DIOGO ROBL

HEMICELULASES E PROTEÍNAS ACESSÓRIAS DE FUNGOS FILAMENTOSOS E DE ACTINOMICETOS PARA DESCONSTRUÇÃO DE BIOMASSA LIGNOCELULÓSICA

Tese apresentada ao Programa De Pós Graduação Interunidades em Biotecnologia USP/ Instituto Butantan/IPT, para obtenção do Título de Doutor em Biotecnologia.

São Paulo
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Área de concentração: Biotecnologia

Orientador: Prof. Dr. Gabriel Padilla

Coorientador: Dr. José Geraldo da Cruz Pradella

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To my parents and my sisters for all dedication, comprehension, compassion and support during these years.

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“Science never solves a problem without creating ten more.” (George Bernard Shaw)

ABSTRACT

Robl D. Hemicellulases and acessory proteins from filamentous fungi and actinomycetes for lignocellulose biomass deconstruction. [Ph. D. thesis (Biotechnology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2015.

Endophytic microorganisms (119 fungi and 45 actinomycetes) were screened for hemicellulases production using plate assays and liquid cultivations. Two strains were selected and used in further studies. *Aspergillus niger* DR02 strain which produced high concentration of xylanase and an *Annulohypoxylon stigyum* DR47 strain which produced high concentration of pectinase and β-glucosidase. In *A.niger* fed-batch submerged cultivation approaches were developed using liquor from hydrothermal sugar cane pretreatment, and maximum xylanase activities obtained were 458.1 U/mL for constant fed-batch mode. For *A. stygium* DR47 media optimization and bioreactor cultivation using citrus bagasse and soybean bran were explored and revealed a maximum production of 6.26 U/mL of pectinase at pH 4.0 and 10.13 U/mL of β-glucosidase at pH 5.0. Improved hemicellulase production was also done by genetic modifications at carbon catabolic repression levels in *A. niger*. Deletion of *creA* gene resulted in a higher expression of some hemicellulolytic genes and a higher specific enzyme production. Introduction of the constitutively active *xlnR* gene in a Δ*creA* strain increased fungal growth, but did not result in a higher expression/production of most hemicellulases on xylose. Mass spectrometric studies were done in enzymatic extracts produced and contributed to understand the enzymatic supplementation of Celluclast 1.5L. Proteomic analysis detected several important enzymes in plant biomass degradation, *A. niger* secretome showed xylanolytic enzymes (GH10, GH11, and GH62), cellobiohydrolase (G6 and GH7), β-glucosidase, β-xylosidase (GH3), and feruloyl esterase (CE1). *A. stygium* presented β-glucosidases (GH3) L-α-arabinofuranosidase (GH54) and a catalase. In addition the extracts produced were tested for an enzyme formulation with Celluclast 1.5L. *A. stygium* extracts were not efficient when combined with *A. niger* extract. Statistical data supported the development of a mixture based mainly by the commercial cellulose preparation with rich xylanse extract from *A. niger*.

Keywords: Hemicellulases. Fungi. Actinomycetes. Endophytics. Sugar cane.

RESUMO

Robl D. Hemicelulases e proteínas acessórias de fungos filamentosos e de actinomicetos para desconstrução de biomassa lignocelulósica. [Tese (Doutorado em Biotecnologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2015.

Microrganismos endofíticos (119 fungos e 45 actinomicetos) foram selecionados para a produção de hemicelulases através de testes em placa e de cultivo líquido. Duas linhagens foram selecionadas para posteriores estudos, *Aspergillus niger* DR02 que produzia altas concentrações de xilanase e *Annulohypoxylon stigyum* DR47 que produzia altas concentrações de pectinase e β-glucosidase. Para o fungo *A.niger* abordagens de batelada alimentada em cultivo submerso foram desenvolvidas, através licor do tratamento hidrotérmico do bagaço de cana de açúcar. Máximos valores de atividade de xilanase foram obtidos (458,1 U/mL) com alimentação constante. Para a linhagem *A. stygium* DR47 a otimização de meio de cultura e cultivo em biorreatores utilizando bagaço cítrico e farelo de soja foram explorados e revelaram máxima produção de 6,26 U/mL de pectinase a pH 4,0 e 10,13 U/mL de β-glucosidase a pH 5,0. Melhoramento da produção de hemicelulase foi realizado através de modificações genéticas ao nível de repressão catabólica de carbono em *A. niger*. Deleção do gene *creA* resultou em maior expressão dos genes hemicelulolíticos e maior produção específica de algumas glicohidrolases. A clonagem da versão ativa e constitutiva do gene *xlnR* em uma linhagem Δ*creA* aumentou o crescimento fúngico, mas não resultou em aumento de expressão/produção da maioria da hemicelulases em xilose. Estudos de espectrometria de massas foram realizados nos extratos enzimáticos produzidos e contribuíram para entender a suplementação da Celluclast 1.5L. Análises da proteômica detectaram várias enzimas importantes na degradação de biomassa, o secretoma do *A. niger* mostrou enzimas endo- e exo-xilanolíticas (GH10, GH11, e GH62), celobiohidrolase (G6 e GH7), β-glucosidase, β-xilosidase (GH3), e feruloil esterase (CE1). *A. stygium* apresentou β-glucosidases (GH3) L-α-arabinofuranosidase (GH54) e catalase. Além disso, os extratos produzidos foram testados para uma formulação com Celluclast 1.5L. Os extratos de *A. stygium* não foram eficientes quando combinados com o extrato de *A. niger*. Ferramentas estatísticas permitiram o desenvolvimento de uma mistura baseada principalmente na celulase comercial e o extrato rico em xilanase de *A. niger*.

Palavras-chaves: Hemicelulases. Fungos. Actinomicetos. Endofíticos. Cana de açúcar.

FIGURE LIST

TABLE LIST

ABBREVIATION LIST

AB: Apple bagasse

AFEX: Ammonia fiber explosion

aguA : α-glucuronidase A

AraR: Arabinoloytic transcriptional activator

BGL: *Annulohypoxylon stygium* DR47 β-glucosidase extract

BT2: Partial β-tubulin

CB: Citrus bagasse

CCD: central composite design

CCR: Carbon catabolite repression

CE: CO_2 explosion

CMC: Carboxymethylcellulose

CreA : Carbon catabolite repressor

CTAB: Cetyltrimethylammonium bromide

DEB: Delignified sugar cane bagasse

DF: Degrees of freedom

EB: Steam exploded bagasse

EGDA: Esculin gel diffusion assay

FDR: False discovery rate

FPase: Filter paper activity

H2S: Histone

HB: Hydrothermal bagasse

HF: Furfural

HL: Hydrothermal pretreatment liquor

HLPC: High- performance liquid chromatography

HMF: 5-hydroxymethylfurfural

HT: Hydrothermal treatment

ITS: Internal Transcribed Spacer

Lf: Lack of fit

MEA: Malt extract agar

MESP: Minimum ethanol selling price

MS: Mean square

PDA: Potato dextrose agar

Pe: Pure error

PEC*: Annulohypoxylon stygium* DR47 pectinase extract

pNP : *p*-nitrophenyl

ppgkA : Phosphoglycerate kinase promoter

R: Regression

r: Residual

SB: Soybean bran

SE: Steam explosion

SmF: Submerged fermentation

SS: Sum of squares

STR: Stirred tank reactor

SUC: Sucrose

T: Total

TSA: Tryptic soy Agar

WB: wheat bran

XDH: xylitol dehydrogenase

XI: xylose isomerase

xlnD : β-xylosidase D

XlnR: Xylanolytic transcriptional activator

XR: Xylose reductase

XYL: *Aspergillus niger* DR02 xylanase extract

xynB : Endoxylanase B

SYMBOL LIST

F: Flow rate

S_o : Carbon source concentration at start of batch phase

S_{inlet} : Inlet carbon source concentration

μ_{crit} : Set value of specific growth rate

Y_{xs} : Cell mass yield from consumed carbon source

V_o : Initial volume of culture broth

X_o : Cell dry weight in beginning of fed

V_{inlet} : Inlet volume of culture media

r_{sx} : Carbon source uptake rate

μ_{max} : Maximum specific growth rate

V_p : Bioreactor volume after solution pulse

S_p : Carbon source concentration after solution pulse

$\sum carbon$: Mass of carbon source added to the bioreactor

SUMMARY

CHAPTER 1 - RESEARCH PRESENTATION

1.1 Introduction

Nowadays, the emphasis in the use of bioethanol is on reducing pollution and helping to achieve the goals of the Kyoto protocol (1). Biofuel as a partial substitute for petroleum can prevent to a great extent the global, environmental and political issues related to the use of fossil fuels. Although burning ethanol produces gas emissions, the net effect does not result in increasing CO_2 concentrations in the atmosphere, since production of plant biomass removes CO_2 from the atmosphere (2). The abundant and renewable supply of plant biomass makes this material an outstanding candidate for bioethanol production.

In nature, lignocellulosic materials are degraded by a consortium of microorganisms that synthesize many hydrolytic enzymes able to loosen and degrade these substrates. Improvement in the efficiency of hydrolysis of lignocellulosic materials has been traditionally focused on cellulose, which is the most abundant plant polysaccharide (3). However, the presence of hemicellulose and lignin can restrict cellulose hydrolysis. The hemicellulases, such as pectinases and xylanases, stimulate cellulose hydrolysis by removal of the non-cellulosic polysaccharides that coat the cellulose fibers (3).

An extensively effort have been done on second generation bioethanol during the last decade to select and obtain good enzyme producers microorganisms, and high performance cellulase/hemicellulase. The current thesis presents data of hemicellulolytic microorganisms screening, enzyme production optimization, genetic fungal modifications and cellulolytic enzymes preparation supplementation aiming to enhance plant biomass deconstruction.

This work is consists by a literature review of the challenges faced in the 2nd bioethanol production and five experimental chapters on the production and application of hemicellulases in sugar cane bagasse hydrolysis. These chapters describe the work developed in partnership between Institute of Biomedical Sciences (ICB- USP), Brazilian Bioethanol Sciences and Technology Laboratory (CTBE) and Fungal Biodiversity Centre (CBS-KNAW).

1.2 Aim of the thesis

The aim of this project was to produce enzymatic complexes and obtain of a hemicellulolytic cocktail from endophytic fungi and actinomycetes applied to plant biomass deconstruction.

Specific aims of this thesis:

a. Select hemicellulases producers microorganisms for lignocellulose biomass degradation

b. Optimize enzyme production from selected strains by kinetics determination, statistical tools and study different conditions in submerse cultivation.

c. Characterize hydrolytic parameters of the enzymatic extract produced

d. Proteomic study of the enzymatic extracts.

e. Genetic modification of *A. niger* strain to improve xylanase production.

f. Study the formulation of cocktails by combination of commercial and produced extracts.

CHAPTER 2 - PRODUCTION OF SECOND GENERATION BIOETHANOL: FROM BASIC SCIENCE TO INDUSTRIAL CHALLENGES

2.1 Introduction

There are several aspects that must be explored to enable an effective bioethanol production, which can be of economic or techno-scientific nature. Economic issues related to the ethanol demand and electricity production by using lignocellulosic biomass must be considered in second generation ethanol production (4). Societal factors, such as geopolitics and oil refinement capacity, influence the oil price and consequently make biofuel plants investments profitable or not (5).

Next to high costs of the (hemi-)cellulases needed to depolymerize the biomass, the most important economic factor is the biomass pre-treatment process required to achieve efficient enzymatic hydrolysis to monomers. According to Macrelli et al. (6) factors such as electricity price and enzyme cost have considerable impact on the 2nd generation bioethanol production.

Genetically modified organisms, integrated 1st and 2nd generation bioethanol production, new biomass pretreatments and in house enzyme production provide potential ways to reduce bioethanol production costs. In this review we summarize difficulties facing cost effective second-generation biofuel production from basic science to industrial challenges.

2.2 Feed stock on 1st and 2nd generation bioethanol

The difference between 1st and 2nd generation bioethanol is the biomass used for its production. 1st generation bioethanol uses cereal and sugar crops, while 2nd generation bioethanol uses lignocellulosic biomass and other agricultural waste products (7). Lignocellulosic raw materials that can be used for 2nd generation bioethanol production are classified as agricultural residues, forest residues, energy crops and industrial/municipal waste and their use depends on each particular situation (8).

Certain features of wild plants made them desirable for domestication millennia ago and they still form the majority of today's food crops. Additional crop properties, such as cell wall composition, growth rate, suitability for growth in different geographical regions and source use efficiencies, are used to characterize the potential of future bioenergy crops (9).

Optimization of bioenergy crops as a source for biofuel production is ongoing, including the generation of genomic information and resources that will be essential for accelerating their domestication. Most of these efforts targeted improved growth on low

quality lands to minimize competition with food crops over land. Moreover, maximizing yield of biomass per unit of land area is highly desirable since it minimizes the overall land use. The combination of plant genomes with gene functions and expression studies has identified potential candidate genes that could be modified to improve plant properties (10, 11). These genes are involved in cellulose and hemicellulose synthesis or participate in morphological growth characteristics such as height and branch thickness (12). *Agrobacterium tumefaciens* or gene-gun-mediated gene transfer are used to efficiently transform many crops such as rice, maize, sorghum, poplar and switch grass (13), to develop the improved characteristics using GMO plants.

Next to this research and innovation prospective, there is a need for a practical system that will enable us to generate and harvest enough energy from crops to replace some of the energy obtained from fossil resources. Aleman grass (*Echinochloa polystachya*), Elephant grass (*Pennisetum purpureum*), foxtail millet (*Setaria italica*), miscanthus (*Miscanthus giganteus*), sweet sorghum (*Sorghum bicolor*), sugarcane and switchgrass (*Panicum virgatum*) are grass species with C_4 photosynthesis, which is the most efficient photosynthetic CO_2 assimilation. Their characteristics make them ideal energy crops (14). Other lignocellulosic feedstocks such as agro industrial residues and urban wastes can be used in the 2^{nd} ethanol generation. The characteristics of each biomass such as composition, cultivation/harvest, processing, annual availability and economic market, affect the development of a suitable process.

At the moment, biofuel production by processing lignocellulosic biomass is more expensive than processing sugar cane and maize. This originates from high costs involved in pretreatment and hydrolysis. To be economically viable the energy used to process lignocellulosic biomass should be significantly lower than the energy produced (15). Transgenic approaches may be used to increase the sugar content of the biomass, by producing sugars that cannot metabolized by the plant, which eventually increases ethanol yield. For instance, introducing a bacterial isomerase to sugar cane that converts sucrose into isomaltose increased the sugar yield up to two-fold compared to regular plants (16).

At present miscanthus, (17-19) switchgrass, corn stover and willow are already being used in lignocellulosic bioethanol assays as feedstocks (20). Furthermore, *Setaria viridis* is a C_4 grass species, which is a promising candidate for a biofuel feedstock model plant, due to its compact structure and short life cycle (21).

There is an increasing interest in using waste and residues as feedstocks. Recently Saucedo-Luna (22) showed that bagasse of *Agave tequilana* has the potential to be used as

ligno-cellulosic material for ethanol production, while Ha et al. (23) showed the same for beer remnants. While several studies demonstrated that diverse feedstocks could be used for 2nd generation biofuel production, the strongly varying composition of these diverse feed stocks puts very high demands on versatile pretreatment and hydrolyzing enzyme mixtures used for saccharification.

Bioethanol production already increased since 1980 but this increase became much stronger since 2005 and currently the United States and Brazil are the main world producers (Figure 1). However, the production of 2nd generation bioethanol is still in its infancy, mainly due to higher production costs and lack of a commercially viable manufacturing technology. To solve these problems demonstration plants have been operated for several years, like in Salamanca, Spain (Abengoa); in Örnsköldsvik, Sweden (SEKAB), and in Ottawa, Canada (Raízen). Only recently, in 2014, commercial 2nd generation plants have been opened. The first commercial plant, project LIBERTY, a partnership between POET and DSM aims to produce initially 20 million gallons bioethanol per year from corn crop residues in Emmetsburg, Iowa US. Also, Abengoa Bioenergy opened its first bioethanol plant on Hugoton, Kansas, US with the capacity to produce up to 25 million gallons per year. In Brazil GranBio opened a commercial plant in São Miguel dos Campos to produce ethanol from sugar cane straw, while others plants are under construction (e.g. Piracicaba Brazil, Raízen).

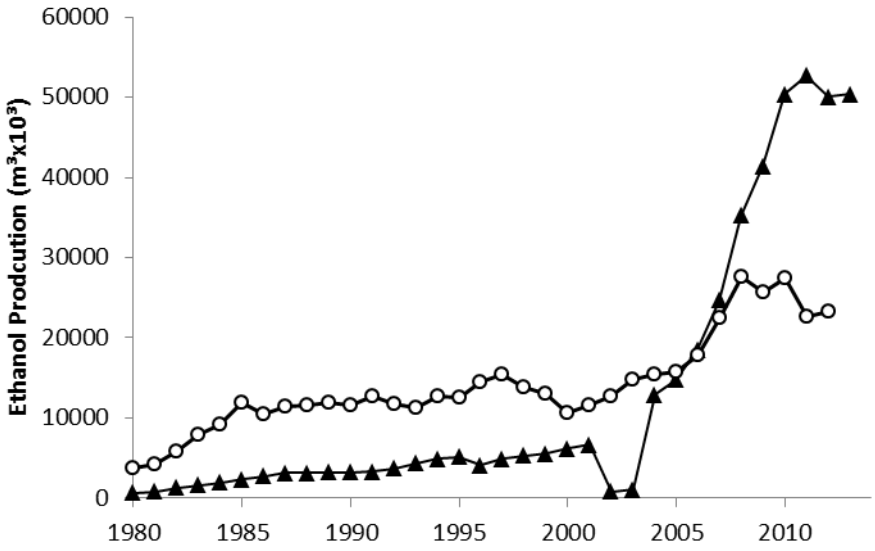

Figure 1 Ethanol production (m^3x10^3) from 1980 until 2013 in United State (▲) and Brazil (○) Source: UNICA (24) and RFA (25).

2.3 **Biomass structure as bioethanol production building blocks**

Photosynthesis is the process that captures solar energy and stores it in the form of cell wall polymers. These polymers can be storage compounds (e.g. starch) or cell wall polysaccharides (e.g. cellulose). Plant cell walls are the main components of lignocellulosic biomass. The plant cell wall is composed of microfibers of cellulose into a matrix of polysaccharides, phenolic compounds and structural proteins (26) (Figure 2). These components are linked by several covalent and non-covalent interactions, which creates a highly organized network (27). Dicotyledonous plants have primary cell walls with equal amounts of glucan and xyloglucan imbedded in pectin. In contrast, cereals and other grasses contain glucuronoarabinoxylans and lack pectin (28).

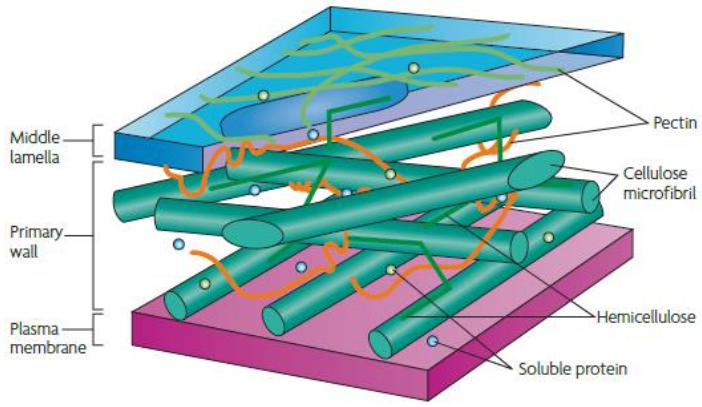

Figure 2 Cell-wall structure of a plant. Cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. Source: Sticklen (28).

Cellulose is linear homopolysaccharide consisting of glucose units linked by β 1→4 glycosidic bonds. The chains can perform hydrogen bonds between themselves and form a stable crystalline structure (29). Hemicelluloses interact through hydrogen bonds with the cellulose microfibers (30). The term hemicellulose is used for a heterogeneous class of polymers that represent 15-35% of plant biomass and contains pentoses, hexoses and uronic acids (31). Xylan is the most abundant hemicellulose and consists of β-1,4-xylose units branched with acetyl, arabinofuranosyl and 4-methyl-O glucuronoyl residues. In addition, it can be attached to lignin through aromatic ester linkages (32).

The third compound of lignocellulose is lignin, a phenylpropane polymer that connects with the cell wall polysaccharides mainly by hydroxycinnamic acids, like *p*-coumaric acid and ferulic acid. These acids are found principally in esters bonds with xylan arabinose, and glucuronoyl residues (33). Lignin is a highly recalcitrant component and hampers the chemical and biological degradation of the plant cell wall (34)

The bonds between polysaccharides and lignin impact in a negative way the hydrolysis to sugars, which indicates that delignification is a critical point in the conversion of biomass into biofuels (32). The removal of lignin and hemicellulose can result in a better accessibility of the cellulose fibers for enzymes (34).

The composition and structure of the plant cell has been described for several crops, such as sugar cane, wheat straw and corn stover (35-37). However, it is still unclear how the enzymes act exactly on the polysaccharides in the feedstocks and how the structure of these feedstocks changes during the enzymatic hydrolysis. A better understanding of this process could help to develop specific strategies on pretreatment and hydrolysis steps for each biomass used.

2.4 **Biomass pretreatment**

Pretreatment of the lignocelulosic materials is required for efficient biomass degradation. The main goal of this step is remove the lignin, reduce the crystallinity of cellulose and open up the overall structure of the biomass (38). The pretreatment choice must consider the improvement of accessibility for the enzymes in the hydrolysis, reduction of the production of inhibitors, low costs and few wastes, for each lignocellulose feedstock (34).

The pretreatments can be divided into four types: physical, physic-chemical, chemical and biological. Physical pretreatment includes milling, irradiation and pyrolysis. These treatments improve the digestibly of the biomass, although they cannot remove the lignin (34).

Most of physical-chemical treatments employ an explosive decompression to open up the biomass structure, like steam explosion (SE), ammonia fiber explosion (AFEX) or CO_2 explosion (CE) (38). The hydrothermal treatment (HT) is also considered a physical-chemical treatment and consists of cooking the lignocellulosic material in hot water. Even though these treatments are promising, problems regarding to formation of degradation products - inhibitors (SE), partial removal of lignin (AFEX, CE and HT) and costs (CE) are still considerable (34, 38)

Several chemical pretreatments have been developed and generally consist of the addition of a chemical compound to remove the lignin. This can be an alkaline or acid solution, a solvent, ozone or oxygen (34). In this class of pretreatments, costs related to the equipment (resistant to corrosion), residues generated (e.g. neutralization in the acid hydrolysis), high amounts of inhibitors (e.g. 5-hydroxymethylfurfura and furfural in the acid hydrolysis), expensive process (e.g. ozone) remain a challenge to overcome.

Biological pretreatments are rarely used due to their low treatment rate. They consist of the partial degradation of lignin and hemicellulose by fungi and actinomycetes. This process requires low energy and no chemicals, but is currently not considered in commercial applications (34, 38).

The formation of inhibitors is one of the biggest obstacles to overcome in pretreatments. Three classes of inhibitors can be formed during pretreatments, furaldehydes such as furfural (HF) and 5-hydroxymethylfurfural (HMF), organic acids, and phenolic substances. HF and HFM are formed by an acid-catalyzed dehydration of pentoses and hexoses respectively. A further degradation reaction can form levunic acid and formic acid from HF and HMF (39). Organic acids, like acetic acid and ferulic acid, are derived from the partial degradation of hemicellulose while phenolic compounds are derived from lignin. The negative impact of these compounds on 2nd generation bioethanol production is related to cell metabolism and enzymatic hydrolysis. Reduced fungal cellulase production/activity, biomass saccharification and alcoholic fermentation can be caused by the inhibitors originating from the pretreatment of lignocelullose (40-42). The production of these substances is mainly dependent on temperature, pH, biomass composition and time of pretreatment. Most of the studies showed that furaldehydes causes oxidizing consequences, principally into DNA and proteins/enzymes and can consequentially reduce the metabolic flux (39, 43). Furfural showed to be an inhibiting agent, decreasing cellulase and β-glucosidase production in *T. reesei* RUT C30 (40). Ximenes et al. (44) showed that phenols, such as vanillin, syringaldehyde, trans-cinnamic acid, and hydroxybenzoic acid, are major inhibitors of cellulose hydrolysis. Some inhibitors can be metabolized at low concentrations by microorganisms, such as furfural that can be transformed into the less toxic substances, such as furoic acid by *S. cerevisiae* and *T. reesei* (40, 43).

2.5 **Enzymatic hydrolysis**

A major aspect of the second generation bioethanol production is the depolymerization of cellulose and hemicellulose into fermentable sugars using cellulolytic and hemicellulolytic enzymes. This step, also called saccharification, is considered the most costly step of the process, due to the amount of enzymes needed and their high production cost (45).

Second generation bioethanol may favorably compete with bioelectricity production when sugarcane trash is used and when low cost enzyme and improved technologies become commercially available. Innovations to achieve a cost effective and efficient commercial saccharication process are receiving considerable attention (46).

Although filamentous fungi and bacteria are both able to produce extracellular hydrolytic enzymes, fungi produce a wider range of enzymes and are more commonly used in the production of glycohydrolases. The genome of some important microorganisms used for biomass degradation have been sequenced and analyzed and provided information about the diversity of their enzyme sets and lead to the discovery of new industrial enzymes (

Table 1).

Table 1 Glycohydrolase producers and their genomic status.

	Genome size		
Organism	**Mp**	**Status**	**Reference**
Trichoderma reesei	33	Completed	(47)
Trichoderma harzianum	37.6	Completed	(48)
Aspergillus niger	34	Completed	(49)
Aspergillus fumigatus	30	Completed	(50)
Penicillium janthinellum	35.2	Complete	*
Myceliophthora thermophile	38.7	Completed	(51)
Phanerochaete chrysosporium	30	Completed	(52)
Lentinus tigrinus	-	Incomplete	*
Lentinus edode	-	Incomplete	*
Pleurotus ostreatus	34.4	Complete	*
Coniophora puteana	43	Complete	(53)
Bacillus subtilis	4.2	Complete	(54)
Bacillus pumilis	3.7	Draft	(55)
Streptomyces griseorubens	7.6	Draft	(56)
Clostridium cellulolyticum	4.1	Draft	*
Clostridium thermocellum	3.8	Complete	(57)
Cellulomonas flavigena	4.1	Complete	(58)
Acidothermus cellulolyticus	2.4	Complete	(59)

* http://jgi.doe.gov/

Many filamentous fungi obtain their nutrients by decomposing plant biomass through the secretion of a mixture of plant biomass degrading enzymes such as cellulases, hemicellulases, ligninases and pectinases (60). *Trichoderma reesei* is a filamentous fungus

broadly used for production of cellulases. However, it is a poor producer of other relevant enzymes like β-glucosidase and hemicellulases. Nowadays, several commercial enzymes mixtures are available for plant biomass saccharificcation and *T. reesei* and *A. niger* are the main microorganisms used for their production (Table 2). According to Gusakov et al. (61) enzyme extracts from a unique strain or species may contain poorly balanced ratios of each enzyme required for complete biomass degradation. Consequently, a combination of extracts/enzymes is needed for suitable application in biofuels.

Table 2 Currently available commercial enzymes for saccharification of industrial lignocellulosic biomass.

Enzyme complex	**Producing Company**	**Production host**	**Main enzymatic activity and mentioned ref**
ACCELLERASE® 1500	Genencor	*Trichoderma reesei*	Endoglucanase, xylanase and β-glucosidase (62)
ACCELLERASE® TRIO	Genencor	*Trichoderma reesei*	Endoglucanase, xylanase and β-glucosidase
ACCELLERASE® XC	Genencor	*Penicillium funiculosum*	Endoglucanase and xylanase (63)
SPEZYME CP	Genencor	*Trichoderma reesei*	Cellulase (63)
Multifect xylanase	Genencor	*Trichoderma reesei*	Xylanase (3)
Multifect pectinase	Genencor	*Aspergillus niger*	Pectinase and xylanase (3)
Celluclast 1.5L	Novozymes	*Trichoderma reesei*	Cellulase (3)
Novozyme 188	Novozymes	*Aspergillus niger*	β-glucosidase (3)
Cellic® CTec3	Novozymes	-	Cellulases, β-glucosidases, hemicellulase (64, 65)
Cellic HTec3	Novozymes	-	Endoxylanase with cellulase background (65)
CodeXyme®4	Codexis	-	Cellulase
CodeXyme®4X	Codexis	-	Cellulase and hemicellulase
Pyrolase® Cellulase	Verenium	-	Spectrum β-glycosidase
Xylathin® xylanase	Verenium	-	Xylanase

Reducing the cost of biomass saccharification requires more efficient enzymes cocktails, which would enable a reduction in the enzyme load and an increase in the yield of sugar release. Most studies that aim to improve the hydrolysis of the lignocellulose materials focus on cellulases, even though hemicellulose and lignin create restrictions to cellulose hydrolysis. Enzymes like xylanase and pectinase can stimulate biomass saccharification by the removal of those polysaccharides that overlie the cellulose fibers (3). One way to obtain more efficient commercial enzyme mixtures is to supplement them with additional enzymes produced by other fungi. Ravalason et al. (66), showed that the supplementation of a *T. reesei* cellulase cocktail with *Fusarium verticillioides* enzymes increased wheat straw hydrolysis.

Inhibition of cellulase activity by glucose represents another hurdle for the efficient enzymatic hydrolysis of plant biomass. In industrial hydrolysis high solid contents are added and consequently higher glucose concentrations are produced, which can inhibit the cellulases and decrease hydrolysis yields (67). To solve this problem, the focus is on cellulolytic enzymes that are not inhibited by glucose and on technologies to remove the monosaccharides during hydrolysis.

The hydrolysis duration also affects the cost of the saccharification step. According to Dias et al. (68) which simulated the integrated bioethanol production process from sugarcane, long hydrolysis periods requires large hydrolysis reactors and provide only small improvements on the yield. The authors suggested that the best conditions for the 2nd generation bioethanol integrated production are periods of 24–48 h of hydrolysis and higher solids loading during the hydrolysis step.

Economic analysis of such scenarios showed that the enzymes strongly impact on the final price of 2nd generation bioethanol. Macrelli et al. (6) analyzed 14 scenarios of 2nd generation bioethanol production from sugar cane and suggested that a decrease of 50% in enzyme cost could lower the minimum ethanol selling price (MESP) by 18-20%. According to Dias et al. (4) as 2nd generation bioethanol competes with bioelectricity production, this biofuel will be feasible when low cost enzymes are commercially available. In this study, applicable to the Brazilian situation, higher enzymes cost impact significantly on the internal return rate.

2.6 **Bioethanol production process**

Lignocellulosic biofuel production involves deconstruction of cell wall polymers into component sugars (pretreatment and saccharification), and fermentation of the sugars to

biofuels. Lignocellulosic biomass degradation products contain a mixture of hexoses (D-glucose) and pentose sugars (D-xylose and L-arabinose), resulting from the hemicellulose part of biomass. However, wild type ethanol fermentation organisms, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, can only use hexoses (69, 70). The main challenge is to construct a microorganism able to metabolize both hexoses (glucose, galactose, mannose) and pentoses (xylose, arabinose) generated in the saccharification step with high alcoholic yield (70). In nature, there are several microorganisms which are able to produce ethanol from xylose, like *Scheffersomyces shehatae, Scheffersomyces stipites, Pachysolen tannophilus* and *Spathaspora passalidarum* (71-74), although no report presented a robust enough microorganism for efficient ethanol production (75).

Sugar- and starch-based ethanol plants operate exclusively with *S. cerevisiae* due its characteristics such as high ethanol yield and high productivity (76). For this reason strategies on pentose utilization by *S. cerevisiae* have been extensively performed, by the functional expression of a variety of foreign genes from natural pentose-fermenting microorganisms (77). Those sugars can be converted in *S. cerevisiae* metabolism in the pentose phosphate pathway after initial conversion by heterologous reduction/oxidation-based and isomerization-based pathways (78). Metabolism of xylose by *S. cerevisiae* was achieved by cloning xylose reductase (XR) and xylitol dehydrogenase (XDH) and over expressing xylose isomerase (XI) (70). The metabolism of arabinose was only suitable for ethanol fermentation by the *Escherichia coli* arabinose pathway in which the original L-arabinose isomerase was replaced by that of *Bacillus subtilis* (76, 79, 80).

However, the transport of pentoses is still a barrier in second generation bioethanol production. A good understanding of the role of the transporters, engineering of more suitable transporters and combine introduction in *S. cerevisiae* with xylose and arabinose pathway are needed to obtain conversion results similar to those of glucose. The *S. cerevisiae* sugar transporters have lower affinity for xylose than for glucose, which can limit the ethanol pathway flux and affect productivity. Tanino (81) improved xylose consumption and ethanol production of a *S. cerevisiae* XI-based metabolic pathway by overexpressing sugar transporters, like HXT1, GXF1 and GXS1. Also directing the metabolic flux to ethanol production and minimizing unwanted product formation like acetic acid and latic acid, can increase productivity. Gonçalves et al. (82) studied the effect of the HXT1, HXT2, HXT5 and HXT7 permeases on ethanol fermentation from xylose and/or glucose media. Different behavior suggested that the HXT1 transporter could be used for xylose/glucose blends, while the HXT7 permease would be a better choice for xylose-rich fractions.

Furthermore, the fermentation product, ethanol, is toxic for the fermenting microorganism putting an upper limit on the concentration that can be achieved. For instance, *S. cerevisiae* cannot tolerate ethanol concentrations exceeding 25% v/v (83). As a result ethanol yield will be limited and the obtained product must be concentrated by distillation, which is an expensive step. However, most other organisms are even less tolerant to ethanol than *S. cerevisiae* (84). Other qualities, like tolerance to acetic acid and phenolic compounds are also required (85). The biggest difficulty in modifying the microorganisms of this step is related to the current operation mode of the alcoholic plants. Biomass recycling and lack of sterilization requires strains that are genetically stable and can suppress the environmental microorganism contaminations.

2.7 **Genetic manipulation**

Modification of the genetic content of the organisms used in the 2nd generation bioethanol process is a promising tool to solve several techno/economic limitations. Genetic manipulation can be applied from the beginning of the process, biomass cultivation, until the end, alcoholic fermentation.

Modifying lignin content, composition, hydrophobicity and cross-linking in plants can improve the enzymatic hydrolysis of their cell walls. Down-regulation of the lignin biosynthetic pathway enzymes would create a potential way to decrease pretreatment costs (86). A second strategy to this is to prevent that plant carbon sources are consumed by lignin synthesis. Moreover, modifying the monomeric composition of lignin even without the need to reduce its entire content helps to improve biomass digestibility. As an example, over expression of the ferulate 5-hydroxylase gene in poplar results in higher content of syringyl lignin and easier digestion in comparison with wild type plants (87). However, it is necessary to insure that lignin manipulation does not disturb the plants defense system and integrity (88).

Many enzymes and other proteins, carbohydrates, lipids, industrial polymers and pharmaceuticals are already industrially produced in plants (88, 89). In order to produce cost effective enzymes for use in cellulosic hydrolysis, heterologous expression of plant cell wall hydrolyzing enzymes in plants is a new opportunity. However, efficient expression needs codon optimization of the coding region, which is widely used for the heterologous expression of microbial proteins. Moreover, accumulation of biomass hydrolysis enzymes in sub-cellular compartments to prevent misfolding in the new environment may be required (90, 91). An oxidizing environment and the presence of numerous chaperons with few

proteases make the endoplasmic reticulum a potential compartment for targeting enzymes, which results in more stable enzymes than when they are secreted to cytosol (92, 93). Another way to increase enzyme production in plants is to engineer the chloroplast genome instead of the nuclear genome. The chloroplast genome in most flowering plants is inherited maternally, which allows transgene containment. Furthermore, it has already been shown that targeting xylanase to both chloroplast and peroxisomes results in higher levels of production compared to targeting the enzyme to either of them (94). Therefore targeting to several compartments simultaneously might increase the enzyme production level. A recent study by Zhang et al. (95) reported production of high levels of endo-1,4-β-glucanase from *Acidothermus cellulolyticus* (E1) in rice seeds (*Oryza sativa* L. ssp. *japonica*). The activity level of the obtained enzyme without purification and enrichment is already close to some commercially produced enzymes.

Increasing the production of cellulolytic and hemicellulolytic enzymes by microorganisms using genetic modification has been done for several years. In addition, classical random mutation methods (UV and chemical) were used to obtain better producing strains, such as the well-known hypercellulolytic *T. reesei* RUT-C30 strain (96). Also other fungal species have been mutated using chemical and physical agents, resulting in more hypercellulolytic strains, such as *Penicillium echinulatum* 9A02S1 (97) and *Acremonium cellulolyticus* CF-2612 (98).

Studies with *Aspergillus niger* revealed that expression of genes encoding cellulases and hemicellulases is modulated by the carbon catabolic repression protein CreA and by positively acting regulators (e.g. XlnR and AraR) that respond to the presence of specific sugars (99-101). Therefore studies were performed to produce CreA-derepressed strains of several fungi able to produce higher levels of enzymes. Fujii et al. (102) constructed a *creA* disruptant strain of *A. cellulolyticus* with enhanced cellulase and xylanase production. Nakari-setalä et al. (103) obtained *T. reesei cre1* mutants, which is the homolog of *creA* that produced more cellulase and xylanase than the wild type in bioreactors.

Heterologous expression of glycohydrolases genes for use of the corresponding enzymes in biofuel production was widely explored. Production of recombinant enzymes does not generate enough amount of enzyme to be applied as the base catalytic enzyme on plant biomass degradation. In this way recombinant enzymes present better applicability on supplementation of base/crude cellulolytic mixtures. Also, the effect of a single enzyme on hydrolysis can be studied better when recombinant or partial purified enzymes are used (104). Supplementation of recombinant enzymes should correct unbalanced base cocktails or

introduce lacking enzyme activities such as arabinofuranosidases, β-xylosidase, β-glucosidase and esterases. Delabona et al. (105) supplemented a cellulolytic extract of *T. harzianum* with a recombinant α-L-arabinofuranosidase and demonstrated synergistic effects of the supplementation on sugar cane bagasse hydrolysis.

2.8 **Conclusions**

Fulfilling the urgent need to substitute fossil fuels with clean and sustainable resources requires acceleration in research and development of biofuels. Moreover this product must be supported by policies and regulation, so that it can compete economically with the fossil fuels. Improving transgenic microorganisms, biomass pretreatment, biomass degrading enzymes and improving efficiency of pentoses conversion for fermentation seem to be promising approached for an economically viable saccharification and fermentation procedure. However, determination and/or developing potential feedstock crops and efficient land use, which will not compete with agriculture or forestry purposes is another necessity for sustainable bioethanol production. Furthermore, enhanced technology and development exchange and global scientific collaborations are essential to decrease bottlenecks of this development.

CHAPTER 3 - THE CAPABILITY OF ENDOPHYTIC MICRORGANISMS FOR PRODUCTION OF HEMICELLULASES AND RELATED ENZYMES

3.1 Introduction

Cellulolytic and hemicellulolytic enzymes have been extensively investigated as tools to achieve viable second-generation ethanol production. The hemicellulases include accessory enzymes, which are a group of enzymes capable of increasing the yield of reducing sugars during enzymatic hydrolysis of lignocellulosic substrates. The definition of the accessory enzymes has evolved over time. Enzymes such as the β-glucosidases were originally classified as accessories, but today are considered essential in enzymatic cocktails, following elucidation of their mechanisms of action during substrate degradation (61, 106, 107).

The main accessory enzymes are currently considered to be α-L-arabinofuronosidase, hemicellulolytic esterases, β-mannanases, α-glucoronidases, β-xylosidases, pectinases, and xylanases. Several studies have shown that cellulase enzymes supplementation can improve the enzymatic hydrolysis of lignocellulosic biomass, in terms of speed and hydrolysis yield. One of the main issues is that crude multi-enzyme cocktails obtained from a single fungus strain, usually isolated from soil, are not ideal in biotechnological applications, since either cellulase activities are not expressed at sufficient levels, or the enzyme complexes are not well balanced in terms of the individual enzymes (61).

For this reason, strains isolated from unusual environments have been sought as alternative sources of hydrolytic enzymes (108, 109). Endophytic microorganisms are potentially amongst the most interesting microorganisms for screening for the production of industrial biocompounds. These microorganisms are ubiquitous in plants, inhabiting plant tissues without inducing any apparent symptoms in their hosts (110). The fact that these microorganisms are present within plant tissues could explain their capacity to produce substances that could have useful industrial, agricultural, and medicinal applications (111).

The endophytic fungi that have been reported to be xylanase producers include *Alternaria alternata* (112), *Hymenoscyphus ericae* (113), and *Aspergillus terreus* (114). De Almeida et al. (115) selected strains from the *Acremonium* endophyte species for hemicellulases and cellulases production. From 14 plant species, Suto et al. (116) isolated 155 strains of fungi that produced xylanases. Harnpicharnchai et al. (117) purified a thermotolerant β-glucosidase from an endophytic *Periconia* sp. Other studies have involved the selection of new isolates using extracellular enzymes as selection parameters for plant

growth promotion. de Olieveira et al. (118) investigated fungi isolated from *Annona* spp., while Luz et al. (119) employed isolates from *Passiflora edulis*.

Endophytic strains may therefore constitute a valuable source of biological material that deserves to be studied and explored for the production of cellulolytic and hemicellulolytic enzymes. In this context, the present work concerns the selection of endophytic fungi and actinomycetes as producers of hemicellulases and related enzymes with different enzymatic profiles, for use in the deconstruction of lignocellulosic biomass.

3.2 **Material and methods**

3.2.1 Microorganisms

Hemicellulase bioprospection was performed using a fungus culture collection maintained at the Microbiology and Molecular Biology Laboratory of the Federal University of Paraná (LabMicro/UFPR) and an endophytic actinomycetes collection maintained at the Bioproducts Laboratory (ICB/USP). A total of 119 Brazilian filamentous fungi were selected, previously isolated from *Eucalyptus benthamii*, *Platanus orientalis, Glycine max, Solanum tuberosum*, *Saccharum officinarum*, and decaying paper. A strain of *Aspergillus niger* ATCC 64973 was used as a positive control in plate assays. Also it was screened 45 actinomycetes isolated from *Citrus reticulate, Citrus sinensis, Theobroma cacao, G. max, S. officinarum*, *Catharanthus roseus* and soil.

3.2.2 Agro-industrial waste materials

The liquor (HL) and hydrothermal bagasse HB were derived from the hydrothermal pretreatment of sugar cane bagasse. The process consisted of suspending an amount of bagasse (10% w/w, dry basis) in water and loading it into a laboratory-scale reactor (7.5 L total volume, Model 4554, Parr, USA). The temperature was raised from room temperature (25 ^{o}C) to 190 ^{o}C, over a period of 1 h. After 10 min, the reactor was cooled to ambient temperature and the pentose-rich liquor (HL) was collected with the aid of a laboratory-scale screen filter (Nutsche filter, POPE Scientific, USA). Material composition was determined by acid hydrolysis with sulfuric acid and high performance liquid chromatography (HPLC) analyze (Dionex Ultimate 3000, equipped with Aminex HPX-87H 300 mm X 7.8 mm X 9 µm column, at 50 oC, 0.5 mL/min flow, mobile phase H_2SO_4 0.005M, Shodex IR detector at 40 oC, 50 µL injection volume), as described by Sluiter et al. (120). Total soluble lignin was determined by the method described by Gouveia et al. (121). The sugar cane bagasse was

obtained from a local mill (Usina Vale do Rosário, Orlândia, SP, Brazil).Ppretreated delignified sugar cane bagasse (DEB) was prepared and characterized by Rocha, Goncalves (122). The soybean bran (SB) was obtained from Agricola (São Carlos, Brazil) and was characterized by Rodrigues-Zuniga et al. (123).

3.2.3 Hemicellulolytic plate assay

The selection of hemicellulolytic strains was performed by cultivation on solid medium as described by Kasana et al. (124) containing 0.2% beechwood xylan (Sigma) or aqueous liquor diluted in deionized water at a volume ratio of 25%. The fungi strains were first grown on malt extract agar (MEA) for 5 days at 29 °C, and then inoculated onto the test media and incubated for 72 h at 29 °C. The pH was adjusted to 5.0, and 0.1% Triton X-100 (Merck) was added as a colony growth limiter. The hydrolysis halos were revealed by application of Congo Red (1%) for 15 min, followed by washing with 1 M NaCl for 10 min (124). The hydrolysis rates were calculated by dividing the diameters of the hydrolysis halos by the diameters of the colony halos. Same procedure was performed for bacterial strains but with previous growth on Tryptic Soy Agar (TSA) and pH adjusted to 7.0.

3.2.4 β-glucosidase plate assay

The strains were grown for 5 days in liquid medium (125) with carboxymethylcellulose (CMC, 1%) as sole carbon source, in 10 mL tubes (200 rpm, 29 °C). Fungal cultivation pH was 5.0 and the actinomycetes cultivation pH was 7.0 The biomass was separated by centrifugation, and the extract was subjected to an esculin gel diffusion assay (EGDA), as described by Saqib and Whitney (126), for 5 h at 37 °C. The plate was then placed on ice, and measurement was made of the dark brown zone formed by the action of β-glucosidase on esculin.

3.2.5 Shake flask cultures

The composition of the main culture medium was adapted from Mandels and Reese (127) for fungal cultivation: 1 mL Tween 80; 0.3 g L-1 urea; 2.0 g L-1 KH_2PO_4; 1.4 g L-1 $(NH_4)_2SO_4$; 0.4 g L-1 $CaCl_2.2H_2O$; 0.3 g L-1 $MgSO_4.7H_2O$; 1.0 g L-1 proteose peptone; 5.0 mg L-1 $FeSO_4.7H_2O$; 1.6 mg L-1 $MnSO_4.4H_2O$; 1.4 mg L-1 $ZnSO_4.7H_2O$; 2.0 mg L-1 $CoCl_2$.6H2O; 10 g L-1 carbon source. For actinomycetes it was used the medium described by Nascimento et al. (128) : 1 g/L de proteose peptona, 0.1% (v/v) de tween 80, g/L: $NaNO_3$

1.2 g/L; KH_2PO_4 3.0 g/L; K_2HPO_4 6.0 g/L; $MgSO_4.7H_20$ 0.2 g/L; $CaCl_2$ 0.05 g/L; $MnSO_4.7H_2O$ 0.01 g/L; $ZnSO_4.7H_2O$ 0.001 g/L; 10 g/L carbon source. As carbon source, it was used 10 g/L of DEB plus SB, at a 3:1 ratio (129). The previously selected strains were grown on PDA for fungi and on TSA for bacteria for 3 days at 29 °C, after which one 0.5 cm diameter disc was removed from each colony edge, transferred to an Erlenmeyer flask containing 20 mL of medium, and incubated for 144 h at 29 °C and 200 rpm. The best six strains were selected for growth using the same medium described above, but with the carbon source changed to citrus pectin or beechwood xylan. Samples were removed for determination of enzyme activities and protein contents, as described below.

3.2.6 Enzymatic assays

Measurement of enzymatic activities (in International Units, U) was performed using different substrates in order to determine global and single activities. Filter paper activity (FPase) was determined as described by Xiao et al. (130). All the polysaccharides were purchased from Sigma Aldrich or Megazyme, and were assayed at 0.5% in a 10 min reaction. The polysaccharides used were: Beechwood xylan; Birchwood xylan; Rye arabinoxylan; Wheat arabinoxylan; Sugar beet arabinan; CMC; Barley β-glucan; Tamarind xyloglucan; Icelandic moss lichenan; Laminarin from *Laminaria digitata*; Chitosan from shrimp shells; Konjac glucomannan; Carob galactomannan; 1,4 β-mannan and citrus pectin. CMC was assayed in a 30 min reaction. The enzymatic activity was determined from the amount of reducing sugars released from the different polysaccharide substrates, using the DNS method (131) with glucose as standard. The activities of β-glucosidase, β-xylosidase, β-mannosidase, α-L-arabinofuranosidase, and cellobiohydrolase II were measured using the respective p-nitrophenol residues (pNP) (Sigma-Aldrich, USA). The assays employed 10 μL of diluted centrifugation supernatant and 90 μL of the respective pNP (0.5 mM, diluted in citrate buffer), and the mixtures were incubated for 10 min at 50 °C. The reactions were stopped by adding 100 μL of 1 M Na_2CO_3, and the absorbance was measured at 400 nm using a Tecan Infinite® 200 instrument (Männedorf, Switzerland). All the assays utilized an epMotion® 5075 automated pipetting system (Eppendorf) and were performed at pH 5,0 with 50 mM citrate buffer for fungal extracts and at pH 7.0 with 50 mM phosphate buffer for actinomycetes extracts. One unit of glycohydrolases activity corresponds to 1 μmol of glucose or pNP released per minute.

3.2.7 Fungal morphological identification

Initial fungus identification was performed using macro and micro morphological characteristics (132-134). The analysis of fungal reproductive structures by optical microscopy was carried out as described by Kern and Blevins (135).

3.2.8 Fungal DNA extraction

An approximately 1 cm^2 colony of 5-day-old cultures was transferred to a 2 mL Eppendorf tube containing 300 µL CTAB (cetyltrimethylammonium bromide) buffer (2% (w/v) CTAB, 1.4 M NaCl, 100 mM Tris–HCl, pH 8.0, 20 mM EDTA, and 0.2% (v/v) β-mercaptoethanol) and about 80 mg of a 2:1 (w/w) mixture of silica gel H (Merck) and Celite™ 545 (Macherey Nagel & Co). The cells were disrupted manually with a sterile pestle for about 5 min. Subsequently, 200 µL CTAB buffer was added, and the mixture was vortexed and then incubated for 10 min at 65 °C. After the addition of 500 µL chloroform, the solution was mixed and centrifuged for 5 min at 20,500 x *g*. The supernatant was transferred to a new tube, together with 2 volumes of ice-cold 96% ethanol. The DNA was allowed to precipitate for 30 min at −20 °C, after which centrifugation was performed for 5 min at 20,500 x *g*. After washing with cold 70% ethanol and drying at room temperature, the pellet was resuspended in 97.5 µL TE buffer together with 2.5 µL RNAse (20 U/mL), and incubated for 5 min at 37 °C, before storage at −20 °C (136).

3.2.9 Fungal DNA amplification and sequencing

The rDNA Internal Transcribed Spacer (ITS) region was amplified using ITS5 and ITS4 primers (137). Partial β-tubulin (BT2) gene was amplified using Bt2a and Bt2b primers (138). The sequencing of β-tubulin gene was performed for some strains to confirm the ITS phylogeny clustering. Amplicons were cleaned with a GFX™ PCR DNA purification kit (GE Healthcare, UK). Sequencing was performed on an ABI 3130 automatic sequencer (Applied Biosystems). The Staden sequence analysis package (v. 1.6.0) was used to edit and align the sequences (139). Sequence analysis was performed using BLASTn sequence alignment software, run against the NCBI (National Center for Biotechnology Information) database. The phylogenetic trees were constructed with 1000 bootstrap replicates using MEGA v4.0.2 software (140), with application of the neighbor-joining method (141), the Jukes-Cantor

distance correction model (142). The nucleotide sequences used in this study were obtained/submitted to GenBank (Appendix, Appendix A - Table 27).

3.3 **Results**

3.3.1 Agro-industrial waste material composition

The sugar cane hydrothermal pretreatment liquor showed the following composition (g/L): xylo-oligosaccharides (9.98), xylose (4.70), glucose (0.55), arabinose (0.77), cellobiose (0.0), furfural (1.05), hydroxymethylfurfural (0.18), acetic acid (1.47), formic acid (0.23), and total soluble lignin (3.15). Despite the presence of inhibitors, this liquor demonstrated to be a potential carbon source for the screening of enzyme producers and the production of hemicellulases. The DEB was composed of 77.89% cellulose, 7.09% hemicellulose, and 16.22% lignin. The SB consisted of 34% cellulose, 18.13% hemicellulose, 9.78% lignin, and 43.22% protein. The media prepared using these waste materials were therefore able to provide a suitable ratio of cellulose and hemicellulose for the synthesis of glycohydrolases, as well as a good source of nitrogen.

3.3.2 Plate screening

A total of 120 fungal strains were bioprospected and used for calculation of hydrolysis rates (Appendix, Appendix B - Table 28). The media containing liquor were stained with Congo Red, revealing the yellow hydrolysis halos (Figure 3). A total of 73 strains were unable to grow on the medium, while only 35 were able to both grow and produce halos. On the other hand, in the case of the medium with xylan, only two strains, one *Aspergillus* sp. and one *Diaphorte* sp. were unable to grow, while 102 strains grew and produced halos. It was therefore demonstrated that the xylose/xylo-oligomers liquor produced by a simple pretreatment was able to sustain the growth of a significant number of the fungi tested.

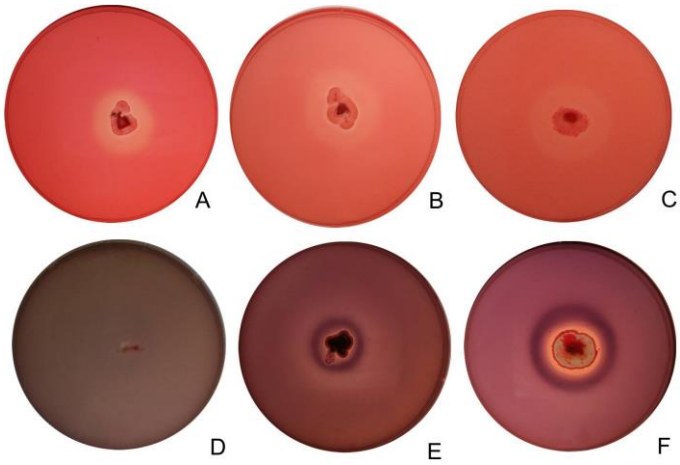

Figure 3 Hydrolysis results following staining with Congo Red, using xylan agar (A, B, and C) and liquor agar (D, E, and F). The organisms used were *Penicillium* sp. DR65 (A, D), *Aspergillus* sp. DR06 (B, E), and *Fusarium* sp. DR15 (C, F).

Selection of β-glucosidase producers employed the EGDA to determine β-glucosidase in the fungal culture extracts, with positive extracts forming dark-colored halos (Figure 4). Of the 119 extracts tested, 63 produced measurable halos, 27 showed dark precipitates although measurement was not possible, and 40 strains were negative for β-glucosidase production. The plate screening and EGDA results were used to select 56 strains for a second screening employing shake flask cultivations. Some of these strains were negative in the hemicellulolytic and β-glucosidase tests, and were used as controls to ensure selection consistency.

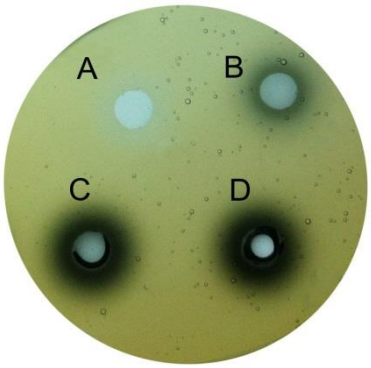

Figure 4 Enzymatic extracts applied in EGDA. Blank (A), positive control *A. niger* ATCC 64973 (B), *Aspergillus* sp. DR24(C) and *Annulohypoxylon stygium* DR47 (D).

Of 45 actinomycetes tested, 15 strains were not able to grow on media with licor (25% v/v) and only 23 grew and produced halos in the presence of this waste. All the bacterial

strains were able to grow on media containing xylan and 25 produced hydrolysis (Appendix, Appendix C- Table 29). The EGDA assay revealed that only 11% actinomycetes were positive for β-glucosidase production which suggested that these microorganisms are not relevant source β-glucosidase. Twelve strains were chosen for shake flask cultivations selection.

3.3.3 Fungal shake flask screening

The strains were grown using DEB+SB (3:1) at 29 °C on a rotary shaker at 200 rpm for 96 h. The results obtained for some of the strains are presented in Figure 5. Low β-glucosidase activities were detected up to 48 h of fermentation, while high activity levels were observed at 96 h. This was expected, since several filamentous fungi are known to begin to produce detectable amounts of this enzyme after 72 h of growth (143).

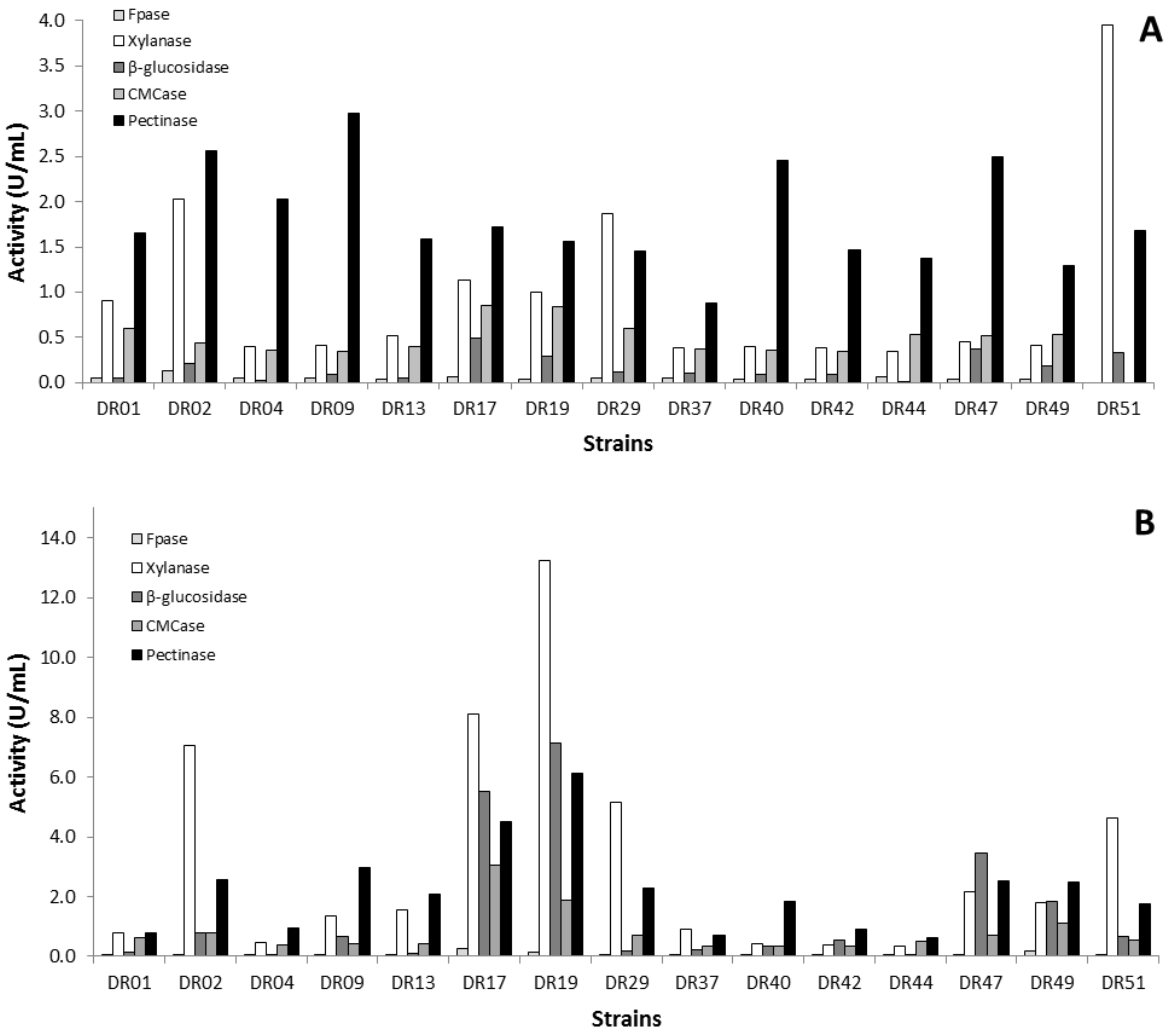

Figure 5 Enzymatic activities of some fungi pre-selected strains, grown in shake flasks with DEB+SB (3:1), after 48 h (A) and 96 h (B).

The CMCase and FPase activities were low for all the strains, as expected because selection was performed using materials rich in hemicelluloses. High xylanase production was detected at 48 h for many strains, but the largest peaks occurred at 96 h. Pectinase production showed little variation between 48 and 96 h, although amounts of the enzyme nonetheless increased over the course of the fermentation. Strains morphologically similar to *Aspergillus fumigatus* (DR08, DR03, DR29, and DR31) were excluded due to possible pathogenicity, which could preclude their use in industrial applications.

3.3.4 Fungal glycohydrolase profile

In order to identify fungi that produced enzyme with different profiles, and hence obtain a more efficient enzymatic extract, 12 strains were selected according to their morphology and enzymatic profiles. A new fermentation with DEB+SB was performed, and samples were taken daily for measurement of xylanase, β-glucosidase, and pectinase activities. The samples that showed the highest glycohydrolase activity were tested using different substrates (Table 3).

Table 3 Glycohydrolases activities (U/mL) of twelve selected strains grown using DEB+SB

Strains	**DR02**	**DR06**	**DR07**	**DR17**	**DR19**	**DR20**	**DR26**	**DR40**	**DR45**	**DR47**	**DR48**	**DR49**
Time (h)	**120**	**120**	**144**	**96**	**72**	**144**	**144**	**120**	**144**	**96**	**144**	**144**
Birchwood xylan	4.50	1.38	0.55	10.32	3.22	0.41	0.60	0.38	0.77	0.96	0.50	4.53
Beechwood xylan	3.94	2.30	0.95	5.54	3.64	0.63	0.77	0.44	1.11	1.51	1.34	4.03
Rye arabinoxylan	2.93	2.19	0.62	4.13	2.00	0.47	0.53	0.38	0.64	1.30	0.44	3.71
Wheat arabinoxylan	0.53	0.53	0.36	0.86	0.30	0.36	0.55	0.43	0.45	0.37	0.42	0.27
Arabinan	0.46	0.50	0.46	0.48	0.48	0.48	0.47	0.47	0.48	0.49	0.48	0.47
CMC	0.27	0.39	0.17	0.26	0.16	0.19	0.18	0.16	0.30	0.22	0.19	0.66
β-glucan	1.84	3.63	2.26	4.16	0.53	0.48	2.29	0.47	2.06	2.63	1.35	3.46
Xyloglucan	0.52	0.65	0.46	0.73	0.47	0.49	0.48	0.47	0.58	0.69	0.43	0.58
Lichenan	1.04	1.81	1.24	2.00	0.66	0.79	1.08	0.37	1.02	1.11	1.14	1.44
Laminarin	0.68	0.54	0.76	1.70	0.72	0.59	1.11	0.67	0.72	0.80	1.90	1.44
Chitosan	0.63	0.57	0.52	0.53	0.42	0.54	0.68	0.66	0.64	0.61	0.49	0.63
Glucomannan	1.02	2.28	0.84	1.83	0.78	0.48	0.95	0.64	1.85	1.64	1.31	1.91
Galactomannan	0.75	1.41	0.49	1.79	0.55	0.56	0.53	0.48	1.30	1.24	1.25	1.63
1,4 β-mannan	0.65	1.34	0.47	1.22	0.52	0.53	0.50	0.46	1.34	1.44	0.94	1.22
Pectin	0.63	0.84	0.69	0.44	0.65	0.87	0.86	0.69	0.77	0.69	0.80	1.14
pNP β-D-xylopyranoside	0.13	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.10
pNP β-D-mannopyranoside	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
pNP β-D-cellobioside	0.24	0.03	0.47	0.46	0.15	0.12	0.03	0.18	0.41	0.37	0.35	0.18
pNP α-L-arabinofuranoside	0.15	0.02	0.01	0.01	0.00	0.00	0.01	0.02	0.07	0.06	0.02	0.10
pNP β-D-glucopyranoside	1.16	0.11	2.85	5.75	0.62	1.19	0.23	0.82	3.44	2.52	1.33	0.68

The strains DR17 and DR19 (*Trichoderma* sp.), and DR02 (*Aspergillus* sp.) presented the highest xylanolytic activities for birchwood xylan, beechwood xylan, and rye arabinoxylan. Despite the fact that the strains DR17 and DR19 belong to the same genus, and have similar morphologies, they presented different enzymatic profiles (Table 1). Selection was made of six strains (DR02, DR17, DR19, DR40, DR47, and DR49) that showed enzymatic activities for a wider range of substrates, were morphologically different, and presented distinct enzymatic profiles. These strains were cultured in shake flasks containing xylan and pectin as inducer carbon sources. Samples were taken daily for measurements of xylanase, β-glucosidase, and pectinase activities. The fungal extracts that showed highest glycohydrolase activities were tested using different substrates (Table 4).

Table 4 Glycohydrolases activities (U/mL) of six selected fungal strains grown on pectin and xylan.

Strain	***A. niger* DR02**		***T. atroviride* DR17**		***T. atroviride* DR19**		***A. stygium* DR40**		***Alternaria* sp. DR47**		***T. wortmannii* DR49**	
Carbon source	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**
Time (h)	**144**	**144**	**120**	**120**	**96**	**144**	**120**	**144**	**120**	**120**	**120**	**120**
Birchwood xylan	0.72	21.34	0.00	2.22	0.59	2.99	1.39	4.68	1.12	1.32	0.74	4.85
Beechwood xylan	1.56	15.04	0.60	2.72	0.49	2.88	1.57	6.87	0.00	2.59	1.44	6.00
Rye arabinoxylan	1.41	11.15	0.00	2.73	0.61	2.76	1.75	5.98	0.46	1.57	1.12	4.07
Wheat arabinoxylan	0.86	3.88	0.80	0.87	0.77	0.87	0.41	0.59	0.15	0.34	0.55	0.37
Arabinan	0.52	1.46	0.77	0.48	0.43	0.49	0.55	0.85	0.89	1.11	1.04	0.95
CMC	1.32	1.24	0.57	0.56	1.25	0.49	0.36	0.56	0.92	1.28	1.42	4.57
β-glucan	2.51	14.03	0.89	1.03	0.69	0.89	0.71	5.67	0.52	1.49	4.16	1.89
Xyloglucan	0.47	1.63	0.31	0.47	0.26	0.48	0.49	2.42	0.76	1.38	0.94	0.94
Lichenan	1.22	4.66	0.55	0.61	0.74	0.71	0.74	2.37	0.27	0.87	2.14	1.43
Laminarin	1.79	1.50	1.72	1.92	1.32	1.78	1.79	4.28	0.66	0.60	3.78	3.20
Chitosan	0.60	1.86	0.76	0.63	0.96	0.57	0.88	0.58	0.00	0.00	1.39	1.09
Glucomannan	1.34	1.90	0.96	0.72	0.53	0.63	0.74	1.23	0.56	0.79	1.41	1.00
Galactomannan	1.11	1.45	0.49	0.49	0.68	0.45	0.50	0.99	0.61	0.23	1.20	0.95
1,4 β-mannan	0.87	1.78	0.56	0.59	0.59	0.49	0.54	0.90	0.81	0.54	1.31	1.09
Pectin	0.58	0.55	5.09	0.71	4.24	0.49	3.92	1.55	7.72	1.31	1.81	0.72
pNP β-D-xylopyranoside	0.16	0.00	0.00	0.05	0.00	0.02	0.03	0.14	0.01	0.02	0.13	2.85
pNP β-D-mannopyranoside	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.01	0.04	0.03
pNP β-D-cellobioside	0.58	0.00	0.00	0.02	0.00	0.03	0.06	0.50	0.05	0.27	1.15	1.50
pNP α-L-arabinofuranoside	0.33	0.21	0.00	0.01	0.00	0.01	0.02	0.67	0.63	0.24	0.57	0.91
pNP β-D-glucopyranoside	3.09	0.22	0.05	0.24	0.22	0.30	0.67	1.48	0.52	1.37	1.86	3.13

The fungi xylanolytic profiles differed among the strains and the carbon sources used. The DR17 strain produced xylanases with the same affinity for birchwood xylan, beechwood xylan, and rye arabinoxylan, when cultivated in the presence of beechwood xylan. However, this was not observed when the same *Trichoderma* sp. was grown using DEB+SB. Some strains showed higher activity for beechwood xylan than for birchwood xylan (DR49 and DR40), and vice versa (DR02). The DR02 strain showed the highest activity for rye arabinoxylan. The DR40 strain only produced xylanase when the fungus was grown in the presence of xylan, in contrast to other strains such as DR19, DR49, and DR17, for which DEB and SB also induced the production of xylanases.

The production of β-glucanases was high for DR02 and DR40 strains when cultivated on xylan, for DR17 when grown on DEB+SB, and for DR49 on pectin. However, when these extracts were tested using xyloglucan, all the activities decreased, indicating less affinity for the hydrolysis of β-glucan with branched xylose residues.

A similar phenomenon occurred in the testing of lichenan, which is a linear glucan with more β-1,3 bonds than β-glucan. This indicates that the β-glucanases present in these extracts had lower lichenanase activity. Furthermore, when the DR40 and DR49 strains were grown on xylan, they showed activity against laminarin, indicating the presence of enzymes able to hydrolyze the β-D-glucosyl (1→6) β-D-glucose bond. For almost all fungi, with the exception of DR02 and DR49, the production of polygalacturonase was only induced in the presence of pectin. The best producers were the strains DR47 (7.72 U/mL) and DR17 (5.09 U/mL).

The production of β-glucosidase showed no consistent induction pattern for the three carbon sources tested. DR17 and DR47 produced more β-glucosidase on DEB+SB, while DR02 produced more on pectin, and DR49 on xylan. None of the fungi showed measurable activities for β-1,4-D-glucosaminidase or α-mannosidase.

When the *Talaromyces* sp. DR49 strain was grown on xylan, it was able to produce multiple accessory proteins such as xylosidase, arabinofuranosidase, cellobiohydrolase II, and β-glucosidase. This strain might therefore be promising for the production of hemicellulases. High CMCase activity was measured when this fungus was cultivated on xylan, but it did not present high activities against β-glucan. However, opposite result was found when this strain was grown on DEB+SB.

The hydrolytic action of the fungal extracts against mannan polymers was low for all the strains. Nevertheless, activities for heteromannans (glucomannan and galactomannan) were higher than for β-1,4-mannan. This could be explained by the presence of β-(1→4)-

glucanase activity in the extracts in the case of glucomannan, and the presence of α-1,6-galactosidase in the case of galactomannan.

3.3.5 Fungal identification

Strain identification was performed using morphological characteristics as well as sequencing of the ITS regions of the ribosomal DNA gene and (in some cases) the partial β-tubulin gene. Result of identification was summarized in Table 5.

The best xylanase producer strain, DR02, previously isolated from *Platanus orientalis*, was identified according to morphology (rough dark brown conidia, spherical vesicles and biseriate conidiophores) as *Aspergillus* section *Nigri*. The ITS regions and partial BT2 sequencing were performed and submitted to GenBank (accession number KC311839, KC311845). The phylogenetic trees, built with reference strains of *Aspergillus Nigri* section species, showed that the DR02 isolate clustered with *A. niger* (Figure 6). Higher value of *A. niger* BT2 clustering confirm the ITS result, the strain DR02 belongs to the *Aspergillus niger* species.

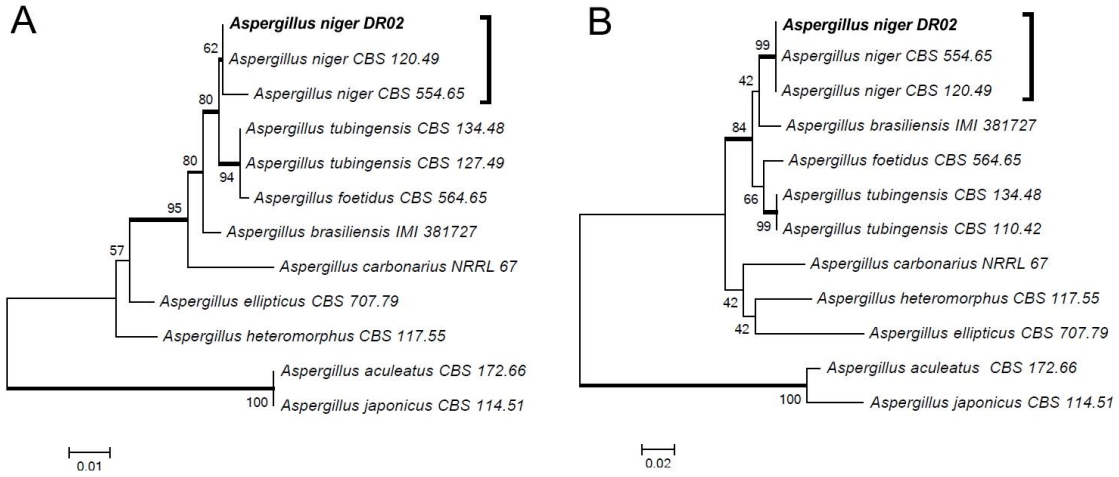

Figure 6 Phylogenetic tree of *Aspergillus* section *Nigri* based on confidently ITS (A) and partial BT2 (B) sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in bold. Strains in bold indicate isolates of this study.

Table 5 Fungi strains identification by morphology and sequencing of ITS region and partial β-tubulin gene.

Strains	**Source**	**Morphological identification**	**Gene**	**Closer Microorganism**	**GenBank number**	**E-Value**	**Identity**	**Strain GenBank number**
DR02	*Plantanus orientalis*	*Aspergillus* section *Nigri*	ITS	*Aspergillus niger* ATCC 1015	JX535496	0.0	100%	KC311839
DR02	*Plantanus orientalis*	*Aspergillus* section *Nigri*	β-tubulin	*Aspergillus niger* DAOM 23922	EU907906	0.0	100%	KC311845
DR47	*Eucaliphytus benthamii*	Xylariaceae	ITS	*Annulohypoxylon stygium* 2713	EU272517	0.0	99%	KC311843
DR47	*Eucaliphytus benthamii*	Xylariaceae	β-tubulin	*Annulohypoxylon stygium YMJ 90041409*	AY951666	0.0	100%	KC311846
DR49	Decaying paper	*Penicillium* sp.	ITS	*Penicillium kloeckeri KUC 1286*	HM469393	0.0	100%	KC311844
DR49	Decaying paper	*Penicillium* sp.	β-tubulin	*Talaromyces wortmannii W35*	AY533533	3E-160	97%	KC311847
DR40	*Eucaliphytus benthamii*	*Alternaria* sp.	ITS	*Alternaria alternata* ATCC MYA-4642	JQ320281	0.0	100%	KC311842
DR17	*Eucaliphytus benthamii*	*Trichoderma* sp.	ITS	*Trichoderma atroviride* ATCC 20476	JQ745258	0.0	100%	KC311840
DR19	*Eucaliphytus benthamii*	*Trichoderma* sp.	ITS	*Trichoderma atroviride* ATCC 20476	JQ745258	0,0	100%	KC311841

The DR47 strain, which is a good pectinase and β-glucosidase producer, did not present reproductive structures under the microculture technique. As the classical methods did not lead to conclusive results, sequencing of the rDNA ITS regions was performed (GenBank accession number KC311843). The blast alignment suggested that the DR47 isolate belonged to the *Annulohypoxylon stygium* species (EU272517, with 99% similarity). A separation of two groups in the ITS tree constructed with *Annulohypoxylon* and related species was found. One group revealed that the DR47 isolated clustered with *A. stygium* and *Annulohypoxylon urceolatum*, but was closer to *A. stygium.* The second group consisted on *Annuhypoxylon* spp. and *Hypoxylon investiens* (Figure 7A). Sáchez-Ballesteros et al. (144) analyzed the ITS1-5.8S-ITS region, and found that *Annulohypoxylon* spp. cluster inter-mingled with species of the genus *Hypoxylon* section *Hypoxylon*. Therefore, sequencing of partial BT2 was also performed (GenBank accession number KC311846) as suggested by Hsieh et al. (145). The phylogenetic tree was built and the DR47 isolated was clustered with *A. stygium* species, with a high bootstrap value, and was closer to *A. stygium* than to *Annulohypoxylon stygium* var. *annulatum* (Figure 7B). Besides, *H. investiens* was consistently separated from *Annulohypoxylon.*

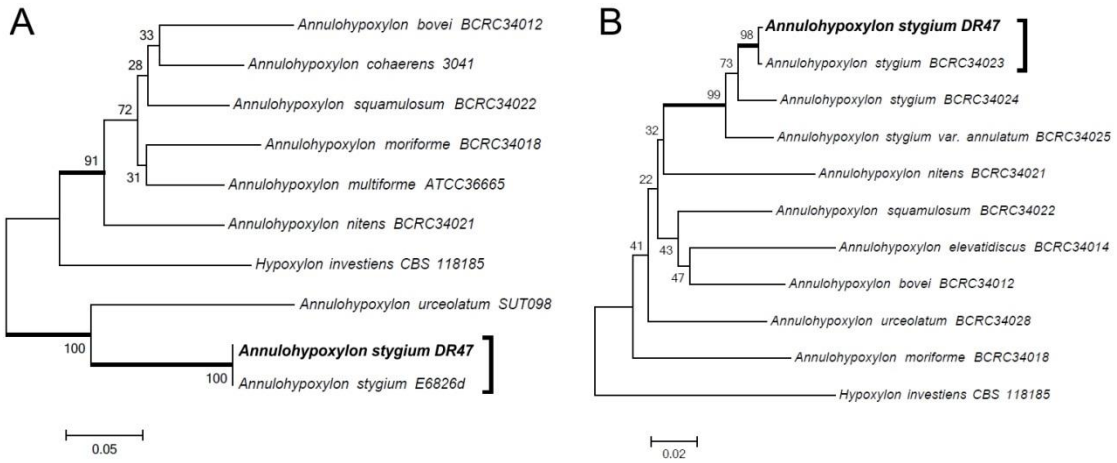

Figure 7 Phylogenetic tree of *Annulohypoxylon* and related species based on confidently ITS (A) and partial BT2 (B) sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in in bold. Strains in bold indicate isolates of this study.

The DR49 strain, previously isolated from spoiled books, was identified as *Talaromyces* sp. The Blast alignment of the ITS regions (GenBank accession number

KC311844) and partial BT2 (GenBank accession number KC311847) sequences suggest similarity with to *Talaromyces wortmanni*. The trees based on ITS and BT2 sequencing built with close related *Talaromyces* spp. corroborated with the blast aligned. The *Talaromyces* sp. DR49 strain was clustered with *Talaromyces wortmannii* with high bootstrap values in both trees (Figure 8).

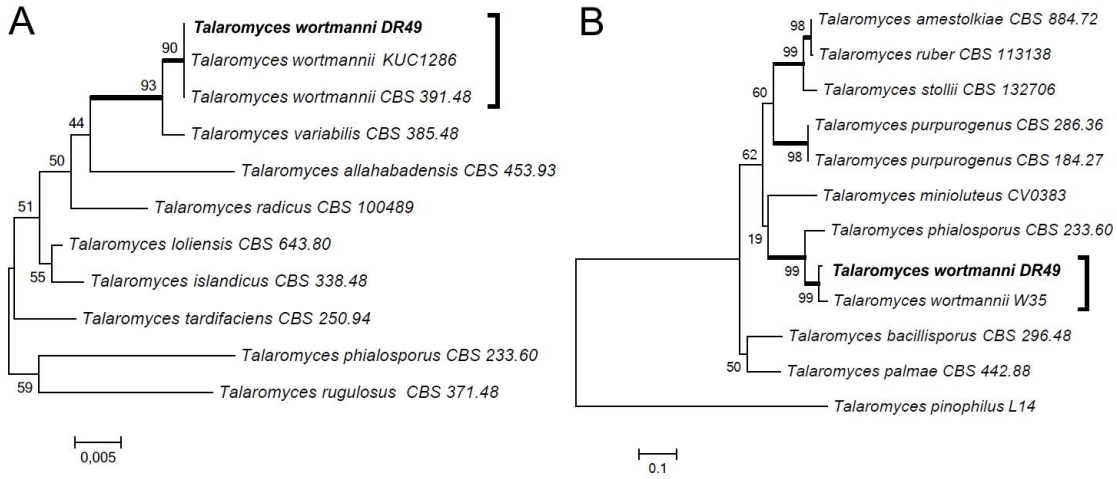

Figure 8 Phylogenetic tree of *Talaromyces* and close related species based on confidently ITS (A) and partial BT2 (B) sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in bold. Strains in bold indicate isolates of this study.

The DR40 strain, isolated from *E. benthamii*, was previous identified by macro and micro morphology as *Alternaria* sp. The sequencing of rDNA ITS (GenBank accession number KC311842), suggested that the DR40 isolate belonged to the *Alternaria alternata* species (JQ320281, with 100% similarity) while no amplicon of the BT2 gene was obtained for this strain. The tree based on rDNA ITS sequencing built with correlated species showed no resolution among the strains of the *Alternata* species group (Figure 9A). Previous work has also found no genetic variation between the small-spored *Alternaria* species in ITS sequences (146, 147). According to Andrew et al. (148), taxonomical differentiation of the small-spored species within the *Alternata* group is difficult, not only because there are few distinguishing morphological characteristics, but also because these characteristics are strongly influenced by the environment. Moreover, the same authors could not solve *Alternaria* spp. that belongs to the *Alternata* group using a phylogenic multilocus approach.

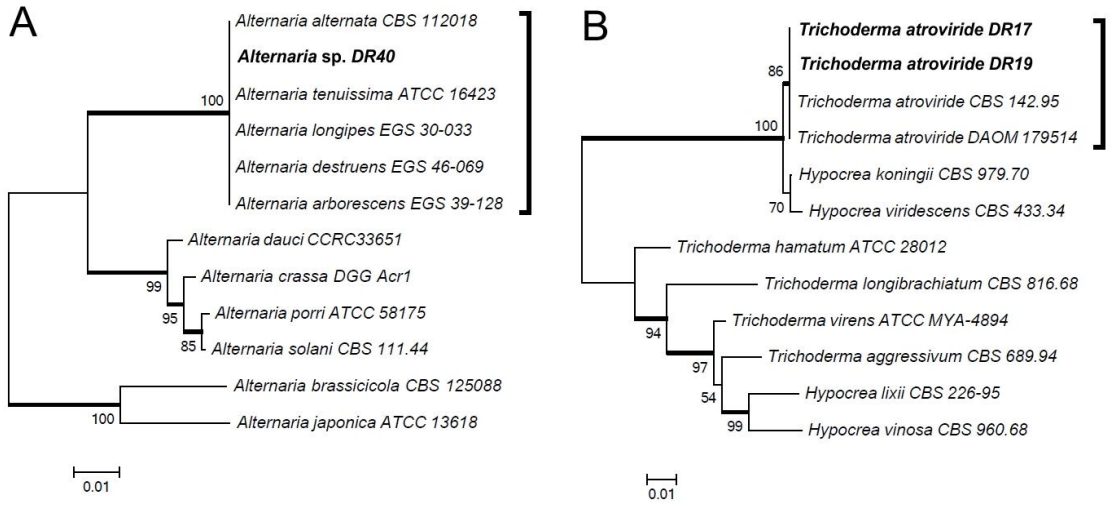

Figure 9 Phylogenetic tree of *Alternaria* (A) and *Trichoderma* (B) species based on confidently ITS sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in bold. Strains in bold. Strains in bold indicate isolates of this study.

The DR17 and DR19 strains were also endophytic isolates from *E. benthamii*, and were morphologically identified as *Trichoderma* sp. The ITS1-5,8S-ITS2 sequences for *Trichoderma* sp. DR17 and *Trichoderma* sp. DR19 (GenBank accession numbers (KC311840, KC31184) aligned with the database *Trichoderma atroviride* strain DAOM 179514 with 100% similarity (EU280125). The tree based on rDNA ITS sequencing (Figure 9B) formed two groups, and the DR17 and DR19 isolates were clustered with the *Viride* clade (*T. atroviride*, *Hypocrea koningii* and *Hypocrea viridescens*), and were closer to the *T. atroviride* species.

3.3.6 Bacteria shake flask screening

All bacterial strains presented rich grow on DEB+SB media at 29ºC, 200 rpm, pH 7.0 and enzymatic activities are shown in Figure 10. Low titration of β-glucosidase, FPase, CMCase and pectinase were detected during 48 and 96 h of cultivations for all the strains. The low β-glucosidase production was already present on plate assay. Among 45 strains, 5 showed β-glucosidase activity, but with few intensity. However, two strains highlighted to xylanse production, DR61 and DR69, at 48h. Also for xylanolytic activity, the strains DR63 presented potential at 96h.

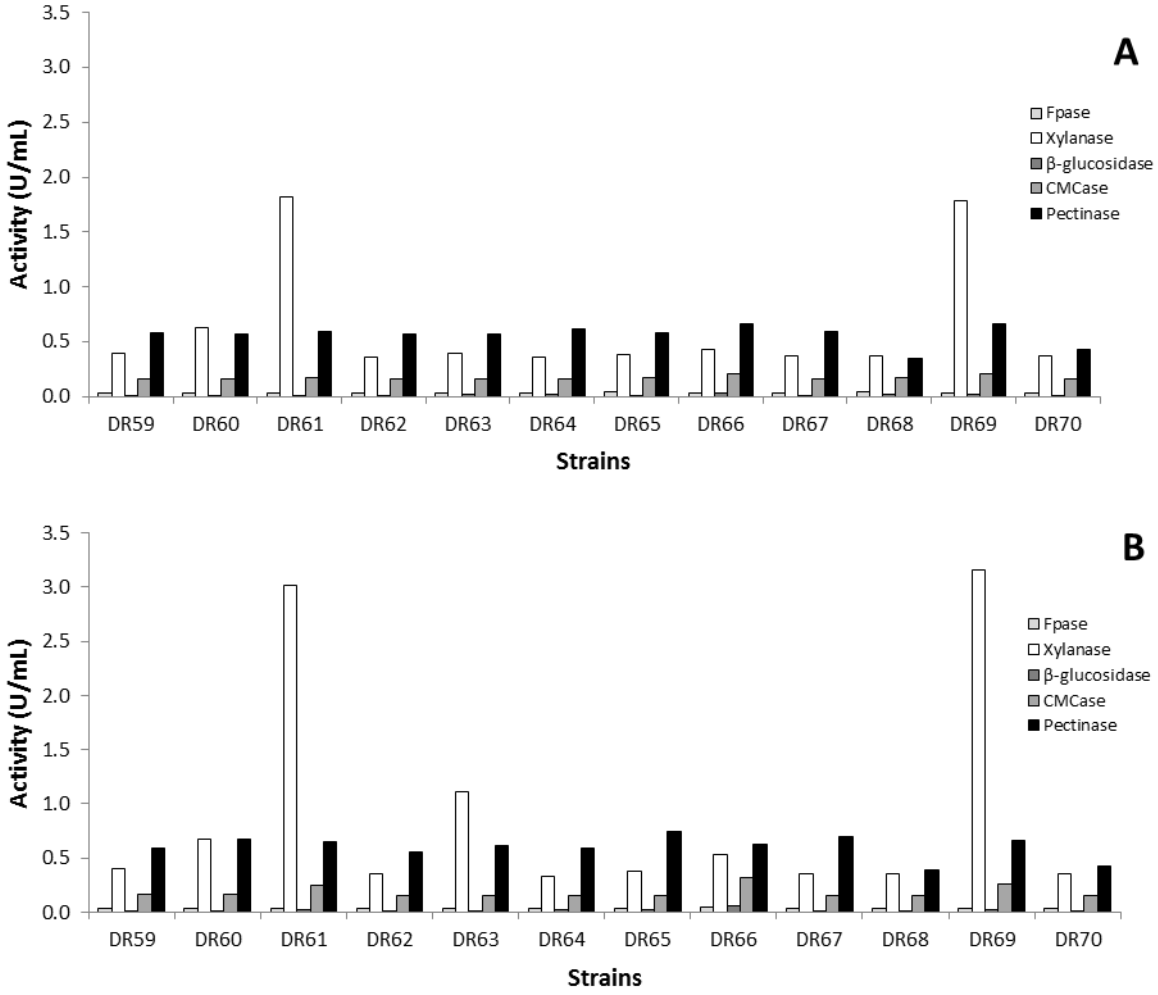

Figure 10 Enzymatic activities of actinomycetes pre-selected strains, grown in shake flasks with DEB+SB (3:1), after 48 h (A) and 96 h (B).

3.3.7 Actinomycetes glycohydrolase profile

In accordance with previous results 4 strains that presented potential for xylanase production (DR61, DR63, DR69 and DR66) were selected for cultivation under other inductors substrates (xylan and pectin). All strains belong to *Streptomyces* genera and have their extracts tested for degradation of several hemicellulolytic substrates (Table 6). It was used the extract from the time point that showed the highest activity pick of glycohydrolase over time of culture.

Table 6 Glycohydrolases activities (U/mL) of six selected actinomycetes strains grown on pectin, xylan and DEB.

Strain	**DR61**			**DR63**			**DR66**			**DR69**		
Carbon source	**DEB+SB**	**Pectin**	**Xylan**	**DEB+SB**	**Pectin**	**Xylan**	**DEB+SB**	**Pectin**	**Xylan**	**DEB+SB**	**Pectin**	**Xylan**
Time (h)	144	96	144	144	144	144	144	96	144	96	144	144
Birchwood xylan	0.73	0.53	4.66	1.22	1.00	10.99	0.76	0.53	2.56	3.16	2.52	9.98
Beechwood xylan	1.51	0.89	8.60	1.96	1.20	19.26	1.33	0.66	4.36	3.48	1.82	14.68
Rye arabinoxylan	0.99	0.28	2.26	1.31	1.70	3.74	1.18	0.53	2.15	1.80	2.05	3.27
Wheat arabinoxylan	0.69	0.44	0.27	0.67	0.18	0.00	0.61	0.47	0.24	0.65	0.60	0.48
Arabinan	0.38	0.52	0.93	0.40	0.11	0.41	0.41	0.41	0.40	0.35	1.09	1.27
CMC	0.51	0.69	1.02	0.46	0.56	0.93	1.61	0.60	1.01	0.84	0.77	1.01
β-glucan	0.48	0.44	0.82	0.37	0.30	0.31	1.89	0.54	0.95	2.18	1.30	0.57
Xyloglucan	0.53	0.42	0.59	0.36	0.24	0.37	0.83	0.58	0.38	0.35	1.05	0.41
Lichenan	0.67	0.65	1.44	0.46	0.64	1.27	2.45	0.62	1.48	2.26	1.52	0.81
Laminarin	0.43	0.50	1.16	0.41	0.73	2.39	0.47	0.43	0.40	0.40	2.22	0.62
1,4 β-mannan	0.38	0.31	0.79	0.38	0.54	0.71	0.64	0.39	0.40	0.39	0.48	0.77
Glucomannan	0.36	0.66	0.57	0.49	1.10	0.44	1.03	0.61	0.78	0.65	0.87	0.93
Galactomannan	0.36	0.52	0.77	0.41	0.50	0.75	0.67	0.39	0.37	0.36	0.46	0.08
Pectin	0.68	0.64	0.91	0.69	0.82	1.01	0.75	0.72	0.74	0.66	0.74	0.98
pNP β-D-xylopyranoside	0.01	0.03	0.01	0.02	0.03	0.02	0.03	0.05	0.03	0.02	0.03	0.01
pNP β-D-mannopyranoside	0.05	0.04	0.02	0.02	0.03	0.03	0.04	0.04	0.02	0.02	0.04	0.01
pNP β-D-cellobioside	0.01	0.04	0.01	0.01	0.04	0.07	0.03	0.04	0.04	0.03	0.06	0.07
pNP α-L-arabinofuranoside	0.04	0.07	0.02	0.02	0.06	0.01	0.05	0.07	0.07	0.02	0.05	0.02
pNP β-D-glucopyranoside	0.01	0.00	0.01	0.01	0.02	0.02	0.08	0.00	0.03	0.02	0.02	0.03

High levels of xylanase activity were detected for the 4 strains, although the strains DR63 and DR69 highlighted. The xylanase production was associated with xylan presence in the media (Table 6) and on hydrolysis a higher affinity against beechwood xylan was presented, probably due to the selection on xylan agar plates. Some strains, such as DR66 e DR69 were able to produce enzymes with activity to β-D-glucosil-(1→4)-β-D-glucose links from different substrates (β-glucano, lichenana and CMC). The strains DR63 produced hydrolytic activity against hetero/homo mannan and pectin. No expressive levels of arabinofuranosidase, xylosidase, manosidase and arabinase were presented under the conditions tested.

In despite of the fact that good production of xylanase were detected principally in DR63 e DR69 strains, these microorganisms revealed small diversity of glycohydrolases when compared with fungal strains. This fact associated with infrastructure conditions led the decision of not work further with the actinomycetes in the thesis.

3.4 Discussion

High activity, good stability, and low cost are key requirements of enzymes employed for large-scale hydrolysis of lignocellulosic biomass into sugar. Agro-industrial wastes can be useful materials for enzyme development, improvement, and production. The liquor derived from sugar cane bagasse hydrothermal pretreatment is a low cost feedstock (149) rich in xylose and xylo-oligosaccharides which are capable of inducing the expression of xylanases and accessory proteins in fungi such as *A. niger* (99). Other materials, such as steam-exploded delignified bagasse and soybean bran, have also been used as inexpensive culture media to achieve high xylanase, cellulase, and β-glucosidase activities employing *Trichoderma harzianum* P49P11 (129).

The full hydrolysis of lignocellulosic biomass requires several types of glycohydrolases that enable the release of saccharides and other compounds from the recalcitrant substrate. However, plant species are highly diverse in terms of cell wall structure and composition, which increases the attraction of formulating specific biomass-degrading enzymatic cocktails. The sugar cane cell wall polysaccharide is mainly composed of xyloglucan and arabinoxylan, closely associated with cellulose, as well as pectin, β-glucan and less branched xylan strongly bound to cellulose (35).

Several studies have shown that supplementing cellulases with other enzymes can assist in the enzymatic hydrolysis of lignocellulosic biomass. Xylanases and β- xylosidases improved the hydrolysis yield when combined with cellulases and β-glucosidades (125, 150, 151). The addition of pectinase to Celluclast 1.5 L increased the hydrolysis of pretreated corn stover (3). Supplementation of cellulolytic cocktails with α-L-arabinofuranosidase and xylanase also showed a synergistic effect in the hydrolysis of wheat straw (152).

The production of glycohydrolases is closely related to the nature of the carbon source, since microbial metabolism is greatly influenced by the composition of the medium (which also hampers the screening of strains). Each strain has a distinct metabolic profile, while the enzymatic profile is also distinct and depends on the medium and the cultivation time.

Physiological variations are the result of the adaptation and evolution of microorganisms, considering their hosts, original habitats, and other factors. The strain *A. stigyum* DR47 belongs to the Xylariaceae family, members of which are frequently encountered as endophytes and saprophytes (153). Gazis and Chaverri (154) isolated several endophytic Xylariaceae strains and one strain of *Annulohypoxylon* sp. from *Hevea brasiliensis*. Wei et al. (155) cultivated an *A. stigyum* strain on Avicel and confirmed the production of β-glucosidase, although only low levels of cellulases were detected.

Most *Alternaria* species are saprophytes commonly found in soil or on decaying plant tissues, and some species are opportunistic plant pathogens (156). However, endophytic strains of *Alternaria* spp. have been isolated from eucalyptus plants such as *Eucalyptus globulus* (157) and *Eucalyptus citriodora* (158). Strains of *A. alternata* are able to produce endopolygalactunorase (159) in the presence of pectin, and β-glucosidase in the presence of saccharose (160).

A. niger is known worldwide for its ability to produce an extensive range of extracellular glucohydrolases, including xylanases, pectinases, and β-glucosidase (161). This characteristic is associated with the ability of the fungus to propagate and colonize a variety of environments, principally those rich in decomposing plant materials (162). The fungus was recently reported to be endophytic in several plant species (163, 164). However, this work is the first report of *A. niger* as an endophytic fungus in *P. orientalis.*

There have been no previous reports in Brazil concerning *T. wortmannii* isolated from decaying materials. Lee et al. (165) first described β-xylosidase activity in a *T. wortmannii* strain previously isolated from Japanese red pine and larch woods in Korea (166). Lee et al.(165) obtained a β-xylosidase production of 3.82 U/mL for cultivation on xylan, in good

agreement with the β-xylosidase activity (2.85 U/mL) found in the present work for the DR49 strain grown on xylan.

Trichoderma spp. are present in soil as saprophytes, and have also been found as endophytic organisms (167). Many species from this genus are good cellulase and xylanase producers, such as *T. harzianum* (129) and *T. reesei* (168-170). *T. atroviride* strains are good producers of these glycohydrolases, and can produce high amounts of β-glucosidase (107).

Actinomycetes are an important class of bacteria with industrial interest. The strains used in this work were identified by sequencing of 16S DNAr region by Andrielli (171). Among the 4 strains, only DR66 was isolated from soil e belong to *Streptomyces olindeses* species. The strains DR61 and DR69 are endophytics of *C. roseus* and were identified as *Streptomyces globisporus* and *Streptomyces roseochromogenus* respectively. Only the strain DR63 is from an unknown origin and identified as *Streptomyces* sp.. Even though *S. olindenses* is capable to produce the antitumor cosmomycin D (172), *S. globisporus* is known as a producer of N-Acetylmuramidase (173) and *S. roseochromogenus* of the antibiotic roseomycin (174), none of these species have been described as producer of plant biomass degradation enzymes.

Microorganisms play an essential role in the degradation of cellulose and hemicellulose standing out the endophytic microorganisms which are excellent sources of hydrolytic enzymes. Evidently, during the endophytic phase, the use of these enzymes must be related to the mutualistic relationship with the host plant (175). However, although the association between plants and endophytic microorganisms is ecologically important, little is known about the physiological characteristics of the interaction.

An important aspect of enzymatic studies involving endophytic microorganisms is the involvement of these in the decomposition of plant material (176, 177). Since they are already present in the senescent plant tissues, they may be able to initiate the decomposition process before it becomes dominated by saprophytic species. This could suggest not only that the production of hydrolytic enzymes by endophytic species might be important for the nutrition of the microorganisms during the endophytic stage, but also that these enzymes are produced and secreted at the surface of the tissues, where they can compete for the substrate during the saprophytic stage. Kumaresan and Suryanarayanan (178) investigated the ability of endophytic fungi from mangrove leaves of different ages to produce hydrolytic enzymes. It was found that endophytic species occurring at relatively low levels in living leaves were

more prevalent after leaf fall, increasing the involvement of these fungi in decomposition of the plant material.

An important consideration is the range of substrates that can be utilized by endophytic microorganisms. Studies have shown that endophytes are capable of metabolizing *in vitro* most substrates found in plants, and produce enzymes including proteases, amylases, phenol oxidases, lipases, laccases, polyphenol oxidases, cellulases, mannanases, xylanases, and pectin lyase (177, 179).

3.4 **Conclusions**

The balanced use of microbial enzymes in biomass deconstruction required the understanding of the role played by these glycohydrolases, and also depends on an economic process development. Therefore, biochemical characterization of new reported glycohydrolases producer strains, as well as a bioprocess development of the selected strains in large scale, must be conducted to evaluate the enzyme applicability on the biomass deconstruction, principally on sugar cane bagasse. The present work demonstrated that it is possible to select endophytic strains that can produce glycohydrolases with activities against a wide range of target substrates. This will enable the future formulation of specific enzymatic cocktails for an efficient biomass deconstruction.

CHAPTER 4 - ENHANCING OF SUGAR CANE BAGASSE HYDROLYSIS BY *Annulohypoxylon stygium* GLYCOHYDROLASES

4.1 Introduction

The complexity of lignocellulose material makes this biomass highly recalcitrant to decomposition for biotechnology applications e.g. production of biofuels. Development of enzyme extracts and purified enzyme combinations can produce hydrolytic cocktails to improve hydrolysis processes, increase product yields with shorter reaction times and reduced feedstock and bioreactor capital investment (106).

It is known that the cellulolytic complex used in the enzymatic hydrolysis benefited, in terms of yield and conversion speed, when supplemented with accessory enzymes as, for example, hemicellulases and pectinases (3, 61). For example, the addition of a commercial pectinase at cellulolytic enzyme extracts increased the hydrolysis yield of corn stover pretreatment with acid (3), and delignified/exploded sugar cane bagasse (105).

The β-glucosidase supplementation of cellulolytic systems is commonly used, considering that the major cellulolytic extracts are not well balanced for an efficient saccharification, like for *Trichoderma reseei* (3). Besides, the supplementation with a β-glucosidase from another fungus can be employed to reduce the cellobiose inhibition over cellobiohydrolases and endoglucanases (180). However, high glucose concentrations and thermal stability can affect the commercial use of β-glucosidases (181).

New fungi strains and consequently new enzymes can be the key for a better biomass hydrolysis process principally regarding to broader substrate specificities and improved biophysical properties. Thereby, microorganisms hydrolases from diverse environments have been extensively searched, as desert (182), rain forest soils (183) and microbial endophytes of plants (112).

The ascomycete fungus *Annulohypoxylon stygium* is an ascomycete that belongs to the *Xilariaceae* family. Members of this genera are commonly find as endophytic or saprophytic (153), but have been briefly studied for glycohydrolases production. In *A. stygium* there is a report of β-glucosidase activity (155). However, others activities has been reported with *A. stygium* such as pectinases, xylanases and β-glucanases when grown using xylan, pectin and sugar cane bagasse substrates (Chapter 3).

The enzyme production cost for biomass deconstruction is related mainly with the productivity system, the amount of enzyme produced by time unit and reactor volume (184). The development of strategies that can produce several glycohydrolases could be an

alternative for the enzymes production cost reduction. The industrial agro wastes have biotechnological potential and can be employed in byproducts production and shows to be a great opportunity to achieve satisfactory prices.

This study aimed to develop a process for the production and use of β-glucosidase and pectinase from *A. stygium* strain DR47 with and explore these enzymes for improved sugar cane bagasse deconstruction. *A. stygium* strain DR47 was chose among 119 fungi strains due to its capacity of production high concentration of β-glucosidase and pectinase, and also because there are few information about this fungi on the literature.

4.2 **Materials and methods**

4.2.1 **S***trains*

Strain *A. stygium* DR47 is an endophytic fungal strain of *Eucalyptus benthamii* and was selected on previous assays (Chapter 3).

4.2.2 Agro-industrial waste materials

Steam exploded bagasse (EB), DEB and HB were produced as described in chapter 3. SB and wheat bran (WB) were obtained from Agricola (São Carlos, Brazil) and were characterized by Rodrigues-Zuniga et al. (123). Apple bagasse (AB) was obtained from Yakult S.A. (Lages, Brazil) and the citrus bagasse (CB) was purchased by Hildebrand (São Carlos, Brazil). Both AB and CB are the residues obtained directly from the juice extractor.

4.2.3 Pre-culture and production media

The composition of the medium was adapted from Mandels and Reese (127). The pH was adjusted to 5.0 and the culture medium was sterilized at 121 °C for 20 min. The composition of the production medium was the same as that of the pre-culture medium, except for the type of carbon source. Seven different carbon sources were evaluated at 10 g/L: HB, EB, DEB, SB, WB, AB and CB.

4.2.4 Shake flask cultures

Inoculum was prepared by adding 20 mL of sterilized distilled water and Tween 80 (0.01%) to mature colonies of *A. stygium* DR47 grown on PDA plates (7 days at 29 °C). The biomass was transferred to Erlenmeyer flasks containing 180 mL of pre-culture medium and incubated for 48 h at 29 °C on a rotary shaker at 200 rpm. A volume of 20 mL of this pre-

culture was transferred to 500 mL Erlenmeyer flasks containing 180 mL of the production medium and incubated at 29 °C on a rotary shaker at 200 rpm for 144 h.

4.2.5 Buffered cultures

Cultivations with phthalate buffer were realized in order to minimize the pH medium alterations. Phthalate buffer (50mM) was added as descripted by Ferreira et al. (185) in the pre-culture and in the production media. Controls without buffer were performed and all the assays were done in triplicated and analyzed by Tukey test.

4.2.6 Experimental design

To select the best carbon source to support optimum β-glucosidase and pectinase activities, central composite design (CCD) design was done with data from shake flask experiments. The data analysis and the medium optimization were performed with Minitab (Release 14) statistical software (Minitab Inc., USA). Cultivations were realized with phthalate buffer (50mM) as descripted by Ferreira et al. (185) in order to minimize the pH alterations. It was tested the best carbon source for pectinase production (CB), the best carbon source for β-glucosidase production (SB) and also sucrose (SUC) as a β-glucosidase inductor. Delabona et al. (129) verified that sucrose could induce the β-glucosidase production on a mixture composition of DSB, SB and SUC by *Trichoderma harzianum*. The complete factorial experimental design was performed with 3 factors, 2 levels, 2 axial points and 6 replicates of the central point, totalizing 20 experiments. All variables were studied on the levels 4.05 g/L (-1), 15.95 g/L (+1), 10 g/L (0), 0 g/L (-1.68) and 20 g/L (+1.68) and the results were fitted to the quadratic model. The data were not transformed for the analysis.

4.2.7 Bioreactor cultures

Bioreactor cultures were conducted in a 3.0 L Bioflo 115 stirred tank reactor (STR) (New Brunswick Scientific Co., USA) equipped with automatic control of temperature (29 °C), pH (5.0), agitation rate (200 – 500 rpm) and aeration rate (0.3 – 1.0 L/min). The pH was controlled by the automatic addition of either 0.4 M H_2SO_4 0.4M or 1:3 (v/v) NH_4OH:H_2O. The dissolved O_2 level was kept above 30% of air saturation by automatic adjustment of aeration and agitation. Foaming was manually controlled by the addition of polyglycol antifoaming agent (FluentCane 114, DOW Chemical, Brazil). A working of volume of 1 L was inoculated with 10% (v/v) inoculum from the pre-culture same as described previously.

Samples were periodically withdrawn, centrifuged at 10,000 x *g*, 10 °C for 15 min and analyzed for protein content and enzymatic activities.

4.2.8 Crude enzyme characterization: influence of ph temperature and thermal stability

Culture supernatants produced under optimal STR production conditions were assayed for β-glucosidase and pectinase activities at different reaction temperatures (20 - 80 °C) in 50 mmol/L sodium citrate buffer (pH 5.0). The effect of pH on enzyme activities (at 50 °C for β-glucosidase and 37 °C for pectinase) was determined using 50 mmol/L citrate-phosphate buffer (pH 3.0 - 8.0). For thermal stability determination, crude supernatant obtained under the optimal production conditions was incubated at 40, 45, 50 and 60 °C for 24 h, in the absence of substrate. The residual enzyme activity was measured after different time intervals. Measurement of enzyme activity was performed under standard pH and temperature conditions.

4.2.9 Enzymatic activity assays

Total cellulolytic activity was measured as Filter paper activity (FPase), as described by Ghose (186). Others enzymatic activities were measured as described on chapter 3.

4.2.10 Total protein determination

Total protein in centrifuged supernatants was determined using the Bio-Rad protein assay reagent following manufacturer's instructions (Bio-Rad Laboratories, USA). Bovine serum albumin was used as standard.

4.2.11 Sugar cane bagasse hydrolysis

Hydrothermal pre-treated sugar cane bagasse (HB) was subjected to enzymatic saccharification combining two different enzyme preparations produced in bioreactor with a commercially available enzyme preparation (Celluclast 1.5L, Novozymes). The enzymatic hydrolysis were performed with 5% (w/v) of HB and sodium azide 0.02% (v/v) in 50 mM citrate buffer, pH 5.0. The reactions were carried out in 2 mL Eppendorf tubes using a Thermomixer microplate incubator (Eppendorf, Germany) operated at an agitation speed of 1000 rpm for 24 h. First a saturation curve for each extract was performed using a fixed Celluclast 1.5L loading of 10 FPU/g of bagasse at 40 °C, 50 °C and 60 °C. Then the effects of partial replacement of Celluclast 1.5L by the enzymatic extracts produced were assessed in the HB hydrolysis with a total fixed concentration of 12 mg of protein per g of bagasse.

Samples were centrifuged at 10,000 x *g* for 15 min (5418 Centrifuge, Eppendorf) filtrated (Sepak C18, Waters) and carbohydrate concentrations were either determined by the DNS method or by HPLC as described by Rocha et al. (122).

4.2.12 Proteomic analyses

Proteomic analysis of fungal extracts from bioreactor cultures grown at pH 4.0 and pH 5.0 was performed by liquid chromatography coupled in-line to mass spectrometry. A volume of supernatant containing 10 µg of total proteins was first separated by 1D SDS-PAGE. Each sample was run in three lanes on the gel, and each lane was then divided into six slices (70-100, 55-70, 40-55, 35-40, 25-35, and 5-25 KDa). The slices were de-stained, reduced and alkylated by carboxymethylation and then in-gel digested overnight using sequencing-grade modified trypsin (Promega, USA) (187). Each gel slice was re-suspended in 12 µL of 0.1% formic acid and an aliquot (4.5 µL) of the resulting peptide mixture was separated using an RP-nanoUPLC C18 column (nanoAcquity, 100 µm x 100 mm, Waters) coupled to a Q-Tof Ultima mass spectrometer (Waters) fitted with a nano-electrospray source operated at a flow rate of 0.6 µL/min. The gradient was 2–90% acetonitrile in 0.1% formic acid over 60 min. The instrument was operated in 'top three' mode, in which one MS spectrum is acquired, followed by MS/MS of the three most intense peaks detected. The spectra were acquired using MassLynx v.4.1 software and the raw data files were converted into a peak list format (mgf), without summing the scans, using Mascot Distiller v.2.3.2.0 2009 software (Matrix Science Ltd.) and then searched against the NCBI taxonomical database for fungi using the MASCOT v.2.3.01 search engine (Matrix Science Ltd.). Carbamidomethylation was used as a fixed modification and oxidation of methionine was used as a variable modification, with one trypsin missed cleavage and a tolerance of 0.1 Da for precursors and fragment ions. Scaffold v.3.6.1 (Proteome Software Inc., Portland, OR) was used to validate the MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability, as specified by the Peptide Prophet algorithm (188). Peptide identifications were also required to exceed specific database search engine thresholds. Protein identifications were accepted if they showed greater than 90.0% probability and contained 2 or more identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (189). Proteins that contained similar peptides and could not be differentiated using MS/MS analysis alone were grouped together for parsimony.

4.3 **Results**

4.3.1 Effect of carbon source on enzyme production in shake flasks

A. stygium was initially grown in shake flasks in order to evaluate the influence of different carbon sources on β-glucosidase and pectinase production. Insoluble carbon sources rich in cellulose (HB, EB and DEB), hemicellulose (SB and WB) and pectin (AB and CB) were used at concentrations of 1% (w/v). Figure 11 shows the pectinase and β-glucosidase activities as a time course over 144 hrs of fermentation. Evaluation of carbon sources indicated that pectinase production was strictly associated when the fungus was grown using pectin as the carbon source (AB and CB). The use of CB resulted in the highest pectinase production after 48 h.

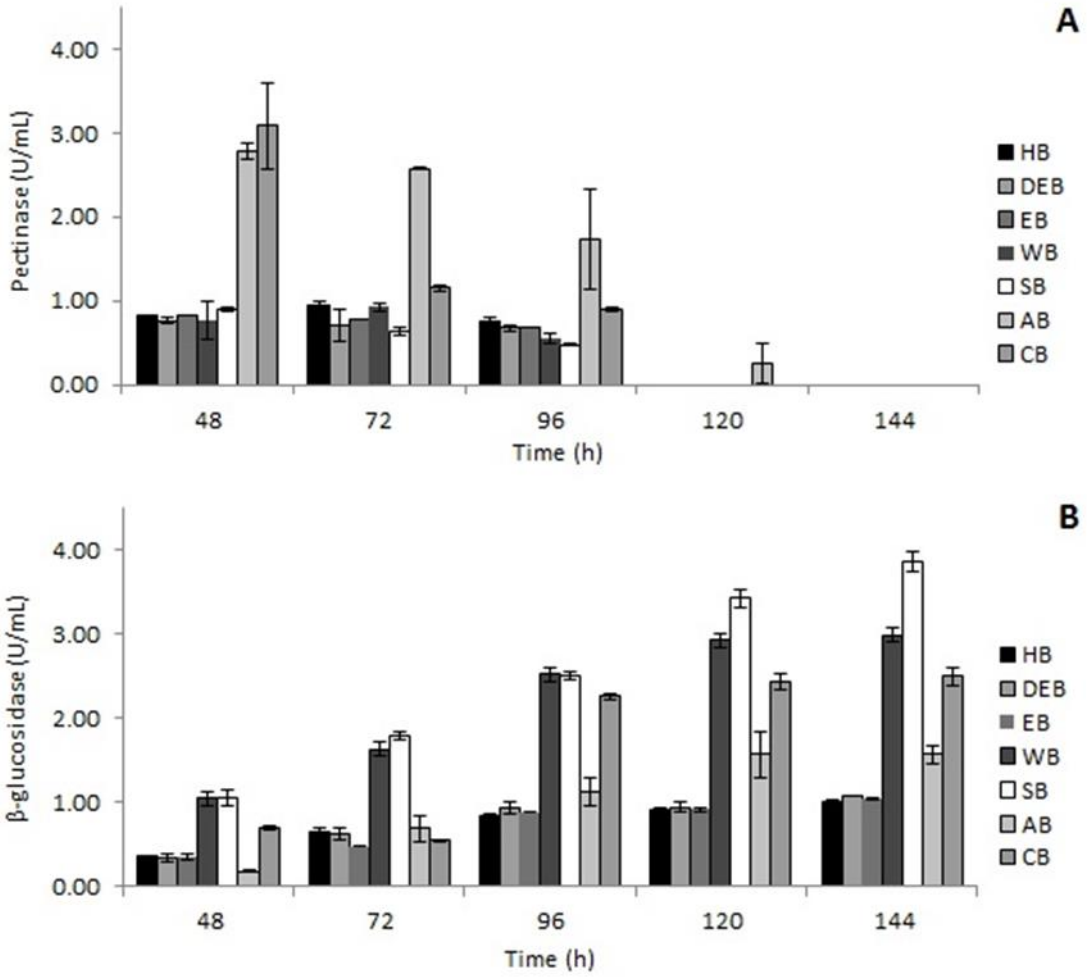

Figure 11 Influence of different carbon sources on the pectinase (A) and β-glucosidase (B) production by *Annulohypoxylon stygium* DR47 during submerged fermentation in flasks.

Evaluation of β-glucosidase production under industrial agro-industrial wastes showed that the highest enzyme titrates were obtained on substrates rich in hemicellulose and pectin (Figure 1B), but not when the fungus was grown on sugar cane bagasse. The best results were in SB (3.9 U/mL), WB (3.0 U/mL) and CB (2.5 U/mL) after 144 h. This result may indicate that the β-glucosidase production may not be totally associated to the carbon source, and might be related with the fungal growth, once that the hemicellulose and pectin are polysaccharides with easier degradability than cellulose. The SB was a good source to produce β-glucosidase and CB was a good source to produce pectinase and β-glucosidase.

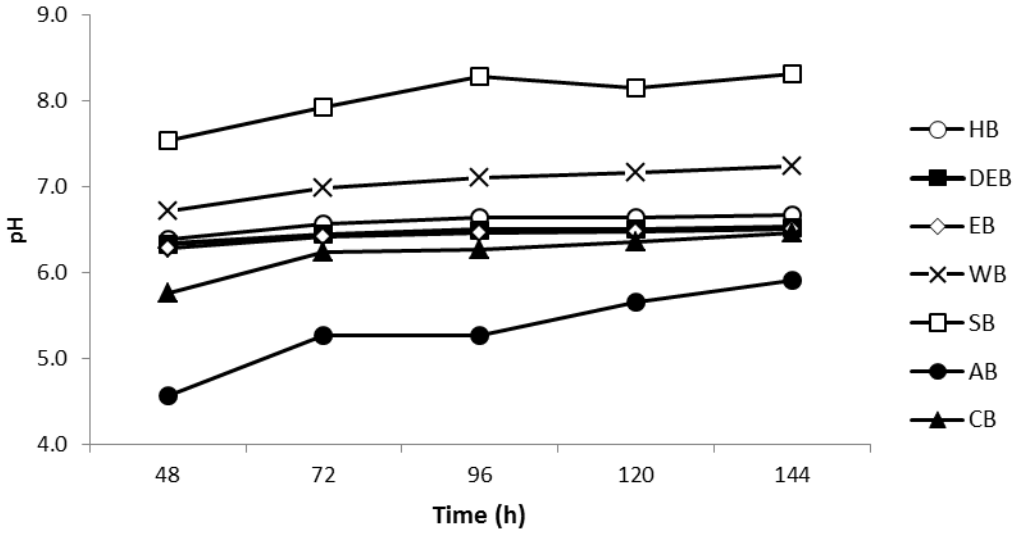

Figure 12 Influence of different carbon sources on pH cultivation of *Annulohypoxylon stygium* DR47 during submerged fermentation in flasks.

Figure 12 shows the influence of different carbon sources on pH cultivation. It is known that GH biosynthesis is tightly regulated by the environment pH (190). Depending on the carbon source the effect on pH variation is greater such as for SB. In order to avoid the influence of pH variation due to higher concentration of carbon sources, tests with phthalate buffer was carried out. The assays was performed to prove that the buffer do not interfere negatively on the enzyme production for CB and SB at pH 5.0 . Figure 3 shows the kinetic production of pectinase and β-glucosidase during 144 h. The buffer was efficient for cultivation with CB during six days. For SB cultures the buffer was able to keep the pH close to 5.0 for 48 h, after the pH increased, but remained always below than the control. Tukey mean test revealed that the pectinase activity did not differ at 95% of probability (Table 7). This corroborated with the pH results that showed low variation among the treatments. The

buffer influenced positively the pectinase activity, probably due to the medium buffering in the range 5.0 at 48 h (Figure 13). In this way, the pH control was necessary to study the influence of SB in the pectinase production.

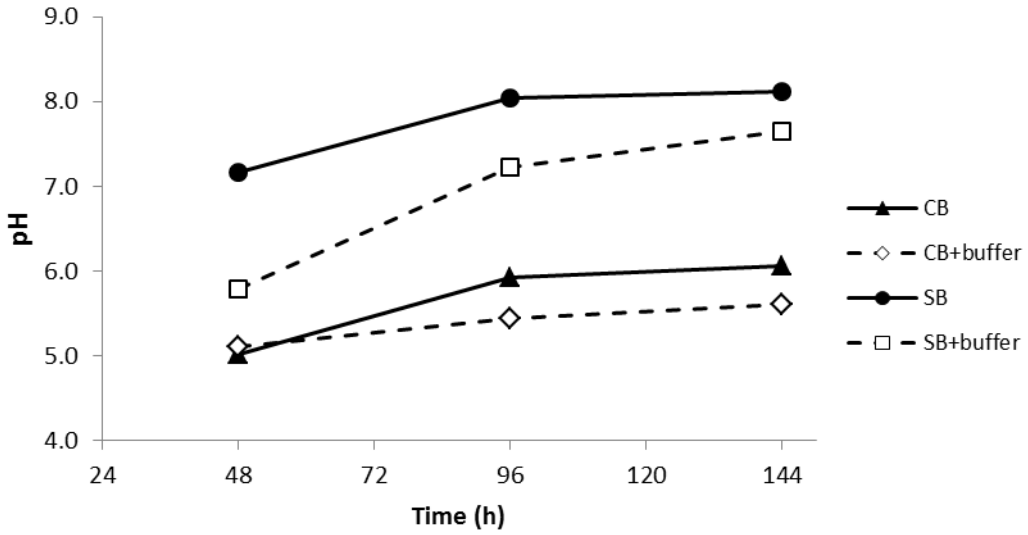

Figure 13 Influence of buffer phthalate on the β-glucosidase and pectinase production by *Annulohypoxylon stygium* DR47 during submerged fermentation in flasks.

Regarding to the β-glucosidase activity, the buffer did not influence positively or negatively ($p>0.05$) in the enzyme production for the both carbon source tested (Table 7.). Furthermore, the experimental design was performed with phthalate buffer in order to minimize the pH effect in the enzymes production.

Table 7 Pectinase and β-glucosidase activity of *Annulohypoxylon stygium* DR47 cultives on SB and CB (10 g/L, 29ºC, 200 rpm) with and without phthalate buffer.

	Pectinase (U/mL) [(1)]		**β-glucosidase (U/mL) [(2)]**	
	48 h		**144 h**	
	No buffer	Buffer	No buffer	Buffer
SB	0,80 ± 0,02 a	2,12 ± 0,09 b	3,51 ± 0,05 a	3,31 ± 0,01 a
BC	5,67 ± 0,18 c	5,07 ± 0,41 c	2,68 ± 0,11 a	2,96 ± 0,70 a

Means calculated from 3 replications. Data not transformed. Means followed by the same small letter do not differ among them by Tukey test at 5%.

4.3.2 Optimal media composition design

Media formulation and optimization are required for the commercial success of any biotechnology process. In this study media for the production of β-glucosidase and pectinase using two feedstocks (CB and SB), together with the low cost sugar saccharose was developed using experimental desing.

Table 8 summarizes the different combinations of SB, BC and SUC concentrations used to culture *A. stygium* and the maximum activities of pectinase at 48 h and β-glucosidase at 144 h. Maximum pectinase activity obtained in these experiments ranged from 2.24 (run 1) to 7.05 U/mL (run 8), with maximum β-glucosidase activities ranging from 1.08 (run 7) to 7.58 U/mL (run 13).

Table 8 Pectinase (48 h) and β-glucosidase (144 h) activities for *Annulohypoxylon stygium* DR47 central composite design experiment using shaking flaks (29 C, pH 5.0, 200 rpm).

Run number	**SB (g/L)**	**CB (g/L)**	**SUC (g/L)**	**Pectinase (U/mL)**	**β-glucosidase (U/mL)**
1	**4.05**	**4.05**	**4.05**	**2.24**	**3.44**
2	15.95	4.05	4.05	3.51	6.06
3	4.05	15.95	4.05	3.83	4.95
4	15.95	15.95	4.05	6.3	7.16
5	4.05	4.05	15.95	5.05	2.34
6	15.95	4.05	15.95	6.25	4.56
7	**4.05**	**15.95**	**15.95**	**5.6**	**1.8**
8	**15.95**	**15.95**	**15.95**	**7.05**	**2.76**
9	0.00	10.00	10.00	2.47	1.67
10	20.00	10.00	10.00	5.72	4.8
11	10.00	0.00	10.00	3.93	4.95
12	10.00	20.00	10.00	2.8	4.66
13	**10.00**	**10.00**	**0.00**	**2.76**	**7.58**

14	10.00	10.00	20.00	3.98	2.19
15	10.00	10.00	10.00	4.7	4.94
16	10.00	10.00	10.00	4.3	4.8
17	10.00	10.00	10.00	4.41	4.28
18	10.00	10.00	10.00	4.42	4.81
19	10.00	10.00	10.00	3.76	5.01
20	10.00	10.00	10.00	3.64	5.36

The influence of medium composition on pectinase and β-glucosidase biosynthesis was estimated by examining the statistical significance of each component. In terms of pectinase activity at 48 h, three substrates (SB, BC and SUC) did not show a statistically significant influence ($p>0.1$) on enzyme activity and the results did not fit well to the quadratic model used and presented lack of fit (Table 9) In this way, pectinase activity was measured at 96 h, but the values obtained were much lower than 48 h (data not shown).

Table 9 Central composite design ANOVA of pectinase production by the *Annulohypoxylon stygium* DR47.

Source of variation	**Sum of squares (SS)**	**Degrees of freedom (DF)**	**Mean square (MS)**	**F value**	**p value**
Regression (R)	21.4	9	2.377	1.92*	0.162
Linear	18.9	3	0.628	0.510	0.686
Quadratric	1.0	3	0.327	0.260	0.850
Interaction	1.6	3	0.518	0.420	0.744
Residual (r)	12.4	10	1.239		
Lack of fit (Lf)	11.5	5	2.307	13.46**	0.006
Pure error (Pe)	0.857	5	0.171		
Total (T)	33.8	19			
R^2					
F listed values (95% of confidence)	0.663		*$F_{9,10}$ (95%) **$F_{5,5}$ (95%)	3.02 5.50	

**F* test for statistical significance of the regression=MSR/MSr. **F test for lack of fit=MSLf/MSPe

Statistical analyses of β-glucosidase activity showed a significant positive influence of the components and enabled the definition of a reliable quadratic model with determination coefficient of 97.6% (Table 10).

Table 10 Central composite design ANOVA of β-glucosidase production by the *Annulohypoxylon stygium* DR47.

Source of variation	**Sum of squares (SS)**	**Degrees of freedom (DF)**	**Mean square (MS)**	**F value**	**p value**
Regression (R)	276.1	9	30.674	42.72*	0.000
Linear	225.2	3	18.378	25.600	0.000
Quadratric	29.8	3	9.934	13.840	0.001
Interaction	21.1	3	7.030	9.790	0.003
Residual (r)	7.2	10	0.718		
Lack of fit (Lf)	3.5	5	0.708	0.97**	0.512
Pure error (Pe)	3.641	5	0.728		
Total (T)	283.2	19			
R^2	0.976				
F listed values (95% of confidence)			*$F_{9,10}$ (95%)	3.02	
			**$F_{5,5}$ (95%)	5.50	

. **F* test for statistical significance of the regression=MSR/MSr. **F test for lack of fit=MSLf/MSPe

The contour plots for β-glucosidase activity showed that higher amounts of SB and CB are associated with the increased of enzyme activity production and that SUC produces a negative effect when associated with SB and BC (Figure 14).

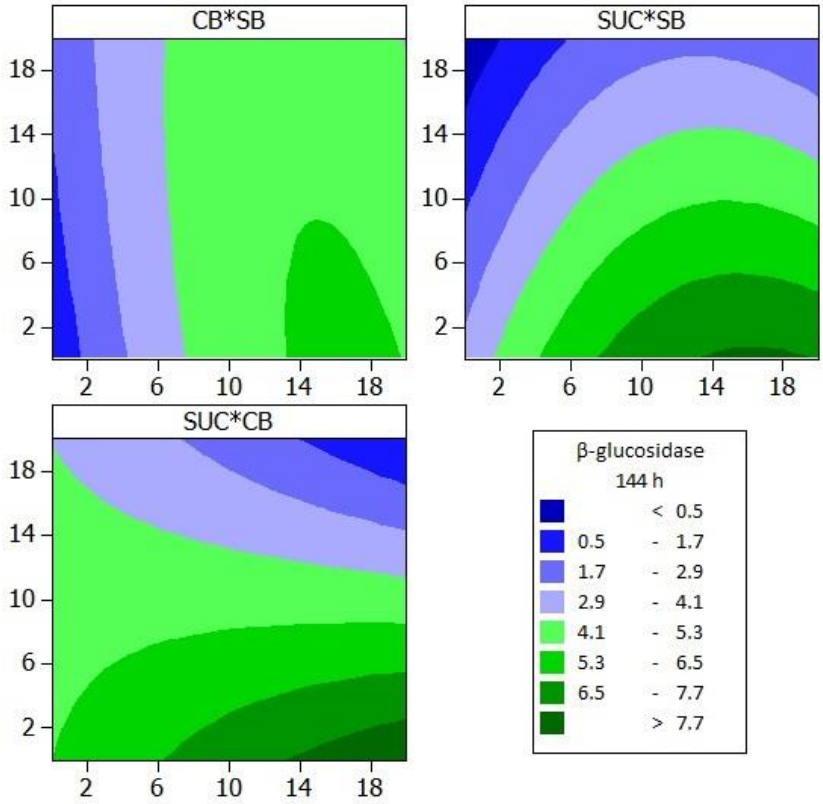

Figure 14 Contour plots of β-glucosidase activity for the *Annulohypoxylon stygium* DR47 central composite design, using the culture medium components (g/L) citrus bagasse (CB), sucrose (SUC), and soybean bran (SB). Hold values 10 (g/L) for which component.

The aim of the composition design was to obtain one media for the production of both enzymes. Therefore, the Minitab response optimizer was used with the data of pectinase activity at 48 h and β-glucosidase at 144 h. Even though the pectinase data had not fit the quadratic model well (p=0.162), the model was used for optimization since pectinase activity was highest at this time point.

The response media optimization was performed by giving equal weights for each response variable; maximum concentration of each component was 2% (w/v) and sought to maximize the values of pectinase and β-glucosidase. The best composition was SB 20 g/L, CB 20g/L and SUC 2.42 g/L. The predicted enzyme activities were 5.32 U/mL of pectinase at 48 h and 7.41 U/mL of β-glucosidase at 144 h with composite desirability of 84.6%.

The optimized media was then tested at three growth temperatures (25°C, 29°C and 32°C) to refine glycohydrolases production. The enzyme activities corroborated with the results obtained of cultures grown at 29 °C (Table 11), indicating that the experimental data

fitted to the model tested. In addition, changing temperature as a growth parameter did not statistically influence ($p>0.05$) enzyme activity (by post-hoc Tukey test). Moreover, an increase in β-glucosidase production and a decrease in the pectinase production were observed at higher growth temperatures, so a temperature of 32 °C that gave median activities for the two enzymes was selected for further STR experiments.

Table 11 Pectinase and β-glucosidase activities for *Annulohypoxylon stygium* DR47 growth in the optimized media at different temperatures using shaking flaks (pH 5.0, 200 rpm).

	Pectinase (U/mL) [(1)]	**β-glucosidase (U/mL) [(2)]**
	48 h	**144 h**
26°C	5.19 ± 0.38 a	6.99 ± 0.86 a
29°C	5.29 ± 0.50 a	8.14 ± 0.55 a
32°C	4.84 ± 0,48 a	9.02 ± 0,51 a

Means calculated from 3 replications. Data not transformed. Means followed by the same small letter do not differ among them by Tukey test at 5%.

4.3.3 Batch bioreactor

Cultivations at pH 4.0, 5.0 and 6.0 were performed to evaluate the kinetics of enzyme production in a controlled batch environment, especially to the effects of oxygen and mass transfer. Experiments in bioreactors were conducted in duplicated for pH 5.0 (Figure 15).

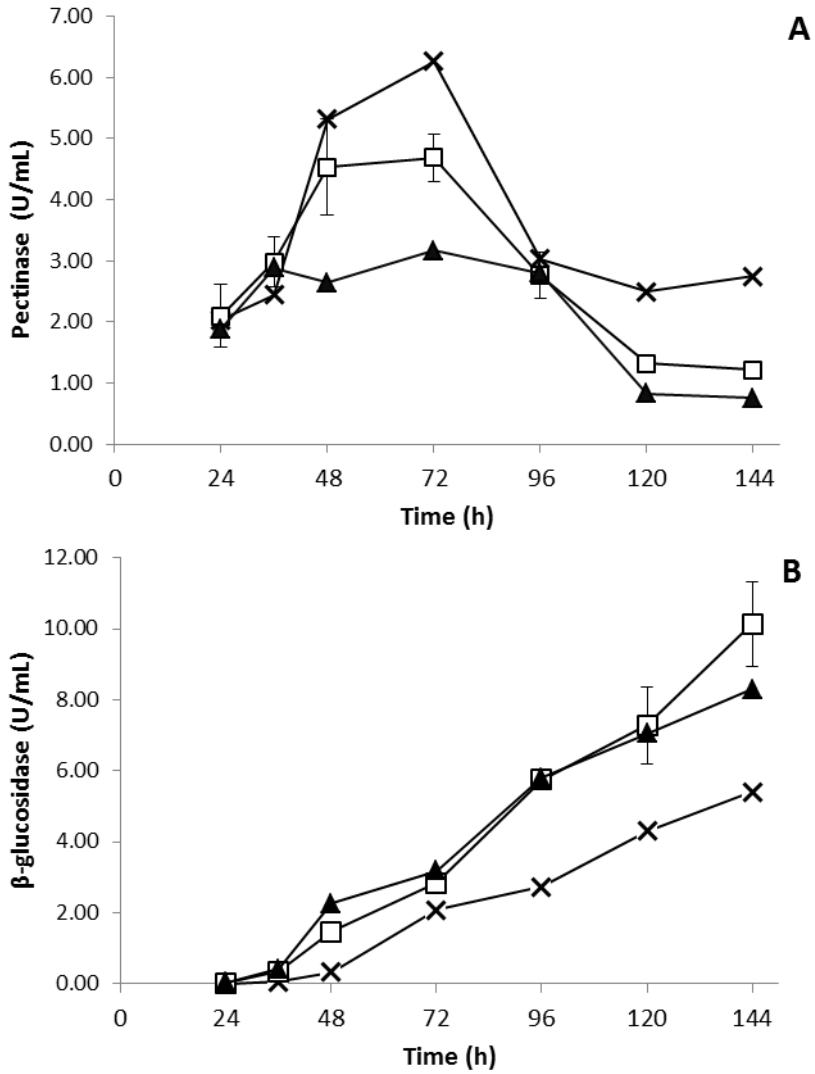

Figure 15 Pectinase (A) and β-glucosidase (B) activities of *Annulohypoxylon stygium* DR47 cultivation on STR in pH 4.0 (X), pH 5.0 (□) and pH6.0 (▲) at 32 °C.

Both enzymes showed similar production profiles in batch bioreactor fermentations to shake flask fermentations. Pectinase production peaked at 72 h (6.26 U/mL) at pH 4.0 (Figure 15A), which indicates that values above 5.0 can influence negatively in the pectinase production. Moreover, β-glucosidase activity was higher (10.13 U/mL) at 144 h at pH 5.0 (Figure 15B).

4.3.4 Multienzyme characterization: influence of temperature and ph and thermal stability

Pectinase activity of the extract produced in STR was measured at different temperatures and ranges of pH (Figure 16). Extracts had highest pectinase activity across a

range of temperatures from 35 °C to 50 °C, but optimally at 45 °C. The same extract showed highest activity at pH 5.0 and maintained 90% of the relative activity between pH 4.0 - 5.5.

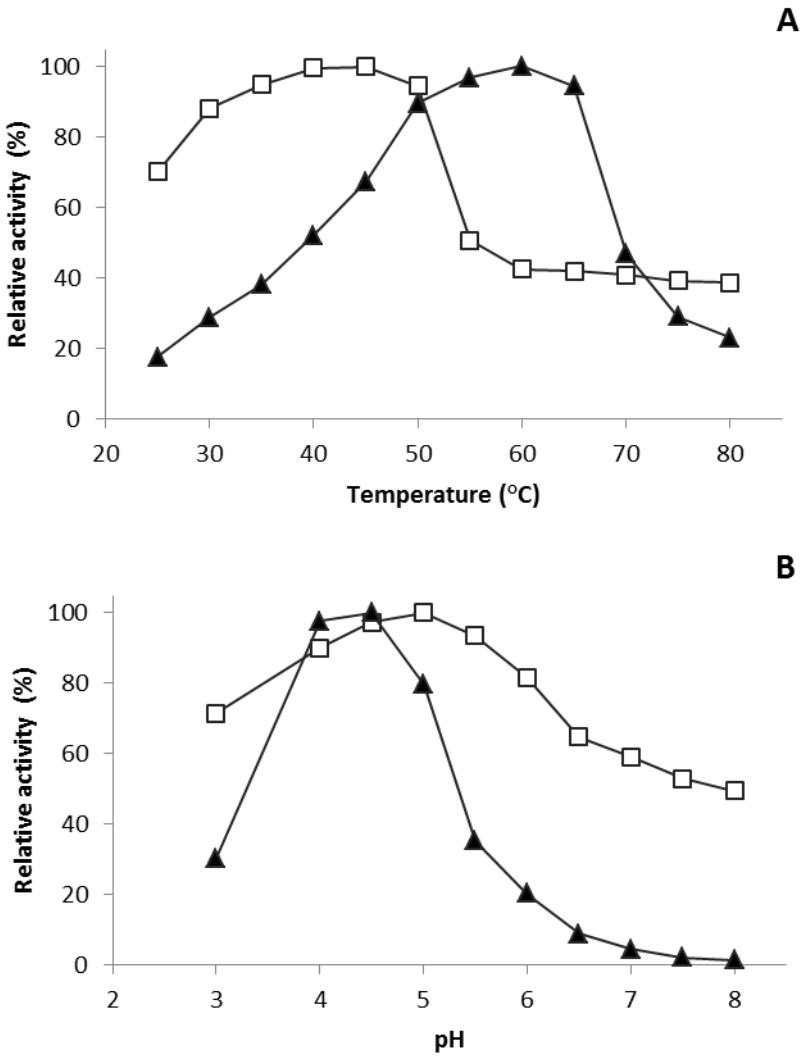

Figure 16 Residual activity expressed as a percentage of the maximum enzymatic activity produced by *Annulohypoxylon stygium* DR47 growth in STR. Pectinase (□) and β-glucosidase (▲) activity under different temperature (A) and pH (B).

β-glucosidase activity of the extract produced in STR was measured at different temperatures and ranges of pH (Figure 16). Extract had highest β-glucosidase activity at 60 °C, with 95 % of the maximum activity remaining between 55 °C and 65 °C. Enzymes with activities higher than 50 °C could be the key for a more efficient biomass hydrolysis, minimizing process problems. This extract gave greatest β-glucosidase activity at slightly acidic conditions, with an optimum pH around 4.5.

The thermal stability is another important parameter for the potential application of fungi in large-scale biomass hydrolysis processes. In terms of thermal stability, pectinase showed low stability, at 40 °C only 30% of activity was lost after 3 hrs of incubation, while 80% of the activity had been lost after incubation at 50 °C for 30 min (Figure 17). However, β-glucosidase was very thermal stable, retaining about 96.5% activity after 24 hrs incubation at 50 °C (Figure 17). The enzyme also retained about 50.2% activity after 9 hrs incubation at at 60 °C.

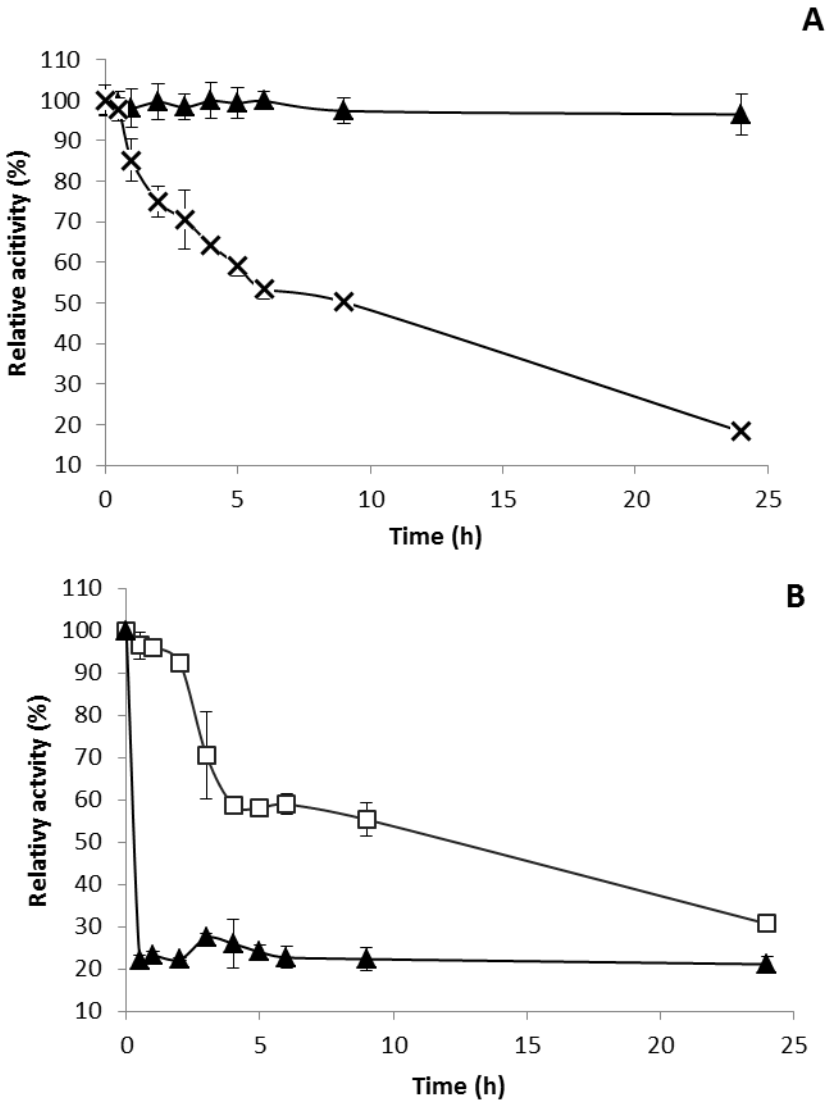

Figure 17 Residual activity expressed as a percentage of the maximum activity of β-glucosidase (A) and pectinase (B), produced by *Annulohypoxylon stygium* DR47 growth in STR. The thermal stability of β-glucosidase activity at 50 °C (▲) and 60 °C (X) and pectinase at 40 °C (□) and 50 °C (▲).

4.3.5 Sugar cane bagasse hydrolysis

The enzymatic extracts produced in bioreactors at pH 4.0 and pH 5.0, were rich in pectinase and β-glucosidase. These extracts were used to supplement a commercially available cellulolytic extract (Celluclast 1.5L) and were tested for HB hydrolysis. The major glycohydrolases from sugar cane bagasse were measured (Table 12). The extracts produced in this study presented low cellulolytic activities, but significant amounts of β-glucanases activities were observed. Also, low activities of other enzymes such as arabinofuranosidase, β-glucanase also were measured.

Table 12 Specific enzymes activities for some important glycohydrolases of *Annulohypoxylon stygium* DR47 extracts and Celluclast 1.5L.

Activity (U/mg)	Extract pH 5.0	Extract pH 4.0	Celluclast 1.5L
FPAse	0.14	0.20	1.71
β-glucanase	22.55	7.44	62.64
Pectinase	1.30	16.12	0.10
β-glucosidase	17.19	5.77	1.20
Xylanase	0.84	2.25	8.75
Xyloglucanase	1.68	0.52	30.81
Cellobiohydrolase	1.21	0.79	0.33
β-xylosidase	0.13	0.05	0.08
α-L-arabinofuranosidase	0.03	0.03	0.01
β-galactosidase	0.19	0.40	0.01

Celluclast 1.5L presents low amount of β-glucosidase and pectinase activity, which is well documented in *T. reseei* cellulolytic complexes. The hydrolysis saturation curves (Figure 18) indicated that the addition of A. *stygium* DR47 extracts increased sugar cane hydrolysis. Pectin extract supplementation presented similar behavior at 40 °C and at 50 °C, and the β-glucosidase showed higher hydrolysis at 50 °C. Besides, a saturation load can be visualized from 13 mg of protein/g of bagasse for the extract rich in pectinase and 10 mg of protein/g of bagasse for the extract rich in β-glucosidase.

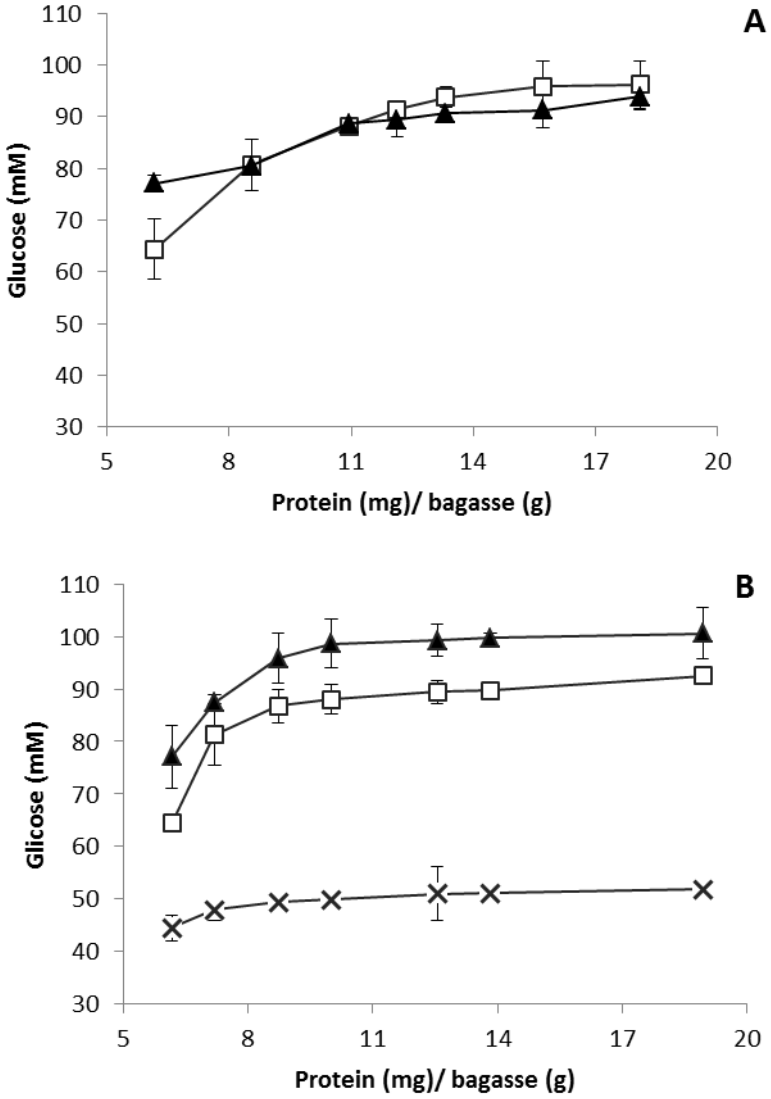

Figure 18 Hydrolysis saturation curve at 40 °C (□), 50 °C (▲) and 60 °C (X) of the Celluclast 1.5L supplementation with *Annulohypoxylon stygium* DR47 extracts growth in STR at pH 4.0 (A) and 5.0 (B).

The protein load in a biomass hydrolysis influences directly in the process cost. For this reason low protein loads, combining different types of enzymes has been studied to improve the saccharification step. In this way, other hydrolysis assay was performed aiming to keep the protein load at 12 mg of protein/g of bagasse and to replace part of the cellulolytic extract by the extracts produced in this study.

Table 13 Hydrolysis analyses of the partial replacement of Celluclast 1.5L by *Annulohypoxylon stygium* DR47 extracts.

	Celluclast 1.5L	Celluclast 1.5L + Extract pH 4.0 + Extract pH 5.0	Extract pH 4.0 + Extract pH 5.0
Monosaccharaides (g/L)	13.775	13.530	1.644
Glucose (g/L)	12.556	12.161	0.836
Xylose (g/L)	1.219	1.369	0.808
Arabinose (g/L)	0.000	0.000	0.000
Cellobiose (g/L)	0.000	0.161	0.184
Acetic acid (g/L)	0.138	0.148	0.065

4.3.6 Proteomic analysis

Proteomic analyses were performed aiming to describe the secreted proteins of *A. stygium* and to understand the effect of supplementation on Celluclast 1.5L. Two STR batch conditions were tested, at pH 4.0 and 5.0, for sugar cane bagasse hydrolysis.

For the extract produced at pH 5.0, were assignment 256 peptides, distributed in 38 protein hits. Several β-glucosidases (GH3) based on 9 peptide matches, which were similar to those enzymes from other fungi including *Neurospora crassa*, *Pyrenophora tritici-repentis* and *Paracoccidiodes* sp., were present (Appendix, Appendix D - Table 30). Besides 5 proteins were identified being two of them β-glucosidases and one a β-glucosidase precursor (GH3). The false discovery rate (FDR) was 5.1% for the protein and 2.0% for the peptide. In the extract produced at pH 4.0 were detected 185 peptides, distributed by 35 protein hits. Four proteins were identified, two L-α-arabinofuranosidase (GH54), a catalase and a carboxypeptidase base on 9 unique peptides (Appendix, Appendix E - Table 31). The FDR was 5.6% for the protein and 2.8% for the peptide.

A comparison of the secretomes using a Fischer exact test ($p<0.05$) reveled significant differences between proteins expressed under different fermentation conditions. For example, β-glucosidase (higher at pH 5.0) and L-α-arabinofuranosidase and catalase (higher at pH 4.0) (Table 14).

Table 14 Comparation of CAZy enzymes and proteins by n° of total peptides of LC/MS-MS from the supernatant of *Annulohypoxylon stygium* grown at pH 5.0 and 4.0

Identified Proteins	Family	Accession Number	Fisher's Exact Test (p-value)	Extract pH 5.0			Extract pH 4.0		
				1º	2º	3º	1º	2º	3º
Similar to α-L-arabinofuranosidase [Aspergillus nidulans FGSC A4]	GH54	gi|67522228	95% (0.018)	2	4	2	3	7	5
Hypothetical protein SNOG_11881 [Phaeosphaeria nodorum SN15]	GH3	gi|169617407 (+6)	95% (0.048)	9	6	11	4	3	3
Carboxypeptidase S1 [Pyrenophora tritici-repentis Pt-1C-BFP]	-	gi|189192809 (+1)	95% (0.0037)	3	4	2	5	7	7
Beta-glucosidase [Penicillium brasilianum]	GH3	gi|145688454	95% (0.0086)	4	8	5	0	0	3
Hypothetical protein SS1G_05679 [Sclerotinia sclerotiorum 1980]	AA	gi|156053664	95% (0.0017)	3	1	3	7	5	6
Hypothetical protein CIMG_03314 [Coccidioides immitis RS]	GH47	gi|119186533	0% (0.58)	4	2	2	1	2	3
Cel3b [Trichoderma reesei]	GH3	gi|31747166	95% (0.045)	5	6	4	2	1	1
Chitinase 1 precursor [Neurospora crassa OR74A]	GH18	gi|164427228 (+5)	0% (0.16)	4	5	4	2	2	1
Beta-glucosidase 2 precursor [Pyrenophora tritici-repentis Pt-1C-BFP]	GH3	gi|189202078	0% (0.48)	2	2	3	1	1	2
Beta-glucosidase [Paracoccidioides sp. 'lutzii' Pb01]	GH3	gi|295670726	95% (0.021)	4	7	6	0	2	2
Peroxidase_2 [Botryotinia fuckeliana B05.10]	AA2	gi|154315332	0% (0.42)	4	3	2	0	5	0
ATP-dependent dna-binding helicase (RAD3/XPD subfamily) [Encephalitozoon cuniculi GB-M1]	-	gi|19074028	0% (0.58)	1	0	0	0	0	0
Hypothetical protein [Podospora anserina S mat+]	GH3	gi|171685516	95% (0.00013)	3	6	7	0	0	0
Alpha-1.2-mannosidase [Podospora anserina S mat+]	GH92	gi|171681924	0% (0.39)	2	3	1	3	2	1
Catalase [Claviceps purpurea]	-	gi|3157413	95% (0.000058)	0	0	0	3	4	4
Elastinolytic metalloproteinase Mep [Neosartorya fischeri NRRL 181]	-	gi|119485809	95% (0.0022)	4	5	2	0	0	0

Beta-galactosidase. putative [Aspergillus clavatus NRRL 1]	GH35	gi|121701157	95% (0.016)	1	0	1	4	1	3
Hypothetical protein CHGG_02841 [Chaetomium globosum CBS 148.51]	CE1	gi|116207096	0% (0.21)	1	1	0	1	1	2
Unnamed protein product [Aspergillus oryzae RIB40]	-	gi|83773422 (+12)	95% (0.0021)	0	0	0	2	3	2
Hypothetical protein [Podospora anserina S mat+]	GH55	gi|171688470	0% (0.62)	1	2	1	1	2	0
Hypothetical protein BC1G_07110 [Botryotinia fuckeliana B05.10]	GH3	gi|154310381 (+2)	0% (0.50)	1	1	2	0	1	1
Endochitinase [Verticillium albo-atrum VaMs.102]	GH18	gi|302404074	0% (0.11)	0	3	1	0	0	0
Predicted protein [Laccaria bicolor S238N-H82]	-	gi|170084953	0% (0.58)	0	0	1	0	0	0
Conserved hypothetical protein [Uncinocarpus reesii 1704]	-	gi|258575447	0% (0.11)	3	1	0	0	0	0
Hypothetical protein PICST_54418 [Scheffersomyces stipitis CBS 6054]	-	gi|150863946 (+4)	0% (0.66)	0	0	1	1	0	0
Hypothetical protein CHGG_04379 [Chaetomium globosum CBS 148.51]	GH92	gi|116195562 (+1)	95% (0.030)	0	0	0	2	1	1
Hypothetical protein [Podospora anserina S mat+]	-	gi|171686504	0% (0.42)	0	0	0	0	0	1
Chain A. Glycoside Hydrolase Family 15 Glucoamylase From Hypocrea Jecorina	GH15	gi|261825113 (+1)	0% (0.19)	1	0	2	0	0	0
Beta-tubulin [Blastocladiella emersonii]	-	gi|117422544 (+150)	0% (0.18)	0	0	0	0	0	2
Pc12g11110 [Penicillium chrysogenum Wisconsin 54-1255]	GH3	gi|255932921	0% (0.58)	1	0	0	0	0	0
Unnamed protein product [Aspergillus niger]	GH3	gi|134076323 (+2)	0% (0.34)	1	1	0	0	0	0
Tranlsation elongation factor 1a [Trichaptum abietinum]	-	gi|13162245 (+1)	0% (0.42)	0	0	0	0	1	0
Nitrate reductase [Aspergillus oryzae]	AA1	gi|1136629 (+3)	0% (0.58)	0	0	1	0	0	0
Probable beta-glucosidase 1 precursor [Neurospora crassa]	GH3	gi|12718377 (+2)	0% (0.34)	1	0	1	0	0	0
ZYRO0D10164p [Zygosaccharomyces rouxii]	-	gi|254581782	0% (0.66)	0	1	0	0	1	0
class III chitinase. putative [Talaromyces stipitatus ATCC 10500]	GH18	gi|242792443	0% (0.58)	0	1	0	0	0	0
Alpha-L-arabinofuranosidase [Talaromyces purpurogenus]	GH54	gi|13991905	95% (0.0052)	0	0	0	0	4	2
Subtilisin-like protease PR1D [Metarhizium acridum]	-	gi|18958207	0% (0.34)	0	0	2	0	0	0

Family 10 xylanase [Cryptovalsa sp. BCC 7197]	GH10	gi|53636303	0% (0.34)	0	2	0	0	0	0
Glucoamylase [Aspergillus oryzae RIB40]	GH15	gi|169770097 (+2)	0% (0.58)	1	0	0	0	0	0
Lactonohydrolase [Cryptococcus neoformans var. neoformans JEC21]	-	gi|58259894	0% (0.42)	0	0	0	1	0	0
Hypothetical protein [Podospora anserina S mat+]	GH35	gi|171683861 (+1)	0% (0.42)	0	0	0	1	0	0
Hypothetical protein CHGG_08330 [Chaetomium globosum CBS 148.51]	GH7	gi|116200349 (+13)	0% (0.58)	0	0	1	0	0	0
YALI0D05049p [Yarrowia lipolytica]	-	gi|50549915	0% (0.58)	0	1	0	0	0	0

In addition, pH influenced the protein profiles regarding to GH families based on unique peptides (Figure 19). The most abundant families were GH3, GH18 and GH15 at pH 5.0 and GH3, GH54, GH35 and GH92 at pH 4.0.

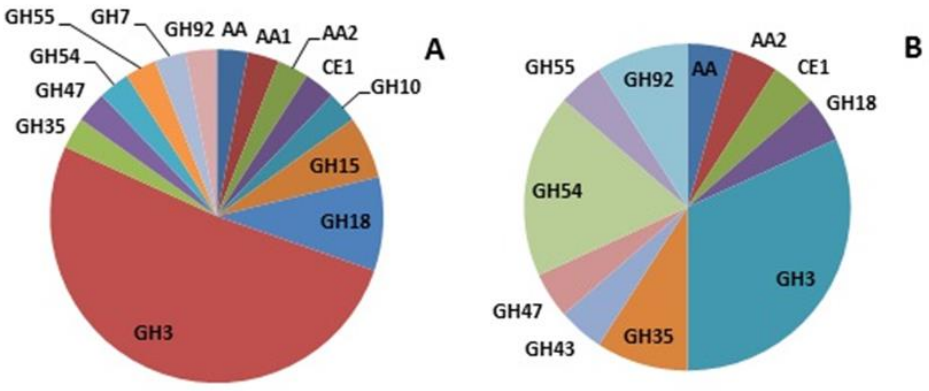

Figure 19 GH's family detected based on unique peptides in *Annulohypoxylon stygium* DR47 extracts growth in STR at pH 5.0 (A) and 4.0 (B).

At pH 4.0 pectinase activity was highest and when added to Celluclast 1.5L biomass hydrolysis was increased, although no polygalacturonase was detected in the secretome (Appendix, Appendix E - Table 31). Enzymes that were detected such as α-L-arabinofuranosidase are able to hydrolyze bonds in hemicellulose and could have contributed to the increase of sugar release during HB hydrolysis. Even though the extract of pH 4.0 indicated the presence of β-galactosidase in the secretome and also enzymatic activity, which could not explain the increase in hydrolysis by this enzyme since no galactose was presented in HB. The secretome analyses from the fermentation at pH 5.0 (Appendix, Appendix D -

Table 30) revealed the presence of β-glucosidase which corroborated the enzymatic activity profile (Table 12), once that main activity detected was β-glucosidase followed of β-glucanase.

4.4 **Discussion**

Media formulation and optimization are tools needed to ensure the success of an industrial bioprocess. In this study we developed a media for the production of β-glucosidase and pectinase using two feedstocks (CB and SB), together with a low cost sugar, saccharose. Citrus-process wastes are used as substrates for the bio-production of other products including citric acid (191), flavor (192) and phytases (193). These feedstocks are also well known sources for pectinolytic enzymes production, but less so for other glycohydrolases. Mamma et al. (194) used citrus peel to produce pectinolytic, cellulolytic and xylanolytic enzymes from *Aspergillus niger*, *Fusarium oxysporum*, *Neurospora crassa* and *Penicillium decumbens* under solid-state fermentation conditions. In the same way, wastes from soybean manufacturing processes have also been extensively as sources of enzymes for biomass degradation. Vitcosque et al. (195) and Delabona et al.(129) used soybean bran to produce cellulases, xylanases and β-glucosidases by *A. niger* and *T. harzianum* to hydrolyze pretreated sugar cane bagasse.

Although the optimized media formulation did support high titres of enzyme production for both β-glucosidase and pectinase, it was not possible to perform the fermentation at the same pH to support the maximum activities of both enzymes. Growth in bioreactors led a high titration of pectinase and β-glucosidase at pH 4.0 and 5.0. At pH above 5.0 we found pectinase activity production was reduced (Figure 15A). Acunaarguelles, Gutierrezrojas (196) also demonstrated that pectinase activity produced by *A. niger* also declined above pH 5.0 due to denaturation.

Like any enzyme catalyzed reaction, the rate of hydrolysis catalyzed by glycosidases is influenced by temperature and pH. As far as we know, there are only reports of β-glucosidase activity but not the effects of temperature and pH in fungi including *A. stygium*, *Hypoxylon* spp. and *Xylaria* spp. (155) *Daldinia eschscholzii* is another specie of the family *Xylariaceae* where β-glucosidase activity has been characterized (197), and was shown to have optimum activity at pH 5.0 and 50 °C. The extracts produced from *A. stygium* DR47 in this study showed activity over wide ranges of temperature and pH consistent with these previously reported fungi.

The partial replacement of Celluclast 1.5L with the enzymatic extracts of *A. stygium* DR47 showed equivalent saccharides released (Figure 18). Enzyme extracts from *A. stygium* DR47 could be used to formulate an enzyme mixture for biomass deconstruction as a commercially viable alternative to commercial celullases currently on the market. However, further experiments are required to establish the optimal hydrolysis conditions as well as optimization of supplementations amounts of the extract produced.

The proteomics study of the secreted proteins (i.e. enzymes) could explain results from the saccharification assay once that additional enzymes such as β-glucosidase and α-L-arabinofuranosidase could be detected. It is known that the β-glucosidase supplementation can increase biomass hydrolysis once it consumes the cellobiose and reduces the inhibitory effect against cellulases (3, 198).

Gonçalves et al. (199) and Goldbeck et al. (200) verified that a recombinant α-L-arabinofuranosidase (GH54) in the presence of the endo-xylanase (GH11) gave synergistic effects of xylose and xylooligosaccharides release from pretreated sugarcane bagasse. GH3 was the most abundant family in both enzyme extracts produced. Several studies have suggested the importance of this class of enzyme on biomass deconstruction, for example in *P. decumbens* proteome (201) and in the metatranscriptome of bee gut (202). The GH3 CAZy family is also known as an important enzyme in biomass saccharification. This class of enzyme is responsible for the breakdown of diverse oligosaccharides found in many types of biomass and has unusually broad substrate specificities, for example, oligosaccharides with diverse carbon-chain lengths and monomer residues.

Also the presence of a catalase in the pH 4.0 extract could indicate a better assimilation of biomass by *A. stygium* in cultivation at pH 4.0, and increase of enzymatic hydrolysis in the supplementation of Celluclast 1.5L. According to Bourdais et al. (203) catalase activity is specifically required to efficiently assimilate lignocellulose in *Podospora anserine,* as hydrogen peroxide participates in the degradation of biomass complex but can be responsible to cell damage and cell death.

There is a paucity of information on enzymes from *A. stygium* or related species in protein databases. This may explain why pectinase activity was detected in the protein extract but not in the proteomic data, which opens up the exciting possibility that the pectinases of *A. stygium* may be novel, with distant homology to pectinase sequences in the protein databases. Future work will now concentrate on using genomics and transcriptomics, in conjunction with proteomics to characterize the pectinases.

4.5 **Conclusion**

A. stygium DR47 showed to be a potential candidate for glycohydrolases production when grown using citrus pulp and soybean bran in STR. Proteomic analysis of the secretome of *A. stygium* DR47 revealed other glycohydrolase families, such as GH3, GH18, GH35, GH54 and GH92, never previously reported in this fungus. The substrate specificities and relative rates of hydrolytic activities of these new enzymes will be explored to develop an enzyme cocktail with superior saccharification yields.

CHAPTER 5 - XYLANASE PRODUCTION BY ENDOPHYTIC *Aspergillus niger* USING PENTOSE-RICH HYDROTHERMAL LIQUOR FROM SUGARCANE BAGASSE

5.1 Introduction

Aspergillus niger, a member of the black Aspergilli group of fungi, is extensively used in industry for many process including the production of citric acid and a wide range of enzymes (191, 204) due to its high rate of protein secretion and its fermentation capabilities (205). The production of fungal xylanases has been extensively studied, and submerged fermentation (SmF) and solid-state fermentation processes have been developed for various fungal species (206-208). Nevertheless, SmF remains the preferred choice for industrial production of xylanase and other cellulolytic enzymes because it can be easily controlled and scaled up to large industrial bioreactors (209). Xylanase enzymes have been proposed for use in applications such as bio-bleaching in the pulp and paper industry (210), as well as in bakeries and the food industry (204). Accessory enzyme activities provided by β-xylosidase, β-mannosidase, α-L-arabinofuranosidase, endoxylanase, pectinase, and esterase have been reported to enhance enzymatic cellulolytic hydrolysis and increase the release of free carbohydrate from biomass, because these enzymes are able to break linkages between cellulose fibrils embedded in the hemicellulose-lignin matrix. Addition of accessory enzyme activity has been used to enhance the hydrolysis of corn stover (3), wheat straw (211), and sugarcane bagasse (151).

The development of an economic process for second generation ethanol production from lignocellulosic material depends on several factors, and the cost of enzyme production is still one of the main challenges (184). The pretreatment of biomass is also crucial for successful enzymatic deconstruction and subsequent alcoholic fermentation. Hydrothermal pretreatment involves the use of water at high temperature (160–200 °C) for several minutes in order to solubilize hemicellulose and lignin (212). Imman et al. (213) obtained high levels of hemicellulose in the liquid phase and improvement in the enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse. The liquor from the hydrothermal sugarcane bagasse pretreatment contains high concentrations of xylose and xylo-oligosaccharides (Chpater 3), and could be used as a substrate for xylanase production. Michelin et al. (214) used the liquor from the hydrothermal pretreatment of wheat straw to produce xylanase with *Aspergillus ochraceus*.

On chapter 3 it was screened hemicellulase producers using the pentose-rich liquor from hydrothermal pretreatment of sugarcane bagasse. Even though this material is a promising feedstock for xylanase induction, due to its high contents of xylo-oligomers and xylose, the high concentrations of compounds such as furfurals, organic acids, and soluble phenols can hamper fungal growth. Among the various microorganisms, the endophytic *A. niger* DR02 strain has emerged as a potential producer due to its high rate of xylanase secretion and relatively high resistance to toxic compounds.

Bioprocess engineering tools such as fed-batch fermentation have been used for many years to produce fungal cellulolytic enzymes and this operational mode is believed to minimize catabolite repression (209). In principle, the use of this cultivation strategy should also help to mitigate the inhibitory effects of pentose-rich liquor.

The aim of this work was to development of a submerged fermentation process to produce hemicellulase using the endophytic *A. niger* DR02 strain grown on the pentose-rich liquor from sugarcane bagasse hydrothermal pretreatment. This strain was previously selected (Chapter 3) and was chosen by its capacity of production high concentration of xylanase. The enzymatic cocktails produced using batch and fed-batch procedures were characterized and used as cellulolytic enzyme supplements in order to enhance the enzymatic hydrolysis of pretreated sugarcane bagasse.

5.2 **Materials and methods**

5.2.1 Strain

The *Aspergillus niger* DR02 strain is an endophytic organism isolated from *Platanus orientalis* and was selected on previous assays (Chapter 3).

5.2.2 Components of the culture media

Sugarcane bagasses, SB, WB and HL were obtained as described chapter 3. A fraction of the HL produced was detoxified by overliming followed by adsorption on activated charcoal, as described by Marton (215). The resulting material is denoted DHL in the present work. The compositions of HL and DHL were performed as describe in chapter 3.

5.2.3 Pre-culture and production media

The composition of the inoculum culture medium was adapted from Mandels and Reese (127), using 10 g/L of glucose as carbon source. The composition of the production medium was the same as that of the pre-culture medium, except for the type of carbon source.

The insoluble carbon sources (HB, BEX, DEB, SB, and WB) were evaluated at concentrations of 10 g/L. HL was tested at concentrations of 10, 20, 30, 40, 50, and 60% (v/v, in water), and DHL was tested at 80% (v/v). The culture media used in the fed-batch experiments are described below. The pH of the culture media was adjusted to 5.0, and the media were sterilized at 121 °C for 20 min.

5.2.4 Shake flask experiments

Suspensions of conidia, prepared by adding sterilized distilled water and Tween 80 to the organism grown on PDA plates, were transferred to Erlenmeyer flasks containing 200 mL of inoculum culture medium (3×10^6 spores/mL of medium) and incubated for 48 h at 29 °C on a rotary shaker at 200 rpm. Aliquots (20 mL) of this pre-culture were transferred to 500 mL Erlenmeyer flasks containing 180 mL of the production medium and incubated at 29 °C on a rotary shaker at 200 rpm for 144 h.

5.2.5 Bioreactor experiments

Experiments were conducted using a 1 L working volume bioreactor (Bioflo 115, New Brunswick Scientific Co., USA) equipped with automatic control of temperature (29 °C), pH (5.0), agitation rate (200–500 min^{-1}) and aeration rate (0.3–1.0 L min^{-1}). The pH was controlled by the automatic addition of either H_2SO_4 (0.4 M) or NH_4OH:H_2O (1:3, v/v), and the dissolved O_2 level was kept above 30% of air saturation by automatic adjustment of aeration and agitation within the ranges indicated previously. Foaming was manually controlled as required using sterilized polyglycol antifoaming agent (Fluent Cane 114, Dow Chemical, Brazil). The bioreactor was inoculated with 10% (v/v) of inoculum broth prepared as described above. Samples were periodically withdrawn, centrifuged at 10,000 x g for 15 min at 10 °C, and analyzed for protein content and enzymatic activity, as described below.

5.2.6 Batch experiments

Batch experiments were performed in duplicate using initial HL concentrations of 10, 30, and 50% (in water). Sampling and conditions were as described above. The maximum exponential growth rate, μ_{max}, was estimated from the slope of the plot of ln X against time (t).

5.2.7 Fed-batch experiments

Fed-batch procedures were evaluated using pulsed feed, constant feed, and exponential feed modes. The feed flows were calculated as described by Diniz et al. (216), and are detailed below. The kinetic parameters used for the flow calculation were obtained in the previous batch cultures. The cell maintenance factor was disregarded in the calculations. All experiments were started in batch mode, and the feed flows were initiated when the rate of the automatically-controlled agitation to maintain dissolved oxygen levels started to decrease, indicating that the carbon source was becoming limited.

5.2.8 Theoretical calculations

5.2.8.1 Pulsed feed

Three pulses (with volume V_{inlet}) of a concentrated HL solution (S_{inlet}, Table 16) were delivered to the bioreactor as soon as the xylose + glucose concentration (total sugar concentration) dropped below 2 g/L, which occurred at 36, 72, and 90 h. In Equation 1, the parameters V_p and S_p are, respectively, the bioreactor volume and the carbohydrate concentration in the culture broth required after the concentrated HL solution pulse.

$$V_{inlet} = \frac{V_p\, S_p}{S_{inlet}} \qquad \text{(Equation 1)}$$

5.2.8.2 Constant feed

The concentrated HL solution (S_{inlet}, Table 2) was fed to the bioreactor at a constant flow rate, F, such that there was no accumulation of carbon source in the broth (in other words, all the carbon source provided was consumed). The carbon source mass balance in the bioreactor is described by Equation 2.

$$\frac{ds}{dt} = FS_{inlet} - Vr_{sx} \qquad \text{(Equation 2)}$$

If there is no accumulation of substrate, so $\frac{ds}{dt} = 0$, and if the substrate consumption rate is described as:

$$r_{sx} = \frac{\mu X}{Y_{xs}}$$ (Equation 3).

Then the Equation 2 can be rewritten as:

$$F = \frac{\mu_{crit} X_o V_o}{S_{inlet} Y_{xs}}$$ (Equation 4).

In order to ensure a carbon-limited regime, μ_{crit} in Equation 4 was set at a fraction of μ_{max} determined from the slope of the plot of ln *X* against time (*t*) (216).

5.2.8.3 Exponential feed

Concentrated HL solution (S_{inlet}, Table 16) was fed to the bioreactor at a flow rate (*F*) that increased exponentially with time (*t*), such that there was no accumulation of carbon source in the broth (all the carbon source provided was consumed). In this situation, if it is assumed that $\frac{ds}{dt} = 0$, the mass balance gives the following equation describing the variation of the flow rate (*F*) with time (*t*) (216):

$$F = \frac{\mu_{crit} X_o V_o e^{\mu_{crit} t}}{S_{inlet} Y_{xs}}$$ (Equation 5).

In order to have a carbon-limited regime, μ_{crit} in Equation 5 was set at a fraction of μ_{max} determined from the slope of the plot of ln *X* against time (*t*) (216).

Other parameter values used in Equations 1, 2, and 5, together with the conditions, are provided in Table 16.Table 16 Parameters for fed-batch cultivation of *A. niger* DR02 on pentose-rich liquor (HL) from the hydrothermal pretreatment of sugarcane bagasse.

5.2.9 Enzymatic assays

All enzymatic activities were measured as described on chapter 3.

5.2.10 Protein concentration

Total protein was measured as described on chapter 4.

5.2.11 Biomass concentration

The dry cell weight concentration of fungal biomass in the bioreactor experiments was obtained by centrifuging 5 mL of the culture broth at 10,000 x *g* for 10 min, washing and then

re-suspending the sediment in deionized water, centrifuging again, and drying at 105 °C to a constant weight.

5.2.12 Crude enzyme characterization: influence of ph temperature

The constant fed-batch cultivation extract at 144 h was assayed for xylanase activity at different reaction temperatures (25 - 80 °C) in 50 mmol/L sodium citrate buffer (pH 5.0). The effect of pH on enzyme activity (at 50 °C) was determined using 50 mmol/L citrate-phosphate buffer (pH 3.0 - 9.0).

5.2.13 Enzymatic hydrolysis

The hydrothermally pretreated sugarcane bagasse (HB) was subjected to enzymatic saccharification using a combination of the enzymatic preparations produced in the bioreactor and a commercial enzyme preparation (Celluclast 1.5L, Novozymes, Denmark). Enzymatic hydrolysis of HB suspended at 5% (w/v) in 50 mM citrate buffer (pH 5.0), amended with 0.02% (v/v) sodium azide, was performed at 10 FPU/g HB. The xylanase dose-response curve was obtained using 2 mL Eppendorf tubes kept at 1000 min^{-1} for 24 h at 50 ^{o}C (Thermomixer, Eppendorf). Investigation of the kinetics of HB hydrolysis (using Celluclast 1.5L supplemented with the enzymatic preparations produced in the bioreactor) was performed using 50 mL Erlenmeyer flasks at 250 min^{-1} for 72 h at 50 ^{o}C (Innova 22R, New Brunswick Scientific). The samples were centrifuged at 10,000 x *g* for 15 min (Model 5418 centrifuge, Eppendorf), filtered (Sep-Pak C18, Waters), and the carbohydrate concentrations were determined by HPLC, as described by Rocha et al. (122). The hydrolysis experiments were carried out in triplicate and the data were calculated as means and standard deviations.

5.2.14 Mass spectrometric analysis of the A. niger secretome

Mass spectrometric analyses were performed as described on chapter 4, using samples obtained from the constant fed-batch reactor at 144 h of cultivation (the time at which the maximum xylanase titer was achieved).

5.3 **Results**

5.3.1 Use of different carbon sources for A. niger DR02 growth and enzyme induction

The various substrates were tested at different concentrations in order to assess enzyme production by *A. niger* DR02 in shake flask experiments. The solid substrates were

HB, DEB, EX, WB and SB. The liquid substrates were HL and DHL. The results (Figure 20) represent the means and standard deviations (error bars) for triplicate runs.

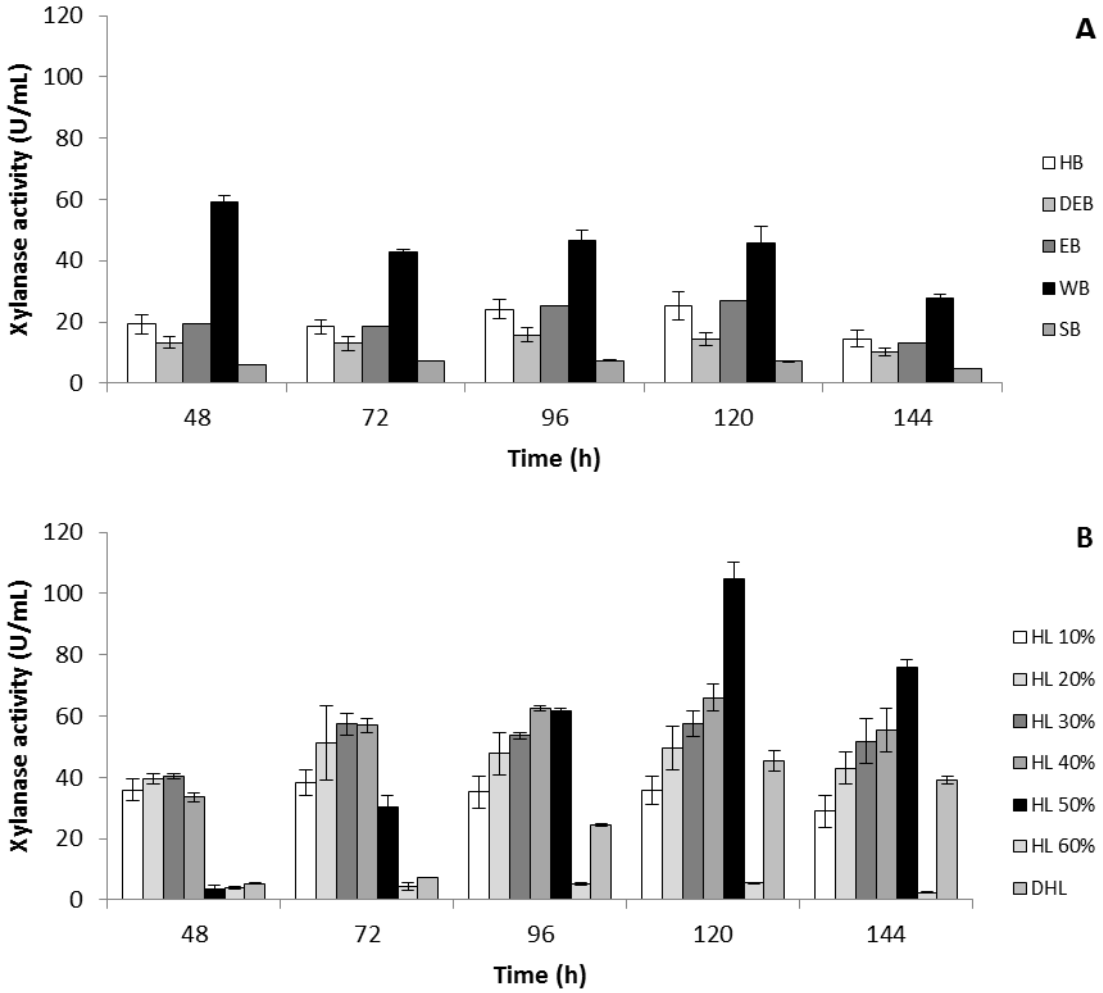

Figure 20 Evolution with time of xylanase activity for *A. niger* DR02 shake flask cultivation using (A) solid (HB: hydrothermally pretreated sugarcane bagasse; DEB: delignified steam-explosion pretreated sugarcane bagasse; EB: steam-explosion pretreated sugarcane bagasse; WB: wheat bran; SB: soybean bran) and (B) liquid (HL: pentose liquid from hydrothermal pretreatment of sugarcane bagasse) carbon sources.

In the case of the solid carbon sources (HB, BED, BEX, WB, and SB), maximum xylanase production (59 U /mL) was achieved at 48 h of fermentation using WS (wheat bran) at a concentration of 10 g/L, with a temperature of 29 ^{o}C and agitation at 200 min^{-1} (Figure 20A).

Different concentrations of HL were tested by diluting it with water at 10, 20, 30, 40, 50, and 60% (v/v). Detoxified hydrothermal liquor (DHL) was used for comparison. Among the different carbon sources, the raw HL pentose liquor diluted in water at a concentration of 50% (v/v) provided the highest xylanase activity, with a value exceeding 100 U/mL at 120 h of cultivation (Figure 20B). Application of the detoxification technique removed a large quantity of inhibitors (Table 15), enabling *A. niger* to grow in the undiluted liquor, although maximum xylanase production did not reach the values obtained for raw aqueous HL diluted at 20, 30, 40, and 50% (v/v) (Figure 20B). The diluted pentose-rich liquor (HL) was therefore selected for use in bioreactor xylanase production experiments.

Table 15 Composition of raw (HL) and detoxified (DHL) pentose-rich liquor from the hydrothermal pretreatment of sugarcane bagasse.

Substance (g/L)	**HL**	**DHL**
Glucose	0.54 ± 0.07	0.00 ± 0.00
Xylose	4.7 ± 0.41	2.48 ± 0.21
Cellobiose	0.00 ± 0.00	0.00 ± 0.00
Arabinose	0.77 ± 0.10	0.59 ± 0.01
Acetic acid	1.47 ± 0.18	2.43 ± 0.30
Formic acid	0.23 ± 0.10	0.18 ± 0.08
HMF	0.18 ± 0.01	0.00 ± 0.00
HF	1.05 ± 0.06	0.00 ± 0.00
Xylo-oligomers	9.98 ± 1.13	4.01 ± 0.45
Soluble lignin	3.15 ± 0.49	1.32 ± 0.20

5.3.2 Effect of HL dilution using batch bioreactor experiments

A. niger DR02 was cultivated in 30 and 50% (v/v) aqueous HL solution in order to evaluate the influence of dilution on xylanase production in a controlled bioreactor system. These assays were performed in duplicate and the results are summarized in Figure 21 and Figure 22. The xylo-oligomers were totally consumed, with induction of xylanase biosynthesis. Measurement of the uptake of free carbohydrates (xylose, glucose, and arabinose) revealed consumption profiles similar to those of the xylo-oligomers (Figure 21 A

and B) which indicated that xylo-oligomer hydrolysis was not the limiting step for carbohydrate assimilation.

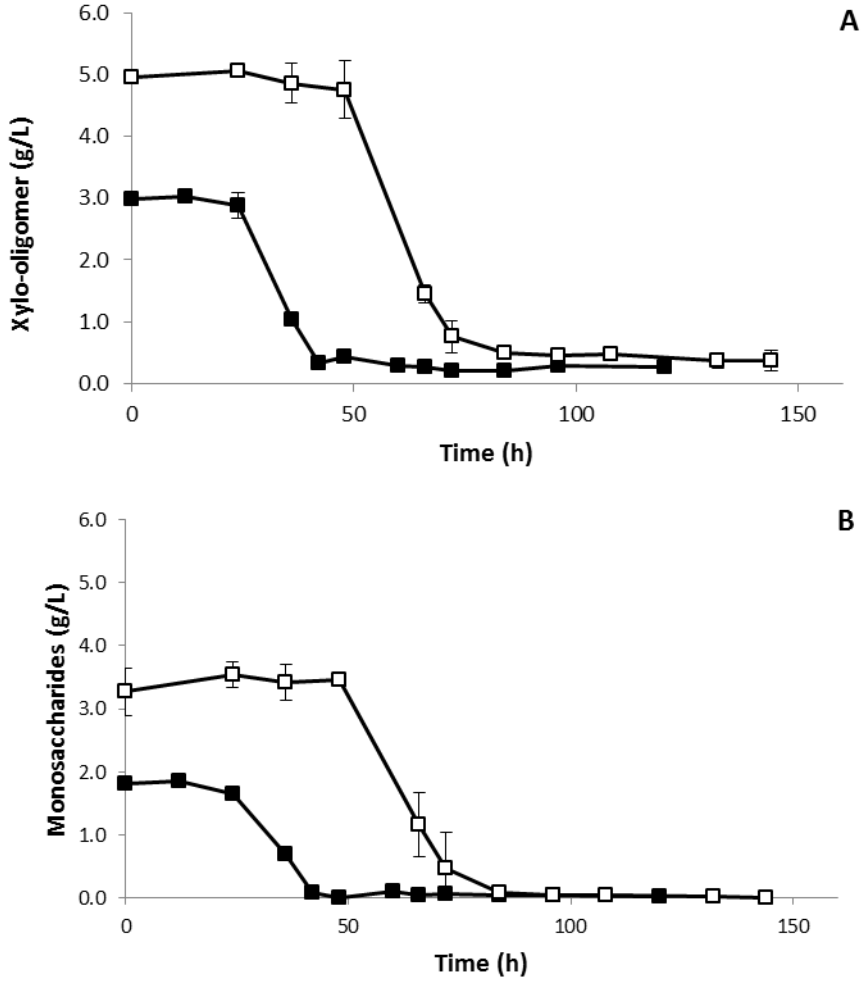

Figure 21 Evolution with time of xylo-oligomers and monosaccharides concentration for batch cultivation of *A. niger* DR02 on pentose-rich liquor from hydrothermal pretreatment of sugarcane bagasse, diluted at 30% (v/v) (■) and 50% (v/v) (□).

The xylanase titers in the cultures increased when the xylo-oligomers and monossacharides, mainly xylose, were exhausted (Figure 21), and reached maximum values of 137.9 U/mL (at 120 h) and 229.3 U/mL (at 144 h) for the HL diluted at 30 and 50% (v/v), respectively (Figure 22A). The exhaustion of the carbon sources was therefore associated with the production of the enzyme. This corroborates previous work that found hemicellulase induction of gene expression at low xylose concentrations (1 mM), because at these levels fungal metabolism was not subject to carbon catabolite repressor, the CreA protein (99).

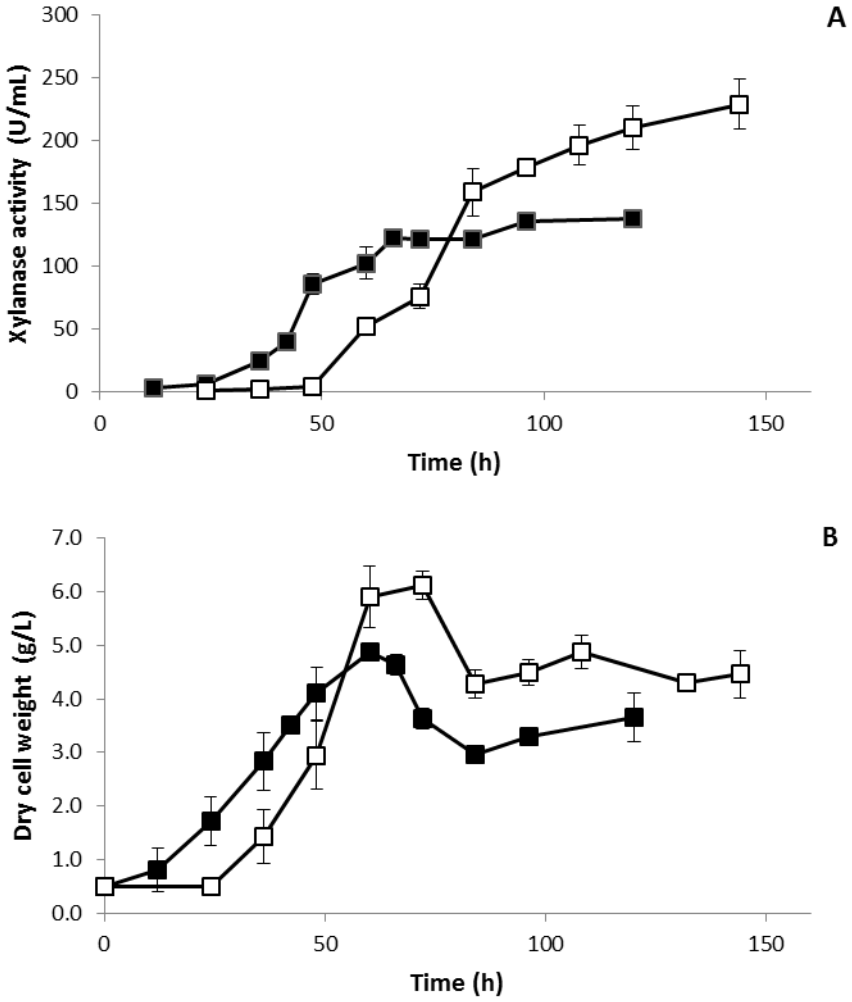

Figure 22 Evolution with time of xylanase activity and dry cell weight concentration for batch cultivation of *A. niger* DR02 on pentose-rich liquor from hydrothermal pretreatment of sugarcane bagasse, diluted at 30% (v/v) (■) and 50% (v/v) (□).

The dry cell weight concentration (Figure 22B) was used to calculate maximum specific growth rates (μ_{max}) of 0.048 and 0.069 h^{-1} for HL at 30 and 50% (v/v), respectively. The lag phase was longer at the higher pentose liquor concentration, probably due to higher amounts of acetic acid, furfural, hydroxyl-methyl furfural (HMF), and soluble lignin, which are known to negatively interfere in microorganism growth.

5.3.3 Fed-batch bioreactor

Fed-batch experiments were conducted as described above. The results are summarized in Figure 23 and Figure 24, which displays the mean values and standard deviations. Cultivations were carried out with initial HL concentrations of 30% (v/v) using

pulsed feed and exponential feed, and 50% (v/v) using constant feed (Table 16). The various operational modes employed the same total mass of carbon added to the system, which amounted to 22.4 g of carbon source in the reactor. All other bioreactor variables (pH, temperature, and minimum dissolved oxygen) were kept constant at the same levels for all cultivation runs. Hence, any observed differences must have been due to the different culture medium feeding regimes.

Table 16 Parameters for fed-batch cultivation of *A. niger* DR02 on pentose-rich liquor (HL) from the hydrothermal pretreatment of sugarcane bagasse.

Fed-batch mode	S_o (g/L)	S_{inlet} (g/L)	μ_{crit} (h^{-1})	Y_{xs} (g/g)	V_o (L)	X_o (g/L)	V_{inlet} (L)	r_{sx} (g/Lh)	$\sum carbon$ (g)	*Equation*
Pulsed feed	6.72	110.4	------	0.57	1	3.517	0.0467	------	22.4	(1)
Constant feed	11.2	110.4	0.0212	0.57	1	3.517	-----	0.113	22.4	(4)
Exponential feed	6.72	110.4	0.0212	0.57	1	3.517	-----	------	22.4	(5)

These experiments were performed to evaluate the performance of the different cultivation methods and to determine their potential to minimize the effects of inhibitors and mitigate the carbon catabolite repression effect. This was expected to lead to higher enzyme productivity and increase the enzyme titer. The cultivations were carried out under a carbon-limited regime in fed-batch experiments employing constant and exponential feeding. In order to achieve this condition, the specific growth rate was set at a value well below the maximum specific growth rate calculated in the previous batch experiments. In these experiments, a set value of μ_{crit} = 0.0212 h^{-1} was used for calculation of the volumetric flow rate profiles, and volumetric flow rates were calculated according to Equations 2 and 5.

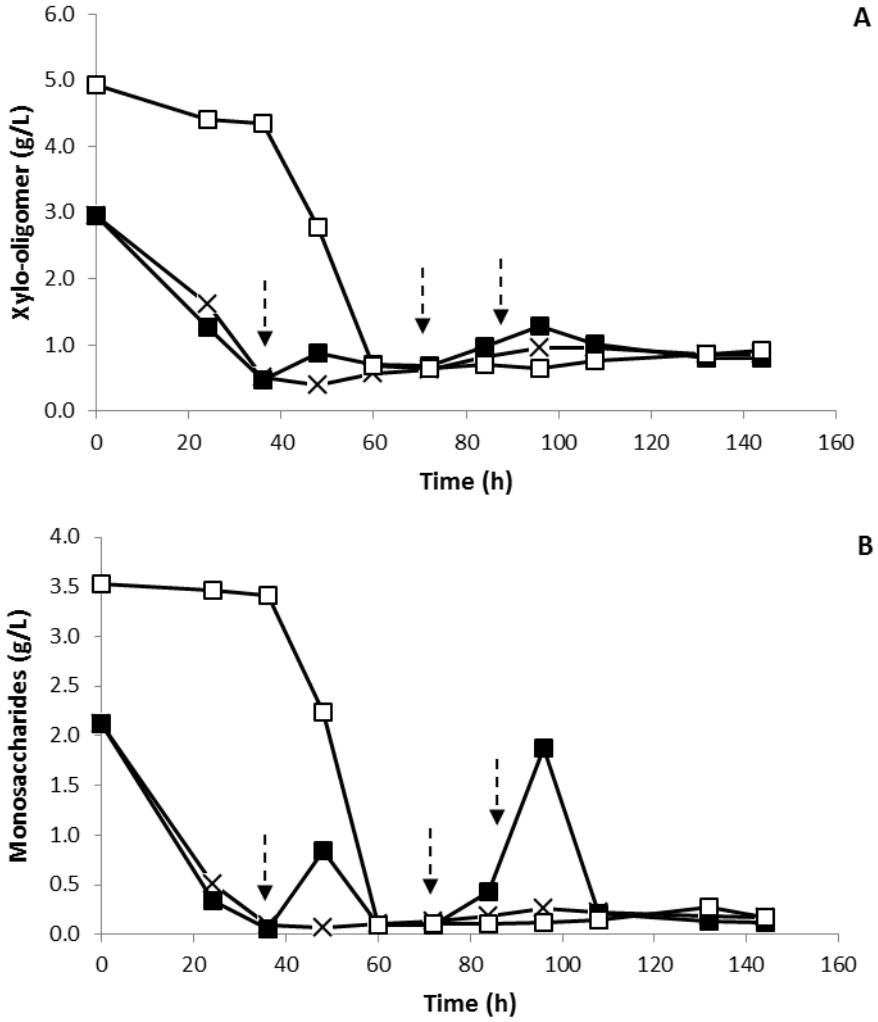

Figure 23 Evolution with time of xylo-oligomers (A) and monosaccharides (B) for fed-batch cultivation of *A. niger* DR02 on pentose-rich liquor from hydrothermal pretreatment of sugarcane bagasse, using exponential feed (X), constant feed (□), and pulsed feed (■) (arrows indicate the time of the pulse).

The experiments were started in batch mode with concentrated HL solution (S_{inlet} = 110.4 g/L, Table 16) and the feed to the system was initiated when the agitation rate began to decrease. This occurred at 36 h for the experiments employing an initial HL concentration of 30% (v/v) and 60 h for cultivation with an initial HL concentration of 50% (v/v). The decrease in agitation rate was associated with exhaustion of the available xylo-oligomers (Figure 23A) and free sugars (Figure 23B) in the culture media, and the feeding profiles used in the constant and exponential feeding experiments produced the desired effect, which was to maintain free carbohydrate concentrations at very low levels (below 0.1 g/L, Figure 23). The

pulsed fed-batch mode showed higher carbohydrate concentrations, as expected due to the nature of its operation (Figure 23).

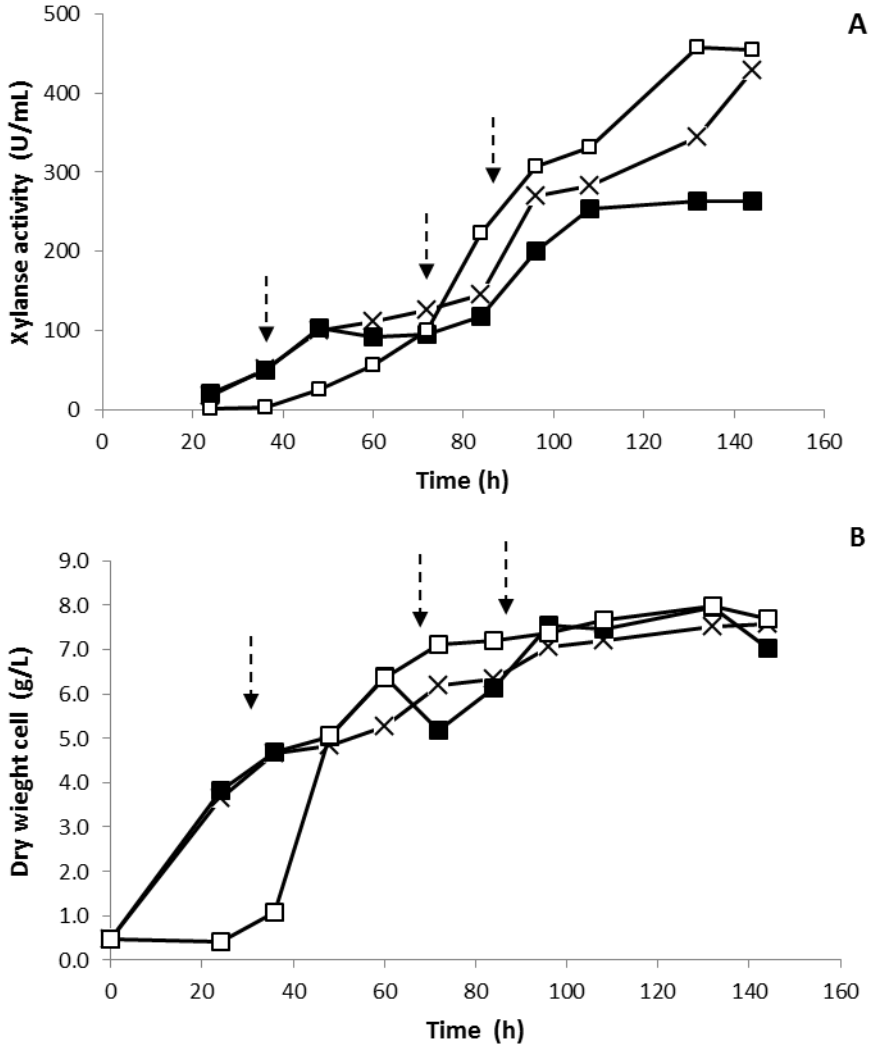

Figure 24 Evolution with time of xylanase activity (A) and dry weight cell (B) for fed-batch cultivation of *A. niger* DR02 on pentose-rich liquor from hydrothermal pretreatment of sugarcane bagasse, using exponential feed (X), constant feed (□), and pulsed feed (■) (arrows indicate the time of the pulse).

Although the same amount of carbon source was provided in all the experiments, the xylanase activity produced was highly influenced by the feeding profile (Figure 24A). Maximum xylanase activities were 458.1 U/mL for constant feeding, 428.1 U/mL for exponential feeding, and 264.37 U/mL for pulsed feeding. In the pulsed feeding mode, the concentrations of xylose and its derivatives (Figure 23) reached values at which the synthesis of xylanase could have experienced CreA repression (99). The xylanase activity was not

linked to the amount of biomass produced. In all cultivations, the biomass concentration reached around 8-7 g/L, but different enzymatic activities were obtained (Figure 24B), which reinforces the hypothesis of carbon catabolite repression. Moreover, acetic acid, HMF, and furfural were consumed by 66 h, and remained at undetectable levels up to the end of the experiments (data not shown). Therefore, the use of carbon-limited fed-batch cultivation may have in some way acted to alleviate the repression in *A. niger* DR02, overcoming possible negative effects of toxic compounds produced during bagasse pretreatment.

5.3.4 Enzymatic hydrolysis and characterization of the enzyme complex

Xylanase activity of the constant feeding fed-batch cultivation was measured at different temperatures and ranges of pH (Figure 25). Extracts had highest xylanase activity across a range of temperatures from 45 °C to 55 °C, but optimally at 50 °C. The same extract showed highest activity at pH 6.0 and maintained 80% of the relative activity between pH 5.0 - 6.5

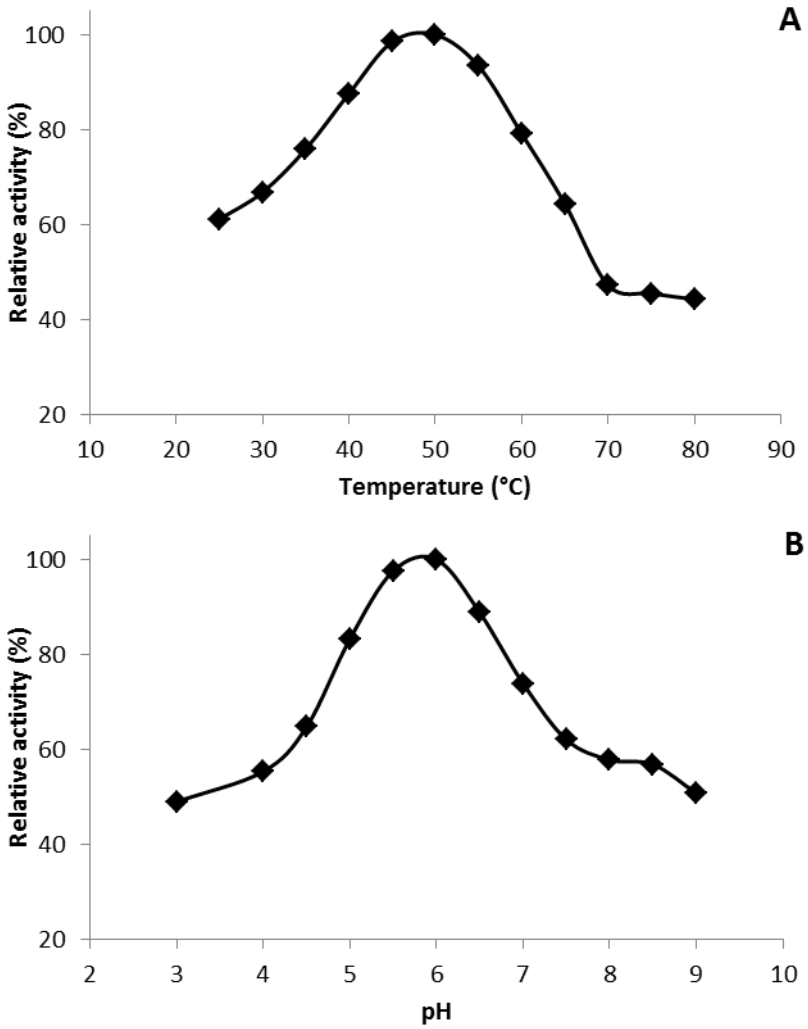

Figure 25 Xylanse residual activity expressed as a percentage of the maximum enzymatic activity produced by *Aspergillus niger* DR02 under different temperature (A) and pH (B).

The *A. niger* DR02 enzyme complex was evaluated for its effectiveness as a supplement to a commercial cellulolytic enzyme complex (Celluclast 1.5L) used for hydrolysis of the hydrothermally pretreated sugarcane bagasse (HB). The results are summarized in Figure 26. The hemicellulolytic extract showed a low protein concentration (0.55 g/L), even after membrane concentration using Amicon Ultra-15 centrifugal filter units with 10 kDa cut-off (Millipore) to increase the specific activities of the enzymes assayed.

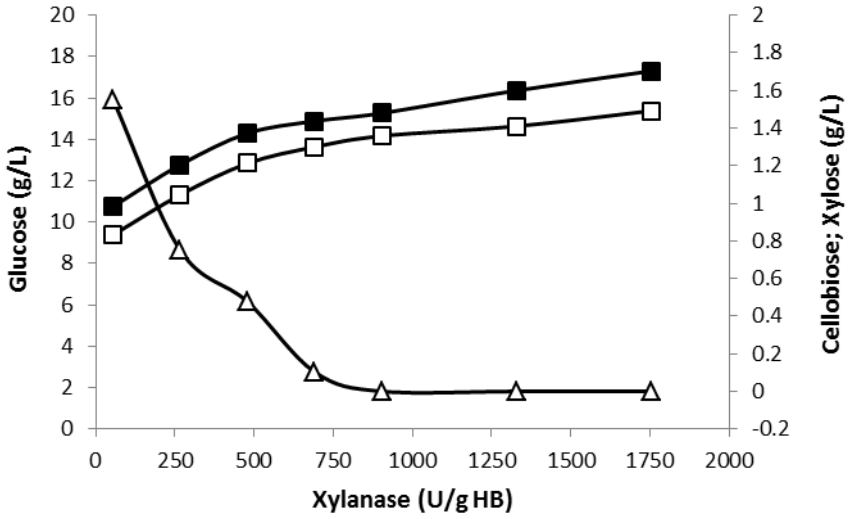

Figure 26 Influence of *A. niger* DR02 enzyme extract load (xylanase U/g of pretreated sugarcane bagasse) on total reducing sugar release, glucose (□), xylose (■) and cellobiose (△).

The dose-response curve (Figure 26) indicated that the extract had a positive effect on HB enzymatic hydrolysis, with a tendency towards saturation at enzyme loads above around 1000 IU of xylanase/g of HB, in terms of glucose and xylose production. Moreover, above this enzyme load, no cellobiose was measured in the system, clearly indicating an effect of β-glucosidase supplementation. Subsequently, enzymatic hydrolysis of HB was performed above the saturation point, with addition of the *A. niger* DR02 enzyme complex to Celluclast 1.5L. This supplementation resulted in the production of around 20 g/L of total carbohydrate (glucose, xylose, and arabinose) at 72 h of cultivation (Figure 27). The increased hydrolysis was reflected in the increased cellulose and hemicellulose hydrolysis, which shows that the extract acted synergistically. No cellobiose accumulation was observed during the supplemented enzymatic hydrolysis of HB (Figure 27), in agreement with previous findings concerning the β-glucosidase activity of *A. niger* DR02 (Chapter 3).

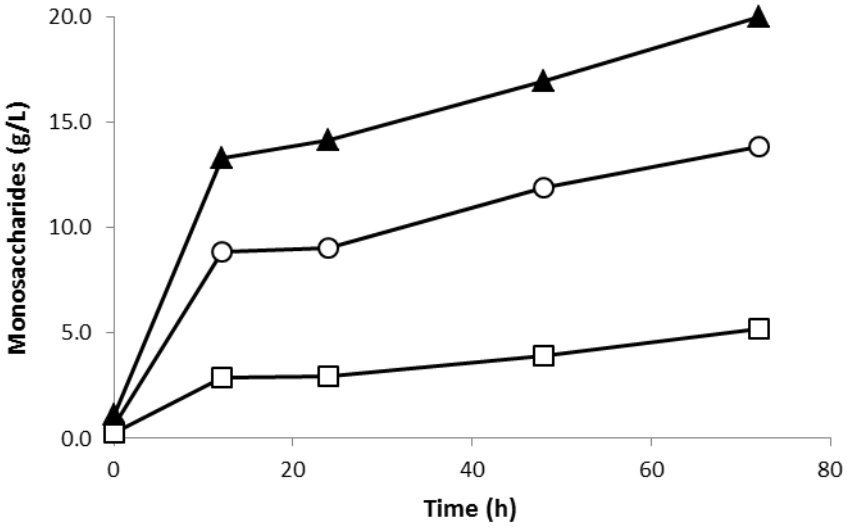

Figure 27 Monosaccharide concentration evolution during enzymatic hydrolysis of pretreated sugarcane bagasse with the *A. niger* DR02 enzyme extract (□), Celluclast 1.5L (○), and Celluclast 1.5L supplemented with the *A. niger* DR02 enzyme extract (▲).

In order to understand the performance of enzymatic hydrolysis due to supplementation with the *A. niger* DR02 cocktail obtained from growth of the organism using the HL constant fed-batch procedure, a panel analysis of activity against relevant substrates (Table 17) was performed, together with a proteomic analysis of the enzyme complex produced (Table 18).

The panel of specific enzyme activities demonstrated that the vast majority of the enzymes secreted by *A. niger* DR02 belonged to the xylanase class. This was not unexpected because it is well known that *A. niger* species are potential xylanolytic enzyme producers and that biosynthesis of these enzymes should be favored by the presence of xylo-oligomers in the HL fed to the bioreactor. However, a number of other important glycohydrolytic activities were present in the enzyme complex produced (Table 17), as a result of which a more accurate analysis of the fungal secretome was performed.

Table 17 Panel analysis of specific enzyme activities of some important glycohydrolases in *A. niger* DR02 extracts and Celluclast 1.5L

Specific activity (U/mg protein)	***Aspergillus niger* DR02 enzyme extract**	**Celluclast 1.5L**
FPAse	0.56	1.71
Cellobiohydrolase	1.36	0.33
β-glucosidase	4.55	1.20
Xylanase	1158.28	8.75
Pectinase	2.38	0.10
β-glucano	81.90	62.64
Xyloglucano	7.73	30.81
Arabinofuranosidase	0.45	0.01
β-xylosidase	1.94	0.08

A proteomic study of *A. niger* was employed to identify the enzymes secreted by the fungus grown using HL in constant fed mode, and to understand the effect of supplementation of Celluclast 1.5L with this extract. The LC-MS/MS spectra were analyzed with Mascot Ions Search software (Matrix Science, UK) for protein identification, using a database containing all non-redundant proteins derived from the NCBI fungi database (http://www.ncbi.nlm.nih.gov/blast). Scaffold v.3.6.1 software (Proteome Software Inc., Portland, OR) was used to validate the MS/MS peptide and protein identifications. This method enabled the unambiguous assignment of 730 peptides, of which 69 were unique peptides distributed by 32 protein hits. The false discovery ratio (FDR) calculated for peptide matches above the identity threshold was 0.63%, indicating a high level of confidence. This strategy enabled the identification of enzymes that degraded cellulose, hemicellulose, and starch, distributed amongst 24 different glycoside hydrolase families. Other enzymes such as esterases, lyases, and oxyreductases were also present in the enzymatic extract. No enzymes involved in lignin and pectin degradation were detected. The total number of peptides and the number of different unique peptides, as well as their classifications and peptide sequences, are detailed in Table 18. A complete table is provided in Appendix F - Table 32 (Appendix).

The previous enzymatic characterization of the extract produced in constant fed mode indicated strong activity of hemicellulolytic enzymes (Table 17). This was corroborated by

the proteomic analyses, which showed the presence of several enzymes related to xylan hydrolysis, such as xylosidase (GH3), endo-1,4-β-xylanase (GH10 and GH11), and α-L-arabinofuranosidase (GH54 and GH62). Furthermore, important hemicellulolytic enzymes whose activities had not been measured previously were detected by MS/MS, including feruloyl esterase (CE1). Although the cellulolytic activity of the extract was low (Table 17), compared to the hemicellulolytic activity, several enzymes related to cellulose degradation were present, such as endoglucanases (GH5 and GH12), cellobiohydrolases (GH6 and GH7), and β-glucosidase (GH3). The vast majority of the protein was identified as belonging to the endo-1,4-β-xylanase GH10 Cazy family (Table 18), in agreement with the panel specific activity that indicated that xylanase was the major measured enzyme activity (Table 17).

Table 18 Proteomic analysis of the supernatant from fed-batch bioreactor cultivation of *A. niger* DR02 on pentose-rich liquor from the hydrothermal pretreatment of sugarcane bagasse.

Protein accession numbers	**Cazy ID**	**Protein name**	**No. of unique peptides**	**No. of total peptides**
gi|145242946	GH3	β-glucosidase M [*A. niger* CBS 513.88]	6	12
gi|145230215	GH3	Exo-1,4-β-xylosidase xlnD [*A. niger* CBS 513.88]	12	58
gi|126046487	GH3	β-glucosidase [*A. niger*]	15	40
gi|145238644	GH5	Endo-β-1,4-glucanase B [*A. niger* CBS 513.88]	4	19
gi|145236118	GH5	Mannan endo-1,4-β-mannosidase F [*A. niger* CBS 513.88]	4	23
gi|145230537	GH5	Endo-β-1,4-glucanase A [*A. niger* CBS 513.88]	2	2
gi|134083538	GH5	Unnamed protein product [*A. niger*]	2	5
gi|134076801	GH6	Unnamed protein product [*A. niger*]	6	66
gi|145246118	GH6	1,4-β-D-glucan	2	46

		cellobiohydrolase [*A. niger* CBS 513.88]		
gi|156712284	GH7	1,4-β-cellobiosidase [*Thermoascus aurantiacus*]	2	3
gi|254212110	GH7	Cellobiohydrolase A [*A niger*]	5	58
gi|145230535	GH7	1,4-β-D-glucan cellobiohydrolase B [*A. niger* CBS 513.88]	8	34
gi|292495278	GH10	Endo-1,4-β-xylanase	22	1036
gi|13242071	GH11	Xylanase [*A. niger*]	4	138
gi|145250953	GH11	Endo-1,4-β-xylanase B precursor [*A. niger* CBS 513.88]	2	16
gi|145249126	GH12	Endoglucanase A [A. niger CBS 513.88]	7	135
gi|145243632	GH13	α-amylase, catalytic domain [*A. niger* CBS 513.88]	2	3
gi|145235763	GH15	Glucoamylase [*A. niger* CBS 513.88]	11	54
gi|145230419	GH16	Glycosidase crf1 [A. niger CBS 513.88]	2	6
gi|145233743	GH27	α-galactosidase B [*A. niger* CBS 513.88]	4	12
gi|134057627	GH30	Unnamed protein product [*A. niger*]	3	3
gi|134055627	GH31	Unnamed protein product [*A. niger*]	2	8
gi|134076816	GH43	Unnamed protein product [*A. niger*]	4	10
gi|145230794	GH47	Mannosyl-oligosaccharide α-1,2-mannosidase 1B [*A. niger* CBS 513.88]	2	2
gi|1168267	GH54	α-N-arabinofuranosidase B	2	4

5.4 **Discussion**

Xylanase production by *A. niger* has been extensively studied and is known to be highly variable depending on the microorganism strain, sources of carbon, nitrogen, and other macro- and microelements, and process conditions. The highest values found in the literature for *A. niger* were obtained using wheat bran as sole carbon source (126.9 U/mL; (217)) or combined with other wastes (996.3 U/mL; (218)). However, Brazilian agribusiness envisages the use of waste that has low value but high availability, such as sugarcane residues. In addition, the production of enzymes using substrates closely related to those that will be used for hydrolyses should favor the synthesis of enzymes that are well suited to the biomass deconstruction step (219). In earlier work concerning the production of xylanase from lignocellulosic materials, Irfan et al. (220) obtained 68.5 U/mL of xylanase activity using pretreated sugarcane bagasse. Low xylanase titers were also observed using wheat straw as carbon source for *A. ochraceus* (214) and corncob for *A. ochraceus* and *A. terricola* (221), indicating the need for strain screening and the development of enzyme activity for specific types of biomass. In recent work, strains were screened in order to produce suitable amounts of cellulolytic, hemicellulolytic, and accessory enzymes from endophytic microorganisms using specific substrates. This resulted in identification of the present *A. niger* DR02 strain (among 119 different filamentous fungi) as having a high capacity to grow on the pentose-rich liquor derived from hydrothermal treatment of sugarcane bagasse at 190 ^{o}C for 10 min (Chapter 3). This pentose-rich liquor contains very high levels of xylo-oligosaccharides and free xylose (10 and 5 g/L, respectively) (Table 15).

It is known that the synthesis of hemicellulolytic enzymes is controlled at the transcription level and that the carbohydrates in the medium play a role in glycohydrolase production. The expression of the genes related to endoxylanase and other hemicellulases is repressed in the presence of high glucose or xylose concentrations, due to the action of the CreA protein (99). In addition, the transcriptional activator XlnR directs the expression of these genes by inducing the biosynthesis of xylose (222). It is therefore reasonable to suppose that the levels of xylose compounds in HL were responsible for the high xylanase induction in the constant fed-batch culture and lower enzyme titers in the pulse-fed culture. The proteomic analyses supported this notion, because enzymes such as XlnD and AglB were detected (Table 18), which genes can be induced in the presence of xylose and arabinose at low concentrations via XlnR (223). Chemical analysis of HL composition also showed the

presence of strong inhibitors of microorganism growth such as acetic acid (1.4 g/L), formic acid (0.2 g/L), furfural (1 g/L), and soluble lignin (3 g/L). Despite the fact that most of these components were assimilated by *A. niger* during the batch experiments (data not shown), it was found that at the higher HL concentration (50%, v/v) there was a longer lag phase during batch cultivation, which was probably caused by the higher levels of the inhibitors. The acetic acid in the culture media could have been assimilated during *A. niger* growth and incorporated into metabolic pathways, hence avoiding its accumulation, as seen previously for oxalic acid in the case of *A. niger* (224). Nevertheless, it appears that *A. niger* was able to adapt to this demanding situation by producing greater numbers of cells and higher xylanase activity as the HL concentration was increased (Figure 22).

The fed-batch procedure is a common bioprocess technique employed to obtain larger quantities of product by overcoming substrate inhibition or oxygen limitation (or both) in submerged bioreactor cultivations. The data illustrated in Figure 22 were used to calculate the maximum specific growth rates (μ_{max}) from the plots of the natural log of the biomass concentration against time, giving values of 0.048 and 0.069 h^{-1} at HL concentrations of 30 and 50% (v/v), respectively. These values were used as a basis to design fed-batch process flow rate profiles, employing mass balance equations, where μ_{crit} was set at a value well above the μ_{max} value in order to limit the cultivation in terms of carbon source availability. This assumed that the carbohydrates in the culture media would become exhausted, so that the cells would be obliged to consume other carbon sources such as acetic acid, formic acid, furfural, and (to a lesser extent) soluble lignin. It was shown experimentally that all the carbohydrates (xylose, glucose, and xylo-oligomers), organic acids, and furfural delivered to the system were totally consumed. On the other hand, it was observed that soluble lignin accumulated during the course of the fermentation, resulting in a final concentration of around 2.5 g/L of this component in the culture broth.

The enzymatic extract rich on xylanase activity obtained from constant feeding fed-batch cultivation, presented similar physic-chemical characteristics with founding in the literature. Khonzue et al. (225) verified that the optimum conditions of xylanase activity of *A. niger* extract cultivated on SB, WB was from 50 °C to 60 °C and pH 5.5. Costa-Ferreira (226) also showed that the xylanase optimum conditions of a *A. niger* extract cultivated on xylan was pH 5.5 and 55 °C.

The enzymatic cocktail from *A. niger* has been used previously to provide β-glucosidase supplementation to *Trichoderma* cellulases. Fortes Gottschalk et al. (151) found

that the *Aspergillus* feruloyl esterase enzyme complex showed a synergic effect when combined with the *Trichoderma reesei* cellulase complex for the enzymatic hydrolysis of pretreated sugarcane bagasse. Even though *A. niger* secretome indicated a set of enzymes able to degraded the sugar cane bagasse, such as endoglucanase, cellobiohydrolases, β-glucosidase and xylanases, it lacks cellulolytic enzymes from other CAZy families and principally lacks a stronger cellulolytic activity concentration in *A. niger* extract. The present work employed a series of experiments to investigate the *A. niger* enzyme complex and assess its role in the supplementation of Celluclast 1.5L. Xylanase contributed most of the activity in an enzymatic complex obtained from *A. niger* cultivated using 50% (v/v) hydrothermally pretreated sugarcane bagasse liquor in fed-batch mode. This was confirmed using proteomic analyses, which also indicated the presence of arabinofuranosidase, β-xylosidase, cellobiohydrolase, β-glucosidase, and feruloyl esterase, which could have contributed to increase hydrolysis of sugar cane bagasse.

5.5 **Conclusions**

In summary, it was demonstrated that a fed-batch carbon-limited approach was able to achieve one of the highest xylanase concentrations reported in the literature. It was possible to use an inexpensive waste material (pentose-rich liquor derived from the hydrothermal treatment of sugarcane bagasse at 190 ^{o}C) containing high levels of xylo-oligomers as the main carbon source for production of a hemicellulolytic enzyme cocktail from a filamentous fungus. The extract produced presented several glycohydrolases important on plant biomass degradation and that the supplementation in Celluclast 1.5L increase HB hydrolysis.

CHAPTER 6 - GENETIC MODIFICATION OF *Aspergillus niger* STRAIN TO IMPROVE XYLANASE PRODUCTION

6.1 Introduction

The global interest in production of second generation bio-ethanol stimulates the investigation of low-cost cellulolytic and hemicellulolytic enzyme processes applied for biomass deconstruction. Several fungi such as *Aspergillus* spp. and *Trichoderma* spp., possess the potential to produce a wide range of plant polysaccharide degradation enzymes and their application already became a reality in the industry.

Fungal metabolism is greatly influenced by the composition of the medium and the production of glycohydrolases is related to the carbon source used (Chapter 3). In this way it is possible to induce and modulate glycohydrolase production by optimizing media design and cultivation methods. The filamentous fungus *Aspergillus niger* is widely used for enzyme production (191, 227). This species belong to the black Aspergilli, members of which possess good industrial characteristics like high protein secretion and metabolic capabilities (205).

Xylanase production has been studied for several years and abundant information on submerged and solid cultivation processes was obtained (206-208). In *A. niger* xylanase production depends highly on the cultivation mode, carbon source used and carbon source concentration (8, 9).

Bioprocess tools, such as cultivation optimization, are approaches to improve hemicellulase production in *A. niger*. However, this increase of productivity is limited by technical issues, such as high media viscosity, and by fungal physiology. In *A. niger* carbon catabolite repression (CCR) occurs in the presence of high (mM-level) concentrations of glucose or other monosaccharides, and is mediated by the catabolic repression protein CreA (228). The presence of monosaccharides in some feed stocks, can result in initial repression of enzyme production, until these sugars have been consumed by the fungus (229). In *A. niger* the synthesis of many hemicellulolytic enzymes is controlled by the transcriptional activator XlnR (230). de vries et al. (99) demonstrated that the expression of hemicellulolytic genes is balanced between induction by XlnR and repression by CreA. Both effects were caused by xylose, but while the induction through XlnR appears to be concentration independent, the level of repression mediated by CreA is directed related to the concentration of xylose.

Mono- and oligosaccharide rich feed stocks such as molasses and pentose-rich fractions derived from the sugar and alcohol industry are suitable candidates for fungal cultivations aiming the production of glycohydrolases. However, monosaccharides such as

xylose, glucose, arabinose and galactose play a role in CCR mediated by CreA and can inhibit hemicellulolytic enzyme production. Therefore strategies to obtain derepressed strains that produce higher hemicellulase/cellulase were performed for several fungi species. Disruption of *creA* in *Acremonium cellulolyticus*, *T. reesei*, *A. niger* and *Aspergillus nidulans* resulted in an improvement in enzyme production (102, 103, 229, 231).

The function of several regulators involved in the production of hemicellulolytic enzymes has been studied (99, 228, 232, 233). In this study, to improve production of plant biomass degrading enzymes, we aimed to construct an *A. niger* strain derepressed for hemicellulase synthesis and that includes a constitutively active version of XlnR to further enhance hemicellulase production.

The first idea was to use the *A. niger* DR02 strains due to its high capacity for xylanase production under HL. However, this is a wild type strain. Efforts to obtain an auxotrophic strain were done but unsuccessfully. We also used resistance to hygromycin as an alternative to the auxotroph. Although it was not possible to obtain transformants from *A. niger* DR02 strain. In this way, strains from CBS-KNAW Fungal Biodiversity Centre, which posse auxotroph markers, were used.

6.2 **Materials and methods**

6.2.1 Strains

The *A. niger* strains used in this study are listed in Table 19 and are all derived from *A. niger* N400 (CBS120.49). For spore production strains were grown on minimal medium agar with 1% inulin at 30 °C for 1 week. Spores were harvested using ACES buffer: 10 mM *N*-(2-acetamido)-2-amino-ethanesulfonic acid, Tween 80 (0.02%), pH 6.8.

Table 19 Strains used in this study.

Strain	Genotype	Reference
NW249	*cspA1*; Δ*argB*; *pyrA6*; *nicA1*; *leuA1*	Jalving et al. (234)
FP712	*cspA1*; Δ*argB; pyrA6; nicA1*; *leuA1; pyrG* : Δ*creA*	van den Brink et al. (229)
FP422.1 until FP422.14	*cspA1*; Δ*argB* :: pIM2102 (*argB*+); *pyrA6; nicA1*, *leuA1*, *pyrG* : Δ*creA*, pPGKXLN (*xlnR* - L668stop, Δaa 669-836)	This study

6.2.2 Agro industrial wastes

Hydrothermally pretreated sugarcane bagasse was prepared as described in chapter 3. Wheat bran (WB) and alfalfa meal were obtained according to van de Brink et al. (235)

6.2.3 Construction of active and constitutive xlnr mutants

A new expression vector pPGKXLN was built based on pANXABF (236) by the replacement of the arabinofuranosidase gene (*abf*) for the xylanolytic activator gene (*xlnR)* using *Nco*I/*Hind*III. This vector possesses a new strong *A. niger* promoter, p*pgkA* (Phosphoglycerate kinase, An08g02260) and the *A. nidulans trpC* terminator (237). The xylanolytic activator gene used, *xlnR* : (L668stop, Δaa 669-836), was obtained by amplification with GoTaq Long PCR Master Mix. The glucose inhibitory region was removed according to Hasper, Trindade (238) and a stop codon was added, resulting in an active protein even under glucose conditions. A co-transformation with pIM2102 (239) was done to *A. niger* NW249, and the mutants were selected for arginine prototrophy after transformation.

6.2.4 Molecular biology methods

Standard methods were used to DNA manipulation, cloning DNA, digestion and DNA isolation (240). *A. niger* transformation method was done as describe by de Bekker, Wiebenga (241).

6.2.5 Growth profile

Growth profiling on solid medium was performed on minimal medium (242) containing agar (1.5%) and plant biomass (3%) or soluble polysaccharides (1%) or sugars (25 mM). Spores were harvested with ACES solution and 1000 spores were added to the center of the petri dishes with the different carbon sources. The cultures were incubated in the dark at 30°C. The growth test was conducted in duplicate.

6.2.6 Southern blot

The *xlnR* copy number was determined by a quantitative Southern blot. Genomic DNA (10 µg) was digested with *Sal*I for 12 h at 37 °C. *Sal*I has two restriction sites in the p*pgkA* : *xlnR* (L668stop, Δaa 669-836) region and one in the genomic *xlnR* gene. After blotting, DNA was hybridized to a conserved region of both *xlnR* versions. The DNA gel blotting was conducted using standard methods (240). The DNA was blotted to nylon Hybond N+ membranes (GE Healthcare, Little Chalfont, UK) and subjected to hybridization and

detection by using Amersham ECL Direct Labeling and Detection System (GE Healthcare, Little Chalfont, UK) according to manufactory instructions. The copy number was than determined by a relative comparison of intensity of the bands by the software UN-SCAN-IT (Silk Scientific, EUA)

6.2.7 Liquid cultivation

Fungal strains were cultivated in 100 ml Erlenmeyer flasks containing 20 mL of medium to obtain the growth profile on glucose and xylose. The composition of culture medium was adapted from literature (127) using 20 g/L of carbon source, plus proteose peptone (1 g/L), Tween 80 (0.1%). The cultures were incubated at 30 °C, 200 rpm. The inoculum was done with 3 x 10^6 spores/mL of media. When necessary, the medium was supplemented with 0.2 g/L arginine, 0.2 g/L leucine, 0.2 g/L uridine and/or 1 mg/L nicotinamide. After incubation the mycelia was harvested, dried and frozen in liquid nitrogen at -70 °C for RNA isolation.

6.2.8 Hemicellulolytic genes expression

RNA was isolated by Trizol method and purified though NucleoSpin RNA Clean-up kit (Macherey-Nagel) with further DNase treatment. cDNA was obtained with ThermoScript RT-PCR (Invitrogen) and qPCR was performed according to Patyshakuliyeva et al. (243). qPCR reactions were performed using an ABI 7500 fast real-time PCR system with ABI Fast SYBR Master Mix (Applied Biosystems, Foster City, CA, USA). The *A. niger* genes studied were: xylanolytic activator (*xlnR*), endoxylanase B (*xynB*), β-xylosidase D (*xlnD*) and α-glucuronidase A (*aguA*). Histone gene (H2S) was used as reference gene. The sequences of all primers for qPCR analysis were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). Optimal primer concentrations and efficiency were also obtained (Table 20). Two biological and three technical replicates were analyzed.

Table 20 Primers used for RT-qPCR.

Primer description	Nucleotide sequence 5' → 3'	Amplicon size (bp)	Primer concentration (nM)	Primer efficiency	
				E value (%)	R^2 value
AguA forward	TTCGAGGAGAACGTCGTGATC	61	300	100.5	0.9958
AguA reverse	GCTCGCGCACTTGGAAGT		900		
XlnR forward	CCTCTTCCCTCGCCATCTC	59	300	105.1	0.9945
XlnR reverse	CTGGAAAACGGATGCAAGCT		300		
XnlD forward	CACCTACCAATGGCACCTGAA	66	300	99.4	0.9877
XnlD reverse	TGCTCAATATCATCGCGAGAGA		300		
XynB forward	GGTCCGTCCGCCAGAAC	60	900	99.4	0.9976
XynB reverse	CATTGAAGTGGTTGGAGGTGGTA		300		
H2B forward	AGACCTCTGTGAGGCTCATCCT	54	300	99.6	0.9934
H2B reverse	CCGACACCGCGTGCTT		900		

6.2.9 Hemicellulolytic enzyme activities

Total xylanase activity was measured by the amount of reducing sugars released from beechwood xylan using the DNS method (131) with xylose as standard. β-Glucosidase, β-xylosidase, β-mannosidase, α-L-arabinofuranosidase and cellobiohydrolase were measured using the respective *p*-nitrophenyl (pNP) substrates (Sigma-Aldrich) according to Zhang, Hong (244). The assays employed 10 µL of diluted centrifugation supernatant and 90 µL of the respective pNP substrate (0.5 mM) in 50 mM sodium citrate buffer pH 5.0 and were performed at 50°C. The reactions were stopped by adding 100 µL of 1 M Na_2CO_3. The absorbance was measured using a microtiter plate reader (FLUOstar Optima; BMGLabTech).

6.2.10 Protease activity

Protease activity was determined using the Pierce Fluorescent protease Assay kit (Thermo scientific). The total protease activity was expressed as function of the trypsin amount (mg trypsin/mL)

6.2.11 Biomass and sugar measurement

The dry weight of fungal biomass was obtained by filtrating 20 mL of the culture broth on Whatman paper filter grade 1, washing with deionized water, filtrated again, and drying at 105 °C to a constant weight

Supernatant samples were analyzed for sugar consumption by HPLC. Samples were filtered (Sep-Pak C18, Waters), and the carbohydrate concentrations were determined by a

system equipped with a Dionex CarboPac PA-10 (2-mm inner diameter [ID] by 250 mm) column in combination with a CarboPac PA guard column (1 mm [ID] by 25 mm) and a Dionex ED1 PAD detector (Dionex Co., Sunnyvale, CA). Standards of xylose and glucose were used to quantify these monosaccharides.

6.3 **Results**

6.3.1 Development of xlnR expression strains

The *A. niger* Δ*creA* strain (FP712) was used for the transformation of the constitutively active x*lnR*. Transformants were purified through single spore cultures and 13 of them were subjected to liquid cultivation. Transformants were analyzed for the level of xylanase and β-xylosidase activity after 48 h shake flash cultivation (100 mL) on beechwood xylan (1%) (Figure 28).

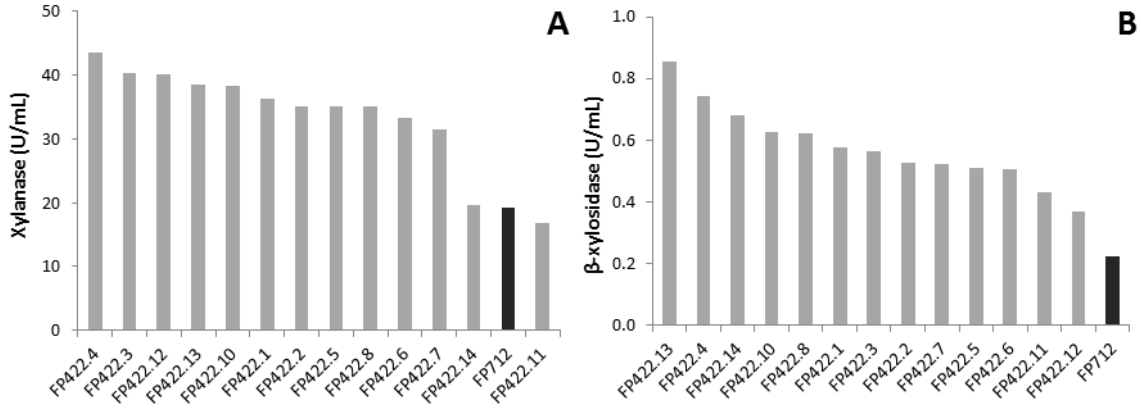

Figure 28 Xylanase (A) and β-xylosidase (B) activities (U/mL) of the cultures (48h, 30°C, 200 rpm) on xylan of the transformants in gray and *A. niger* FP712 (Δ*creA*) in black

All transformants had elevated levels of β-xylosidase compared to the parent strain, and FP422.13 strain almost quadrupled this activity. The same profile was obtained for xylanase, where 12 mutants presented higher xylanase activity than the Δ*creA* strain and strain FP422.4 double xylanase activity. For this reason two mutants (FP422.4; FP422.13) were selected for a more detailed study on beechwood xylan cultivation.

6.3.2 Enzyme activity of A. niger FP422.4 and FP422.13

Enzyme activity of the transformants was studied by sampling at different time points. Cultivations were carried out in triplicate in 250 mL flasks with 100 mL of Mandels media with beechwood xylan (1% w/v) at 30 °C, pH 5.0 and 200 rpm. Samples were taken at 48, 96

and 144h, and xylanase, β-xylosidase, arabinofuranosidase and β-glucosidase activities were measured.

Higher xylanase activities were detected at 48h, while for β-xylosidase, arabinofuranosidase and β-glucosidase activities gradually increased. For both transformants the enzymatic activities were higher than for the Δ*creA* strain (Figure 29).

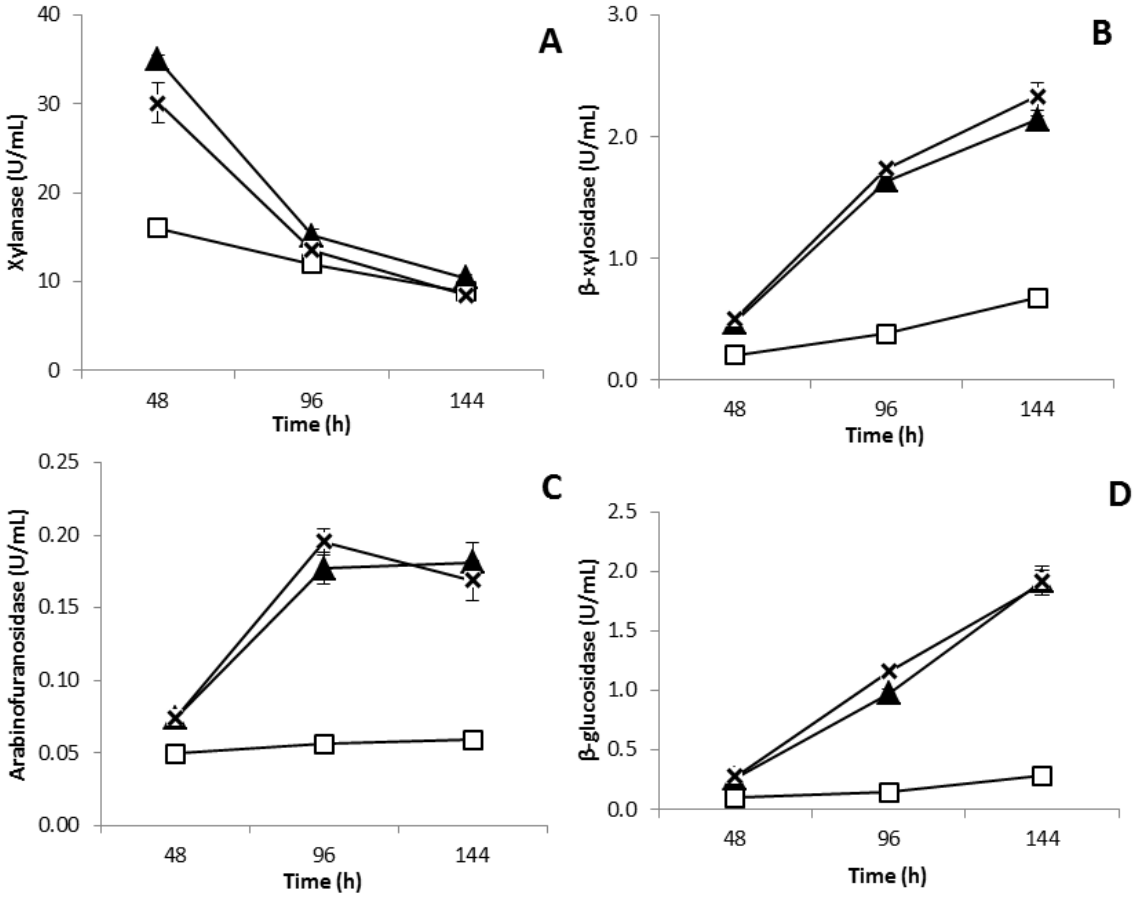

Figure 29 Xylanase (A), β-xylosidase (B), arabinofuranosidase (C) and β-glucosidase (D) activities of the cultivation of *A. niger* transformants, FP422.13 (X), FP422.4 (▲), FP712 (□) on beechwood xylan (1%)

6.3.3 Copy number determination

Southern blot results revealed that only in the strain FP422.13 it was possible to detect the presence of a 2796 bp band related to the cloning performed. Using intensities comparison the copy number of p*pgkA* : *xlnR* was determined as 1 in relation of the copy numbers of the wild *xlnR* (Figure 30).

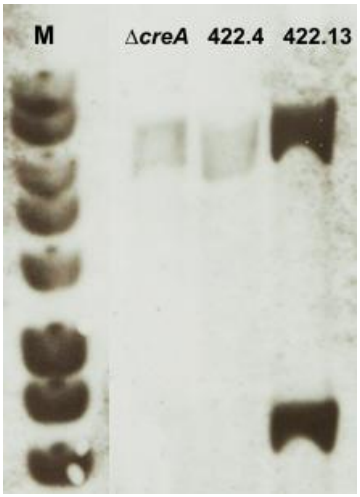

Figure 30 Southern blot gel to determine the copy number of the transformants. Ladder BenchTop 1 kb DNA Promega (M).

6.3.4 Comparison of gene expression in the parent and mutant strains

The expression of several (hemi-)cellulolytic genes was studied to assess the effect of the strain manipulations in more detail. The strains NW249, FP712 and FP422.13 were cultivated in 2% (w/v) glucose and xylose medium for 48 and 72 h. These time points were selected based on initial experiments, where maximum xylanase and β-xylosidase activity were detected at these time points (data not shown).

The results of dry weight (Figure 31A and Figure 31B) indicated that FP422.13 grew better than NW249 and FP712 on glucose and xylose. Sugar consumption measured by HPLC (Figure 31C and Figure 31D) revealed a higher monosaccharide consumption rate by FP422.13 and NW249 on both glucose and xylose.

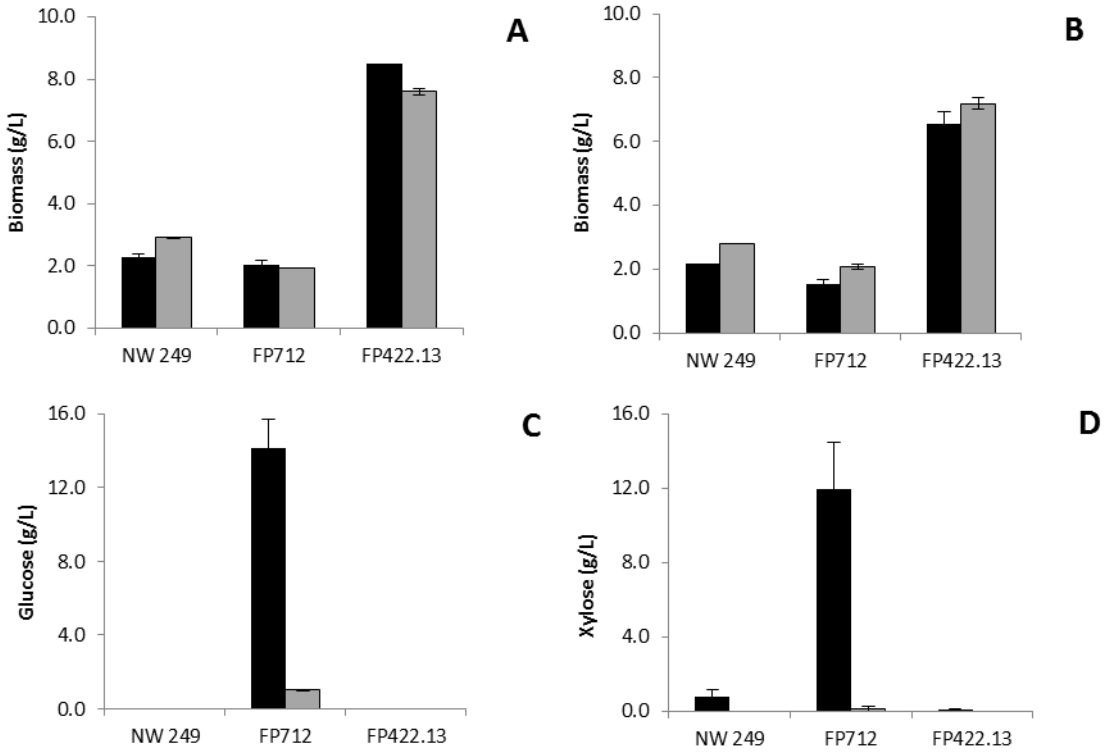

Figure 31 Culture parameters of *A. niger* strains cultivated on glucose 2% (A and C) and on xylose 2% (B and D) at 48h (black) and 72h (gray): Dry biomass (A and B) and sugar consumption (C and D).

Higher expression was detected for *xlnR* at 48h for FP422.13 and at 72h for the wild type and FP712 (Figure 32A and Figure 32B). In all the strains, expression levels of the genes encoding endoxylanase B (*xynB*), β-xylosidase D (*xlnD*) and α-glucuronidase A *guA*) were highly dependent on the presence of xylose (Figure 32). Surprisingly, the strain containing the constitutively active *xlnR* had lower expression levels of *xynB*, *xlnD* and *aguA* when compared to the Δ*creA* strain.

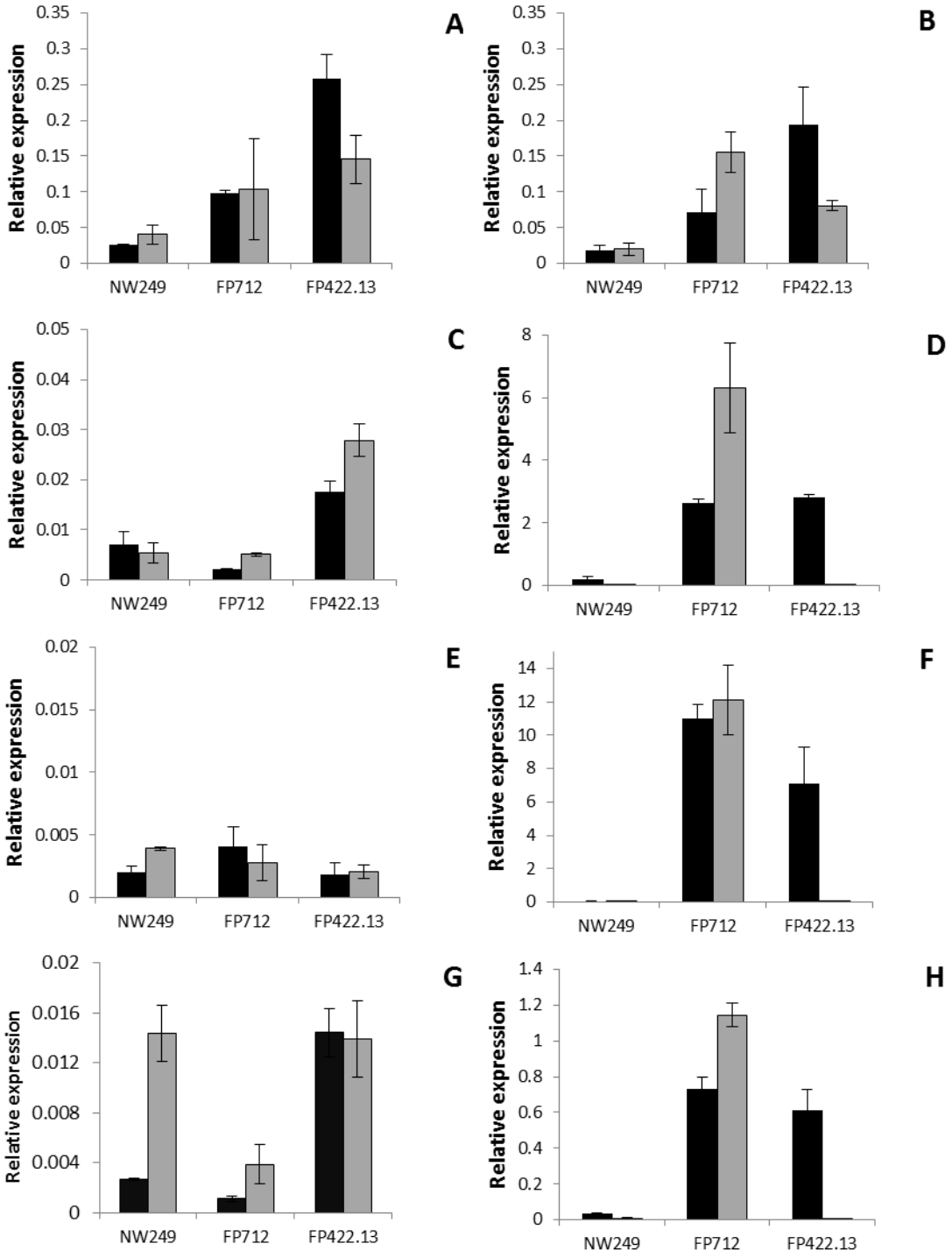

Figure 32 Gene expression in cultures of *A. niger* on glucose 2% (A, C, E and G) and xylose 2% (B, D, F and H) at 48h (black) and 72h (gray): *xlnR* (A and B), *xlnD* (C and D), *xynB* (E and F) and *aguA* (G and H).

Xylanase and β-xylosidase activities were measured (Figure 33) and correlated with the expression levels of the corresponding genes. The presence of xylose in the media is

essential for a good production of hemicellulases (Figure 34). Other glycohydrolase activities whose genes are controlled by XlnR and/or AraR were also measured, such as α-arabinofuranosidase, cellobiohydrolase, α-galactosidase and β-glucosidase (Figure 34). AraR is a transcriptional regulator homolog to XlnR which coordinated the synthesis of arabinolytic enzymes and also arabinose metabolism enzymes. It presents an overlap of functions with XlnR in regulation of some enzymes (232). The enzymatic activities (Figure 34) also follow the pattern of xylanase and β-xylosidase and revealed that the Δ*creA* strain produced overall more enzymes by gram of biomass.

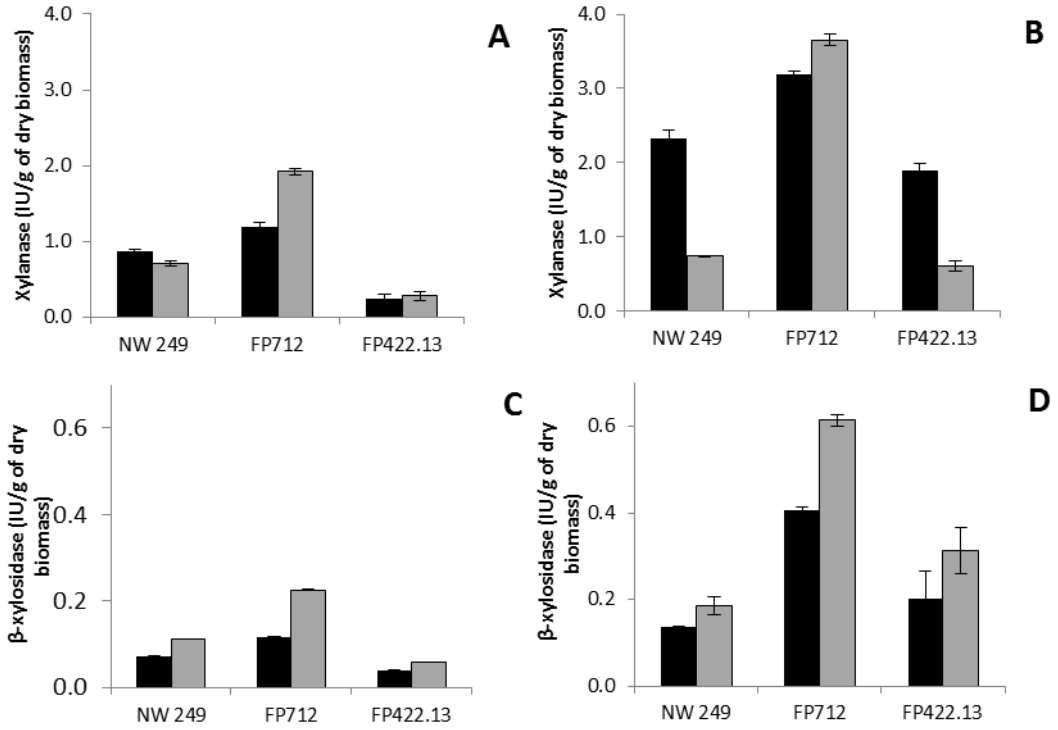

Figure 33 Enzymatic activity in cultures of *A. niger* on glucose 2% (A and C) and xylose 2% (B and D) at 48h (black) and 72h (gray): xylanase (A and B) and β-xylosidase (C and D).

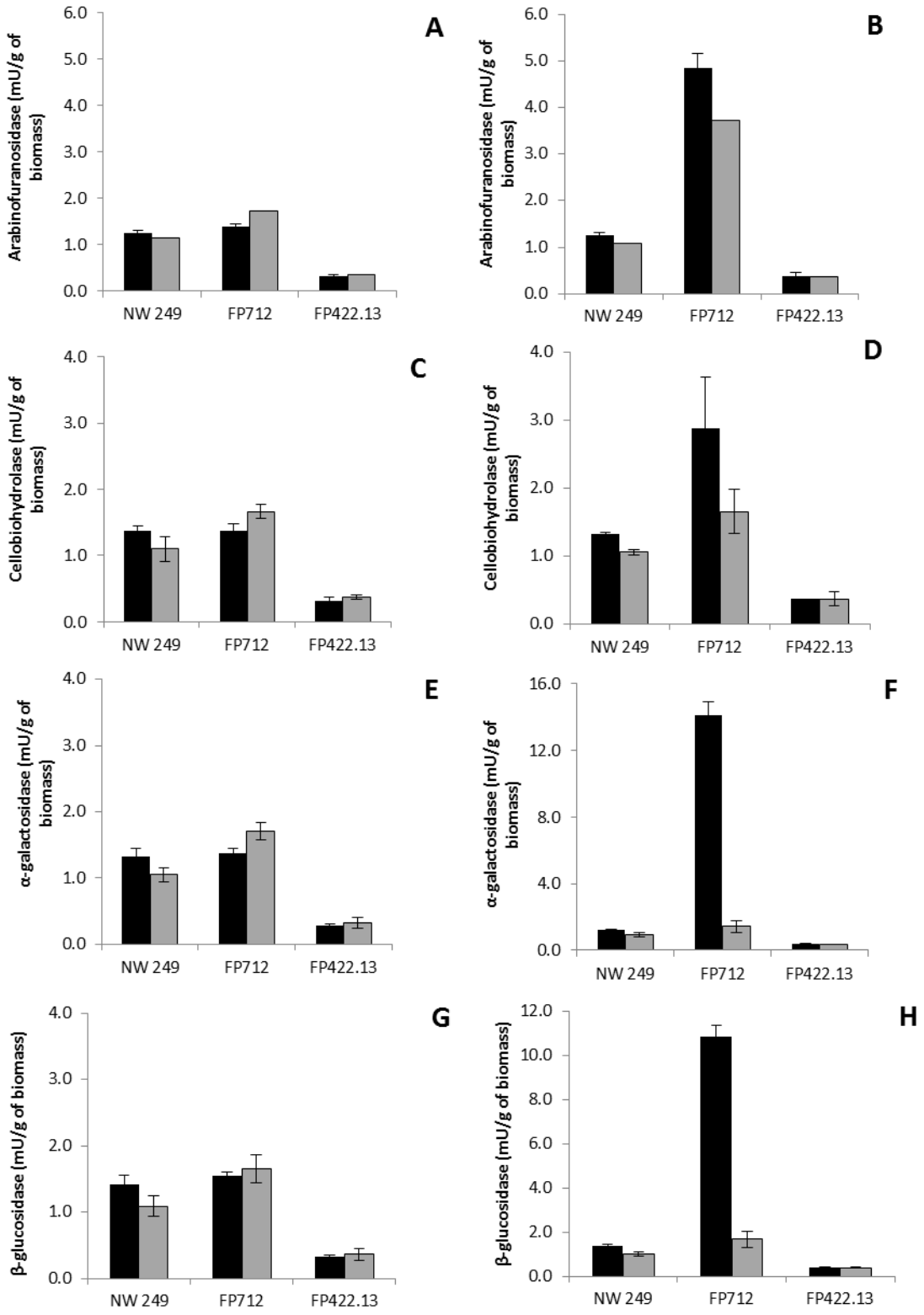

Figure 34 Enzymatic activity in cultures of *A. niger* on glucose 2% (A, C, E and G) and xylose 2% (B, D, F and H) at 48h (black) and 72h (gray): Arabinofuranosidase (A and B), cellobiohydrolase (C and D), α-galactosidase (E and F) and β-glucosidase (G and H).

The culture productivity was also calculated (Table 21) and expressed as U/L.h. In glucose medium no differences between the strains with respect to xylanase and β-xylosidase activity was detected. However, on xylose cultivations the deletion of *creA* resulted in a positive effect on the production of xylanase and β-xylosidase. The overexpression of the constitutively active *xlnR* had almost no effect on xylanase production, but almost doubled β-xylosidase production.

Table 21 Productivity (U/L.h) of the cultivations of *A. niger* on glucose 2% and xylose 2%: xylanase (48h) and β-xylosidase (72h).

Glucose

	NW249	FP712	FP422.13
Xylanase	41.08 ± 0.81	49.96 ± 1.33	43.64 ± 1.16
β-xylosidase	6.01 ± 0.01	6.02 ± 0.01	6.01 ± 0.01

Xylose

	NW249	FP712	FP422.13
Xylanase	104.82 ± 2.39	299.75 ± 1.09	281.25 ± 3.05
β-xylosidase	7.20 ± 0.29	17.57 ± 0.19	31.21 ± 0.47

To ensure that the observed effects are not caused by proteolytic degradation of the glycohydrolases, protease activity was measured by Pierce® Fluorescent protease Assay kit (Thermo scientific). However, no protease activity was detected in the samples.

6.3.5 Growth profile

To study the physiological effect of the constitutive and active version of *xlnR* on Δ*creA* strain, a growth experiment was performed using NW249, FP712 and FP422.13 on xylose, glucose, xylans and plant biomass residues (Wheat bran, alfalfa meal, sugar cane bagasse and hydrothermal sugar cane bagasse) (Figure 35).

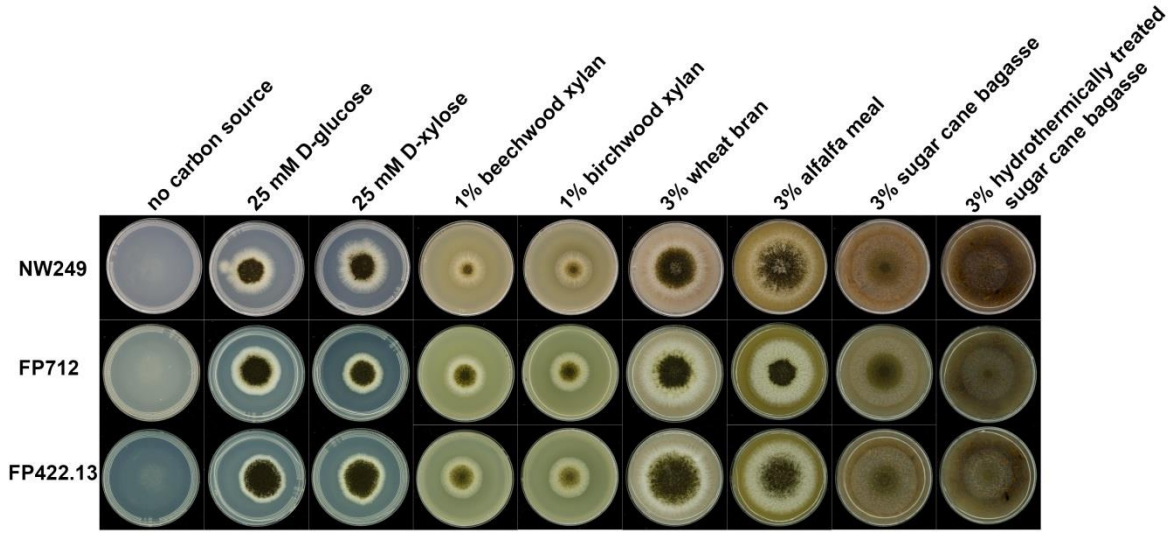

Figure 35 Growth profiles of NW249 (reference strain), FP712 (Δ*creA*) and FP422.13 (Δ*creA*, constitutive and active *xlnR* strain) on a variety of carbon sources. Carbon source concentrations were 25 mM for glucose and xylose, 1% for polysaccharides (beechwood xylan and birchwood xylan) and 3% for plant biomass.

Reduced growth for all strains was observed for sugar cane bagasse and for hydrothermally pretreated sugar cane bagasse. No clearly improved growth for all strains could be seen on beechwood xylan and birchwood xylan. However, on glucose xylose, wheat bran and alfalfa meal, an increase in growth and sporulation was visible in the overexpressing *xlnR* strain (FP422.13), when compared with Δ*creA* strain and parental type.

6.4 **Discussion**

Efforts to understand the physiology, regulation and mechanism of glycohydrolase production in *A. niger* have been performed for several decades. Degraff et al. (228) showed that glucose and xylose in high concentration are able to inhibited several hemicellulolytic enzymes by the wide domain regulatory protein, CreA. Recent studies produced CreA-derepressed strains of several species and increased glycohydrolase production such as for *A. niger*, *A. nidulans* and *A. cellulolyticus* (102, 229, 231). Similar to those, deletion of *cre1*, a homolog of *creA* in *Trichoderma reesei*, was also performed.

A. niger is an important microorganism in bioprocess industry such as in enzyme production, which led for strategies along years to increase enzyme productivity by classical mutation and heterologous protein expression. In *A. niger*, xylanase production is mainly

associated with the amount of xylose presented in the media (99). The regulation of hemicellulolytic and cellulolytic genes is performed at transcriptional level by the xylanolytic activator XlnR (230, 245, 246). Also, xylose plays a role in the XlnR phosphorylation and increase affinity to target DNA (247). High xylose concentrations not only triggers CCR (228), also it is unsuitable as a substrate for enzyme production due to its high costs.

In our study we developed an *A. niger* strain capable to express constitutively an active version of *xlnR* combined with a deficiency in CCR by deletion of the *creA* gene. The overexpression of *xlnR* led to lower enzyme activity (Figure 33) when compared to the Δ*creA* strain. This fact could be explained by the higher xylose consumption by the FP422.13 strain (Figure 31B and Figure 31D). In *A. niger*, D-xylose metabolism occurs by pentose catabolic pathway (PCP). Genes of PCP (*xdhA* and *xkiA*) and from the pentose phosphate pathway (*rpiA* and *talB*) are coregulated by XlnR and AraR (248). In this way, an overexpression of *xlnR* could lead to higher xylose consumption rate and consequently lower enzyme synthesis by an unknown mechanism.

The transcription of *xynB*, *xlnD* and *aguA* are controlled by XlnR (205, 230). Hasper et al. (249) verified that in *A. niger* multiple copies of *xlnR* resulted in slightly increased transcriptional levels of *xyrA*, *xlnD* and *xlnB* after growth in xylose (2%). Hence, higher transcriptional levels of these genes and higher enzymatic activities were expected in FP422.13 cultivations as a consequence of higher *xlnR* expression levels (Figure 32A and Figure 32B). However, it appears that in our experiments overexpression of *xlnR* diverted the xylose and glucose metabolism to biomass formation and not enzyme synthesis (Figure 31). Mach-Aigner et al. (250) demonstrated that transcriptional levels of *xlnR* do not reflect on xylose induction of hemicellulases genes. In this way, the higher xylose consumption by FP422.13 may have minimized the xylose effect on XlnR phosphorylation and consequently lower enzyme synthesis (238, 247).

Our data showed a dominant effect on hemicellulases production of CCR over transcriptional activation. The Δ*creA* strain expressed higher levels of *xynB*, *xlnD* and *aguA* than FP422.13 (Figure 32) and also higher levels of specific enzymatic activities (Figure 33 and Figure 34). This results corroborated with Mach-Aigner (250) findings, which showed that the enhancement of *xlnD* and *aguA* transcript formation was due to *creA* deletion. Moreover, Delmas et al. (251) proposed an induction model in *A. niger* based on sequential expression of responsive genes for lignocellulose degradation. According to the study when CreA repression is alleviated there is expression of a subset of starvation genes, such as *abfB*

and *cbhB*, which confirms the higher specific arabinofuranosidase and cellobiohydrolase activities found for Δ*creA* strain (Figure 5).

In our study, no significant difference was detected between volumetric extracellular enzyme activities (xylanase, arabinofuranosidase and β-glucosidase) of *A. niger* NW249 and FP712 strain when cultivated on pure monosaccharides (data not shown). However, when beechwood xylan was used as carbon source, a clear difference could be visualized between FP712 and FP422.13 strains (Figure 28). In agreement with this results is the study of Bouzid et al. (252), which showed that a *xlnR* multicopy strain of *Aspergillus vadensis* when cultivated on beechwood xylan also increased xylanase volumetric activity. The growth profile (Figure 35) showed similar growth for all strains on xylan, although an increase in growth and sporulation was visible on glucose and xylose. The growth on xylan is slower than on xylose (Figure 35), which support the hypothesis that fungal growth rate and xylose concentration can impact directly on hemicellulases synthesis (251).

The slower growth of the FP712 strain was expected (Figure 31 A and Figure 31B) as it has been reported in literature that strains with nonfunctional *creA* grow more slowly than the wild type (224). This likely happens because this repressor controls many genes, such as those involved in assimilation of polysaccharides and monosaccharides (99, 248, 253), and some genes from central metabolism like acetyl-CoA synthase (*facA*), isocitrate dehydrogenase, isocitrate lyase (*acuD*) e malate synthase (*acuE*) (254). It has been suggested that the main function of CreA is to ensure that only needed genes are expressed. In the Δ*creA* this level of control is removed, resulting in expression of many non-required genes which is a waste of energy and therefore reduced the energy available for growth.

In this study, a dominant effect of *creA* deletion on *xlnR* overexpression in the production of biomass degrading enzymes was observed. In nature, fungi developed regulatory mechanism for efficiently use energy according to the carbon source present in the environment. This efficient regulatory mechanism consists of a network of specific and general regulators (101). This network appears to exist a set of inducer-specific transcriptional activators for induction of the genes, while CreA is the main factor responsible for turning off those genes that are not required.

The depletion of CCR by deletion of *creA* was used to deregulate the system and allowed glycohydrolase synthesis in the presence of monosaccharides. However, genetic modifications on metabolic regulations can prompt the cell to find ways to overcome this new situation. The constitutive copies of *xlnR* led to high xylose consumption during growth on

this monosaccharide. Another explanation could be that the amount of XlnR produced by ΔCreA strain is sufficient to activate the target genes and its overexpression had not much effect on the production of XlnR-regulated activities. Similar results were found by Bouzid et al. (252) with *Aspergillus vadensis*, the insertion of additional copies of *faeA* and *xlnD* presented higher effect on the enzymatic activity than additional copies of *xlnR*.

6.5 Conclusion

Even though the overexpression of *xlnR* did not lead to high hemicellulases production on xylose it was possible to increase enzyme production on xylan. The data indicated that a mechanism not totally elucidated is present and involves fungal growth and inductor. Future approaches such as controlling fungal growth on xylose-rich medium by nutrient limitation on fed batch cultures and study the carbon effect of xylan could support and allow improving *A. niger* to hemicellulose production.

CHAPTER 7 - ENZYMATIC COCKTAIL FORMULATION

7.1 Introduction

Hydrolysis of plant biomass components presents an opportunity and a challenge in biofuels area. These feedstocks are composed by several polysaccharides and organic substances which can be converted by microorganisms into industrially relevant products. The use of several enzymes to loose and break down the recalcitrant plant cell wall are therefore of high importance in the endeavor (255).

The enzymes cost is nowadays the main issue in enzymatic hydrolysis (6), so enzymes with better performance are in demand. Formulation of blends using enzymatic extracts of different microorganisms and recombinant proteins have been extensively studied (3, 106, 151, 200). According to each feedstock used it seems necessary to develop a specific enzymatic preparation, to take into account the variations of cell wall composition between crops.

However, some enzymes such as β-glucosidases are indispensable. This enzyme cleaves cellobiose into glucose and minimizes the inhibitory effect of cellobiose in cellulases (256). With increasing glucose concentration another inhibition occurs in β-glucosidase (200). Ratios between cellulase and β-glucosidase depend mainly on the β-glucosidase type, cellulolytic preparation and biomass. In this way ideal ratios must be defined and optimized for each hemi/ cellulolytic cocktail development.

Plant biomass such as sugar cane bagasse present high proportion of xylan in its composition (122). According to de Souza et al. (35) sugar cane cell wall are mainly composed of unbranched xylan backbone arabinosylated (Ara:Xyl ratios of 1:5). Due to that, the use of xylanase showed synergist effect when supplemented on cellulolytic enzymes preparations (3, 151). The use of hemicellulolytic enzymes depends on the hemicellulose composition (200). Xylanases from *A. niger* have been used to supplement cellulolytic preparations such as celluclast 1.5 and showed positive effect in HB hydrolysis yield (Chapter 5).

On one hand, glycohydrolases play a role on biomass degradation such as feruoyl esterase, which has a described action on the lignocelulosic material (151). On the other hand, the supplementation of unknown acting glycohydrolases such as pectinase, can increase hydrolysis yield. Pectin is absent in pretreated sugar cane bagasse but is present in sugar cane

cell wall. Supplementation of pectinase preparation increases delignificated/exploded sugar cane bagasse (105).

It has been shown in chapter 3 and 4 that a novel fungal species, *A stygium*, produced on industrial agro wastes several glychohydrolases able to improve sugar cane bagasse hydrolysis (Chapter 4). The novelty of extracellular enzymes produced by this fungus makes it a promising candidate for further studies on HB enzymatic hydrolysis. In this chapter, the application of enzymatic extracts produced by *A. niger* and *A. stygium* aiming the development of a more efficient enzymatic mixtures for sugar cane bagasse saccharification, was studied.

7.2 **Materials and methods**

7.2.1 Agro-industrial waste materials

Hydrothermal pre-treated sugar cane bagasse (HB) was obtained and characterized according to chapter 3.

7.2.2 Enzymatic extracts

Three enzymatic extracts used on this chapter were produced previously. One extract, rich in xylanase (XYL), was produced by *A. niger* DR02 under a constant fed batch cultivation using liquor from the hydrothermal pre-treated sugar cane bagasse. The other two were produced by *A. stygium* DR47 using citrus bagasse and soybean bran at pH 4.0 and pH 5.0, and possessed high pectinase (PEC) and β-glucosidase (BGL) activity respectively. A commercially available enzyme preparation, Celluclast 1.5L (Novozymes), was used as a standard cellulolytic cocktail and was supplement with the enzyme extracts produced.

7.2.3 Mini scale sugar cane bagasse hydrolysis

The enzymatic hydrolysis was performed with 5 % (w/v) of HB and sodium azide 0.02% (v/v) in 50 mM citrate buffer, pH 5.0. The reactions were carried out in 2 mL Eppendorf tubes using a Thermomixer microplate incubator (Eppendorf, Germany) operated at an agitation speed of 1000 rpm for 24 h.

7.2.4 Experimental design

To select the best concentration of each enzymatic extract and their effect when supplemented in Celluclast 1.5 L, a central composite design (CCD) with six replicates in the central point was performed with data from saturation curve experiments (Chapter 4 and 5).

The data analysis and the cocktail optimization were performed with software Statistica 10.0 (Statsoft, Inc., Tulsa, OK, USA). Hydrolysis were conducted using a constant concentration of Celluclast 1.5L (10 FPU/g of HB) and the maximum enzyme extracts used corresponded to the saturation point determined in Chapter 4 and 5. The complete factorial experimental design was performed with 3 factors, 2 levels, 2 axial points and 6 replicates of the central point, totalizing 20 experiments. The range of values considered, varying XYL, BGL and PEC, are presented on Table 22, and the results were fitted to the quadratic model. The data were not transformed for the analysis.

Table 22 Coded factor levels and real values considered for each variable in the study.

Variables (U/g of HB)	Level -1.68	Level -1	Central point	Level +1	Level +1.68
XYL	0.0	202.7	500.0	797.3	1000.0
BGL	0.0	2.0	5.0	8.0	10.0
PEC	0.0	16.2	40.0	63.8	80.0

7.2.5 Inhibition hydrolysis

To access the behavior of the extracts on sugar cane bagasse hydrolysis, different concentrations of glucose ranging from 0 to 72 g/L were added to 2 mL hydrolysis solution. The blends of enzymes developed in the statistical design analysis were used to assess its performance.

7.2.6 Hydrolysis kinetics

Hydrolysis in 50mL shake flasks (20mL volume) with 10% of HB during 72h were carried out. One set of experiments was conducted using the mixture developed in statistical design analysis.

7.2.7 Sugar measurement

Samples were centrifuged at 10,000 x *g* for 15 min (5418 Centrifuge, Eppendorf), filtrated (Sepak C18, Waters) and carbohydrate concentrations were determined by HPLC as described by Rocha et al. (122).

7.3 **Results**

7.3.1 Sugar cane bagasse characterization

Hydrothermal pretreatment of the sugar cane bagasse resulted in the following composition (%): cellulose (56.03 ± 0.26), hemicellulose (4.59 ± 0.40), lignin (36.36 ± 0.34) and ashes (4.24 ± 0.14).

7.3.2 Central composite design (CCD)

Enzymatic formulation and optimization are required to achieve better hydrolysis results. In the present study, a hemicellulolytic preparation was developed using CCD.

Table 23 Sugar release in central composite design experiments for the enzymatic hydrolysis of pretreated sugarcane bagasse (HB 5%, 50 ºC, pH 5.0).

Run number	Glucose (g/L)	Xylose (g/L)	Cellobiose (g/L)	Arabinose (g/L)	PEC (U/g of HB)	BGL (U/g of HB)	XYL (U/g of HB)
1	11.56	1.11	0.31	0.00	16.2	2.0	202.7
2	13.08	1.32	0.26	0.00	16.2	2.0	797.3
3	13.35	1.26	0.34	0.00	16.2	8.0	202.7
4	**15.20**	**1.48**	**0.34**	**0.00**	**16.2**	**8.0**	**797.3**
5	10.86	1.33	0.32	0.00	63.8	2.0	202.7
6	14.12	1.66	0.34	0.00	63.8	2.0	797.3
7	11.70	1.39	0.35	0.00	63.8	8.0	202.7
8	14.50	1.73	0.36	0.00	63.8	8.0	797.3
9	**9.52**	**0.97**	**0.28**	**0.00**	**40.0**	**5.0**	**0.0**
10	14.16	1.58	0.31	0.00	40.0	5.0	1000.0
11	12.19	1.40	0.27	0.00	40.0	0.0	500.0
12	13.32	1.44	0.31	0.00	40.0	10.0	500.0
13	12.90	1.14	0.28	0.00	0.0	5.0	500.0
14	12.16	1.63	0.35	0.00	80.0	5.0	500.0
15	13.53	1.47	0.33	0.00	40.0	5.0	500.0
16	12.77	1.40	0.29	0.00	40.0	5.0	500.0
17	13.09	1.49	0.34	0.00	40.0	5.0	500.0
18	12.62	1.42	0.29	0.00	40.0	5.0	500.0
19	13.25	1.46	0.30	0.00	40.0	5.0	500.0
20	13.64	1.45	0.33	0.00	40.0	5.0	500.0

Table 23 summarizes the different combinations of xylanase, β-glucosidase and pectinase concentrations used to hydrolyzed HB and the maximum sugar concentration obtained. Maximum glucose release obtained in these experiments ranged from 9.52 (run 9) to

15.27 g/L (run 4). Xylose and cellobiose presented minor variations between the runs and no arabionose was detected.

The influence of hemicellulolytic preparation HB hydrolysis was estimated by examining the statistical significance of each component and their interactions as shown in the Pareto chart (Figure 36). The largest effect is linear from xylanase, followed by the linear effect of β-glucosidase, both affecting positively the glucose yield. The only significant quadratic term was xylanase but with negative effect. Pectinase concentration presented no statistically significant influence on hydrolysis (p>0.05).

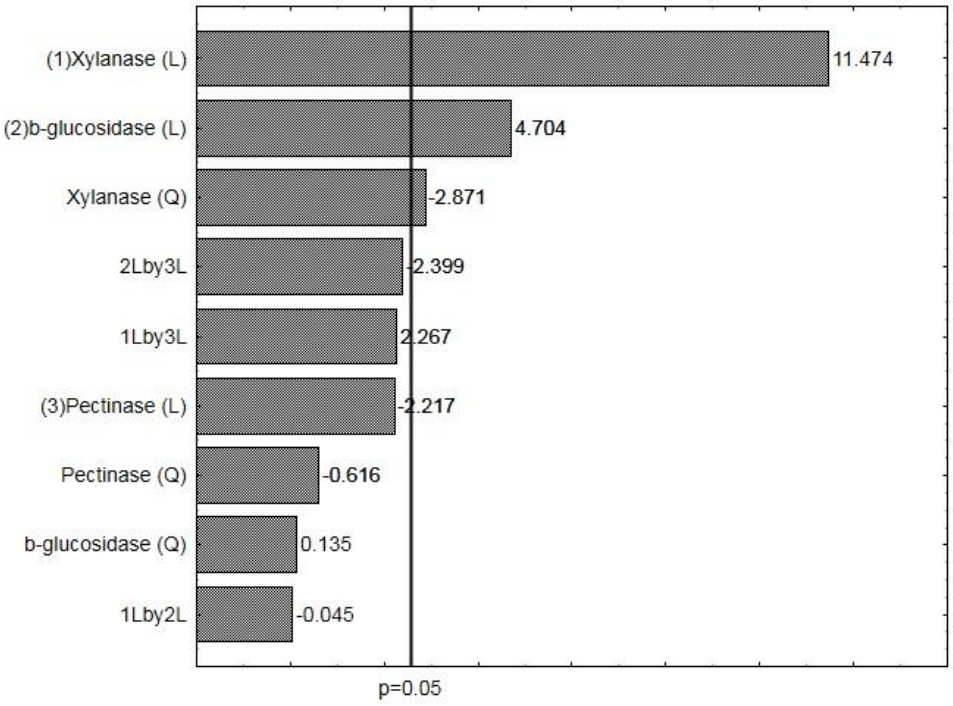

Figure 36 Pareto chart of standardized effects (p=0.05) of glucose released (g/L) after pretreated sugarcane bagasse (HB) hydrolysis

Table 22 presents the analysis of variance (ANOVA) for HB hydrolysis models. In this analyze only significant coefficients were taken into account.

Table 24 ANOVA for the hydrolysis models describing glucose release.

Source of variation	Sum of squares (SS)	Degrees of freedom (DF)	Mean square (MS)	*F* value
Regression (R)	27.0	3	8.996	24.92 *
Residual (r)	5.8	16	0.361	
Lack of fit (Lf)	4.9	11	0.449	2.69 **
Pure error (Pe)	0.833	5	0.167	
Total (T)	32.8	19		
R^2	0.824			
F listed values (95% of confidence)			*$F_{3,16}$ (95%)	3.24
			**$F_{11,5}$ (95%)	4.70

**F* test for statistical significance of the regression=MSR/MSr. **F test for lack of fit=MSLf/MSPe

A quadratic model was proposed for glucose release as a function of xylanse (XYL) and β-glucosidase (BGL) concentration (Equation 6). The quadratic model proposed fitted with experimental data. *F*-value for regression (24.92) was found to be 7-fold higher than the listed value at a 95% level of confidence. This value was considered very satisfactory for predicting the model used to describe HB hydrolysis based on glucose release:

$$Glucose\ (g/L) = -0.304XYL^2 + 1.267XYL + 0.520BGL + 13.087 \quad \text{(Equation 6)}$$

Therefore, the proposed model was used to plot response surface and for enzymatic extract optimization. Figure 37 shows the surface response of glucose release of HB hydrolysis by XYL and BGL extracts. To maximized glucose released for xylanase concentration, the partial derivate was calculated and a xylanase concentration level of 2.08 was determined. However, xylanse concentration was out of the experimental values range (-1.68 to +1.68). As previously shown on chapter 5, the point +1.68 represented the saturation point of HB hydrolysis (5%) with Cellulclast 1.5L (10FPU/g of HB). For this reason it was used the +1.68 xylanase level (1000 U of xylanse/ g of HB) for further experiments. Statistical analyzes showed that β-glucosidase only had a linear term on equation 6, thus the maximum value for this term was the extreme positive value, +1.68 (10 U of β-glucosidase/ g of HB).

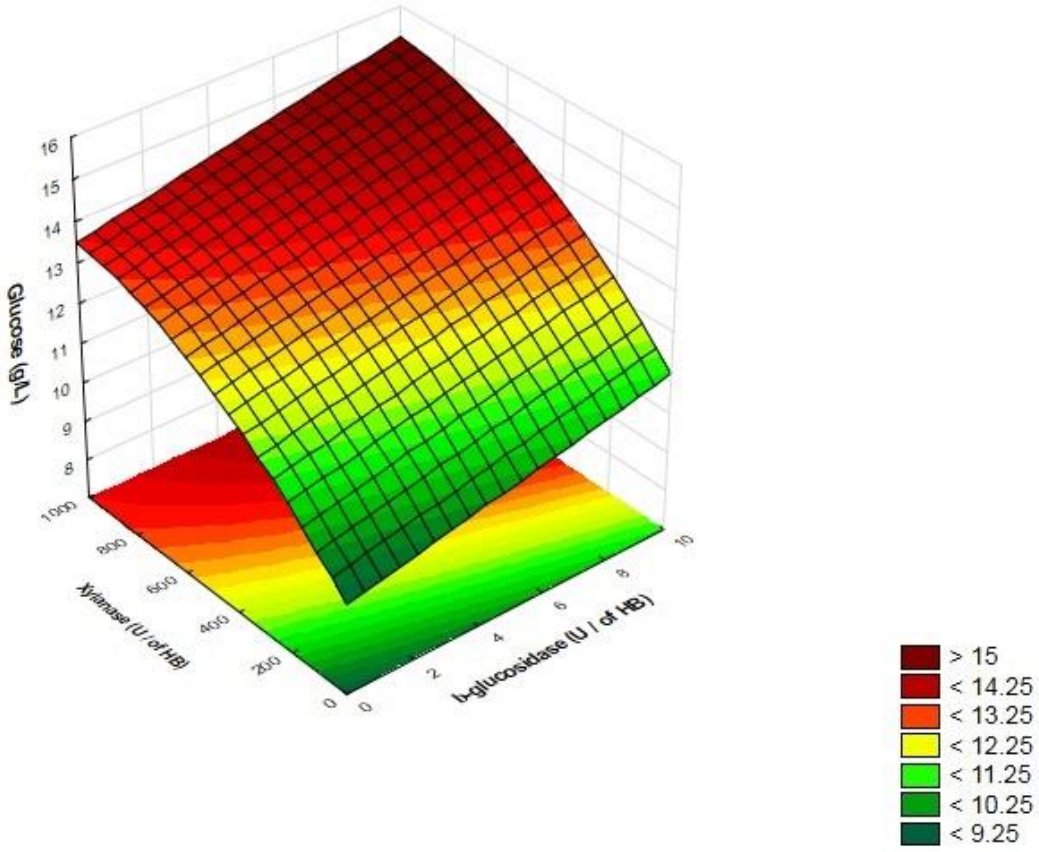

Figure 37 Response surface for glucose release on HB hydrolysis using XYL and BGL extract. The highest response values are indicated in the dark red area.

To validate the statistical model, a 24 h hydrolysis was performed using the optimized enzymatic load. As enzymatic extracts were not pure enzymes and presented a blend of several glycohydrolases, total FPase, β-glucosidase, xylanase, pectinase and protein in the combination were calculated (Table 25). Cellulase activity was not affected by the addition of XYL and BGL, since that these extract were poor in cellulolytic activity.

Table 25 Total enzymatic activities in the enzymatic mixtures.

Enzymatic combinations	FPase (U/g of HB)	β-glucosidase (U/g of HB)	Xylanase (U/g of HB)	Pectinase (U/g of HB)	Protein (mg/g of HB)
Celluclast 1.5L	10.01	6.98	51.07	0.59	5.84
Celluclast 1.5L + XYL	10.49	10.91	1051.29	2.65	8.78
Celluclast 1.5L + BGL	10.09	16.99	51.56	1.35	6.27
Celluclast 1.5L + XYL + BGL	10.57	20.92	1051.78	3.41	9.21

The results analyzed using the Tukey test are presented in Table 26. The addition of XYL and BGL exhibited a positive effect on HB hydrolysis ($p<0.05$). Regarding to BGL addition, no statistical difference was detected on glucose and monosaccharides sum between Celluclast 1.5L+ XYL and Celluclast 1.5L+ XYL + BGL, which could suggest that the extract XYL overlaped the BGL effect.

Table 26 Sugar release (g/L) in the optimized enzymatic extracts mixtures.

Enzymatic combinations	Arabinose (g/L)	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Monossachaides sum (g/L)
Celluclast 1.5L	0.00 ± 0.00 a	1.24 ± 0.04 a	6.51 ± 0.05 a	0.54 ± 0.01 a	8.38 ± 0.01 a
Celluclast 1.5L+ XYL	0.00 ± 0.00 b	0.11 ± 0.01 b	15.51 ± 0.10 b	1.26 ± 0.04 b	17.08 ± 0.06 b
Celluclast 1.5L + BGL	0.00 ± 0.00 c	0.31 ± 0.01 c	8.03 ± 0.11 c	0.60 ± 0.01 a	9.06 ± 0.013 c
Celluclast 1.5L+ XYL + BGL	0.00 ± 0.00 d	0.10 ± 0.01 d	15.75 ± 0.41 b	1.22 ± 0.05 c	17.27 ± 0.45 a

Means calculated from 3 replications. Data not transformed. Means followed by the same small letter do not differ among them by Tukey test at 5%.

7.3.3 Inhibition hydrolysis

The statistical design allowed the development of a cocktail to supplement Celluclast 1.5L. However, it was not clear the effect of A. stygium BGL extract on HB hydrolysis. Statistical design showed a positive of BGL effect but Tukey test of optimized cocktail

showed no significant difference between BGL addition. In literature there is no information about A. stygium enzymes properties, in particular, regarding to inhibition effect.

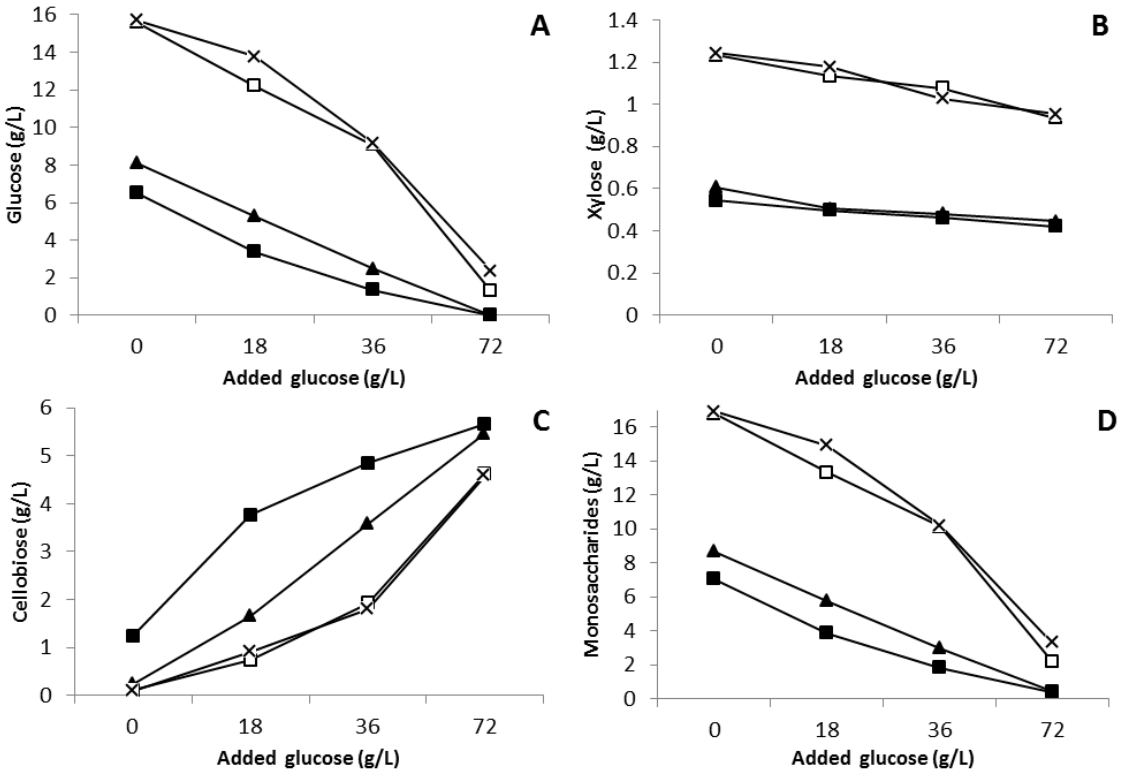

Figure 38 Sugar release in hydrolysis inhibitory test: glucose (A), xylose (B), cellobiose (C) and monosaccharides (D). Celluclast 1.5L(■), Celluclast 1.5L+XYL (□), Celluclast 1.5L +BGL (▲), Celluclast 1.5L+XYL+BGL (X).

Three glucose concentration on HB (5%) hydrolysis, 18, 36 and 72 g/L were tested, aiming to mimic the behavior of the extracts in inhibitory conditions, e.g high pulp concentration. Figure 38 shows the effect of glucose addition in HB hydrolysis. The supplementation with the enzymatic extracts allowed a less inhibitory effect of β-glucosidase by glucose concentration. However, enzyme combinations with XYL presented lower reduction on glucose and xylose released when compared with Celluclast 1.5L and Celluclast 1.5L+ BGL. Surprisingly, BGL and XYL + BGL extracts combinations presented similar results and a major inhibitory effect was visualized above 36 g/L. It appeared that BGL supplementation had a neutral effect in terms of glucose release inhibition. Regarding to hemicellulose degradation, small reduction on hydrolysis was visualized (Figure 38B).

7.3.4 Hydrolysis kinetics

Hydrolysis kinetics was performed using extreme conditions aiming to test more applicable environments to the enzyme mixtures obtained. Two conditions were tested, 5 FPU/ g of HB and 40 FPU/g of HB using 10% of HB (w/v). In both conditions levels of XYL and BGL were kept at 1000 U of xylanase/ g of HB and 10 U of β-glucosidase/ g of HB respectively. Daily samples were taken, sugar measurement were performed and hydrolysis yields were calculated (Figure 39).

Supplementation in low cellulolytic load (5 FPU/g of HB) showed 120% and 238% increase in the cellulose and hemicelullose hydrolysis yields respectively (Figure 39A and Figure 39B). The use of higher Celluclast 1.5L load (40 FPU /g of HB) led to higher HB hydrolysis yields but not proportionally to cellulase increment. A slight difference between Celluclast 1.5L+XYL+BGL and Celluclast 1.5L +XYL could be observed (Figure 39C and Figure 39D). Interestingly no cellobiose accumulation was detected under high Celluclast 1.5 L load (data not shown), which suggest that the slight increase is not caused by β-glucosidase action.

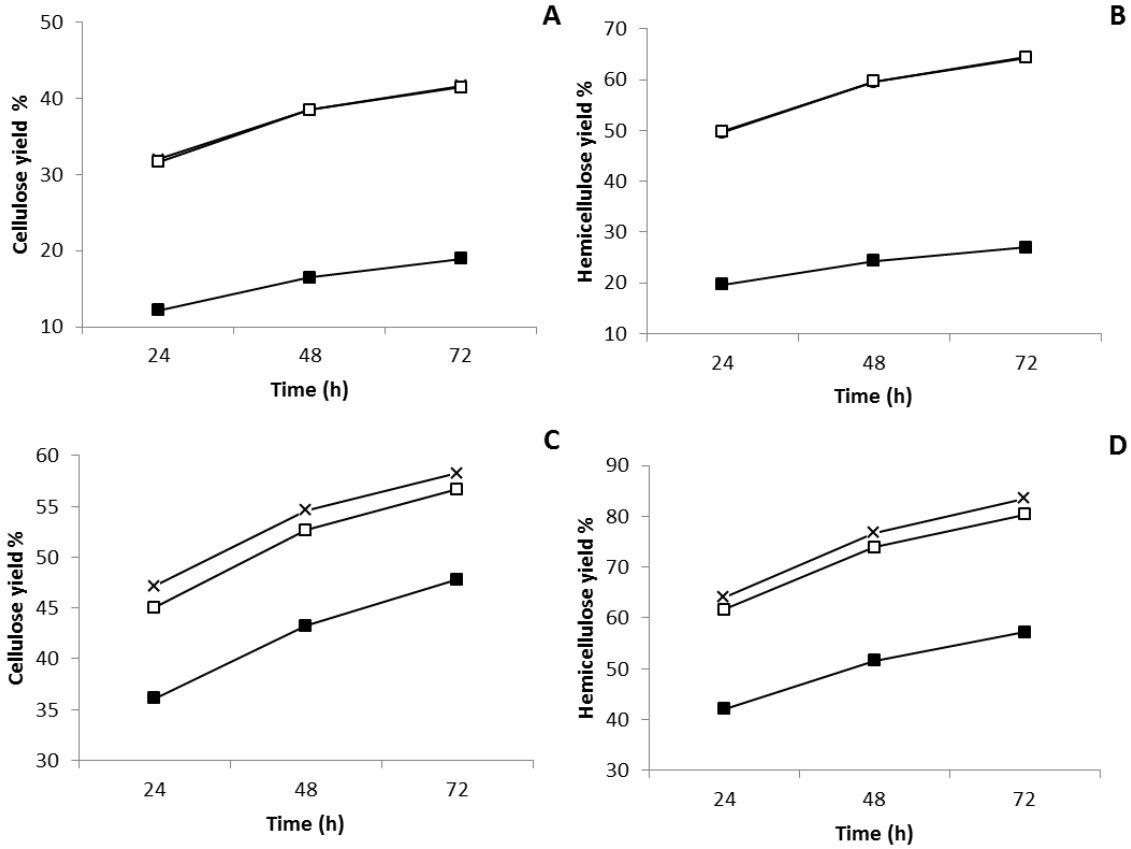

Figure 39 Sugar cane bagasse hydrolysis with 5 FPU/g of HB (A and B) and 40 FPU/g of HB (C and D): Cellulose hydrolysis yield (A and C) and hemicellulose hydrolysis yield (B and D) expressed as the percentage of the theoretical yields. Celluclast 1.5L (■), Celluclast 1.5L +XYL (□), Celluclast 1.5L+XYL+BGL (X).

7.4 **Discussion**

This work aimed to develop an enzymatic preparation using two novel enzymes extracts from *A. stygium* and one xylanase rich enzyme extract from *A. niger*. Supplementation in a commercial cellulolytic extract, Celluclast 1.5L, was performed and all extracts showed positive effect on HB hydrolysis when used individually (Chapter 4 and 5).

Statistical design allowed to access the effect of each extract in Celluclast 1.5L and the combination between them. It is interesting to note that pectinase extract was not statistically significant for glucose release of HB hydrolysis (Figure 36). This data does not corroborate with Delabona et al. (105) and Berlin et al. (3) findings, which supplemented cellulolytic extracts with pectinases preparations and others accessory enzymes and verified a positive effect of the extracts. However, these studies used pectinase preparations from *A. niger*, which is a well known pectinolytic fungus (257).

In chapter 4, it was shown that PEC is able to increase HB hydrolysis when supplemented in Celluclast 1.5L. Proteomic analysis of PEC (Chapter 4) did not detect the presence of pectinases peptides, only pectinase activity. Two hypotheses were raised; one is the low glycohydrolases information on the data base of this fungus; the other was that PEC presented oxidases and glycohydrolases such as catalase and arabinofuranosiadases which could have contributed to the sugar cane bagasse hydrolysis.

Using statistical tools, an equation with the significant terms was developed and corroborated with the experimental data. A maximal glucose release value was suggested, 15.26 g/L, and maximum experimentally value found was 15.74 g/L. Also, Tukey test inferred no statistical differences between Celluclast 1.5L + XYL and Celluclast 1.5L + XYL + BGL. In other words BGL extract had no effect on the supplementation of Celluclast + XYL. This data could suggest that XYL supplies all the required enzymes for Cellulcast 1.5 L supplementation. Proteomic studies of XYL (Chapter 5) showed that this extract possess several important enzymes to HB hydrolysis already described with positive effect in *T. ressei* cellulolytic extract supplementation, such as endo-1,4-β-xylanase, α-L-arabinofuranosidase, feruloyl esterase and β-glucosidase.

No literature data regarding to *A. stygium* β-glucosidase inhibition are reported. In this work, we showed that *A. stygium* β-glucosidase was strongly inhibited by glucose concentration above 23.3 g/L (Figure 38). On one hand Karnchanatat et al. (197) characterized a β-glucosidase from *Daldinia eschscholzii*, Xylariacea member, and verified that glucose was a competitive inhibitor of the enzyme with inhibitor constant (K_i) of 0.79 mM. On the other hand *A. niger* β-glucosidase preparation presented a competitive inhibiton by glucose but with K_i range 2.7 - 3.4 (258). That could explain the stronger inhibiton visualized for the Celluclast 1.5L + BGL combination and the poor performance of this cocktail.

To study the behavior of the combinations, more industrial applicable conditions were tested, using 5 and 40 FPU/g of HB with HB concentration of 10% (w/v). The increase on Celluclast 1.5L load was not proportional to the hydrolysis increment. This data is usually found on lignocellulose hydrolysis and this effect has been explained as a consequence of a product inhibition (3, 151).

The addition of XYL extract allowed reasonable hydrolysis yields even in low Celluclast 1.5L load, which could represent a “low enzyme hydrolysis strategy” once that enzymes costs are still a challenge to overcome (6). HB used is composed by 4.59% of

hemicellulose and the removal of this polysaccharide was important to increase glucose release (Figure 39). Several studies pointed to the importance of xylanase on sugar cane bagasse hydrolysis (151, 200) and others biomass feedstocks such as corn stover (3). Even though a slight increase was detected between Celluclast 1.5L+XYL+BGL and Celluclast 1.5L +XYL, industrially it is not profitable spend on the addition of BGL *A. stygium* extract due to its lower outcome.

7.5 **Conclusions**

The performance of an enzymatic preparation for Celluclast 1.5L supplementation was tested. *A. stygium* extracts were not efficient when combined with *A. niger* DR02 extract (XYL). Statistical data supported the development of a cocktail based mainly by the commercial cellulose preparation with rich xylanse extract XYL.

CHAPTER 8 - FINAL REMARKS AND GENERAL CONCLUSION

This work represents a small part of the intense research effort that has been developed in Brazil in the last years in second ethanol generation technology. This thesis focused efforts to obtain a hemicellulolytic mixture able to increase sugar cane bagasse hydrolysis. Several tools and approaches were performed. New strains able to produced high glycohydrolases titration and novel strains never been used before for plant biomass degradation were selected as described in chapter 3. Bioprocess tools were also used and increased enzyme production of *A. niger* and *A. stygium* in bench scale (Chapter 4 and 5). Secretome of both fungi were done and allowed to clarify hydrolysis in sugar cane bagasse. Aiming to improve xylanase production in *A. niger* deletion of *creA* and over expressing *xlnR* were done, but only led to increase in β-xylosidase productivity (Chapter 6). The formulation of enzymes extracts that showed best hydrolysis performance was based mainly by the commercial cellulose preparation with rich xylanse extract (XYL). It is important to stand out the importance of the hemicellulases and accessory enzymes from others microorganisms in cellulolytic preparations and how combinations of several enzymes can lead to higher plant biomass hydrolysis.

Continuity of this subject in future works should be done to each experimental chapter and could produce large amounts of relevant information for biofuels area. In chapter 3 several microorganisms were not deeply studied and can still present benefits for enzyme industry. In chapter 4, genomic and transcriptomic studies can produce novel data for a better understanding of the uncharacterized *A. stygium* enzymes and the action of these in biomass hydrolysis. In chapter 5, test liquor from different pretreatments and bioreactor cultivations strategies can also be performed to improve xylanase production. In chapter 6, combining of gene expression and fed-batch cultivations of *A. niger* mutants strains may unveil the not totally elucidated process of hemicellulases regulation. Finally, in chapter 7 an embracer hydrolysis approaches could be done, by testing more commercial cellulolytic extracts, recombinants enzymes and with the produced extracts from this study.

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APPENDIX

Appendix A - Table 27 Strains used in the phylogenic analysis. Nucleotide sequences were obtained/submitted to GenBank

Name	Reference	Genes sequenced/Genebank access	Source	Geography
Aspergillu niger	DR02	ITS - KC311839, BT2 - KC311845	*Plantanus orientalis*	Brazil, Curitiba
Annuhypoxylon stygium	DR47	ITS - KC311843, BT2 - KC311846	*Eucalyptus benthamii*	Brazil, Colombo
Penicillium kloeckeri	DR49	ITS - KC311844, BT2 - KC311847	Spoiled books	Brazil, Joinville
Alternaria sp.	DR40	ITS - KC311842	*Eucalyptus benthamii*	Brazil, Colombo
Trichoderma atroviride	DR17	ITS - KC311840	*Eucalyptus benthamii*	Brazil, Colombo
Trichoderma atroviride	DR19	ITS - KC311841	*Eucalyptus benthamii*	Brazil, Colombo
Aspergillus niger	CBS 554.65	ITS - AJ223852, BT2 - GU296687	Tannin-gallic acid fermentation	USA, Connecticut
Aspergillus niger	CBS 120.49	ITS - AJ280006, BT2 - GU296688	Unkown	USA
Aspergillus aculeatus	CBS 172.66	ITS - AJ279988, BT2 - FJ629271	Tropical soil	Unknown
Aspergillus japonicus	CBS 114.51	ITS - AJ279985, BT2 - GU296707	Saito 5087	Unknown
Aspergillus tubingensis	CBS 134.48	ITS - AJ223853, BT2 - GU296696	Unknown	Unknown
Aspergillus tubingensis	CBS 127.49	ITS - AJ280007	*Coffea arabica*, seed	Unknown
Aspergillus tubingensis	CBS 110.42	BT2 - DQ768455	Unknown	Unknown
Aspergillus foetidus	CBS 564.65	ITS - AJ280009 , BT2 - GU296697	Unknown	Japan
Aspergillus brasiliensis	IMI 381727	ITS - AJ280010, BT2 - AM295186	Soil	Brazil, São Paulo, Pedreira
Aspergillus carbonarius	NRRL 67	ITS - U65305, BT2 - EF661097	Unknown	Unknown
Aspergillus heteromorphus	CBS 117.55	ITS - AJ280013, BT2 - GU296704	Culture contaminant	Brazil
Aspergillus ellipticus	CBS 707.79	ITS - AJ280014, BT2 - FJ629279	Soil	Costa Rica
Annulohypoxylon stygium	E6826d	ITS - HQ008900	*Macrocarpaea sodiroana*	Ecuador
Annulohypoxylon stygium	BCRC34024	BT2 - AY951667	Unknown	Tawain
Annulohypoxylon stygium	BCRC34023	BT2 - AY951666	Unknown	Tawain
Annulohypoxylon stygium var *annulatum*	BCRC34025	BT2 - AY951669	Unknown	France
Annulohypoxylon urceolatum	SUT098	ITS - DQ322103	Unknown	Thailand, Songkhla Province
Annulohypoxylon urceolatum	BCRC34028	BT2 - AY951670	Unknown	Tawain
Annulohypoxylon nitens	BCRC34021	ITS - EF026138, BT2 - AY951663	Unknown	Tawain, Taipei

Annulohypoxylon bovei var. *microspora*	BCRC34012	ITS - EF026141, BT2 - AY951654	Unknown	Tawain
Annulohypoxylon squamulosum	BCRC34022	ITS - EF026139, BT2 - AY951665	Unknown	Tawain
Annulohypoxylon moriforme var. *microdiscus*	BCRC34018	ITS - EF026137, BT2 - AY951660	Unknown	Tawain
Annulohypoxylon multiforme	ATCC 36665	ITS - AF201717	*Betula* sp.	-
Annulohypoxylon cohaerens	3041	ITS - EF026140	On *Fagus* sp.	France, Ariège, Rimont
Annulohypoxylon elevatidiscus	BCRC34014	BT2 - AY951656	Unknown	Tawain
Hypoxylon investiens	CBS 118185	ITS - FJ185308, BT2 - FJ185299	Decorticated and blackened branch	Ecuador
Talaromyces wortmannii	KUC1286	ITS - HM469393	Wood	Korea
Talaromyces wortmannii	CBS 391.48	ITS - JN899352	Unknown	Unknown
Talaromyces wortmannii	W35	BT2 - AY533533	Unknown	Unknown
Talaromyces radicus	CBS 100489	ITS - JN899324	Root of seedling of *Triticum aestivum*	Australia, Wagga
Talaromyces allahabadensis	CBS 453.93	ITS - JN899345	Soil of cultivated field	India, Allahabad
Talaromyces tardifaciens	CBS 250.94	ITS - JN899361	Unknown	-
Talaromyces loliensis	CBS 643.80	ITS - JN899379	*Lolium* sp.	New Zealand, Palmerston North
Talaromyces phialosporus	CBS 233.60	ITS - JN899340, BT2 - HQ156949	Milled Californian rice	USA, California
Talaromyces variabilis	CBS 385.48	ITS - JN899343	Cocos fibre	South Africa, Johannesburg
Talaromyces islandicus	CBS 338.48	ITS - JN899318	Unknown source	South Africa, Cape Town
Talaromyces rugulosus	CBS 371.48	ITS - JN89937	*Solanum tuberosum*	USA, , Connecticut
Talaromyces amestolkiae	CBS 884.72	BT2 - JX315622	Manure	France,
Talaromyces ruber	CBS 113138	BT2 - JX965349	PVC/Paper wall covering	-
Talaromyces bacillisporus	CBS 296.48	BT2 - AY753368	*Begonia* sp., leaf	USA, New York city
Talaromyces palmae	CBS 442.88	BT2 - HQ156947	*Begonia* sp., leaf	USA, New York city
Talaromyces minioluteus	CV0383	BT2 - JF910277	Sandy fynbos soil	South Africa, Western Cape
Talaromyces purpurogenus	CBS 286.36	BT2 - JX315639	Parasitic on a culture of *Aspergillus oryzae*	Japan
Talaromyces purpurogenus	CBS 184.27	BT2 - JX315637	Soil	USA, Louisiana
Talaromyces stollii	CBS 132706	BT2 - JX965359	Indoor air form bakery	The Netherlands, Avenhorn
Talaromyces pinophilus	L14	BT2 - EU597716	Litchi	South Africa
Alternaria alternata	CBS 112018	ITS - AY673074	Phaeohyphomycosis	Spain, Santiago de Compostela
Alternaria porri	ATCC 58175	ITS - AF229470	*Allium fistulosum*	USA, Arizona
Alternaria tenuissima	ATCC 16423	ITS - AF229476	Unkown	Unkown

Alternaria longipes	EGS 30-033	ITS - AY278835	Unkown	Unkown
Alternaria arborescens	EGS 39-128	ITS - AF347033	Unkown	Unkown
Alternaria destruens	EGS 46-069	ITS - AY278836	Unkown	Unkown
Alternaria solani	CBS 111.44	ITS - Y17070	*Ageratum houstonianum*, seed	Unkown
Alternaria brassicicola	CBS 125088	ITS - GQ496082	*Brassica oleracea*, leaf	Hungary,Keszthely
Alternaria dauci	CCRC33651	ITS - AF267130	Seed	Unkown
Alternaria crassa	DGG Acr1	ITS - AF229464	Unkown	Unkown
Alternaria japonica	ATCC 13618	ITS - AY376639	Infected radish	Canada
Trichoderma atroviride	CBS 142.95	ITS - AF456917	Gallery of ambrosia beetle, in decayed log of *Quercus* sp.	Slovenia
Trichoderma atroviride	DAOM 179514	ITS - EU280125	Unkown	Unkown
Hypocrea lixii	CBS 226.95	ITS - AF057606	Soil	England
Hypocrea viridescens	CBS 433.34	ITS - AY380905	mouldy apple core	UK
Trichoderma aggressivum	CBS 689.94	ITS - FJ442606	Mushroom compost	England
Trichoderma longibrachiatum	CBS 816.68	ITS - EU401556	Mud	USA, Ohio
Hypocrea koningii	CBS 979.70	ITS - DQ323410	Decaying angiosperm wood	The Netherlands, Groeneveld
Hypocrea vinosa	CBS 960.68	ITS - AF191038	Sand, in lysimeter system	USA, Ohio, Cincinnati
Trichoderma hamatum	ATCC 28012	ITS - X93975	Soil	USA, North Carolina
Trichoderma virens	ATCC MYA-4894	ITS - JX174053	Unkown	Unkown

Appendix B - Table 28 Results of the selection of fungal strains using the sum of the hydrolysis ratios for liquor agar and xylan agar, and calculation of the average halos obtained in the esculin gel diffusion assay (EGDA)

Strain	**Identification[a]**	**Source**	**Hydrolysis ratio using liquor agar[b,c]**	**Hydrolysis ratio using xylan agar[b,c]**	**Ratio sum[b,c]**	**EGDA halo average (mm)[d,e]**
ATCC64973	*A. niger*	-	1.00	1.42	2.42	14.50
DR01	*Aspergillus* sp.	Spoiled books	0.00	1.82	1.82	16.00
DR02	*Aspergillus niger*	*P. orientalis*	2.48	1.59	4.06	17.25
DR03	*Aspergillus* sp.	*E. benthamii*	2.44	1.00	3.44	-
DR04	*Paecilomyces lilacinus*	*G. max*	0.00	1.85	1.85	13.00
DR05	*Trichoderma* sp.	*E. benthamii*	1.00	1.83	2.83	+
DR06	*Aspergillus* sp.	Spoiled books	2.47	1.57	4.04	11.50
DR07	NI	*E. benthamii*	2.89	1.00	3.89	17.25
DR08	*Aspergillus* sp.	*E. benthamii*	2.32	2.13	4.45	+
DR09	NI	*E. benthamii*	1.86	1.61	3.47	19.00
DR100	NI	*S. tuberosum*	0.00	1.38	1.38	+
DR101	NI	*E. benthamii*	0.00	1.36	1.36	-
DR102	NI	*E. benthamii*	0.00	1.36	1.36	-
DR103	*Cladosporium* sp.	*G. max*	0.00	1.35	1.35	+
DR104	*Penicillium* sp.	*G. max*	0.00	1.35	1.35	-
DR105	*Penicillium janthinellum*	*S. officinarum*	0.00	1.33	1.33	14.25
DR106	*Cladosporium* sp.	*G. max*	0.00	1.32	1.32	-

DR107	*Penicillium* sp.	*G. max*	0.00	1.32	1.32	-
DR108	*Aspergillus* sp.	*G. max*	0.00	1.31	1.31	12.00
DR109	*Penicillium* sp.	*G. max*	0.00	1.29	1.29	12.00
DR110	NI	*E. benthamii*	0.00	1.27	1.27	13.50
DR111	*Trichoderma* sp.	*E. benthamii*	0.00	1.21	1.21	-
DR112	*Saccharicola* sp.	*S. officinarum*	0.00	1.20	1.20	-
DR113	*Alternaria* sp.	*P. orientalis*	0.00	1.14	1.14	+
DR114	NI	*E. benthamii*	0.00	1.12	1.12	12.25
DR115	*Bipolaris* sp.	*G. max*	0.00	1.11	1.11	+
DR116	*Rhizomucor* sp.	*G. max*	0.00	1.00	1.00	-
DR117	NI	*E. benthamii*	0.00	1.00	1.00	-
DR118	NI	*E. benthamii*	0.00	1.00	1.00	12.50
DR119	*Beauveria bassiana*	*G. max*	0.00	1.00	1.00	+
DR12	*Penicillium* sp.	*E. benthamii*	2.82	1.55	4.36	+
DR120	*Trichoderma* sp.	*S. officinarum*	0.00	1.00	1.00	+
DR121	*Diaphorte* sp.	*S. officinarum*	0.00	0.00	0.00	-
DR13	*Penicillium* sp.	*G. max*	0.00	1.00	1.00	14.00
DR14	*Aspergillus* sp.	*G. max*	0.00	1.00	1.00	-
DR15	*Fusarium* sp.	*G. max*	1.58	1.40	2.98	-
DR16	NI	*E. benthamii*	1.23	1.54	2.77	-
DR17	*T. atroviride*	*E. benthamii*	1.73	1.05	2.78	+
DR18	*Penicillium* sp.	*G. max*	1.17	1.82	2.99	-
DR19	*T. atroviride*	*E. benthamii*	2.00	1.00	3.00	12.00
DR20	*Coletotrichum gloeosporioides*	*G. max*	0.00	1.00	1.00	20.00
DR21	NI	*E. benthamii*	2.00	1.59	3.59	20.00
DR22	*Acremonium* sp.	Spoiled books	1.50	1.16	2.66	12.00
DR23	*C. gloeosporioides*	*G. max*	1.83	1.38	3.21	17.50
DR24	*Aspergillus* sp.	*G. max*	1.65	1.42	3.07	18.00
DR25	*Aspergillus* sp.	*E. benthamii*	1.92	1.38	3.31	13.25
DR26	*Paecilomyces sp.*	*G. max*	2.33	1.45	3.79	15.00
DR27	*Penicillium* sp.	*E. benthamii*	2.29	1.42	3.71	+
DR28	*Penicillium* sp.	*G. max*	0.00	3.78	3.78	-
DR29	*Aspergillus* sp.	*G. max*	1.32	1.84	3.16	-
DR30	*Aspergillus* sp.	*E. benthamii*	1.65	1.60	3.25	14.50
DR31	*Aspergillus* sp.	*G. max*	2.29	1.24	3.53	-
DR32	NI	*E. benthamii*	0.00	1.00	1.00	-
DR33	*Aspergillus* sp.	*G. max*	2.22	1.24	3.46	16.00
DR34	*Aspergillus* sp.	*G. max*	1.00	2.75	3.75	12.50
DR35	*Aspergillus* sp.	*G. max*	1.00	2.75	3.75	18.25
DR36	*Aspergillus* sp.	*G. max*	2.24	1.40	3.64	16.00
DR37	*Trichoderma* sp.	*G. max*	1.39	1.24	2.64	12.75
DR38	*Trichoderma* sp.	*E. benthamii*	1.37	1.34	2.71	11.00
DR39	*Acremonium* sp.	*G. max*	1.25	1.28	2.53	15.00
DR40	*Alternaria* sp.	*E. benthamii*	0.00	1.28	1.28	12.00
DR41	NI	*S. officinarum*	0.00	1.55	1.55	16.00
DR42	*Alternaria* sp.	*E. benthamii*	1.58	1.08	2.66	13.00
DR43	NI	*E. benthamii*	0.00	1.00	1.00	16.00
DR44	*Aspergillus* sp.	*G. max*	0.00	0.00	0.00	-
DR45	*Alternaria* sp.	*E. benthamii*	0.00	1.22	1.22	14.50
DR46	NI	*E. benthamii*	0.00	1.58	1.58	18.50
DR47	*A. stygium*	*E. benthamii*	1.00	1.72	2.72	20.50
DR48	NI	*E. benthamii*	0.00	1.45	1.45	17.00
DR49	*P. kloeckeri*	Spoiled books	0.00	1.53	1.53	15.00
DR50	*Penicillium* sp.	*G. max*	1.51	1.19	2.70	-

DR51	*Aspergillus* sp.	*E. benthamii*	1.27	1.33	2.60	+
DR52	*Penicillium* sp.	*G. max*	0.00	1.43	1.43	15.00
DR53	*Penicillium* sp.	*G. max*	0.00	1.36	1.36	14.50
DR54	*Aspergillus* sp.	*G. max*	1.56	1.25	2.80	+
DR55	*Aspergillus* sp.	*G. max*	0.00	1.09	1.09	16.50
DR56	*Aspergillus* sp.	*G. max*	0.00	1.35	1.35	17.00
DR57	*Aspergillus* sp.	*G. max*	0.00	1.34	1.34	17.00
DR58	*Aspergillus* sp.	*G. max*	1.00	1.67	2.67	18.50
DR59	NI	*E. benthamii*	1.00	1.63	2.63	+
DR60	*Penicillium* sp.	*G. max*	1.24	1.31	2.55	12.00
DR61	NI	*E. benthamii*	1.00	1.49	2.49	20.00
DR62	*Penicillium* sp.	*G. max*	1.00	1.47	2.47	12.50
DR63	*Coletotrichum* sp.	*G. max*	1.13	1.33	2.47	-
DR64	*Penicillium* sp.	*G. max*	1.00	1.46	2.46	+
DR65	*Penicillium* sp.	*G. max*	1.00	1.46	2.46	-
DR66	NI	*E. benthamii*	1.23	1.17	2.40	+
DR67	NI	*S. officinarum*	0.00	2.35	2.35	13.50
DR68	*Penicillium* sp.	*G. max*	1.37	0.96	2.33	11.00
DR69	NI	*E. benthamii*	0.00	2.00	2.00	-
DR70	*Penicillium* sp.	*G. max*	0.00	2.00	2.00	+
DR71	NI	*E. benthamii*	1.00	1.00	2.00	-
DR72	*Aspergillus* sp.	Spoiled books	0.00	1.94	1.94	+
DR73	NI	*G. max*	0.00	1.87	1.87	-
DR74	NI	*S. officinarum*	0.00	1.86	1.86	-
DR75	*Cladosporium* sp.	*G. max*	0.00	1.74	1.74	-
DR76	*Penicillium* sp.	*G. max*	0.00	1.73	1.73	+
DR77	*Alternaria* sp.	*E. benthamii*	0.00	1.73	1.73	16.00
DR78	*Cladosporium* sp.	*G. max*	0.00	1.70	1.70	-
DR79	NI	*E. benthamii*	0.00	1.67	1.67	+
DR80	*P. lilacinus*	*S. tuberosum*	0.00	1.67	1.67	+
DR81	*P. janthinellum*	*S. officinarum*	0.00	1.58	1.58	-
DR82	NI	*E. benthamii*	0.00	1.58	1.58	-
DR83	NI	Spoiled books	0.00	1.57	1.57	+
DR84	*Cladosporium* sp.	Spoiled books	0.00	1.55	1.55	-
DR85	*Phomopsis* sp.	*S. officinarum*	0.00	1.55	1.55	+
DR86	NI	*E. benthamii*	0.00	1.54	1.54	-
DR87	*P. lilacinus*	*G. max*	0.00	1.54	1.54	+
DR88	*Penicillium* sp.	*G. max*	0.00	1.51	1.51	-
DR89	*Cladosporium* sp.	*G. max*	0.00	1.48	1.48	-
DR90	*Chaetomium* sp.	*G. max*	0.00	1.47	1.47	-
DR91	*Penicillium* sp.	*G. max*	0.00	1.43	1.43	12.00
DR92	*Cladosporium* sp.	*G. max*	0.00	1.43	1.43	-
DR93	NI	Spoiled books	0.00	1.43	1.43	+
DR94	*Aspergillus* sp.	*G. max*	0.00	1.43	1.43	-
DR95	NI	*G. max*	0.00	1.42	1.42	-
DR96	*Alternaria* sp.	*E. benthamii*	0.00	1.41	1.41	17.50
DR97	NI	Spoiled books	0.00	1.41	1.41	11.50
DR98	*Fusarium* sp.	*S. officinarum*	0.00	1.41	1.41	-
DR99	*Fusarium* sp.	*S. officinarum*	0.00	1.39	1.39	17.75

[a]NI = Not identified; [b]0.00 = No growth; [c]1.00 = Growth and absence of hydrolysis halo; [d]+ = Positive unmeasured halo; [e]- = No halo.

Appendix C- Table 29 Results of the selection of actinomycetes strains using the sum of the hydrolysis ratios for liquor agar and xylan agar, and calculation of the average halos obtained in the esculin gel diffusion assay (EGDA)

Strain	Identification[a]	Source	Hydrolysis ratio using liquor agar[b,c]	Hydrolysis ratio using xylan agar[b,c]	Ratio sum[b,c]	EGDA halo average (mm)[d,e]
DR59	*Streptomyces galileus*	Solo	4.80	3.37	8.17	-
DR60	*Streptomyces* sp.	*Theobroma cacao*	4.57	3.27	7.84	-
DR61	*Streptomyce globisporus*	*C. roseus*	4.22	3.21	7.43	-
DR62	*Streptomyces* sp.	*C. sinensis*	3.60	3.20	6.80	-
DR63	*Streptomyces* sp.	Unknown	2.36	4.27	6.63	-
DR64	*Streptomyces* sp.	*C. sinensis*	3.24	3.25	6.49	-
B6P4	*Streptomyces* sp.	*S. officinarum*	2.64	3.83	6.48	-
H4P4	*Streptomyces* sp.	*S. officinarum*	2.31	3.83	6.15	-
A10	*Streptomyces sampsonii*	*C. reticulata*	3.40	2.73	6.13	-
A82	*Streptomyces pseudogriseolus*	*S. officinarum*	3.67	2.31	5.97	-
A12,1(31)	*Streptomyces* sp.	*S. officinarum*	1.94	3.21	5.16	-
A25	*Streptomyces* sp.	*C. sinensis*	2.71	2.35	5.07	-
H4.3 / C7.3	*Streptomyces* sp.	*S. officinarum*	1.94	2.77	4.72	-
G1P1	*S. pseudogriseolus*	*S. officinarum*	1.88	2.77	4.66	-
A01	*Streptomyces* sp.	*C. reticulata*	2.00	2.56	4.56	-
	Streptomyces capoamus	Unknown	2.00	2.40	4.40	-
DR69	*Streptomyces roseochromogenus*	*C. roseus*	1.50	2.80	4.30	12.00
DR66	*Streptomyces olindenses*	Solo	2.00	2.22	4.22	+
ATCC 31267	*Streptomyces avermitilis*	Solo	1.20	3.00	4.20	-
A07	*Nocardiopsis* sp.	*C. sinensis*	1.00	3.13	4.13	-
G10P4	*Streptomyces macrosporeus*	*S. officinarum*	1.40	2.10	3.50	-
A28	*Streptomyces* sp.	*C. sinensis*	2.44	1.00	3.44	-
A18	*Streptomyces* sp.	*C. sinensis*	0.00	3.25	3.25	-
A03	*Nocardiopsis* sp.	*C. reticulata*	0.00	3.00	3.00	-
A12P2	*Streptomyces* sp.	*S. officinarum*	0.00	2.93	2.93	-
DR67	*Streptomyces lividans*	Solo	0.00	2.86	2.86	-
DSM46458	*Streptomyces chartresuts*	Unknown	1.60	1.00	2.60	-
CCT2398	*Streptomyces rimosus*	Unknown	1.33	1.00	2.33	-
A04	*Nocardiopsis* sp.	*C. reticulata*	0.00	2.27	2.27	-
A30	*Streptomyces verne*	*C. sinensis*	0.00	2.05	2.05	-
A11P2	*S. macrosporeus*	*S. officinarum*	1.00	1.00	2.00	-
DR65	*Streptomyces* sp.	*C. sinensis*	1.00	1.00	2.00	+
H4.3 C7.3	*Streptomyces* sp.	*S. officinarum*	1.00	1.00	2.00	-
A08	*Streptomyces* sp.	*C. reticulata*	1.00	1.00	2.00	-
A09	*Streptomyces* sp.	*C. sinensis*	1.00	1.00	2.00	-
A11	*Nocardiopsis* sp.	*C. sinensis*	0.00	1.56	1.56	-
DR70	*Nocardiopsis* sp.	*C. sinensis*	0.00	1.55	1.55	14.50
DR68	*Nocardiopsis* sp.	*C. sinensis*	0.00	1.00	1.00	+
A16	*Nocardiopsis* sp.	*C. sinensis*	0.00	1.00	1.00	-
A23	*Streptomyces* sp.	*C. sinensis*	1.00	1.00	1.00	-
A32	*Streptomyces* sp.	*C. sinensis*	0.00	1.00	1.00	-
A3P1	*Streptomyces albus*	*S. officinarum*	0.00	1.00	1.00	-
A4P1	*Streptomyces pulveraceus*	*S. officinarum*	0.00	1.00	1.00	-
F7P4	*Streptomyces akiyoshiensis*	*S. officinarum*	0.00	1.00	1.00	-
H4P3	*Streptomyces tsukiyonensis*	*S. officinarum*	0.00	1.00	1.00	-

NI = Not identified; [b]0.00 = No growth; [c]1.00 = Growth and absence of hydrolysis halo; [d]+ = Positive unmeasured halo; [e]- = No halo.

Appendix D - Table 30 CAZy enzymes and proteins hits by LC/MS-MS from the supernatant of *Annulohypoxylon stygium* grown at pH 5.0

GH family	Protein name	Protein accession numbers	Protein identification probability	No. unique peptides	No. unique spectra	No total spectra	Peptide sequence
-	ATP-dependent dna-binding helicase (RAD3/XPD subfamily) [Encephalitozoon cuniculi GB-M1]	gi|19074028	94.60%	1	1	1	EGGGPKGEEAR
-	Conserved hypothetical protein [Uncinocarpus reesii 1704]	gi|258575447	94.70%	1	1	4	LTIGGENDTPAR
-	Carboxypeptidase S1 [Pyrenophora tritici-repentis Pt-1C-BFP]	gi|189192809	99.80%	2	3	9	IYESGHEVPFYQPLASLEMFER TLQGFMGAFPQYSR
GH47	hypothetical protein CIMG_03314 [Coccidioides immitis RS]	gi|119186533	94.70%	1	2	10	MYVYDKDR
GH3	YALI0D05049p [Yarrowia lipolytica]	gi|50549915	92.40%	1	1	1	LEGTETR
GH10	family 10 xylanase [Cryptovalsa sp. BCC 7197]	gi|53636303	94.70%	1	1	2	AWDVVNEIFNEDGSLR
GH3	hypothetical protein BC1G_07110 [Botryotinia fuckeliana B05.10]	gi|154310381	94.70%	1	1	4	ELGAAGTVLLK
CE1	hypothetical protein CHGG_02841 [Chaetomium globosum CBS 148.51]	gi|116207096	94.70%	1	1	3	VGLWGFLASER
-	ZYRO0D10164p [Zygosaccharomyces rouxii]	gi|254581782	94.70%	1	1	1	AVKFLETR
GH18	endochitinase [Verticillium albo-atrum VaMs.102]	gi|302404074	94.70%	1	2	5	GLGGSMFWEASGDR
GH55	hypothetical protein [Podospora anserina S mat+]	gi|171688470	94.70%	1	1	5	LVDGISVGSEDLYR
GH3	Cel3b [Trichoderma reesei]	gi|31747166	94.70%	1	1	19	GVDVLLGPVAGPLGR
-	hypothetical protein PICST_54418 [Scheffersomyces stipitis CBS 6054]	gi|150863946	94.70%	1	1	2	KMIQNGLIK
GH3	beta-glucosidase 2 precursor [Pyrenophora tritici-repentis Pt-1C-BFP]	gi|189202078	100.00%	3	3	10	GVAIGEEFR RGVAIGEEFR VGIPQLCLQDGPLGVR
AA2	Peroxidase_2 [Botryotinia fuckeliana B05.10]	gi|154315332	94.70%	1	2	10	HNVLEHDGSISR

GH15	glucoamylase [Aspergillus oryzae RIB40]	gi|169770097	92.90%	1	1	1	LVVDSFR
GH18	class III chitinase. putative [Talaromyces stipitatus ATCC 10500]	gi|242792443	94.70%	1	1	1	SLMDADTSK
GH3	hypothetical protein [Podospora anserina S mat+]	gi|171685516	94.70%	1	1	15	FVAVIGEDAGPNPNGPNSCADR
GH3	hypothetical protein [Podospora anserina S mat+]	gi|171685516	94.70%	1	1	15	IDDMAMR
GH3	probable beta-glucosidase 1 precursor [Neurospora crassa]	gi|12718377	94.70%	1	1	2	GVQEQGVIATIK
GH3	unnamed protein product [Aspergillus niger]	gi|134076323	94.70%	1	1	2	TLDGIIK
GH3	Pc12g11110 [Penicillium chrysogenum Wisconsin 54-1255]	gi|255932921	94.70%	1	1	2	HYIGNEQEMHR
GH3	beta-glucosidase [Penicillium brasilianum]	gi|145688454	100.00%	3	5	21	HYIGNEQEHFR IMAAYFK LDDMAMR
AA	hypothetical protein SS1G_05679 [Sclerotinia sclerotiorum 1980]	gi|156053664	94.70%	1	2	8	GAGPNFGIVTSAVMK
-	predicted protein [Laccaria bicolor S238N-H82]	gi|170084953	94.70%	1	1	1	VSTPEIWHAK
GH54	Similar to α-L-arabinofuranosidase [Aspergillus nidulans FGSC A4]	gi|67522228	98.70%	1	1	10	AYGVFVSPGTGYR
GH7	hypothetical protein CHGG_08330 [Chaetomium globosum CBS 148.51]	gi|116200349	94.70%	1	1	1	YGTGYCDAQCAR
-	elastinolytic metalloproteinase Mep [Neosartorya fischeri NRRL 181]	gi|119485809	99.80%	2	2	12	LTGGPANSNCLNALESGGMGEGWGDFMATAIR LVIDGMALQPCNPNFVQAR
GH18	chitinase 1 precursor [Neurospora crassa OR74A]	gi|164427228	94.70%	1	1	15	IVLGIPLYGR
GH15	Chain A. Glycoside Hydrolase Family 15 Glucoamylase From Hypocrea Jecorina	gi|261825113	94.70%	1	1	3	AIALIGYSK
GH3	hypothetical protein SNOG_11881 [Phaeosphaeria nodorum SN15]	gi|169617407	94.70%	1	2	30	NWEGFSPDPYLSGIAVAESVR
-	subtilisin-like protease PR1D [Metarhizium acridum]	gi|18958207	94.70%	1	1	2	KATIISVK
GH35	beta-galactosidase. putative [Aspergillus clavatus NRRL 1]	gi|121701157	94.70%	1	1	3	LPVPSLWIDILQK

-	hypothetical protein CaO19.11114 [Candida albicans SC5314]	gi|68483177	91.70%	1	1	1	IIVNLNQSKFR
-	hypothetical protein VDBG_06613 [Verticillium albo-atrum VaMs.102]	gi|302410435	89.50%	1	1	1	TLTSPKSSPR
GH92	alpha-1.2-mannosidase [Podospora anserina S mat+]	gi|171681924	94.70%	1	1	6	GYTQGGSNADIVLADAFVK
AA1	nitrate reductase [Aspergillus oryzae]	gi|1136629	93.00%	1	1	1	GRISEELLK
GH3	beta-glucosidase [Paracoccidioides sp. 'lutzii' Pb01]	gi|295670726	99.80%	2	2	17	ARDFVSQLTLAEK DFVSQLTLAEK

Appendix E - Table 31 CAZy enzymes and proteins hits by LC/MS-MS from the supernatant of *Annulohypoxylon stygium* grown at pH 4.0

GH family	**Protein name**	**Protein accession numbers**	**Protein identification probability**	**No. unique peptides**	**No. unique spectra**	**No total spectra**	**Peptide sequence**
GH3	beta-glucosidase [Penicillium brasilianum]	gi|145688454	94.80%	1	2	3	HYIGNEQEHFR
GH3	avenacinase [Rasamsonia emersonii]	gi|24416585	99.40%	1	1	4	GVALGEEFR
-	tranlsation elongation factor 1a [Trichaptum abietinum]	gi|13162245	94.80%	1	1	3	LSSMRLMPSILR
-	carboxypeptidase S1 [Pyrenophora tritici-repentis Pt-1C-BFP]	gi|189192809	99.80%	2	3	20	IYESGHEVPFYQPLASLEMFER TLQGFMGAFPQYSR
GH47	hypothetical protein CIMG_03314 [Coccidioides immitis RS]	gi|119186533	94.80%	1	2	8	MYVYDKDR
CE1	Esterase_lipase CHGG_02841 [Chaetomium globosum CBS 148.51]	gi|116207096	94.80%	1	1	4	VGLWGFLASER
GH3	hypothetical protein BC1G_07110 [Botryotinia fuckeliana B05.10]	gi|154310381	94.80%	1	1	3	ELGAAGTVLLK
-	ZYRO0D10164p Squalene cyclase [Zygosaccharomyces rouxii]	gi|254581782	93.50%	1	1	1	AVKFLETR
GH55	hypothetical protein [Podospora anserina S mat+]	gi|171688470	94.80%	1	1	4	LVDGISVGSEDLYR
GH3	Cel3b [Trichoderma reesei]	gi|31747166	94.80%	1	1	6	GVDVLLGPVAGPLGR

GH35	hypothetical protein [Podospora anserina S mat+]	gi|171683861	94.80%	1	1	1	GPLNEGGLFAER
-	hypothetical protein PICST_54418 [Scheffersomyces stipitis CBS 6054]	gi|150863946	94.80%	1	1	1	KMIQNGLIK
AA2	Peroxidase_2 [Botryotinia fuckeliana B05.10]	gi|154315332	94.80%	1	2	5	HNVLEHDGSISR
-	beta-tubulin [Blastocladiella emersonii]	gi|117422544	94.80%	1	1	2	GHYTEGAELVDSVLDVVRK
-	hypothetical protein [Podospora anserina S mat+]	gi|171686504	94.80%	1	1	1	AAIEFLKR
-	YALI0D17864p [Yarrowia lipolytica]	gi|50550971	89.90%	1	1	1	AGNTEIDAIK
AA	hypothetical protein SS1G_05679 [Sclerotinia sclerotiorum 1980]	gi|156053664	94.80%	1	2	20	GAGPNFGIVTSAVMK
GH54	Similar to α-L-arabinofuranosidase [Aspergillus nidulans FGSC A4]	gi|67522228	99.90%	2	2	27	AYGVFVSPGTGYR QQASWTVR
-	hypothetical protein CTRG_01616 [Candida tropicalis MYA-3404]	gi|255724762	90.70%	1	1	1	LDKVTNK
GH18	chitinase 1 precursor [Neurospora crassa OR74A]	gi|164427228	94.80%	1	1	6	IVLGIPLYGR
-	serine carboxypeptidase (CpdS). putative [Neosartorya fischeri NRRL 181]	gi|119467005	94.80%	1	1	7	QLEFILGR
GH3	hypothetical protein SNOG_11881 [Phaeosphaeria nodorum SN15]	gi|169617407	94.80%	1	1	11	NWEGFSPDPYLSGIAVAESVR
GH92	alpha-1.2-mannosidase [Chaetomium globosum CBS 148.51]	gi|116195562	94.80%	1	1	4	NWVDHSFFTEGK
GH35	beta-galactosidase. putative [Aspergillus clavatus NRRL 1]	gi|121701157	94.80%	1	1	8	LPVPSLWIDILQK
-	catalase [Claviceps purpurea]	gi|3157413	100.00%	3	3	12	AGDRGPTLLEDFIFR GPTLLEDFIFR GVDFTEDPLLQGR
GH3	hypothetical protein BC1G_15815 [Botryotinia fuckeliana B05.10]	gi|154290006	94.80%	1	1	1	QYALTQK

	hypothetical protein VDBG_06613 [Verticillium albo-atrum VaMs.102]	gi|302410435	92.50%	1	1	1	TLTSPKSSPR
-	lactonohydrolase [Cryptococcus neoformans var. neoformans JEC21]	gi|58259894	94.80%	1	1	1	QFNSLNDVSVNPR
GH92	alpha-1.2-mannosidase [Podospora anserina S mat+]	gi|171681924	94.80%	1	1	7	GYTQGGSNADIVLADAFVK
GH43	hypothetical protein NECHADRAFT_53602 [Nectria haematococca mpVI 77-13-4]	gi|302889082	92.70%	1	1	1	IAVHTAPSIEGPWTYK
GH54	alpha-L-arabinofuranosidase [Talaromyces purpurogenus]	gi|13991905	99.80%	2	2	4	QQASWTVR YIAHTGSTVNTQVVTSSSSTTLK
GH3	beta-glucosidase [Paracoccidioides sp. 'lutzii' Pb01]	gi|295670726	94.80%	1	1	4	DFVSQLTLAEK
-	hypothetical protein SS1G_09518 [Sclerotinia sclerotiorum 1980]	gi|156047583	91.70%	1	1	1	QYEDEARQR
-	SNF2 family helicase/ATPase. putative [Talaromyces marneffei ATCC 18224]	gi|212534786	89.80%	1	1	2	KDIGISWINPAK

Appendix F - Table 32 CAZy enzymes and proteins hits by LC/MS-MS from the supernatant from fed-batch bioreactor cultivation of *A. niger* DR02 on pentose-rich liquor from the hydrothermal pretreatment of sugarcane bagasse

Protein accession numbers	Cazy ID	Protein name	Peptide sequence	No. of unique peptides	No. of total peptides
gi|145242946	GH3	β-glucosidase M [*A. niger* CBS 513.88]	GVNLLLGPVVGPLGR KGVNLLLGPVVGPLGR NTNNALPLQTPQLVSVFGYDAK NWEGFSNDPYLTGALVYETVQGVQSSGVGVSTK QASDYGSLLHPSEPQTPYGLFPQSDFSEGVYIDYR SALTDDYSDTLVTNVASK AAFEEAGYK	6	12
gi|145230215	GH3	Exo-1,4-β-xylosidase xlnD [*A. niger* CBS 513.88]	AASLISLFTLDELIANTGNTGLGVSR DDIEQGVIR ELRVPVEVGSFAR ESIAWPGNQLDLIQK GQETPGEDVSLAAVYAYEYITGIQGPDPESNLK	12	58

			LGLPAYQVWSEALHGLDR		
			LVTTQYPASYAEEFPATDMNLRPEGDNPGQTYK		
			NSNNVLPLTEK		
			TLIHQIASIISTQGR		
			VNEDGDWVVFPGTFELALNLER		
			YGLDVYAPNINTFR		
			AVDIVSQMTLAEK		
			DLANWNVETQDWEITSYPK		
			GADIQLGPAAGPLGR		
			GIQDAGVVATAK		
			GQAMGQEFSDK		
			HYIAYEQEHFR		
			ITLQPSEETQWSTTLTR		
gi|126046487	GH3	β-glucosidase [*A. niger*]	LWTPPNFSSWTR	15	40
			NDGALPLTGK		
			NGVFTATDNWAIDQIEALAK		
			NWEGFSPDPALSGVLFAETIK		
			TMHELYLWPFADAIR		
			VAGDEVPQLYVSLGGPNEPK		
			VNQFVNVQR		
			YYYVSEGPYEK		
			AVTDGGAHALIDPHNYGR		
gi|145238644	GH5	Endo-β-1,4-glucanase B [*A. niger* CBS 513.88]	ITDATQWLK	4	19
			VGFIGEYAGGSNDVCR		
			VQFMMER		
			LDYVVSSAEQHDIK		
gi|145236118	GH5	Mannan endo-1,4-β-mannosidase F [*A. niger* CBS 513.88]	STINTGADGLQR	4	23
			TALSTTGVGADLFWQYGDDLSTGK		
			VWGFNDVTSQPSSGTVWYQLHQDGK		
gi|145230537	GH5	Endo-β-1,4-glucanase A [*A. niger* CBS 513.88]	ATVQTITDLGAYAVVDPHNFGR	2	2
			LVPDELTGAADATYMADLK		
			FDGSIITSTSDFK		
gi|134083538	GH5	Unnamed protein product [*A. niger*]		2	5
			LTPDGLTSSFASTYLSDLK		
			DTGFGAQPTTDTGDELADAFVWVKPGGESDGTSDTSSSR		
			GLATNVANFNAWSIDSCPSYTSGNDVCDEK		
gi|134076801	GH6	Unnamed protein product [*A. niger*]	QPTGQSAWGDWCNVK	6	66
			SYINAIAPELSSAGFDAHFITDTGR		
			VPTMGEYLEDIQTQNAAGASPPIAGIFVVYDLPDR		
			YDAHCGYSDALQPAPEAGTWFQAYFEQLLTNANPSL		
gi|145246118	GH6	1,4-β-D-glucan cellobiohydrolase [*A. niger* CBS 513.88]	EILVQYSDVHTLLVIEPDSLANLVTNLNVAK	2	46
			VPSFVWLDTAAK		

gi|156712284	GH7	1,4-β-cellobiosidase [*Thermoascus aurantiacus*]	LNFVTQSSGK YAGTCDPDGCDFNPYR YGTGYCDSQCPR FVTGSNVGSR	2	3
gi|254212110	GH7	Cellobiohydrolase A [*A niger*]	GTCDSESGVPATVEGAHPDSSVTFSNIK MTVVTQFITDGSGSLSEIK MTVVTQFITDGSGSLSEIKR YGGTCDPDGCDFNPYR YGTGYCDSQCPR HGGLEGMGEAMAK	5	58
gi|145230535	GH7	1,4-β-D-glucan cellobiohydrolase B [*A. niger* CBS 513.88]	LGNTDFYGPGLTVDTNSPFTVVTQFITDDGTSSGTLTEIK LGNTDFYGPGLTVDTNSPFTVVTQFITDDGTSSGTLTEIKR LNFVTQGSSK LYLMSDDSNYELFK TLFGDENVFDK YGTGYCDSQCPR GTCSTDSGVPATVEAESPNAYVTYSNIK ADFGALTPENSMK CIGITVWGVADPDSWR DSVFYK	8	34
gi|292495278	GH10	Endo-1,4-β-xylanase	EIAVTELDIAGASSTDYVEVVEACLNQPK GHTLVWHSQLPSWVQSITDK GHTLVWHSQLPSWVQSITDKNTLIEVMK GKIYAWDVVNEIFNEDGSLR GQFSFSGSDYLVNFAQSNNK IAFETAR IYAWDVVNEIFNEDGSLR IYAWDVVNEIFNEDGSLRDSVFYK KWIAAGIPIDGIGSQTHLSAGGGAGISGALNALAGAGTK KYLGNIGDQYTLTK LTGMVSHVK LTGMVSHVKK LYINDYNLDSASYPK NHITTVMQHYK NTLIEVMK SSSTPLLFDSNYNPKPAYTAIANAL VIGEDYVR WIAAGIPIDGIGSQTHLSAGGGAGISGALNALAGAGTK YLGNIGDQYTLTK	22	1036
gi|13242071	GH11	Xylanase [*A. niger*]	GTVTSDGSVYDIYTATR LGMNLGTHNYQIVATEGYQSSGSSSITVQ TNAASIQGTATFTQYWSVR	4	138
gi|145250953	GH11	Endo-1,4-β-xylanase	VGGTVTTSNHFNAWAK NVPEIYGVTNFDQHWSVR	2	16

		B precursor [*A. niger* CBS 513.88]	STGTVDVSAHFQR		
gi|145249126	GH12	Endoglucanase A [A. niger CBS 513.88]	LSSSGASWHTEWTWSGGEGTVK LVSDVSSIPTSVEWK QIATATVGGK SWEVWYGSTTQAGAEQR SYSNSGVTFNK SYSNSGVTFNKK	7	135
gi|145243632	GH13	α-amylase, catalytic domain [*A. niger* CBS 513.88]	YGNIQPIGK IYDVNSNFGTADDLK SLSDALHAR	2	3
gi|145235763	GH15	Glucoamylase [*A. niger* CBS 513.88]	ALYSDAATGTYSSSSSTYSSIVDAVK ATAMIGFGQWLLDNGYTSTATDIVWPLVR ATLDSWLSNEATVAR DANTLLGSIHTFDPEAACDDSTFQPCSPR EVVDSFR FNVDETAYTGSWGRPQR IESDDSVEWESDPNR NGDTSLLSTIENYISAQAIVQGISNPSGDLSSGAGLGEPK QGSLEVTDVSLDFFK SIYTLNDGLSDSEAVAVGR TLVDLFR	11	54
gi|145230419	GH16	Glycosidase crf1 [A. niger CBS 513.88]	SVSITNYNPGSSYTYSDK TLAYSDAQSGTR	2	6
gi|145233743	GH27	α-galactosidase B [*A. niger* CBS 513.88]	IVTAANEVVNLGLK SAVWEEVPELK TPALGWNSWNAYSCDIDADKIVTAANEVVNLGLK WGYNPDWTFDPEHPAEYWSGPTSSGEVFVLMLNSEGEVK	4	12
gi|134057627	GH30	Unnamed protein product [*A. niger*]	LSIDDTSSGHK LSSITAPVQGSGSPGSASTWK VLGSPWSAPGWMK	3	3
gi|134055627	GH31	Unnamed protein product [*A. niger*]	GDEVLFDSSASPLVFQSQYVNLR IPLETMWTDIDYMDK	2	8
gi|134076816	GH43	Unnamed protein product [*A. niger*]	FTGSLVGAYATK TEDFGVSPEGYPNTLR WEVGEWPVVQPVR YQGQGQEIDFGR	4	10
gi|145230794	GH47	Mannosyl-oligosaccharide α-1,2-mannosidase 1B [*A. niger* CBS 513.88]	GPVSDLVQDSSK LSDLTGDTTYADLSQK	2	2
gi|1168267	GH54	α-N-arabinofuranosidase B	AYGVFMSPGTGYR YVSGSLVSGPSFTSGEVVSLR	2	4

Appendix G - The capability of endophytic fungi for production of hemicellulases and related enzymes

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The capability of endophytic fungi for production of hemicellulases and related enzymes

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Abstract

Background: There is an imperative necessity for alternative sources of energy able to reduce the world dependence of fossil oil. One of the most successful options is ethanol obtained mainly from sugarcane and corn fermentation. The foremost residue from sugarcane industry is the bagasse, a rich lignocellulosic raw material uses for the production of ethanol second generation (2G). New cellulolytic and hemicellulytic enzymes are needed, in order to optimize the degradation of bagasse and production of ethanol 2G.

Results: The ability to produce hemicellulases and related enzymes, suitable for lignocellulosic biomass deconstruction, was explored using 110 endophytic fungi and 9 fungi isolated from spoiled books in Brazil. Two initial selections were performed, one employing the esculin gel diffusion assay, and the other by culturing on agar plate media with beechwood xylan and liquor from the hydrothermal pretreatment of sugar cane bagasse. A total of 56 isolates were then grown at 29°C on steam-exploded delignified sugar cane bagasse (DEB) plus soybean bran (SB) (3:1), with measurement of the xylanase, pectinase, β-glucosidase, CMCase, and FPase activities. Twelve strains were selected, and their enzyme extracts were assessed using different substrates. Finally, the best six strains were grown under xylan and pectin, and several glycohydrolases activities were also assessed. These strains were identified morphologically and by sequencing the internal transcribed spacer (ITS) regions and the partial β-tubulin gene (BT2). The best six strains were identified as *Aspergillus niger* DR02, *Trichoderma atroviride* DR17 and DR19, *Alternaria* sp. DR45, *Annulohypoxylon stigyum* DR47 and *Talaromyces wortmannii* DR49. These strains produced glycohydrolases with different profiles, and production was highly influenced by the carbon sources in the media.

Conclusions: The selected endophytic fungi *Aspergillus niger* DR02, *Trichoderma atroviride* DR17 and DR19, *Alternaria* sp. DR45, *Annulohypoxylon stigyum* DR47 and *Talaromyces wortmannii* DR49 are excellent producers of hydrolytic enzymes to be used as part of blends to decompose sugarcane biomass at industrial level.

Keywords: Endophytic fungi, Xylanase, Hemicellulases, Accessory enzymes

Background

In nature, lignocellulosic materials are degraded by a consortium of microorganisms that synthesize many hydrolytic enzymes able to loosen and degrade these substrates. Improvement in the efficiency of hydrolysis of lignocellulosic materials has traditionally focused on cellulose, which is the most abundant plant polysaccharide [1]. However, the presence of hemicellulose and lignin can restrict cellulose hydrolysis. The hemicellulases, such as pectinases and xylanases, stimulate cellulose hydrolysis by removal of the non-cellulosic polysaccharides that coat the cellulose fibers [1].

Cellulolytic and hemicellulolytic enzymes have been extensively investigated as tools to achieve viable second-generation ethanol production. The hemicellulases include accessory enzymes, which are a group of enzymes capable of increasing the yield of reducing sugars during enzymatic hydrolysis of lignocellulosic substrates. The definition of the accessory enzymes has evolved over time. Enzymes such as the β-glucosidases were originally classified as accessories, but today are considered essential in enzymatic cocktails, following elucidation of their mechanisms of action during substrate degradation [2-4].

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The main accessory enzymes are currently considered to be α-L-arabinofuronosidase, hemicellulolytic esterases, β-mannanases, α-glucoronidases, β-xylosidases, pectinases, and xylanases. Several studies have shown that cellulase enzymes supplementation can improve the enzymatic hydrolysis of lignocellulosic biomass, in terms of speed and hydrolysis yield. An issue is that crude multi-enzyme blends obtained from a single fungus strain are not ideal in biotechnological applications. This is because cellulase activities are not expressed at sufficient levels, or the enzyme complexes are not well balanced in terms of the individual enzymes [3].

For this reason, fungi strains isolated from unusual environments have been sought as alternative sources of hydrolytic enzymes [5,6]. Endophytic fungi are potentially amongst the most interesting microorganisms for screening for the production of industrial biocompounds. These microorganisms are ubiquitous in plants, inhabiting plant tissues without inducing any apparent symptoms in their hosts [7]. The fact that these microorganisms are present within plant tissues could explain their capacity to produce substances that could have useful industrial, agricultural, and medicinal applications [8].

The endophytic fungi that have been reported to be xylanase producers include *Alternaria alternata* [9], *Hymenoscyphus ericae* [10], and *Aspergillus terreus* [11]. De Almeida et al. [12] selected strains from the *Acremonium* endophyte species for hemicellulases and cellulases production. From 14 plant species, Suto et al. [13] isolated 155 strains of fungi that produced xylanases. Harnpicharnchai et al. [14] purified a thermotolerant β-glucosidase from an endophytic *Periconia* sp. Other studies have involved the selection of new isolates using extracellular enzymes as selection parameters for plant growth promotion. Silva et al. [15] investigated fungi isolated from *Annona* spp., while Luz et al. [16] employed isolates from *Passiflora edulis*.

Endophytic fungal strains may therefore constitute a valuable source of biological material that deserves to be studied and explored for the production of cellulolytic and hemicellulolytic enzymes. In this context, the present work concerns the selection of endophytic fungi as producers of hemicellulases and related enzymes with different enzymatic profiles, for use in the deconstruction of lignocellulosic biomass.

Results

Agro-industrial waste material composition

The sugar cane hydrothermal pretreatment liquor showed the following composition (g/L): xylo-oligosaccharides (9.98), xylose (4.70), glucose (0.55), arabinose (0.77), cellobiose (0.0), furfural (1.05), hydroxymethylfurfural (0.18), acetic acid (1.47), formic acid (0.23), and total soluble lignin (3.15). Despite the presence of inhibitors, this liquor demonstrated to be a potential carbon source for the screening of enzyme producers and the production of hemicellulases. The DEB was composed of 77.89% cellulose, 7.09% hemicellulose, and 16.22% lignin. The SB consisted of 34% cellulose, 18.13% hemicellulose, 9.78% lignin, and 43.22% protein. The media prepared using these waste materials were therefore able to provide a suitable ratio of cellulose and hemicellulose for the synthesis of glycohydrolases, as well as a good source of nitrogen.

Plate screening

A total of 120 fungal strains were bioprospected and used for calculation of hydrolysis rates (Additional file 1: Table S1). The media containing liquor were stained with Congo Red, revealing the yellow hydrolysis halos (Figure 1). A total of 73 strains were unable to grow on the medium, while only 35 were able to both grow and produce halos. On the other hand, in the case of the medium with xylan, only two strains, one *Aspergillus* sp. and one *Diaphorte* sp. were unable to grow, while 102 strains grew and produced halos. It was therefore demonstrated that the xylose/xylo-oligomers liquor produced by a simple pretreatment was able to sustain the growth of a significant number of the fungi tested.

Selection of β-glucosidase producers employed the EGDA to determine β-glucosidase in the fungal culture extracts, with positive extracts forming dark-colored halos. Of the 119 extracts tested, 63 produced measurable halos, 27 showed dark precipitates although measurement was not possible, and 40 strains were negative for β-glucosidase production. The plate screening and EGDA results were used to select 56 strains for a second screening employing shake flask cultivations. Some of these strains were negative in the hemicellulolytic and β-glucosidase tests, and were used as controls to ensure selection consistency.

Shake flask screening

The strains were grown using DEB + SB (3:1) at 29°C on a rotary shaker at 200 rpm for 96 h. The results obtained for some of the strains are presented in Figure 2. Low β-glucosidase activities were detected up to 48 h of fermentation, while high activity levels were observed at 96 h. This was expected, since several filamentous fungi are known to begin to produce detectable amounts of this enzyme after 72 h of growth [17].

The CMCase and FPase activities were low for all the strains, as expected because selection was performed using materials rich in hemicelluloses. High xylanase production was detected at 48 h for many strains, but the largest peaks occurred at 96 h. Pectinase production showed little variation between 48 and 96 h, although amounts of the enzyme nonetheless increased over the

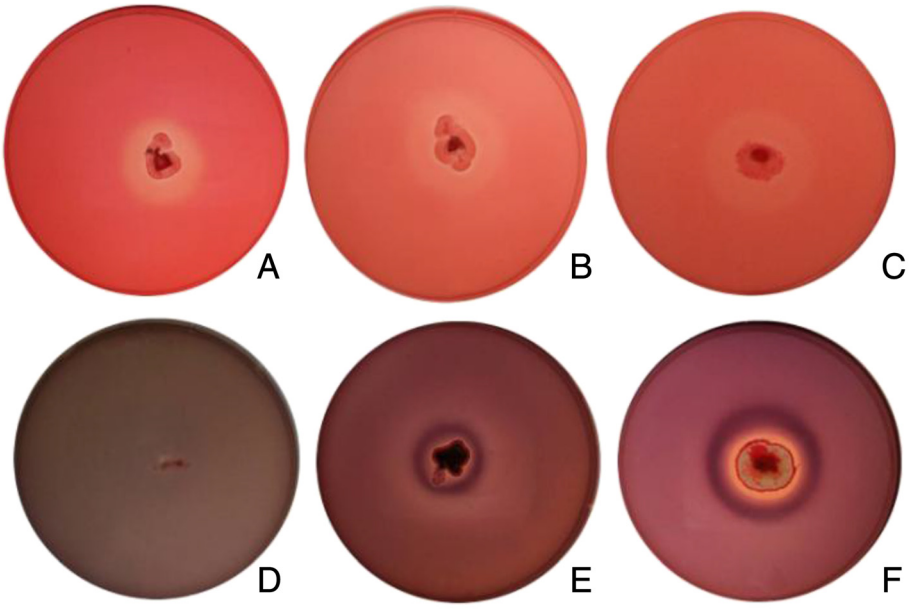

Figure 1 Hydrolysis results following staining with Congo Red, using xylan agar (A, B, and C) and liquor agar (D, E, and F). The organisms used were *Penicillium* sp. DR65 **(A, D)**, *Aspergillus* sp. DR06 **(B, E)**, and *Fusarium* sp. DR15 **(C, F)**.

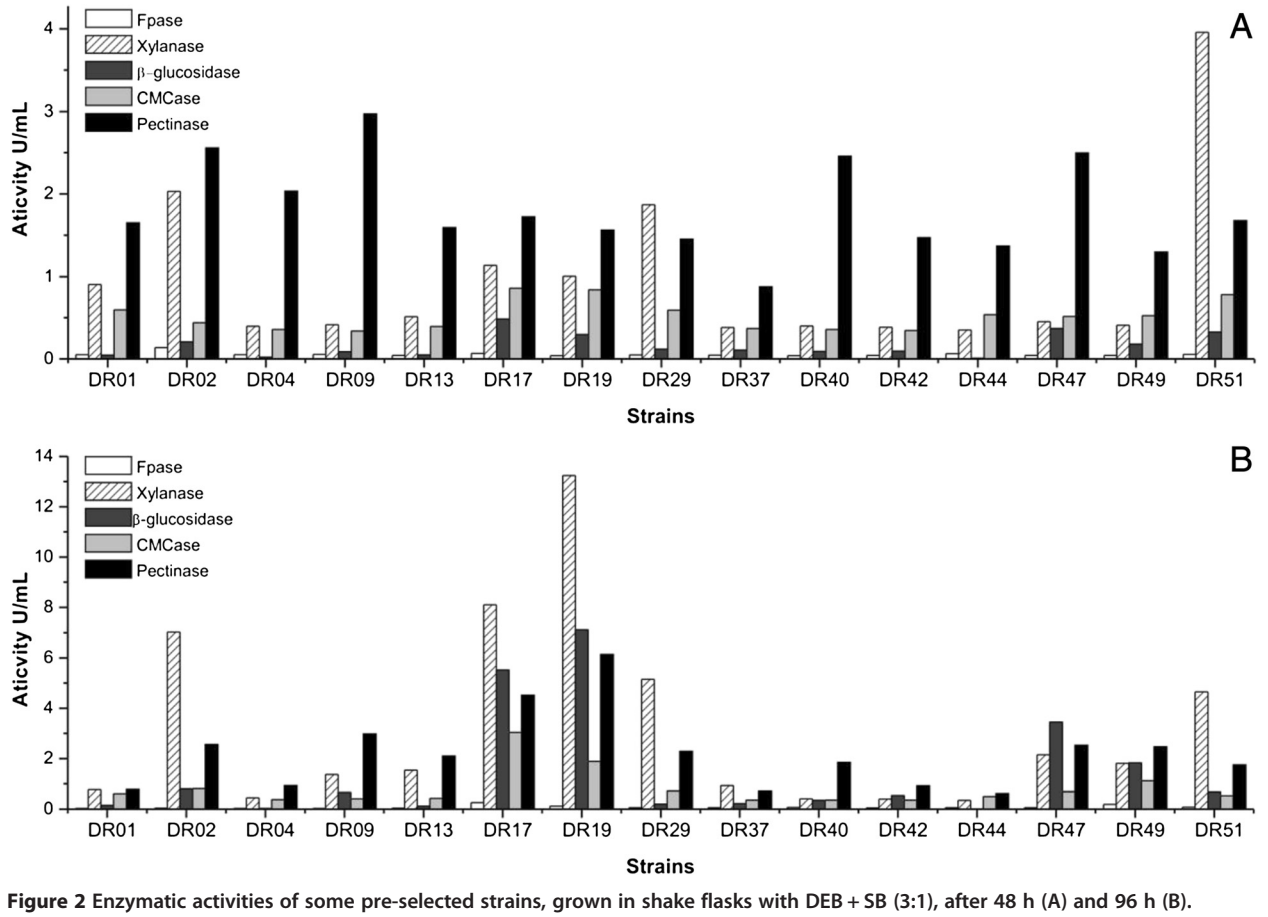

Figure 2 Enzymatic activities of some pre-selected strains, grown in shake flasks with DEB + SB (3:1), after 48 h (A) and 96 h (B).

course of the fermentation. Strains morphologically similar to *Aspergillus fumigatus* (DR08, DR03, DR29, and DR31) were excluded due to possible pathogenicity, which could preclude their use in industrial applications.

Glycohydrolase profile

In order to identify fungi that produced enzyme with different profiles, and hence obtain a more efficient enzymatic extract, 12 strains were selected according to their morphology and enzymatic profiles. A new fermentation with DEB + SB was performed, and samples were taken daily for measurement of xylanase, β-glucosidase, and pectinase activities. The samples that showed the highest glycohydrolase activity were tested using different substrates (Table 1).

The strains DR17 and DR19 (*Trichoderma* sp.), and DR02 (*Aspergillus* sp.) presented the highest xylanolytic activities for birchwood xylan, beechwood xylan, and rye arabinoxylan. Despite the fact that the strains DR17 and DR19 belong to the same genus, and have similar morphologies, they presented different enzymatic profiles (Table 1). Selection was made of six strains (DR02, DR17, DR19, DR40, DR47, and DR49) that showed enzymatic activities for a wider range of substrates, were morphologically different, and presented distinct enzymatic profiles. These strains were cultured in shake flasks containing xylan and pectin as inducer carbon sources. Samples were taken daily for measurements of xylanase, β-glucosidase, and pectinase activities. The fungal extracts that showed highest glycohydrolase activities were tested using different substrates (Table 2).

The fungi xylanolytic profiles differed among the strains and the carbon sources used. The DR17 strain produced xylanases with the same affinity for birchwood xylan, beechwood xylan, and rye arabinoxylan, when cultivated in the presence of beechwood xylan. However, this was not observed when the same *Trichoderma* sp. was grown using DEB + SB. Some strains showed higher activity for beechwood xylan than for birchwood xylan (DR49 and DR40), and vice versa (DR02). The DR02 strain showed the highest activity for rye arabinoxylan. The DR40 strain only produced xylanase when the fungus was grown in the presence of xylan, in contrast to other strains such as DR19, DR49, and DR17, for which DEB and SB also induced the production of xylanases.

The production of β-glucanases was high for DR02 and DR40 strains when cultivated on xylan, for DR17 when grown on DEB + SB, and for DR49 on pectin. However, when these extracts were tested using xyloglucan,

Table 1 Glycohydrolases activities (U/mL) of twelve selected strains grown using DEB + SB

Strains	DR02	DR06	DR07	DR17	DR19	DR20	DR26	DR40	DR45	DR47	DR48	DR49
Time (h)	**120**	**120**	**144**	**96**	**72**	**144**	**144**	**120**	**144**	**96**	**144**	**144**
Birchwood xylan	4.50	1.38	0.55	10.32	3.22	0.41	0.60	0.38	0.77	0.96	0.50	4.53
Beechwood xylan	3.94	2.30	0.95	5.54	3.64	0.63	0.77	0.44	1.11	1.51	1.34	4.03
Rye arabinoxylan	2.93	2.19	0.62	4.13	2.00	0.47	0.53	0.38	0.64	1.30	0.44	3.71
Wheat arabinoxylan	0.53	0.53	0.36	0.86	0.30	0.36	0.55	0.43	0.45	0.37	0.42	0.27
Arabinan	0.46	0.50	0.46	0.48	0.48	0.48	0.47	0.47	0.48	0.49	0.48	0.47
CMC	0.27	0.39	0.17	0.26	0.16	0.19	0.18	0.16	0.30	0.22	0.19	0.66
β-glucan	1.84	3.63	2.26	4.16	0.53	0.48	2.29	0.47	2.06	2.63	1.35	3.46
Xyloglucan	0.52	0.65	0.46	0.73	0.47	0.49	0.48	0.47	0.58	0.69	0.43	0.58
Lichenan	1.04	1.81	1.24	2.00	0.66	0.79	1.08	0.37	1.02	1.11	1.14	1.44
Laminarin	0.68	0.54	0.76	1.70	0.72	0.59	1.11	0.67	0.72	0.80	1.90	1.44
Chitosan	0.63	0.57	0.52	0.53	0.42	0.54	0.68	0.66	0.64	0.61	0.49	0.63
Glucomannan	1.02	2.28	0.84	1.83	0.78	0.48	0.95	0.64	1.85	1.64	1.31	1.91
Galactomannan	0.75	1.41	0.49	1.79	0.55	0.56	0.53	0.48	1.30	1.24	1.25	1.63
1,4 β-mannan	0.65	1.34	0.47	1.22	0.52	0.53	0.50	0.46	1.34	1.44	0.94	1.22
Pectin	0.63	0.84	0.69	0.44	0.65	0.87	0.86	0.69	0.77	0.69	0.80	1.14
pNP β-D-xylopyranoside	0.13	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.10
pNP β-D-mannopyranoside	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
pNP β-D-cellobioside	0.24	0.03	0.47	0.46	0.15	0.12	0.03	0.18	0.41	0.37	0.35	0.18
pNP α-L-arabinofuranoside	0.15	0.02	0.01	0.01	0.00	0.00	0.01	0.02	0.07	0.06	0.02	0.10
pNP β-D-glucopyranoside	1.16	0.11	2.85	5.75	0.62	1.19	0.23	0.82	3.44	2.52	1.33	0.68

Table 2 Glycohydrolases activities (U/mL) of six selected strains grown on pectin and xylan

Strain	*A. niger* DR02		*T. atroviride* DR17		*T. atroviride* DR19		*A. stygium* DR40		*Alternaria* sp. DR47		*T. wortmannii* DR49	
Carbon source	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**	**Pectin**	**Xylan**
Time (h)	**144**	**144**	**120**	**120**	**96**	**144**	**120**	**144**	**120**	**120**	**120**	**120**
Birchwood xylan	0.72	21.34	0.00	2.22	0.59	2.99	1.39	4.68	1.12	1.32	0.74	4.85
Beechwood xylan	1.56	15.04	0.60	2.72	0.49	2.88	1.57	6.87	0.00	2.59	1.44	6.00
Rye arabinoxylan	1.41	11.15	0.00	2.73	0.61	2.76	1.75	5.98	0.46	1.57	1.12	4.07
Wheat arabinoxylan	0.86	3.88	0.80	0.87	0.77	0.87	0.41	0.59	0.15	0.34	0.55	0.37
Arabinan	0.52	1.46	0.77	0.48	0.43	0.49	0.55	0.85	0.89	1.11	1.04	0.95
CMC	1.32	1.24	0.57	0.56	1.25	0.49	0.36	0.56	0.92	1.28	1.42	4.57
β-glucan	2.51	14.03	0.89	1.03	0.69	0.89	0.71	5.67	0.52	1.49	4.16	1.89
Xyloglucan	0.47	1.63	0.31	0.47	0.26	0.48	0.49	2.42	0.76	1.38	0.94	0.94
Lichenan	1.22	4.66	0.55	0.61	0.74	0.71	0.74	2.37	0.27	0.87	2.14	1.43
Laminarin	1.79	1.50	1.72	1.92	1.32	1.78	1.79	4.28	0.66	0.60	3.78	3.20
Chitosan	0.60	1.86	0.76	0.63	0.96	0.57	0.88	0.58	0.00	0.00	1.39	1.09
Glucomannan	1.34	1.90	0.96	0.72	0.53	0.63	0.74	1.23	0.56	0.79	1.41	1.00
Galactomannan	1.11	1.45	0.49	0.49	0.68	0.45	0.50	0.99	0.61	0.23	1.20	0.95
1,4 β-mannan	0.87	1.78	0.56	0.59	0.59	0.49	0.54	0.90	0.81	0.54	1.31	1.09
Pectin	0.58	0.55	5.09	0.71	4.24	0.49	3.92	1.55	7.72	1.31	1.81	0.72
pNP β-D-xylopyranoside	0.16	0.00	0.00	0.05	0.00	0.02	0.03	0.14	0.01	0.02	0.13	2.85
pNP β-D-mannopyranoside	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.01	0.04	0.03
pNP β-D-cellobioside	0.58	0.00	0.00	0.02	0.00	0.03	0.06	0.50	0.05	0.27	1.15	1.50
pNP α-L-arabinofuranoside	0.33	0.21	0.00	0.01	0.00	0.01	0.02	0.67	0.63	0.24	0.57	0.91
pNP β-D-glucopyranoside	3.09	0.22	0.05	0.24	0.22	0.30	0.67	1.48	0.52	1.37	1.86	3.13

all the activities decreased, indicating less affinity for the hydrolysis of β-glucan with branched xylose residues.

A similar phenomenon occurred in the testing of lichenan, which is a linear glucan with more β-1,3 bonds than β-glucan. This indicates that the β-glucanases present in these extracts had lower lichenanase activity. Furthermore, when the DR40 and DR49 strains were grown on xylan, they showed activity against laminarin, indicating the presence of enzymes able to hydrolyze the β-D-glucosyl (1→6) β-D-glucose bond. For almost all fungi, with the exception of DR02 and DR49, the production of polygalacturonase was only induced in the presence of pectin. The best producers were the strains DR47 (7.72 U/mL) and DR17 (5.09 U/mL).

The production of β-glucosidase showed no consistent induction pattern for the three carbon sources tested. DR17 and DR47 produced more β-glucosidase on DEB + SB, while DR02 produced more on pectin, and DR49 on xylan. None of the fungi showed measurable activities for β-1,4-D-glucosaminidase or α-mannosidase.

When the *Talaromyces* sp. DR49 strain was grown on xylan, it was able to produce multiple accessory proteins such as xylosidase, arabinofuranosidase, cellobiohydrolase II, and β-glucosidase. This strain might therefore be promising for the production of hemicellulases. High CMCase activity was measured when this fungus was cultivated on xylan, but it did not present high activities against β-glucan. However, opposite result was found when this strain was grown on DEB + SB.

The hydrolytic action of the fungal extracts against mannan polymers was low for all the strains. Nevertheless, activities for heteromannans (glucomannan and galactomannan) were higher than for β-1,4-mannan. This could be explained by the presence of β-(1→4)-glucanase activity in the extracts in the case of glucomannan, and the presence of α-1,6-galactosidase in the case of galactomannan.

Fungal identification

Strain identification was performed using morphological characteristics as well as sequencing of the ITS regions of the ribosomal DNA gene and (in some cases) the partial β-tubulin gene. The best xylanase producer strain, DR02, previously isolated from *Platanus orientalis*, was identified according to morphology (rough dark brown conidia, spherical vesicles and biseriate conidiophores) as *Aspergillus* section *Nigri*. The ITS regions and partial BT2 sequencing were performed and submitted to

GenBank (accession number KC311839, KC311845). The phylogenetic trees, built with reference strains of *Aspergillus Nigri* section species, showed that the DR02 isolate clustered with *A. niger* (Figure 3). Higher value of *A. niger* BT2 clustering confirm the ITS result, the strain DR02 belongs to the *Aspergillus niger* species.

The DR47 strain, which is a good pectinase and β-glucosidase producer, did not present reproductive structures under the microculture technique. As the classical methods did not lead to conclusive results, sequencing of the rDNA ITS regions was performed (GenBank accession number KC311843). The blast alignment suggested that the DR47 isolate belonged to the *Annulohypoxylon stygium* species (EU272517, with 99% similarity). A separation of two groups in the ITS tree constructed with *Annulohypoxylon* and related species was found. One group revealed that the DR47 isolated clustered with *A. stygium* and *Annulohypoxylon urceolatum*, but was closer to *A. stygium.* The second group consisted on *Annuhypoxylon* spp. and *Hypoxylon investiens* (Figure 4A). Sánchez-Ballesteros et al. [18] analyzed the ITS1-5.8S-ITS region, and found that *Annulohypoxylon* spp. cluster inter-mingled with species of the genus *Hypoxylon* section *Hypoxylon*. Therefore, sequencing of partial BT2 was also performed (GenBank accession number KC311846) as suggested by Hsieh et al. [19]. The phylogenetic tree was built and the DR47 isolated was clustered with *A. stygium* species, with a high bootstrap value, and was closer to *A. stygium* than to *Annulohypoxylon stygium* var. *annulatum* (Figure 4B). Besides, *H. investiens* was consistently separated from *Annulohypoxylon.*

The DR49 strain, previously isolated from spoiled books, was identified as *Talaromyces* sp. The Blast alignment of the ITS regions (GenBank accession number KC311844) and partial BT2 (GenBank accession number KC311847) sequences suggest similarity with to *Talaromyces wortmanni*. The trees based on ITS and BT2 sequencing built with close related *Talaromyces* spp. corroborated with the blast aligned. The *Talaromyces* sp. DR49 strain was clustered with *Talaromyces wortmannii* with high bootstrap values in both trees (Figure 5).

The DR40 strain, isolated from *E. benthamii*, was previous identified by macro and micro morphology as *Alternaria* sp. The sequencing of rDNA ITS (GenBank accession number KC311842), suggested that the DR40 isolate belonged to the *Alternaria alternata* species (JQ320281, with 100% similarity) while no amplicon of the BT2 gene was obtained for this strain. The tree based on rDNA ITS sequencing built with correlated species showed no resolution among the strains of the *alternata* species group (Figure 6A). Previous work has also found no genetic variation between the small-spored *Alternaria* species in ITS sequences [20,21]. According to Andrew et al. [22], taxonomical differentiation of the small-spored species within the *alternata* group is difficult, not only because there are few distinguishing morphological characteristics, but also because these characteristics are strongly influenced by the environment. Moreover, the same authors could not solve *Alternaria* spp. that belongs to the *alternata* group using a phylogenic multilocus approach.

The DR17 and DR19 strains were also endophytic isolates from *E. benthamii*, and were morphologically identified as

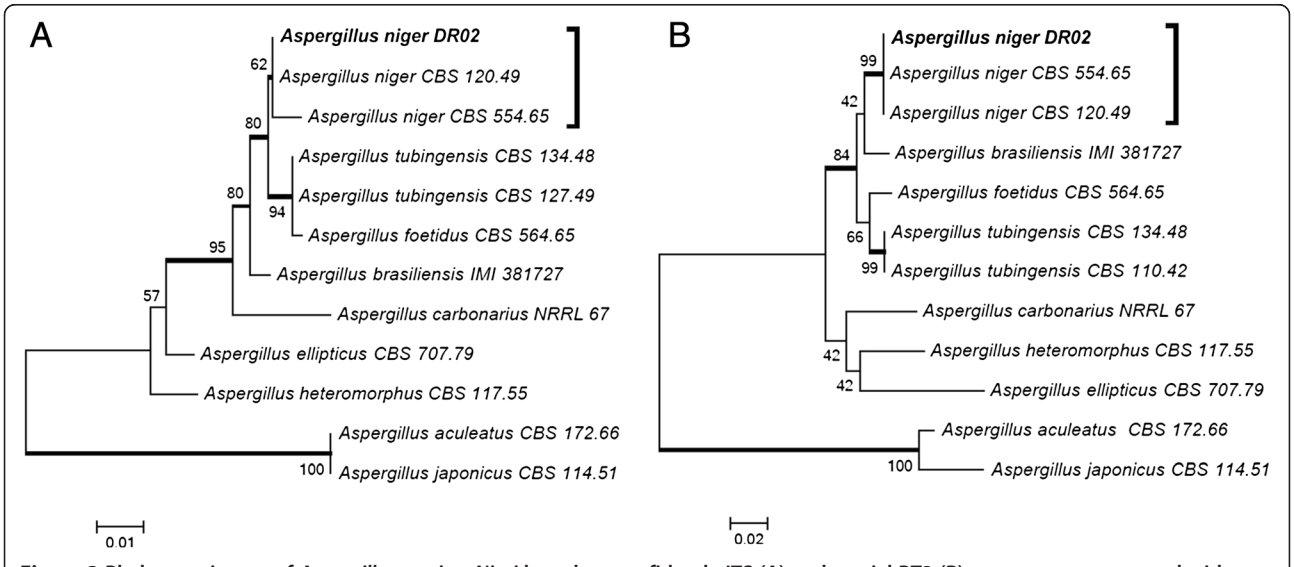

Figure 3 Phylogenetic tree of *Aspergillus* section *Nigri* based on confidently ITS (A) and partial BT2 (B) sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in bold. Strains in bold indicate isolates of this study.

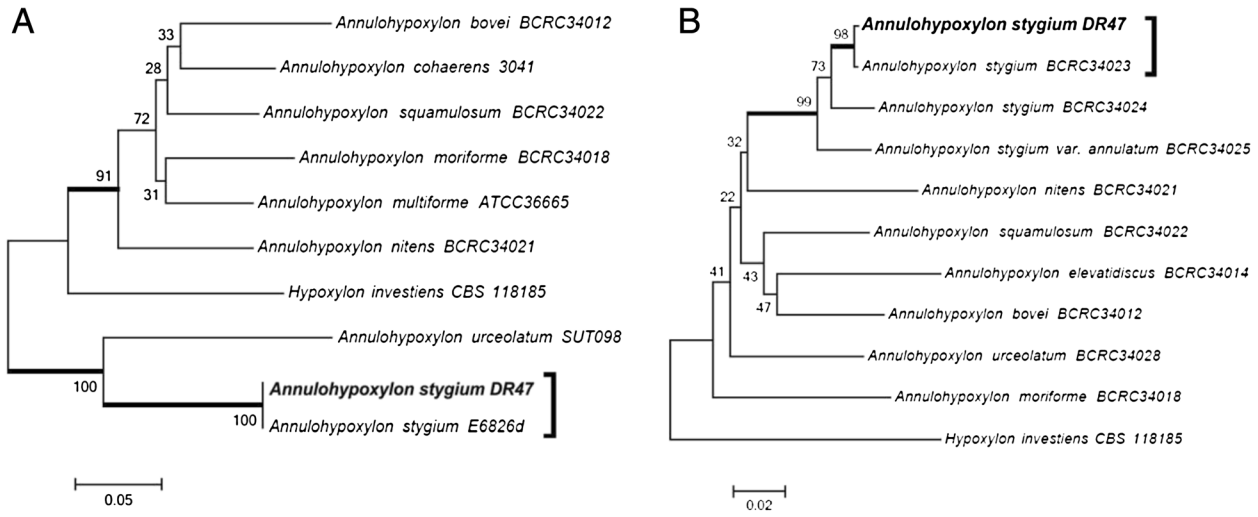

Figure 4 Phylogenetic tree of *Annulohypoxylon* and related species based on confidently ITS (A) and partial BT2 (B) sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in bold. Strains in bold indicate isolates of this study.

Trichoderma sp. The ITS1-5,8S-ITS2 sequences for *Trichoderma* sp. DR17 and *Trichoderma* sp. DR19 (GenBank accession numbers (KC311840, KC31184) aligned with the database *Trichoderma atroviride* strain DAOM 179514 with 100% similarity (EU280125). The tree based on rDNA ITS sequencing (Figure 6B) formed two groups, and the DR17 and DR19 isolates were clustered with the *Viride* clade (*T. atroviride, Hypocrea koningii* and *Hypocrea viridescens*), and were closer to the *T. atroviride* species.

Discussion

High activity, good stability, and low cost are key requirements of enzymes employed for large-scale hydrolysis of lignocellulosic biomass into sugar. Agro-industrial wastes can be useful materials for enzyme development, improvement, and production. The liquor derived from sugar cane bagasse hydrothermal pretreatment is a low cost feedstock [23] rich in xylose and xylo-oligosaccharides which are capable of inducing the expression of xylanases and

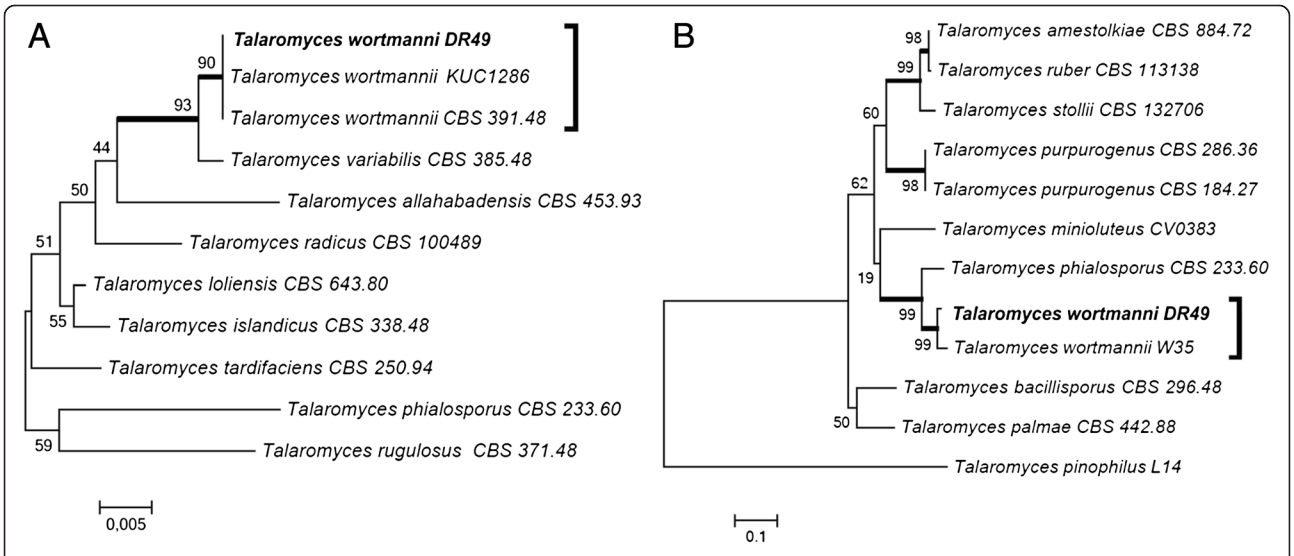

Figure 5 Phylogenetic tree of *Talaromyces* and close related species based on confidently ITS (A) and partial BT2 (B) sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in bold. Strains in bold indicate isolates of this study.

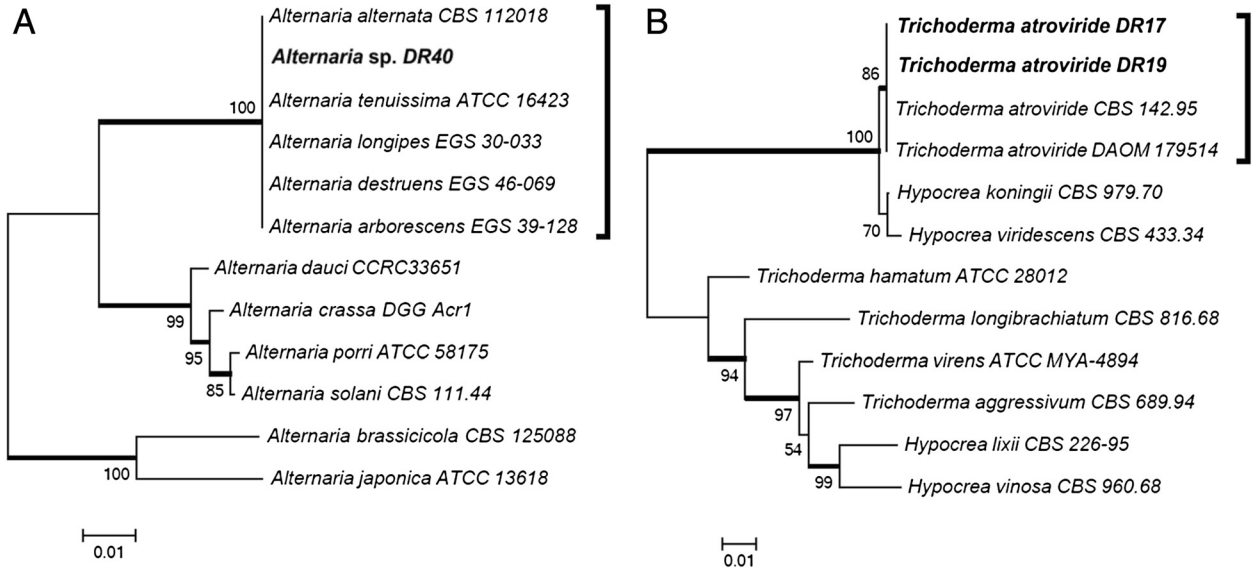

Figure 6 Phylogenetic tree of *Alternaria* (A) and *Trichoderma* (B) species based on confidently ITS sequences constructed with Neighbor-joining implemented in MEGA 4.0.2. Bootstrap values > 80 from 100 resampled datasets are shown with branches in bold. Strains in bold indicate isolates of this study.

accessory proteins in fungi such as *A. niger* [24]. Other materials, such as steam-exploded delignified bagasse and soybean bran, have also been used as inexpensive culture media to achieve high xylanase, cellulase, and β-glucosidase activities employing *Trichoderma harzianum* P49P11 [25].

The full hydrolysis of lignocellulosic biomass requires several types of glycohydrolases that enable the release of saccharides and other compounds from the recalcitrant substrate. However, plant species are highly diverse in terms of cell wall structure and composition, which increases the attraction of formulating specific biomass-degrading enzymatic cocktails. The sugar cane cell wall polysaccharide is mainly composed of xyloglucan and arabinoxylan, closely associated with cellulose, as well as pectin, β-glucan and less branched xylan strongly bound to cellulose [26].

Several studies have shown that supplementing cellulases with other enzymes can assist in the enzymatic hydrolysis of lignocellulosic biomass. Xylanases and β-xylosidases improved the hydrolysis yield when combined with cellulases and β-glucosidades [27-29]. The addition of pectinase to Celluclast 1.5 L increased the hydrolysis of pretreated corn stover [1]. Supplementation of cellulolytic cocktails with α-L-arabinofuranosidase and xylanase also showed a synergistic effect in the hydrolysis of wheat straw [30].

The production of glycohydrolases is closely related to the nature of the carbon source, since fungal metabolism is greatly influenced by the composition of the medium (which also hampers the screening of strains). Each strain has a distinct metabolic profile, while the enzymatic profile is also distinct and depends on the medium and the cultivation time (Table 1).

Physiological variations are the result of the adaptation and evolution of fungi, considering their hosts, original habitats, and other factors. The strain *A. stigyum* DR47 belongs to the Xylariaceae family, members of which are frequently encountered as endophytes and saprophytes [31]. Gazis & Chaverri [32] isolated several endophytic Xylariaceae strains and one strain of *Annulohypoxylon* sp. from *Hevea brasiliensis*. Wei et al. [33] cultivated an *A. stigyum* strain on Avicel and confirmed the production of β-glucosidase, although only low levels of cellulases were detected.

Most *Alternaria* species are saprophytes commonly found in soil or on decaying plant tissues, and some species are opportunistic plant pathogens [34]. However, endophytic strains of *Alternaria* spp. have been isolated from eucalyptus plants such as *Eucalyptus globulus* [35] and *Eucalyptus citriodora* [36]. Strains of *A. alternata* are able to produce endopolygalactunorase [37] in the presence of pectin, and β-glucosidase in the presence of saccharose [38].

A. niger is known worldwide for its ability to produce an extensive range of extracellular glucohydrolases, including xylanases, pectinases, and β-glucosidase [39]. This characteristic is associated with the ability of the fungus to propagate and colonize a variety of environments, principally those rich in decomposing plant materials [40]. The fungus was recently reported to be endophytic in several plant species [41,42]. However, this

work is the first report of *A. niger* as an endophytic fungus in *P. orientalis.*

There have been no previous reports in Brazil concerning *T. wortmannii* isolated from decaying materials. Jang et al. [43] first described β-xylosidase activity in a *T. wortmannii* strain previously isolated from Japanese red pine and larch woods in Korea [44]. Jang et al. [43] obtained a β-xylosidase production of 3.82 U/mL for cultivation on xylan, in good agreement with the β-xylosidase activity (2.85 U/mL) found in the present work for the DR49 strain grown on xylan.

Trichoderma spp. are present in soil as saprophytes, and have also been found as endophytic organisms [45]. Many species from this genus are good cellulase and xylanase producers, such as *T. harzianum* [25] and *T. reesei* [46-48]. *T. atroviride* strains are good producers of these glycohydrolases, and can produce high amounts of β-glucosidase [4].

Conclusions

Microorganisms play an essential role in the degradation of cellulose and hemicellulose standing out the endophytic fungi which are excellent sources of hydrolytic enzymes. Evidently, during the endophytic phase, the use of these enzymes must be related to the mutualistic relationship with the host plant [49]. However, although the association between plants and endophytic fungi is ecologically important, little is known about the physiological characteristics of the interaction.

An important aspect of enzymatic studies involving endophytic fungi is the involvement of these microorganisms in the decomposition of plant material [50,51]. Since the fungi are already present in the senescent plant tissues, they may be able to initiate the decomposition process before it becomes dominated by saprophytic species. This could suggest not only that the production of hydrolytic enzymes by endophytic species might be important for the nutrition of the fungus during the endophytic stage, but also that these enzymes are produced and secreted at the surface of the tissues, where they can compete for the substrate during the saprophytic stage. Kumaresan & Suryanarayanan [52] investigated the ability of endophytic fungi from mangrove leaves of different ages to produce hydrolytic enzymes. It was found that endophytic species occurring at relatively low levels in living leaves were more prevalent after leaf fall, increasing the involvement of these fungi in decomposition of the plant material.

An important consideration is the range of substrates that can be utilized by endophytic microorganisms. Studies have shown that endophytes are capable of metabolizing *in vitro* most substrates found in plants, and produce enzymes including proteases, amylases, phenol oxidases, lipases, laccases, polyphenol oxidases, cellulases, mannanases, xylanases, and pectin lyase [53,54]. The balanced use of microbial enzymes in biomass deconstruction required the understanding of the role played by these glycohydrolases, and also depends on an economic process development. Therefore, biochemical characterization of new reported glycohydrolases producer strains, as well as a bioprocess development of the selected strains in large scale, must be conducted to evaluate the enzyme applicability on the biomass deconstruction, principally on sugar cane bagasse. The present work demonstrated that it is possible to select endophytic fungal strains that can produce glycohydrolases with activities against a wide range of target substrates. This will enable the future formulation of specific enzymatic cocktails for an efficient biomass deconstruction.

Methods

Fungal strains

Hemicellulase bioprospection was performed using a fungus culture collection maintained at the Microbiology and Molecular Biology Laboratory of the Federal University of Paraná (LabMicro/UFPR). A total of 119 Brazilian filamentous fungi were selected, previously isolated from *Eucalyptus benthamii*, *Platanus orientalis*, *Glycine max*, *Solanum tuberosum*, *Saccharum officinarum*, and decaying paper. A strain of *Aspergillus niger* ATCC 64973 was used as a positive control in plate assays. The strains were stored on potato dextrose agar (PDA) slants at 4°C.

Agro-industrial waste materials

The liquor used was derived from the hydrothermal pretreatment of sugar cane bagasse in a reactor (Parr Model 4554, 7.5 L), using 10% (w/w) bagasse, a temperature of 190°C for 10 min, and a 1 h heating gradient. The liquor composition was determined by acid hydrolysis and HPLC [55]. Total soluble lignin was determined by the method described by Gouveia et al. [56]. The sugar cane bagasse was obtained from a local mill (Usina Vale do Rosário, Orlândia, SP, Brazil), and was prepared and characterized by Rocha et al. [57]. The soybean bran (SB) was obtained from Agricola (São Carlos, Brazil) and was characterized by Rodriguez-Zuniga et al. [58].

Hemicellulolytic plate assay

The selection of hemicellulolytic strains was performed by cultivation on solid medium as described by Kasana et al. [59] containing 0.2% beechwood xylan (Sigma) or aqueous liquor diluted in deionized water at a volume ratio of 25%. The strains were first grown on malt extract agar (MEA) for 5 days at 29°C, and then inoculated onto the test media and incubated for 72 h at 29°C. The pH was adjusted to 5.0, and 0.1% Triton X-100 (Merck) was added as a colony growth limiter. The hydrolysis halos were revealed by application of Congo Red (1%)

for 15 min, followed by washing with 1 M NaCl for 10 min [59]. The hydrolysis rates were calculated by dividing the diameters of the hydrolysis halos by the diameters of the colony halos.

β-glucosidase plate assay

The strains were grown for 5 days in liquid medium [29] with carboxymethylcellulose (CMC, 1%) as sole carbon source, in 10 mL tubes (pH 5.0, 200 rpm, 29°C). The biomass was separated by centrifugation, and the extract was subjected to an esculin gel diffusion assay (EGDA), as described by Saqib & Whitney [60], for 5 h at 37°C. The plate was then placed on ice, and measurement was made of the dark brown zone formed by the action of β-glucosidase on esculin.

Shake flask cultures

The composition of the main culture medium was adapted from Mandels & Weber [61], using 10 g/L of pretreated delignified sugar cane bagasse (DEB) plus SB, at a 3:1 ratio [25]. The 56 previously selected fungal strains were grown on PDA for 3 days at 29°C, after which one 0.5 cm diameter disc was removed from each colony edge, transferred to an Erlenmeyer flask containing 20 mL of medium, and incubated for 144 h at 29°C and 200 rpm. The best six strains were selected for growth using the same medium described above, but with the carbon source changed to citrus pectin or beechwood xylan. Samples were removed for determination of enzyme activities and protein contents, as described below.

Enzymatic assays

Measurement of enzymatic activities (in International Units, IU) was performed using different substrates in order to determine global and single activities. Filter paper activity (FPase) was determined as described by Xiao et al. [62]. All the polysaccharides were purchased from Sigma Aldrich or Megazyme, and were assayed at 0.5% in a 10 min reaction. The polysaccharides used were: Beechwood xylan; Birchwood xylan; Rye arabinoxylan; Wheat arabinoxylan; Sugar beet arabinan; CMC; Barley β-glucan; Tamarind xyloglucan; Icelandic moss lichenan; Laminarin from *Laminaria digitata*; Chitosan from shrimp shells; Konjac glucomannan; Carob galactomannan; 1,4 β-mannan and citrus pectin. CMC was assayed in a 30 min reaction. The enzymatic activity was determined from the amount of reducing sugars released from the different polysaccharide substrates, using the DNS method [63] with glucose as standard. The activities of β-glucosidase, β-xylosidase, β-mannosidase, α-L-arabinofuranosidase, and cellobiohydrolase II were measured using the respective p-nitrophenol residues (pNP) (Sigma-Aldrich, USA). The assays employed 10 μL of diluted centrifugation supernatant and 90 μL of the respective pNP (0.5 mM, diluted in citrate buffer), and the mixtures were incubated for 10 min at 50°C. The reactions were stopped by adding 100 μL of 1 M Na_2CO_3, and the absorbance was measured at 400 nm using a Tecan Infinite® 200 instrument (Männedorf, Switzerland). All the assays utilized an epMotion® 5075 automated pipetting system (Eppendorf) and were performed at pH 5,0 with 50 mM citrate buffer. One unit of glycohydrolases activity corresponds to 1 μmol of glucose or pNP released per minute.

Morphological identification

Initial fungus identification was performed using macro and micro morphological characteristics [64-66]. The analysis of fungal reproductive structures by optical microscopy was carried out as described by Kern & Blevins [67].

DNA extraction

An approximately 1 cm^2 colony of 5-day-old cultures was transferred to a 2 mL Eppendorf tube containing 300 μL CTAB (cetyltrimethylammonium bromide) buffer (2% (w/v) CTAB, 1.4 M NaCl, 100 mM Tris–HCl, pH 8.0, 20 mM EDTA, and 0.2% (v/v) β-mercaptoethanol) and about 80 mg of a 2:1 (w/w) mixture of silica gel H (Merck) and Celite™ 545 (Macherey Nagel & Co). The cells were disrupted manually with a sterile pestle for about 5 min. Subsequently, 200 μL CTAB buffer was added, and the mixture was vortexed and then incubated for 10 min at 65°C. After the addition of 500 μL chloroform, the solution was mixed and centrifuged for 5 min at 20,500 × *g*. The supernatant was transferred to a new tube, together with 2 volumes of ice-cold 96% ethanol. The DNA was allowed to precipitate for 30 min at −20°C, after which centrifugation was performed for 5 min at 20,500 × *g*. After washing with cold 70% ethanol and drying at room temperature, the pellet was resuspended in 97.5 μL TE buffer together with 2.5 μL RNAse (20 U/mL), and incubated for 5 min at 37°C, before storage at −20°C [68].

DNA amplification and sequencing

The rDNA Internal Transcribed Spacer (ITS) region was amplified using ITS5 and ITS4 primers [69]. Partial β-tubulin (BT2) gene was amplified using Bt2a and Bt2b primers [70]. The sequencing of β-tubulin gene was performed for some strains to confirm the ITS phylogeny clustering. Amplicons were cleaned with a GFX™ PCR DNA purification kit (GE Healthcare, UK). Sequencing was performed on an ABI 3130 automatic sequencer (Applied Biosystems). The Staden sequence analysis package (v. 1.6.0) was used to edit and align the sequences [71]. Sequence analysis was performed using BLASTn sequence alignment software, run against the NCBI (National Center for Biotechnology Information) database. The phylogenetic trees were constructed with

1000 bootstrap replicates using MEGA v4.0.2 software [72], with application of the neighbor-joining method [73], the Jukes-Cantor distance correction model [74]. The nucleotide sequences used in this study were obtained/submitted to GenBank (Additional file 1: Table S2).

Additional file

Additional file 1: Table S1. Hydrolysis rate of the bioprospected fungal strains. **Table S2.** Nucleotide sequences of fungal strains submitted to GenBank.

Abbreviations

DEB: Deglignified sugar cane bagasse; SB: Soybean bran; CMC: Carboxymethylcellulose; FP: Filter paper; ITS: Internal transcribed spacer; BT2: β-tubulin gene; EGDA: Esculin gel diffusion assay; pNP: p-nitrophenol.

Competing interests

JGP is employed at CTBE; GP at USP, ICP and VAV at UFPR; CMM, PSD, PSC and DR are M.Sc and Ph.D students respectively; JDR posdoctoral at USP.

Authors' contributions

GP, JGP, JDR, DR conceived the study and wrote the paper; DR, CMM, PSD, PSC produced the biological, enzymatic data; JDR and DR performed phylogenetic studies; ICP, VAV, JDR and DR isolated, preserved and identified fungal strains. All authors read and approved the manuscript.

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Appendix H - Enhancing of sugar cane bagasse hydrolysis by *Annulohypoxylon stygium* glycohydrolases

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Enhancing of sugar cane bagasse hydrolysis by *Annulohypoxylon stygium* glycohydrolases

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HIGHLIGHTS

- Process to produce β-glucosidase and pectinase by *Annulohypoxylon stygium*.
- Increase sugarcane bagasse hydrolysis with the produced extract.
- Partial replacement of Celluclast 1.5L by the extracts produced in hydrolysis.
- Understand the supplementation with secretome of the enzymes extract produced.

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ABSTRACT

The aim of this study was to develop a bioprocess for the production of β-glucosidase and pectinase from the fungus *Annulohypoxylon stygium* DR47. Media optimization and bioreactor cultivation using citrus bagasse and soybean bran were explored and revealed a maximum production of 6.26 U/mL of pectinase at pH 4.0 and 10.13 U/mL of β-glucosidase at pH 5.0. In addition, the enzymes extracts were able to replace partially Celluclast 1.5L in sugar cane bagasse hydrolysis. Proteomic analysis from *A. stygium* cultures revealed accessory enzymes, mainly belong to the families GH3 and GH54, that would support enhancement of commercial cocktail saccharification yields. This is the first report describing bioreactor optimization for enzyme production from *A. stygium* with a view for more efficient degradation of sugar cane bagasse.

1. Introduction

The complexity of lignocellulose material makes this biomass highly recalcitrant to decomposition for biotechnology applications e.g. production of biofuels. Development of enzyme extracts and purified enzyme combinations can produce hydrolytic cocktails to improve hydrolysis processes, increase product yields with shorter reaction times and reduced feedstock and bioreactor capital investment (Gao et al., 2010).

It is known that the cellulolytic complex used in the enzymatic hydrolysis benefited, in terms of yield and conversion speed, when supplemented with accessory enzymes as, for example, hemicellulases and pectinases (Berlin et al., 2007; Gusakov et al., 2007). For example, the addition of a commercial pectinase at cellulolytic enzyme extracts increased the hydrolysis yield of corn stover pretreatment with acid (Berlin et al., 2007), and delignified/exploded sugar cane bagasse (Delabona et al., 2013a).

The β-glucosidase supplementation of cellulolytic systems is commonly used, considering that the major cellulolytic extracts are not well balanced for an efficient saccharification, like for *Trichoderma reseei* (Berlin et al., 2007). Besides, the supplementation with a β-glucosidase from another fungus can be employed to reduce the cellobiose inhibition over cellobiohydrolases and endoglucanases (Xiao et al., 2004). However, high glucose concentrations and thermal stability can affect the commercial use of β-glucosidases (Bhatia et al., 2002).

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New fungi strains and consequently new enzymes can be the key for a better biomass hydrolysis process principally regarding to broader substrate specificities and improved biophysical properties. Thereby, microorganisms hydrolases from diverse environments have been extensively searched, as desert (Moreno et al., 2012), rain forest soils (Delabona et al., 2012) and microbial endophytes of plants (Wipusaree et al., 2011).

The ascomycete fungus *Annulohypoxylon stygium* is an ascomycete that belongs to the *Xilariaceae* family. Members of this genera are commonly find as endophytic or saprophytic (Stone et al., 2004), but have been briefly studied for glycohydrolases production. In *A. stygium* there is a report of β-glucosidase activity (Wei et al., 1992). However, others activities has been reported with *A. stygium* such as pectinases, xylanases and β-glucanases when grown using xylan, pectin and sugar cane bagasse substrates (Robl et al., 2013).

The enzyme production cost for biomass deconstruction is related mainly with the productivity system, the amount of enzyme produced by time unit and reactor volume (Klein-Marcuschamer et al., 2012). The development of strategies that can produce several glycohydrolases could be an alternative for the enzymes production cost reduction. The industrial agro wastes have biotechnological potential and can be employed in byproducts production and shows to be a great opportunity to achieve satisfactory prices. This study aimed to develop a process for the production and use of β-glucosidase and pectinase from *A. stygium* strain DR47 with a view to explore use of these enzymes for improved sugar cane bagasse deconstruction.

2. Methods

2.1. Strains

Strain *A. stygium* DR47 is an endophytic fungal strain of *Eucalyptus benthamii* that was obtained from the culture collection of the Microbiology and Molecular Biology Laboratory at the Federal University of Paraná (LabMicro/UFPR). The strain was maintained on potato dextrose agar (PDA) slants at 4 °C.

2.2. Agro-industrial waste materials

Sugar cane bagasse was obtained from a local mill (Usina Vale do Rosário, Orlândia, SP, Brazil), and was prepared and characterized by Rocha et al. (2012). Soybean bran (SB) and wheat bran (WB) were obtained from Agricola (São Carlos, Brazil) and were characterized by Rodriguez-Zuniga et al. (2011). Apple bagasse (AB) was obtained from Yakult S.A. (Lages, Brazil) and the citrus bagasse (CB) was purchased by Hildebrand (São Carlos, Brazil). Both AB and CB are the residues obtained directly from the juice extractor.

2.3. Pre-culture and production media

The composition of the medium was adapted from Mandels and Reese (1960): 1 mL Tween 80; 0.3 g L^{-1} urea; 2.0 g L^{-1} KH_2PO_4; 1.4 g L^{-1} $(NH_4)_2SO_4$; 0.4 g L^{-1} $CaCl_2{\cdot}2H_2O$; 0.3 g L^{-1} $MgSO_4{\cdot}7H_2O$; 1.0 g L^{-1} proteose peptone; 5.0 mg L^{-1} $FeSO_4{\cdot}7H_2O$; 1.6 mg L^{-1} $MnSO_4{\cdot}4H_2O$; 1.4 mg L^{-1} $ZnSO_4{\cdot}7H_2O$; 2.0 mg L^{-1} $CoCl_2{\cdot}6H_2O$; 10 g L^{-1} glucose (carbon source). The pH was adjusted to 5.0 and the culture medium was sterilized at 121 °C for 20 min. The composition of the production medium was the same as that of the pre-culture medium, except for the type of carbon source. Seven different carbon sources were evaluated at 10 g/L: hydrothermal bagasse (HB), EB (steam exploded bagasse), DEB (deslignified/steam exploded bagasse), SB, WB, AB and CB.

2.4. Shake flask cultures

Inoculum was prepared by adding 20 mL of sterilized distilled water and Tween 80 (0.01%) to mature colonies of *A. stygium* DR47 grown on PDA plates (7 days at 29 °C). The biomass was transferred to Erlenmeyer flasks containing 180 mL of pre-culture medium and incubated for 48 h at 29 °C on a rotary shaker at 200 rpm. A volume of 20 mL of this pre-culture was transferred to 500 mL Erlenmeyer flasks containing 180 mL of the production medium and incubated at 29 °C on a rotary shaker at 200 rpm for 144 h.

2.5. Response surface methodology (RSM)

To select the best carbon source to support optimum β-glucosidase and pectinase activities, a rotated full factorial design was done with data from shake flask experiments. The data analysis and the medium optimization were performed with Minitab (Release 14) statistical software (Minitab Inc., USA). Cultivations were realized with phthalate buffer (50 mM) as descripted by Ferreira et al. (2009) in order to minimize the pH alterations. It was tested the best carbon source for pectinase production (CB), the best carbon source for β-glucosidase production (SB) and also sucrose (SUC) as a β-glucosidase inductor. Delabona et al. (2013b) verified that sucrose could induce the β-glucosidase production on a mixture composition of DSB, SB and SUC by *Trichoderma harzianum*. The complete factorial experimental design was performed with 3 factors, 2 levels, 2 axial points and 6 replicates of the central point, totalizing 20 experiments (Appendix A). All variables were studied on the levels 4.05 g/L (−1), 15.95 g/L (+1), 10 g/L (0), 0 g/L (−1.68) and 20 g/L (+1.68) and the results were fitted to the quadratic model. The data were not transformed for the analysis.

2.6. Bioreactor cultures

Bioreactor cultures were conducted in a 3.0 L Bioflo 115 stirred tank reactor (STR) (New Brunswick Scientific Co., USA) equipped with automatic control of temperature (29 °C), pH (5.0), agitation rate (200–500 rpm) and aeration rate (0.3–1.0 L/min). The pH was controlled by the automatic addition of either 0.4 M H_2SO_4 0.4 M or 1:3 (v/v) NH_4OH:H_2O. The dissolved O_2 level was kept above 30% of air saturation by automatic adjustment of aeration and agitation. Foaming was manually controlled by the addition of polyglycol antifoaming agent (FluentCane 114, DOW Chemical, Brazil). A working of volume of 1 L was inoculated with 10% (v/v) inoculum from the pre-culture same as described previously. Samples were periodically withdrawn, centrifuged at 10,000×*g*, 10 °C for 15 min and analyzed for protein content and enzymatic activities.

2.7. Crude enzyme characterization: influence of pH temperature and thermal stability

Culture supernatants produced under optimal STR production conditions were assayed for β-glucosidase and pectinase activities at different reaction temperatures (20–80 °C) in 50 mmol/L sodium citrate buffer (pH 5.0). The effect of pH on enzyme activities (at 50 °C for β-glucosidase and 37 °C for pectinase) was determined using 50 mmol/L citrate–phosphate buffer (pH 3.0–8.0). For thermal stability determination, crude supernatant obtained under the optimal production conditions was incubated at 40, 45, 50 and 60 °C for 24 h, in the absence of substrate. The residual enzyme activity was measured after different time intervals. Measurement of enzyme activity was performed under standard pH and temperature conditions.

2.8. Enzymatic activity assays

Total cellulolytic activity was measured as Filter paper activity (FPase), as described by Ghose (1987). Others enzymatic activities were measured in International Units (U). All polysaccharide substrates were purchased from Sigma Aldrich or Megazyme and were assayed at 0.5% (w/v) in a 10 min reaction at 50 °C, except for pectin which was assayed at 37 °C. Enzyme activity was determined as the amount of reducing sugars released from the different polysaccharide substrates using the DNS method with glucose, xylose or acid galacturonic as standards. The activities of β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, β-galactosidase and cellobiohydrolase were measured at pH 5.0 using the p-nitrophenol assay (pNP) (Sigma–Aldrich, USA). This assay used 10 μL of diluted supernatant and 90 μL of the respective pNP (0.5 mM, diluted in 50 mM citrate buffer at pH 5.0). The mixtures were incubated for 10 min at 50 °C and the reactions stopped by addition of 100 μL 1 M Na_2CO_3. The absorbance was measured at 400 nm using a Tecan Infinite® 200 instrument (Männedorf, Switzerland).

2.9. Total protein determination

Total protein in centrifuged supernatants was determined using the Bio-Rad protein assay reagent following manufacturer's instructions (Bio-Rad Laboratories, USA). Bovine serum albumin was used as standard.

2.10. Sugar cane bagasse hydrolysis

Hydrothermal pre-treated sugar cane bagasse (HB) was subjected to enzymatic saccharification combining two different enzyme preparations produced in bioreactor with a commercially available enzyme preparation (Celluclast 1.5L, Novozymes). The enzymatic hydrolysis were performed with 5% (w/v) of HB and sodium azide 0.02% (v/v) in 50 mM citrate buffer, pH 5.0. The reactions were carried out in 2 mL Eppendorf tubes using a Thermomixer microplate incubator (Eppendorf, Germany) operated at an agitation speed of 1000 rpm for 24 h. First a saturation curve for each extract was performed using a fixed Celluclast 1.5L loading of 10 FPU/g of bagasse at 40 °C, 50 °C and 60 °C. Then the effects of partial replacement of Celluclast 1.5L by the enzymatic extracts produced were assessed in the HB hydrolysis with a total fixed concentration of 12 mg of protein per g of bagasse. Samples were centrifuged at 10,000×*g* for 15 min (5418 Centrifuge, Eppendorf) filtrated (Sepak C18, Waters) and carbohydrate concentrations were either determined by the DNS method or by HPLC as described by Rocha et al. (2012).

2.11. Proteomic analyses

Proteomic analysis of fungal extracts from bioreactor cultures grown at pH 4.0 and pH 5.0 was performed by liquid chromatography coupled in-line to mass spectrometry. A volume of supernatant containing 10 μg of total proteins was first separated by 1D SDS–PAGE. Each sample was run in three lanes on the gel, and each lane was then divided into six slices (70–100, 55–70, 40–55, 35–40, 25–35, and 5–25 KDa). The slices were de-stained, reduced and alkylated by carboxymethylation and then in-gel digested overnight using sequencing-grade modified trypsin (Promega, USA) (Shevchenko et al., 2007). Each gel slice was re-suspended in 12 μL of 0.1% formic acid and an aliquot (4.5 μL) of the resulting peptide mixture was separated using an RP-nanoUPLC C18 column (nanoAcquity, 100 μm × 100 mm, Waters) coupled to a Q-Tof Ultima mass spectrometer (Waters) fitted with a nano-electrospray source operated at a flow rate of 0.6 μL/min. The gradient was 2–90% acetonitrile in 0.1% formic acid over 60 min. The instrument was operated in 'top three' mode, in which one MS spectrum is acquired, followed by MS/MS of the three most intense peaks detected. The spectra were acquired using MassLynx v.4.1 software and the raw data files were converted into a peak list format (mgf), without summing the scans, using Mascot Distiller v.2.3.2.0 2009 software (Matrix Science Ltd.) and then searched against the NCBI taxonomical database for fungi using the MASCOT v.2.3.01 search engine (Matrix Science Ltd.). Carbamidomethylation was used as a fixed modification and oxidation of methionine was used as a variable modification, with one trypsin missed cleavage and a tolerance of 0.1 Da for precursors and fragment ions. Scaffold v.3.6.1 (Proteome Software Inc., Portland, OR) was used to validate the MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability, as specified by the Peptide Prophet algorithm (Keller et al., 2002). Peptide identifications were also required to exceed specific database search engine thresholds. Mascot identifications required ion scores to be at least greater than both the associated identity scores and 30. Protein identifications were accepted if they showed greater than 90.0% probability and contained 2 or more identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated using MS/MS analysis alone were grouped together for parsimony.

3. Results and discussion

3.1. Effect of carbon source on enzyme production in shake flasks

A. stygium was initially grown in shake flasks in order to evaluate the influence of different carbon sources on β-glucosidase and pectinase production. Insoluble carbon sources rich in cellulose (HB, EB and DEB), hemicellulose (SB and WB) and pectin (AB and CB) were used at concentrations of 1% (w/v). Fig. 1 shows the pectinase and β-glucosidase activities as a time course over 144 h of fermentation. Evaluation of carbon sources indicated that pectinase production was strictly associated when the fungus was grown using pectin as the carbon source (AB and CB). The use of CB resulted in the highest pectinase production after 48 h.

Evaluation of β-glucosidase production under industrial agro-industrial wastes showed that the highest enzyme titrates were obtained on substrates rich in hemicellulose and pectin (Fig. 1B), but not when the fungus was grown on sugar cane bagasse. The best results were in SB (3.9 U/mL), WB (3.0 U/mL) and CB (2.5 U/mL) after 144 h. This result may indicate that the β-glucosidase production may not be totally associated to the carbon source, and might be related with the fungal growth, once that the hemicellulose and pectin are polysaccharides with easier degradability than cellulose.

The SB was a good source to produce β-glucosidase and CB was a good source to produce pectinase and β-glucosidase. Citrus-process wastes are used as substrates for the bio-production of other products including citric acid (Rodrigues et al., 2009), flavor (Rossi et al., 2009) and phytases (Spier et al., 2008). These feedstocks are also well known sources for pectinolytic enzymes production, but less so for other glycohydrolases. Mamma et al. (2008) used citrus peel to produce pectinolytic, cellulolytic and xylanolytic enzymes from *Aspergillus niger*, *Fusarium oxysporum*, *Neurospora crassa* and *Penicillium decumbens* under solid-state fermentation conditions. In the same way, wastes from soybean manufacturing processes have also been extensively as sources of enzymes for biomass degradation. Vitcosque et al. (2012) and Delabona et al. (2013b) used soybean bran to produce cellulases,

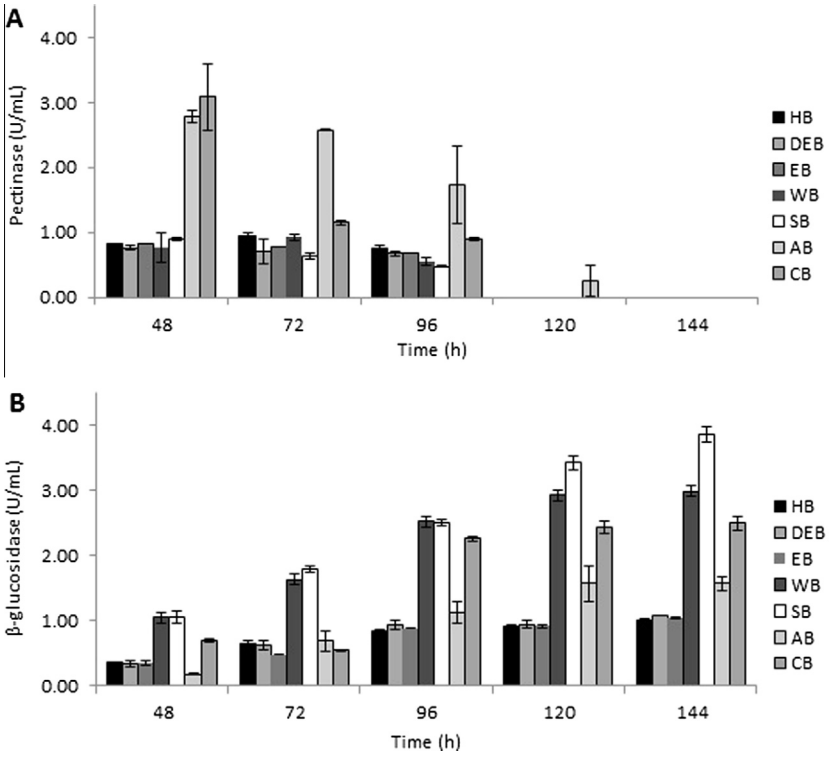

Fig. 1. Influence of different carbon sources on the pectinase (A) and β-glucosidase (B) production by *Annulohypoxylon stygium* DR47 during submerged fermentation in flasks.

Table 1
Pectinase (48 h) and β-glucosidase (144 h) activities for *Annulohypoxylon stygium* DR47 response surface experiment using shaking flaks (29 C, pH 5.0, 200 rpm). The values in bold represent the maximum and minimum activities obtained.

Run number	SB (g/L)	CB (g/L)	SUC (g/L)	Pectinase (U/mL)	β-Glucosidase (U/mL)
1	**4.05**	**4.05**	**4.05**	**2.24**	**3.44**
2	15.95	4.05	4.05	3.51	6.06
3	4.05	15.95	4.05	3.83	4.95
4	15.95	15.95	4.05	6.3	7.16
5	4.05	4.05	15.95	5.05	2.34
6	15.95	4.05	15.95	6.25	4.56
7	**4.05**	**15.95**	**15.95**	**5.6**	**1.8**
8	**15.95**	**15.95**	**15.95**	**7.05**	**2.76**
9	0.00	10.00	10.00	2.47	1.67
10	20.00	10.00	10.00	5.72	4.8
11	10.00	0.00	10.00	3.93	4.95
12	10.00	20.00	10.00	2.8	4.66
13	**10.00**	**10.00**	**0.00**	**2.76**	**7.58**
14	10.00	10.00	20.00	3.98	2.19
15	10.00	10.00	10.00	4.7	4.94
16	10.00	10.00	10.00	4.3	4.8
17	10.00	10.00	10.00	4.41	4.28
18	10.00	10.00	10.00	4.42	4.81
19	10.00	10.00	10.00	3.76	5.01
20	10.00	10.00	10.00	3.64	5.36

xylanases and β-glucosidases by *A. niger* and *T. harzianum* to hydrolyze pretreated sugar cane bagasse.

3.2. Optimal media composition design

Media formulation and optimization are required for the commercial success of any biotechnology process. In this study media for the production of β-glucosidase and pectinase using two feedstocks (CB and SB), together with the low cost sugar saccharose was developed using RSM.

Table 1 summarizes the different combinations of SB, BC and SUC concentrations used to culture *A. stygium* and the maximum activities of pectinase at 48 h and β-glucosidase at 144 h. Maximum pectinase activity obtained in these experiments ranged from 2.24 (run 1) to 7.05 U/mL (run 8), with maximum β-glucosidase activities ranging from 1.08 (run 7) to 7.58 U/mL (run 13).

The influence of medium composition on pectinase and β-glucosidase biosynthesis was estimated by examining the statistical significance of each component. In terms of pectinase activity at 48 h, three substrates (SB, BC and SUC) did not show a statistically significant influence ($p > 0.1$) on enzyme activity and the results did not fit well to the quadratic model used. In this way, pectinase activity was measured at 96 h, but the values obtained were much lower than 48 h (data not shown).

Statistical analyses of β-glucosidase activity showed a significant positive influence of the components and enabled the definition of a quadratic model with determination coefficient of 97.6%. The equation thus obtained was:

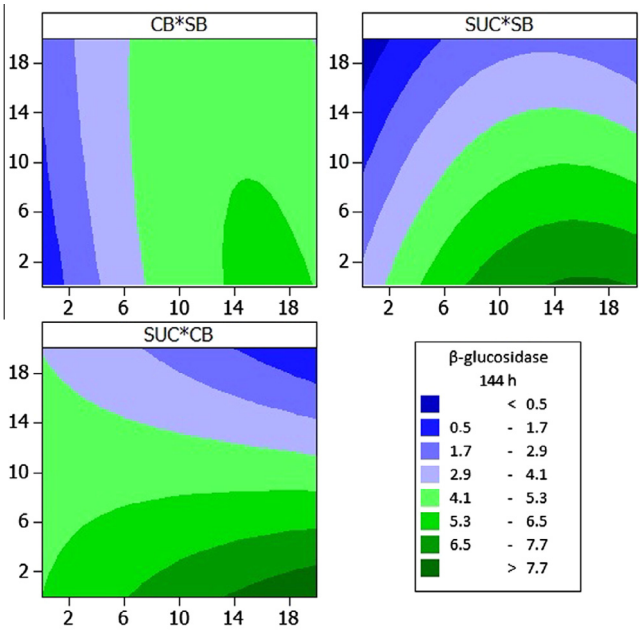

Fig. 2. Contour plots of β-glucosidase activity for the *Annulohypoxylon stygium* DR47 response surface experiment, using the culture medium components (g/L) citrus bagasse (CB), sucrose (SUC), and soybean bran (SB). Hold values 10 (g/L) for which component.

$$\beta\text{-glucosidase (U/mL)} = 0.7844 + 0.6243 * SB + 0.2594 * CB + 0.0092 * SUC - 0.0171 * SB^2 - 0.0014 * CB^2 - 0.0006\ SUC^2 - 0.0059 * SB * CB - 0.0058 * SB * SUC - 0.0176 * BC * SUC$$

The contour plots for β-glucosidase activity showed that higher amounts of SB and CB are associated with the increased of enzyme activity production and that SUC produces a negative effect when associated with SB and BC (Fig. 2).

The aim of the composition design was to obtain one media for the production of both enzymes. Therefore, the Minitab response optimizer was used with the data of pectinase activity at 48 h and β-glucosidase at 144 h. Even though the pectinase data had not fit the quadratic model well ($p = 0.162$), the equation were used for optimization since pectinase activity was highest at this time point.

$$\text{Pectinase (U/mL)} = 0.7650 - 0.0123 * SB + 0.1042 * CB + 0.2694 * SUC + 0.0073 * SB^2 - 0.0001 * CB^2 + 0.0001 * SUC^2 + 0.0051 * SB * CB - 0.0039 * SB * SUC - 0.0107 * BC * SUC$$

The response media optimization was performed by giving equal weights for each response variable; maximum concentration of each component was 2% (w/v) and sought to maximize the values of pectinase and β-glucosidase. The best composition was SB 20 g/L, CB 20 g/L and SUC 2.42 g/L. The predicted enzyme activities were 5.32 U/mL of pectinase at 48 h and 7.41 U/mL of β-glucosidase at 144 h with composite desirability of 84.6%.

The optimized media was then tested at three growth temperatures (25 °C, 29 °C and 32 °C) to refine glycohydrolases production. The enzyme activities corroborated with the results obtained of cultures grown at 29 °C (Table 2), indicating that the experimental data fitted to the models tested. In addition, changing temperature as a growth parameter did not statistically influence ($p > 0.05$) enzyme activity (by post hoc Tukey test). Moreover, an increase in β-glucosidase production and a decrease in the pectinase production were observed at higher growth temperatures, so a temperature of 32 °C that gave median activities for the two enzymes was selected for further STR experiments.

Table 2
Pectinase and β-glucosidase activities for *Annulohypoxylon stygium* DR47 growth in the optimized media at different temperatures using shaking flaks (pH 5.0, 200 rpm).

Pectinase (U/mL)[A] 48 h		β-Glucosidase (U/mL)[B] 144 h
26 °C	5.19 ± 0.38 a	6.99 ± 0.86 a
29 °C	5.29 ± 0.50 a	8.14 ± 0.55 a
32 °C	4.84 ± 0.48 a	9.02 ± 0.51 a

Means calculated from 3 replications. Data not transformed. Means followed by the same small letter do not differ among them by Tukey test at 5%.
[A] C.V = 10.65%.
[B] C.V = 8.90%.

3.3. Batch bioreactor

Cultivations at pH 4.0, 5.0 and 6.0 were performed to evaluate the kinetics of enzyme production in a controlled batch environment, especially to the effects of oxygen and mass transfer. Experiments in bioreactors were conducted in duplicated for pH 5.0 (Fig. 3).

Both enzymes showed similar production profiles in batch bioreactor fermentations to shake flask fermentations. Pectinase production peaked at 72 h (6.26 U/mL) at pH 4.0 (Fig. 3A), which indicates that values above 5.0 can influence negatively in the pectinase production. Moreover, β-glucosidase activity was higher (10.13 U/mL) at 144 h at pH 5.0 (Fig. 3B).

Although the optimized media formulation did support high titres of enzyme production for both β-glucosidase and pectinase, it was not possible to perform the fermentation at the same pH to support the maximum activities of both enzymes. Growth in bioreactors led a high titration of pectinase and β-glucosidase at pH 4.0 and 5.0. At pH above 5.0 we found pectinase activity production was reduced (Fig. 3A). Acunaarguelles et al. (1995) also demonstrated that pectinase activity produced by *A. niger* also declined above pH 5.0 due to denaturation.

3.4. Multienzyme characterization: influence of temperature and pH and thermal stability

Pectinase activity of the extract produced in STR was measured at different temperatures and ranges of pH (Appendix B). Extracts had highest pectinase activity across a range of temperatures from 35 °C to 50 °C, but optimally at 45 °C. The same extract showed highest activity at pH 5.0 and maintained 90% of the relative activity between pH 4.0–5.5.

β-Glucosidase activity of the extract produced in STR was measured at different temperatures and ranges of pH (Appendix B). Extract had highest β-glucosidase activity at 60 °C, with 95% of the maximum activity remaining between 55 °C and 65 °C. Enzymes with activities higher than 50 °C could be the key for a more efficient biomass hydrolysis, minimizing process problems. This extract gave greatest β-glucosidase activity at slightly acidic conditions, with an optimum pH around 4.5.

The thermal stability is another important parameter for the potential application of fungi in large-scale biomass hydrolysis processes. In terms of thermal stability, pectinase showed low stability, at 40 °C only 30% of activity was lost after 3 h of incubation, while 80% of the activity had been lost after incubation at 50 °C for 30 min (Fig. 4). However, β-glucosidase was very thermal stable, retaining about 96.5% activity after 24 h incubation at 50 °C (Fig. 4). The enzyme also retained about 50.2% activity after 9 h incubation at 60 °C.

Like any enzyme catalyzed reaction, the rate of hydrolysis catalyzed by glycosidases is influenced by temperature and pH. As far

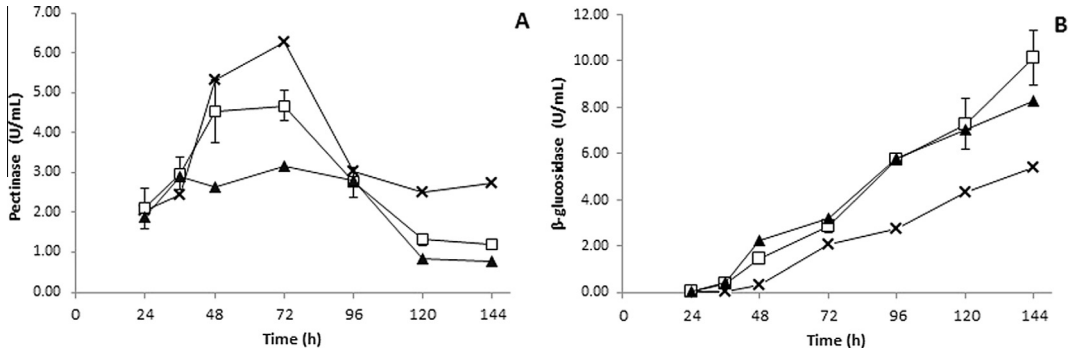

Fig. 3. Pectinase (A) and β-glucosidase (B) activities of *Annulohypoxylon stygium* DR47 cultivation on STR in pH 4.0 (X), pH 5.0 (□) and pH 6.0 (▲) at 32 °C.

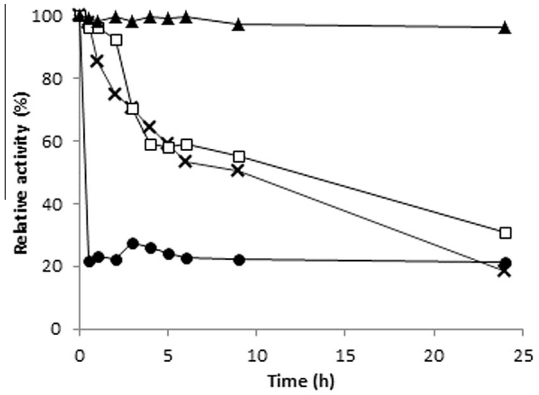

Fig. 4. Residual activity expressed as a percentage of the maximum enzymatic activity produced by *Annulohypoxylon stygium* DR47 growth in STR. The thermal stability of pectinase at 40 °C (□) and 50 °C (●); β-glucosidase activity at 50 °C (▲) and 60 °C (X).

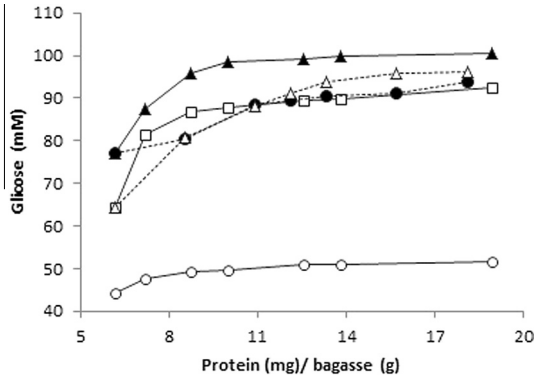

Fig. 5. Hydrolysis saturation curve of the Celluclast 1.5L supplementation with *Annulohypoxylon stygium* DR47 enzymes. Extracts produced at pH 4.0 (dotted line): 40 °C (△), 50 °C (●); pH 5.0 (continuous line): 40 °C (□), 50 °C (▲) and 60 °C (○).

as we know, there are only reports of β-glucosidase activity but not the effects of temperature and pH in fungi including *A. stygium*, *Hypoxylon* spp. and *Xylaria* spp. (Robl et al., 2013; Wei et al., 1992). *Daldinia eschscholzii* is another specie of the family *Xylariaceae* where β-glucosidase activity has been characterized (Karnchanatat et al., 2007), and was shown to have optimum activity at pH 5.0 and 50 °C. The extracts produced from *A. stygium* DR47 in this study showed activity over wide ranges of temperature and pH consistent with these previously reported fungi.

3.5. Sugar cane bagasse hydrolysis

The enzymatic extracts produced in bioreactors at pH 4.0 and pH 5.0, were rich in pectinase and β-glucosidase. These extracts were used to supplement a commercially available cellulolytic extract (Celluclast 1.5L) and were tested for HB hydrolysis. The major glycohydrolases from sugar cane bagasse were measured (Table 3). The extracts produced in this study presented low cellulolytic activities, but significant amounts of β-glucanases activities were observed. Also, low activities of other enzymes such as arabinofuranosidase, β-glucanase also were measured.

Table 3
Specific enzymes activities for some important glycohydrolases of *Annulohypoxylon stygium* DR47 extracts and Celluclast 1.5L.

Activity (U/mg)	Extract pH 5.0	Extract pH 4.0	Celluclast 1.5L
FPAse	0.14	0.20	1.71
β-Glucanase	22.55	7.44	62.64
Pectinase	1.30	16.12	0.10
β-Glucosidase	17.19	5.77	1.20
Xylanase	0.84	2.25	8.75
Xyloglucanase	1.68	0.52	30.81
Cellobiohydrolase	1.21	0.79	0.33
β-Xylosidase	0.13	0.05	0.08
α-L-Arabinofuranosidase	0.03	0.03	0.01
β-Galactosidase	0.19	0.40	0.01

Table 4
Hydrolysis analyses of the partial replacement of Celluclast 1.5L by *Annulohypoxylon stygium* DR47 extracts.

	Celluclast 1.5L	Celluclast 1.5L + extract pH 4.0 + extract pH 5.0	Extract pH 4.0 + extract pH 5.0
Monosaccharides (g/L)	13.775	13.530	1.644
Glucose (g/L)	12.556	12.161	0.836
Xylose (g/L)	1.219	1.369	0.808
Arabinose (g/L)	0.000	0.000	0.000
Cellobiose (g/L)	0.000	0.161	0.184
Acetic acid (g/L)	0.138	0.148	0.065

Celluclast 1.5L present low amount of β-glucosidase and pectinase activity, which is well documented in *T. reseei* cellulolytic complexes. The hydrolysis saturation curves (Fig. 5) indicated that the addition of *A. stygium* DR47 extracts increased sugar cane hydrolysis. Pectin extract supplementation presented similar behavior at 40 °C and at 50 °C, and the β-glucosidase showed higher hydrolysis at 50 °C. Besides, a saturation load can be visualized from 13 mg of protein/g of bagasse for the extract rich in pectinase and 10 mg of protein/g of bagasse for the extract rich in β-glucosidase.

The protein load in a biomass hydrolysis influences directly in the process cost. For this reason low protein loads, combining different types of enzymes has been studied to improve the

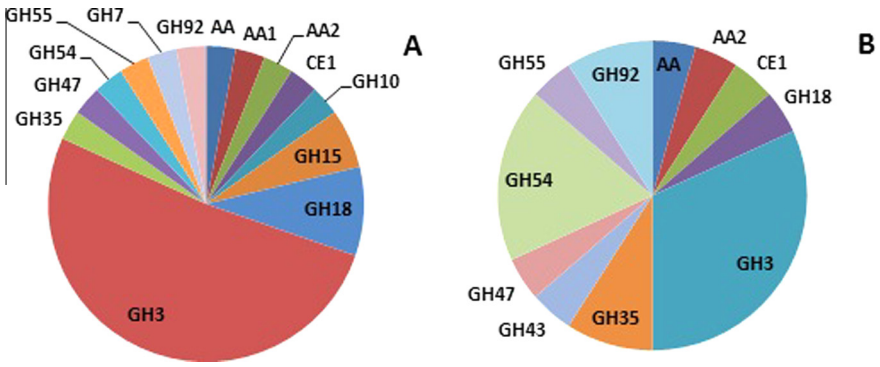

Fig. 6. GH's family detected based on unique peptides in *Annulohypoxylon stygium* DR47 extracts growth in STR at pH 5.0 (A) and 4.0 (B).

saccharification step. In this way, other hydrolysis assay was performed aiming to keep the protein load at 12 mg of protein/g of bagasse and to replace part of the cellulolytic extract by the extracts produced in this study.

The partial replacement of Celluclast 1.5L with the enzymatic extracts of *A. stygium* DR47 showed equivalent saccharides released (Table 4). Enzyme extracts from *A. stygium* DR47 could be used to formulate an enzyme mixture for biomass deconstruction as a commercially viable alternative to commercial celullases currently on the market. However, further experiments are required to establish the optimal hydrolysis conditions as well as optimization of supplementations amounts of the extract produced.

3.6. Proteomic analysis

Proteomic analyses were performed aiming to describe the secreted proteins of *A. stygium* and to understand the effect of supplementation on Celluclast 1.5L. Two STR batch conditions were tested, at pH 4.0 and 5.0, for sugar cane bagasse hydrolysis.

For the extract produced at pH 5.0, were assignment 256 peptides, distributed in 38 protein hits. Several β-glucosidases (GH3) based on 9 peptide matches, which were similar to those enzymes from other fungi including *N. crassa*, *Pyrenophora tritici-repentis* and *Paracoccidiodes* sp., were present (Appendix C). Besides 5 proteins were identified being two of them β-glucosidases and one a β-glucosidase precursor (GH3). The false discovery rate (FDR) was 5.1% for the protein and 2.0% for the peptide. In the extract produced at pH 4.0 were detected 185 peptides, distributed by 35 protein hits. Four proteins were identified, two L-α-arabinofuranosidase (GH54), a catalase and a carboxypeptidase base on 9 unique peptides (Appendix D). The FDR was 5.6% for the protein and 2.8% for the peptide.

A comparison of the secretomes using a Fischer exact test ($p < 0.05$) reveled significant differences between proteins expressed under different fermentation conditions. For example, β-glucosidase (higher at pH 5.0) and L-α-arabinofuranosidase and catalase (higher at pH 4.0) (Appendix E). In addition, pH influenced the protein profiles regarding to GH families based on unique peptides (Fig. 6). The most abundant families were GH3, GH18 and GH15 at pH 5.0 and GH3, GH54, GH35 and GH92 at pH 4.0.

At pH 4.0 pectinase activity was highest and when added to Celluclast 1.5L biomass hydrolysis was increased, although no polygalacturonase was detected in the secretome (Appendix D). Enzymes that were detected such as α-L-arabinofuranosidase are able to hydrolyze bonds in hemicellulose and could have contributed to the increase of sugar release during HB hydrolysis. Even though the extract of pH 4.0 indicated the presence of β-galactosidase in the secretome and also enzymatic activity, which could not explain the increase in hydrolysis by this enzyme since no galactose was presented in HB. The secretome analyses from the fermentation at pH 5.0 (Appendix C) revealed the presence of β-glucosidase which corroborated the enzymatic activity profile (Table 3), once that main activity detected was β-glucosidase followed of β-glucanase.

The proteomics study of the secreted proteins (i.e. enzymes) could explain results from the saccharification assay once that additional enzymes such as β-glucosidase and α-L-arabinofuranosidase could be detected. It is known that the β-glucosidase supplementation can increase biomass hydrolysis once it consumes the cellobiose and reduces the inhibitory effect against cellulases (Berlin et al., 2007; Gruno et al., 2004).

Gonçalves et al. (2012) and Goldbeck et al. (2014) verified that a recombinant α-L-arabinofuranosidase (GH54) in the presence of the endo-xylanase (GH11) gave synergistic effects of xylose and xylooligosaccharides release from pretreated sugarcane bagasse. GH3 was the most abundant family in both enzyme extracts produced. Several studies have suggested the importance of this class of enzyme on biomass deconstruction, for example in *P. decumbens* proteome (Cattaneo et al., 2014) and in the metatranscriptome of bee gut (Lee et al., 2014). The GH3 CAZy family is also known as an important enzyme in biomass saccharification. This class of enzyme is responsible for the breakdown of diverse oligosaccharides found in many types of biomass and has unusually broad substrate specificities, for example, oligosaccharides with diverse carbon-chain lengths and monomer residues.

Also the presence of a catalase in the pH 4.0 extract could indicate a better assimilation of biomass by *A. stygium* in cultivation at pH 4.0, and increase of enzymatic hydrolysis in the supplementation of Celluclast 1.5L. According to Bourdais et al. (2012) catalase activity is specifically required to efficiently assimilate lignocellulose in *Podospora anserine,* as hydrogen peroxide participates in the degradation of biomass complex but can be responsible to cell damage and cell death.

There is a paucity of information on enzymes from *A. stygium* or related species in protein databases. This may explain why pectinase activity was detected in the protein extract but not in the proteomic data, which opens up the exciting possibility that the pectinases of *A. stygium* may be novel, with distant homology to pectinase sequences in the protein databases. Future work will now concentrate on using genomics and transcriptomics, in conjunction with proteomics to characterize the pectinases.

4. Conclusion

A. stygium DR47 showed to be a potential candidate for glycohydrolases production when grown using citrus pulp and soybean bran in STR. Proteomic analysis of the secretome of *A. stygium*

DR47 revealed other glycohydrolase families, such as GH3, GH18, GH35, GH54 and GH92, never previously reported in this fungus. The substrate specificities and relative rates of hydrolytic activities of these new enzymes will be explored to develop a commercially viable enzyme cocktail with superior saccharification yields.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.11.082.

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