or more effective enzymes, and to improve the production of (hemi)cellulases in homologous or heterologous systems. Additional knowledge on the mode of action of enzymes is also necessary as well as utilization of recycling techniques to increase enzyme productivity. Furthermore, studies must concentrate efforts on the search for fermentative microorganisms that process pentoses in high yields, which may represent further increases in production efficiencies. Consolidated Bioprocessing (CPB) is an additional alternative to reduce costs, although much more complex. The various different types of implementation, integration and optimization of the best techniques and parameters will lead to enhanced efficiency of second generation bioethanol production.

Acknowledgements

We acknowledge the Brazilian institutions CAPES for the scholarship granted to the first author and FAPEMIG and CNPq for the resources provided.

References and Recommended Reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

** of outstanding interest

1. Lima M, Gomez L, Steele-King C, Simister R, Bernardinelli O, Carvalho M, Rezende C, Labate C, deAzevedo E, McQueen-Mason S, et al.: Evaluating the

composition and processing potential of novel sources of Brazilian biomass for sustainable biorenewables production. *Biotechnology for Biofuels* 2014, 7:10.

2. UNICA: Brazilian Sugarcane Industry Association - Sugarcane Production and Processing. 2014.

3. Njoku SI, Ahring BK, Uellendahl H: Pretreatment as the crucial step for a cellulosic ethanol biorefinery: Testing the efficiency of wet explosion on different types of biomass. *Bioresource Technology* 2012, 124:105-110.

4. Xu Z, Huang F: Pretreatment Methods for Bioethanol Production. *Applied Biochemistry and Biotechnology* 2014, 174:43-62.

5. Casey E, Mosier N, Adamec J, Stockdale Z, Ho N, Sedlak M: Effect of salts on the Co-fermentation of glucose and xylose by a genetically engineered strain of Saccharomyces cerevisiae. *Biotechnology for Biofuels* 2013, 6:1-10.

6. Zhang Z, Liu B, Zhao Z: Efficient acid-catalyzed hydrolysis of cellulose in organic electrolyte solutions. *Polymer Degradation and Stability* 2012, 97:573-577.

7. Brummer V, Skryja P, Jurena T, Hlavacek V, Stehlik P: Suitable Technological Conditions for Enzymatic Hydrolysis of Waste Paper by Novozymes(R) Enzymes NS50013 and NS50010. *Applied Biochemistry and Biotechnology* 2014, 174:1299-1308.

8. Poovaiah CR, Nageswara-Rao M, Soneji JR, Baxter HL, Stewart CN: Altered lignin biosynthesis using biotechnology to improve lignocellulosic biofuel feedstocks. *Plant Biotechnology Journal* 2014: doi: 10.1111/pbi.12225.

**This review discusses about the recalcitrance of lignin and how it has been modified and the consequences for the host plant and biomass saccharification.

9. van den Brink J, de Vries R: Fungal enzyme sets for plant polysaccharide degradation. *Applied Microbiology and Biotechnology* 2011, 91:1477-1492.

10. Van Dyk JS, Pletschke BI: A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. *Biotechnology Advances* 2012, 30:1458-1480.

11. Kumar D, Murthy GS: Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. *Biotechnol Biofuels* 2013, 6:63.

12. Robl D, Delabona Pd, Mergel C, Rojas J, Costa Pd, Pimentel I, Vicente V, da Cruz Pradella JG, Padilla G: The capability of endophytic fungi for production of hemicellulases and related enzymes. *BMC Biotechnology* 2013, 13:94.

13. Hu J, Arantes V, Pribowo A, Saddler J: The synergistic action of accessory enzymes enhances the hydrolytic potential of a "cellulase mixture" but is highly substrate specific. *Biotechnology for Biofuels* 2013, 6:112.

14. Kim IJ, Lee HJ, Choi IG, Kim KH: Synergistic proteins for the enhanced enzymatic hydrolysis of cellulose by cellulase. *Applied Microbiology and Biotechnology* 2014, 98:8469-8480.

** This review focuses on the action of non-GH proteins which enhance lignocellulosic biomass degradation.

15. Yan X, Geng A, Zhang J, Wei Y, Zhang L, Qian C, Wang Q, Wang S, Zhou Z: Discovery of (hemi-) cellulase genes in a metagenomic library from a biogas digester using 454 pyrosequencing. *Applied Microbiology and Biotechnology* 2013, 97:8173-8182.

16. Youssef NH, Couger MB, Struchtemeyer CG, Liggenstoffer AS, Prade RA, Najar FZ, Atiyeh HK, Wilkins MR, Elshahed MS: The genome of the anaerobic fungus Orpinomyces sp. strain C1A reveals the unique evolutionary history of a remarkable plant biomass degrader. *Appl Environ Microbiol* 2013, 79:4620-4634.

17. Couturier M, Navarro D, Olive C, Chevret D, Haon M, Favel A, Lesage-Meessen L, Henrissat B, Coutinho PM, Berrin JG: Post-genomic analyses of fungal lignocellulosic biomass degradation reveal the unexpected potential of the plant pathogen Ustilago maydis. *BMC Genomics* 2012, 13:57.

18. Culleton H, McKie V, de Vries RP: Physiological and molecular aspects of degradation of plant polysaccharides by fungi: What have we learned from Aspergillus? *Biotechnology Journal* 2013, 8:884-894.

19. Amore A, Giacobbe S, Faraco V: Regulation of cellulase and hemicellulase gene expression in fungi. *Curr Genomics* 2013, 14:230-249.

20. de Almeida MN, Falkoski DL, Guimaraes VM, Ramos HJD, Visser EM, Maitan-Alfenas GP, de Rezende ST: Characteristics of free endoglucanase and glycosidases multienzyme complex from Fusarium verticillioides. *Bioresource Technology* 2013, 143:413-422.

**This is the first report of endoglucanase in *Fusarium verticillioides* and the first study reporting multienzyme complex in a *Fusarium* species.

21. Sarkar N, Ghosh SK, Bannerjee S, Aikat K: Bioethanol production from agricultural wastes: An overview. *Renewable Energy* 2012, 37:19-27.

22. Tiwari R, Singh S, Nain PK, Rana S, Sharma A, Pranaw K, Nain L: Harnessing the hydrolytic potential of phytopathogenic fungus Phoma exigua ITCC 2049 for saccharification of lignocellulosic biomass. *Bioresour Technol* 2013, 150:228-234.

23. Falkoski D, Guimarães V, de Almeida M, Alfenas A, Colodette J, de Rezende S: Characterization of Cellulolytic Extract from Pycnoporus sanguineus PF-2 and Its Application in Biomass Saccharification. *Applied Biochemistry and Biotechnology* 2012, 166:1586-1603.

*The authors discovered that the phytopathogen *Pycnoporus sanguineus* has a great potential as an enzyme producer for biomass saccharification.

24. Falkoski DL, Guimaraes VM, de Almeida MN, Alfenas AC, Colodette JL, de Rezende ST: Chrysoporthe cubensis: A new source of cellulases and hemicellulases to application in biomass saccharification processes. *Bioresour Technol* 2013, 130:296-305.

*This paper shows that the phytopathogenic fungus *Chrysoporthe cubensis* secretes many accessory enzymes which release greater sugar after sugarcane bagasse saccharification compared to a commercial cocktail.

25. Visser EM, Falkoski DL, de Almeida MN, Maitan-Alfenas GP, Guimarães VM: Production and application of an enzyme blend from Chrysoporthe cubensis and Penicillium pinophilum with potential for hydrolysis of sugarcane bagasse. *Bioresource Technology* 2013, 144:587-594.

*The authors report a great enzyme blend for sugarcane bagasse hydrolysis with high solids loading.

26. de Almeida M, Guimarães V, Falkoski D, Paes GT, Ribeiro J, Jr., Visser E, Alfenas R, Pereira O, de Rezende S: Optimization of Endoglucanase and Xylanase Activities from Fusarium verticillioides for Simultaneous Saccharification and Fermentation of Sugarcane Bagasse. *Applied Biochemistry and Biotechnology* 2014, 172:1332-1346.

*This is the first report on the fermentation performance of the fungi *Fusarium verticillioides* and *Acremonium zeae*. Both fungi presented high ethanol yields for

glucose and xylose fermentation and were able to produce ethanol directly from sugarcane bagasse.

27. Kolasa M, Ahring BK, Lübeck PS, Lübeck M: Co-cultivation of Trichoderma reesei RutC30 with three black Aspergillus strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. *Bioresource Technology* 2014, 169:143-148.

28. Delabona PdS, Farinas CS, da Silva MR, Azzoni SF, Pradella JGdC: Use of a new Trichoderma harzianum strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. *Bioresource Technology* 2012, 107:517-521.

29. van den Brink J, Maitan-Alfenas GP, Zou G, Wang C, Zhou Z, Guimarães VM, de Vries RP: Aspergillus niger and Trichoderma reesei enzymes have a synergistic effect on saccharification of wheat straw and sugarcane bagasse. *Biotechnology Journal* 2014, 9:1329-1338.

*The authors show that producing *A. niger* and *T. reesei* enzymes using complex substrates is a promising strategy to obtain better enzyme mixtures for plant biomass saccharification at lower cost.

30. Weiss N, Borjesson J, Pedersen LS, Meyer A: Enzymatic lignocellulose hydrolysis: Improved cellulase productivity by insoluble solids recycling. *Biotechnology for Biofuels* 2013, 6:5.

31. Ota M, Sakuragi H, Morisaka H, Kuroda K, Miyake H, Tamaru Y, Ueda M: Display of Clostridium cellulovorans xylose isomerase on the cell surface of Saccharomyces cerevisiae and its direct application to xylose fermentation. *Biotechnology Progress* 2013, 29:346-351.

32. Li Z, Qu H, Li C, Zhou X: Direct and efficient xylitol production from xylan by Saccharomyces cerevisiae through transcriptional level and fermentation processing optimizations. *Bioresource Technology* 2013, 149:413-419.

33. Zheng J, Tashiro Y, Yoshida T, Gao M, Wang Q, Sonomoto K: Continuous butanol fermentation from xylose with high cell density by cell recycling system. *Bioresource Technology* 2013, 129:360-365.

34. Hasunuma T, Kondo A: Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. *Biotechnology Advances* 2012, 30:1207-1218.

35. Moreno AD, Ibarra D, Ballesteros I, González A, Ballesteros M: Comparing cell viability and ethanol fermentation of the thermotolerant yeast Kluyveromyces marxianus and Saccharomyces cerevisiae on steam-exploded biomass treated with laccase. *Bioresource Technology* 2013, 135:239-245.

36. Lin T-H, Huang C-F, Guo G-L, Hwang W-S, Huang S-L: Pilot-scale ethanol production from rice straw hydrolysates using xylose-fermenting Pichia stipitis. *Bioresource Technology* 2012, 116:314-319.

37. Wirawan F, Cheng C-L, Kao W-C, Lee D-J, Chang J-S: Cellulosic ethanol production performance with SSF and SHF processes using immobilized Zymomonas mobilis. *Applied Energy* 2012, 100:19-26.

38. Elliston A, Collins SRA, Wilson DR, Roberts IN, Waldron KW: High concentrations of cellulosic ethanol achieved by fed batch semi simultaneous saccharification and fermentation of waste-paper. *Bioresource Technology* 2013, 134:117-126.

Figures

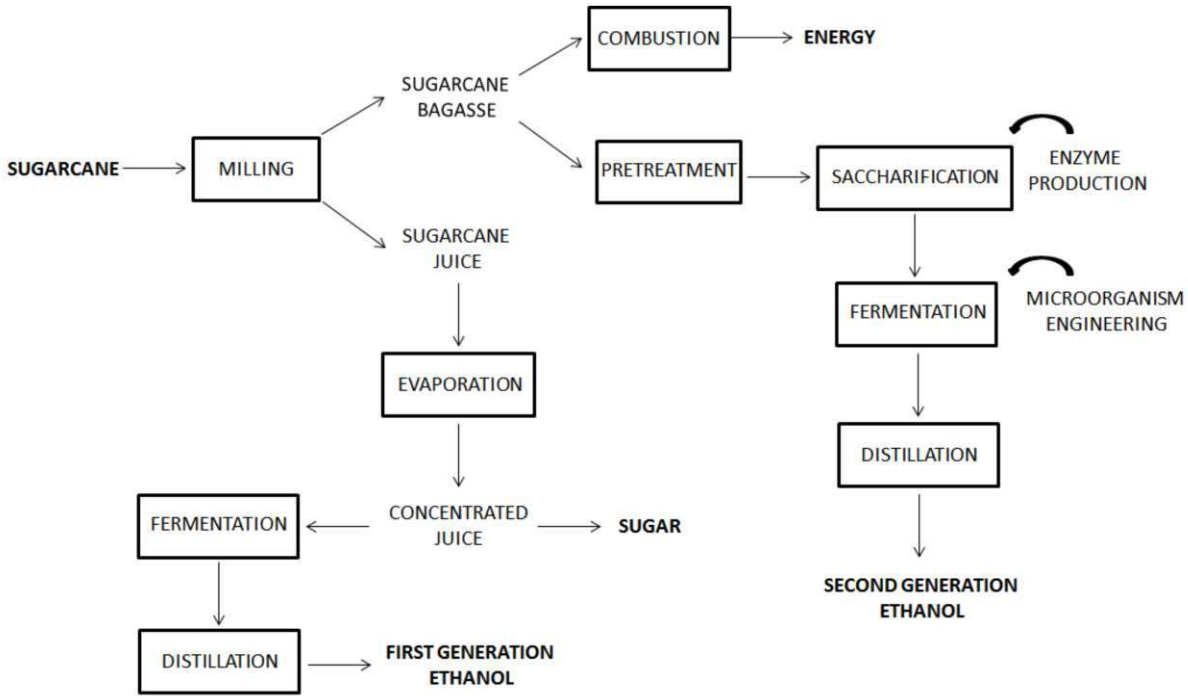


Figure 1: Utilization of sugarcane for food industry and bioethanol production.

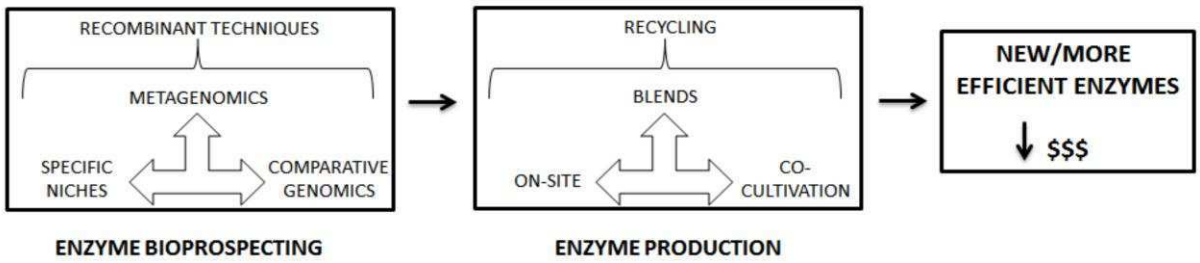


Figure 2: Strategies to reduce costs of second generation ethanol production concerning to enzyme bioprospecting and production.

CHAPTER 2

The influence of the pretreatment method on saccharification of sugarcane bagasse by various enzyme cocktails

Authors: Gabriela Piccolo Maitan-Alfenas, Evan Michael Visser, Rafael Ferreira Alfenas, Bráulio Ris G. Nogueira, Guilherme Galvão de Campos, Adriane Ferreira Milagres, Ronald P. de Vries, Valéria Monteze Guimarães

Submitted to: BioMed Research International

The influence of the pretreatment method on saccharification of sugarcane bagasse by various enzyme cocktails

Gabriela Piccolo Maitan-Alfenas[1,2], Evan Michael Visser[1], Rafael Ferreira Alfenas[3], Bráulio Ris G. Nogueira[1], Guilherme Galvão de Campos[4], Adriane Ferreira Milagres[4], Ronald P. de Vries[2,5], Valéria Monteze Guimarães[1,*]

[1]Department of Biochemistry and Molecular Biology, Universidade Federal de Viçosa, Av. PH Rolfs, s/n, 36570-900, Viçosa, MG, Brazil

[2]CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

[3]Department of Forest Engineering, Universidade Federal de Mato Grosso, Av. Alexandre Ferronato, 1200, 78557-267, Sinop, MT, Brazil

[4] Department of Biotechnology, Escola de Engenharia de Lorena, Universidade de São Paulo, 12602-810, Lorena, SP, Brazil

[5]Fungal Molecular Physiology, Utrecht University, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

* Corresponding Author

Email: vmonteze@ufv.br

Telephone: (+55 31) 3899 2374

Fax: (+55 31) 3899 2373

Abstract

An appropriate pretreatment should ideally maximize enzymatic hydrolysis to produce more available sugars for posterior fermentation into ethanol. The best pretreatment is a function of the biomass composition and the enzymatic cocktail utilized for hydrolysis. In this study, saccharification of acid- and alkali-pretreated sugarcane bagasse was compared using the enzymatic extract from the pathogen fungus *Chrysoporthe cubensis* and three commercial enzymatic mixtures. Overall, the alkaline pretreatment promoted the best saccharification yields (glucose, xylose, cellobiose and xylobiose release) when the different enzyme sources were applied for biomass hydrolysis. The *C. cubensis* extract promoted higher release of glucose

and xylose than the commercial mixtures when sugarcane bagasse was pretreated with sodium hydroxide. Moreover, the *C. cubensis* enzymatic extract was able to produce high specific enzyme activities when compared to the commercial cocktails mainly concerning to endoglucanase (331.84 U/mg of protein), β-glucosidase (29.48 U/mg of protein), β-xylosidase (2.95 U/mg of protein), pectinase (127.46 U/mg of protein) and laccase (2.49 U/mg of protein).

1. Introduction

Lignocellulosic biomass is the most abundant organic material in the world and it has the potential to be a very promising alternative source of fuels and chemicals. Enzymatic hydrolysis of biomass for its conversion into liquid fuels requires the action of cellulases and hemicellulases, since lignocellulose consists of a network of cellulose and hemicellulose bound by lignin [1]. For cellulose degradation, three enzymes typically act in synergy: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.176) and β-glucosidase (EC 3.2.1.21) [2]. For hemicellulose hydrolysis, a more complex set of enzymes is necessary. For xylan hydrolysis, the major hemicellulose polymer, the action of endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and some accessory enzymes are required to increase sugars yields in the hydrolysis step. Examples of these accessory enzymes are α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetylxylan esterase (EC3.1.1.72) and feruloyl esterase (3.1.173) [3-5].

To achieve complete conversion of lignocellulosic biomass to ethanol, there are still some hurdles to overcome. The major bottlenecks of this process remain the pretreatment methods, which have to be efficient and economical, and the high costs of enzymes, which have to be sufficiently robust to produce high yields of fermentable sugars [6, 7].

The comparison of various pretreatment methods for enzymatic saccharification allows for measuring their efficiency in relation to a particular biomass [8]. Additionally, the used pretreatment must improve the availability of sugars, prevent carbohydrate degradation, avoid inhibitor formation and be low cost [9].

Dilute acid pretreatments are normally used to degrade the hemicellulosic fraction and increase biomass porosity, improving the enzymatic hydrolysis of

cellulose. The advantages of this pretreatment are its low cost and the availability of commonly used acids. The disadvantage of acid pretreatments is the formation of furan and short chain aliphatic acid derivatives, which are considered strong inhibitors in microbial fermentation [10-13]. The most commonly used pretreatment methods for biomass hydrolysis are acid-based, mainly because most fungal enzymes, which are the main players in enzymatic saccharification, have optimal pH values in the range of 4.0-5.0 [14].

Alkaline pretreatments are well known for removing lignin. The hydrolysis of ester linkages between hemicellulose residues and lignin promotes an increase of porosity in the biomass, and as a result cellulose and hemicellulose become more accessible to enzyme action [9, 15]. The retention of cellulose and hemicellulose allows for hydrolysis of a much larger fraction of the pretreated biomass, with the release of glucose from cellulose and additional pentose sugars from hemicellulose, in an environment free of strong acids and fermentation inhibitors [16]. Under these conditions, the degradation of sugars is minimal [17]. Although hydroxides are not expensive, this process consumes a lot of water for washing the sodium (or calcium) salts that incorporate into the biomass and present difficult removal, while some enzyme inhibitors can be generated during lignin depolymerization [18].

The high costs and/or low efficiencies of the enzymes used for biomass hydrolysis are one of the main obstacles of lignocellulosic ethanol production. Enzymes must be stable, efficient, highly active and low cost. The majority of the cellulases applied in industries are produced by fungi of the genus *Trichoderma*. However, the amount of β-glucosidase secreted by *Trichoderma* species is very low, which can compromise complete cellulose hydrolysis due to the cellobiose accumulation [19]. *Penicillium* and *Aspergillus* are also good producers of cellulases, with higher levels of β-glucosidases, but they present lower FPase activity - total cellulase activity [20].

It is well known that plant pathogenic fungi produce extracellular enzymes to degrade plant cell walls, and there is a close relationship between hydrolase secretion capacity and the virulence of these microorganisms [21]. *Chrysoporthe cubensis* is able to produce high titers of cellulases and hemicellulases, mainly β-glucosidase, xylanase and some interesting accessory enzymes, and the efficiency of *C. cubensis* enzymatic extract in the saccharification of alkali-pretreated sugarcane

bagasse is around 60% for glucan and 90% for xylan degradation, respectively [16, 20].

Few studies evaluate the effect of different enzymatic cocktails in the hydrolysis efficiency of acid- or alkali-pretreated biomass. In this study, saccharification of acid- and alkali-pretreated sugarcane bagasse was compared using the enzymatic extract from the pathogen fungus *C. cubensis* and three commercial enzyme mixtures.

2. Methods

2.1. Materials

Substrates including *p*-nitrophenyl-β-D-glucopyranoside (pNPGlc), *p*-nitrophenyl-β-D-xylopyranoside (pNPXyl), ρ-nitrophenyl-β-D-mannopyranoside (pNPMan), *p*-nitrophenyl-β-D-galactopyranoside (pNPGal), *p*-nitrophenyl-α-D-arabinofuranoside (pNPAra), *p*-nitrophenyl-β-D-cellobioside (pNPCel), carboxymethylcellulose (CMC), xylan from birchwood, locust bean gum, polygalacturonic acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and also the chemical reagents monopotassium phosphate, ammonia nitrate, magnesium sulfate, calcium chloride, cuprum sulfate, sodium acetate, sodium carbonate, dinitrosalicylic acid (DNS) and potato dextrose agar (PDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Yeast extract was obtained from Himedia Laboratories Co. (Mumbai, Maharashtra, India). The chemical reagents NaOH, H_2SO_4 and potassium sodium tartrate were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). The commercial enzymatic mixtures Multifect® CL, Multifect® XL and Accellerase® 1500 were purchased from Dupont/Genencor International Inc. (Rochester, NY, USA). Sugarcane bagasse was kindly donated by Jatiboca Sugar and Ethanol Plant, Urucânia, MG, Brazil. Wheat bran was obtained on the local market. All others reagents used in this study were of analytical grade.

2.2. Microorganism

C. cubensis LPF-1 used in this study was obtained from the mycological collection of the Forest Pathology Laboratory, Federal University of Viçosa, MG, Brazil. This fungus was maintained on PDA plates at 28 °C and periodically

subcultured. The inoculum was prepared by growing the fungus under submerged fermentation in 250 mL Erlenmeyer flasks containing 100 mL of medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2.0. Each flask was inoculated with 10 agar plugs cut out of a 5 day-old colony of *C. cubensis* grown on PDA plates and incubated in a rotary shaker for 5 days, at 150 rpm and 28 °C. The culture obtained was aseptically homogenized using a Polytron® device and immediately used to inoculate the solid culture media. For enzyme production via solid state fermentation (SSF), 250 mL Erlenmeyer contained 12.5 g of wheat bran and 18.75 mL of the culture media (final moisture of 60%) consisting of, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2.0. Furthermore, $MnCl_2$ (0.1 mg/L), H_3BO_3 (0.075 mg/L), Na_2MoO_4 (0.02 mg/L), $FeCl_3$ (1.0 mg/L) and $ZnSO_4$ (3.5 mg/L) were also added to the medium as trace elements. The flasks were autoclaved at 120 °C for 20 min and then inoculated with 5 mL (containing 1.5 x 10^7 spores/mL) of inoculum obtained as aforementioned. The flasks were maintained at 28 °C in a controlled temperature chamber and enzyme extraction was performed after 7 days of fermentation. Enzymes secreted during SSF were extracted and solubilized in sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate), with agitation of 150 rpm for 60 min at room temperature. Solids were separated by filtration through nylon cloth followed by centrifugation at 15,000g for 10 min; and the clarified supernatants were frozen and stored for subsequent enzymatic analysis.

2.3. Protein Analysis

Protein concentration in the enzymatic extract from *C. cubensis* and in the commercial enzymatic mixtures was determined by the Coomassie Blue binding method using bovine serum albumin (BSA) as a standard [22].

2.4. Enzymatic Assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50 °C. They were performed in triplicate and the mean values were calculated. Relative standard deviations of the measurements were below 5%. FPase and endoglucanase activities were determined using Whatman No. 1 filter paper (1 x 6 cm, 50 mg) and 1.25% (w/v) CMC as substrates, respectively, according to

previously described standard conditions [23]. The total reducing sugars released during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method [24] using glucose as the standard. Xylanase, mannanase and pectinase activities were determined using xylan from birchwood (1% w/v), locust bean gum (0.4% w/v) and polygalacturonic acid (0.25% w/v) as substrates, respectively. The enzymatic reactions were initiated by the addition of 100 µL of the appropriately diluted enzyme solution to 400 µL of the polysaccharide substrate solution prepared in buffer. The reaction mixtures were incubated for 30 min and the total reducing sugar content released was determined via the DNS method using xylose, mannose and galacturonic acid as standards.

β-Glucosidase, β-xylosidase, β-mannosidase, α-galactosidase, α-arabinofuranosidase and cellobiohydrolase activities were measured using ρPNGlc, ρNPXyl, ρNPMan, ρNPGal, ρNPAra and ρNPCel as substrates, respectively. The reaction mixtures contained 100 µL of the appropriately diluted enzyme solution, 125 µL of the synthetic substrate solution (4 mM at final concentration) and 275 µL of buffer. The reaction mixtures were incubated for 30 min and stopped by addition of 0.5 mL of a sodium carbonate solution (0.5 M). Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released was estimated using a standard curve.

Laccase activity was determined by monitoring the oxidation of the substrate ABTS. The reaction mixtures contained 100 µL of the appropriately diluted enzyme solution, 350 µL of the buffer and 50 µL of 10 mM ABTS. This mixture was incubated for 10 min and, at the end of the incubation period, absorbance was immediately measured at 420 nm. Laccase activity was calculated by the Lambert-Beer principle, using a molar extinction coefficient of $3.6 \times 10^4\ M^{-1}cm^{-1}$.

For all activity assays, one unit of enzymatic activity (U) was defined as the amount of enzyme that released 1 µmol of the corresponding product (glucose equivalent, xylose, mannose, galacturonic acid and *p*-nitrophenol) per minute, under the assay conditions used.

2.5. Biomass pretreatments

Sugarcane bagasse was washed and dried in an oven at 70 °C until reaching a constant mass, after which it was further milled (particle size less than 1 mm) and submitted to alkaline or acid pretreatments prior to being employed in saccharification

experiments. Sodium hydroxide and sulfuric acid, both at concentrations of 1.0%, were used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v). The pretreatments were performed in an autoclave at 120 °C for 60 min. Pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at - 20 °C.

2.6. Chemical composition of the bagasse samples

Approximately 3 g of milled samples were extracted with 95% ethanol for 6 h in a Soxhlet apparatus. Extracted samples were hydrolyzed with 72% (w/w) sulfuric acid at 30 °C for 1 h (300 mg of sample and 3 mL of sulfuric acid) as described by Ferraz et al [25]. Acid was diluted by addition of 79 mL of water and the mixture was heated to 121 °C for 1 h. The resulting material was cooled and filtered through a number 3 porous glass filter. Solids were dried to a constant weight at 105 °C, from which the insoluble lignin content was determined. Soluble lignin in the filtrate was determined by UV spectroscopy at 205 nm. An absorptivity value of 105 L/g.cm was used to calculate the amount of acid-soluble lignin present in the hydrolyzate. Concentrations of monomeric sugars in the soluble fraction were determined by HPLC using a BIO-RAD HPX-87H column at 45 °C eluted at 0.6 mL/min with 5 mM sulfuric acid. Sugars were detected with a temperature-controlled RI detector.

2.7. Sugarcane bagasse saccharification

The crude enzymatic extract from *C. cubensis* and the commercial cocktails (Multifect® CL, Multifect® XL and Accellerase® 1500) were applied in a biomass saccharification experiment. The *C. cubensis* enzymatic extract was concentrated 5-fold before the experiment using an Amicon Ultrafiltration system (Millipore Co. Billerica, MA, USA) with a membrane filter (Cut-off Mr 10,000 Da). Enzymatic saccharification of alkali- and acid-pretreated sugarcane bagasse was performed in 125 mL Erlenmeyer flasks with 50 mL working volume, at an initial solid concentration of 8% dry matter (w/v) in 50 mM sodium acetate buffer at pH 5.0. Enzyme loading was specified as 10 FPase units per gram of biomass with the addition of sodium azide (10 mM) and tetracycline (40 μgmL^{-1}) to the reaction mixture to inhibit microbial contamination. The reaction was carried out in an orbital shaker at

250 rpm and 50 °C for 72 h. Samples (1.0 mL) were taken from the reaction mixture at different time intervals for process monitoring. These samples were immediately heated to 100 °C to denature the enzymes, cooled and then centrifuged for 5 min at 15,000g.

2.8. Analysis of hydrolysis products

Products of the saccharification assays were analyzed by high performance liquid chromatography (HPLC) using a Shimadzu series 10A chromatograph. The HPLC was equipped with an Aminex HPX-87P column (300 x 7.8 mm) and refractive index detector. The column was eluted with water at a flow rate of 0.6 mL/min and it operated at 80 °C.

3. Results and Discussion

3.1. Activities of the enzymatic extracts

In order to establish a comparison between the enzymatic profile of the pathogenic fungus *C. cubensis* and the commercial cocktails (Multifect® CL, Multifect® XL and Accellerase® 1500), the activities of cellulases, hemicellulases, pectinase and laccase were determined (Table 1).

The commercial mixtures consist of highly concentrated industrial enzyme preparations and contain different additives to maintain all proteins in a very stable solution. In contrast, the extract from *C. cubensis* was prepared at the laboratory level in bench top fermenters. For this reason, and also to level the amount of enzymatic volumes, the extract from *C. cubensis* was concentrated 5 times.

The measured activities were then indexed to the protein content and expressed in specific activity. The *C. cubensis* extract contains higher endoglucanase activity, 1.5, 6.0 and 2.0-fold compared to Multifect® CL, Multifect® XL and Accellerase® 1500 commercial cocktails, respectively. This is significant since endoglucanase is believed to be the first enzyme to act on the cellulose structure, releasing substrates for other cellulases [26]. The *C. cubensis* cocktail also presents high β-glucosidase activity, 3.0 and 24.0-fold compared to Multifect® CL and Multifect® XL, respectively. β-Glucosidase is necessary to release glucose and avoid the accumulation of cellobiose. The *C. cubensis* extract contains higher β-xylosidase activity, 2.5-fold compared to Multifect® XL and 15-fold compared to Multifect® CL

and Accellerase® 1500, respectively. β-Glucosidase and β-xylosidase constitute the activities that are often insufficiently present in commercial cocktails, limiting their use for cellulose and hemicellulose hydrolysis. These two enzymes from *C. cubensis* could therefore be used to supplement commercial cocktails to avoid product inhibition that commonly retards or stops the action of enzymes during biomass hydrolysis.

In addition, the *C. cubensis* extract contains higher hemicellulolytic and pectinolytic activities than the other commercial mixtures. These accessory enzymes support the complete hydrolysis of biomass and they contribute to better action of the enzymatic mixture, since these enzymes are fundamental for the synergistic effect. The hydrolysis of biomass not only depends on the presence and isolated action of cellulases, but efficient degradation is a function of a balanced proportion of different enzymes that act in synergy to breakdown the complex structure of the lignocellulose [5]. When enzymes act in synergy, the total effect is greater than the sum of the effects of the individual components [27].

Furthermore, the *C. cubensis* extract contains significant laccase activity, 2.49 U/mg protein, while in the other enzyme mixtures only traces of this activity could be measured. Laccase can assist in degradation of lignin, which is an obstacle to the biomass hydrolysis process. Extracts rich in laccase positively contribute for higher saccharification and fermentation yields since these enzymes can attack phenolic compounds derived from lignin degradation during the pretreatment which act as inhibitors [28].

3.2. Biomass pretreatment

After acid or alkaline pretreatment, the sugarcane bagasse was filtered and the solid fraction was dried for moisture content determination and compositional analysis (Table 2).

Both pretreatment methods were responsible for approximately 30% of biomass loss (data not shown). The content of biomass which is lost in this step is associated with the severity of the specific pretreatment method, but it always occurs and affects the final yield, increasing costs of the finished product [18].

The compositional analysis of the raw and the pretreated sugarcane bagasse samples shows that after the acid pretreatment the cellulose content decreased

slightly (90% recovery) unlike in the alkaline pretreatment. The acid pretreatment can degrade some portions of the cellulose structure, contributing to reduce the crystallinity and the polymerization degree of this polysaccharide. The hemicellulose content in the alkali-pretreated sugarcane bagasse decreased only slightly (81% recovery), while a significant part of this fraction was removed in the acid pretreatment (27% recovery). The lignin content was reduced after the alkaline pretreatment (44% recovery), but the acid pretreatment did not result in a significant change in the lignin amount (93% recovery). These effects of acid and alkaline pretreatments, especially on hemicellulose and lignin degradation, respectively, are in agreement with a previous study performed by Harrison et al. [8].

The material provided by alkaline pretreatment contained approximately 20% more hemicellulose than the acid pretreated sugarcane bagasse and therefore could yield more xylose after biomass saccharification. However, glucose release would be expected to be very similar for both pretreatments since the amount of cellulose was 42.88% and 47.99% for acid and alkali-pretreated sugarcane bagasse, respectively. Nevertheless, the removal of lignin by the alkaline pretreatment, which is crucial for effective enzymatic hydrolysis of the biomass, could be responsible for a larger release of glucose. Lignin acts as a shield limiting the hydrolysis rate of the digestible portions in the plant cell wall and it can also absorb proteins in solution [29]. Almost 70% of all enzymes added for hydrolysis can become unproductive due to the nonspecific adsorption by lignin [30, 31]. Therefore, the higher amount of lignin in the acid pretreated sugarcane bagasse contributes to nonspecific adsorption of cellulases and hemicellulases, leading to a reduced release of monosaccharides after biomass saccharification.

3.3. Saccharification of pretreated sugarcane bagasse by commercial enzyme mixtures

Three commercial enzymatic cocktails were applied for saccharification of pretreated sugarcane bagasse with the same enzyme loading of 10 FPase units/g of dried pretreated bagasse. Overall, the saccharification assays resulted in higher release of sugars from the alkaline pretreated sugarcane bagasse than the acid pretreated biomass (Figure 1). Multifect® CL and Accelerase® 1500, commercial enzymatic mixtures containing high cellulase activities, were able to promote the

release of 5.30 g/L and 3.19 g/L of glucose from the alkaline pretreated sugarcane bagasse, respectively, but only 1.29 g/L and 0.37 g/L of xylose, respectively (Fig 1A and 1C). In contrast, hydrolysis of the same bagasse with Multifect® XL, a commercial enzymatic mixture containing higher xylanase activity, released 8.83 g/L of xylose and 1.90 g/L of glucose (Fig. 1B).

The hydrolysis of acid pretreated sugarcane bagasse using Multifect® CL and Multifect® XL extracts promoted a lower release of glucose and xylose (Fig. 1D and 1E). However, Accelerase® 1500 achieved a higher release of xylose after saccharification of the acid pretreated sugarcane bagasse and this was the only situation that the acid pretreatment showed to be more efficient (Fig 1F).

Multifect® CL was the only cocktail resulting in significant levels of cellobiose (4.07 g/L) and xylobiose (3.68 g/L) after saccharification of alkali-pretreated sugarcane bagasse, suggesting that the amount of β-glucosidase and β-xylosidase in this cocktail is not sufficient to convert all oligosaccharides to glucose and xylose (Fig 1A).

3.4. Saccharification of pretreated sugarcane bagasse by *C. cubensis* enzymatic extract

Concerning utilization of the *C. cubensis* extract, the saccharification assays also resulted in higher release of sugars from the alkaline pretreated sugarcane bagasse than the acid pretreated biomass (Figure 2). Beyond that, the *C. cubensis* extract was more efficient to promote the release of glucose and xylose from alkaline pretreated bagasse compared to the commercial cocktails. These results suggest that, under these conditions, the *C. cubensis* extract presents an adequate balance between the different enzymatic activities involved in biomass degradation. Falkoski et al. [20] compared the action of enzymes from *C. cubensis* and the Multifect® CL on the saccharification of alkali-pretreated sugarcane bagasse with 2% solids loading, and found that the non-commercial mixture was more effective for biomass hydrolysis than the commercial cocktail. Here, this study was expanded to include two other commercial enzyme mixtures and also to use higher solids loading, since the higher solids concentration in the hydrolysis step will positively influence the fermentation and distillation yields. The *C. cubensis* extract maintained a superior hydrolysis capacity compared to the commercial cocktails in both low and high solids

loading. The *C. cubensis* extract presents numerous characteristics that could explain this better performance. The large amount of β-glucosidase prevents accumulation of cellobiose during the hydrolysis step, and therefore there is no end-product inhibition of other cellulases, which results in higher saccharification yields [19]. Furthermore, the *C. cubensis* extract also contains a high concentration of endoglucanase which may contribute to a better action of the other cellulases due to the rapid formation of their substrates. Another explanation could be related to the fact that the *C. cubensis* extract has more accessory enzymes when compared to the commercial mixtures. Although these enzymes are not able to produce glucose directly, they play an important role in stimulating cellulose hydrolysis by facilitating the access of cellulolytic enzymes to the cellulose fraction, and thus increasing the monosaccharide concentrations for posterior fermentation [32, 33]. Moreover, the action of these enzymes can release pentoses and hexoses which may also be fermented to ethanol or other higher-value products. Finally, the *C. cubensis* extract has a higher laccase activity when compared to the commercial mixtures. Laccases assist in removing the residual lignin of the lignocellulosic biomass as well as in oxidation of phenolic compounds which inhibit the cellulase enzymes, facilitating the access of cellulases to the cellulose fraction [34-37].

The results show that when the enzyme loading was adjusted based on FPase activity (10 FPase units/g of biomass) for the alkaline pretreated sugarcane bagasse hydrolysis (Fig. 1A-B and 2A), the *C. cubensis* extract was able to promote similar release of glucose (5.32 g/L) compared to Multifect® CL (5.30 g/L), which is cocktail rich in cellulases, and similar release of xylose (9.00 g/L) compared to and Multifect® XL (8.83 g/L), which is a rich xylanase mixture.

Concerning acid pretreatment (Fig. 2B), the released amount of sugars by the *C. cubensis* enzyme extract was 2.94 g/L of glucose and 1.71 g/L of xylose. The dilute acid pretreatment eliminates or reduces the need for hemicellulases [38] and *C.cubensis* extract is very rich in hemicellulases and accessory enzymes. However, *C. cubensis* enzymes presented a better performance for the saccharification of acid pretreated sugarcane bagasse than the commercial cocktails, being only behind Multifect® CL for glucose release and Multifect® XL for xylose release (Fig. 1D-E).

Small levels of cellobiose, 0.87 g/L and 0.20 g/L, remained after the action of the *C. cubensis* extract on alkali and acid-pretreated sugarcane bagasse,

respectively, while xylobiose was not detected after the hydrolysis of pretreated bagasse (Fig. 2).

Overall, under the experimental conditions of this study, the hydrolysis yields achieved by the *C. cubensis* extract and the commercial cocktails were reasonable. The *C. cubensis* extract was able to convert 12.5% of glucan and 44% of xylan after saccharification of alkali-pretreated sugarcane bagasse. For the hydrolysis of acid pretreated sugarcane bagasse, *C. cubensis* enzymes promoted 7.7% and 25% of glucan and xylan conversions, respectively. The saccharification yields were lower when compared to previous studies [16, 20]. This may be explained by three factors: 1- the sugarcane bagasse used in this study was obtained from industry and it contains more lignin than the sugarcane bagasse used in the previous studies. Lignin is one of the major obstacles for high saccharification yields because it can promote non-specific linkages of enzymes that harm their action; 2 - the saccharification experiments of this work utilized 8% biomass loading instead of 2%. The utilization of high substrate concentrations generates excessively viscous mixtures that complicate homogenization and the action of enzymes; 3 - the enzymatic cocktails were used alone, i.e., there was no testing of enzyme blends.

Therefore, for this study, the *C. cubensis* enzymatic extract was the best enzyme source for glucose and xylose release, compared to the commercial mixtures, when sugarcane bagasse was pretreated with sodium hydroxide. It is important to emphasize that the *C. cubensis* extract presents lower costs since it is obtained from a simple carbon source and no technical improvement was performed to ameliorate the protein secretions. In fact, the objective of this work was not to establish the best conditions for sugarcane bagasse saccharification, but instead to test and compare two pretreatment methods for different enzymatic cocktails. Indeed, the best enzymatic mixture is the one which presents the most appropriate set of enzymes for the pretreatment applied to the biomass to generate higher sugar yields with higher solids loading.

4. Conclusions

There are several biomass pretreatment methods being studied and each method has its advantages and disadvantages. Different pretreatments can be only compared for a specific biomass and a specific enzymatic cocktail used in the

saccharification step. At the same time, there are several available enzymatic mixtures for biomass hydrolysis and they can be compared with respect to costs, efficiency and solids loading used in the saccharification step. For the sugarcane bagasse studied in this work, the alkaline pretreatment promoted the best saccharification yields (glucose and xylose release) when *C. cubensis* was used as the enzyme source. Moreover, *C. cubensis* was able to produce high specific enzyme activities when compared to the commercial cocktails. The extract from *C. cubensis* showed great potential to be applied in biomass hydrolysis processes.

Acknowledgements

We acknowledge the Brazilian institutions CAPES for the scholarship granted to the first author and FAPEMIG and CNPq for the resources provided to complete this experiment. We also acknowledge Dupont/Genencor for the enzymatic mixtures provided.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. V. S. H. Suhardi, B. Prasai, D. Samaha and R. Boopathy, "Combined biological and chemical pretreatment method for lignocellulosic ethanol production from energy cane," *Renewable Bioresources*, vol. 1, no. 1, 2013.
2. S. Horn, G. Vaaje-Kolstad, B. Westereng and V. Eijsink, "Novel enzymes for the degradation of cellulose," *Biotechnology for Biofuels*, vol. 5, no. 1, pp. 1-13, 2012.
3. R. P. de Vries, H. C. M. Kester, C. H. Poulsen, J. A. E. Benen and J. Visser, "Synergy between enzymes from Aspergillus involved in the degradation of plant cell wall polysaccharides," *Carbohydrate Research*, vol. 327, no. 4, pp. 401-410, 2000.
4. J. van den Brink and R. de Vries, "Fungal enzyme sets for plant polysaccharide degradation," *Applied Microbiology and Biotechnology*, vol. 91, no. 6, pp. 1477-1492, 2011.

5. J. S. Van Dyk and B. I. Pletschke, "A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy," *Biotechnology Advances*, vol. 30, no. 6, pp. 1458-1480, 2012.
6. L. J. Jonsson, B. Alriksson and N. O. Nilvebrant, "Bioconversion of lignocellulose: inhibitors and detoxification," *Biotechnol Biofuels*, vol. 6, no. 1, pp. 16, 2013.
7. J. D. McMillan, E. W. Jennings, A. Mohagheghi and M. Zuccarello, "Comparative performance of precommercial cellulases hydrolyzing pretreated corn stover," *Biotechnol Biofuels*, vol. 4, pp. 29, 2011.
8. M. D. Harrison, Z. Zhang, K. Shand, I. M. O'Hara, W. O. S. Doherty and J. L. Dale, "Effect of pretreatment on saccharification of sugarcane bagasse by complex and simple enzyme mixtures," *Bioresource Technology*, vol. 148, no. 0, pp. 105-113, 2013.
9. Y. Sun and J. Cheng, "Hydrolysis of lignocellulosic materials for ethanol production: a review," *Bioresource Technology*, vol. 83, no. 1, pp. 1-11, 2002.
10. A. T. W. M. Hendriks and G. Zeeman, "Pretreatments to enhance the digestibility of lignocellulosic biomass," *Bioresource Technology*, vol. 100, no. 1, pp. 10-18, 2009.
11. K. H. Kim, M. Tucker and Q. Nguyen, "Conversion of bark-rich biomass mixture into fermentable sugar by two-stage dilute acid-catalyzed hydrolysis," *Bioresource Technology*, vol. 96, no. 11, pp. 1249-1255, 2005.
12. P. Kumar, D. M. Barrett, M. J. Delwiche and P. Stroeve, "Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production," *Industrial & Engineering Chemistry Research*, vol. 48, no. 8, pp. 3713-3729, 2009.
13. Z. L. L. Liu and M. Z. Song, "Genomic adaptation of Saccharomyces cerevisiae to inhibitors for lignocellulosic biomass conversion to ethanol," *Applied mycology*, pp. 136-155, 2009.
14. M. Dashtban, H. Schraft and W. Qin, "Fungal bioconversion of lignocellulosic residues; opportunities & perspectives," *Int J Biol Sci*, vol. 5, no. 6, pp. 578-595, 2009.

15. C. A. Cardona, J. A. Quintero and I. C. Paz, "Production of bioethanol from sugarcane bagasse: Status and perspectives," *Bioresour Technol*, vol. 101, no. 13, pp. 4754-4766, 2010.
16. E. M. Visser, D. L. Falkoski, M. N. de Almeida, G. P. Maitan-Alfenas and V. M. Guimarães, "Production and application of an enzyme blend from Chrysoporthe cubensis and Penicillium pinophilum with potential for hydrolysis of sugarcane bagasse," *Bioresource Technology*, vol. 144, no. 0, pp. 587-594, 2013.
17. R. Sharma, V. Palled, R. Sharma-Shivappa and J. Osborne, "Potential of Potassium Hydroxide Pretreatment of Switchgrass for Fermentable Sugar Production," *Applied Biochemistry and Biotechnology*, vol. 169, no. 3, pp. 761-772, 2013.
18. V. Chaturvedi and P. Verma, "An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products," *3 Biotech*, vol. 3, no. 5, pp. 415-431, 2013.
19. X. Jiang, A. Geng, N. He and Q. Li, "New isolate of Trichoderma viride strain for enhanced cellulolytic enzyme complex production," *Journal of Bioscience and Bioengineering*, vol. 111, no. 2, pp. 121-127, 2011.
20. D. L. Falkoski, V. M. Guimarães, M. N. de Almeida, A. C. Alfenas, J. L. Colodette and S. T. de Rezende, "Chrysoporthe cubensis: A new source of cellulases and hemicellulases to application in biomass saccharification processes," *Bioresource Technology*, vol. 130, no. 0, pp. 296-305, 2013.
21. G. E. Kikot, R. A. Hours and T. M. Alconada, "Contribution of cell wall degrading enzymes to pathogenesis of Fusarium graminearum: a review," *Journal of Basic Microbiology*, vol. 49, no. 3, pp. 231-241, 2009.
22. M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal Biochem*, vol. 72, pp. 248-254, 1976.
23. T. K. Ghose, "Measurement of Cellulase Activities," *Pure and Applied Chemistry*, vol. 59, no. 2, pp. 257-268, 1987.
24. G. L. Miller, "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar," *Analytical Chemistry*, vol. 31, no. 3, pp. 426-428, 1959.

25. A. Ferraz, J. Baeza, J. Rodriguez and J. Freer, "Estimating the chemical composition of biodegraded pine and eucalyptus wood by DRIFT spectroscopy and multivariate analysis," *Bioresource Technology*, vol. 74, no. 3, pp. 201-212, 2000.
26. D. H. X. Quay, F. D. A. Bakar, A. Rabu, M. Said, R. M. Illias, N. M. Mahadi, O. Hassan and A. M. A. Murad, "Overexpression, purification and characterization of the Aspergillus niger endoglucanase, EglA, in Pichia pastoris," *African Journal of Biotechnology* vol. 10, pp. 2101-2111, 2011.
27. M. Kostylev and D. Wilson, "Synergistic interactions in cellulose hydrolysis," *Biofuels*, vol. 3, no. 1, pp. 61-70, 2011.
28. T. Kudanga and M. Le Roes-Hill, "Laccase applications in biofuels production: current status and future prospects," *Appl Microbiol Biotechnol*, vol. 98, no. 15, pp. 6525-6542, 2014.
29. B. Yang and C. E. Wyman, "Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose," *Biotechnology and Bioengineering*, vol. 86, no. 1, pp. 88-98, 2004.
30. A. Berlin, N. Gilkes, A. Kurabi, R. Bura, M. Tu, D. Kilburn and J. Saddler, "Weak lignin-binding enzymes: a novel approach to improve activity of cellulases for hydrolysis of lignocellulosics," *Applied Biochemistry and Biotechnology*, vol. 121-124, pp. 163-170, 2005.
31. Y. Lu, B. Yang, D. Gregg, J. N. Saddler and S. D. Mansfield, "Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues," *Applied Biochemistry and Biotechnology*, vol. 98-100, pp. 641-654, 2002.
32. J. Hu, V. Arantes and J. Saddler, "The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect?," *Biotechnology for Biofuels*, vol. 4, no. 1, pp. 1-14, 2011.
33. A. Várnai, L. Huikko, J. Pere, M. Siika-aho and L. Viikari, "Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood," *Bioresource Technology*, vol. 102, no. 19, pp. 9096-9104, 2011.

34. Q. Chen, M. N. Marshall, S. M. Geib, M. Tien and T. L. Richard, "Effects of laccase on lignin depolymerization and enzymatic hydrolysis of ensiled corn stover," *Bioresource Technology*, vol. 117, no. 0, pp. 186-192, 2012.
35. A. Gutiérrez, J. Rencoret, E. M. Cadena, A. Rico, D. Barth, J. C. del Río and Á. T. Martínez, "Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks," *Bioresource Technology*, vol. 119, no. 0, pp. 114-122, 2012.
36. A. Rico, J. Rencoret, J. C. Del Rio, A. T. Martinez and A. Gutierrez, "Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock," *Biotechnol Biofuels*, vol. 7, no. 1, pp. 6, 2014.
37. E. Ximenes, Y. Kim, N. Mosier, B. Dien and M. Ladisch, "Inhibition of cellulases by phenols," *Enzyme and Microbial Technology*, vol. 46, no. 3–4, pp. 170-176, 2010.
38. B. C. Saha, L. B. Iten, M. A. Cotta and Y. V. Wu, "Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol," *Process Biochemistry*, vol. 40, no. 12, pp. 3693-3700, 2005.

Tables and Figures

Table 1: Comparative analysis of cellulases, hemicellulases, pectinase and laccase activities present in the crude extract from *Chrysoporthe cubensis* and the commercial cocktails Multifect® CL, Multifect® XL and Accelerase®1500.

Units of enzymatic activity/ mg of Protein				
Enzyme	***C.cubensis* extract**	**Multifect® CL**	**Multifect® XL**	**Accellerase® 1500**
Fpase	2.66±0.015	23.50±0.019	0.83±0.018	10.95±0.020
Endoglucanase	331.84±0.065	208.32±0.112	53.76±0.024	178.37±0.019
β-Glucosidase	29.48±0.031	10.02±0.020	1.21±0.018	33.42±0.086
Cellobiohydrolase	0.85±0.017	2.11±0.041	0.83±0.05	6.63±0.036
Xylanase	183.04±0.019	313.17±0.002	3,135.02±0.061	97.03±0.009
β-Xylosidase	2.95±0.034	0.20±0.04	1.13±0.001	0.20±0.041
β-Manosidase	1.69±0.057	n.d.	0.002±0	n.d.
Mannanase	14.12±0.033	2.33±0.056	3.17±0.098	3.18±0.21
Pectinase	127.46±0.011	10.40±0.012	20.54±0.036	7.76±0.004
α-Galactosidase	6.25±0.051	0.03±0.016	0.18±0.005	n.d.
α-Arabinofuranosidase	8.70±0.134	0.38±0.002	0.20±0.028	n.d.
Laccase	2.49±0.077	n.d.	n.d.	n.d.

n.d., not detected

Table 2: Compositional analysis of the raw and the pretreated sugarcane bagasse. The amounts of cellulose, hemicellulose and lignin are based on dry weight.

	Composition (g)		
Sample	**Cellulose**	**Hemicellulose**	**Lignin**
Sugarcane bagasse	34.01±0.86	22.71±0.45	30.07±1.67
Sugarcane bagasse after acid pretreatment	30.70±0.74	6.23±0.08	27.90±0.69
Sugarcane bagasse after alkaline pretreatment	34.63±1.89	18.38±1.05	13.36±0.10

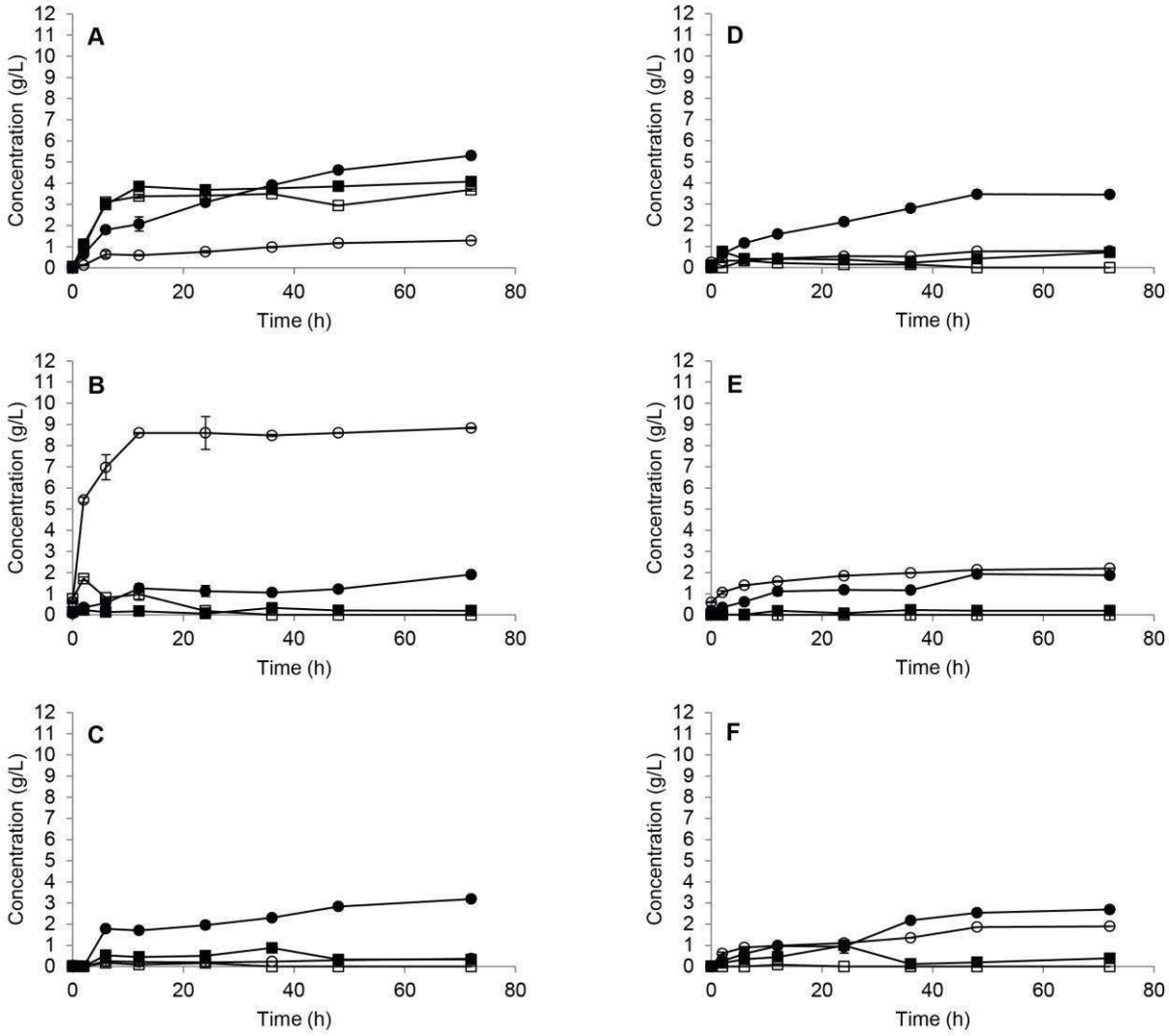


Figure 1: Saccharification of sugarcane bagasse after 72 hours at 50 °C using commercial enzyme mixtures. Alkali-pretreated sugarcane bagasse hydrolysis using: (**A**) Multifect® CL, (**B**) Multifect® XL and (**C**) Accelerase® 1500. Acid-pretreated sugarcane bagasse hydrolysis using: (**D**) Multifect® CL, (**E**) Multifect® XL and (**F**) Accelerase® 1500. Sugars released: (■) cellobiose, (□) xylobiose, (●) glucose and (○) xylose.

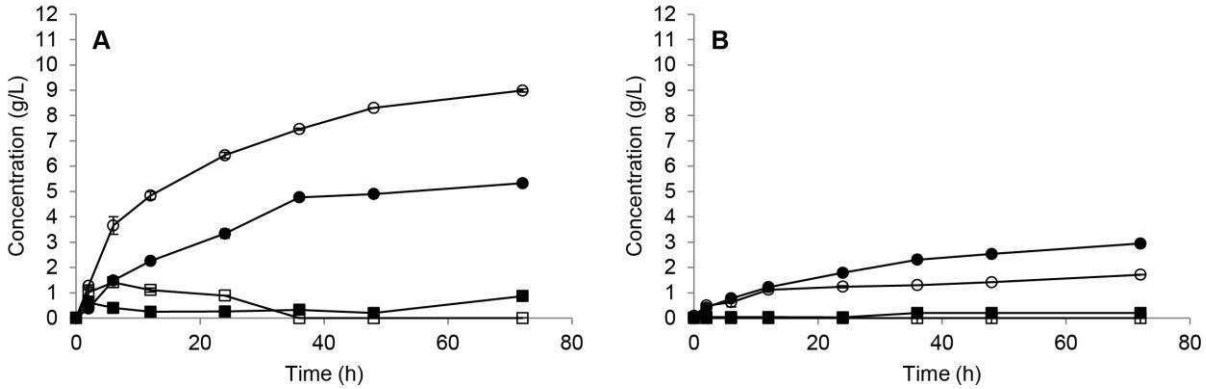


Figure 2: Saccharification of sugarcane bagasse after 72 hours at 50 °C using the *C. cubensis* extract. (A) Alkali-pretreated sugarcane bagasse hydrolysis. (B) Acid-pretreated sugarcane bagasse hydrolysis. Sugars released: (■) cellobiose, (□) xylobiose, (●) glucose and (○) xylose.

CHAPTER 3

Screening of fungal diversity for selection of enzymes that optimize sugarcane bagasse hydrolysis

Authors: Gabriela P. Maitan-Alfenas, Valéria M. Guimarães, Ronald P. de Vries

Screening of fungal diversity for selection of enzymes that optimize sugarcane bagasse hydrolysis

Gabriela P. Maitan-Alfenas[1,2], Valéria M. Guimarães[1], Ronald P. de Vries[2,3,*]

[1]Department of Biochemistry and Molecular Biology, Universidade Federal de Viçosa, MG, Brazil

[2]CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

[3]Fungal Molecular Physiology, Utrecht University, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

* Corresponding Author

Email: r.devries@cbs.knaw.nl

Telephone: +31 302122600

Fax: +31 302512097

Abstract

One strategy used for seeking new and/or more efficient enzymes is comparative genomic analysis of different microorganisms which allows for the screening of several candidates of interest in a short period of time. In this study, the genomic potential of candidate fungi for sugarcane bagasse hydrolysis was evaluated and the most interesting enzymes were expressed in *A. vadensis*. Nine enzymes from three different fungi, *Aspergillus terreus*, *Nectria haematoccoca* and *Phaeosphaeria nodorum*, were successfully cloned and expressed by the heterologous system: two cellobiohydrolases, two endoglucanases, one xyloglucan hydrolase/endoglucanase and four β-xylosidases. The β-xylosidases presented optima activities at pH 4.5-5.0 and 55-60°C. They were inhibited by xylose, SDS, $HgCl_2$ and $CuSO_4$ and activated by β-mercaptoethanol, MnO_2 and EDTA in different proportions. The nine enzymes produced in this work represent potential for a better degradation of sugarcane bagasse during the second generation ethanol production process.

1. Introduction

Sugarcane bagasse has great potential for biotechnological conversion processes, being the most available feedstock in Brazil (Moniruzzaman et al., 2013). This country is the largest producer of sugarcane in the world, where the 2013/2014 harvest was 653.319 million tons (UNICA 2014). As a general rule, in Brazil one ton of raw sugarcane generates 260 kg of bagasse (Lima et al., 2014). However, about 50% of this residue is used in distilleries as a source of energy and the remainder is stockpiled (UNICA, 2014). Therefore, sugarcane bagasse can be used for second generation ethanol (2G ethanol) production.

The process of 2G ethanol production consists basically of four steps: pretreatment, hydrolysis, fermentation and distillation. The hydrolysis or saccharification step, which is the conversion of polysaccharides into monomeric sugars, is still one of the major bottlenecks of the total process. Complete hydrolysis of biomass is very complex since it is constituted of a network of cellulose and hemicellulose bound to lignin (Suhardi et al., 2013).

For cellulose degradation, at least three enzymes acting in synergy are necessary: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.176) and β-glucosidase (EC 3.2.1.21) (Horn et al., 2012). For the hydrolysis of hemicellulose, more enzymes are necessary since its structure is more complex. For example, for xylan degradation, the action of several enzymes is required such as endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), and accessory enzymes like α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetylxylan esterase (EC3.1.1.72) and feruloyl esterase (3.1.173) (Van Dyk and Pletschke, 2012, van den Brink and de Vries, 2011, de Vries et al., 2000).

Microorganisms are very important for the production of lignocellulosic enzymes. Enzymes present in the available enzymatic cocktails for biomass saccharification are produced mostly by filamentous fungi such as *Trichoderma spp.* and *Aspergillus spp*., native or genetically modified (de Almeida et al., 2011; Singhvi et al., 2011). However, the amount of β-glucosidase secreted by *Trichoderma* is very low and *Aspergillus* normally present low FPase activity – total cellulase activity (Jiang et al., 2010, Falkoski et al., 2013). Beyond that, several studies have reported the use of enzymes from plant pathogenic fungi since they produce extracellular

enzymes for plant cell wall degradation to obtain important nutrients and to enable their penetration into the plant tissue (Kikot et al., 2009, Falkoski et al., 2013).

The available commercial enzyme mixtures are not able to totally degrade the different types of biomass. Therefore, studies have concentrated on efforts to identify novel and/or more efficient enzymes. One of the strategies used for this search is comparative genomic analysis of different microorganisms. With this approach, the analysis of the genome of a microorganism predicts its proteome which allows for the screening of several candidates of interest in a short period of time (Battaglia et al., 2011, Coutinho et al., 2009). In addition, recombinant technology in homologous and heterologous systems can be used to improve the enzymatic production.

The main goal of this work was to identify new enzymes that aid in efficient sugarcane bagasse degradation using comparative genomic and phylogeny methods. These enzymes were investigated in candidate filamentous fungi from the fungal collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands. The most interesting enzymes were expressed in *Aspergillus vadensis*. This species has several advantages for recombinant protein production, such as low levels of extracellular proteases and no acidification of the culture medium (de Vries et al., 2004). The potential of *A. vadensis* as a host for recombinant protein production was examined by Culleton et al. (2014a) with the cloning and expression of two homologous genes encoding *α*-L-arabinofuranosidase (GH54) and endo-1,4-*β*-D-glucanase (GH12).

2. Materials and Methods

2.1. Materials

Substrates including *p*-nitrophenyl-β-D-cellobioside (pNPβCel), *p*-nitrophenyl-β-D-xylopyranoside (pNPβXyl), carboxymethylcellulose (CMC), sucrose and dinitrosalicylic acid (DNS) and the enzyme Accu Taq^{TM} LA DNA Polymerase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Xyloglucan was obtained from Megazyme (Wicklow, Ireland). Two samples of sugarcane bagasse (SCB) were studied: one was kindly donated by Jatiboca Sugar and Ethanol Plant, Urucânia, MG, Brazil (SCBI) and the other was obtained from the Center for Research and Breeding of Sugarcane (SCBC) of the Federal University of Viçosa, Viçosa, MG, Brazil.

2.2. Strains, Media and Culture Conditions

The strains used for this study are listed in Table 1. MM (*Aspergillus* Minimal Medium) contained the following (per liter): 6.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, 200 µl of trace elements (10 g of EDTA/liter, 4.4 g of $ZnSO_4*7H_2O$/liter, 1.01 g of $MnCl_2*4H_2O$/liter, 0.32 g of $CoCl_2*6H_2O$/liter, 0.315 g of $CuSO_4*5H_2O$/liter, 0.22 g of $(NH_4)_6Mo_7O_{24}*4H_2O$/liter, 1.47 g of $CaCl_2*2H_2O$/liter, and 1.0 g of $FeSO_4*7H_2O$/liter). Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C in an orbital shaker at 250 rpm. Agar was added at 1.5% (w/v) for solid medium. Precultures for *A. vadensis* protoplast formation were grown overnight at 30°C in MM with 0.5% (w/v) yeast extract, 0.2% (w/v) Casamino Acids and 2% (w/v) D-glucose, after the inoculation of $5*10^6$ spores/ml.

2.3. Molecular Biology Methods

Standard methods were used for DNA manipulations, cloning, DNA digestion reactions and DNA isolations (Sambrook et al., 1989).

2.4. Comparative Genomic Analysis

Comparative genomic analysis was performed to identify candidate fungi that possess an enriched enzymatic potential for sugarcane bagasse hydrolysis. For this purpose, the CAZy - Carbohydrate Active Enzymes (www.cazy.org, Lombard et al., 2013) content of fungal genomes was analyzed for enzymes involved in the degradation of polysaccharides present in bagasse. Therefore, some GH (Glucoside Hydrolases) families were indicated to be more interesting for study: GH6, GH7, GH10, GH11, GH12, GH39, GH54, AA9 and GH62.

2.5. Phylogeny of Enzymes

Phylogeny was used to identify enzymes that are significantly different from those present in currently available commercial enzyme mixtures. For this, the amino acid sequences corresponding to the genes of interest were obtained from different databases: JGI (genome.jgi.doe.gov), ASPDG (www.aspgd.org), PFAM (pfam.xfam.org) and CAZY (www.cazy.org). The sequences were aligned with MAFFT (Katoh et al., 2009) or CLUSTAL X2 (Larkin et al., 2007). The resulting alignment was loaded into MEGA 5.0 (Tamura et al., 2011) to enable bootstrap

analysis using two methods - Maxmimum Likelihood and Minimal Evolution - in parallel to obtain a high confidence phylogenetic tree. Sequences from *Aspergillus spp.* and *Trichoderma reesei* (*Hypocrea jecorina*) were used as reference sequences to constitute clade(s) formed by the well-characterized enzymes.

2.6. Compositional Analysis of Sugarcane Bagasse

The chemical composition of the SCBI and SCBC was determined using a modified Klason lignin method derived from the TAPPI Standard Method T222 om-98 (TAPPI, 1999). Extractive-free biomass (0.3 g) was incubated at 30°C with 3 ml of 72% H_2SO_4 for 1 h with occasional mixing. The slurry was then transferred into a penicillin bottle containing 84 ml of deionized water and the flask was sealed with a rubber stopper and aluminum seal. The bottle was placed in an autoclave calibrated at 118°C for 1 h, then the slurry was filtered through a medium coarseness sintered glass filter for gravimetric determination of acid-insoluble lignin. Concentrations of biomass sugars (arabinose, galactose, glucose, xylose, and mannose) in the filtrate were quantified using high-performance liquid chromatography (HPLC), while acid-soluble lignin was determined by absorption measurements at 205 nm (TAPPI, 1991). The HPLC system Dionex DX-300 (Dionex Co. – Sunnyvale, CA, USA) was equipped with a Carbopac PA1 column and a pulsed amperometric detector with a gold electrode. Prior to injection, samples were filtered through 0.45 µm HV filters and a volume of 20 µl was loaded into the chromatograph system. The column was pre-equilibrated with a 300 mM NaOH solution and elution was carried out at a flow rate of 1.0 $ml.min^{-1}$ at room temperature.

2.7. Growth Profiles

Filamentous fungi were obtained from the stock at - 45°C and inoculated in Malt Extract Agar (MEA) plates at 37°C until a desirable growth. The freshly growing strains were then grown on solid MM with four different carbon sources at 3% (w/v) concentration on small petri-dishes in duplicate. The tested carbon sources were: washed and non-washed SCBI and washed and non-washed SCBC. The inoculation of growth profile plates was done with 10 µl of the spore solution (spores of a full plate harvested in 10 ml of ACES buffer: 10 mM N-(2-acetamido)-2-aminoethanesulfonic acid, 0.02% Tween 80, pH 6.8.) or plugs of mycelium. The

plates were incubated at 25°C and analyzed every day for score (measurement of diameter, note of sporulation and note of colony appearance). The growth profile was stopped as soon as the largest colony reached the edge of the plate and pictures were taken for comparisons.

2.8. Cloning in *Aspergillus vadensis*

DNA isolation was performed using the MoBio UltraClean Microbial DNA isolation Kit (Cat No. 12224-50). DNA was measured in NanoDrop (NanoDrop 1000 Spectrophotometer, Thermo Scientific) for correct dilution. The genes corresponding to the enzymes of interest were amplified by PCR using specific primers (Table 2). PCR reactions were carried out using Accu Taq^{TM} and conditions supplied. The PCR products were inserted into pGEM®-T Easy vector (Promega) and plasmids were verified by sequencing. The confirmed genes were cloned into the vector carrying the promoter p*pg*kA of *A. niger* CBS 513.88 (Culleton et al., 2014b) and the heterologous production of these proteins was performed in the host *A. vadensis*. The transformation was performed as described by de Vries et al. (2004), with $2*10^6$ protoplasts, 0.5 µg of the selection gene (pGW635, carrying the *A. niger* pyrA gene for selection) and 20 µg of the cotransforming DNA (carrying each gene of interest under the control of p*pg*kA).

2.9. Enzymatic Profile

The enzyme secretion profile was performed after cultivation of *A. vadensis* transformants in MM with 2% (w/v) sucrose and 0.1% (w/v) TWEEN® 80 in a shaker at 30°C and 250 rpm. The enzymatic activities were evaluated at days 3 and 4 after cultivation of the transformants.

Enzymatic assays of recombinant enzymes were performed in triplicate using different substrates in 96 well plates on a BMG labtech plate reader. The activities of cellobiohydrolase and β-xylosidase were evaluated with the synthetic substrates pNPβCel and pNPβXyl, respectively, using *p*-nitrophenol for a standard curve. For the enzymatic reaction, 40 µl of the supernatant, 10 µl of the respective pNP substrate (0.1% w/v) and 50 µl of sodium acetate buffer (100 mM, pH 5.0) were combined and incubated at 30°C for 60 min. Reactions were stopped by adding 100 µl of 0.25 M Na_2CO_3 and the absorbance was measured at 410 nm. The

endoglucanase and xyloglucan hydrolase activities were evaluated using the substrates CMC and xyloglucan, respectively. The amount of reducing sugars released using the DNS reagent was determined by the method of Miller (1959) and the standard curve was built with the respective monosaccharide. The polysaccharides were used at 1% (w/v) concentration in 100 mM sodium acetate buffer (pH 5.0) and 10 µl of the supernatant was added to 90 µl of the substrate. The mixtures were incubated at 37°C for 60 min. Absorbance was measured at 540 nm. One unit of enzyme activity is defined as the amount of protein required to release one µmol of product per minute under the assay conditions.

2.10. Enzymatic Characterization

2.10.1. Effects of pH and temperature

The influences of pH and temperature on β-xylosidase activities were determined using the standard enzymatic assay, except that the pH values were modified for a range of 2.0 to 8.0 (McIlvaine, 1921) and the temperature values ranged from 20 to 70°C. Thermal stability was investigated by incubating the enzymatic solutions in 100 mM sodium acetate buffer, pH 5.0, at temperatures of 50 and 60°C, for 48 hours. Aliquots of the enzymes were collected at specific times and submitted to the standard assay, measuring the remaining activity. The relative activities were calculated in relation to β-xylosidase activity without pre-incubation, which was considered to be 100%. Results of the analysis are presented as mean ± SD for three measurements.

2.10.2. Effect of Ions, Sugars and Reducing Agents

The effects of ions, sugars and reducing agents on β-xylosidase activities were assayed by the standard method with the addition of the ion or sugar or reducing agent at a final concentration of 2 and 10 mM. The data presented for all enzyme activity assays are mean values ± SD of measurements performed in triplicate.

3. Results

3.1. Phylogenomic Analysis

Sixty seven fungi with a sequenced genome were analyzed for their CAZy content (data not shown). As the aim of this study was to find novel enzymes to aid in sugarcane bagasse hydrolysis, seven fungi were selected since they contained a high number of genes encoding cell wall polysaccharide degrading enzymes in their genome (Table 3). It is important to emphasize that the selection was based on the total number of genes for all the defined GH families and not based on individual analysis for each GH family. Nine GH families were selected to be studied: families GH6 and GH7 contain cellulases such as endoglucanases and cellobiohydrolases. Families GH10 and GH11 contain endoxylanases and family GH12 contains endoglucanases and xyloglucan hydrolases. Family GH39 contains β-xylosidases and was chosen since it is not yet well characterized. Family GH54 comprises α-L-arabinofuranosidases, while family GH62 contains arabinoxylan arabinofuranohydrolases. Finally, family AA9 (formerly GH61) is composed by copper-dependent lytic polysaccharide monooxygenases.

A bioinformatics analysis was applied to determine if the enzymes encoded by the selected genes were likely to have different properties from already characterized enzymes. Therefore, phylogenetic trees were constructed for each GH family of interest using reference sequences encoding characterized enzymes from *Aspergillus spp.* and *Trichoderma reesei* (*Hypocrea jecorina*) and amino acid sequences from the seven fungi previously selected (Figures 1-9, Supplemental Data). Sequences that are distant from the reference sequences are probably related to new enzymes since they do not cluster with the well characterized proteins.

3.2. Growth profiling of selected fungi for plant biomass degrading potential

To better decide which fungi should be the best candidates for novel enzymes, the phylogenetic data were associated with growth profile analysis. The seven fungi were grown on four carbon sources (Figure 1). The results show that *N. haematococca*, *A. terreus* and *P. nodorum* grew better than the other fungi on both samples of sugarcane bagasse, presenting faster growth than the other candidates. In terms of colony density, the growth was better on SCBC. However, there was no difference in the diameter of colonies on washed and non-washed sugarcane

bagasse samples (Table 1, Supplemental Data). The compositional analysis of the two samples of sugarcane bagasse revealed that the SCBI presents around 10% less cellulose and 5% more hemicellulose and lignin than the SCBC (Table 2, Supplemental Data). These results suggest that the degradation of the SCBI could be more difficult, but this was not observed in our study. Normally, growth on plates using biomass as carbon source is associated with the capacity of the microorganism to degrade this feedstock. Therefore, *N. haematococca*, *A. terreus* and *P. nodorum* were best able to degrade sugarcane bagasse and were selected as sources for novel enzymes.

3.3. Cloning and expression of the selected genes in *Aspergillus vadensis*

Amino acid sequences from the selected CAZy families for the three fungi were analyzed for the presence of signal peptides in the protein, while the DNA sequences were analyzed for suitable restriction sites for cloning of the genes. This resulted in the cloning of five, six and seven genes from *N. haematococca*, *A. terreus* and *P. nodorum*, respectively.

The selection of these genes also included the distance of the protein sequence from the reference sequences in the phylogenetic trees. Based on these criteria, four genes were selected for GH6, one gene for GH7, five genes for GH12, two genes for GH39 and two genes for GH62 (Figures 1-9, Supplemental Data). Besides, based on previous proteomics studies (data not shown), four additional genes from *A. terreus* were selected: one from GH3 (β-xylosidase), one from GH5 (endoglucanase) and two from GH43 (both β-xylosidases). Details on the eighteen cloned sequences are listed in Table 2.

The selected genes were amplified by PCR from the gDNA of the three different fungi. After sequencing, the identification and confirmation of the genes were performed using the BLAST program at NCBI. For the expression of the selected genes, the corresponding fragments were cloned behind the p*pg*kA promoter of *A. niger* and the expression vectors were transformed in *A. vadensis*.

3.4. Enzymatic Profile of *A. vadensis* transformants

A. vadensis transformants were grown and had their enzyme activity compared to the reference strain, *A. vadensis* CBS 137441. The reference strain has

the plasmid pGW635 for selection and no cotransforming DNA, grown as a negative control. Therefore, if the introduced enzyme activity is present in the target transformant and no activity is found for CBS 137441, the transformation worked and the gene of interest is present in the genomic DNA of the *A. vadensis* transformant. For homologous or heterologous recombination, expression vectors are normally integrated into chromosomal DNA of *Aspergillus* for protein expression (Fleissner and Dersch, 2010).

The enzyme activities after 3 and 4 days of growth are presented in Table 4. Measurements of expression were performed in triplicate and the average was calculated to give the standard deviation for each transformant. During growth on MM with sucrose, very few proteins are produced by *A. vadensis* and this condition was selected for heterologous protein production (Culleton et al., 2014b). It can be observed that the enzymatic activities were detected for nine transformants among the eighteen possibilities. All six genes from *A. terreus* were successfully cloned in *A. vadensis*. For *N. haematococca* and for *P. nodorum*, only one and two genes were cloned, respectively. In total, nine enzymes that possibly represent new possibilities for efficient sugarcane bagasse saccharification were obtained.

3.5. Characterization of β-xylosidases

The four β-xylosidases from *A. terreus*, here nominated as Xyl09052, Xyl10193, Xyl01089 and Xyl00093, were biochemically characterized as shown in Figure 2 and Table 5. β-xylosidase is one of the enzymes that is normally lacking in the available enzymatic cocktails, limiting their use for complete hydrolysis of biomass. Therefore these enzymes were chosen for further studies.

Overall, the maximal activity performed by the four enzymes occurred in the pH range of 4.5-5.0 and temperature range of 55-60°C (Fig. 2A and 2B). These data are correlated with previous studies which showed that the β-xylosidase from *Aspergillus sp.* BCC125 expressed in *P. pastoris* exhibited maximum activity at pH 4.0-5.0 and 60°C (Wongwisansri et al., 2013), while the β-xylosidase from *A. niger* GS1 expressed in *A. niger* AB4.1 achieved best activity at pH 3.6 and 70°C (Amaro-Reyes et al., 2011).

The β-xylosidases showed no thermal stability at 60°C (data not shown), but at 50°C the half-life values were 8 h 30 min, 5 h 10 min, 1 h 40 min and 1 h 24 min for Xyl09052, Xyl10193, Xyl01089 and Xyl00093, respectively (Fig. 2C).

All four β-xylosidases were inhibited by xylose, SDS, $HgCl_2$ and $CuSO_4$ and the enzymes were activated by β-mercaptoethanol, MnO_2 and EDTA in different proportions (Table 5). The denaturing action of SDS probably affected the integrity of the enzyme tridimensional structures which are fundamental for their catalytic activity and activation by β-mercaptoethanol can be explained by the fact that some reduced chemical ligations in the enzyme structures are favorable for the catalytic activities (Maitan-Alfenas et al., 2014).

4. Conclusion

The capacity of *A. vadensis* for recombinant production of enzymes for biomass degradation has remained largely unexplored. In this study, the potential of this fungus as a host for recombinant protein production was examined by cloning and expressing nine heterologous genes from three different fungi encoding plant biomass degrading enzymes. These enzymes represent a possibility for a better degradation of sugarcane bagasse during the 2G ethanol production process. Further studies need to be developed to characterize these new enzymes and their mode of action, but it can be suggested that *A. vadensis* is a potential host for the production of recombinant proteins for industrial applications.

Acknowledgements

The first author was supported by a grant of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (1527/12-6), Brazil.

References

Amaro-Reyes, A., García-Almendárez, B.E., Vázques-Mandujano, D.G., Amaya-Llano, S., Castaño-Tostado, E., Guevara-González, R.G., Loera, O., Regalado, C., 2011. Homologue expression of a β-xylosidase from native *Aspergillus niger*. Journal of Industrial Microbiology and Biotechnology 38, 1311-1319.

Battaglia, E., Benoit, I., van den Brink, J., Wiebenga, A., Coutinho, P.M., Henrissat, B., de Vries, R.P., 2011. Carbohydrate-active enzymes from the zygomycete fungus *Rhizopus oryzae*: a highly specialized approach to carbohydrate degradation depicted at genome level. BioMed Central Genomics, 12:38.

Coleman, J.J., Rounsley, S.D., Rodriguez-, M., Kuo, A., Wasmann, C.C., Grimwood, J., , J., Taga M., White, G.J., Zhou, S., Schwartz, D.C., Freitag, M., Ma, L.J., Danchin, E.G.J., Henrissat, B., coutinho, P.M., Nelson, D.R., Straney, D., Napoli, C.A., Barker, B.M, Gribskov, M., Rep, M., Kroken, S., Molnár, I., Rensing, C., Kennell, J.C., Zamora, J., Farman, M.L., Selker, E.U., Salamov, A., Shapiro, H., Pangilinan, J., Lindquist, E., Lamers, C., Grigoriev, I.V., Geiser, D.M., Covert, S.F., Temporini, E., vanEtten, H.D., 2009. The genome of *Nectria haematococca*: contribution of supernumerary chromosomes to gene expansion. PLoS Genet 5:e1000618.

Coutinho, P.M., Andersen, M.R., Kalenova, K., vanKuyk, P.A., Benoit, I., Gruben, B.S., Trejo-Aguilar, B., Visser, H., van Solingen, P., Pakula, T., Seiboth, B., Battaglia, E., Aguilar-Osorio, G., de Jong, J.F., Ohm, R.A., Aguilar, M., Henrissat, B., Nielsen, J., Stalbrand, H., de vries, R.P., 2009. Post-genomic insights into the plant polysaccharide degradation potential of *Aspergillus nidulans* and comparison to *Aspergillus niger* and *Aspergillus oryzae*. Fungal Genetics and Biology, 46, 61-69.

Culleton, H., McKie, V.A., de Vries, R.P., 2014a. Overexpression, purification and characterisation of homologous α-L-arabinofuranosidase and endo-1,4-β-D-glucanase in *Aspergillus vadensis*. Journal of Industrial Microbiology and Biotechnology 41, 1697-1708.

Culleton, H., Bouzid, O., McKie, V.A., de Vries, R.P., 2014b. New promoters to improve heterologous protein production in *Aspergillus vadensis*. Current Biotechnology 3, 244-251.

de Almeida, M.N., Guimarães, V.M., Bischoff, K.M., Falkoski, D.L., Pereira, O.L, Gonçalves, D.S.P., Rezende, S.T., 2011. Cellulases and Hemicellulases from endophytic *Acremonium* species and its application on sugarcane bagasse hydrolysis. Applied Biochemistry and Biotechnology, 165, 594-610.

de Vries R.P., Kester H.C.M., Poulsen C.H., Benen J.A.E., Visser J., 2000. Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. Carbohydrate Research 327, 401-410.

de Vries, R.P., Burgers, K., van de Vondervoort, P.J.I., Frisvad, J.C., Samson, R.A., Visser, J., 2004. A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. Journal of Applied and Environmental Microbiology, 70, 3954-3959.

Espagne E., Lespinet O., Malagnac F., Da Silva C., Jaillon O., Porcel B.M., Couloux A., Ségurens B., Poulain J., Anthouard V., Grossetete S., Khalili H., Coppin E., Déquard-Chablat M., Picard M., Contamine V., Arnaise S., Bourdais A., Berteaux-Lecellier V., Gautheret D., de Vries R.P., Battaglia E., Coutinho P.M., Danchin E.G.J., Henrissat B., El Khoury R., Sainsard-Chanet A., Boivin A., Pinan-Lucarré B., Sellem C.H., Debuchy R., Wincker P., Weissenbach J., Silar P., 2008. The genome sequence of the model ascomycete fungus *Podospora anserina*. Genome Biol 9: R77.

Falkoski, D.L., Guimarães, V.M., de Almeida, M.N., Alfenas, A.C., Colodette, J.L., de Rezende, S.T, 2013. *Chrysoporthe cubensis*: A new source of cellulases and hemicellulases to application in biomass saccharification processes. Bioresource Technology 130, 296-305.

Fedorova N.D., Khaldi N., Joardar V.S., Maiti R., Amedeo P., Anderson M.J., Crabtree J., Silva J.C., Badger J.H., Albarraq A., Angiuoli S., Bussey H., Bowyer P., Cotty P.J., Dyer P.S., Egan A., Galens K., Fraser-Liggett C.M., Haas B.J., Inman J.M., Kent R., Lemieux S., Malavazi I., Orvis J., Roemer T., Ronning C.M., Sundaram J.P., Sutton G., Turner G., Venter J.C., White O.R., Whitty B.R., Youngman P., Wolfe K.H., Goldman G.H., Wortman J.R., Jiang B., Denning D.W., Nierman W.C., 2008. Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PLoS Genet 4:e1000046.

Fleissner, A., Dersch, P., 2010. Expression and export: recombinant protein production systems for *Aspergillus*. Applied Microbiology and Biotechnology 87, 1255-1270.

Hane J.K., Lowe, R.G.T., Solomon, P.S., Tan K.C., Schoch, C.L., Spatafora, J.W.B., Crous, P.C., Kodira, C., Birren, B.W., Galagan, J.E., Torriani, S.F.F., McDonald, B.A., OLIVER, R.P., 2007. Dothideomycete-plant interactions illuminated

by genome sequencing and EST analysis of the wheat pathogen *Stagonospora nodorum*. Plant Cell 19: 3347-3368.

Horn, S.J., Vaaje-Kolstad, G., Westereng, B., Eijsink, V.G.H., 2012. Novel enzymes for the degradation of cellulose. Biotechnology for Biofuels 5, 45.

Jiang, X., Geng, A., He, N., Li, Q., 2010. New isolate of *Trichoderma viride* strain for enhanced cellulolytic enzyme complex production. Journal of Bioscience and Bioengineering 111, 121-127.

Katoh, K., Asimenos, G., Toh, H., 2009. Multiple alignment of DNA sequences with MAFFT. Methods in Molecular Biology, *537*, 39-64.

Kikot, G.E., Hours, R.A., Alconada, T.M., 2009. Contribution of cell wall degrading enzymes to pathogenesis of *Fusarium graminearum*: a review. Journal of Basic Microbiology 49, 231–241.

Kubicek C.P., Herrera-Estrella A., Seidl-Seiboth V., Martinez, D.A., Druzhinina I.S., Thon M., Zeilinger S., Casas-Flores S., Horwitz B.A., Mukherjee P.K., Mukherjee M., Kredics L., Alcaraz L.D., Aerts A., Antal Z., Atanasova L., Cervantes-Badillo M.G., Challacombe J., Chertkov O., McCluskey K., Coulpier F., Deshpande N., von Döhren H., Ebbole D.J., Esquivel-Naranjo E.U., Fekete E., Flipphi M., Glaser F., Gómez-Rodríguez E.Y., Gruber S., Han C., Henrissat B., Hermosa R., Hernández-Oñate M.,Karaffa L., Kosti I., Le Crom S., Lindquist E., Lucas S., Lübeck M., Lübeck P.S., Margeot A., Metz B., Misra M., Nevalainen H., Omann M., Packer N., Perrone G., Uresti-Rivera E.E., Salamov A., Schmoll M., Seiboth B., Shapiro H., Sukno S., Tamayo-Ramos J.A., Tisch D., Wiest A., Wilkinson H.H., Zhang M., Coutinho P.M., Kenerley C.M., Monte E., Baker S.E., Grigoriev I.V., 2011. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biology 12(4):R40.

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J., Higgins D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

Lima, M.A., Gomez, L.D., Steele-King, C.G., Simister, R., Bernardinelli, O.,Carvalho, M.A., Rezende, C.A., Labate, C.A., de Azevedo, E.R., McQueen-Mason, J., Polikarpov, I. (2014) Evaluating the composition and processing potential

of novel sources of Brazilian biomass for sustainable biorenewables production. Biotechnology for Biofuels, 7:10.

Lombard, V., Ramulu, H.G., Drula, E., Coutinho, P.M., Henrissat, B., 2013. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Research 42, D490-D495.

Maitan-Afenas, G.P., Lage, L.G.A., de Almeida, M.N., Visser, E.M., de Rezende, S.T., Guimarães, V.M., 2014. Hydrolysis of soybean isoflavones by *Debaryomyces hansenii* UFV-1 immobilised cells and free β-glucosidase. Food Chemistry 146, 429-436.

Martinez D., Larrondo L.F., Putnam N., Gelpke M.D., Huang K., Chapman J., Helfenbein K.G., Ramaiya P., Detter J.C., Larimer F., Coutinho P.M., Henrissat B., Berka R.,Cullen D., Rokhsar D., 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnology 22,695-700.

McIlvaine, T. (1921). A buffer solution for colorimetric comparison. Journal of Biological Chemistry, 49, 183-186.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426-428.

Moniruzzaman M., Alam, M.Z., Sujan, S.M.A., Hossain, M., Jamal, M.S., 2013. Enzymatic saccharification of bagasse: Effects of different pre-treatment methods. International Journal of Renewable Energy Research 3, 230-234.

Sambrook, J., Fritsch, E. F., Maniatis., T., 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Singhvi, M.S., Adsul, M.G., Gokhale, D.V., 2011. Comparative production of cellulases by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose. Bioresource Technology,102, 6569-6572.

Suhardi V. S. H., Prasai B., Samaha D., Boopathy R., 2013. Combined biological and chemical pretreatment method for lignocellulosic ethanol production from energy cane. Renewable Bioresources 1:1.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28, 2731-2739.

TAPPI, 1991. Technical Association of the Pulp and Paper Industry Tappi Useful Methods (UM-250), Norcross.

TAPPI, 1999. Technical Association of the Pulp and Paper Industry. Tappi Standard Methods (T-222 om-98), Atlanta.

UNICA. 2014. Brazilian Sugarcane Industry Association - Sugarcane Production and Processing.

van den Brink, J., de Vries, R.P., 2011. Fungal enzyme sets for plant polysaccharide degradation. Applied Microbiology and Biotechnology 91, 1477-1492.

van Dyk, J.S., Pletschke, B.I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes – factors affecting enzymes, conversion and synergy. Biotechnology Advances 30, 1458–1480.

Wongwisansri, S., Promdonkoy, P., Matetaviparee, P., Roongsawang, N., Eurwilaichitr, L., Tanapongpipat, S., 2013. High-level production of thermotolerant β-xylosidase of *Aspergillus* sp. BCC125 in *Pichia pastoris*: Characterization and its application in ethanol production. Bioresource Technology 132, 410-413.

Tables and Figures

Table 1: Strains used for this work.

Species	Strain	Reference
Aspergillus terreus	NIH 2624	Fedorova et al., 2008
Trichoderma virens	Gv29-8_version2	Kubicek et al., 2011
Podospora anserina	S_mat+	Espagne et al., 2008
Nectria haematococca	mpVI	Coleman et al., 2009
Phaeosphaeria nodorum	SN_15	Hane et al., 2007
Chaetomium globosum	CBS 148.51	
Phanerochaete chrysosporium		Martinez et al., 2004

Table 2: Genes of interest and specific primers used for cloning.

Strain	Gene	GH Family	Forward Primer (5'- 3')	Reverse Primer (3'- 5')*
Aspergillus terreus	ATEG_08700	GH7	CCATGGTACGCAAATTGACACCG	AAGCTTCTAatgatgatgatgatgatgCCGCCGCTTGCCACGATAAGT
	ATEG_09052	GH3	CCATGGATTCTTCCAATTCCCG	AAGCTTCTAatgatgatgatgatgatgTGCCTTGGTGGCGTTCCGCGGCG
	ATEG_05003	GH5	CCATGGAGCTCTCGCTGCTCGCCG	AAGCTTCTAatgatgatgatgatgatgCAGACACTGCGAGTACCAGTCG
	ATEG_10193	GH43	CCATGGAGTGTTTTGCAAGACTCCTG	AAGCTTCTAatgatgatgatgatgatgAGCTAAATTCATGTTGAACTGGC
	ATEG_01089	GH39	CCATGGCTGTCTTTTCCAATATAG	AAGCTTCTAatgatgatgatgatgatgAAAGCCAAATTCAAAAGCATACGC
	ATEG_00093	GH43	CCATGGCGAACGACGACCACGAGT	GTTAACTAatgatgatgatgatgatgCTTCAAGTGGATATCTCCCTTCTCG
Nectria haematococca	Nec_47861	GH39	CCATGGCTCTTTCCAACATTCTCGTCTC	AAGCTTTTAatgatgatgatgatgatgCTGAACAAACTCAAAGGCGTAAGC
	Nec_29778	GH12	CCATGGAGTCCGCCATCGTCGCAG	AAGCTTTTAatgatgatgatgatgatgGTATTCAACATCAGCGTGATAGTTG
	Nec_48733	GH12	CCATGGAGTTCTTTGGTGTTGTTTCGG	AAGCTTTTAatgatgatgatgatgatgGTTGACGGAGATGGTGTAGGCAGA
	Nec_106477	GH12	CCATGGAGCTGACCTCCTTCCTTG	AAGCTTCTAatgatgatgatgatgatgGTTGCTGTTGACCTTGATGG
	Nec_122895	GH12	CCATGGTGAAGTTTGCGTTTTACCTCG	AAGCTTCTAatgatgatgatgatgatgCAAGTTGATCCTCAGCGACAGC
Phaeosphaeria nodorum	PN_07147	GH12	CCATGGAGCTCACACTCTTTACTCTGG	AAGCTTCTAatgatgatgatgatgatgGGATTGGCTCATCGTGTACGCGC
	PN_09915	GH62	CCATGGGCGGTCTTTTCAACTCTG	GTTAACTAatgatgatgatgatgatgCGTAAACTGCTTGAAGAATTCCC
	PN_10764	GH62	CCATGGTGGGATTCACACTCCTTGC	GTTAATCAatgatgatgatgatgatgAGCTTGTGTGATAAGACCCAGG
	PN_09852	GH6	CCATGGAGAGTATTTTCGCCGCTGTCC	AAGCTTTTAatgatgatgatgatgatgAAGCGCTGGCTTAGCATTCTTGACC
	PN_11171	GH6	CCATGGTCCAACTCACTCAATTCGTTC	GTTAATTAatgatgatgatgatgatgGTACGTCGGTGTCAAGCTTGCATCC
	PN_12155	GH6	CCATGGAGGCTGTTTCTTTCCTCGCTGTTG	AAGCTTTTAatgatgatgatgatgatgAAATGCGGGGCTTGCGTTGCTC
	PN_05106	GH6	CCATGGTGTCCAACGCTCTCCTCAC	AAGCTTCTAatgatgatgatgatgatgAGCAAGGGGAGGGTTGGCG

*The sequence *atgatgatgatgatgatg* in the reverse primers is related to the histidine tag.

Table 3: Comparison of gene numbers in selected CAZy families based on genome content.

	Number of genes								
Species	**GH 6**	**GH 7**	**GH 10**	**GH11**	**GH12**	**GH 39**	**GH 54**	**AA9**	**GH 62**
Aspergillus terreus	2	4	4	2	5	1	1	11	3
Trichoderma virens	1	2	2	4	3	1	2	3	4
Podospora anserina	4	6	8	6	2	0	0	33	2
Nectria haematococca	1	3	3	3	4	1	1	9	2
Phaeosphaeria nodorum	4	4	8	7	3	1	1	25	5
Chaetomium globosum	4	5	6	9	3	0	0	25	9
Phanerochaete chrysosporium	1	7	6	1	2	0	0	10	0

Table 4: Enzymatic activities of *A. vadensis* transformants (best transformant for each cloned gene). The data are the mean of assays performed in triplicate.

		Enzymatic Activity (U/ml)	
Gene	**Enzyme**	**Day 3**	**Day 4**
ATEG_08700	Cellobiohydrolase	1.27±0.002	4.16±0.008
ATEG_09052	β-xylosidase	0.61±0.005	1.53±0.016
ATEG_05003	Endoglucanase	1.78±0.095	1.53±0.093
ATEG_10193	β-xylosidase	0.87±0.002	0.05±0.001
ATEG_01089	β-xylosidase	0.34±0.003	0.45±0.002
ATEG_00093	β-xylosidase	1.62±0.016	3.56±0.020
Nec_48733	Endoglucanase	1.26±0.036	0.17±0.027
PN_07147*	Endoglucanase	1.72±0.032	1.42±0.052
PN_07147*	Xyloglucan hydrolase	1.42±0.121	1.44±0.022
PN_09852	Cellobiohydrolase	4.21±0.013	4.63±0.053

*The activity of the same enzyme was tested against two different substrates.

Table 5: Relative activity of β-xylosidases from *A. terreus* submitted to different effectors. Relative activities were calculated in relation to the β-xylosidase activities without pre-incubation which was considered to be 100%.

	Relative Activity (%)			
Ion/Sugar/Reducing Agent	**Xyl09052**	**Xyl10193**	**Xyl01089**	**Xyl00093**
Lactose	59.04±0.10	75.13±0.04	82.14±0.15	87.36±0.02
Xylose	24.94±0.07	31.90±0.12	30.03±0.02	38.83±0.01
Galactose	73.59±0.02	99.09±0.24	89.02±0.12	93.77±0.14
Sacarose	74.32±0.12	101.81±0.03	78.11±0.01	67.91±0.22
Glucose	72.85±0.07	91.25±0.11	57.87±0.02	80.11±0.13
2 mM SDS	99.80±0.15	94.92±0.12	96.37±0.24	94.57±0.09
10 mM SDS	30.51±0.08	46.91±0.21	14.73±0.03	2.64±0.03
2 mM NaCl	80.40±0.17	100.34±0.01	68.92±0.18	86.41±0.04
10 mM NaCl	80.18±0.23	98.16±0.15	67.23±0.09	82.21±0.12
2 mM β-mercaptoethanol	65.88±0.11	102.68±0.23	82.63±0.12	72.70±0.17
10 mM β-mercaptoethanol	103.07±0.14	121.77±0.14	119.91±0.03	119.85±0.12
2 mM EDTA	103.5±0.22	116.24±0.12	108.33±0.15	232.16±0.31
10 mM EDTA	132.90±0.17	146.88±0.24	135.17±0.23	235.42±0.29
2 mM $MgCl_2$	99.96±0.12	99.04±0.02	88.48±0.18	109.50±0.21
10 mM $MgCl_2$	100.06±0.03	71.10±0.22	87.14±0.09	138.65±0.06
2 mM $HgCl_2$	0.00	0.00	0.00	0.00
10 mM $HgCl_2$	0.00	0.00	0.00	0.00
2 mM CuSO4	23.76±0.08	46.93±0.10	6.78±0.01	0.00
10 mM $CuSO_4$	8.63±0.11	44.95±0.02	0.00	0.00
2 mM MnO_2	98.96±0.13	117.94±0.03	103.72±0.05	181.29±0.19
10 mM MnO_2	203.19±0.37	152.88±0.32	126.21±0.16	246.99±0.31
2 mM $CaCl_2$	80.55±0.04	93.17±0.11	103.62±0.32	105.32±0.18
10 mM $CaCl_2$	80.13±0.07	90.21±0.01	101.02±0.21	150.83±0.01
2 mM $ZnCl_2$	53.31±0.01	89.67±0.21	48.68±0.014	170.90±0.16
10 mM $ZnCl_2$	43.51±0.14	85.70±0.12	43.83±0.-15	208.60±0.36

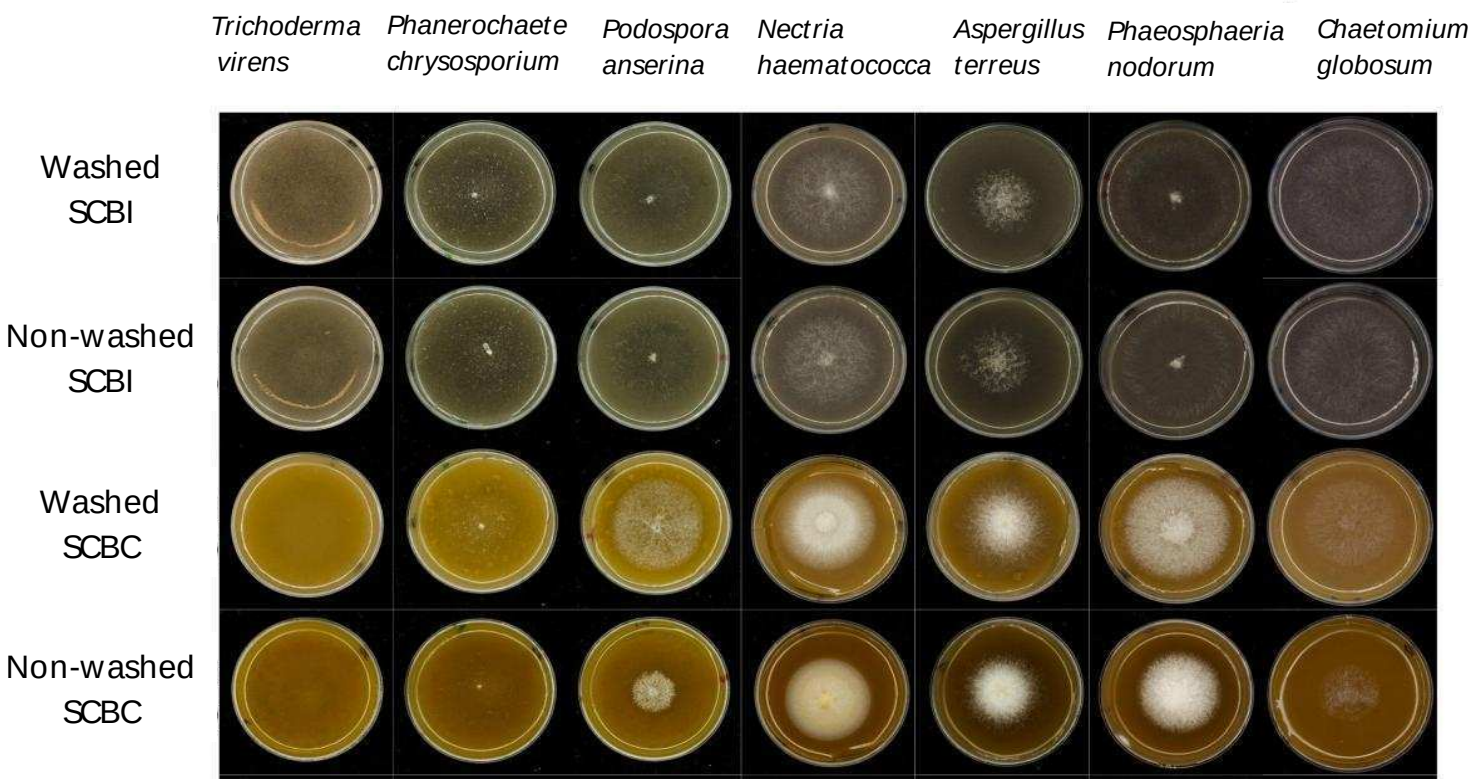


Figure 1: Growth profile of seven candidate fungi using sugarcane bagasse as carbon source.

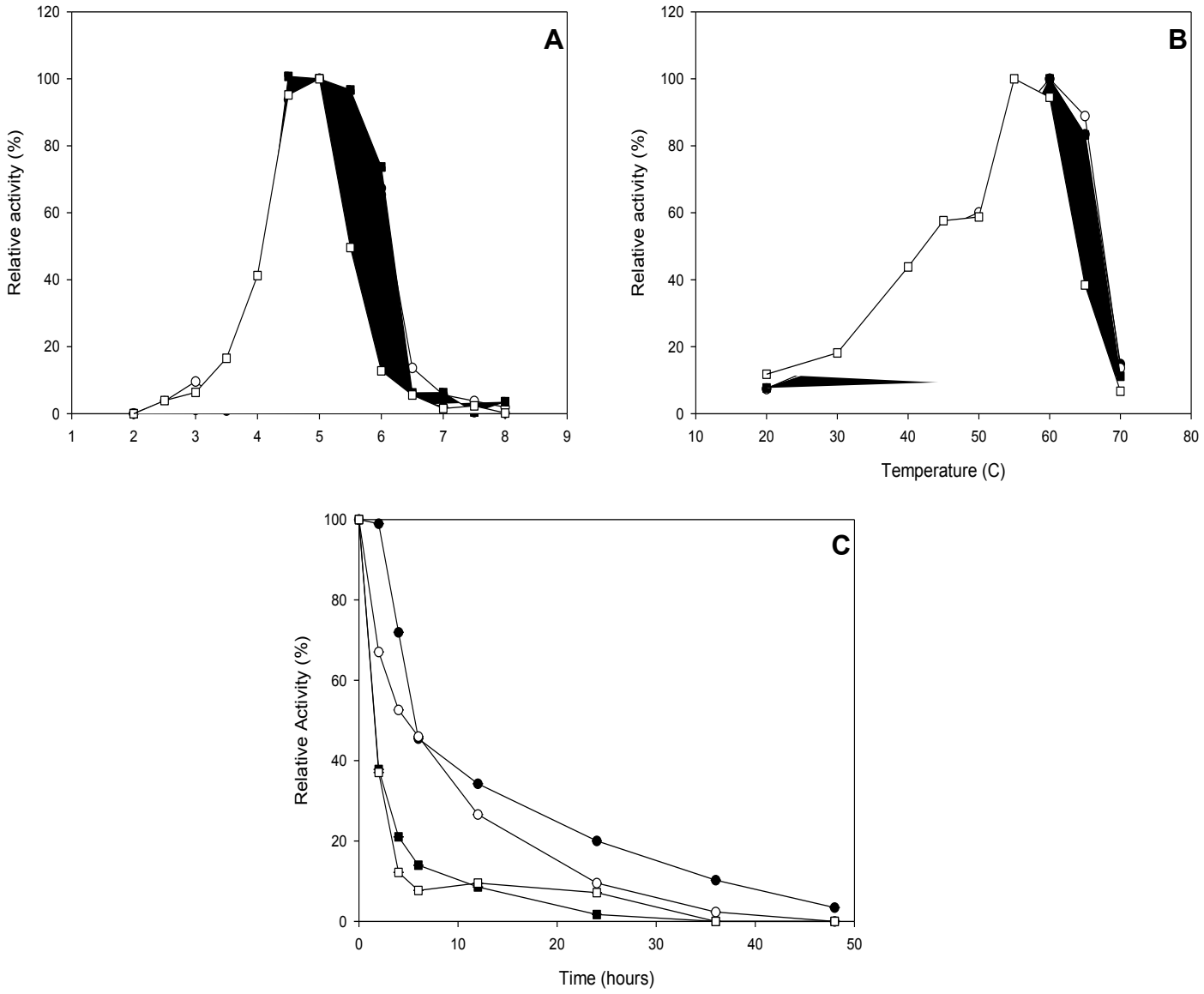


Figure 2: Effect of pH and temperature on β-xylosidase activities from *A. terreus*. (**A**) Effect of pH on activity of (●) Xyl09052, (○) Xyl10193, (■) Xyl01089 and (□) Xyl00093. (**B**) Effect of temperature on activity of (●) Xyl09052, (○) Xyl10193, (■) Xyl01089 and (□) Xyl00093. Relative activities were calculated in relation to activities determined at optima pH and temperature. (**C**) Thermostability at 50°C for (●) Xyl09052, (○) Xyl10193, (■) Xyl01089 and (□) Xyl00093. Relative activities were calculated in relation to β-xylosidase activities without pre-incubation, which was considered to be 100 %.

Supplemental Data

Table 1: Diameter of fungal colonies after 3 days of growth on MM with 3% (w/v) sugarcane bagasse.

	Diameter of colony (cm)			
Strains	**Washed SCBI**	**Non-washed SCBI**	**Washed SCBC**	**Non-washed SCBC**
N. haematococca	1.05±0.05	1.2±0.00	0.7±0.00	0.65±0.05
A. terreus	1.05±0.05	1.25±0.05	1.2±0.00	1.05±0.05
P. nodorum	0.95±.05	0.95±.05	0.75±0.05	0.7±0.00

Table 2: Compositional analysis of two samples of sugarcane bagasse (SCBI and SCBC).

Sugarcane Bagasse	**% Cellulose**	**% Hemicellulose**	**% Lignin**	**% Total**
SCBI	42.85	31.45	23.65	97.95
SCBC	51.80	26.60	18.45	96.85

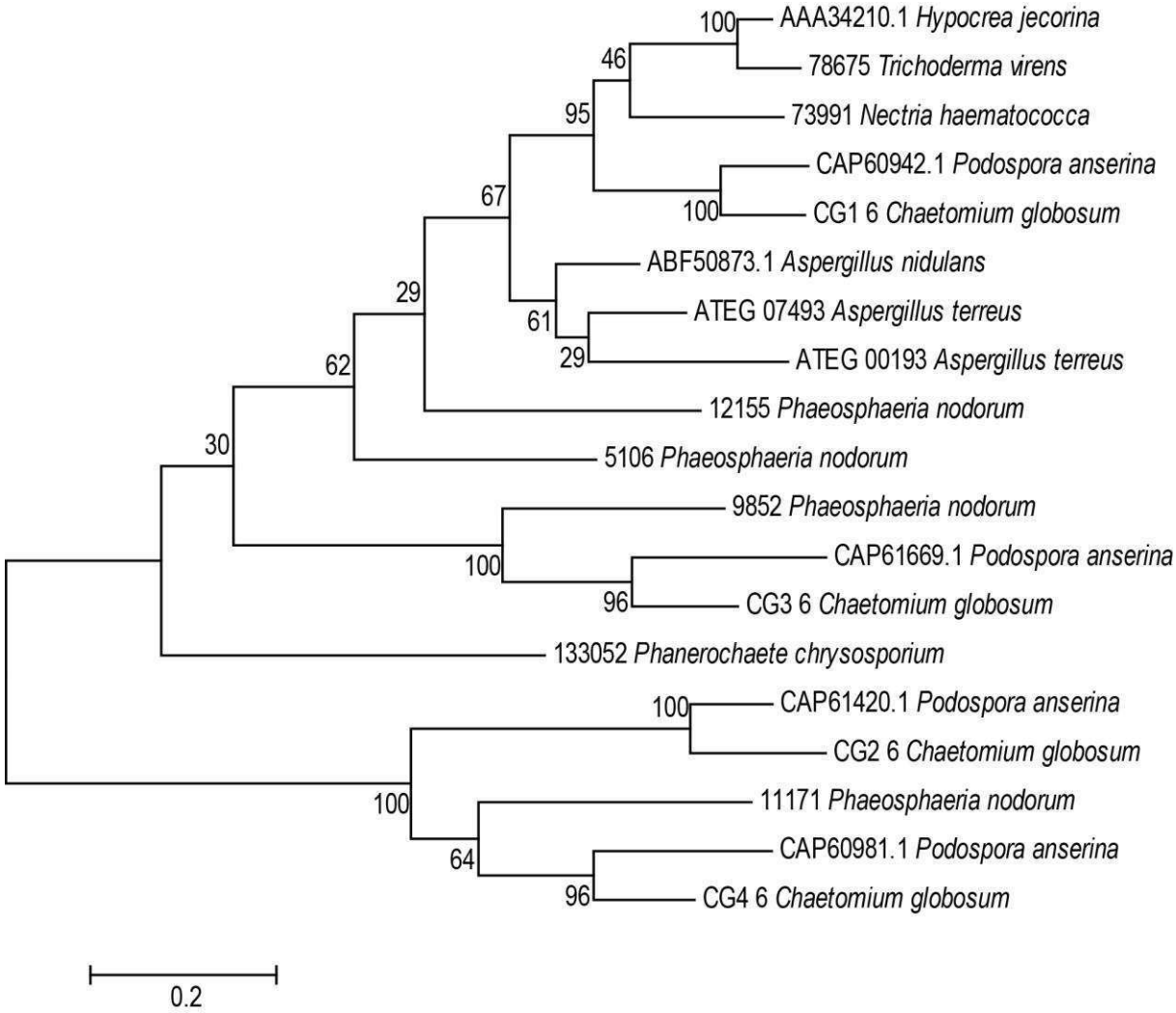


Figure 1: Phylogenetic tree of Family GH6. The alignment of the aminoacid sequences was done using MAFFT. The tree was constructed with Maximum Likelihood method based on the Poisson correction model. Evolutionary analysis were conducted in MEGA5. Bootstrap with 200 replicates.

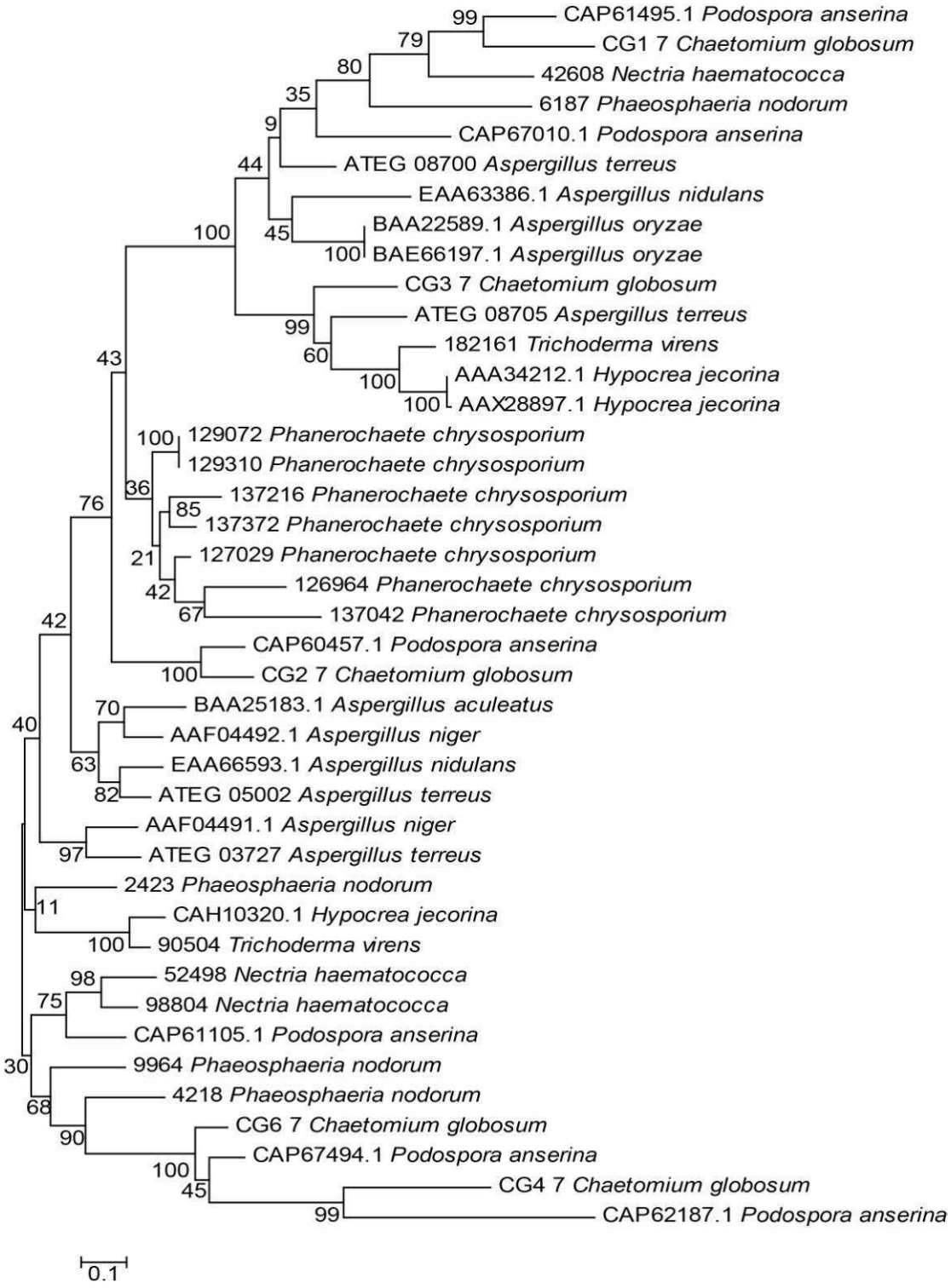


Figure 2: Phylogenetic tree of Family GH7. The alignment of the aminoacid sequences was done using MAFFT. The tree was constructed with Maximum Likelihood method based on the Poisson correction model. Evolutionary analysis were conducted in MEGA5. Bootstrap with 200 replicates.

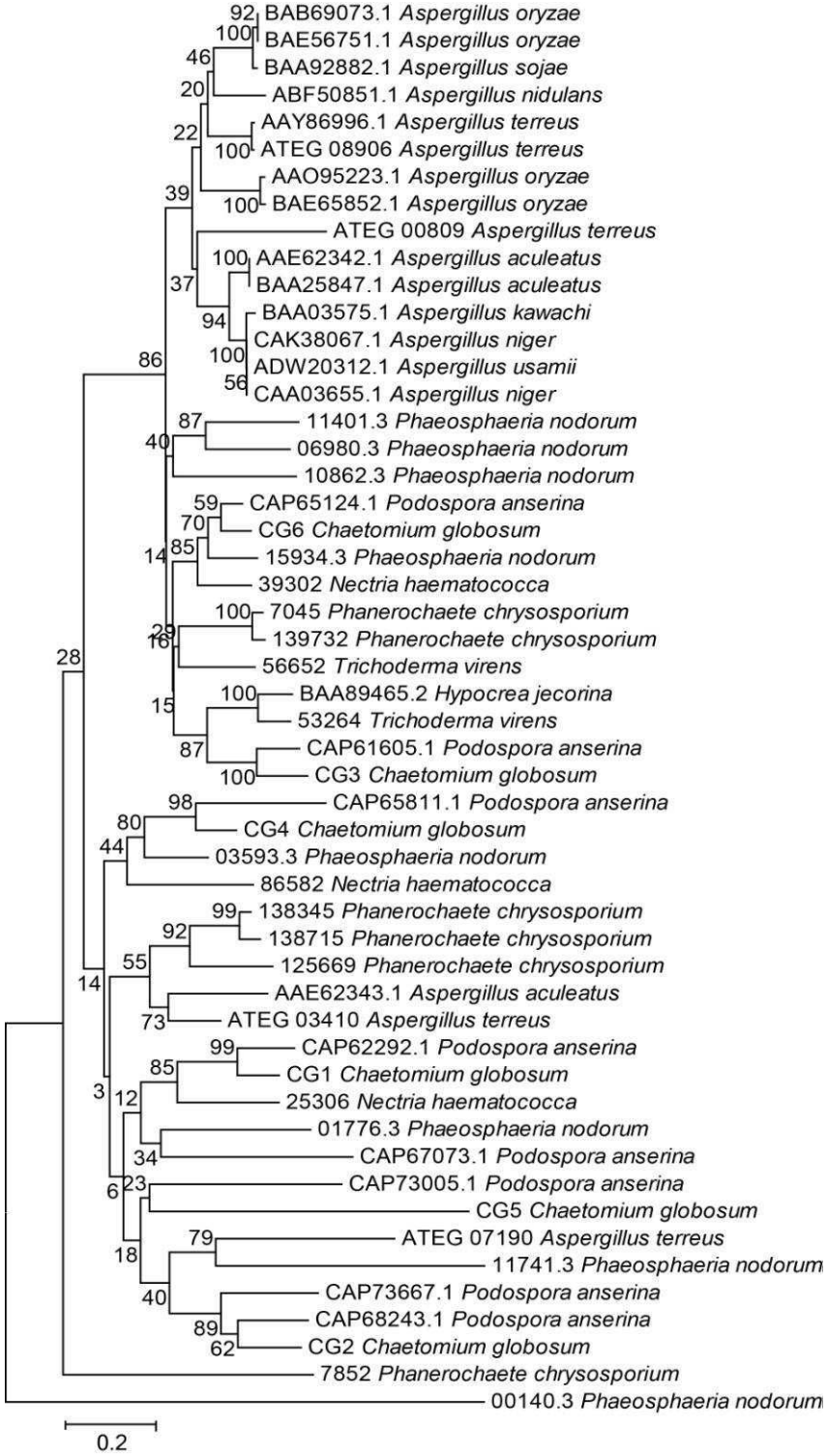


Figure 3: Phylogenetic tree of Family GH10. The alignment of the aminoacid sequences was done using CLUSTAL X2. The tree was constructed with Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analysis were conducted in MEGA5.

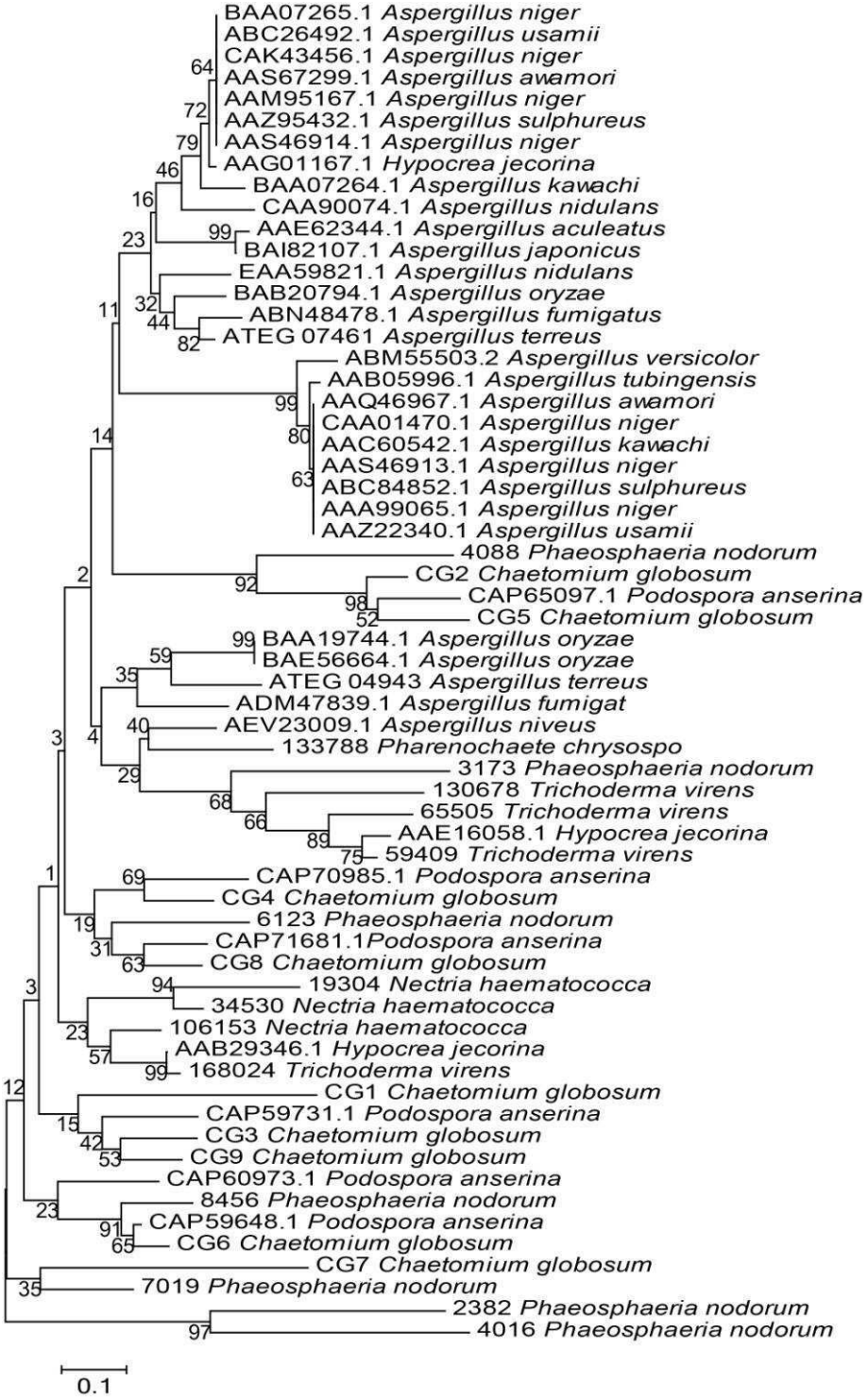


Figure 4: Phylogenetic tree of Family GH11. The alignment of the aminoacid sequences was done using CLUSTAL X2. The tree was constructed with Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analysis were conducted in MEGA5.

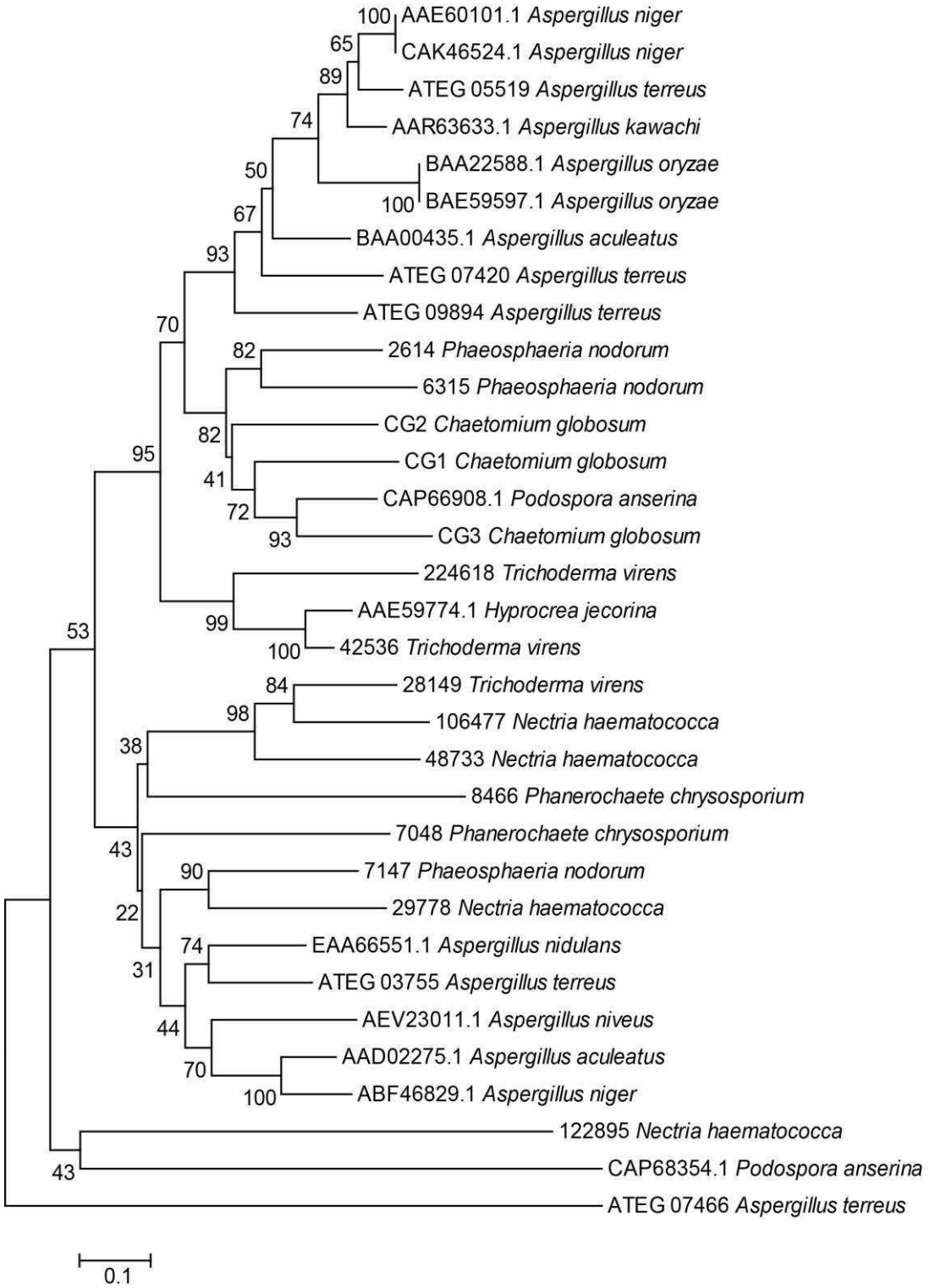


Figure 5: Phylogenetic tree of Family GH12. The alignment of the aminoacid sequences was done using CLUSTAL X2. The tree was constructed with Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analysis were conducted in MEGA5.

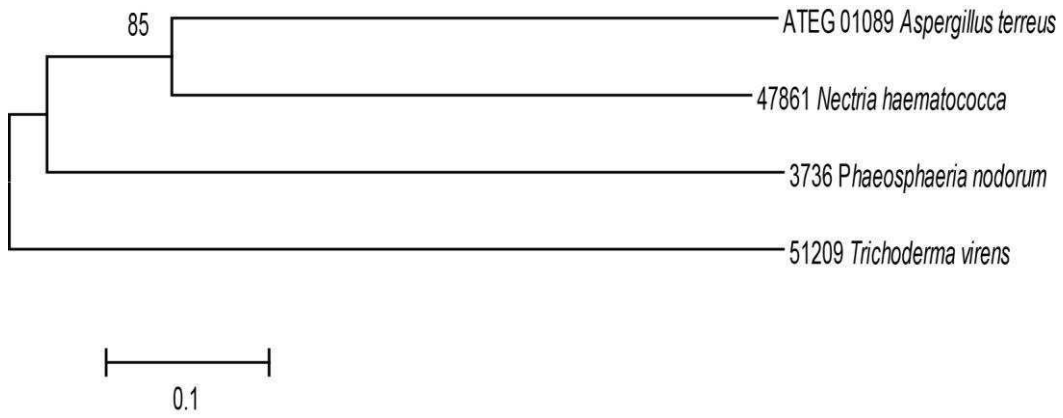


Figure 6: Phylogenetic tree of Family GH39. The alignment of the aminoacid sequences was done using CLUSTAL X2. The tree was constructed with Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analysis were conducted in MEGA5.

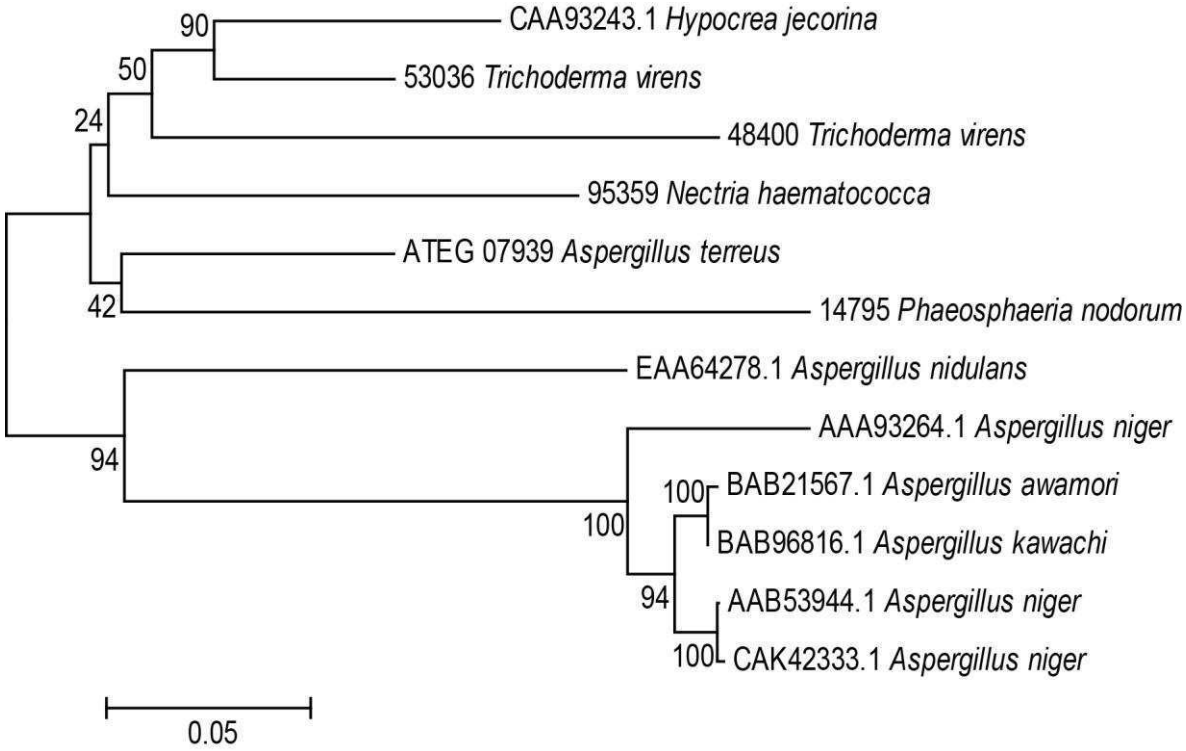
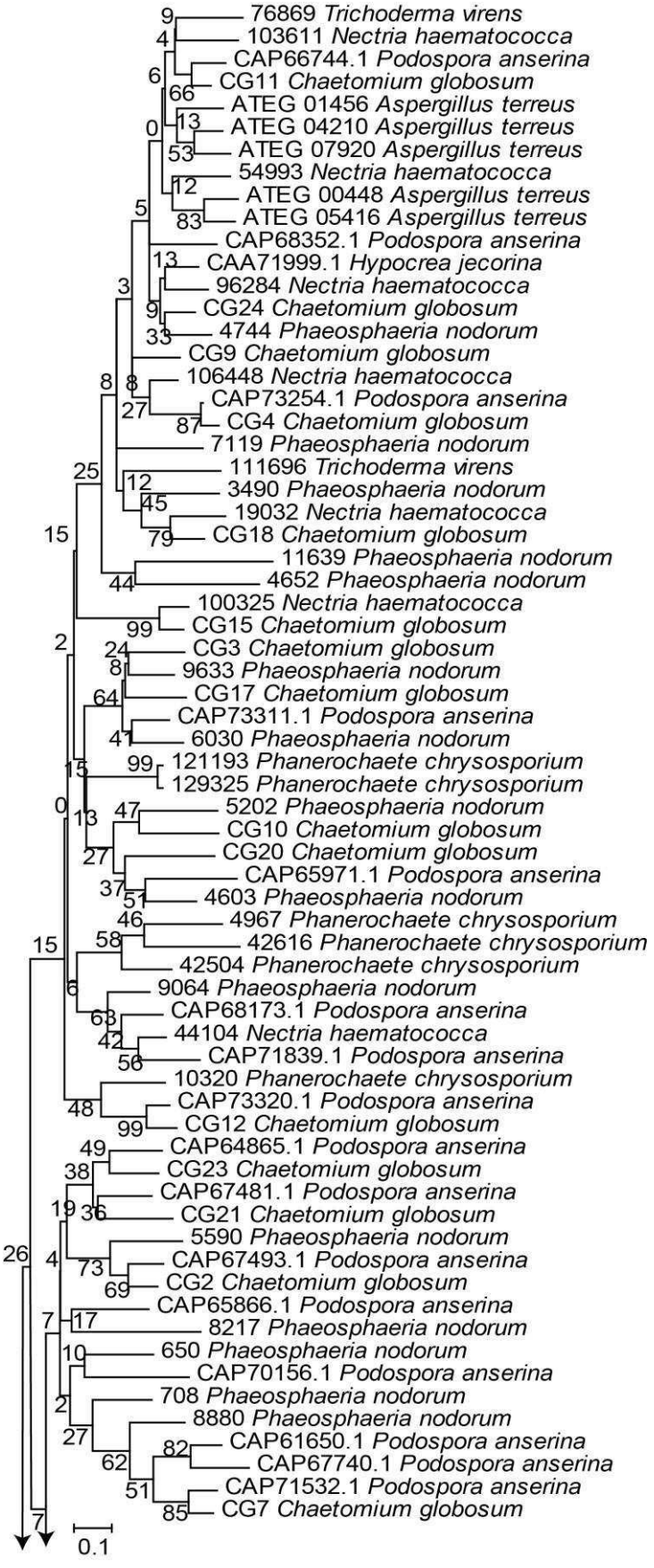


Figure 7: Phylogenetic tree of Family GH54. The alignment of the aminoacid sequences was done using CLUSTAL X2. The tree was constructed with Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analysis were conducted in MEGA5.


76869 Trichoderma virens
103611 Nectria haematococca
CAP66744.1 Podospora anserina
CG11 Chaetomium globosum
ATEG 01456 Aspergillus terreus
ATEG 04210 Aspergillus terreus
ATEG 07920 Aspergillus terreus
54993 Nectria haematococca
ATEG 00448 Aspergillus terreus
ATEG 05416 Aspergillus terreus
CAP68352.1 Podospora anserina
CAA71999.1 Hypocrea jecorina
96284 Nectria haematococca
CG24 Chaetomium globosum
4744 Phaeosphaeria nodorum
CG9 Chaetomium globosum
106448 Nectria haematococca
CAP73254.1 Podospora anserina
CG4 Chaetomium globosum
7119 Phaeosphaeria nodorum
111696 Trichoderma virens
3490 Phaeosphaeria nodorum
19032 Nectria haematococca
CG18 Chaetomium globosum
11639 Phaeosphaeria nodorum
4652 Phaeosphaeria nodorum
100325 Nectria haematococca
CG15 Chaetomium globosum
CG3 Chaetomium globosum
9633 Phaeosphaeria nodorum
CG17 Chaetomium globosum
CAP73311.1 Podospora anserina
6030 Phaeosphaeria nodorum
121193 Phanerochaete chrysosporium
129325 Phanerochaete chrysosporium
5202 Phaeosphaeria nodorum
CG10 Chaetomium globosum
CG20 Chaetomium globosum
CAP65971.1 Podospora anserina
4603 Phaeosphaeria nodorum
4967 Phanerochaete chrysosporium
42616 Phanerochaete chrysosporium
42504 Phanerochaete chrysosporium
9064 Phaeosphaeria nodorum
CAP68173.1 Podospora anserina
44104 Nectria haematococca
CAP71839.1 Podospora anserina
10320 Phanerochaete chrysosporium
CAP73320.1 Podospora anserina
CG12 Chaetomium globosum
CAP64865.1 Podospora anserina
CG23 Chaetomium globosum
CAP67481.1 Podospora anserina
CG21 Chaetomium globosum
5590 Phaeosphaeria nodorum
CAP67493.1 Podospora anserina
CG2 Chaetomium globosum
CAP65866.1 Podospora anserina
8217 Phaeosphaeria nodorum
650 Phaeosphaeria nodorum
CAP70156.1 Podospora anserina
708 Phaeosphaeria nodorum
8880 Phaeosphaeria nodorum
CAP61650.1 Podospora anserina
CAP67740.1 Podospora anserina
CAP71532.1 Podospora anserina
CG7 Chaetomium globosum
0.1

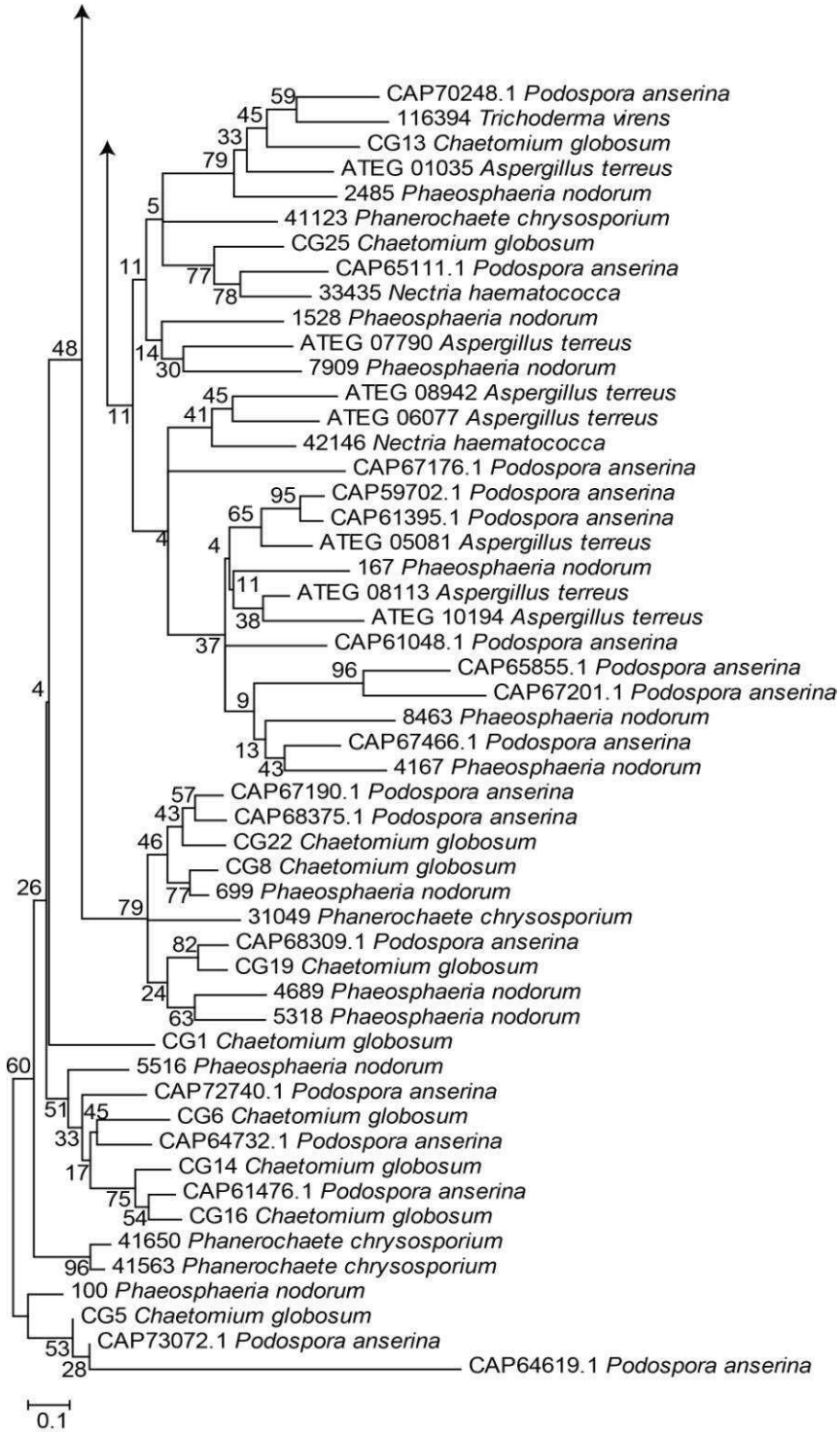


Figure 8: Phylogenetic tree of Family AA9. The alignment of the aminoacid sequences was done using CLUSTAL X2. The tree was constructed with Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analysis were conducted in MEGA5.

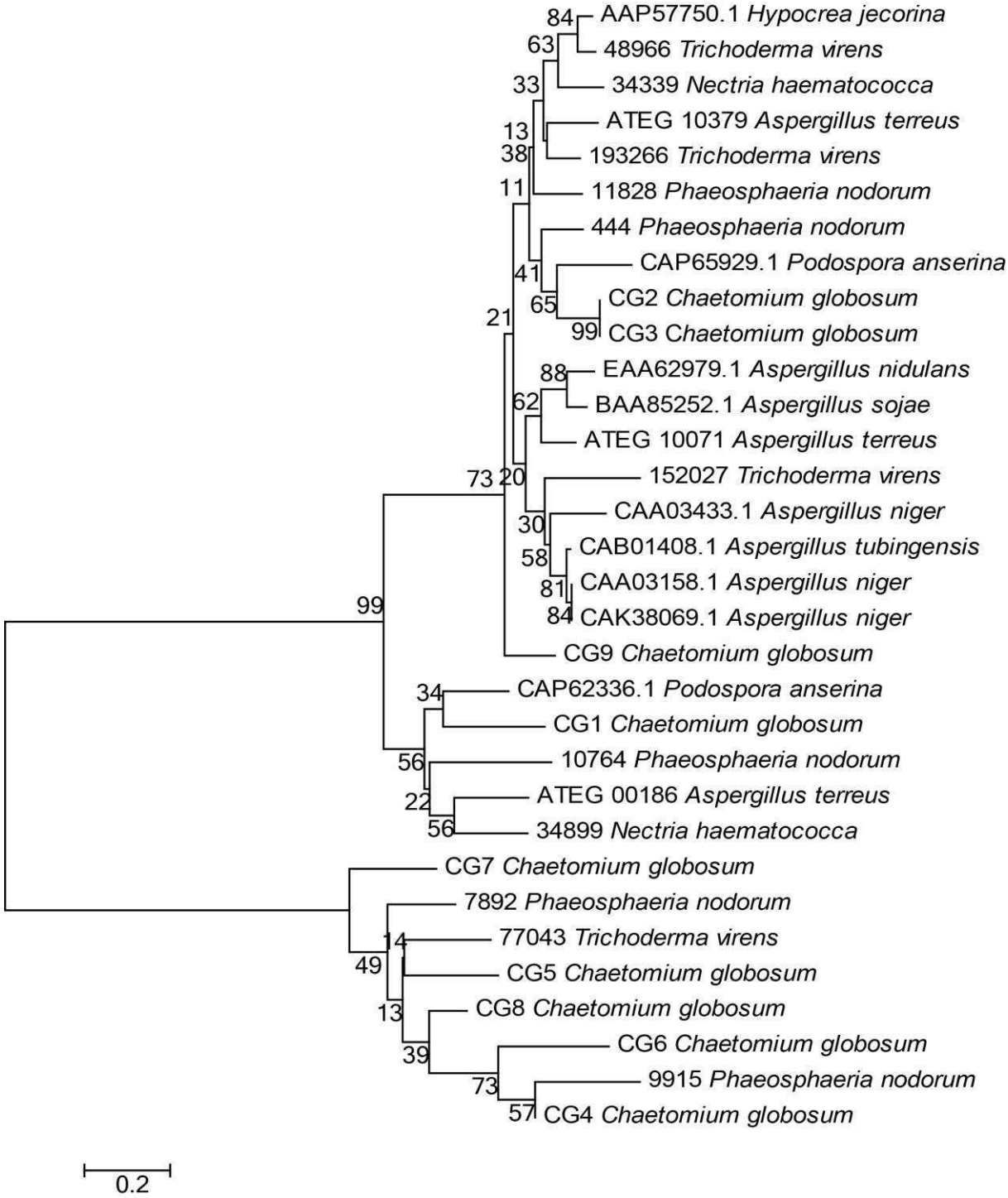


Figure 9: Phylogenetic tree of Family GH62. The alignment of the aminoacid sequences was done using CLUSTAL X2. The tree was constructed with Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analysis were conducted in MEGA5.

CHAPTER 4

Purification, characterization and biotechnological application of xylanases from *Aspergillus nidulans* expressed in *Pichia pastoris*

Authors: Gabriela P. Maitan-Alfenas, Mariana B. Oliveira, Humberto J. O. Ramos, Ronald P. de Vries, Valéria M. Guimarães

Purification, characterization and biotechnological application of xylanases from *Aspergillus nidulans* expressed in *Pichia pastoris*

Gabriela P. Maitan-Alfenas[1,2], Mariana B. Oliveira[1], Humberto J. O. Ramos[1], Ronald P. de Vries[2,3], Valéria M. Guimarães[1]

[1]Department of Biochemistry and Molecular Biology, Universidade Federal de Viçosa, MG, Brazil

[2]CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

[3]Fungal Molecular Physiology, Utrecht University, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Abstract

Two xylanases from *Aspergillus nidulans* cloned in *Pichia pastoris*, here denominated as Xyn1818 and Xyn3613, were expressed and, after purification, mass spectrometry was performed for confirmation of the enzyme identities. Xyn1818 and Xyn3613 achieved maximal activities at 60°C and pH 7.5, and at 50°C and pH 6.0, respectively. Xyn1818 showed to be very thermostable, maintaining 50% of its original activity after 49 hours incubated at 50°C. Xyn1818 presented higher activity against wheat arabinoxylan while Xyn3613 had the best activity against xylan from beechwood. Both enzymes were completely inhibited by SDS and $HgCl_2$. Sugarcane bagasse saccharification results showed that commercial enzymatic cocktails were able to release more glucose and xylose after supplementation with these xylanases and synergy occurred for xylanase and endoglucanase activities.

1. Introduction

Hemicellulose is the second most abundant organic material in the world and it presents a very complex structure composed of various different residues (Lee et al., 2014, van den Brink and de Vries, 2011). Xylan, the major hemicellulose polymer, is hydrolyzed by β-1,4-endoxylanase, β-1,4-xylosidase and accessory enzymes (Maitan-Alfenas et al., 2014a, Van Dyk and Pletschke, 2012, de Vries and Visser, 2001, de Vries et al., 2000). β-1,4-Endoxylanases (E.C. 3.2.1.8) are glucosyl

hydrolases that catalyze the hydrolysis of β-1,4-xylosidic linkages of the xylan backbone (Dodd and Cann, 2009). They are produced by several organisms but the main commercial sources are filamentous fungi (Polizeli et al., 2005).

β-1,4-Endoxylanases from fungi belong to two different glucoside hydrolase families: GH10 and GH11, and these two groups differ from each other in substrate specificity (Polizeli et al., 2005, Biely et al., 1997). Overall, concerning catalytic domains, GH10 endoxylanases present an α/β8 TIM-barrel topology and GH11 endoxylanases have a jelly-roll structure (Paes et al., 2012, van den Brink and de Vries, 2011). Both groups of xylanases catalyze the hydrolysis of xylan through retention of the anomeric configuration, with two glutamate residues being implicated in the catalytic mechanism (Motta et al., 2013). However, GH10 endoxylanases present a broader substrate specificity and are very important for complete degradation of substituted xylans (Pollet et al., 2010, Biely et al., 1997).

Xylanases can be used for various biotechnological applications such as bioconversion of lignocellulose into fermentable sugars, clarification of juices, preparation of animal feed and pulp bleaching (Shi et al., 2013, Li et al., 2000). However, for industrial applications, xylanases need to present desirable properties such as stability in a wide range of pH and temperature values, high specific activities and low costs (Taibi et al., 2012, Qiu et al., 2010). Therefore, molecular techniques must be used to obtain xylanases with ideal characteristics for commercial purposes. Genes encoding several xylanases have been cloned in homologous and heterologous hosts but the heterologous system is the main tool for the production of xylanases at industrial levels (Ahmed et al., 2009, Pérez et al., 2002, Kulkarni et al., 1999).

Heterologous expression in yeast systems has many advantages, including the production of soluble and correctly folded recombinant proteins that have undergone post-translational modifications, the capacity of growth until higher cell densities and the ability to secrete proteins (Daly and Hearn, 2005). *Pichia pastoris* has become a well described and widely applied expression host for xylanases production, mainly because this yeast presents a very high expression under its own promoters (Ahmed et al., 2009). Furthermore, there is no secretion of endogenous lignocellulolitic enzymes in significant amounts by *P. pastoris* and the recombinant proteins are almost pure heterologous enzyme preparations (Mellitzer et al., 2012). In

addition, proteins can be obtained by inexpensive large-scale cultivation of *P. pastoris* which reduces process costs (Tolner et al., 2006).

In this work, the two *A. nidulans* genes, AN1818.2 and AN3613.2, previously cloned in *P. pastoris* (Bauer et al., 2005, 2006) were expressed and the correspondent xylanases, here denominated as Xyn1818 (GH10) and Xyn3613 (GH11), respectively, were produced, purified and biochemically characterized. Their ability to supplement commercial enzymatic cocktails for use in sugarcane bagasse hydrolysis was also evaluated.

2. Materials and Methods

2.1. Materials

Substrates including *p*-nitrophenyl-β-D-glucopyranoside (pNPβGlc), *p*-nitrophenyl-β-D-xylopyranoside (pNPβXyl), ρ-nitrophenyl-β-D-mannopyranoside (pNPβMan), ρ-nitrophenyl-α-D-mannopyranoside (pNPαMan), *p*-nitrophenyl-β-D-galactopyranoside (pNPβGal), *p*-nitrophenyl-α-D-galactopyranoside (pNPαGal), *p*-nitrophenyl-α-D-arabinofuranoside (pNPαAra), *p*-nitrophenyl-β-D-cellobioside (pNPβCel), carboxymethylcellulose (CMC), Avicel®, xylan from birchwood, xylan from beechwood, oat spelt xylan, dinitrosalicylic acid (DNS), methanol, dithiothreitol, iodoacetamide and ammonium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Wheat arabinoxylan was obtained from Megazyme (Wicklow, Ireland). Yeast extract was purchased from Himedia Laboratories Co. (Mumbai, Maharashtra, India). The chemical reagents NaOH and potassium sodium tartarate were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). The commercial enzymatic mixtures Multifect® CL and Accellerase® 1500 were purchased from Genencor International Inc. (Rochester, NY, USA). Sugarcane bagasse was kindly donated by the Jatiboca Sugar and Ethanol Plant, Urucânia, MG, Brazil. All others reagents used in this study were of analytical grade.

2.2. *Pichia pastoris* strains

The *P. pastoris* strains used in this study were previously described by Bauer et al. (2005, 2006). The strains were grown in 25 ml of buffered complex glycerol medium (BMGY) in 250 ml shaker flasks at 28°C and 250 rpm for 16 hours. Aliquot cells were diluted to an OD 1.0 (600 nm) with 25 ml of buffered complex methanol

medium (BMMY) and incubated for 72 hours with daily addition of 150 µl of 100% methanol. The culture was centrifuged at 5000 rpm for 10 minutes according to the Pichia Expression Kit Manual (Invitrogen) and stored at -20°C for posterior analysis.

2.3. Protein Analysis

Protein concentration in the enzymatic extract of *P. pastoris* and in the commercial enzymatic mixtures was determined by the Coomassie Blue binding method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.4. Xylanase Assay

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50°C. They were performed in triplicate and the mean values were calculated. Relative standard deviations of the measurements were below 5%. Xylanase activity was determined using xylan from beechwood (1% w/v at final concentration) as substrate. The enzymatic reactions were initiated by the addition of 100 µl of the appropriately diluted enzyme solution to 400 µl of the polysaccharide substrate solution prepared in buffer. The reaction mixtures were incubated for 15 min and the total reducing sugar content released was determined at 540 nm by DNS method (Miller, 1959) using xylose as standard. One enzyme unit (U) was defined as the amount of enzyme necessary to produce 1 µmol of xylose equivalent per minute.

2.5. Xylanase Purification

The stored crude extracts from *P. pastoris* were centrifuged at 15,000 g for 30 minutes at 4°C. Histidine tags were used for the purification of the recombinant proteins by nickel affinity chromatography. Xylanase purification was carried out at room temperature under native and denaturing conditions according to protocols described in The QIAExpressionist™ Mannual (Fifth Edition, March 2001).

2.6. SDS–PAGE and zymogram analysis for xylanolytic activities

SDS–PAGE was performed using a 12% (w/v) polyacrylamide gel with a 5% stacking gel and the Mini-Protean II system (BioRad) according to the method previously described by Laemmli (1970), with some modifications. Sample preparation for zymogram analysis was performed by mixing 0.5 U of enzyme with 5

µl of loading buffer containing SDS 2% (w/v). The mixture was applied in a SDS-PAGE containing 1% of birchwood xylan and, after running, the gel was divided into two parts. One part, containing the molecular marker obtained from BioRad (BioRad Precision Plus Protein Unstained Standard), was stained with Comassie Brilliant Blue. The other part of the gel containing only the enzymatic samples was washed twice for 30 min in 20% isopropanol (v/v) to remove SDS and allow refolding of the proteins in the gel. The gel was washed again for 30 min in 100 mM acetate buffer, pH 5, to remove 2-propanol and immediately incubated in same buffer for 15 min at 50°C for development of xylanase activity. After that, the gel was submerged in 0.1% (w/v) Congo red solution for 30 min and destained with 1 M NaCl until pale-red hydrolysis zones appeared against a red background. Acetic acid at 0.5% concentration was added to expose the bands and the gel turned to a dark blue color.

2.7. Protein Digestion and Identification by Mass Spectrometry (MS)

Protein spots were removed manually from the gels, reduced by DTT (Dithiothreitol) and alkylated by iodoacetamide. Digestion was carried out with trypsin in a 50 mM ammonium bicarbonate buffer, pH 7.8, containing 20 ng/µl of sequencing grade trypsin (Promega) at 37°C overnight. Peptides were extracted from the spots with 30 µl of 50 mM ammonium bicarbonate solution, followed by incubation for 10 min with occasional vortex mixing. After that, the supernatant was collected and transferred to a 0.5 ml plastic microcentrifuge tube. This extraction was performed two more times. The volume of the extract was completely dried by evaporation in a speed-vac. Tryptic peptides were solubilized in 30 µl of MS grade water (Sigma-Aldrich) containing trifluoroacetic acid 0.1% (v/v).

For protein identification by mass spectrometry, the tryptic peptides were analyzed using a MALDI-TOF/TOF mass spectrometer model Ultraflex III (Bruker Daltonics). Samples of the tryptic peptides were mixed with α-cyano-4-hydroxyl cinnamic acid in a proportion of 1:1. The mass spectra obtained were processed using FlexAnalysis software (Bruker Daltonics) and a peak list (mgf format) was used for identification of the proteins by *MS MS ions search* using the Mascot software against the NCBI protein databases. For the search, the following parameters were considered: a mass tolerance of 75 ppm for the parental ion, fixed modification for

carbamidomethylation of cysteine residues and variable modification for oxidation of the methionine residues. The sequences obtained were confirmed by *de novo* sequencing manually and the trypic cleavage partners of the proteins were analyzed by interpretation of the spectra using the flexAnalysis software.

2.8. Enzymatic Characterization

2.8.1. Effects of pH and temperature

The influences of pH and temperature on xylanase activities were determined using the standard enzymatic assay, except that the pH values were modified to a range of 2.0 to 14.0, using different buffer solutions, and the temperature values ranged from 25 to 70°C.

The pH stability of xylanases was determined by pre-incubating enzyme solutions in the pH range of 2.0 to 14.0 for 30 min, on ice. After pre-incubation, the mixture was used for determining residual activity, according to standard assay, using xylan from beechwood as the substrate. Thermal stability was investigated by pre-incubating the enzymatic solutions in 100 mM sodium acetate buffer, pH 5.0, at temperatures of 50 and 60°C, for 48 hours. Aliquots of the enzymes were collected at specific times and submitted to the standard assay, measuring the remaining activity. The relative activities were calculated in relation to xylanase activity without pre-incubation, which was considered to be 100%. Results of the analysis are presented as mean ± SD for three measurements.

2.8.2. Substrate Specificity

Enzymatic assays were performed with various synthetic, natural and polymeric substrates. FPase and endoglucanase activities were determined using Whatman No. 1 filter paper (1 x 6 cm, 50 mg) and 1% CMC/1% Avicel® as substrates, respectively, according to previously described standard conditions (Ghose, 1987). The total reducing sugars liberated during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose as the standard. Xylanase activity using xylan from beechwood, oat spelt xylan and arabinoxylan, all at 1% (w/v) concentration, was determined as the standard assay. β-Glucosidase, β-xylosidase, α and β-mannosidase, α and β-galactosidase, α-arabinofuranosidase and cellobiohydrolase activities were measured using ρPNβGlc,

ρNPβXyl, ρNPα/βMan, ρNPα/βGal, ρNPαAra and ρNPβCel as substrates, respectively. The reaction mixtures contained 100 µl of the appropriately diluted enzyme solution, 125 µl of the synthetic substrate solution (4 mM at final concentration) and 275 µl of 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 min and it was stopped by addition of 0.5 ml of a 0.5 M sodium carbonate solution. Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released was estimated using a standard curve. The data presented for all enzyme activity determinations are mean values ± SD of three measurements.

2.8.3. Effect of Ions and Reducing Agents

The effects of ions and reducing agents on xylanase activities were assayed by the standard method. Reaction mixtures contained 100 µl of the appropriately diluted enzyme solution with an adequate amount of the ion or reducing agent for a final concentration of 2 and 10 mM (except for β-mercapthoethanol that presented a final concentration of 1 mM) and 400 µl of xylanase solution prepared in buffer. The reaction was incubated for 15 minutes and the reducing sugars were measured at 540 nm, as described before. The data presented for all enzyme activity assays are mean values ± SD of measurements performed in triplicate.

2.9. Sugarcane bagasse pretreatment

Sugarcane bagasse was washed and dried in an oven at 70°C until reaching a constant mass, after which it was further milled (particle size less than 1 mm) and submitted to alkaline pretreatment prior to being employed in saccharification experiments. Sodium hydroxide at 1.0% (w/v) concentration was used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v). The pretreatment was performed in an autoclave at 120°C for 60 min. Pretreated bagasse was separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at - 20°C.

2.10. Sugarcane bagasse saccharification

The purified xylanases and the commercial cocktails containing cellulases (Multifect® CL and Accellerase® 1500) were applied in a biomass saccharification

experiment. Enzymatic saccharification of alkali-pretreated sugarcane bagasse was performed in 125 ml Erlenmeyer flasks with 20 ml working volume, at an initial solid concentration of 8% dry matter (w/v) in 100 mM sodium acetate buffer at pH 5.0. The saccharification assays contained: the individual commercial cocktails (10 FPase units (FPU)/g of biomass); or the individual commercial cocktails (10 FPU/g biomass) supplemented with Xyn1818 or Xyn3613 (15 U/g biomass); or the individual commercial cocktails (10 FPU/g biomass) supplemented with Xyn1818 and Xyn3613 (7.5 U/g biomass for each enzyme). Sodium azide (10 mM) and tetracycline (40 µg/ml) were added to the reaction mixture to inhibit microbial contamination. The reactions were carried out in duplicate in an orbital shaker at 250 rpm and 50°C for 72 hours. Samples (0.5 ml) were taken from the reaction mixture at different time intervals for process monitoring. These samples were immediately heated to 100°C to denature the enzymes, cooled and then centrifuged at 15,000 g for 5 min.

2.11. Analysis of hydrolysis products

Products of the saccharification assays were analyzed by high performance liquid chromatography (HPLC) using a Shimadzu series 10A chromatograph. The HPLC was equipped with an Aminex HPX-87P column (300x7.8 mm) and refractive index detector. The column was eluted with water at a flow rate of 0.6 ml/min and it operated at 80°C.

The total reducing sugars were measured by the DNS method (Miller, 1959) using a mix of glucose and xylose as standard.

2.12. Synergism Tests

To investigate the presence of synergy of each xylanase activity (Xyn1818 and Xyn3613) and enzyme activities present in the commercial cocktails (Multifect® CL and Accellerase® 1500), the theoretical activities were calculated based on the enzyme loadings used for the saccharification experiments. This theoretical value was compared to the actual measured activity and synergism was expressed as a multiple of the theoretical activity.

3. Results

3.1. Purification and identity confirmation of the xylanases Xyn1818 and Xyn3613

After 72 h of cultivation of *P. pastoris*, the xylanase activities in the crude extract were 80.45 U/ml and 35.64 U/ml for Xyn1818 and Xyn3613, respectively. Expressed in terms of the protein content, the specific activities were 311.82 U/mg of protein and 105.13 U/mg of protein for Xyn1818 and Xyn3613, respectively. The expected molecular masses were 35 kDa for Xyn1818 and 24 kDa for Xyn3613, which were confirmed on the zymogram (Figure1).

After enzyme purification, the active elution fractions from affinity chromatography were pooled and the proteins were visualized on SDS-PAGE. The fraction containing Xyn1818 presented four protein bands on gel while the fraction corresponding to Xyn3613 showed three protein bands (data not shown). Mass spectrometry was then performed to confirm the identity of the proteins, since in both cases one main protein band correctly related to the molecular mass of the Xyn1818 and Xyn3613 was visualized, and the other protein bands presented lower molecular mass. The mass spectrometry results showed that the proteins visualized on gel for Xyn1818 and Xyn3613 were identified as xylanases from *A. nidulans* of 35 and 24 kDa, respectively. In addition, the tryptic ions generated for the bands of the gel were compared and it was observed that they shared common peptides that were confirmed by *de novo* sequencing (Figure 2). Thus, it was verified that the four protein bands presented by Xyn1818 were confirmed as Xyn1818 and the three protein bands related to Xyn3613 were identified as Xyn3613. These results confirm that both xylanases, Xyn1818 and Xyn3613, were pure and the extra protein bands were fragments of these enzymes.

3.2. Biochemical characterization of Xyn1818 and Xyn3613

Xyn1818 exhibited substantial activity against xylan from beechwood within a pH range of 5.0-9.0 and temperature range of 50-60°C, while Xyn3613 showed the highest activities in the pH range 5.0-8.0 and the same temperature range. The optima pH and temperature were 7.5 and 60°C for Xyn1818, and Xyn3613 achieved maximal activity at pH 6.0 and 50°C (Figure 3A-D). Since Xyn1818 and Xyn3613 presented more than 75% of their activities at pH 5.0, the standard activity assay was

performed at this pH value. These data are in accordance with previous studies which reported that recombinant xylanases from *Aspergillus terreus*, *Plectosphaerella cucumerina*, and *Alternaria* sp. HB186**,** also expressed in *P. pastoris*, presented maximal activity at pH 5.0 and 60°C (Chantasingh et al., 2006), pH 6.0 and 40°C (Zhang et al., 2007) and pH 6.0 and 50°C, respectively (Mao et al., 2012).

Xyn1818 and Xyn3613 showed significant stability over a wide pH range (Figure 3A-B). After pre-incubation for 30 min in pH values below 4.0 and above 10.0, Xyn1818 recovered over 65% and 90% of its initial activity, respectively, when this enzyme returned to pH 5.0. The results show that Xyn1818 was able to refold and retrieve its activity after pre-incubation in extreme pH ranges. Similar results were observed for Xyn3613 that recovered over 90% of its initial activity after 30 min of pre-incubation in pH values lower than 4.0 and higher than 9.0.

Concerning thermal stability (Figure 3E-F), Xyn1818 showed to be very stable at 50°C. This enzyme presented about 70% of its original activity after 2 h of pre-incubation at this temperature and this residual activity was maintained until 6 h of pre-incubation. This enzyme still retained more than 30% of its original activity after 100 h of pre-incubation, and after 170 h the residual activity was 25%. However, at 60°C, Xyn1818 lost activity after 9 hours of pre-incubation. Xyn3613 presented lower thermal stability compared to Xyn1818, since it retained only 28% of its initial activity after 6 hours of incubation at 50°C and it lost all activity after 30 minutes at 60°C. Xyn1818 and Xyn3613 presented half-life values of 49 h 20 min and 1 h 10 min, respectively, at 50°C, and at 60°C the half-life values were 20 min and 5.8 min, respectively. These stabilities presented by both enzymes for a wide pH range and reasonable temperature levels are desirable for industrial applications.

Activity of Xyn1818 and Xyn3613 against several substrates is shown in Table 1. The xylanases did not hydrolyze the aryl synthetic substrates containing α- or β-linkages and different monosaccharides, indicating that these enzymes exhibit only endo activity. This result was expected since the cloned genes correspond to xylanases from GH10 and GH11 families (Bauer et al., 2006). The exception referred to the ability of Xyn1818 to hydrolyze pNPβCel, to a lesser extent. In relation to the natural substrates, both xylanases were able to hydrolyze the different xylans. Xyn1818 presented higher activity against wheat arabinoxylan, followed by xylan

from beechwood and oat spelt xylan, while Xyn3613 had the best activity against xylan from beechwood. The structure of the different xylans varies between plant species and tissues, and also during cellular differentiation (Ebringerova et al., 2005). Arabinoxylan is a highly decorated form of xylan since it presents a more complex structure with many arabinofuranose decorations (Correia et al., 2011). In oat spelt xylan the most abundant carbohydrate is xylose followed by arabinose, while in xylan from beechwood about 95% of the carbohydrate consists of xylose (Li et al., 2000). Indeed, Xyn1818 is a GH10 xylanase which presents a broader substrate specificity with the ability to degrade the substituted portions of xylans (van den Brink and de Vries, 2011, Pollet et al., 2010, Biely et al., 1997), unlike Xyn3613 that is a GH11 and requires a higher number of unsubstituted backbone residues, being less active on highly branched xylans (Bauer et al., 2006).

The enzymes showed distinct sensitivities to ions and reducing agents (Table 2). Xyn1818 and Xyn3613 were completely inhibited by SDS and $HgCl_2$ in both tested concentrations. $CuSO_4$ completely inhibited Xyn1818 at both tested concentrations, but total inactivation of Xyn3613 was achieved only at 10 mM concentration. The denaturing action of SDS probably affected the integrity of the enzyme tridimensional structure which is fundamental for its catalytic activity (Maitan-Alfenas et al., 2014b). The ion Cu^{2+} is known to be a strong inhibitor of xylanases (Zhang et al., 2007). Mao et al. (2012) showed that Cu^{2+} and Hg^{2+} ions inhibited most of the xylanase activity of *Alternaria* sp. expressed in *P. pastoris*. Xyn1818 presented no activation by the tested compounds, but $MgCl_2$ and $MnCl_2$ were able to activate Xyn3613 in both tested concentrations. β-Mercaptoethanol, a reducing agent, promoted the activation of Xyn3613 which can be explained by the fact that some reduced chemical ligations in the enzyme structure are favorable for the catalytic activity (Maitan-Alfenas et al., 2014b).

3.3 Synergy Experiments

The results present in Figure 4 show the synergy between enzyme activities present in the cocktails and the recombinant xylanase activities. Synergism was observed for xylanase and endoglucanase activities. The synergism was higher for endoglucanase activity, varying between 3.3 and 9.9-fold the theoretical activity. For xylanase activity, synergism enhanced the theoretical activity by 1.1 to 2.9-fold. The

increment in activities was higher for Multifect® CL compared to Accellerase® 1500. This indicates that the enzymes contained in Multifect® CL present a more significant synergistic effect for endoglucanase and xylanase activities. The synergism consequence for endoglucanase activity is very interesting since the addition of xylanases (Xyn1818 and/or Xyn3613) is able to increase cellulase activity presented by the commercial cocktails. However, the xylanases were not able to hydrolyze CMC when tested for substrate specificity. Therefore, xylanases potentiate endoglucanase activities, a characteristic of the synergistic effect.

3.4. Saccharification Experiments

Alkali-pretreated sugarcane bagasse was hydrolyzed by commercial cocktails rich in cellulase activities (Multifect® CL and Accellerase® 1500) with or without the supplementation by Xyn1818 and/or Xyn3613 (Figure 5).

Concerning sugarcane bagasse saccharification by Multifect® CL, increased glucose release (6.94 g/l) was obtained when the commercial cocktail was supplemented with both xylanases, Xyn1818 and Xyn3613. Xylanases are not able to produce glucose directly but they stimulate cellulose hydrolysis since they act in the hemicellulose fraction and facilitate the access of cellulolytic enzymes to the cellulose portion which increases the glucose concentration (Hu et al., 2011, Várnai et al., 2011). Concerning xylose release from sugarcane bagasse saccharification, Multifect® CL without supplementation and the mixture Multifect® CL with both xylanases, Xyn1818 and Xyn3613, were able to promote the higher yields, 3.36 and 3.37 g/l, respectively. In this case, the expected effect of the xylanases was not visualized because only xylose was quantified and other xyloogosaccharides were not measured for further conclusions.

When sugarcane bagasse saccharification was performed by Accellerase® 1500, the supplemented mixtures were responsible for higher glucose release. Accellerase®1500 without supplementation promoted the release of 6.66 g/l of this sugar while the mixture Accellerase® 1500 with Xyn3613 was able to release 8.3 g/l of glucose, the higher value. For xylose release, the supplementation with Xyn3613 promoted the best yield, 4.54 g/l. These results show that Xyn3613 (GH11) presents a better potential for sugarcane bagasse hydrolysis when used as a supplement for the commercial mixture Accellerase® 1500. Indeed, GH11 xylanases have been

used for a long time as biotechnological tools in various industrial applications due to several of their interesting properties including high substrate selectivity and high catalytic efficiency, small size (around 20 kDa), and variety of optimum pH and temperature and they are considered true xylanases (Paes et al., 2012).

The saccharification assays performed with Accellerase® 1500 resulted in higher releases of glucose and xylose when compared to the experiments performed by Multifect® CL. This indicates that Accellerase® 1500, when used as cellulases source, leads to higher sugar release from alkali-pretreated sugarcane bagasse, with or without supplementation.

The total reducing sugars were measured after 72 h of saccharification experiments (Table 3). These results confirm that when Multifect® CL was used for sugarcane bagasse hydrolysis, the best yield was achieved by supplementation of this cocktail with both xylanases, Xyn1818 and Xyn3613. For the experiments performed with Accellerase® 1500, the maximal release of sugars occurred when the cocktail was supplemented with Xyn3613.

Saccharification yields are represented in Table 3. For Multifect® CL, the supplementation with both xylanases, Xyn1818 and Xyn3613, presented the best yields, 16.25% of glucan conversion and 14.45% of xylan conversion. In fact, the xylanase activity improved almost 3-fold when Multifect® CL was supplemented with Xyn1818 and Xyn3613, and the synergy enhanced the endoglucanase activity more than 8-fold for the same enzymatic mixture (Figura 4). For Accellerase® 1500, the supplementation with Xyn3613 rendered the best results, where 19.40% and 19.63% for glucan and xylan conversions were observed, respectively. Actually, the synergism results show that xylanase activity was improved by about 1.5-fold while this increment in endoglucanase activity was over 4-fold for the same enzyme mixture (Figure 4). Therefore, synergism could be one of the factors that contributes to better saccharification yields. When enzymes act in synergy, the total effect is greater than the sum of the effects of the individual components (Kostylev and Wilson, 2011). Complementing crude enzyme extracts shows promise since it can result in synergistic effects to improve biomass saccharification efficiency (Visser at al., 2013).

4. Conclusion

Two xylanases from *A. nidulans* expressed in *P. pastoris,* Xyn1818 (GH10) and Xyn3613 (GH11), were studied and their ability to degrade different types of xylans was demonstrated. These enzymes showed to be stable in a wide pH range and reasonable temperatures and they were able to act in synergy with xylanases and endoglucanases present in commercial enzymatic cocktails. They also presented satisfactory results when used in supplementation with commercial enzyme cocktails for alkali-pretreated sugarcane bagasse hydrolysis. Therefore, the xylanases Xyn1818 and Xyn3613 present great potential for biotechnological conversions, mainly supplementation of commercial cocktails for biomass saccharification in the second generation ethanol production process.

Acknowledgements

We acknowledge the Brazilian institutions CAPES for the scholarship granted to the first and second authors and FAPEMIG and CNPq for the resources provided to complete this experiment

References

Ahmed S., Riaz, S., Jamil, A., 2009. Molecular cloning of fungal xylanases: an overview. Applied Microbiology and Biotechnology 84, 19–35.

Bauer, S., Vasu, P., Mort, A. J., Somerville, C. R., 2005. Cloning, expression, and characterization of an oligoxyloglucan reducing end-specific xyloglucanobiohydrolase from *Aspergillus nidulans*. Carbohydrate Research *340*, 2590-2597.

Bauer, S., Vasu, P., Persson, S., Mort, A. J., Somerville, C. R., 2006. Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. Proceedings of the National Academy of Sciences of the United States of America 103, 11417-11422.

Biely P., Vrsanska M., Tenkanen M., Kluepfel D.. 1997. Endo-beta-1,4-xylanase families: differences in catalytic properties. Journal of Biotechnology 57, 151–166.

Bradford M.M., 1976. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. Analytical Biochemistry 72, 248-254.

Chantasingh, D., Pootanakit, K., Champreda, V., Kanokratana, P., Eurwilaichitr, L., 2006. Cloning, expression, and characterization of a xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris*. Protein Expression and Purification 46, 143-149.

Correia, M.A.S., Mazumder, K., Brás, J.L.A., Firbank, S.J., Zhu, Y., Lewis, R.J., York, W.S., Fontes, C.M.G.A., Gilbert, H.J., 2011. Structure and Function of an Arabinoxylan-specific Xylanase. The Journal of Biological Chemistry 286, 22510-22520.

Daly, R., Hearn, M.T., 2005. Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production. Journal of Molecular Recognition 18,119-138.

de Vries R.P., Kester H.C.M., Poulsen C.H., Benen J.A.E., Visser J., 2000. Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. Carbohydrate Research 327, 401-410.

de Vries R.P., Visser J., 2001. Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. Microbiology and Molecular Biology Reviews 65(4):497–522.

Dodd, D., Cann, I.K.O., 2009. Enzymatic deconstruction of xylan for biofuel production. Global Change Biology Bioenergy 18, 2-17.

Ebringerova, A., Hromadkova, Z., Heinze, T., 2005. Polysaccharides 1: Structure, Characterization and Use. Heinze, T., editor. pp. 1–67, Springer-Verlag, Berlin.

Ghose T.K., 1987. Measurement of cellulase activities. Pure & Applied Chemistry 59, 257-268.

Hu, J., Arantes, V., Saddler, J., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnology for Biofuels 4, 36

Kostylev M., Wilson D., 2011. Synergistic interactions in cellulose hydrolysis. Biofuels 3, 61-70.

Kulkarni N., Shendye, A., Rao, M., 1999. Molecular and biotechnological aspects of xylanases. FEMS Microbiology Reviews 23, 411–456.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during Assembly of Head of Bacteriophage-T4. *Nature, 227*(5259), 680-685.

Lee, H.V., Hamid, S.B.A., Zain, S.K., 2014. Conversion of Lignocellulosic Biomass to Nanocellulose: Structure and Chemical Process. The Scientific World Journal, http://dx.doi.org/10.1155/2014/631013.

Li, K., Azadi, P., Collins, R., Tolan, J., Kim, J.S., Eriksson, K.E.L., 2000. Relationships between activities of xylanases and xylan structures. Enzyme and Microbial Technology 27, 89-94.

Maitan-Alfenas, G.P., Visser, E.M., Guimarães, V.M., 2014a. Enzymatic Hydrolysis of Lignocellulosic Biomass: Converting Food Waste in Valuable Products. Current Opinion in Food Science, http://dx.doi.org/10.1016/j.cofs.2014.10.001.

Maitan-Alfenas, G.P., Lage, L.G.A., de Almeida, M.N., Visser, E.M., de Rezende, S.T., Guimarães, V.M., 2014b. Hydrolysis of soybean isoflavones by *Debaryomyces hansenii* UFV-1immobilised cells and free b-glucosidase. Food Chemistry 146, 429-436.

Mao, L., Meng, P., Zhou, C., Ma, L., Zhang, G., Ma, Y., 2012. Molecular cloning and heterologous expression of an acid stable xylanase gene from *Alternaria* sp. HB186. World Journal of Microbiology and Biotechology 28, 777-784.

Mellitzier, A., Weis, R., Glieder, A., Flicker, K., 2012. Expression of lignocellulolytic enzymes in Pichia pastoris. Microbial Cell Factories 11, 61-71.

Miller G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31, 426-428.

Motta, F., Andrade, C., Santana, M., 2013. A Review of Xylanase Production by the Fermentation of Xylan: Classification, Characterization and Applications. Chandel, A. K. and Silva, S. S. (Editors). Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization: InTech, cap. 10.

Paes, G., Berrin, J.G., Beaugrand, J., 2012. GH11 xylanases: Structure/function/properties relationships and applications. Biotechnology Advances 30, 564-592.

Pérez, J., Muñoz-Dorado, J., de la Rubia, T., Martínez, J., 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview., International Microbiology: the Official Journal of the Spanish Society for Microbiology 5, 53–63.

Polizeli M.L., Rizzatti A.C., Monti R., Terenzi H.F., Jorge J.A., Amorim D.S., 2005. Xylanases from fungi: properties and industrial applications. Applied Microbiology and Biotechnology 67, 577–591.

Pollet A., Delcour J.A., Courtin C.M., 2010. Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families. Critical Reviews in Biotechnology 30, 176–191.

Qiu Z., Shi P., Luo H., Bai Y., Yuan T., Yang P. A., 2010. A xylanase with broad pH and temperature adaptability from Streptomyces megasporus DSM 41476, and its potential application in brewing industry. Enzyme and Microbial Technology 46, 506–512.

Shi, H., Zhang, W., Li, X., Huang, Y., Wang, L., Wang, Y., Ding, H., Wang, F., 2013. A novel highly thermostable xylanase stimulated by Ca2+ from *Thermotoga thermarum*: cloning, expression and characterization. Biotechnology for Biofuels 6, 26.

Taibi Z., Saoudi B., Boudelaa M., Trigui H., Belghith H., Gargouri A., 2012. Purification and biochemical characterization of a highly thermostable xylanase from Actinomadura sp. strain Cpt20 isolated from poultry compost. Applied Biochemistry and Biotechnology 166, 663–679.

Tolner, B., Smith, L., Begent, R.H., Chester, K.A., 2006. Production of recombinant protein in *Pichia pastoris* by fermentation. Nature Protocols 1,1006–1021.

Várnai, A., Huikko, L., Pere, J., Siika-aho, M., Viikari, L., 2011. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresource Technolology 102, 9096-9104.

van den Brink, J., de Vries, R.P., 2011. Fungal enzyme sets for plant polysaccharide degradation. Applied Microbiology and Biotechnology 91, 1477-1492.

Van Dyk, J.S., Pletschke, B.I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes – factors

affecting enzymes, conversion and synergy. Biotechnology Advances 30, 1458–1480.

Visser, E.M., Falkoski, D.L., de Almeida, M.N., Maitan-Alfenas, G.P., Guimarães, V.M., 2013. Production and application of an enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* with potential for hydrolysis of sugarcane bagasse. Bioresource Technology 144,587-594.

Zhang, G.M., Huang, J., Huang, G.R., Ma, L.X., Zhang, X.E., 2007. Molecular cloning and heterologous expression of a new xylanase gene from *Plectosphaerella cucumerina*. Applied Microbiology and Biotechnology, 74, 339-346.

Tables and Figures

Table 1: Hydrolysis of several substrates by the xylanases Xyn1818 and Xyn3613.

		Xyn1818	Xyn3613
Substrate	**Final Concentration**	**Activity (U mL-1)±DP**	
ρNPβXyl	1 mM	0	0
ρNPαGal	1 mM	0	0
ρNPβCel	1 mM	0.41±0.006	0
ρNPβGlc	1 mM	0	0
ρNPαAra	1 mM	0	0
ρNPβGal	1 mM	0	0
ρNPβMan	1 mM	0	0
ρNPαMan	1 mM	0	0
xylan from beechwood	1%	31.12±0.022	23.64±0.014
oat spelt xylan	1%	19.64±0.043	7.32±0.006
wheat arabinoxylan	1%	47.57±0.031	10.62±0.007
Avicel®	1%	0	0
CMC	1%	0	0

Table 2: Relative activity of the xylanases Xyn1818 and Xyn3613 submitted to different ions and reducing agents. Relative activities were calculated in relation to the xylanase activity without pre-incubation which was considered to be 100%.

		Xyn1818	Xyn3613
Ion/Reducing Agent	**Concentration (mM)**	**Relative Activity (%)±DP**	
$HgCl_2$	10	0	0
	2	0	0
$ZnCl_2$	10	56.12±0.009	73.65±0.016
	2	58.07±0.002	98.24±0.024
NaCl	10	97.88±0.001	93.24±0.020
	2	92.76±0.001	114.44±0.036
$MgCl_2$	10	86.79±0.032	129.97±0.019
	2	83.65±0.035	113.59±0.015
EDTA	10	57.62±0.016	76.68±0.027
	2	64.34±0.012	90.17±0.014
SDS	10	0,00	0,00
	2	0,00	0,00
$MnCl_2$	10	93.18±0.097	119.70±0.022
	2	101.58±0.039	116.52±0.017
$CaCl_2$	10	90.28±0.061	95.44±0.012
	2	90.14±0.029	113.21±0.040
$CuSO_4$	10	0	0
	2	0	93.66±0.009
β-mercapthoethanol	1	87.64±0.013	122.66±0.013

Table 3: Total reducing sugars (the results are mean values ± SD of three measurements) and percentage of glucan and xylan conversions after 72 hours of sugarcane bagasse saccharification by the different enzyme mixtures.

Enzymatic Mixture	Reducing Sugars ± SD	Saccharification Yield – Conversion	
	(µmol/ml)	Glucan (%)	Xylan (%)
Multifect CL	68.90±0.026	14.03	14.45
Multifect CL + 1818	68.36±0.052	14.38	12.94
Multifect CL + 3613	71.84±0.084	14.73	12.73
Multifect CL + 1818 + 3613	72.30±0.116	16.25	14.45
Accellerase	74.93±0.062	15.55	16.82
Accellerase + 1818	79.19±0.050	17.53	17.25
Accellerase + 3613	80.33±0.029	19.40	19.63
Accellerase + 1818 + 3613	69.61±0.005	18.00	17.04

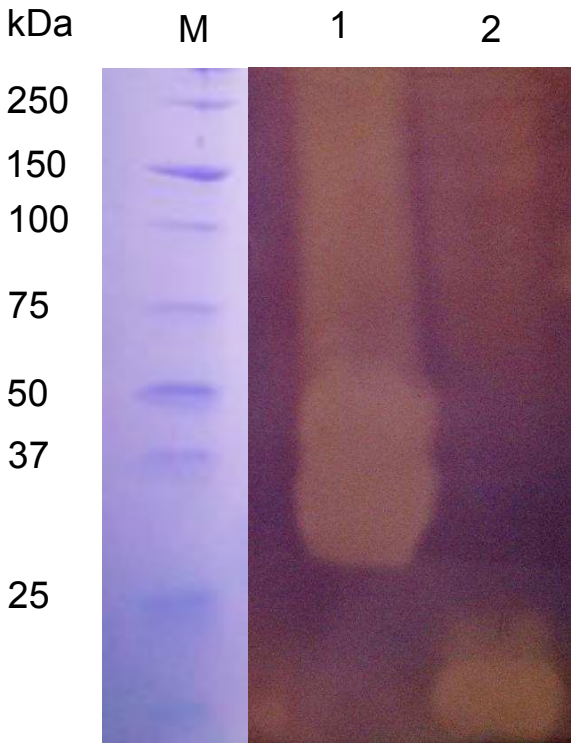


Figure 1: Zymogram of xylanases (SDS-PAGE 12% containing 1% of birchwood xylan). M - molecular mass marker stained with Comassie Brilliant Blue. 1 - Crude extract containing Xyn1818. 2 - Crude extract containing Xyn3613.

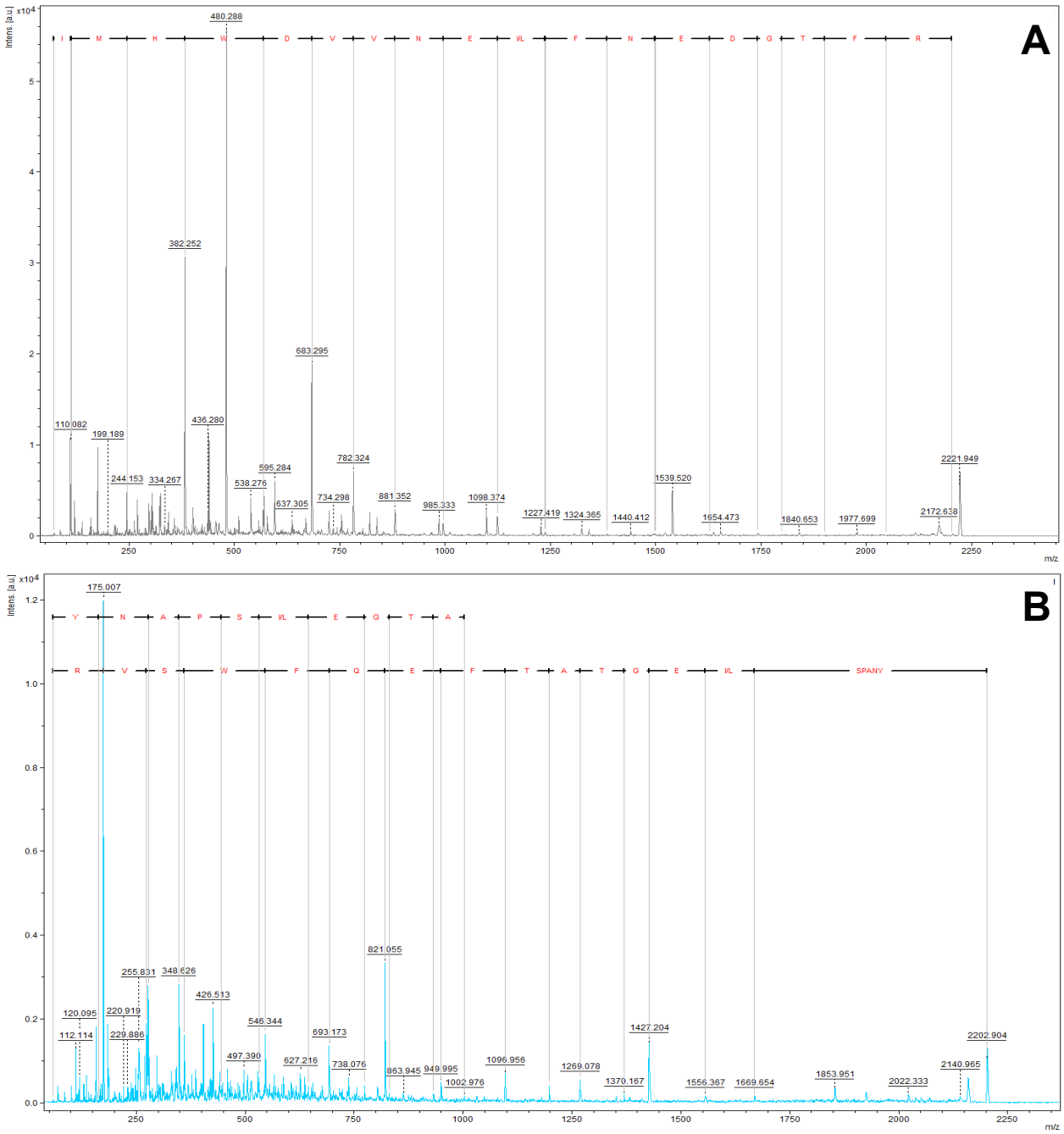


Figure 2: Mass spectrometry: *de novo* sequencing for (**A**) Xyn1818 fragments and (**B**) Xyn3613 fragments.

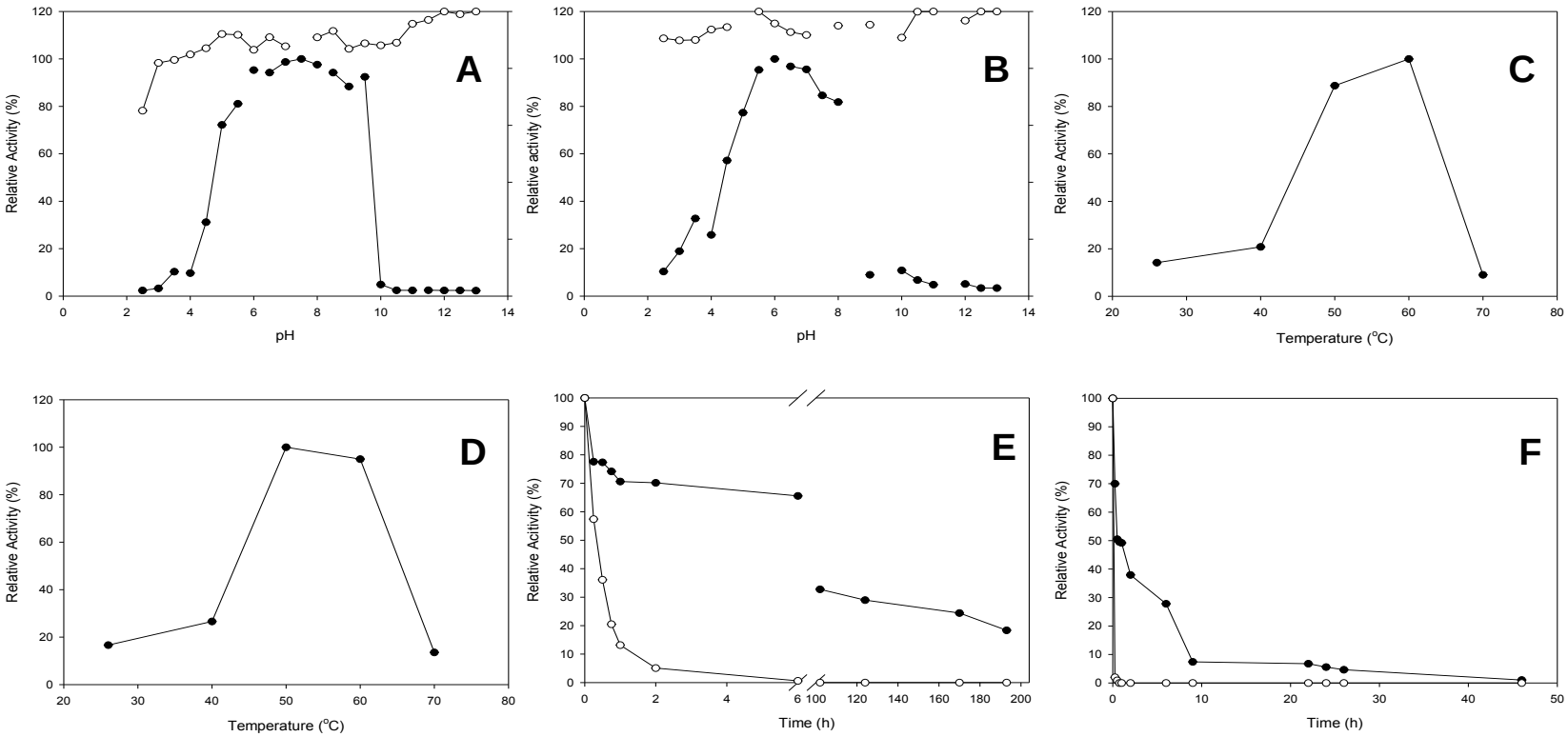


Figure 3: Effect of pH and temperature on the xylanases from *A. nidulans*. (**A**) Effect of pH on activity (●) and stability (○) of Xyn1818. (**B**) Effect of pH on activity (●) and stability (○) of Xyn3613. (**C**) Effect of temperature on Xyn1818. (**D**) Effect of temperature on Xyn3613. (**E**) Thermostability at 50 (●) and 60°C (○) for Xyn1818. (**F**) Thermostability at 50 (●) and 60°C (○) for Xyn3613. Relative activities were calculated in relation to xylanase activities without pre-incubation, which was considered to be 100 %.

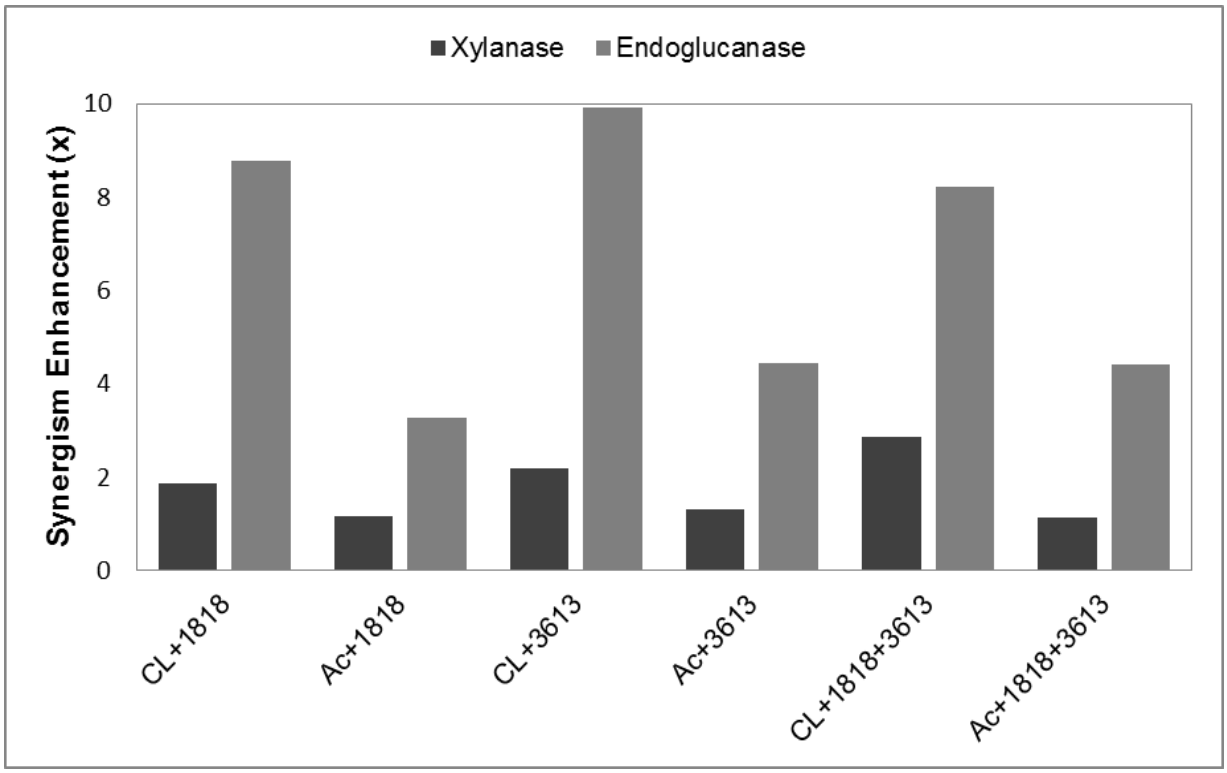


Figure 4: Synergism between the xylanases Xyn1818 and Xyn3613 and the enzymes present in the enzymatic cocktails Multifect® CL (represented as CL) and Accellerase® 1500 (represented as Ac). Synergism is expressed as a multiple of the theoretical activity.

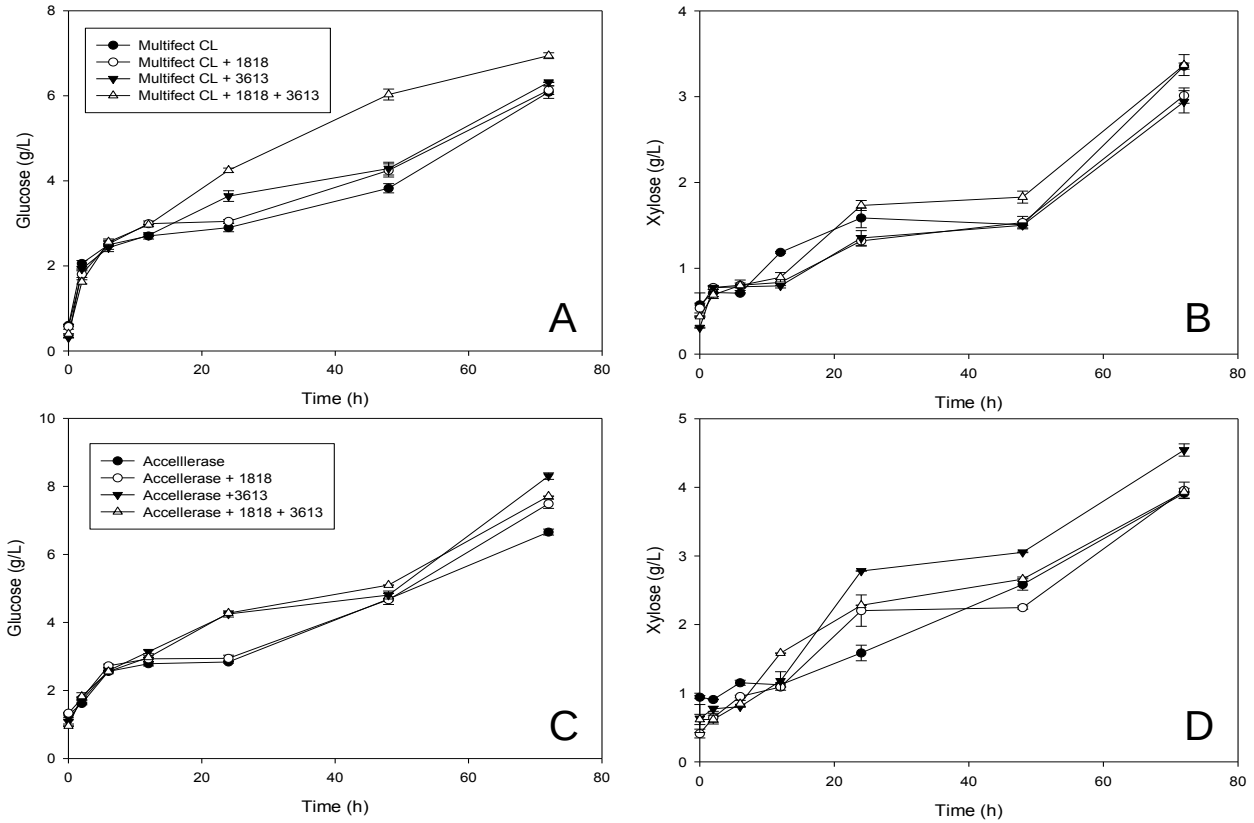


Figure 5: Saccharification assays of alkali-pretreated sugarcane bagasse. (A) Glucose and (B) xylose release using Multifect® CL as the cellulase source. (C) Glucose and (D) xylose release using Accellerase® 1500 as the cellulase source.

CHAPTER 5

Synergistic effect of *Aspergillus niger* and *Trichoderma reesei* enzyme sets on the saccharification of wheat straw and sugarcane bagasse

Authors: Joost van den Brink, Gabriela Piccolo Maitan-Alfenas, Gen Zou, Chengshu Wang, Zhihua Zhou, Valéria Monteze Guimarães and Ronald P. de Vries

Published on: Biotechnology Journal, DOI: 10.1002/biot.201400317.

Synergistic effect of *Aspergillus niger* and *Trichoderma reesei* enzyme sets on the saccharification of wheat straw and sugarcane bagasse

Joost van den Brink[1], Gabriela Piccolo Maitan-Alfenas[2], Gen Zou[3], Chengshu Wang[3], Zhihua Zhou[3], Valéria Monteze Guimarães[2] and Ronald P. de Vries[1]

[1] CBS Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands
[2] Department of Biochemistry and Molecular Biology, Federal University of Viçosa, Brazil
[3] Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China

Correspondence: Prof. Ronald de Vries, CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
E-mail: r.devries@cbs.knaw.nl

Abbreviations

WS, wheat straw; SCB, sugarcane bagasse; FP, filter paper; CMC, carboxymethyl cellulose

Abstract

Plant-degrading enzymes can be produced by fungi on abundantly available low-cost plant biomass. However, enzymes sets after growth on complex substrates need to be better understood, especially with emphasis on differences between fungal species and the influence of inhibitory compounds in plant substrates, such as monosaccharides. In this study, *Aspergillus niger* and *Trichoderma reesei* were evaluated for the production of enzyme sets after growth on two 'second generation' substrates: wheat straw and sugarcane bagasse. *A. niger* and *T. reesei* produced different sets of (hemi-)cellulolytic enzymes after growth on wheat straw and sugarcane bagasse. This was reflected in an overall strong synergistic effect in releasing sugars during saccharification using *A. niger* and *T. reesei* enzyme sets. *T. reesei* produced less hydrolytic enzymes after growth on non-washed sugarcane

bagasse. The sensitivity to non-washed plant substrates was not reduced by using CreA/Cre1 mutants of *T. reesei* and *A. niger* with a defective carbon catabolite repression. The importance of removing monosaccharides for producing enzymes was further underlined by the decrease in hydrolytic activities with increased glucose concentrations in WS media. This study showed the importance of removing monosaccharides from the enzyme production media and combining *T. reesei* and *A. niger* enzyme sets to improve plant biomass saccharification.
Keywords: *Aspergillus niger*, *Trichoderma reesei*, saccharification, plant biomass, synergy

Practical applications

The production costs of plant-degrading enzyme mixtures can be lowered by growing fungi on abundantly available low-cost substrates. This study supported this strategy by showing that the enzyme sets produced by *Aspergillus niger* and *Trichoderma reesei* grown on wheat straw or sugarcane bagasse were fine-tuned in releasing sugars from these substrates. Even better enzyme mixture was achieved when both enzyme sets were combined and monosaccharides were removed from the enzyme production media. This study therefore provides leads to improve industrial enzyme productions for plant biomass saccharification.

1- Introduction

Sustainable production of fuels and chemicals from plant biomass requires efficient enzymatic hydrolysis of plant polymers into fermentable sugars. The feasibility of biofuels and biochemical production is largely dependent on cost-efficiency of the process. Besides materials and transport, an important part of the overall production costs are the commercial enzyme mixtures [1, 2]. One possibility to reduce the costs of plant-degrading enzyme mixtures is on-site enzyme production [3, 4]. This process avoids transport costs and extensive down-stream process to purify and stabilize the enzymes produced. Furthermore, common substrates to grow enzyme producing fungi on, such as mono-, or disaccharides, are less abundant and more expensive than plant biomass substrates. Moreover, compared to mono-, or disaccharides, fungi grown on complex substrates are able to fine-tune their enzyme production to the available fractions within the biomass. One major challenge will be

that the produced enzymes have to degrade not just cellulose but all fractions within plant biomass (*e.g.* cellulose, hemicellulose, and pectin [5, 6]). Using plant biomass for enzyme production has the advantages that they are tailor made for releasing sugar from the same substrates. It should be noted that on average individual fungi do not aim to fully degrade plant biomass and, therefore, different fungi often target different components of the biomass. Consequently, each fungal species has different waste fractions after hydrolysis.

The filamentous fungi *Trichoderma reesei* and *Aspergillus niger* are common enzyme producers for plant polysaccharide degradation [5, 6]. *T. reesei* has a long history as a specific producer of cellulolytic enzymes [7]. Already in the fifties of the previous century, *T. reesei* was recognized to be a great producer of cellulases [8]. In the seventies, *T. reesei* strains such as QM9414 were created using random mutagenesis with increased cellulase production [9]. Several other mutagenetic studies have been performed which resulted in hypercellulolytic strains, of which RUT-C30 is the most renowned one [10]. Major features of RUT-C30 were its derepressed carbon regulation and it presented high specific activities with emphasis on endo- and exoglucanase activities [7]. However, *T. reesei* has a relatively low β-glucosidase activity which limits the release of monosaccharides during saccharification of plant biomass [11]. Therefore, enzyme sets of *T. reesei* for cellulose degradation are often complemented with β-glucosidases. Many studies improved the release of glucose by producing heterologous β-glucosidases from other fungi, such as *Aspergilli* [12-14]. This complementation was also commercialized by combining *T. reesei*'s Celluclast® and *A. niger*'s β-glucosidase Novozym188® [15].

Unlike *T. reesei*, strain development of *A. niger* has not specifically focused on cellulolytic enzymes. *A. niger* produces many different enzymes when exposed to plant biomass such as wheat straw (WS) or sugarcane bagasse (SCB) [16, 17]. Growth on WS revealed that *A. niger* produced a higher percentage of plant-degrading enzymes in comparison to *T. reesei* [18]. Furthermore, *A. niger* strains have been used for the production of a range of plant-degrading enzymes such as pectinases [19, 20], xylanases [21, 22], mannanases [23, 24], and esterases [25, 26]. However, compared to *T. reesei*, fewer *A. niger* strains with fine-tuned derepression of carbon regulation are available. Production of hydrolytic enzymes in *A. niger* has

shown to be strongly dependent on the presence of the monosaccharides glucose and xylose [27]. For instance, a mixture of glucose and xylose repressed the expression of feruloyl esterase, α-glucuronidase, endoxylanase and β-xylosidase. The genes encoding these xylanolytic enzymes and others were expressed in the presence of glucose and/or xylose after deletion of the main central carbon regulator CreA [28, 29]. Not many studies have focused on the overall effect on the saccharification of plant biomass by enzymes of *A. niger* CreA deletion strains after growth on complex substrates.

The majority of studies have been focused on one aspect of plant biomass saccharification, e.g. one class of enzymes, one fungal platform, or one plant substrate. The aim of this study is to compare and understand the saccharification efficiency of the plant-degrading enzyme sets produced by two fungal platforms, *A. niger* and *T. reesei*, during growth on two major agricultural waste streams, WS and SCB. The saccharification capacity of both enzyme sets will be analyzed separately and combined. The two *T. reesei* strains studied in this comparison were the high-cellulase producing strain QM9414^{ref} and the hypercellulolytic RC30-8^{cre1} strain [30, 31], in which carbon regulation was largely derepressed. Furthermore, *A. niger* strains with (NW249^{ref}, [32]) or without (FP712^{creA}) the general carbon repressor CreA were taken along in this comparison.

2- Material and Methods

2.1- *T. reesei* and *A. niger* strains

The *Trichoderma reesei* strains used were QM9414^{ref} and RC30-8^{cre1}. RC30-8^{cre1} had hypercellulolytic properties due to a truncated *cre1* gene causing impaired CRE1-mediated carbon catabolite repression [30, 31]. *Aspergillus niger* NW249^{ref} (*cspA1, ΔargB, pyrA6, leuA1, nicA1*) was used as reference [32]. The *creA* deletion in this strain (FP712^{creA}; *cspA1, pyrA6, nicA1, leuA1, PyrG:ΔCreA*) was created using standard DNA manipulations and molecular biology techniques [33]. Plasmid pXY1.1 was used containing 3'- and 5'- flanking regions of CreA bordering *A. oryzae* PyrG gene [34]. *A. niger* mutants were selected for uridine prototrophy after transformation. Deletion of CreA was confirmed by PCR using internal and external primers for NW249^{ref} and *creA* deletion mutant (Table 1). *Aspergillus niger* strains NW249^{ref} and

FP712^{creA} were deposited at the CBS Fungal Biodiversity Centre as CBS 137562 and CBS 137561, respectively.

2.2- Materials

Two sources of plant biomass were used for growth and saccharification experiments: WS [35] and SCB [36]. The plant biomasses were used in a 3% concentration. WS contained 36% glucan and 21% xylan [35], while SCB contained 53% glucan and 19% xylan [36]. After sterilization, 'non-washed' WS medium contained 1.6 ± 0.1 mM dissolved sugars as measured by reducing sugar assay using 3,5-dinitrosalicylic acid solution. Similarly, 'non-washed' SCB medium contained 33.4 ± 1.9 mM dissolved sugars. While only glucose was present in 'non-washed' SCB medium, glucose and xylose were present in equal amounts in 'non-washed' WS medium according to enzymatic determination using Megazyme kits (K-FRGLQR and K-XYLOSE). 'Washed' substrates meant that the easily accessible sugars were removed by making a 3% solution with demineralized water, which was heated for 20 min at 121°C, followed by a filter step using Whatman filter papers, quality 4, and a thorough washing of the solid fraction with demineralized water until no detectable amounts of sugars were present in the filtrates of washed media.

2.3- *T. reesei* and *A. niger* growth

All *A. niger* and *T. reesei* strains were initially grown on Malt Extract Agar plates. Spore solutions were made using ACES buffer: 10 mM N-(2-acetamido)-2-aminoethanesulfonic acid, 0.02% Tween 80, pH 6.8. The cultivation using $1 \cdot 10^6$ spores $\cdot mL^{-1}$ were performed in 500 ml shake flasks with 100 mL media. *A. niger* strains were grown for 4 days at 30°C and 250 rpm on an Aspergillus minimal media containing 3% pretreated plant biomass and appropriate supplements [37]. *T. reesei* strains were grown for 4 days at 28°C and 250 rpm on a Trichoderma minimal media containing 3% pretreated plant biomass [38].

2.4- Saccharification

Culture filtrates were incubated together with 50 mM NaAc buffer (pH 5.0) medium consisting of 3% pretreated plant biomass. Compared to the individual incubations, 50% of each culture filtrate was used with the combinations of *A. niger*

and *T. reesei* culture filtrates. Samples of the saccharification were taken every day for sugar analysis.

After centrifugation (3220 *g* for 5 min), the supernatant was used for saccharification product analysis. The released sugars were quantified by high performance liquid chromatography (HPLC) with a Shimadzu series 10A chromatograph (Shimadzu Corporation, Kyoto, Japan). The HPLC was equipped with an Aminex HPX-87P column (300 x 7.8 mm) and refractive index detectors. The column was eluted with water at a flow rate of 0.6 $mL \cdot min^{-1}$ at 80°C. Prior to injection, samples were filtered through a 0.45 µm filter and a volume of 20 µL was loaded. Standards (arabinose, glucose, xylose, cellobiose and xylobiose) were also analyzed to quantify these saccharides.

The combined incubations were compared to the theoretical release of sugars when the *A. niger* and *T. reesei* mixture would have been the sum of individual saccharification (0.5•*A. niger* + 0.5•*T. reesei*). This assumption was confirmed by a WS saccharification experiment using dilutions of *A. niger* NW249 culture filtrate after 4 days growth on WS (data not shown). Significance was tested using a Student's *t*-test with Excel 2010 (Microsoft, WA, USA), employing a two-tailed test.

2.5- Enzymatic assays

Cellulase, xyloglucanase and xylanase activities were determined against filter paper, carboxymethyl cellulose (Sigma-Aldrich, Germany), tamarind xyloglucan (Megazyme, Ireland), and beechwood xylan (Sigma-Aldrich, Germany), respectively. The assays contained a total volume of 200 µL using 10-50 µL of culture filtrates and 150 µL of 1% substrate in 50 mM sodium acetate buffer pH 5.0. The samples were incubated in microtiter plates for 30-120 min at 50°C. Subsequently, 100 µL of supernatant was mixed with 150 µL 3,5-dinitrosalicylic acid (DNS) solution. After an incubation of 30 min at 95°C, absorbance was measured at 540 nm in a microtiter platereader (FLUOstar OPTIMA, BMG LabTech). The activities were calculated using a standard curve ranging from 0 to 2 $g \cdot L^{-1}$ glucose.

Enzyme activities in the culture filtrates were measured using *p*-nitrophenol linked substrates (β-D-cellobioside N5759, β-D-glucopyranoside N7006, β-D-xylopyranoside N2132, α-L-arabinofuranoside N3641 of Sigma-Aldrich, Germany). The assays contained a total volume of 100 µL using 10-40 µL of the culture filtrates,

10 µL of 0.1 % *p*-nitrophenol linked substrates, and 25 mM sodium acetate buffer pH 5.0. Samples were incubated in microtiter plates for 30-120 min at 37°C. Reactions were stopped by addition of 100 µL 0.25 M Na_2CO_3. Absorbance was measured at 405 nm in a microtiter platereader (FLUOstar OPTIMA, BMG LabTech). The activities were calculated using a standard curve ranging from 0 to 80 nmol *p*-nitrophenol per assay volume.

Protein concentrations were determined using Modified Lowry Protein Assay Reagent Kit adapted for 96-wells microtiter plates (no. 23240, Pierce).

Significance of the fold changes between activities was tested using a Student's *t*-test with Excel 2010 (Microsoft, WA, USA), employing a two-tailed test.

3- Results

3.1- Saccharification activities against WS and SCB

A. niger and *T. reesei* strains were grown on wheat straw (WS) and sugarcane bagasse (SCB). Subsequently, as an initial experiment, saccharification activities of each culture filtrate were determined against both substrates. WS-grown cultures produced higher activities against WS compared to SCB-grown cultures (Figure 1). Conversely, SCB-grown cultures had higher activities against SCB. The difference in saccharification between WS and SCB culture filtrates was smallest for the *A. niger* strains. Also, CreA mutant FP712^{creA} did not show large differences compared to its reference NW249^{ref}, even though deletion of CreA had a strong effect on well-defined substrates (Supplemental Figure 1). The difference in activities was the largest for *T. reesei* RC30-8^{cre1} with WS-saccharification activities of 5.7 ± 0.7 µg·min^{-1}·mL^{-1} for WS cultures and 2.4 ± 0.1 µg·min^{-1}·mL^{-1} for SCB cultures. SCB-saccharification activities of RC30-8^{cre1} were 3.6 ± 0.8 µg·min^{-1}·ml^{-1} for WS cultures and 8.0 ± 0.1 µg·min^{-1}·mL^{-1} for SCB cultures. This initial experiment showed that enzymes produced on plant biomass were tailored to saccharification of the available substrate.

3.2- Hydrolytic activities during growth on WS and SCB

(Hemi-)cellulolytic activities were determined to better understand the differences between *A. niger* and *T. reesei*. Previous experiments indicated that growth on non-washed substrates produced enzyme sets with low saccharification activities (data not shown). Media with non-washed WS and SCB contained

dissolved sugars, 1.6 ± 0.1 mM and 33.4 ± 1.9 mM, respectively. Therefore, the strains were also grown on media with washed substrates (see Materials and Methods). The highest hydrolytic activities were measured after 4 days of growth (Figure 2).

Activities against filter paper (FP) and carboxymethyl cellulose (CMC) were measured as indicators of general cellulolytic activity. The CMC activities of the four strains were under most conditions around 0.1 – 0.2 μmol·min^{-1}·mL^{-1}, with exception of *T. reesei* strains after growth on SCB. On washed SCB, *T. reesei* QM9414^{ref} had a 3.6-fold (p < 0.0001) increase in activity compared to *A. niger* NW249^{ref}, while *T. reesei* RC30-8^{cre1} had a 8.5-fold (p < 0.0001) increase. *T. reesei* RC30-8^{cre1} also had a 1.6-fold (p = 0.048) increase in activity after growth on non-washed SCB. The FP activities were similar between all four strains after growth on non-washed WS (10 – 20 nmol·min^{-1}·mL^{-1}) and washed WS (45 – 75 nmol·min^{-1}·mL^{-1}). Similar to CMC activities, *T. reesei* QM9414^{ref} and RC30-8^{cre1} had, respectively, 11.2-fold and 18.8-fold (p < 0.0001) increase in FP activities compared to *A. niger* NW249^{ref}.

The activities against short gluco-oligosaccharides revealed low values for *T. reesei* and high values for *A. niger*. NW249^{ref} on washed SCB produced the highest cellobiohydrolase and β-glucosidase activities of 23 ± 1 and 49 ± 5 μmol·min^{-1}·mL^{-1}, respectively. In contrast to FP activity, cultivation on washed WS did not strongly induce cellobiohydrolase and β-glucosidase activities. The activities of NW249^{ref} and FP712^{creA} on non-washed WS and SCB were even higher than on washed WS. This indicated that other activities then CMCase, cellobiohydrolase and β-glucosidase contributed to FP activity after growth on washed WS. Concerning *T. reesei* activities, QM9414^{ref} and RC30-8^{cre1} showed more than 15-fold (p < 0.0004) decrease in activities compared to *A. niger* NW249^{ref} after growth on WS, and 4- to 6-fold (p < 0.0001) decrease in activities after growth on SCB.

General hemicellulolytic activities were measured against tamarind xyloglucan and beechwood xylan. As observed for endoglucanase, xyloglucanase activities were under most conditions around 0.1 – 0.5 μmol·min^{-1}·mL^{-1}, with exception of *T. reesei* strains after growth on SCB. RC30-8^{cre1} on non-washed and washed SCB had, respectively, 9.8-fold and 8.7-fold (p < 0.0001) increase in activities compared to *A. niger* NW249^{ref}. Concerning xylanase activity, *A. niger* and *T. reesei* produced the highest activities after growth on non-washed and washed SCB. The highest

xylanase activities was produced by *T. reesei* QM9414^{ref} and RC30-8^{cre1} after growth on washed SCB with a 3- to 4-fold (p < 0.0001) increase compared to *A. niger* NW249^{ref}.

Hemicellulolytic activities against short oligosaccharides showed less variation between strains and growth conditions. β-Xylosidase activities were between 20– 40 μmol·min^{-1}·mL^{-1}, with exception of the cultures on washed SCB. While *T. reesei* QM9414^{ref} produced low β-xylosidase activities on washed SCB, *A. niger* NW249^{ref} and *T. reesei* RC30-8^{cre1} had the highest β-xylosidase activities of 63 ± 3 and 52 ± 4 μmol·min^{-1}·ml^{-1}, respectively. Similarly, α-arabinofuranosidase activities were mostly stable with values between 11– 25 μmol·min^{-1}·mL^{-1}. Only *T. reesei* QM9414^{ref} and RC30-8^{cre1} after growth on washed SCB produced higher α-arabinofuranosidase activities (2.8-fold and 1.9-fold (p < 0.0001) increase, respectively) compared to *A. niger* NW249^{ref}.

These results showed that (hemi-)cellulolytic activities varied between species, strains, and growth conditions. Except low cellobiohydrolase and β-glucosidase activities for *T. reesei*, variation between the strains was small after growth on non-washed and washed WS. In contrast, growth on washed SCB caused strong differences between the strains with often the highest activities for *T. reesei* RC30-8^{cre1}. When SCB was not washed, the high activities of RC30-8^{cre1} strongly decreased for the substrates CMC, β-glucopyranoside, beechwood xylan, β-xylopyranoside, and α-arabinofuranoside.

3.3- Saccharification of WS and SCB using *A. niger* and *T. reesei* enzyme mixtures

The importance of the measured enzyme activities for the saccharification capacity was studied in detail by following the release of sugars from WS and SCB. The (hemi-)cellulolytic activities of *A. niger* and *T. reesei* seemed to complement each other with high activities of *A. niger* against short gluco-oligosaccharides and high activities of *T. reesei* against more complex substrates containing glucan and xylan polysaccharides. Therefore, *A. niger* and *T. reesei* enzyme sets were combined to study the synergistic effect in releasing sugars. According to measurements of total amounts of reduced sugars, the sugar release from the substrates steadily increased in all conditions during 5 days of incubation (Supplemental Figure 2). The

HPLC sugar analysis at day 5 only detected significant amounts of glucose and xylose, but no other sugars such as xylobiose and cellobiose (Figure 3).

The enzyme sets produced by *A. niger* released more sugars from non-washed WS than *T. reesei* sets. Enzymes from *A. niger* NW249^{ref} released 0.57 ± 0.02 glucose and 0.52 ± 0.05 g·L^{-1} xylose, while those from *T. reesei* RC30-8^{cre1} released 0.30 ± 0.01 glucose and 0.41 ± 0.01 g·L^{-1} xylose. The lower sugar release by *T. reesei* sets correlated with the lower enzyme activities of *T. reesei* sets on non-washed WS. The enzyme sets of CreA/Cre1 mutants did not release more sugars then those of their reference strains. In fact, FP712^{creA} released 0.28 ± 0.03 g·L^{-1} glucose, while NW249^{ref} released 0.57 ± 0.02 g·L^{-1} glucose. Mixtures of *A. niger* and *T. reesei* enzymes act synergistically when the release of sugars is higher than the sum of the individual saccharification (bold lines in Figure 3). Interestingly, the combination of *A. niger* FP712^{creA} and *T. reesei* RC30-8^{cre1} enzymes showed a 1.9-fold higher release of sugars (1.52 ± 0.03 g·L^{-1}) from non-washed WS compared to the sum of the individual enzyme sets (0.78 g·L^{-1}).

Washing WS improved sugar release using the individual enzyme sets. Especially xylose release was strongly increased using *T. reesei* enzyme sets. For instance, *T. reesei* RC30-8^{cre1} enzymes had a 2-fold higher release of xylose from washed WS (0.84 ± 0.09 g·L^{-1}) compared to non-washed WS. Even though xylose was released in the highest levels from WS, the synergistic effect of *A. niger* and *T. reesei* enzymes was the strongest for the release of glucose. A mixture of *A. niger* FP712^{creA} and *T. reesei* RC30-8^{cre1} enzymes showed a 4-fold higher release of glucose (0.88 ± 0.05 g·L^{-1}) compared to individual enzyme sets. In total, FP712^{creA} and RC30-8^{cre1} enzyme mixtures released 1.78 ± 0.13 g·L^{-1} sugar from washed WS. Again the CreA/Cre1 mutants did not produce improved enzyme sets after growth on WS.

Like non-washed WS saccharification, the enzyme sets produced by *A. niger* strains released more sugars from non-washed SCB than *T. reesei* sets. *A. niger* NW249^{ref} released 0.40 ± 0.08 glucose and 0.27 ± 0.05 g·L^{-1} xylose, while RC30-8^{cre1} released 0.14 ± 0.01 glucose and 0.14 ± 0.01 g·L^{-1} xylose. While glucose and xylose release was reasonable balanced in WS saccharification, a mixture of *A. niger* and *T. reesei* enzymes released far more glucose then xylose from SCB. Moreover, the synergistic effect was very strong between *A. niger* and *T. reesei* enzymes. The

mixture of *A. niger* NW249^{ref} and *T. reesei* QM9414^{ref} enzymes showed a 4-fold higher release of glucose (0.88 ± 0.01 g·L^{-1}) compared to the sum of the individual enzyme sets (0.21 g·L^{-1}).

Washing SCB significantly improved the release of glucose by *T. reesei* enzymes. In fact, glucose release using RC30-8^{cre1} enzymes improved 7-fold when washing SCB (0.98 ± 0.02 g·L^{-1} with washed SCB compared to 0.14 ± 0.01 g·L^{-1} glucose with non-washed SCB). Conversely, *A. niger* enzyme sets were not improved by washing SCB. Similar to non-washed SCB, the synergistic effect of *A. niger* and *T. reesei* enzymes was the strongest for the release of glucose. The mixture of *A. niger* FP712^{creA} and *T. reesei* RC30-8^{cre1} enzymes showed a 1.8-fold higher release of glucose (1.15 ± 0.01 g·L^{-1}) compared to the sum of the individual enzyme sets (0.63 g·L^{-1}). The total amount of sugars released from washed SCB (1.61 ± 0.03 g·L^{-1}) was similar to the total amount of sugars released from WS.

3.4- Sensitivity to extracellular glucose on enzyme production by *A. niger* and *T. reesei*

In particular *T. reesei* showed large differences in activities and saccharification capacity between washed and non-washed SCB. These clear differences were not observed between washed and non-washed WS. Non-washed SCB contained 33 mM of glucose after sterilization, while non-washed WS contained less than 2mM dissolved sugar. To confirm the influence of glucose on enzyme production, WS media was supplemented with different concentrations of glucose (1 – 50 mM). *T. reesei* RC30-8^{cre1} was compared to *A. niger* FP712^{creA} for β-xylosidase, α-arabinofuranosidase, α-glucosidase, and β-galactosidase activities. The enzymes corresponding to these activities have been described to be under regulation of carbon catabolite repression (CCR) [27, 39].

The addition of small amounts of glucose (2.5 mM and 5 mM) had a positive effect on the enzyme activities for both strains (Figure 4). Overall, increasing the glucose concentration to 10, 20, 30, or 40 mM caused a decrease in activities. The addition of 50 mM glucose caused non-detectable or very low activities for both strains. β-Xylosidase activities of FP712^{creA} and RC30-8^{cre1} decreased steeply with increased glucose up to no detectable activities for media containing 30 mM glucose. However, in the presence of 20 mM glucose, the activity of *A. niger* FP712^{creA} was

higher than *T. reesei* RC30-8^{cre1} (0.55 ± 0.03 and 0.07 ± 0.01 μmol·min^{-1}·mg total protein^{-1}, respectively). Similarly, α-arabinofuranosidase activities showed low values at 30 mM for both strains, but high activity for FP712^{creA} and low activity for RC30-8^{cre1} at 20 mM (1.97 ± 0.04 and 0.42 ± 0.03 μmol·min^{-1}·mg total protein^{-1}, respectively). The difference for α-glucosidase activities was even stronger with low activities of RC30-8^{cre1} at 20 mM glucose and high activities for FP712^{creA} up to 40 mM glucose. β-Galactosidase activities, after growth on WS media with 20 mM glucose, also showed a large difference between *A. niger* FP712^{creA} and *T. reesei* RC30-8^{cre1} (0.54 ± 0.02 and 0.03 ± 0.00 μmol·min^{-1}·mg total protein^{-1}, respectively).

These data showed lower enzyme production for both strains in the presence of high amounts of glucose. However, *T. reesei* RC30-8^{cre1} was more sensitive to the presence of this sugar. Corresponding to the glucose level in non-washed SCB, the differences were especially visible within 10 to 40 mM glucose.

4- Discussion

Plant-degrading enzyme mixtures are produced during growth of fungi on low-cost abundant plant biomass. This strategy has the advantage that the produced enzyme mixtures are better adapted for saccharification of the substrate the fungus was grown on. Specifically, *T. reesei* grown on SCB produced enzyme sets which were clearly more active against SCB then WS, and *vice versa*. An inherent disadvantage of using crude substrates is the complexity of composition. The composition in lignin, cellulose, and hemicellulose differs between type of substrate, harvesting location, and pretreatment [40]. Moreover, the pretreatment can release metabolites with a negative effect on fungal growth and/or enzyme production. In our study, non-washed SCB media contained 33 mM glucose after sterilization at 121°C. Although glucose is a good substrate for growth of *A. niger* and *T. reesei*, this monosaccharide will repress the production of extracellular hydrolytic enzymes [27]. For this reason, strains were used with a CCR deficiency such as *A. niger* strain FP712^{creA} without the general repressor CreA. Deletion of CreA allowed production of hydrolytic enzymes in the presence of 1% (or 50 mM) glucose [28, 41]. This effect of deregulation was clearly shown in our study by growth of NW249^{ref} and FP712^{creA} on xylan media with or without 1% glucose (Supplemental Figure 1). However, albeit some variation in enzyme activities between *A. niger* strains, the difference of

NW249^{ref} and FP712^{creA} between non-washed and washed substrates was not as clear as for the two *T. reesei* strains. After growth on WS and SCB, the CreA mutant did not increase hemicellulolytic activities or released more sugar from WS and SCB saccharifications. This lack in improvement might be explained by an already low sensitivity to glucose of *A. niger* NW249^{ref}. In contrast, *T. reesei* strains were sensitive to the presence of glucose. Non-washed SCB contained high amounts of glucose which decreased the hemicellulolytic activities and saccharification capacity. However, hypercellulolytic *T. reesei* strains with a CCR deficiency has described to be derepressed at 5% glucose (w/v) [9, 42]. This concentration is much higher than the initial 33 mM glucose found in non-washed SCB. The lower enzyme activities in non-washed SCB might be related to limited induction of (hemi-)cellulolytic enzymes. Deregulation of activation through a mutation of the regulator Xyr1 showed a strong increase in expression of (hemi-)cellulolytic genes [43]. It will be interesting to study the enzyme production of this CCR-deficient constitutive Xyr1 strain after growth on complex substrates.

Mixing enzymes of *A. niger* and *T. reesei* is known to have a positive effect on the saccharification of cellulose substrates [15]. Even though both fungi have a similar strategy in degrading plant biomass, they secrete enzymes of different CAZy families on the same substrate [18]. Our study supported that *T. reesei* produced enzyme sets with low activity against short gluco-oligosaccharides [44]. *A. niger* however produced high cellobiohydrolase and β-glucosidase activities after growth on both non-washed and washed substrates. Furthermore, only *A. niger* produced high xylanase activities after growth on non-washed SCB, and only *T. reesei* produced high xyloglucanase activities after growth on washed SCB. These differences in activities correlate with differences in releasing sugars from WS and SCB. In line with the enzyme data, *T. reesei* released in general less sugar after growth on non-washed substrates. Under these conditions the synergistic effect with *A. niger* was also the highest with a 2-fold increase in sugars release from non-washed WS and a 3-fold increase in sugars release from non-washed SCB. Mixing of *A. niger* and *T. reesei* enzymes had the largest effect on the release of glucose. For instance, after growth on washed WS, the addition of a half portion of FP712^{creA} enzymes to a half portion of RC30-8^{cre1} enzymes improved the glucose release more than 4-fold. Apparently the complementation benefited mostly cellulose degradation

and not so much xylose release from xylan. Our analysis did not detect any cellobiose during saccharification using *T. reesei* enzymes. This indicates that the complementary effect of *A. niger* enzymes was not only due to activities against short gluco-oligosaccharides. Cellulose degradation has been described to profit from the presence of xyloglucanases, xylanases, and esterases [45-47], which is an effect that likely also affected our study. Genome-scale analysis of the secreted *A. niger* and *T. reesei* enzymes, such as proteomics, will pin-point in more detail the differences between the two species.

In conclusion, combining enzyme sets from two fungi, *Aspergillus niger* and *Trichoderma reesei,* improves saccharification of WS and SCB. This improvement went beyond the previous described compensation of *T. reesei*'s low β-glucosidase activity. This study also showed that it is important to consider the presence of monosaccharides when using agricultural plant biomass as a growth substrate. Even with *ΔCreA*/*ΔCre1* strains, a high quantity of monosaccharides did inhibit enzyme production. However, after removing monosaccharides from the media, producing *A. niger* and *T. reesei* enzymes using complex substrates is a promising strategy to obtain better enzyme mixtures for plant biomass saccharification at lower cost.

Acknowledgements

JvdB was supported by a grant of the The Netherlands Organisation for Scientific Research (NWO) of the China–Netherlands Joint Scientific Thematic Research Programme (jstp.10.005) to RPdV. GPMA was supported by a grant of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (1527/12-6), Brazil.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

References

1 Kumar, D., Murthy, G.S., Impact of pretreatment and downstream processing technologies on economics and energy in cellulosic ethanol production. *Biotechnol. Biofuels* 2011, *4*, 27.

2. Macrelli, S., Mogensen, J., Zacchi, G., Techno-economic evaluation of 2nd generation bioethanol production from sugar cane bagasse and leaves integrated with the sugar-based ethanol process. *Biotechnol. Biofuels* 2012, *5*, 22.
3. Sorensen, A., Teller, P.J., Lubeck, P.S., Ahring, B.K., Onsite enzyme production during bioethanol production from biomass: screening for suitable fungal strains. *Appl. Biochem. Biotechnol.* 2011, *164*, 1058-1070.
4. Gyalai-Korpos, M., Mangel, R., Alvira, P., Dienes, D. et al., Cellulase production using different streams of wheat grain- and wheat straw-based ethanol processes. *J. Ind. Microbiol. Biotechnol.* 2011, *38*, 791-802.
5. van den Brink, J., de Vries, R.P., Fungal enzyme sets for plant polysaccharide degradation. *Appl. Microbiol. Biotechnol.* 2011, *91*, 1477-1492.
6. Culleton, H., McKie, V., de Vries, R.P. Physiological and molecular aspects of degradation of plant polysaccharides by fungi: what have we learned from *Aspergillus*? *Biotechnol. J.* 2013, *8*, 884-894.
7. Peterson, R., Nevalainen, H., *Trichoderma reesei* RUT-C30 - thirty years of strain improvement. *Microbiology* 2012, *158*, 58-68.
8. Reese, E.T., History of the cellulase program at the U.S. army Natick Development Center. *Biotechnology and bioengineering symposium* 1976, 9-20.
9. Montenecourt, B.S., Eveleigh, D.E., Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl. Environ. Microbiol.* 1977, *34*, 777-782.
10. Eveleigh, D.E., Montenecourt, B.S., Increasing yields of extracellular enzymes. *Advances in applied microbiology* 1979, *25*, 57-74.
11. Jourdier, E., Cohen, C., Poughon, L., Larroche, C. et al., Cellulase activity mapping of *Trichoderma reesei* cultivated in sugar mixtures under fed-batch conditions. *Biotechnol. Biofuels* 2013, *6*, 79.
12. Wang, B., Xia, L., High efficient expression of cellobiase gene from *Aspergillus niger* in the cells of *Trichoderma reesei*. *Bioresour. Technol.* 2011, *102*, 4568-4572.
13. Nakazawa, H., Kawai, T., Ida, N., Shida, Y. et al., Construction of a recombinant *Trichoderma reesei* strain expressing *Aspergillus aculeatus* beta-

glucosidase 1 for efficient biomass conversion. *Biotechnol. Bioeng.* 2012, *109*, 92-99.

14. Ma, L., Zhang, J., Zou, G., Wang, C. et al., Improvement of cellulase activity in *Trichoderma reesei* by heterologous expression of a beta-glucosidase gene from *Penicillium decumbens*. *Enzyme Microb. Technol.* 2011, *49*, 366-371.
15. Rosgaard, L., Pedersen, S., Cherry, J.R., Harris, P. et al., Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose. *Biotechnol. Prog.* 2006, *22*, 493-498.
16. Delmas, S., Pullan, S.T., Gaddipati, S., Kokolski, M. et al, Uncovering the genome-wide transcriptional responses of the filamentous fungus *Aspergillus niger* to lignocellulose using RNA sequencing. *PLoS Genet.* 2012, *8*, e1002875.
17. de Souza, W.R., de Gouvea, P.F., Savoldi, M., Malavazi, I. et al., Transcriptome analysis of *Aspergillus niger* grown on sugarcane bagasse. *Biotechnol. Biofuels* 2011, *4*, 40.
18. Ries, L., Pullan, S.T., Delmas, S., Malla, S. et al., Genome-wide transcriptional response of *Trichoderma reesei* to lignocellulose using RNA sequencing and comparison with *Aspergillus niger*. *BMC Genomics* 2013, *14*, 541.
19. Zeni, J., Cence, K., Grando, C.E., Tiggermann, L. et al., Screening of pectinase-producing microorganisms with polygalacturonase activity. *Appl. Biochem. Biotechnol.* 2011, *163*, 383-392.
20. Dinu, D., Nechifor, M.T., Stoian, G., Costache, M. et al., Enzymes with new biochemical properties in the pectinolytic complex produced by *Aspergillus niger* MIUG 16. *J. Biotechnol.* 2007, *131*, 128-137.
21. Pal, A., Khanum, F., Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. *Bioresour. Technol.* 2010, *101*, 7563-7569.
22. Kang, S.W., Park, Y.S., Lee, J.S., Hong, S.I. et al., Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Bioresour. Technol.* 2004, *91*, 153-156.
23. Ademark, P., de Vries, R.P., Hagglund, P., Stalbrand, H. et al., Cloning and characterization of *Aspergillus niger* genes encoding an alpha-galactosidase

and a beta-mannosidase involved in galactomannan degradation. *Eur. J. Biochem.* 2001, *268*, 2982-2990.

24. Ademark, P., Varga, A., Medve, J., Harjunpaa, V. et al., Softwood hemicellulose-degrading enzymes from *Aspergillus niger*: purification and properties of a beta-mannanase. *J. Biotechnol.* 1998, *63*, 199-210.
25. Faulds, C.B., Mandalari, G., Lo Curto, R.B., Bisignano, G. et al., Synergy between xylanases from glycoside hydrolase family 10 and family 11 and a feruloyl esterase in the release of phenolic acids from cereal arabinoxylan. *Appl. Microbiol. Biotechnol.* 2006, *71*, 622-629.
26. Garcia-Conesa, M.T., Kroon, P.A., Ralph, J., Mellon, F.A. et al., A cinnamoyl esterase from *Aspergillus niger* can break plant cell wall cross-links without release of free diferulic acids. *Eur. J. Biochem.* 1999, *266*, 644-652.
27. Stricker, A.R., Mach, R.L., de Graaff, L.H., Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). *Appl. Microbiol. Biotechnol.* 2008, *78*, 211-220.
28. de Vries, R.P., Visser, J., de Graaff, L.H., CreA modulates the XlnR-induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. *Research in microbiology* 1999, *150*, 281-285.
29. Mach-Aigner, A.R., Omony, J., Jovanovic, B., van Boxtel, A.J. et al, D-Xylose concentration-dependent hydrolase expression profiles and the function of CreA and XlnR in *Aspergillus niger*. *Appl. Environ. Microbiol.* 2012, *78*, 3145-3155.
30. Seidl, V., Gamauf, C., Druzhinina, I.S., Seiboth, B. et al., The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. *BMC Genomics* 2008, *9*, 327.
31. Zou, G., Shi, S., Jiang, Y., van den Brink, J. et al., Construction of a cellulase hyper-expression system in *Trichoderma reesei* by promoter and enzyme engineering. *Microb. Cell Fact.* 2012, *11*, 21.
32. Vankuyk, P.A., Diderich, J.A., MacCabe, A.P., Hererro, O. et al., *Aspergillus niger* mstA encodes a high-affinity sugar/H+ symporter which is regulated in response to extracellular pH. *Biochem. J.* 2004, *379*, 375-383.

33. Kusters-van Someren, M.A., Harmsen, J.A., Kester, H.C., Visser, J., Structure of the *Aspergillus niger* pelA gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. *Curr. Genet.* 1991, *20*, 293-299.
34. Yuan, X.L., Goosen, C., Kools, H., van der Maarel, M.J. et al., Database mining and transcriptional analysis of genes encoding inulin-modifying enzymes of *Aspergillus niger*. *Microbiology* 2006, *152*, 3061-3073.
35. van den Brink, J., van Muiswinkel, G.C., Theelen, B., Hinz, S.W. et al., Efficient plant biomass degradation by thermophilic fungus *Myceliophthora heterothallica*. *Appl. Environ. Microbiol.* 2013, *79*, 1316-1324.
36. Visser, E.M., Falkoski, D.L., de Almeida, M.N., Maitan-Alfenas, G.P. et al., Production and application of an enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* with potential for hydrolysis of sugarcane bagasse. *Bioresour. Technol.* 2013, *144*, 587-594.
37. de Vries, R.P., Burgers, K., van de Vondervoort, P.J., Frisvad, J.C. et al., A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. *Appl. Environ. Microbiol.* 2004, *70*, 3954-3959.
38. Penttila, M., Nevalainen, H., Ratto, M., Salminen, E. et al., A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 1987, *61*, 155-164.
39. Ribeiro, D.A., Cota, J., Alvarez, T.M., Bruchli, F. et al., The *Penicillium echinulatum* secretome on sugar cane bagasse. *PLoS One* 2012, *7*, e50571.
40. de Vries, R.P., Visser, J., Regulation of the feruloyl esterase (faeA) gene from *Aspergillus niger*. *Appl. Environ. Microbiol.* 1999, *65*, 5500-5503.
41. Montenecourt, B.S., Eveleigh, D.E., Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*, in: Brown, R., Jurasek, L. (Ed.), *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis, Advances in Chemistry Series*, American Chemical Society, Washington D.C. 1979, *181*, 289.
42. Derntl, C., Gudynaite-Savitch, L., Calixte, S., White, T. et al., Mutation of the Xylanase regulator 1 causes a glucose blind hydrolase expressing phenotype in industrially used *Trichoderma* strains. *Biotechnol. Biofuels* 2013, *6*, 62.

43. Ryu, D.D., Mandels, M., Cellulases: biosynthesis and applications. *Enzyme and Microbial Technology* 1980, *2*, 11.
44. Zhang, J., Siika-Aho, M., Tenkanen, M., Viikari, L., The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. *Biotechnol. Biofuels* 2011, *4*, 60.
45. Selig, M.J., Knoshaug, E.P., Adney, W.S., Himmel, M.E. et al., Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. *Bioresour. Technol.* 2008, *99*, 4997-5005.
46. Hu, J., Arantes, V., Saddler, J.N., The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnol. Biofuels* 2011, *4*, 36.

Tables and Figures

Table 1: Primers used in this study to check CreA deletion in *A. niger* mutant.

Loci	FW	RV
CreA 3' flank reference	TAGGAGTACCATGTGATTTGAA	ATCTGCATTTGCCGTCTATCC
CreA 3' flank mutant	TAGGAGTACCATGTGATTTGAA	GTGGTTGAAACTGGCTTATTAG
CreA 5' flank reference	CGATCGCAGAGGGGGCACTTGTAG	CCAACAATACGGGGCACGCACTTC
CreA 5' flank mutant	TGTGGGAAATGAGGCAGCGGTTAC	CCAACAATACGGGGCACGCACTTC

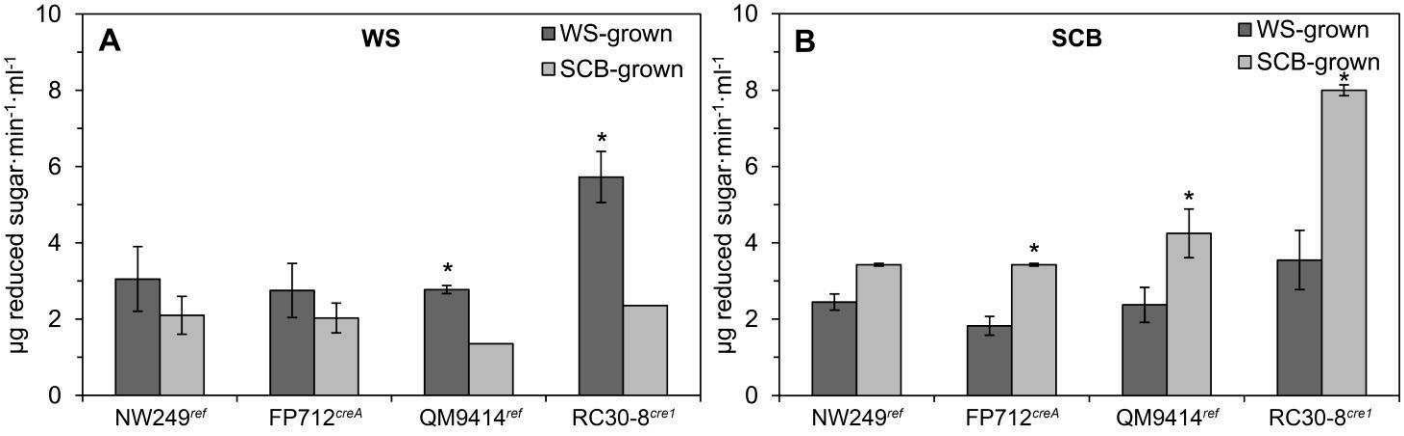


Figure 1: Saccharification activities against WS (A) and SCB (B) of *A. niger* and *T. reesei* enzyme sets after growth on WS and SCB. *A. niger* (NW249^{ref} and FP712^{creA}) and *T. reesei* (QM9414^{ref} and RC30-8^{cre1}) strains were grown in medium containing washed WS and SCB for 4 days. The activities are given in µg·min^{-1}·mL^{-1}. The standard deviations are based on two independent cultivations with a variation of technical replications within 10 percent. Significance between WS-grown and SCB-grown cultures are indicated by asterisks (* $p < 0.05$).

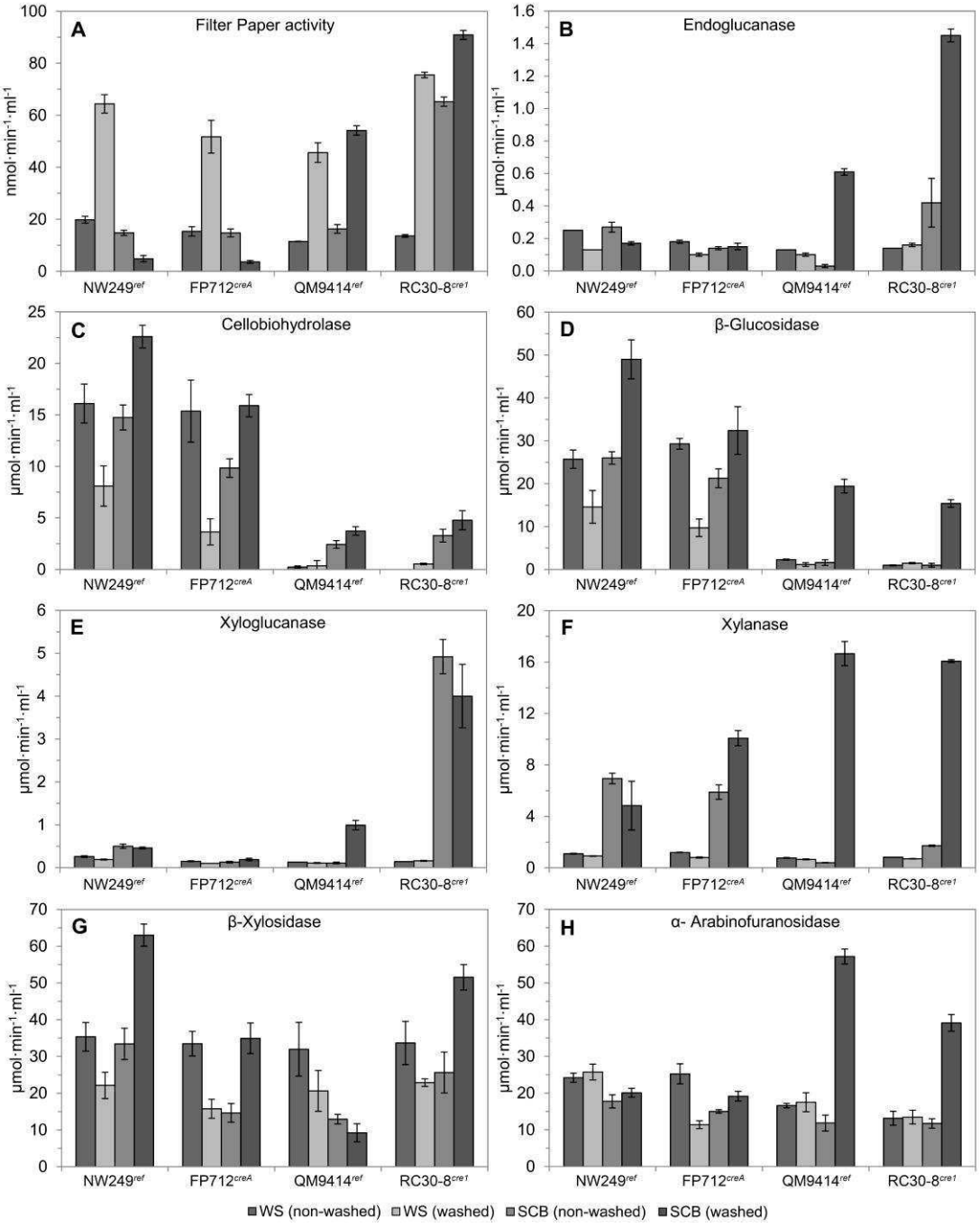


Figure 2: Extracellular enzyme activities of *A. niger* (NW249^{ref} and FP712^{creA}) and *T. reesei* (QM9414^{ref} and RC30-8^{cre1}) strains during growth on wheat straw (WS) or sugarcane bagasse (SCB). The strains were grown on non-washed and washed substrates for 4 days. The measured activities were filter paper activity (A), endoglucanase (B), cellobiohydrolase (C), β-glucosidase (D), xyloglucanase (E), xylanase (F), β-xylosidase (G), and α-arabinofuranosidase (H). The standard deviations are based on two independent cultivations and, at least, four technical replicates.

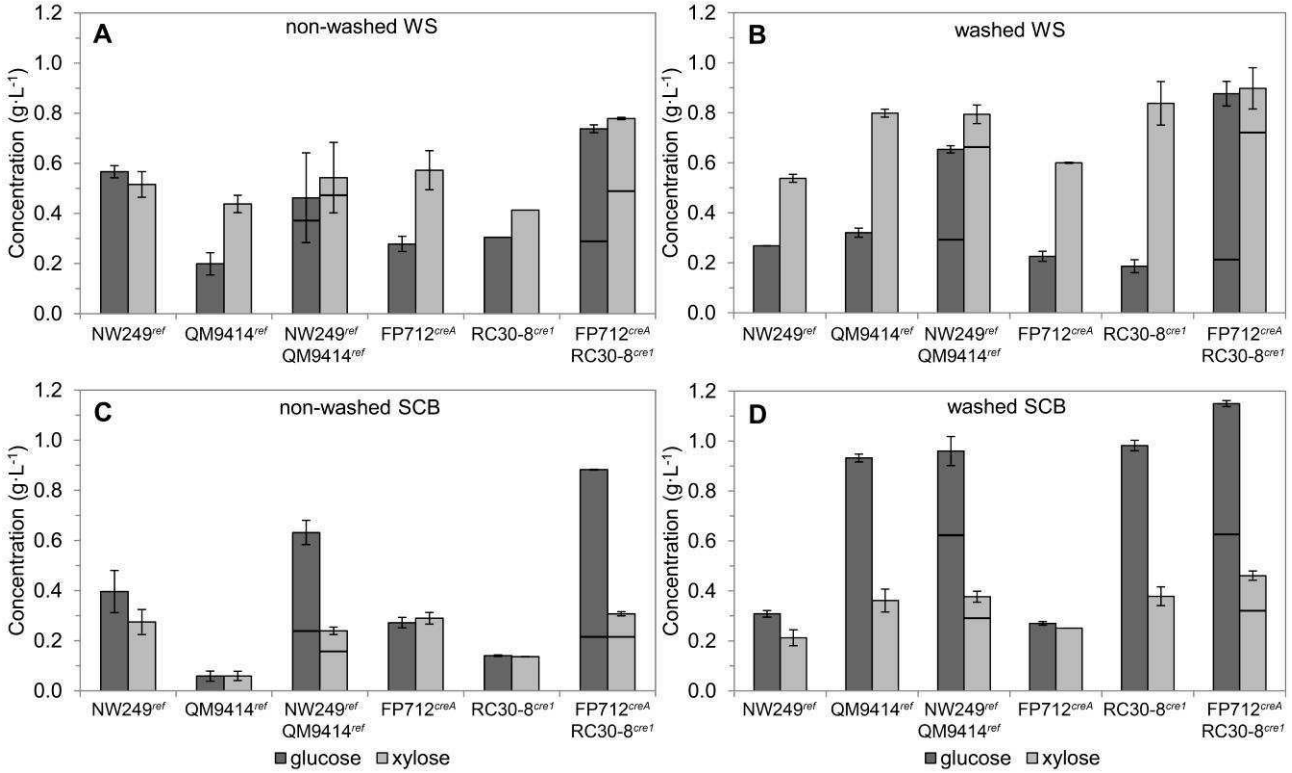


Figure 3: Glucose and xylose concentrations after saccharification of non-washed WS (A), washed WS (B), non-washed SCB (C), and washed SCB (D). The substrates were incubated for 5 days with combinations of enzyme sets of *A. niger* NW249^{ref}, *A. niger* FP712^{creA}, *T. reesei* QM9414^{ref}, and *T. reesei* RC30-8^{cre1}. The standard deviations are based on two independent incubations. The bold line represents the theoretical release of sugars when the *A. niger* and *T. reesei* mixture would have been the sum of individual saccharification (0.5·*A. niger* + 0.5· *T. reesei*).

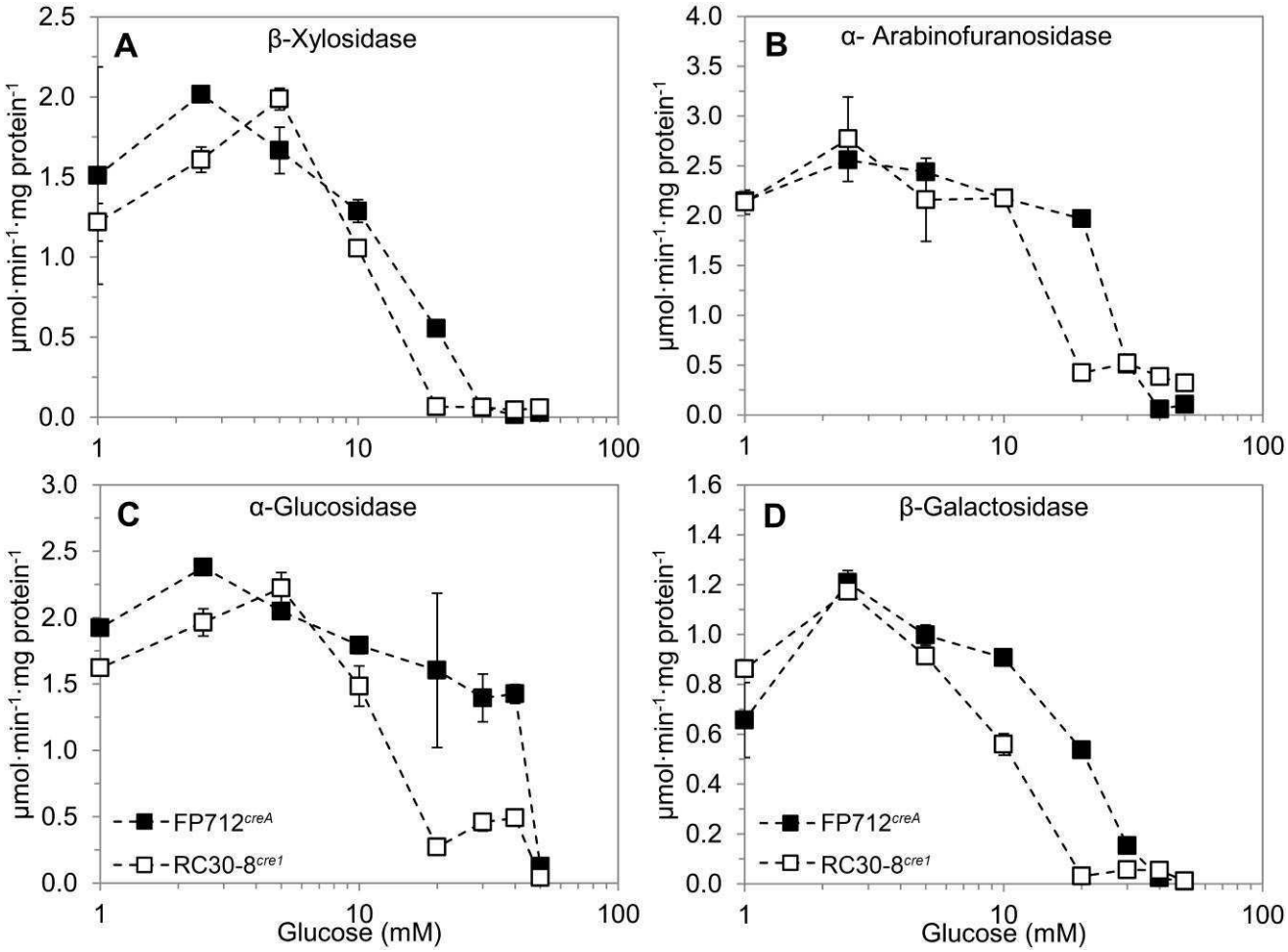


Figure 4: Extracellular enzyme activities of *A. niger* FP712^{creA} and *T. reesei* RC30-8^{cre1} after 4-days growth on 3% wheat straw with addition of 1–50 mM glucose. The measured activities were β-xylosidase (A), α-arabinofuranosidase (B), α-glucosidase (C), and β-galactosidase (D). The standard deviations are based on two independent cultivations and, at least, four technical replicates.

Supplemental Data

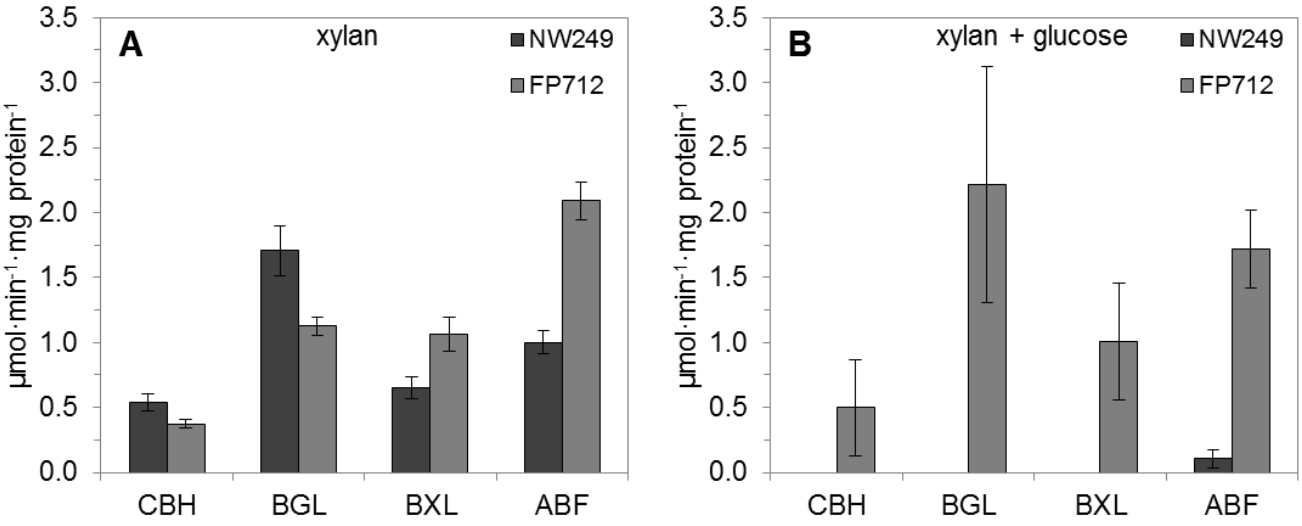


Figure 1. Enzyme activities of A. niger NW249^{ref} and FP712^{creA} after growth on xylan (A) or xylan with glucose (B).

Aspergillus niger strains NW249^{ref} (*cspA1, ΔargB, pyrA6, leuA1, nicA1*) and FP712^{creA} (*cspA1, pyrA6, nicA1, leuA1, PyrG:ΔCreA*) were grown on minimal media with 2% beechwood xylan (Sigma-Aldrich X4252) with or without 1% glucose. Shakeflasks with 50 ml media were inoculated with ± 500 spores and grown for three days at 200 rpm and 30°C. The culture filtrates were tested for their *in vitro* activities against *p*-nitrophenol linked substrates (CBH: β-D-cellobioside N5759, BGL: β-D-glucopyranoside N7006, BXL: β-D-xylopyranoside N2132, ABF: α-L-arabinofuranoside N3641 of Sigma-Aldrich, Germany). The activities are given in µmol *p*NP·min^{-1}·mg protein^{-1}. The standard deviations are based on two independent cultivations and, at least, four technical replicates.

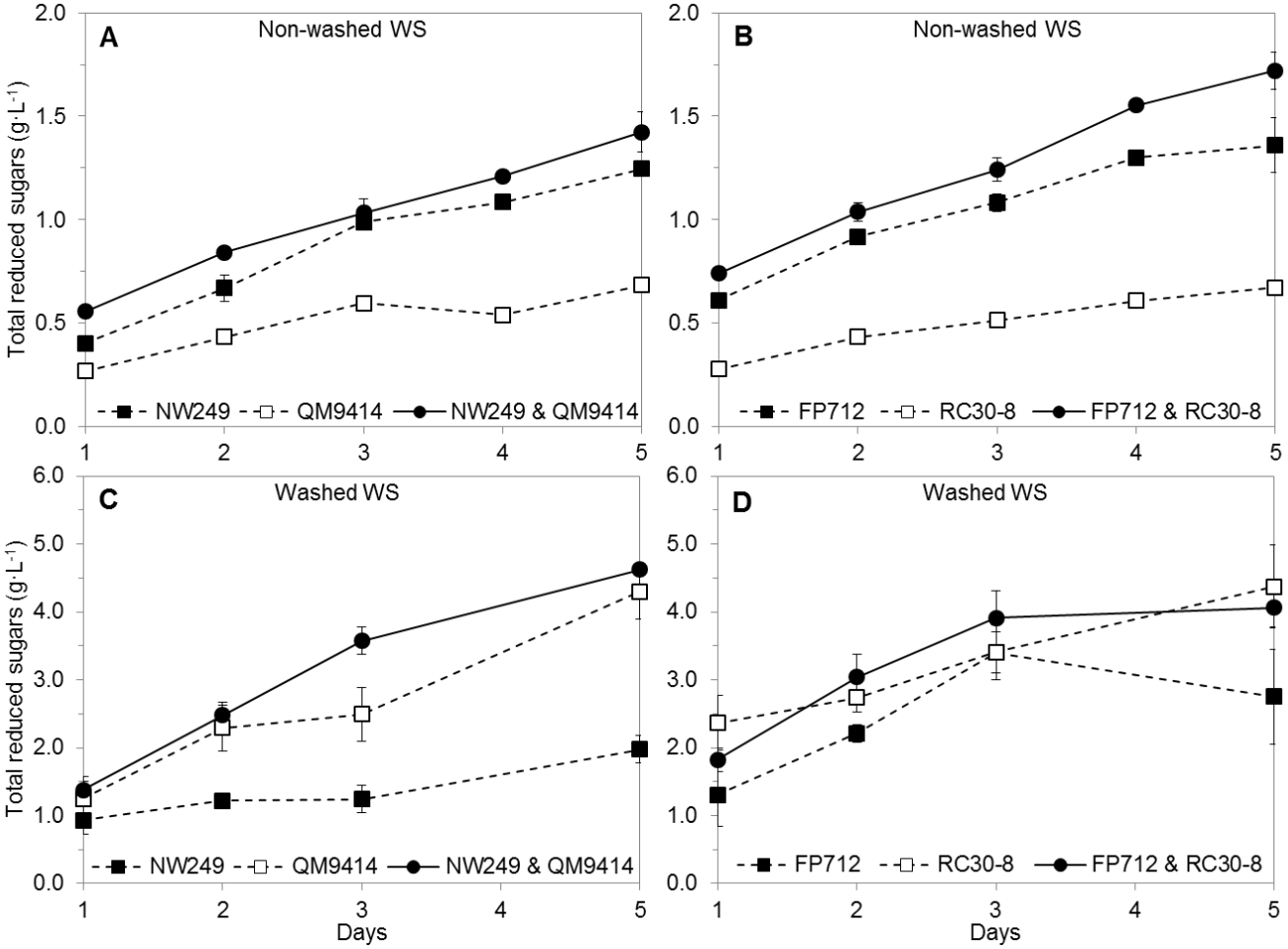


Figure 2. Total amount of reduced sugars after saccharification of non-washed (A & B) or washed (C & D) wheat straw using *A. niger* and *T. reesei* strains.

The substrates were incubated for 5 days with combinations of enzyme mixtures of *A. niger* NW249^{ref}, *A. niger* FP712^{creA}, *T. reesei* QM9414^{ref}, and *T. reesei* RC30-8^{cre1}. Shakeflasks of 500 ml with 100 ml media were inoculated with 1•10^6 spores. *A. niger* strains were grown for 4 days at 30 °C and 250 rpm on Aspergillus Minimal Media containing 3% pretreated plant biomass and appropriate supplements. *T. reesei* strains were grown for 4 days at 28 °C and 250 rpm on Trichoderma Minimal Media containing 3% pretreated plant biomass. The culture filtrates were incubated together with 50 mM NaAc buffer (pH 5.0) medium consisting of 3% pretreated plant biomass. A 50%/50% blend was used for mixtures of *A. niger* and *T. reesei*. Samples of the saccharification were taken every day for sugar analysis. The total amount of reduced sugars was measured using 3,5-dinitrosalicylic acid (DNS). The concentrations were calculated using a standard curve ranging from 0 to 2 g•L^{-1} glucose. The standard deviations are based on two independent cultivations and, at least, four technical replicates.

GENERAL CONCLUSIONS

- The saccharification yields depend on the pretreatment, the enzymatic mixture and the composition of the biomass;
- Fungi are the main sources of commercial enzymes;
- New enzymes are necessary to reduce costs of the second ethanol production process and phylogenomics is an efficient alternative for their bioprospection;
- Hemicellulases are very important for glucose release since they support the access of cellulases to cellulose fraction;
- On-site production is a promising alternative for costs reduction of 2G ethanol production and monosaccharides play an important role on enzyme sets production.