Universidade de Brasília

Departamento de Biologia Celular

Programa de Pós-graduação em Biologia Molecular

Tratamento hidrotérmico de bagaço de cana-de-açúcar como ferramenta para aumentar a produção de holocelulases por *Aspergillus niger*

Caio de Oliveira Gorgulho Silva

Brasília - DF, fevereiro de 2018

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Tese apresentada ao Programa de Pós-Graduação em Biologia Molecular do Departamento de Biologia Celular da Universidade de Brasília como pré-requisito para a obtenção do título de Doutor em Ciências Biológicas (área de concentração Biologia Molecular).

Orientador: Prof. Dr. Edivaldo Ximenes Ferreira Filho

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Resumo

Este trabalho teve como objetivo investigar a utilização das frações líquida (licor) e sólida obtidas a partir de tratamento hidrotérmico do bagaço de cana-de-açúcar (BCA) como fontes de carbono para a produção de holocelulases pelo fungo *Aspergillus niger* DCFS11. Numa primeira etapa, as condições do tratamento hidrotérmico foram investigadas por meio de análise fatorial visando a utilização dos licores resultantes como substratos para indução de hemicelulases. O tratamento de baixa severidade e utilizando baixa concentração de sólidos (170°C, 30 min, 1 % m/m BCA) foi selecionado como condição ótima devido à alta e rápida indução de xilanases pelo licor resultante. Uma variedade de mono e oligossacarídeos responsáveis pela indução de hemicelulases foi identificada nos licores por métodos analíticos (cromatografia líquida e espectrometria de massas). Numa segunda etapa, secretomas de *A. niger* cultivado na presença de bagaço *in natura* (BNT), bagaço tratado (BPT), licor (LIC) e bagaço tratado + licor (BPT+LIC) foram comparados por análise bioquímica e proteômica quantitativa. Os secretomas produzidos nas condições BPT, LIC e BPT+LIC mostraram-se superiores em termos de atividades de holocelulases, termoestabilidade e eficiência na sacarificação enzimática de BCA, além de apresentarem maior abundância de dezenas de celulases, hemicelulases e pectinases em relação ao secretoma produzido na condição BNT. Este trabalho demonstrou o potencial do tratamento hidrotérmico do BCA como ferramenta para aumentar a produção de holocelulases por *A. niger*, uma vez que gerou substratos menos recalcitrantes para crescimento microbiano na forma de carboidratos solúveis (licor) ou polissacarídeos mais acessíveis nos sólidos tratados.

Abstract

The goal of this work was to investigate the use of the liquid (liquor) and solid fractions arising from hydrothermal pretreatment of sugarcane bagasse (SCB) as a carbon source for the production of holocellulases by *Aspergillus niger*. Initially, hydrothermal pretreatment parameters were investigated by factorial design aiming the use of preteatment liquors as a substrate for hemicellulase induction. Pretreatment of low severity and low SCB loading (170 °C, 30 min, 1 % w/w SCB) was selected as the optimum condition due to the high and fast xylanase induction promoted by the resulting liquor. Several mono and oligosaccharides responsible for hemicellulase induction were identified in liquors by analytical methods (liquid chromatography and mass spectrometry). Thereafter, secretomes of *A. niger* cultivated in the presence of untreated SCB (BNT), pretreated bagasse (BPT), liquor (LIC) and pretreated bagasse + liquor (BPT+LIC) were compared by biochemical and quantitative proteomic analyses. Secretomes produced under conditions BPT, LIC and BPT+LIC were superior to BNT in terms of holocellulase activities, thermostability, efficiency in enzymatic saccharification of SCB. They also provided a greater abundance of dozens of cellulases, hemicellulases and pectinases in comparison to the secretome produced in the presence of BNT. This work demonstrated the potential of hydrothermal treatment of SCB as a tool to increase the production of holocellulases by *A. niger*, since it generated less recalcitrant substrates for microbial growth in the form of soluble carbohydrates (liquor) or more accessible polysaccharides in pretreated solids.

Sumário

Lista de tabelas

Capítulo II

Capítulo III

Capítulo IV

Anexo I

Anexo II

Lista de figuras

Capítulo I

Capítulo II

Capítulo III

Capítulo IV

Capítulo I

Introdução

1. Revisão Bibliográfica

1.1. Biorrefinaria de lignocelulose e o potencial do bagaço de cana-de-açúcar

O conceito atual de biorrefinaria de biomassa lignocelulósica consiste na conversão deste tipo de material vegetal, incluindo resíduos agroindustriais, florestais, municipais e lavouras dedicadas à bioenergia (ex: gramíneas do gênero *Miscanthus*), a um amplo espectro de bioprodutos (entre eles biocombustíveis, biomateriais e outros biocompostos) e energia (calor e eletricidade) através de processos sustentáveis do ponto de vista ambiental, econômico e social (Jungmeier, Hingsamer, *et al.*, 2013; Jungmeier, Stichnothe, *et al.*, 2013; Silva *et al.*, 2017).

O conceito de biorrefinaria abarca diversos tipos de processos para a conversão da biomassa lignocelulósica, dentre eles as vias químicas (ex: hidrólise ácida), termoquímicas (ex: gaseificação, pirólise) e bioquímicas (hidrólise enzimática e fermentação) (De Jong e Jungmeier, 2015). A via bioquímica é a estratégia que permite maior recuperação de carboidratos totais da biomassa e é compatível com a posterior fermentação destes açúcares a etanol (Silva *et al.*, 2017).

O processo tipicamente empregado para a obtenção de açúcares fermentescíveis a partir dos polissacarídeos estruturais da biomassa lignocelulósica e posterior conversão destes a bioetanol ou outros bioprodutos é realizado em três principais etapas: (1) pré-tratamento da biomassa, (2) hidrólise enzimática e (3) fermentação/conversão química de monossacarídeos a produtos finais. A etapa de pré-tratamento é necessária para tornar a biomassa menos recalcitrante para a posterior sacarificação enzimática. Estes processos serão discutidos nas seções a seguir.

Os processos de conversão enzimática de lignocelulose, por requererem condições amenas de temperatura e pH, apresentarem alta especificidade de conversão, gerarem pouca perda de carboidratos por reações não desejáveis e serem processos amigáveis ao meio ambiente, se sobressaem em relação a processos químicos, como a hidrólise da biomassa com ácido concentrado (Wong *et al.*, 1988; Schäfer *et al.*, 2005). Apesar

disto, o uso de enzimas para conversão de biomassa em escala industrial ainda encontra obstáculos importantes, como o alto custo para produção de coquetéis enzimáticos eficientes e os baixos rendimentos de conversão, mesmo após pré-tratamento da biomassa (Arantes e Saddler, 2010).

Uma das estratégias discutidas atualmente para reduzir o custo das enzimas empregadas na etapa de sacarificação é utilizar a própria biomassa recebida na biorrefinaria como substrato para sua produção por organismos lignocelulolíticos, numa abordagem designada como produção local e integrada (Silva e Ferreira Filho, 2017). O capítulo II desta tese foca na discussão da abordagem de produção integrada de enzimas e no potencial do uso de biomassas pré-tratadas, ao invés de biomassas *in natura*, como substrato para produção enzimática em biorrefinarias.

Resíduos agroindustriais lignocelulósicos (ex: palha e bagaço de cana-de-açúcar, palha e sabugo de milho, casca do grão da soja, resíduos do processamento do algodão, entre outros) apresentam grande potencial para serem convertidos a energia e bioprodutos de valor agregado, inclusive para serem utilizados como substrato para produção de enzimas, uma vez que representam matérias-primas renováveis, abundantes, baratas e cuja acumulação pode trazer impactos ambientais. Além disso, o potencial dos resíduos lignocelulósicos também reside no fato de não serem materiais FRPHVWtYHLV HYLWDQGR R GHEDWH *versus* DOLPHQWRV **FRPHVWtYHLV´HQIUHQWDGR** SHOD produção de bioetanol de primeira geração a partir de sacarose e amido e biodiesel a partir de óleos vegetais comestíveis (Fitzpatrick *et al.*, 2010).

O bagaço de cana-de-açúcar corresponde ao resíduo da moagem do colmo da cana-de-açúcar gerado nas usinas de álcool e açúcar. Considerando que cerca de 280 kg de bagaço são gerados a partir de cada tonelada de cana-de-açúcar moída (Cardona *et al.*, 2010), a previsão é que 180 milhões de toneladas deste resíduo sejam geradas na safra de 2017/18 no Brasil (Conab, 2017). Sendo o resíduo agroindustrial mais abundante no país, o bagaço de cana-de-açúcar apresenta grande potencial para ser utilizado como matéria prima para biorrefinarias de lignocelulose. O capítulo V desta tese discute o papel central que o Brasil pode desempenhar em um cenário global de crescentes demandas por biomassa, bioenergia, biocombustíveis e outros bioprodutos, uma vez que produz quantidade substancial de biomassa lignocelulósica a partir de sua atividade agroindustrial intensa.

1.2. Estrutura geral da parede celular vegetal e composição do bagaço de cana-de-açúcar

A parede celular vegetal corresponde a um complexo arranjo de polissacarídeos e lignina normalmente chamado de estrutura lignocelulósica. Sua porção polissacarídica, designada holocelulose, é composta por celulose, hemiceluloses e pectina (De Siqueira *et al.*, 2010). A composição do bagaço de cana-de-açúcar normalmente varia entre 38,4 ± 45,5 % de celulose, 22,7 ± 27 % de hemicelulose, 19,1 ± 32,4 % de lignina e até 9,1 % de extraíveis (Canilha *et al.*, 2012). Os extraíveis não são componentes estruturais da parede celular e se referem a materiais hidrofóbicos como lipídeos de membrana, ceras e compostos secundários.

1.2.1. Celulose

A celulose corresponde a um polissacarídeo linear composto por resíduos de glicose conectados entre si SRUOLJDo}HVJOLFRVtGLFDVGRWLSR-1,4. A maior parte da celulose é encontrada na forma cristalina, estrutura onde as cadeias deste polissacarídeo são empacotadas através de ligações de hidrogênio intra e intermoleculares, formando microfibrilas e impedindo a penetração de água e enzimas no seu interior. A Figura 1 ilustra a estrutura da celulose cristalina. As microfibrilas de celulose normalmente apresentam um número mínimo de 18 cadeias empacotadas em estrutura cristalina, podendo conter até 80 cadeias empacotadas (Jarvis, 2018). Regiões com cadeias semi-ordenadas e desordenadas, denominadas regiões paracristalinas e amorfas, respectivamente, estão presentes ao longo das microfibrilas, sendo estas mais acessíveis às moléculas de água e ação de celulases (Kulasinski *et al.*, 2014). Cadeias presentes na superfície das microfibrilas interagem de forma íntima com cadeias de hemiceluloses, como glicuronoarabinoxilana e xiloglicana, e pectinas através de ligações de hidrogênio (Jarvis, 2018).

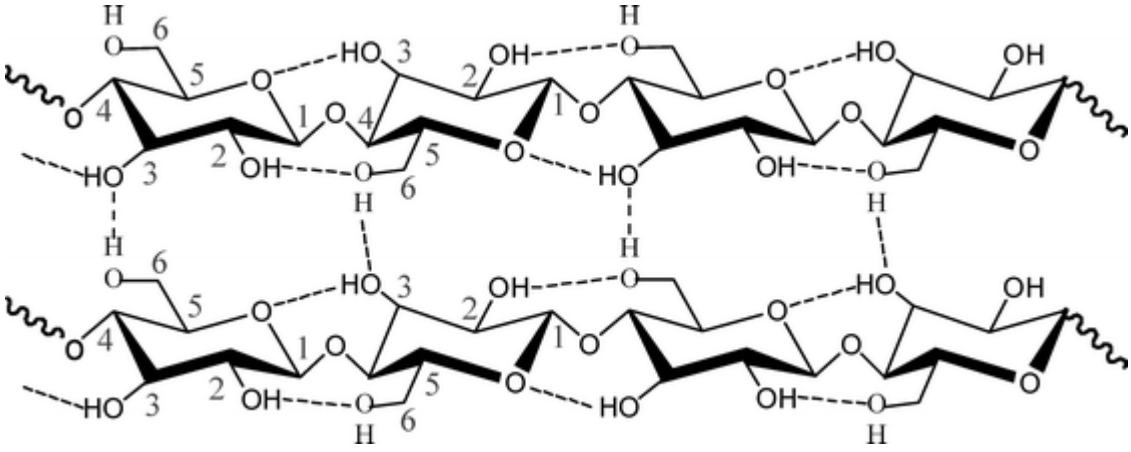
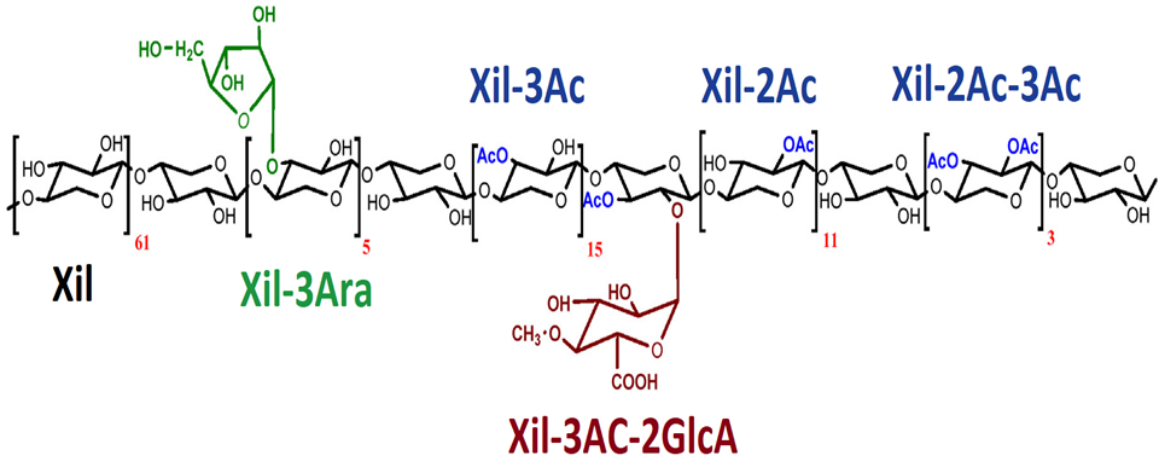


Figura 1. Estrutura esquemática da celulose. As ligações de hidrogênio intra e intermoleculares são ilustradas por linhas pontilhadas. Figura extraída de Xu *et al.* (2010).

1.2.2. Hemiceluloses

As hemiceluloses são um conjunto de polissacarídeos heterogêneos da parede celular vegetal que inclui as xilanas, xiloglicanas, mananas e -glicanos de ligações PLVWDVH -1,4) (Scheller e Ulvskov, 2010). A porção hemicelulósica do bagaço de cana-de-açúcar é composta principalmente por xilana, com menores teores de xiloglicano, -1,3-β-1,4-glicanos e manana (De Souza *et al.*, 2013). A xilana corresponde a uma cadeia principal formada por resíduos de xilose conectados entre si por ligações glicosídicas do tipo -1,4 e normalmente apresenta diversos tipos de ramificações laterais (correspondendo a heteroxilanas). A xilana presente no bagaço de cana-de-açúcar é designada glicuronoarabinoxilana acetilada, apresentando ramificações de α-L-arabinofuranose (ligadas aos resíduos de xilose da cadeia principal através de ligações do tipo . -1,3 RX . -1,2), ácido (4-O-metil-)glicurônico (ligados aos UHVtGXRVGH[LORVHSRUOL-1,2) e grupos acetil (conectados por ligações éster aos carbonos C-2 e/ou C-3 dos resíduos de xilose). Um esquema da estrutura da glicuronoarabinoxilana acetilada presente no bagaço de cana-de-açúcar é apresentado na Figura 2, acompanhado da abundância relativa de cada componente estrutural como descrito por De Carvalho *et al.* (2017). A glicuronoarabinoxilana presente no bagaço de cana-de-açúcar também apresenta unidades de ácido ferúlico e ácido *p*-cumárico conectadas aos resíduos de α-L-arabinofuranose por ligações tipo éster. A esterificação com ácido ferúlico permite que haja conexão covalente da xilana com outros polissacarídeos (pectina ou outras cadeias de xilana) e com a lignina através de ligações cruzadas, contribuindo para a rigidez e recalcitrância da estrutura lignocelulósica

(Christov e Prior, 1993; Benoit *et al.*, 2006; Scheller e Ulvskov, 2010). A Figura 3 ilustra possíveis ligações cruzadas entre xilana e lignina envolvendo a presença de ácido ferúlico.



Fragmentos estruturais	Designação	Abundância relativa (por 100 unidades Xil*p*)
→4)-β-D-Xil*p*-(1→	(Xil)	65
→4)[3-O-Ac]-β-D-Xil*p*-(1→	(Xil-3Ac)	15
→4)[2-O-Ac]-β-D-Xil*p*-(1→	(Xil-2Ac)	11
→4)[3-O-Ac] [2-O-Ac]-β-D-Xil*p*-(1→	(Xil-2Ac-3Ac)	3
→4)[α-L-Araf(1→)]-β-D-Xil*p*-(1→	(Xil-3Ara)	5
→4)[4-O-Me-α-D-Glc*p*A-(1→2)][3-O-Ac]-β-D-Xil*p*-(1→	(Xil-3Ac-2GlcA)	1

Figura 2. Representação esquemática da estrutura da glicuronoarabinoxilana acetilada presente no bagaço de cana-de-açúcar. A tabela mostra a abundância relativa dos componentes estruturais do polissacarídeo. β-D-Xil*p* = β-D-xilopiranose; Ac = grupo acetil; α-L-Ara*f* = α-L-arabinofuranose; 4-O-Me-α-D-GlcA = ácido 4-O-metil-α-D-glicurônico. Esquema extraído e modificado de De Carvalho *et al.* (2017).

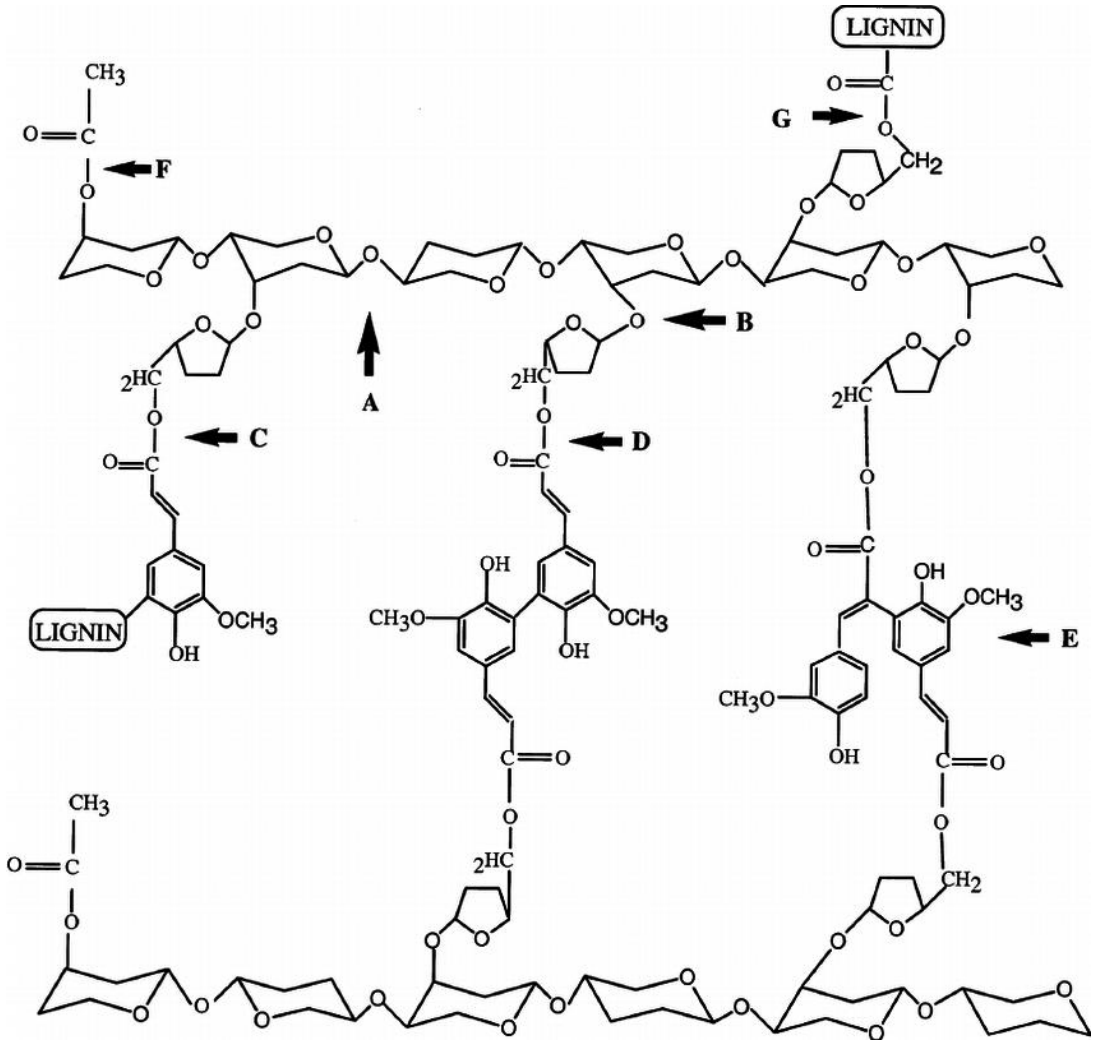


Figura 3. Ilustração de ligações cruzadas entre cadeias de arabinoxilana e lignina. A: cadeia principal da arabinoxilana, B: ligação xilose-arabinose, C: 5-O-feruloil-lignina, D: ponte diferúlica (dímero 5-5), E: ponte diferúlica (dímero 8-5), F: grupo acetil, G: arabinose-lignina. Extraído de Mathew e Abraham (2004).

Xiloglicanos são polissacarídeos cuja cadeia principal é composta por unidades de glicose unidas por ligações -1,4 e que apresentam ramificações de xilose conectadas SRU OLJDo}HV-1,6. Os resíduos laterais de xilose podem ser ligados a resíduos de arabinose ou galactose, e este último, por sua vez, pode ser acetilado e/ou conectado a um resíduo de fucose (Scheller e Ulvskov, 2010). O bagaço de cana-de-açúcar apresenta xiloglicano com baixos níveis de fucose em sua estrutura (De Souza *et al.*, 2013).

-Glicanos de ligações mistas são polissacarídeos compostos por resíduos de glicose FRQHFWDGRVSRUOLJDo}HVGOLFRVtGLFDVDFDGDRXPDLVXQLGDGHVSRUOLJDo}HV 1,3. Por não formarem microfibrilas como a celulose, este polissacarídeo é mais facilmente extraído e hidrolisado por tratamentos físico-químicos. -Glicanos de ligações mistas também estão presentes na cana-de-açúcar (De Souza *et al.*, 2013).

Como componente hemicelulósico minoritário, o bagaço de cana-de-açúcar também apresenta manana e glicomanana em baixas quantidades, havendo baixa detecção de manose em hidrolisados deste material (Sun *et al.*, 2004; De Souza *et al.*, 2013). Mananas lineares se referem a cadeias formadas por resíduos de manose conectados entre si SRU OLJDo}HV-1,4. Galactomananas apresentam ramificações laterais de galactose conectados à cadeia principal de manose por ligações do tipo . -1,6. No caso das glicomananas, a cadeia principal é formada por resíduos de manose e glicose conectados por ligações -1,4 sem um padrão de sequência específico, e que também podem apresentar ramificações de galactose, formando a estrutura da chamada galactoglicomanana. Em todos os tipos de manana, grupos acetil também podem estar ligados à cadeia principal (Moreira e Filho, 2008; Scheller e Ulvskov, 2010). A Figura 4 ilustra estruturas modelo de xiloglicanos, β-glicanos de ligações mistas e galacto(glico)mananas.

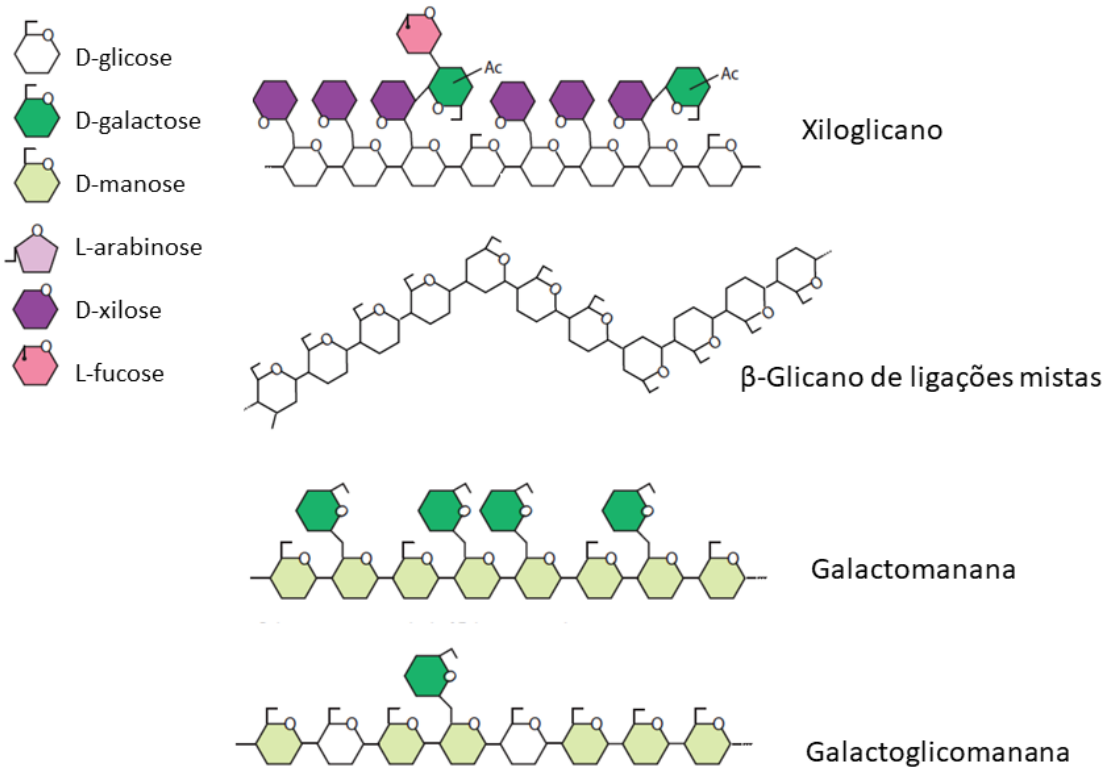


Figura 4. Ilustração de estruturas modelo de hemiceluloses. Extraído e modificado de Scheller e Ulvskov (2010).

1.2.3. Pectinas

As pectinas são polissacarídeos ricos em ácido galacturônico, classificadas em principalmente 4 tipos: homogalacturonana, xilogalacturonana, rhamnogalaturonana I e

rhamnogalaturonana II. A Figura 5 ilustra a estrutura geral dos diferentes tipos de pectina. A homogalacturonana, ou ácido poligalacturônico, corresponde a uma cadeia linear de resíduos de ácido galacturônico conectados entre si SRU OLJDo}HV-1,4, que normalmente apresentam metilação e acetilação. Xilogalacturonana corresponde à homogalacturonana com ramificações de xilose conectadas à cadeia principal por OLJDo}HV-1,3. As rhamogalacturonanas I e II são materiais pécticos mais complexos e apresentam ramificações mais longas e elaboradas que os demais polissacarídeos da parede celular. Rhamnogalacturonanas II contêm cadeia principal formada por ácido galacturônico com cadeias laterais ramificadas que podem conter até 12 tipos de açúcares, incluindo rhamnose, apiose, xilose, arabinose, galactose, fucose entre outros, conectados por até 20 tipos de ligações diferentes. As rhamnogalacturonanas I são polissacarídeos cuja cadeia principal é formada por unidades intercaladas de ácido galacturônico e rhamnose. Cadeias laterais longas e ramificadas contendo galactose (galactanas), arabinose (arabinanas) ou combinações destes açúcares (arabinogalactanas) são ligadas aos resíduos da rhamnose da cadeia principal da rhamnogalacturonana I. Tais cadeias laterais podem ser esterificadas com ácido ferúlico e estão conectadas à lignina ou outros polissacarídeos através de pontes diferúlicas (Mohnen, 2008). Homogalacturonana e rhamnogalacturonana I com ramificações de arabinogalactanas estão presentes na estrutura da parede celular do colmo de cana-de-açúcar (De Souza *et al.*, 2013).

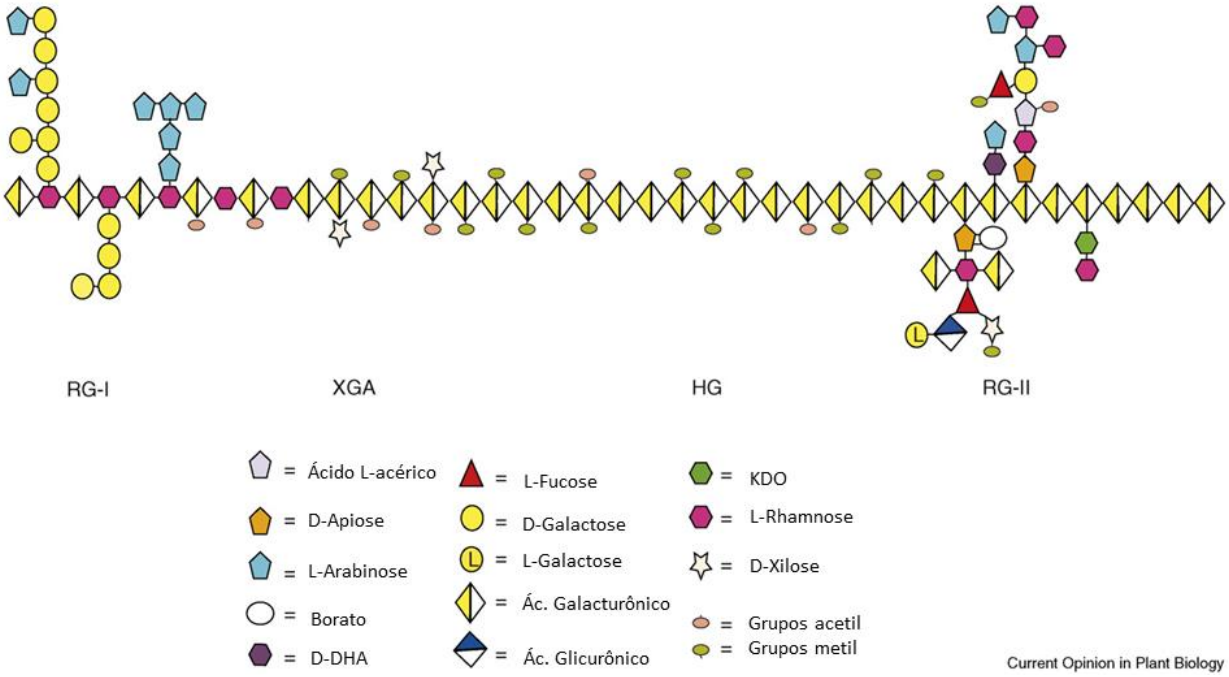


Figura 5. Esquema da estrutura geral de diferentes tipos de pectina, sendo elas: homogalacturonana (HG), xilogalaturonana (XGA), rhamnogalacturonana I (RG-I) e rhamnogalacturonana II (RG-II). Esquema extraído e modificado de Mohnen (2008).

1.2.4. Lignina

A lignina é um complexo fenólico, amorfo, hidrofóbico e altamente recalcitrante à degradação química e biológica. A lignina forma uma trama tridimensional que envolve os demais componentes da parede celular e atua como barreira física que compromete a hidrólise enzimática da porção polissacarídica da estrutura. É sintetizada a partir de três álcoois fenólicos precursores: álcool *p*-cumarílico (ou *p*-hidroxifenil propanol), álcool coniferílico (ou guaiacil propanol) e álcool siringílico (ou siringil propanol), que dão origem às ligninas do tipo *p*-hidroxifenil (H), guaiacil (G) e siringil (S), respectivamente (Figura 6). As unidades fenólicas são conectadas entre si por diversos tipos de ligação C-C e éter, sem uma ordem padronizada. A lignina de gramíneas, como a cana-de-açúcar, é composta pelos três tipos de precursores (H, G e S), enquanto coníferas são mais ricas em lignina guaiacil e madeiras angiospermas são compostas principalmente por ligninas G e S (Grabber, 2005; Lawoko, 2005; 5XL[XHxDV H 0DUWtQH] ; Sánchez, 2009).

Por sua característica hidrofóbica, a lignina pode causar a adsorção de enzimas à sua superfície através de interações eletrostáticas e hidrofóbicas, reduzindo a eficiência da sacarificação de biomassa por coquetéis enzimáticos (Sammond *et al.*, 2014; Ko,

Ximenes, *et al.*, 2015; Lu *et al.*, 2016). Modelos preditivos podem ser utilizados para identificar enzimas com menor propensão à adsorção à lignina (Sammond *et al.*, 2014) e engenharia proteica pode ser utilizada para modificar as características da superfície de enzimas de modo a minimizar perdas por adsorção inespecífica à lignina (Whitehead *et al.*, 2017).

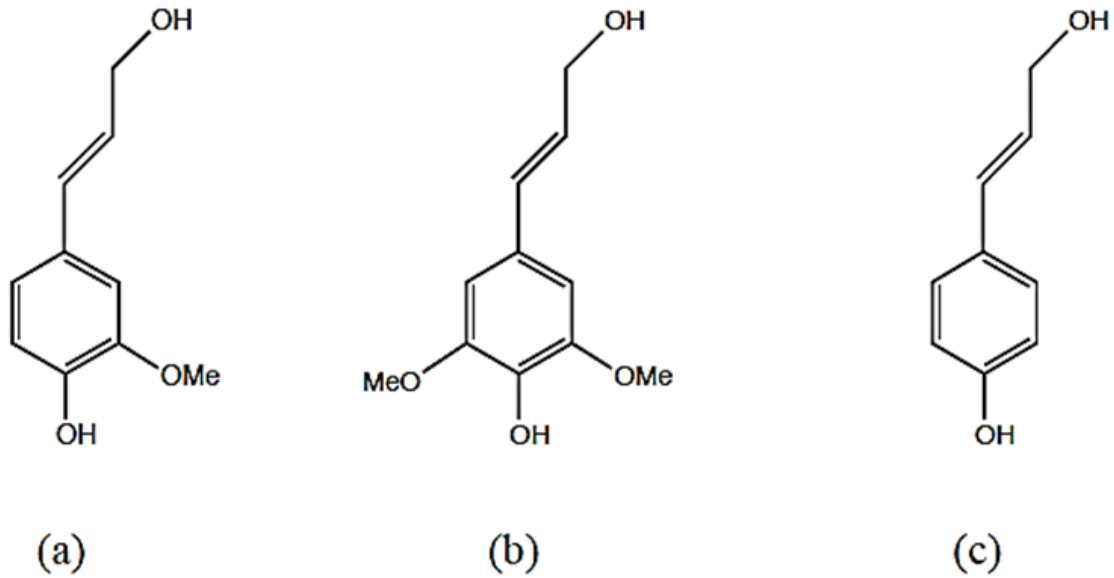


Figura 6. Unidades fenólicas precursoras da lignina. (a) álcool coniferílico, (b) álcool siringílico e (c) álcool *p*-cumarílico. Extraído de Ferhan (2016).

1.3. Holocelulases

As holocelulases são um grupo de enzimas que atuam na desconstrução de polissacarídeos estruturais da parede celular vegetal. Estas enzimas são sintetizadas por diversos microrganismos, dentre eles os saprófitos, como o fungo filamentoso *Aspergillus niger*, durante a decomposição de resíduos vegetais.

As holocelulases são tradicionalmente classificadas de acordo com a similaridade de sequência de aminoácidos, sendo organizadas em famílias dentro de quatro grandes grupos de enzimas: glicosídeo hidrolases (GH), polissacarídeo liases (PL), carboidrato esterases (CE) e atividades auxiliares (AA), que incluem oxiredutases ativas sobre carboidratos (Lombard *et al.*, 2013).

Considerando a composição do bagaço de cana-de-açúcar detalhada acima, um complexo arsenal enzimático é necessário para sua completa desconstrução, como descrito a seguir.

Apesar de a celulose apresentar estrutura química simples, várias enzimas de diferentes famílias são necessárias para sua completa degradação até glicose, sendo elas endoglicanases, exoglicaQDVHV -glicosidases e monooxigenases líticas de polissacrídeos (LPMOs). As endoglicanases (pertencentes às famílias GH 5, 6, 7, 9, 12, 44 ou 51) atuam sobre porções desordenadas das microfibrilas de celulose, clivando ligações glicosídicas no interior das cadeias do polissacarídeo através de mecanismo do tipo endo. A ação de endoglicanases diminui o grau de polimerização das cadeias de celulose, levando à liberação de celooligômeros solúveis e à formação de novas extremidades de cadeia. Exoglicanases (famílias GH 5, 6, 7 e 48) atuam a partir das extremidades redutoras ou não-redutoras das cadeias de celulose localizadas na superfície das microfibrilas, liberando glicose (enzimas chamadas celodextrinases) ou celobiose (enzimas chamadas celobiohidrolases ± CHB) como produtos finais. As CBHs (tipo de exoglicanase mais comum e estudada) são consideradas enzimas processivas ± apresentam estrutura em forma de túnel ao redor do sítio catalítico por onde uma cadeia de celulose se acomoda e desliza durante a reação de hidrólise, havendo liberação progressiva de unidades de celobiose (Lynd *et al.*, 2002; Annamalai *et al.*, 2016). Endo e exoglicanases frequentemente apresentam, anexado ao módulo catalítico, um módulo de ligação ao carboidrato (CBM), que atua no reconhecimento do substrato, na aproximação do módulo catalítico à superfície das microfibrilas e, em alguns casos, na ruptura não catalítica da celulose cristalina, promovendo amorfogênese da celulose (Boraston *et al.*, 2004; Arantes e Saddler, 2010). As enzimas -glicosidases (GH 1, 3, 5) atuam na hidrólise de celooligosacarídeos solúveis e celobiose, liberando glicose (Lynd *et al.*, 2002). LPMOs fúngicas (família AA9) são enzimas oxidativas que, na presença de moléculas ou enzimas doadoras de elétrons, atuam na clivagem oxidativa de ligações glicosídicas da celulose, gerando novas extremidades para ação de exoglicanases (Monclaro e Ferreira Filho, 2017).

A glicuronoarabinoxilana acetilada presente no bagaço apresenta uma alta complexidade química, com uma variedade de monossacarídeos constituintes, substituintes laterais não sacarídicos e tipos de ligações glicosídicas, requerendo um arsenal de enzimático complexo atuando em sinergismo para sua desconstrução completa. Endoxilanases (GH 10, 11, 43, 8 e 5) clivam ligações internas da cadeia principal da xilana, liberando xilooligossacarídeos e xilobiose como produtos principais. β-Xilosidases (GH 3, 43, 54, 39 e 116) clivam xilooligossacarídeos e xilobiose a

unidades de xilose. Os resíduos de L-arabinose e ácido (4-O-metil)glicurônico conectados à cadeia principal da xilana são hidrolisados pela ação de α-L-arabinofuranosidases (GH 43, 51, 54, 62 e 3) e α-glicuronidases (GH 47 e 115), respectivamente. As moléculas de ácido ferúlico conectadas às unidades de arabinose são removidas pela ação de feruloil esterases (CE 1 e 5). Os grupos acetil esterificados à cadeia principal, por fim, são extraídos pela ação da enzima acetilxilana esterase (CE 1 a 8) (Moreira *et al.*, 2011).

Considerando a degradação de xiloglicanos, endoglicanases específicas para xiloglicano (GH 12, 5, 9, 74 e 44) atuam na hidrólise da cadeia principal, enquanto α-D-xilosidases (GH 31), β-D-galactosidases (GH 35, 1, 2) e α-L-fucosidases (GH 95) específicas para xiloglicanos removem substituições laterais deste polissacarídeo (Moreira *et al.*, 2011). β-Glicanos de ligações mistas são hidrolisados em oligossacarídeos por lichenases (GH 16), enzimas que correspondem a endoglicanases capazes de reconhecer ligações β-1,4 próximas a ligações β-1,3 na cadeia deste polissacarídeo (Elgharbi *et al.*, 2013). Para a hidrólise de galactomanana, endo-β-1,4-mananases e β-manosidases atuam em sinergismo na hidrólise da cadeia principal, enquanto α-galactosidases (GH 3, 27 e 36) e acetilmanana esterases promovem a remoção de substituintes laterais de galactose e acetil, respectivamente (Moreira e Filho, 2008).

Uma ampla gama de hidrolases, liases e esterases são necessárias para a completa desconstrução da homogalacturonana e rhamnogalacturonana I presentes no bagaço de cana-de-açúcar. Pectina metil esterases (CE 8) e pectina acetil esterases (CE 12 e 13) catalisam a desesterificação de grupos metil e acetil ligados à cadeia de ácido poligalacturônico, liberando metanol e acetato, respectivamente. Poligalacturonases (GH 28) catalisam a hidrólise das ligações glicosídicas do tipo α-1,4 presentes no interior (endopoligalacturonases) ou na extremidade não-redutora (exopoligalacturonases) das cadeias de ácido poligalacturônico. Pectato liases e pectina liases (PL 1) atuam na clivagem de cadeias de ácido poligalacturônico não esterificadas e altamente esterificadas, respectivamente, por mecanismo de transeliminação, gerando produtos monoméricos e oligoméricos insaturados (Δ-4,5-D-galacturonatos). Rhamnogalacturonana hidrolases (GH 28) e rhamnogalacturonana liases (PL 4 e 11) atuam na clivagem de ligações internas da cadeia principal da rhamnogalacturonana I

por mecanismo hidrolítico e de transeliminaçao, respectivamente. α-L-Rhamnosidases (GH 28, 78 e 106) e rhamnogalacturonana galacturonohidrolases (GH 28) catalisam a clivagem hidrolítica da cadeia principal da rhamnogalacturonana I a partir da extremidade não-redutora, liberando rhamnose e monogalacturonato, respectivamente. Rhamnogalacturonana acetil esterases (CE 12) removem grupos acetil da cadeia principal da rhamnogalaturonana I (Pedrolli *et al.*, 2009). Endo-arabinases e α-L-arabinofuranosidases (GH 43, 51, 54 e 62) atuam na hidrólise das cadeias de arabinana ligadas à cadeia principal da rhamnogalacturonana I (Cartmell *et al.*, 2011). Feruloil esterases (CE 1 e 5), além de atuarem sobre arabinoxilana, também tem papel importante na remoção de moléculas de ácido ferúlico anexadas às cadeias de arabinana da rhamnogalacturnana I (De Vries *et al.*, 2002). Xilogalacturonases (GH 28) atuam na hidrólise de xilogalacturonanas, liberando dímeros de xilose-galacturonato (Pedrolli *et al.*, 2009).

1.4. Tratamento hidrotérmico da biomassa para redução da recalcitrância de materiais lignocelulósicos

Diversos aspectos da estrutura da parede celular vegetal tornam-na recalcitrante à degradação enzimática, tanto no contexto de decomposição natural de biomassa vegetal quanto na conversão de biomassa em nível industrial. Dentre os fatores que contribuem para sua recalcitrância estão: a estrutura cristalina da celulose, a presença de lignina, a ligação cruzada entre polissacarídeos e lignina (complexos lignina-carboidrato), as substituições laterais das hemiceluloses e pectinas, a associação íntima de hemiceluloses e pectina à superfície das microfibrilas de celulose e a estrutura compacta do complexo lignocelulósico (baixa porosidade e área superficial). Estes aspectos dificultam o acesso das holocelulases aos polissacarídeos e suas ligações glicosídicas (Hu e Ragauskas, 2012).

No processo de conversão de biomassa lignocelulósica a açúcares fermentescíveis, uma etapa de pré-tratamento da biomassa é tradicionalmente aplicada para reduzir a recalcitrância da estrutura ao ataque enzimático. Tecnologias de pré-tratamento de biomassa têm sido continuamente desenvolvidas e aprimoradas de modo a atingir os seguintes objetivos: alta recuperação de carboidratos presentes na biomassa original, alta digestibilidade dos polissacarídeos em etapas subsequentes de hidrólise enzimática, nula ou baixa geração de moléculas inibitórias a microrganismos e enzimas, baixa

demanda energética e baixo custo operacional (Galbe e Zacchi, 2012; Silva e Ferreira Filho, 2017).

O capítulo II fornece uma breve descrição das principais tecnologias de pré-tratamento atualmente disponíveis. Dentre estas tecnologias, os tratamentos hidrotérmicos de biomassa se destacam por uma série de fatores, incluindo a utilização de apenas água como reagente e a não adição de catalisadores químicos (ex: ácidos, álcalis ou solventes). Estas tecnologias são consideradas amigáveis ao meio ambiente, não necessitam de reatores construídos com materiais especiais resistentes à corrosão, não requerem etapas de recuperação de catalisadores (por não haver uso deles) e requerem menor quantidade de químicos necessários para a neutralização da biomassa tratada quando comparadas aos tratamentos ácidos ou alcalinos. Os tratamentos hidrotérmicos, entretanto, demandam energia para manter a água ou vapor em altas temperaturas (Yang *et al.*, 2017).

Existem duas principais tecnologias de tratamento hidrotérmico de biomassa: (1) explosão a vapor e (2) tratamento hidrotérmico tradicional, também designado como *Liquid Hot Water* (LHW), *Hot Compressed Water* (HCW), autohidrólise, hidrotermólise ou fracionamento aquoso. No tratamento por explosão a vapor, a biomassa é incubada com vapor de água quente e pressurizado, seguido de uma etapa de descompressão. Já no tratamento hidrotérmico tradicional (objeto de estudo deste trabalho), a água permanece no estado líquido durante todo o processo e não há etapa de descompressão explosiva ao final do tratamento.

O tratamento hidrotérmico tradicional consiste no cozimento da biomassa em água sob altas temperatura e pressão. A pressão no interior do reator de tratamento torna-se superior ao ponto de saturação do vapor, mantendo a água no estado líquido durante o processo, mesmo em temperaturas acima de 100 °C. As temperaturas empregadas normalmente variam entre 160 e 230 °C e o tempo de residência, de minutos a horas (Hu e Ragauskas, 2012; Silveira *et al.*, 2015). Durante o tratamento hidrotérmico de materiais lignocelulósicos, diversas reações de fracionamento ocorrem, dentre elas a solubilização de parte das hemiceluloses, liberação de substituintes das hemiceluloses, dissolução de uma pequena parte da lignina, extração de pequenas moléculas e degradação de proteínas (Vegas *et al.*, 2008). A solubilização da hemicelulose é a principal característica desta tecnologia de pré-tratamento de biomassa. Apesar de ser considerado um tratamento do tipo neutro, por não fazer uso de ácidos ou álcalis, as

reações que ocorrem durante o tratamento hidrotérmico são resultado da acidificação do meio aquoso utilizado durante o processo. Sob altas temperaturas, o pH e o pKa da água são alterados, e esta passa a atuar como um ácido fraco através da formação de íons hidrônio (H_3O^+) resultantes de sua autoionização. Este é o principal fator que leva à despolimerização da hemicelulose pela quebra de suas ligações glicosídicas. Além disso, grupos acetil e outros substituintes laterais da xilana, como ácido glicurônico e sua forma metilada (ácido 4-O-metilglicurônico), bem como ácido galacturônico advindo da hidrólise de pectinas, são liberados para o meio aquoso e constituem uma fonte *in situ* de ácidos orgânicos que contribuem para a acidificação do meio e a hidrólise das cadeias de hemicelulose (Hu e Ragauskas, 2012; Silveira *et al.*, 2015).

Ao longo do tratamento hidrotérmico da biomassa, a xilana é quebrada em xilooligossacarídeos de alta massa molecular, que são progressivamente quebrados em oligossacarídeos de menor massa e assim por diante, até a formação de xilose monomérica. Da mesma forma, os substituintes laterais (arabinose, ácido glicurônico, ácido 4-O-metil-glicurônico, ácido ferúlico, ácido *p*-coumárico e grupos acetil) são progressivamente liberados no meio de reação. Em tratamentos de maior severidade, a solubilização da xilana em oligômeros solúveis é maior, assim como a quebra dos oligômeros em xilose monomérica. A xilana e os oligômeros de alta massa molecular são pouco solúveis em água. À medida que os oligômeros são hidrolisados a oligômeros menores, sua solubilidade aumenta e eles passam para a fase aquosa. Ao final do tratamento, oligômeros com baixa solubilidade re-precipitam sobre a superfície da biomassa. A alta temperatura e a acidificação do meio aquoso durante o tratamento promovem a reação de desidratação de moléculas de xilose monomérica (perda de três moléculas de água), levando à formação de furfural. O furfural, por sua vez, pode ser convertido a ácido fórmico sob condições severas. A dinâmica de hidrotermólise da xilana tem a seguinte sequência:

$$X_n \rightarrow XO_A \rightarrow XO_M \rightarrow XO_B \rightarrow X \rightarrow F \rightarrow AF \quad (1)$$

onde X_n corresponde à xilana, XO_A, XO_M e XO_B denotam xilooligômeros de alta, média e baixa massa molecular, X é xilose, F é furfural e AF, ácido fórmico (Garrote *et al.*, 2007; Kabel *et al.*, 2007; Hu e Ragauskas, 2012). O furfural e o ácido fórmico são potentes inibidores de fermentação e de crescimento microbiano. Uma das vantagens dos pré-tratamentos hidrotérmicos em relação a pré-tratamentos ácidos é a geração de

menor concentração de produtos de degradação de monossacarídeos (Hu e Ragauskas, 2012).

Por apresentar estrutura fibrilar, o solvente tem menor acesso às ligações glicosídicas da celulose cristalina, e esta permanece pouco alterada após o tratamento hidrotérmico. As porções desordenadas da celulose, entretanto, são mais acessíveis e a aplicação de tratamentos severos normalmente aumenta o índice de cristalinidade da celulose por promover solubilização da porção amorfa (Yu e Wu, 2010; Xiao *et al.*, 2011). Moléculas de glicose advindas da celulose e outras hexoses solubilizadas a partir da hemicelulose e da pectina (manose e galactose) também sofrem degradação durante o tratamento hidrotérmico, levando à formação de 5-hidroximetilfurfural (HMF), um potente inibidor microbiano. O HMF, por sua vez, pode ser degradado a ácido fórmico e ácido levulínico (Hu e Ragauskas, 2012).

Considerando a lignina, o tratamento hidrotérmico é capaz de solubilizar parte da porção denominada lignina solúvel em ácido, enquanto a lignina insolúvel em ácido (chamada de lignina Klason) permanece no sedimento sólido. A porção solubilizada durante a autohidrólise corresponde a derivados de lignina solúveis em água e de baixa massa molecular. A lignina que permanece nos sólidos é redistribuída num processo simultâneo de despolimerização e repolimerização, e gotas esféricas de lignina aparecem sobre a superfície da biomassa pré-tratada, provavelmente em um processo espontâneo para minimizar o contato da lignina (hidrofóbica) com a solução aquosa. A repolimerização ou condensação da lignina envolve tanto porções da lignina solubilizadas quanto produtos de degradação de açúcares (furfural e HMF), que se polimerizam à lignina gerando a chamada pseudo-lignina (Ko, Kim, *et al.*, 2015).

As reações de hidrotermólise da biomassa ocorrendo no tratamento hidrotérmico tradicional e no tratamento por explosão à vapor são similares. A principal diferença de resultado entre os dois tratamentos é a concentração final dos produtos de hidrotermólise, sendo resultado da maior carga de sólidos empregada no tratamento por explosão à vapor. Por utilizar uma quantidade de água menor, o licor resultante do tratamento por explosão à vapor apresenta maior concentração tanto de carboidratos quanto de inibidores, o que pode inviabilizar seu uso como meio de cultura para microrganismos sem que haja diluição ou aplicação de métodos de destoxificação. Por utilizar maior quantidade de água, os licores resultantes de tratamento hidrotérmico

tradicional são mais diluídos que os gerados no tratamento por explosão à vapor, tendo menor concentração de inibidores mas também de carboidratos (Hu e Ragauskas, 2012).

De acordo com o descrito acima, o tratamento hidrotérmico de biomassa lignocelulósica resulta numa fração líquida rica em produtos de degradação da hemicelulose (chamada de licor ao longo deste trabalho) e uma fração sólida com menor teor de hemicelulose, mais rica em celulose e lignina, menos recalcitrante e mais suscetível à hidrólise enzimática por holocelulases (Hongdan *et al.*, 2013).

1.5. Utilização de licores ricos em hemicelulose

Diferentes abordagens para a valorização de licores de pré-tratamento de biomassa já foram propostos, dentre elas: produção de etanol por microrganismos capazes de fermentar pentoses, incluindo linhagens de *Saccharomyces cerevisiae* geneticamente modificadas (Erdei *et al.*, 2013), produção de biogás por digestão anaeróbica (Rabelo *et al.*, 2011), produção de biohidrogênio (Kaparaju *et al.*, 2009), produção de acetona-butanol-etanol (ABE) (Qureshi *et al.*, 2010), produção de xilitol (Su *et al.*, 2015), produção de lipídeos (Zheng *et al.*, 2012), produção de aminoácidos (Gopinath *et al.*, 2011), produção de furanos (Peleteiro *et al.*, 2016) e refinamento do licor para obtenção de oligossacarídeos pré-bióticos com aplicação alimentícia e farmacêutica (Otieno e Ahring, 2012). A utilização de licores de tratamento hidrotérmico de biomassa como substrato solúvel para produção de enzimas, principalmente holocelulases, por fungos filamentosos também já foi proposta por diferentes autores (Milagres e Prade, 1994; Gyalai-Korpos *et al.*, 2011; Michelin *et al.*, 2012; Ottenheim *et al.*, 2014; De Sousa Paredes *et al.*, 2015; Robl *et al.*, 2015). O uso de licores para produção de holocelulases é revisado no capítulo II e alvo de investigação no capítulo III desta tese.

1.6. Aspergillus niger

Aspergillus niger (Divisão *Ascomycota*, Classe *Eurotiomycetes*, Ordem *Eurotiales*, Família *Trichocomaceae*, gênero *Aspergillus*, sessão *Nigri*) é um fungo filamentoso economicamente importante por ser tradicionalmente utilizado pela indústria de biotecnologia para produção de ácidos orgânicos, principalmente ácido cítrico, e uma variedade de enzimas com aplicações industriais (Baker, 2006; Pel *et al.*, 2007).

A produção industrial de ácido cítrico por *A. niger* é um dos bioprocessos de maior eficiência atualmente realizados pela indústria biotecnológica. O ácido cítrico tem

aplicações em diversos ramos industriais, dentre eles a indústria alimentícia, farmacêutica e cosmética (Schuster *et al.*, 2002; Baker, 2006).

A. niger é capaz de secretar uma grande variedade de enzimas hidrolíticas e oxidativas necessárias para se nutrir de biopolímeros como amido, proteínas e polissacarídeos estruturais da parede celular vegetal. Diversas enzimas de *A. niger* são produzidas comercialmente, dentre elas: amilases utilizadas na produção de etanol e xarope de glicose a partir de amido de milho; glicose oxidase para determinação de glicose em kits de diagnóstico; e holocelulases utilizadas no processamento de frutas pela indústria de sucos e vinhos (pectinases), na indústria de panificação (hemicelulases) e na conversão de biomassa lignocelulósica visando a produção de etanol de segunda geração (Schuster *et al.*, 2002).

A consolidação de *A. niger* como fungo modelo para processos fermentativos se deve em parte pela longa tradição de seu uso seguro. Os principais produtos de *A. niger*, incluindo o ácido cítrico e as diversas enzimas citadas acima, são aprovados pelo órgão americano *Food and Drug Administration* (FDA), conferindo a *A. niger* o status de organismo seguro para uso alimentício e industrial (GRAS, *generally regarded as safe*). Apesar disso, antes de ser utilizada para aplicações industriais, cada nova linhagem de *A. niger* isolada ou desenvolvida deve ser testada quanto à produção de toxinas. *A. niger* apresenta genes putativos envolvidos na síntese das micotoxinas fumonisina e ocratoxina, e algumas linhagens são capazes de produzi-las (Schuster *et al.*, 2002; Pel *et al.*, 2007).

Quatro linhagens de *A. niger* têm o genoma completamente sequenciado e publicamente disponível, sendo elas CBS 513.88, ATCC 1015, An76 e NRRL3. *A niger* CBS 513.88 foi selecionada após mutagênese clássica para superprodução de amilases e corresponde à cepa parental de linhagens atualmente utilizadas na produção industrial de enzimas (Pel *et al.*, 2007). *A. niger* ATCC 1015 é uma cepa selvagem utilizada como modelo para produção de ácido cítrico e corresponde à cepa parental da linhagem ATCC 11414 utilizada na produção industrial deste ácido orgânico (Baker, 2006). *A. niger* An76 tem sido estudada por sua elevada capacidade de produção de endoxilanases e β-xilosidases com aplicações na indústria de polpa de celulose e papel (Gong *et al.*, 2016). *A. niger* NRRL3 (https://genome.jgi.doe.gov/Aspni_NRRL3_1) é uma cepa selvagem precursora da linhagem N402, atualmente estudada com foco na produção de

holocelulases para degradação de biomassa lignocelúlósica (Borin *et al.*, 2015; Borin *et al.*, 2017; Daly *et al.*, 2017).

A disponibilidade de genoma sequenciado torna análises proteômicas e transcriptômicas mais acessíveis e acuradas. Diversos trabalhos transcriptômicos e proteômicos de *A. niger* cultivado em açúcares simples, polissacarídeos purificados e biomassas lignocelulósicas complexas já foram realizados, permitindo uma compreensão cada vez mais detalhada das proteínas e vias metabólicas envolvidas na degradação de parede celular vegetal e na importação e utilização de seus produtos de hidrólise, além das vias de regulação deste processo (Tsang *et al.*, 2009; Souza *et al.*, 2011; Souza *et al.*, 2013; Van Munster *et al.*, 2014; Borin *et al.*, 2015; Florencio *et al.*, 2016; Shi *et al.*, 2016; Borin *et al.*, 2017; Daly *et al.*, 2017; Kowalczyk *et al.*, 2017).

A capacidade de *A. niger* de secretar um amplo espectro de holocelulases, a disponibilidade de diferentes genomas sequenciados, a aplicação consolidada em bioprocessos fermentativos industriais e o seu tradicional uso como microrganismo seguro à saúde humana contribuem para a escolha desta espécie como organismo modelo para avaliar o efeito de tratamento hidrotérmico do bagaço de cana-de-açúcar sobre a produção de holocelulases. A linhagem de *A. niger* utilizada neste trabalho, depositada sob código DCFS 11, foi isolada a partir de amostras de solo do bioma Cerrado, fitofisionomia cerradão floresta.

2. Objetivos

O objetivo geral deste trabalho foi investigar o uso do tratamento hidrotérmico do bagaço de cana-de-açúcar como ferramenta para gerar substratos menos recalcitrantes para o cultivo de *A. niger* DCFS11 e aumentar a produção de holocelulases com aplicação industrial. Para avaliar o potencial de utilização das frações líquida (licor) e sólida geradas no tratamento como fontes de carbono, as seguintes etapas foram realizadas:

1) Otimização dos parâmetros do tratamento hidrotérmico do bagaço de cana-de-açúcar (sendo eles temperatura, tempo de incubação e concentração de sólidos) através de planejamento fatorial de modo a gerar licores adequados para uso como fonte de carbono solúvel para produção de hemicelulases (xilanases e α-L-arabinofuranosidases) por *A. niger*, sem necessidade de etapas de destoxificação (Capítulo III).

2) Caracterização dos licores através de métodos analíticos para identificação de compostos possivelmente responsáveis pela indução de hemicelulases (Capítulo III).
3) Utilizar licor produzido na condição otimizada de tratamento hidrotérmico como fonte de carbono solúvel para escalonamento da produção de hemicelulases em fermentador (Capítulo III).
4) Avaliar o efeito do tratamento hidrotérmico sobre a produção de holocelulases através da comparação dos secretomas de *A. niger* quando cultivado na presença de bagaço de cana-de-açúcar *in natura* e das frações sólida e líquida (licor) geradas no tratamento como fontes de carbono (Capítulo IV).
5) Comparar secretomas através de análise proteômica quantitativa (*label-free*), ensaios de atividade holocelulásica, zimografia e sacarificação enzimática de biomassa (Capítulo IV).

3. Justificativas

As tecnologias de pré-tratamento de materiais lignocelulósicos são intensamente estudadas como ferramenta para diminuir a recalcitrância à sacarificação enzimática e aumentar o rendimento de produção de etanol a partir de tais materiais. Apesar disso, menos atenção é endereçada à utilização de pré-tratamento de biomassa lignocelulósica como ferramenta para aumentar a produção de holocelulases por microrganismos quando utilizando este material como substrato.

As investigações quanto ao efeito do tratamento hidrotérmico do bagaço de cana-de-açúcar sobre a produção de holocelulases por *A. niger* propostas nesta tese visam contribuir para a redução no custo de produção destas enzimas e, consequentemente, contribuir para a viabilidade econômica da conversão enzimática do bagaço de cana-de-açúcar em biorrefinarias.

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Capítulo II

A Review of Holocellulase Production Using Pretreated Lignocellulosic Substrates

Artigo de revisão publicado na revista BioEnergy Research (Editora Springer), volume 10(2), páginas 592-602, ano 2017. Fator de Impacto 2,487. Capes Qualis B1 (Ciências Biológicas I).

Este artigo foca no uso de resíduos lignocelulósicos como fonte de carbono para produção local e integrada de holocelulases por fungos filamentosos em biorrefinarias e na importância da aplicação de tecnologias de pré-tratamento de biomassa para obtenção de substratos mais acessíveis que favoreçam o crescimento fúngico e a produção de enzimas. As principais metodologias de pré-tratamento de biomassa atualmente disponíveis são brevemente descritas e uma revisão sobre o efeito da aplicação destas tecnologias sobre a produção de holocelulases por fungos filamentosos é apresentada. As tecnologias de pré-tratamento de biomassa são mostradas como importantes ferramentas para a redução de custos associados à produção de coquetéis enzimáticos para sacarificação de lignocelulose, um tópico fundamental para a viabilidade econômica de biorrefinarias.

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A Review of Holocellulase Production Using Pretreated Lignocellulosic Substrates

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Abstract The economic viability of enzyme-based lignocellulosic biomass biorefineries depends on the low cost of holocellulose-degrading enzymes necessary for decomposing biomass into fermentable sugars and other value-added products. The high costs of commercial enzymes and the high enzyme loadings required for biomass hydrolysis motivates the use of lignocellulose as feedstock for on-site, integrated production of holocellulases in biorefineries. However, due to high recalcitrance, raw lignocellulose limits fungal growth and enzyme production. Pretreatment technologies can enhance enzyme production when employing lignocellulosic materials as substrate. This review provides a brief description of currently available pretreatment technologies and illustrates the potential of pretreating lignocellulosic wastes for enzyme production with filamentous fungi.

Keywords Filamentous fungi · Cellulase · Hemicellulase · Enzyme cost · On-site production · Biorefinery

Introduction

Lignocellulosic biomass is regarded as an alternative, renewable, low-cost feedstock for the production of green chemicals, materials, and biofuels, primarily in the form of bioethanol. Lignocellulose, i.e., plant cell walls, is composed of a complex array of holocellulose (cellulose, hemicellulose and pectin) and lignin with their proportions varying according to plant species, variety, and tissue. The economic viability of enzyme-based lignocellulosic biomass biorefineries depends on the low cost of holocellulose-degrading enzymes used for decomposing biomass into fermentable sugars and other value-added products [1]. Owing to its complex and intricate structure, efficient deconstruction of biomass usually requires a pretreatment step, which makes it less recalcitrant for posterior enzymatic or microbial conversion. Based on the current biomass pretreatment technologies available, high enzyme loadings are still required for efficient hydrolysis of lignocellulose.

This review examines a few variables impacting the production of holocellulases in the context of biorefineries, such as the location of enzyme production, the material used as feedstock, and the advantages of pretreating lignocellulosic feedstocks for enzyme induction. We provide examples of biomass pretreatments as tools for enhancing holocellulase production. A brief description of currently available pretreatment technologies is also given.

On-Site versus Off-Site Approaches to Enzyme Production

The high enzyme loadings required for biomass hydrolysis have increased the interest in on-site enzyme production approaches, in which biocatalysts are synthetized in facilities annexed to cellulosic ethanol plants or biorefineries [2–4]. On-site production schemes represent an alternative to off-site approaches, which consist of a central factory that supplies multiple biorefineries with commercial enzyme preparations. As a result of simplified logistics, an on-site scheme can reduce the costs and greenhouse gas (GHG) emissions associated with enzyme production, circumventing downstream protein separation processes and the transportation required

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for off-site schemes. Microfiltration and ultrafiltration steps are applied to off-site enzymes to remove cell mass (usually filamentous fungi) and increase protein concentration. These steps lead to protein loss (approximately 15%), reducing final protein yields and increasing final costs [2]. Off-site approaches also require the addition of enzyme stabilizers (usually sucrose or glycerol) to keep enzymes viable during transportation [5]. In contrast, enzymes produced on-site are applied directly to the biomass hydrolysis step as a crude enzyme cocktail, i.e., the whole fermentation broth, including cell mass and residual feedstock. Mycelium from the enzyme fermentation process does not negatively impact the subsequent saccharification of biomass [4]. In fact, *Trichoderma reesei* mycelium-bound enzymes can effectively improve biomass saccharification [6]. The profit margin for off-site producers as well as taxes associated with commercialization also contribute to the higher cost of commercial enzymes [2, 7]. The scale of enzyme production has an important impact on production costs and favors the off-site approach. Increasing the production capacity of a bioethanol or biorefinery plant will decrease on-site enzyme production costs [2]. As pretreatment technologies and the composition of enzymatic cocktails improve, enzyme loadings necessary for efficient biomass hydrolysis will decrease, reducing enzyme expenditures. Independent of the approach used for production, a reduction in enzyme production costs can be achieved by increasing enzyme productivity (the amount of enzyme produced by volume and time) and fermentation yields (amount of enzyme produced according to feedstock weight). Classical mutagenesis, improving enzyme cocktail composition from synergy studies, introducing heterologous holocellulase genes into host organisms, regulating protein expression and secretion by selecting proper promoter regions and signal peptides, and optimizing enzymes by direct evolution techniques represent alternative, cost-reducing strategies [4].

Simple Sugars versus Lignocellulosic Feedstocks

Several papers have described industrial cellulase production using simple sugars, primarily in the form of glucose, as feedstock [2, 3, 8]. When employing glucose, metabolically derepressed microorganisms such as *T. reesei* RUT-C30 must be used for enzyme synthesis [5]. Without metabolic de-repression, glucose would inhibit holocellulase gene expression via the carbon catabolite repression mechanism. Glucose production from corn starch in the USA is energy and GHG intensive and represents the major contributor to GHG emissions and the costs associated with cellulase production [2, 3]. Lignocellulosic feedstocks constitute an alternative method for reducing enzyme costs as they are largely available and much cheaper than glucose. As an example, the price (in $/t) of corn straw is approximately 1/10 that of glucose from cornstarch. Furthermore, the use of lignocellulosic feedstocks renders the process 100% second generation as no food or animal feed is used as substrate. On-site enzyme production using lignocellulosic feedstocks is referred to as integrated production [5].

Lignocellulose can be employed in submerged or solid-state fermentations for holocellulase production. Submerged fermentations are more frequently used as they allow for better parameter monitoring and ease of handling. However, solid-state fermentations are an alternative for reducing production costs owing to their lower energy input, effluent generation, usually higher volumetric productivity, and higher product concentration [9].

A number of studies have demonstrated that lignocellulosic feedstocks induce higher enzyme titers and more diverse holocellulolytic arsenals than simple sugar feedstocks. When simple sugars and de-repressed organisms are employed, the ability of filamentous fungi to secrete complex enzyme arsenals becomes underused [5]. Lau et al. [1] demonstrated that ammonia fiber expansion (AFEX)-pretreated corn stover was a more potent inducer of *T. reesei* RUT-C30 cellulases than lactose at the same weight of sugar. Enzymes induced with corn stover released 2.5- and 7-fold more glucose and xylose from biomass, respectively, than lactose-induced enzymatic broth. Proteomic analysis revealed that corn stover induced significantly higher secretion of several enzymes compared to lactose. These were lytic cellulose monooxigenases (AA 9), endoglucanases (GH 12), endoxylanases (GH 10 and 11), β-xylosidases (GH 3), α-arabinofuranosidases (GH 54 and 62), acetyl xylan esterases, α-glucuronidases (GH 67), and polygalacturonases (GH 28) [1]. In organisms that are catabolite-repressed, the discrepancy in holocellulase induction by simple sugars and lignocellulose is higher. Transcriptomic analysis of *Aspergillus niger* grown on steam exploded sugarcane bagasse revealed that the expression of genes encoding cellulases like endoglucanases, cellobiohydrolases, and β-glucosidases were upregulated compared to fructose. The same was observed in hemicellulases such as endoxylanases, β-xylosidase, β-mannosidase, arabinofuranosidase, α-galactosidase, α-glucuronidase, feruloyl esterase, and acetyl xylan esterase [10]. Brown et al. [11] observed that *A. nidulans* enacts a carbon starvation stress response when transferred from fructose to pretreated sugarcane bagasse. This carbon starvation triggered the expression of several carbohydrate-active enzymes, mainly lytic polysaccharide monooxigenases (AA9) and hemicellulases (GH 2, 3, 10, 11, 43 and 62).

In the integrated production approach, the same feedstock that is converted to ethanol or other value-added products can be used as a carbon source for enzyme synthesis. The cultivation of microbes on a given lignocellulosic substrate is likely to result in an enzymatic arsenal specifically suited for the hydrolysis of that material. In contrast, enzymes produced commercially off-site are designed to hydrolyze a broad

spectrum of substrates with varying chemical compositions [5]. Regarding their efficiency in biomass hydrolysis, there are examples demonstrating that integrated production of enzymes can be as affective or superior to commercial enzymes. Johnson [5] showed that enzymes produced using an integrated approach at low enzyme loadings resulted in equal or superior sugar yields compared to commercial enzymes on the hydrolysis of pretreated wheat straw. Similarly, Juhász et al. [12] showed that enzymes produced in-house by *T. reesei* RUT-C30 on pretreated corn stover performed better than commercial enzymes on the hydrolysis of that specific material. However, this tendency is not always observed. In some cases, commercial enzymes performed better than enzymes produced on-site, while in others, enzymes obtained from cultivation on a given lignocellulosic substrate performed better on the hydrolysis of other substrates [12, 13].

In the same way that raw lignocellulose hinders enzymatic hydrolysis, recalcitrance is also observed with use of untreated lignocellulosics as substrate for enzyme production [14, 15]. It has been observed that pretreatment can enhance microbial growth and holocellulase production when lignocellulose is used as feedstock. All examples cited in this review employ pretreated substrates. In the following sections, we provide a brief description of currently available biomass pretreatment technologies as well as literature examples employing biomass pretreatment as a tool for enhancing holocellulase production.

Pretreatment Technologies

A number of pretreatment technologies have been developed with the primary aim of increasing cellulose digestibility by cellulases as well as ethanol yields following fermentation. Not all pretreatment methods, however, have been tested to obtain better substrates for the induction of holocellulases. Here, we present those methods for which there is data regarding enzyme production.

Hydrothermal Pretreatments

Hydrothermal pretreatments use only water, in the liquid or vapor state, as reagent [16, 17]. Liquid hot water (LHW) pretreatment involves cooking the lignocellulosic material in water under high temperature and pressure. In steam explosion, also known as steam pretreatment, biomass is subjected to hot, high-pressure saturated steam followed by a rapid and explosive decompression step [18]. Key features of hydrothermal pretreatments are hemicellulose solubilization mainly into oligosaccharides, the release of hemicellulose substituents, and partial cleavage of ester linkages between lignin and carbohydrates [19]. Hydrothermal pretreatments may solubilize portions of cellulose that are amorphous, preserving its crystalline form. Under severe conditions, sugar and lignin-derived inhibitors accumulate. Hydrothermal pretreatments result in a liquid hydrolysate (liquor) rich in hemicellulose hydrolysis products. A solid fraction also results with higher cellulose and lignin proportions that are less recalcitrant to further enzymatic and microbial conversion [20, 21].

Acid Pretreatments

Dilute acid pretreatment (DAP) and acid-catalyzed steam pretreatment are the most prominent acidic pretreatment technologies. DAP consists of cooking biomass in a dilute acid solution. In acid-catalyzed steam pretreatment, the biomass is soaked in a dilute, acidic solution or impregnated with an acidic gas prior to being submitted to pressurized steam, as described above. Acid pretreatments are effective in removing hemicellulose from biomass with low cellulose and lignin solubility [16]. Hemicellulose is hydrolyzed into monosaccharides, rather than oligosaccharides. A major drawback of acid hydrolysis is the considerable loss of fermentable sugars due to degradation reactions. This leads to a higher concentration of inhibitory molecules (e.g., furfural, hydroxymethylfurfural—HMF, formic acid and levulinic acid) than any other pretreatment technology.

Alkaline Pretreatments

Alkaline pretreatments are characterized by effectively removing lignin from biomass. This is achieved through the breakdown of lignin-carbohydrate complexes (LCCs) and the bonds within lignin itself. Here, hemicellulose is solubilized to a lesser extent than in hydrothermal or acid pretreatments. The hydrogen bonds between glucan chains are partially disrupted, making cellulose more accessible. Alkaline pretreatments are performed at milder conditions (room temperature–180 °C), generating less inhibitors than other pretreatments. Hydroxide solutions such as NaOH, KOH, $Ca(OH)_2$, and ammonia in aqueous or anhydrous forms are usually used as reagents [16, 22]. AFEX is a prominent alkaline pretreatment technology. It involves cooking the biomass in hot, pressurized liquid anhydrous ammonia. The pressure is rapidly released at the end of the incubation causing the ammonia to vaporize and allowing for its recovery and reuse. Instead of removing lignin, AFEX partially relocates it to the biomass surface. Cellulose allomorphs Iα and Iβ are irreversibly converted into the amorphous-like allomorph IIIi, which is more easily hydrolyzed by cellulases [23, 24]. Safety and environmental concerns arise from the use and storage of ammonia on a large scale, hindering the commercial application of AFEX [25].

Biological Pretreatment

Biological pretreatment seeks to remove lignin from biomass by employing brown, soft, and mainly white rot fungi during solid-state fermentation, exposing holocellulose to subsequent microbial and enzymatic attack. White rot fungi promotes lignin degradation through a variety of oxidative lignolytic enzymes such as lignin peroxidases, manganese peroxidases, and laccases. Generally, some carbohydrate (mainly hemicellulose) is consumed with lignin due to the simultaneous secretion of holocellulases. Fungi mixtures can perform better and faster delignification than single cultures. Biological pretreatment is frequently combined with other physicochemical pretreatments for more efficient reduction of biomass recalcitrance [26].

Ionic Liquid Pretreatment

Ionic liquids (ILs) are able to dissolve biomass by disrupting the strong intermolecular and intramolecular hydrogen bonds that keep cellulose, hemicellulose, and lignin in close association. IL pretreatment simultaneously separates lignin from carbohydrates and reduces cellulose crystallinity without promoting significant depolymerization or degradation reactions [17, 27, 28]. Regenerated cellulose is more susceptible to enzymatic hydrolysis but requires thorough washing steps as ILs are incompatible with holocellulases. Enzyme inactivation depends on IL type, concentration, exposure time, and temperature [28–30]. Increased viscosity, ionic strength [28], and a reduction in thermodynamic water activity in IL-aqueous media [31] are possible causes of decreased activity. Although it is not clear if ILs promote denaturation [32, 33], inhibition was shown to be reversible [28, 31]. The search for IL-tolerant cellulases has been a focus for many groups [29, 34, 35].

Holocellulase Production Using Pretreated Feedstocks

Adjusting the parameters of lignocellulose hydrothermal pretreatment can greatly enhance the production of enzymes by filamentous fungi when cultivated in the presence of those materials. Goldbeck et al. [36] showed that mild severity steam pretreatment of sugarcane bagasse (steam pressure/temperature of up to 1.2 MPa/191.5 °C) increased cellulase production by *Acremonium strictum*. Here, filter paper (FPase), carboxymethylcelulase (CMCase), cellobiase, and β-glucosidase activities were approximately 60, 90, 280, and 24% higher, respectively, in comparison to raw, untreated material. However, high-severity pretreatment (steam >1.2 MPa/191.5 °C) of substrate reduced CMCase and β-glucosidase production, possibly due to the presence of fermentation inhibitors in pretreated biomass [36]. Similarly, corn straw subjected to mild steam pretreatment yielded greatly enhanced FPase production by *T. reesei* YG3 during solid-state fermentation. This increase was up to 36-fold higher than that obtained with untreated straw, while fungal growth was completely inhibited on severely pretreated material [37]. Nevertheless, inhibition could be alleviated by oven-drying or washing the pretreated straw. This reduced the concentration of furfural, phenolic compounds, and weak acids from the material and promoted *T. reesei* growth [37]. Bigelow and Wyman [38] also observed that an intense washing step of LHW-pretreated sugarcane bagasse promoted growth of *T. reesei* RUT-C30 and cellulase production in submerged fermentations. An efficient washing step of pretreated biomass proved to be necessary for the removal of any residual toxic compounds generated during severe pretreatment.

Physical changes in biomass structure provoked by pretreatment play an important role in holocellulase induction. Pretreatment modifies fiber surface morphology from smooth in the untreated form to rough after pretreatment. Pretreatment also reduces average particle size and increases specific surface area, pore volume, and diameter [39]. Also observed is an increase in the accessibility of biomass polysaccharides, promoting better microbial growth and higher enzyme production. Pereira et al. [7] associated the enhanced production of cellulases and xylanases by *Penicilium echinulatum* grown on hydrothermally pretreated bagasse with the increase in biomass-specific surface area led by pretreatment (from 0.65 to 1.07 m^2/g). In accordance, production of FPase, endoglucanase, and β-glucosidase by *T. reesei* under solid-state fermentation on steam-pretreated soybean hulls also increased. This was mainly attributed to an increase in the volume of gas occupying the continuous network of space between and within substrate particles, a property known as bed porosity. The chemical composition of the hulls did not change significantly after pretreatment [40]. The increase in bed porosity led to superior oxygen availability in the solid-state fermentation system, which affected aerobic fungal propagation and enzyme production [40].

A study by Ribeiro et al. [41] compared the secretomes of *P. echinulatum* cultivated in the presence of raw sugarcane bagasse with bagasse derived from DAP, LHW, or steam explosion pretreatments. All pretreatments reducing the pectin and hemicellulose content of biomass provoked a shift toward secreted enzymes involved in cellulose degradation. Proteomic analysis revealed that the great majority of enzymes secreted by *P. echinulatum* in the presence of pretreated bagasses are cellulases belonging to GH families 3, 5, 6, 7, 12, and 17; AA 9; and swollenins. In contrast, the secretome produced in the presence of untreated bagasse, the most heterogenic and complex carbon source tested, presented a greater diversity of GH family members. In addition to the abovementioned cellulases, several hemicellulases;

pectinases; and amylases from GH 10, 11, 13, 43, 62, 28, CE 1, and CE 2 were also secreted. These results indicate that biomass pretreatment can also be used to modulate the secretome of fungi for desired enzymatic activities [41].

The liquid fractions obtained after lignocellulose pretreatment can also be used as a soluble substrate for holocellulase production, mainly as hemicellulases. Hydrothermal pretreatment liquors are rich in hemicellulose hydrolysis products such as monomeric and oligomeric xylose. Soluble carbohydrates, being more easily assimilated by fungi than native polysaccharides, promote faster fungal growth and, at low concentrations, act as inducers of holocellulases. Robl et al. [42] demonstrated that xylanase production by *A. niger* DR02 cultivated on pentose-rich liquor from hydrothermal pretreatment of sugarcane bagasse was high and exceeded that induced by the solids arising from the same pretreatment. Liquor had to be diluted to 50% (*v*/*v*) in order to reduce the concentration of pretreatment-derived inhibitors. In batch fermentations using bagasse liquor as feedstock, xylanase titers only increased after free xylose and xylooligomer concentrations dropped to near depletion. This indicates that carbon source exhaustion is associated with xylanase production by *A. niger* DR02. Fed-batch fermentations designed to keep monosaccharide concentrations low (<1.0 g/L) throughout the entire cultivation allowed xylanase production to reach very high values: up to 458 IU/mL compared to 229 IU/mL obtained under batch mode [42]. Monosaccharides inhibit lignocellulolytic enzyme synthesis in filamentous fungi via the CreA-mediated carbon catabolite repression (CCR) mechanism. CreA or Cre1 are DNA-binding proteins that, in the presence of elevated monosaccharide concentrations, bind to the promoter region of transcription factor genes, blocking their transcription and subsequent expression of regulated holocellulase genes. The consumption of simple sugars from liquor during the initial stages of fungal growth and the maintenance of low concentrations during fed-batch allow the fungus to enter a carbon limitation situation. This is required for carbon catabolite de-repression and holocellulase transcription and secretion [42, 43]. At low levels, plant cell wall-derived monosaccharides induce the binding of positive transcriptional regulators (such as XlnR) to cellulase, hemicellulase, and pectinase genes. This promotes the secretion of a suitable hydrolytic arsenal to degrade the detected substrate [43]. Proteomic analysis of the secretome of *A. niger* DR02 grown on bagasse hydrothermal liquor identified enzymes related mainly to xylan breakdown such as endoxylanases, β-xylosidase, α-L-arabinofuranosidase, and feruloyl esterase. Endoglucanases, cellobiohydrolases, and β-glucosidase were also detected [42]. Similarly, transcriptomic analysis of *A. niger* DSM 26641 grown on palm oil empty fruit bunch hydrothermal hydrolysate revealed three endoxylanases and one α-L-arabinofuranosidase with high expression levels. β-xylosidase, acetyl xylan esterase, and feruloyl esterase genes were expressed at lower levels [44]. Other works reinforce the potential of pretreatment liquors as inducers of holocellulases. Sugarcane bagasse liquor obtained after steam explosion was a strong inducer of xylanase synthesis by *P. janthinellum*, being more effective than pure xylan or xylose at similar concentrations [45]. Gyalai-Korpos et al. [46] showed that *T. reesei* Rut C-30 demonstrated higher cellulase and xylanase activity when grown on diluted and detoxified liquor from wheat straw steam explosion than on washed solids arising from the same pretreatment. Despite this, supplementing liquors with pretreated solids, crystalline cellulose, or even untreated lignocellulosic materials usually enhances the induction potential of liquors [46–50].

Within the context of biorefineries, the economic viability on an industrial scale involves, among other things, the maximum utilization of components from pretreated lignocellulosic feedstock and the recycling of water. Using pretreatment liquors as a culture medium for on-site enzyme production satisfies those two important demands. Fermentation of pretreatment liquors by filamentous fungi allows for more complete utilization of the carbohydrate and non-carbohydrate content of biomass since these organisms show higher metabolic diversity than yeasts. As an example, Cavka and Jönsson [51] showed that *A. niger* and *T. reesei* utilize glucose, mannose, xylose, arabinose, galactose, oligosaccharides, acetic acid, and formic acid present in Norway spruce SO_2-catalyzed steam pretreatment liquor. Meanwhile, *Saccharomyces cerevisiae* and *Pichia pastoris* can only efficiently metabolize glucose and mannose while being unable to consume pentoses and aliphatic acids. Filamentous fungi can also perform biological detoxification of liquors by consuming inhibitors of yeast fermentation and xylooligosaccharides, which are known as inhibitors of cellulase activity [52–54]. Liquor detoxification allows for its reuse in ethanol-producing biorefineries, circumventing its inhibitory effects on subsequent enzymatic hydrolysis of pretreated biomass or ethanolic fermentation. It was shown that *T. reesei* and *A. nidulans* could bio-detoxify steam-pretreatment liquors from willow and corn stover, respectively. This is accomplished by consuming high amounts of acetic acid, formic acid, furfural, and HMF, while simultaneously secreting cellulases [49, 50].

There is no consensus on the use of alkaline pretreatment of biomass for obtaining more suitable substrates for holocellulase induction. A variety of works has shown negative, positive, and neutral effects of alkaline pretreatment for this application (Table 1). Yoon et al. [55] observed a much lower recovery of FPase, CMCase, and β-glucosidase activities from solid-state fermentation of *Pycnoporus sanguineus* grown on NaOH-pretreated sugarcane bagasse than on untreated material. The unfavorable effect of pretreatment was attributed to the increased adsorption of cellulase onto pretreated lignocellulose, which was a consequence of the

Table 1 The effect of alkaline pretreatment of lignocellulosic substrates on the production of cellulases and xylanases by filamentous fungi

Lignocellulosic biomass/ organism	Pretreatment conditions	FPase	CMCase	β-glucosidase	Xylanase	Reference
Soybean hulls/ *T. reesei*	1.0 N NaOH, ambient temperature, 24 h	−50%	−46%	−15%	−100%	[40][a]
Soybean hulls/ *A. oryzae*		−90%	−85%	−90%	−97.3%	
Soybean hulls/ *T. reesei x A. oryzae*		−90%	−93%	−95%	−96.4%	
Sugarcane bagasse/ *T. reesei*	0.25 N NaOH, room temperature, 30 min	Unnafected				[71]
	0.25 N NaOH, 100 °C, 30 min	−100%				
Sugarcane bagasse/ *P. funiculosum*	4% *w/v* NaOH, 121 °C, 20 min	+330%	−56%	+69%		[57]
Sugarcane bagasse/ *P. equinulatum*	16% *w/v* NaOH, 120 °C, 20 min	+35%	+13%	−18%	−32%	[14]
	16% *w/v* NaOH, 0.3% H_2O_2, 0.02% AQ	+140%	+44%	+17%	−35%	
	16% *w/v* NaOH, 0.6% H_2O_2	+62%	+23%	+47%	−34%	
	16% *w/v* NaOH, 0.6% H_2O_2, 0.02% AQ, 0.3% EDTA	+100%	+48%	+24%	−40%	
Soybean hulls/ *T. reesei*	1% *w/v* NaOH, 121 °C, 30 min	+60%			Unaffected	[15]
Wheat straw/ *A. niger*	0.25 M NaOH, 121 °C, 1 h	+160%	+150%	+150%		[72]
Corn cobs/ *A. niger*		+190%	+70%	+120%		
Groundnut shells/ *A. niger*		+190%	+90%	+210%		
Sugarcane bagasse/ *A. niger*		+140%	+120%	+110%		
Sugarcane bagasse/ *T. reesei* PC-3-7	0.1 N NaOH, 120 °C, 15 min		+155%			[73]
	0.3 N NaOH		+190%			
	1.0 N NaOH		+200%			
	3.0 N NaOH		+172%			
Sugarcane bagasse/ *P. janthinellum*	20% *w/v* NaOH solution, 4 h, 25 °C	+22%	+17,5%	+64%	−22%	[56]
	bagasse/$NaClO_2$ 1:0.5; 4 h, 70 °C	+40%	+40,3%	+178%	+25%	
	bagasse/$NaClO_2$ 1:0.25; 4 h, 70 °C	+78%	+24,5%	+277%	+9%	
Sugarcane bagasse/ *T. viride*	20% *w/v* NaOH solution, 4 h, 25 °C	+185%	+45%	+207%	−7.3%	
	bagasse/$NaClO_2$ 1:0.5; 4 h, 70 °C	+189%	+30%	+154%	−16.4	
	bagasse/$NaClO_2$ 1:0.25; 4 h, 70 °C	+214%	+65%	+419%	Unaffected	

All comparisons were made on basis of volumetric enzymatic activities (IU/mL)

AQ anthraquinone, *EDTA* ethylenediaminetetraacetic acid

[a] IU/g dried substrate

increased accessibility of cellulose [55]. Conversely, FPase, CMCase, and β-glucosidase activities arising from submerged cultivation of *P. janthinellum* and *T. viride* on sugarcane bagasse were increased when the substrate was pretreated with NaOH, $NaClO_2$, or H_2O_2 at different conditions [56]. Alkaline pretreatment of biomass is frequently applied in combination to hydrothermal or acid pretreatments. In the work of Castro et al. [57], acid and alkaline pretreatments of sugarcane bagasse alone or in sequence were employed to generate better substrates for cellulase production by *P. funiculosum*. The combined H_2SO_4 and NaOH pretreatments culminated in higher FPase, endoglucanase, and β-glucosidase production than NaOH pretreatment alone. This was likely due to increased glucan content, lower lignin and hemicellulose contents, smaller average particle size, and larger superficial area of the dual-pretreated substrate.

There are few examples in the literature of biological pretreatment of lignocellulose for the purposes of enhanced holocellulase production in subsequent fungal fermentation steps. Ideally, low carbohydrate consumption should take place during biological pretreatment, preserving it for the microorganism to be inoculated. Camassola and Dillon [58] studied the use of sugarcane bagasse arising from biological pretreatment with *P. sajor-caju* PS2011 as feedstock for cellulase and xylanase production by *P. echinulatum* in a posterior fermentation step. *P. sajor-caju* was not selective for lignin during pretreatment and consumed fair amounts of carbohydrates from bagasse. Consequently, holocellulase production by *P. echinulatum* was lower on pretreated material than on integral sugarcane bagasse [58].

To the best of our knowledge, there are no literature reports available on the use of lignocellulose pretreated with ILs as feedstock for holocellulase production. ILs are not only damaging for enzymes, they are also toxic to many microorganisms. We will describe an example of the toxicity of 1-ethyl-3-methylimidazolium chloride ([C2min]Cl) toward

Enterobacter lignolyticus, an anaerobic bacterium capable of lignin degradation [59]. Lag phase was extended and maximum growth rates were reduced in the presence of IL at increasing concentrations. A comparison with NaCl revealed that imidazolium [C2mim] cations, and not Cl anions, are responsible for growth inhibition. [C2min]Cl was neither degraded nor consumed by *E. lignolyticus*, indicating that imidazolium-based ILs are of low biodegradability. The tolerance response of *E. lignolyticus* toward IL involved (i) remodeling of the cell membrane composition towards membrane stabilization; (ii) an increase in the intracellular concentration of compatible solutes such as sugars, amino acids, and peptides, as a strategy to offset the osmotic pressure caused by IL; (iii) up-regulation of drug efflux pumps, which are promiscuous to some extent and may play an important role in [C2mim]Cl tolerance; and (iv) down-regulation of porins leading to a decrease in membrane permeability to IL [59].

As illustrated in the examples above, biomass pretreatment can greatly affect enzyme production due to the chemical and physical changes provoked by the process. Depending on the method and severity, pretreating lignocellulosic substrate can increase enzymatic titers and change the pattern of holocellulase expression. However, if not optimized for this purpose, pretreatment can also have negative effects on the induction of holocellulases. Some pretreatment technologies can generate two different substrates from the same feedstock. Specifically, the solid and liquid fractions (Fig. 1a), which usually induce different sets of enzymes, shifted toward cellulose or hemicellulose degradation, respectively.

It is noteworthy that enzyme induction by lignocellulosic feedstocks does not depend exclusively on pretreatment. The chemical composition of the substrate plays an important role in the composition of the resultant enzyme broth, and this is actually one of the reasons why pretreatment affects the induction profile. As an example, feedstocks with high xylan content, such as corn stover, tend to induce high xylanase and β-xylosidase activities. Galactoglucomanan-rich materials, such as softwood, tend to induce higher expression of β-mananase, β-galactosidade, and galacturonase than hardwood or agricultural wastes [12, 60]. However, depending on the organism, hemicellulases are expressed regardless of the presence of hemicellulose as they are induced by cellulose [60, 61].

Although pretreatment has the potential to enhance holocellulase production, it is an expensive process [16]. Techno-economic studies on the use of pretreated feedstocks in comparison to raw materials for holocellulase production are lacking. These are required to assess whether the advantages of using pretreated biomass actually compensate financially for the viability of biorefineries. The fact that a pretreatment unit is already part of the scope of a biorefinery (seen as fundamental for efficient enzymatic hydrolysis of the feedstock) favors the use of pretreated biomass for the production of holocellulases.

In the cases of using lignocellulose as feedstock for on-site enzyme production, the yield of final product (ethanol or other value-added products) is reduced because part of the incoming sugar biomass is allocated for enzyme synthesis [2]. Even so, recent techno-economic studies [5, 62] have shown the viability of some on-site production scenarios. Barta et al. [62] compared various scenarios involving on-site, integrated cellulase production in a softwood-based ethanol plant. The use of pretreatment liquor from steam explosion of spruce supplemented with molasses as carbon source for *T. reesei* RUT-C30 was shown to be more economically advantageous than relying on commercial enzymes. A study by Johnson [5] estimated that cellulase costs in a corn straw ethanol plant (70,000 t/year for ethanol production) can be significantly reduced from 0.78 to 0.58 to 0.23 $/gallon of ethanol by changing the cellulase production approach from off-site to on-site (using glucose as feedstock) to integrated scheme (using corn straw as feedstock).

Other Alternative Lignocellulosic Feedstocks for On-Site Production

Enzymatically hydrolyzed lignocellulose is also a viable carbon source for enzyme production (Fig. 1b). The biomass hydrolysate is rich in fermentable monosaccharides (mainly glucose), which is normally inoculated with *S. cerevisiae* in the situation of second-generation ethanol production. As an alternative, a portion of the hydrolysate can be diverted and used as a sugar-rich media for enzyme production. Tolan [63] reported that a fraction of the sugar slurry obtained from the enzymatic hydrolysis of pretreated feedstock was used as carbon source for cellulase production at an Iogen's ethanol production facility. In the work of Cunha et al. [64], enzymatically liquefied sugarcane bagasse was used as culture medium for *A. niger* and was able to induce 15 times higher endoglucanase activity (2.5 IU/mL) than the non-liquefied pretreated biomass under solid-state fermentation (0.17 IU/mL).

After enzymatic hydrolysis of biomass polysaccharides, a solid residue remains unhydrolyzed, corresponding mainly to lignin, and unconsumed cellulose and hemicellulose. This material, often called filter cake or lignin cake, still contains residual polysaccharides and may also be used as a substrate for hydrolytic enzyme production under submerged or solid-state fermentation (Fig. 1b). It is possible that fungi able to grow in such materials have enzymes that can hydrolyze the more recalcitrant portions of cellulose and hemicellulose [65]. The reuse of polysaccharides from filter cake as carbon source seems interesting because enzyme synthesis would not compete with ethanol production regarding the feedstock. At the same time, it would add value to this residual fraction by looping it back to the process [65]. Hogan and Mes-Hartree

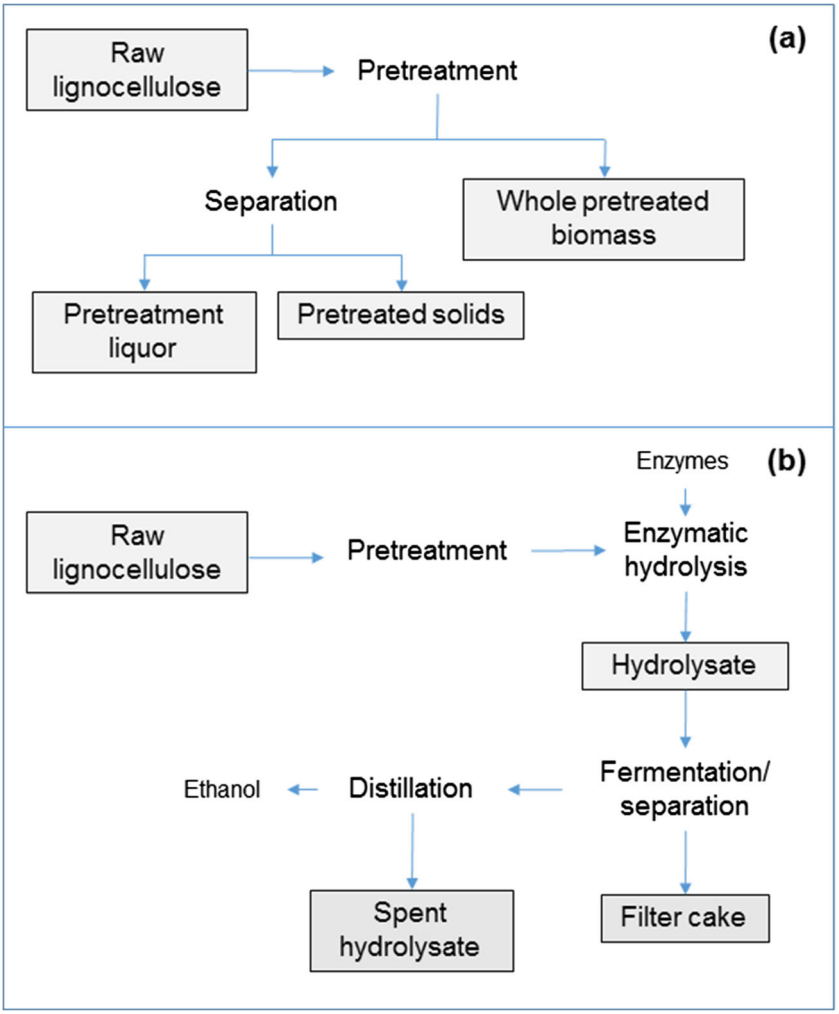

Fig. 1 Possible feedstocks (*gray rectangles*) for on-site, integrated production of holocellulases in lignocellulose biorefineries. Lignocellulose may be used in raw or pretreated forms (**a**). Hydrothermal, acid, and alkaline pretreatments generate solid and liquid fractions, which can both be used as carbon source. Depending on pretreatment severity, detoxification steps may be required to remove inhibitors from pretreated solids and liquor. Downstream processed biomass are also viable options for carbon sources (**b**). Part of the biomass hydrolysate may be diverted for enzyme synthesis. Filter cake and spent hydrolysate, viewed as biorefinery wastes, are also potential substrates for enzyme production as they do not compete with the production of the final products (ethanol or other value-added products)

[66] showed that residual filter cake from the enzymatic hydrolysis of steam-exploded aspenwood induced high levels of cellulase production by *T. harzianum*. The low cellulose content in filter cake was not a problem for enzyme induction. In fact, filter cake obtained after prolonged hydrolysis periods gave higher cellulase yields. In spite of that, high filter cake loadings in the medium were detrimental to enzyme production, possibly due to the higher lignin amounts exposed during the process of fermentation [66]. In the work of Dopplebauer et al. [67], filter cake obtained after enzymatic hydrolysis of pretreated wheat straw induced higher FPase and β-glucosidase activities by *T. reesei* than pretreated straw itself.

Another alternative carbon source is the spent hydrolysate, or thin stillage, obtained after distillation of the fermented biomass hydrolysate (Fig. 1b). Recombinant *S. cerevisiae* strains are capable of utilizing both pentoses and hexoses from lignocellulose. In spite of that, industrial and robust wild type *S. cerevisiae* strains can only efficiently convert glucose and mannose, leaving galactose, xylose, arabinose, and aliphatic acids virtually unconsumed in fermentation broth. Sequential fermentation with filamentous fungi enables the utilization of unmetabolized carbon sources for the production of enzymes or other valuable products [68, 69].

Conclusions

The use of lignocellulose as feedstock for on-site, integrated production of holocellulases may bring a number of advantages over the off-site and simple sugar-based production approach. Lignocellulose biorefining would be completely second generation and enzyme cocktails would also be specifically targeted for the degradation of the incoming biomass. Simplified logistics and low-cost feedstock would reduce enzyme production costs. Lignocellulose, being recalcitrant, usually requires pretreatment steps to make the substrate available for fungal growth and enzyme synthesis. According to Klein-Marcuschamer et al. [70], enzymes still contribute a

large portion of the cost of lignocellulose bioconversion, and significant efforts are still required to lower enzyme costs for biofuel production. Reducing feedstock prices and enzyme fermentation residence times are two important means to reduce enzyme production costs. In this direction, using residues from the agroindustry can be helpful. Pretreatment of the feedstock has already been shown to have a positive effect on reducing enzyme fermentation times, releasing readily available sugars, and making biomass more accessible to fungal growth, thus it can also contribute to lowering enzyme production costs [70].

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Capítulo III

Sugarcane bagasse hydrothermal pretreatment liquors as suitable carbon sources for hemicellulase production by *Aspergillus niger*

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Este trabalho teve como objetivo avaliar o potencial de licores resultantes do tratamento hidrotérmico do bagaço de cana-de-açúcar como fonte de carbono solúvel para produção de hemicelulases por uma linhagem de *A. niger* isolada de amostras de solo do bioma Cerrado. O efeito dos parâmetros do tratamento hidrotérmico (temperatura, tempo de incubação e concentração de bagaço de cana-de-açúcar) sobre a composição dos licores e a produção de endoxilanases e α-L-arabinofuranosidases foi investigado através de planejamento fatorial. Todos os licores gerados induziram maior produção de xilanase e arabinofuranosidase que o bagaço de cana-de-açúcar *in natura* quando utilizados como fonte de carbono. O tratamento hidrotérmico de baixa severidade e baixa concentração de sólidos (170°C, 30 min, 1 % m/m bagaço de cana-de-açúcar) foi selecionado como condição ótima devido à alta e rápida indução enzimática pelo licor resultante sem que houvesse necessidade de métodos de destoxificação. Análises de cromatografia líquida e espectrometria de massas dos licores identificaram uma variedade de mono e oligossacarídeos responsáveis pela indução de hemicelulases.

BioEnergy Research
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Sugarcane Bagasse Hydrothermal Pretreatment Liquors as Suitable Carbon Sources for Hemicellulase Production by *Aspergillus niger*

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Abstract

The aim of this study was to valorize the hemicellulose-rich liquid fraction (liquor) arising from hydrothermal pretreatment of sugarcane bagasse (SCB) through its utilization as an unconventional, soluble carbon source for the production of hemicellulases, namely xylanases and α-L-arabinofuranosidases (ABFases), by *Aspergillus niger* DCFS11. Through the use of factorial design, pretreatment conditions producing liquors optimized for either early- or late-phase enzyme production were identified. Subsequent deep characterization of liquor components using liquid chromatography and mass spectrometry was performed to identify compounds likely responsible for hemicellulase induction. SCB liquors arising from various pretreatment configurations induced up to 2- and 8.6-fold higher xylanase and ABFase production, respectively, by *A. niger* DCFS11 than raw SCB substrate owing to the strong inducing potential of arabinosylated xylooligosaccharides and free arabinose solubilized during pretreatment. Notably, unlike the severe pretreatment conditions required for maximum cellulose saccharification and ethanol yields during biomass conversion, low severity and low biomass loading are required if enzyme production from liquor is desired at early-phase growth with no additional detoxification steps. This suggests that for effective application in biorefineries, separate or multi-step processes would be required to optimize both hemicellulase production by *A. niger* DCFS11 and cellulose digestion. This work demonstrates the potential of hydrothermal pretreatment of lignocellulosic substrates as a tool to increase the production of enzymes by filamentous fungi.

Keywords Hydrothermal pretreatment · Liquor · Sugarcane bagasse · *Aspergillus niger* · Xylanase · α-L-Arabinofuranosidase

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Introduction

The valorization of hemicelluloses from biomass, i.e., their conversion into valuable bio-products, has been considered strategic for the economic viability and full implementation of lignocellulose biorefineries [1]. Traditionally, in biomass refining, hemicellulose is extracted by hydrothermal or acid pretreatments, yielding a hemicellulose-rich liquid fraction as a side stream (pretreatment liquor) and a solid fraction enriched in cellulose and lignin, less recalcitrant for enzymatic saccharification [2].

Among different valorization approaches, biomass pretreatment liquors arise as potential low-cost carbon sources for the production of hemicellulases by filamentous fungi. Previous studies by Michelin et al. [3, 4], Ottenheim et al. [5], Paredes et al. [6], and Robl et al. [7], among others, have successfully explored this liquor valorization approach by using *Aspergillus* species as model hemicellulase producers. This approach is compatible with the concept of integrated enzyme production,

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in which part of the lignocellulosic feedstock (in this case, a liquid stream from biomass processing) coming into a biorefinery is diverted for on-site enzyme synthesis, reducing dependence on expensive commercial enzymes produced off-site [8]. In addition, pretreatment liquors likely induce an enzymatic arsenal especially suited for the hydrolysis of polysaccharides present in the incoming feedstock [8]. Liquor utilization as a soluble carbon source also mitigates problems associated with the use of insoluble substrates (e.g., raw biomass or pretreated solids) for enzyme synthesis in industrial-scale submerged fermentations, such as difficulty in homogenization and aeration of the fermentation broth [6].

Despite these advantages, the utilization of liquors for microbial growth is limited by inhibitory molecules such as furans, phenolic compounds, and weak acids generated during pretreatment [9]. Pretreatment of biomass is usually aimed primarily at the maximization of cellulose enzymatic saccharification yields, which are only achieved after severe conditions that generate microbial inhibitors as a counterpart. Under such conditions, furans are generated from pentose and hexose sugars by cyclodehydration reactions with the formation of furfural and hydroxymethylfurfural (HMF), respectively, which are further converted into formic and levulinic acids as pretreatment severity increases [10, 11]. Acetic acid is released from hemicellulose while phenolic compounds are solubilized from lignin [9]. Detoxification steps are frequently applied with the purpose of lowering inhibitor concentrations, but are undesirable for liquor valorization at the industrial scale [9].

Sugarcane bagasse (SCB) constitutes a promising raw material for Brazilian biorefineries owing to its enormous availability as a waste in sugarcane mill plants. SCB generation in Brazil is expected to reach over 180 million tons in the 2017/2018 season alone [12], and although a portion of this amount is used for cogeneration of heat and power in the plants, the conversion of bagasse into bio-products may add more value to this material. In particular, SCB contains large amounts of hemicelluloses (22–27% according to Canilha et al. [13]) in the form of acetylated glucuronoarabinoxylan (main component), mixed-linkage β-glucans, xyloglucans, and glucomannan [14–16]. This indicates that a complex arsenal of hemicellulases is required for its complete breakdown and that hydrothermal pretreatment of this material generates hemicellulose-rich liquors that may be useful for the induction of such enzymes.

The aim of this study was therefore to establish, with the aid of factorial design, SCB hydrothermal pretreatment conditions that produce suitable liquors for utilization as a carbon source for *Aspergillus niger* DCFS11 cultivation and production of two important hemicellulases for SCB hydrolysis, namely xylanases and α-L-arabinofuranosidases (ABFases), while avoiding the application of liquor detoxification procedures. A deep characterization of liquor composition was also performed by liquid chromatography and mass spectrometry to enable the identification of compounds responsible for hemicellulase induction.

Materials and Methods

Feedstock and Hydrothermal Pretreatment Procedure

SCB was obtained from a local sugarcane mill (Jalles-Machado S/A, Goianésia, State of Goiás, Brazil). Biomass was ground to form a homogeneous fine powder, which was used as feedstock in the pretreatment process and as a carbon source for fungus cultivation.

For the hydrothermal pretreatment procedure, sealable stainless steel cylindrical reactors of internal volume of 300 ml (Swagelok, Cleveland, OH, USA) were filled with SCB and distilled water at different solid loadings and incubated at specific temperatures and periods in a pre-heated fluidized sand bath (Tecam SBL-2, Cole Parmer, Vernon Hills, IL, USA), according to the factorial design displayed in Table 1. Final weight of all pretreatment mixtures was 225 g. After pretreatment, reactors were quenched in cold water. Pretreated slurry was vacuum filtered using a filter paper and the pH and volume of the liquid fraction (liquor) were measured. Liquor was centrifuged (2739*g* for 10 min) and the supernatant was stored at −20 °C for further compositional analyses and culture medium formulation. Pretreatment severity factor (SF) was calculated according to Overend and Chornet [17]: $SF = \log \{IP \times \exp[(T - 100) / \omega]\}$, where IP is the incubation period (min), T is the temperature (°C), and ω is an adjustment parameter, fixed at the value of 14.75. Pretreatment heat-up phase (not determined) was accounted for in the incubation period.

Factorial Design

A central composite rotatable design (CCRD, Table 1) was performed to evaluate the effect of pretreatment variables, i.e., temperature, incubation period, and SCB loading, on the composition of the resulting liquors and the xylanase and ABFase activities produced by *A. niger* when using liquors as the carbon source. Pretreatments were performed in triplicate and the central point was replicated nine times. The significance of the effect of pretreatment variables and the mathematical response prediction models were assessed by *t* test, *F* test, and analysis of variance (ANOVA) employing a 10% significance level. Statistica v. 12 (Statsoft Inc., Tulsa, OK, USA) and Design-Expert v. 7 (Stat-Ease Inc., Minneapolis, MN, USA) software packages were used for statistical analyses.

Compositional Analysis of Raw SCB and Pretreatment Liquors

Raw SCB composition was determined according to Sluiter et al. [18] and the quantification of monosaccharides obtained after two-step acid hydrolysis of biomass was performed by high-performance anion exchange chromatography coupled

Table 1 Experimental design (CCRD) and hemicellulase activities produced by *A. niger* DCFS11 using raw sugarcane bagasse (SCB) and hydrothermal pretreatment liquors as a carbon source. Coded values are show in brackets

Pretreatment/carbon source	Temperature (°C)	Incubation period (min)	SCB loading (% *w*/*w*)	Severity factor (SF)	Xylanase (IU ml^{-1})		α-L-Arabinofuranosidase (IU ml^{-1})		β-1,3-Glucanase (IU ml^{-1})
					Day 2	Day 7	Day 2	Day 7	Day 7
Raw SCB					0.34 ± 0.06	1.79 ± 0.22	0.01 ± 0.00	0.02 ± 0.00	0.09 ± 0.02
1	160 (− 1)	15 (− 1)	3 (− 1)	2.94	2.23 ± 0.03	2.27 ± 0.15	0.04 ± 0.00	0.04 ± 0.00	0.20 ± 0.01
2	180 (+ 1)	15 (− 1)	3 (− 1)	3.53	2.49 ± 0.03	2.56 ± 0.04	0.04 ± 0.00	0.07 ± 0.00	0.24 ± 0.01
3	160 (− 1)	45 (+ 1)	3 (− 1)	3.42	2.30 ± 0.35	2.86 ± 0.03	0.02 ± 0.01	0.08 ± 0.01	0.34 ± 0.01
4	180 (+ 1)	45 (+ 1)	3 (− 1)	4.01	2.55 ± 0.10	3.07 ± 0.13	0.01 ± 0.00	0.08 ± 0.00	0.14 ± 0.01
5	160 (− 1)	15 (− 1)	9 (+ 1)	2.94	2.54 ± 0.20	2.78 ± 0.19	0.07 ± 0.01	0.11 ± 0.01	0.25 ± 0.04
6	180 (+ 1)	15 (− 1)	9 (+ 1)	3.53	2.60 ± 0.05	2.95 ± 0.33	0.08 ± 0.01	0.17 ± 0.02	0.35 ± 0.04
7	160 (− 1)	45 (+ 1)	9 (+ 1)	3.42	2.59 ± 0.03	2.97 ± 0.08	0.05 ± 0.01	0.16 ± 0.01	0.18 ± 0.03
8	180 (+ 1)	45 (+ 1)	9 (+ 1)	4.01	n.d.	3.13 ± 0.25	n.d.	0.04 ± 0.00	0.09 ± 0.04
9 (CP)	170 (0)	30 (0)	6 (0)	3.54	2.81 ± 0.14	3.53 ± 0.11	0.04 ± 0.02	0.14 ± 0.01	0.21 ± 0.02
10 (CP)	170 (0)	30 (0)	6 (0)	3.54	2.65 ± 0.19	3.45 ± 0.15	0.03 ± 0.01	0.13 ± 0.00	0.22 ± 0.02
11 (CP)	170 (0)	30 (0)	6 (0)	3.54	2.52 ± 0.19	3.34 ± 0.23	0.03 ± 0.01	0.12 ± 0.00	0.23 ± 0.05
12	153 (− 1.68)	30 (0)	6 (0)	3.04	2.60 ± 0.03	2.76 ± 0.07	0.08 ± 0.00	0.13 ± 0.00	0.25 ± 0.02
13	187 (+ 1.68)	30 (0)	6 (0)	4.04	2.13 ± 0.33	3.11 ± 0.10	0.01 ± 0.01	0.11 ± 0.00	0.13 ± 0.02
14	170 (0)	5 (− 1.68)	6 (0)	2.76	1.95 ± 0.07	2.28 ± 0.18	0.02 ± 0.00	0.02 ± 0.00	0.21 ± 0.01
15	170 (0)	55 (+ 1.68)	6 (0)	3.80	1.11 ± 0.04	3.09 ± 0.18	n.d.	0.11 ± 0.01	0.14 ± 0.02
16	170 (0)	30 (0)	1 (− 1.68)	3.54	2.53 ± 0.02	2.85 ± 0.07	0.01 ± 0.00	0.04 ± 0.00	0.25 ± 0.02
17	170 (0)	30 (0)	11 (+ 1.68)	3.54	2.54 ± 0.12	3.22 ± 0.06	0.04 ± 0.02	0.14 ± 0.02	0.19 ± 0.04

CP central point, *n.d.* not detected

with pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS-3000DC System equipped with a CarboPac PA-1 column (Dionex Co., Sunnyvale, CA, USA). The elution gradient comprised 15–22 mM NaOH for 30 min, 200 mM NaOH for 15 min, and 15 mM NaOH for 15 min, under a flow rate of 0.2 ml min^{-1}. Standard sugars and NaOH solution were from Sigma Chemical Co. (St. Louis, MO, USA). Monosaccharides and oligosaccharides in liquors were quantified according to Sluiter et al. [19] using the HPAEC-PAD protocol described above. Total phenolic compounds in liquors were assayed by the Folin-Ciocauteu reagent method [20], using vanillin as the standard.

Mass Spectrometry Analysis of Pretreatment Liquors

Pretreatment liquors were analyzed using a high-resolution mass spectrometer (maXis 4G Q-TOF MS, Bruker Daltonics; Billerica, MA, USA) equipped with an electrospray ionization (ESI) source in both positive and negative modes. Instrument settings were end plate offset = 500 V, capillary voltage = 4000 V, nebulizer pressure = 0.4 bar, dry gas flow = 5.0 l min^{-1}, and dry temperature = 180 °C. Detection range was 70–1000 *m/z*. Sodium formate solution (1.0 mM) was used for instrument *m/z* calibration. Liquor samples were diluted 100-fold in methanol 50% (*v/v*) and injected by direct infusion (i.e., without any chromatographic separation) at a flow rate of 10 μl min^{-1}. otofControl v. 3.4 and DataAnalysis v. 4.2 software packages (Bruker Daltonics, Billerica, MA, USA) were used for data acquisition and processing, respectively. Profile Analysis v.2.0 (Bruker Daltonics, Billerica, MA, USA) was used on preprocessing data in order to convert spectral data into ASCII files containing a data matrix of samples versus integral segments, which were submitted to multivariate data analysis using The Unscrambler v.9.7 (CAMO Software, Oslo, Norway) using principal component analysis (PCA) as a statistical tool. The SmartFormula algorithm (Data Analysis 4.2, Bruker Daltonics) was used to elucidate elemental formulas of major ions. Based on their *m/z* ratio and mSigma values, compounds were identified using either Kyoto Encyclopedia of Genes and Genomes (KEGG) [21] or ChEBI [22] database (Compound Crawler, DataAnalysis 4.2, Bruker Daltonics) or with aid of a list of possible lignocellulose pretreatment-derived compounds manually built from literature data.

Microorganism

A. niger strain was isolated from soil samples of the Brazilian biome Cerrado and deposited under strain code DCFS11 in the fungal culture collection at the Enzymology Laboratory, University of Brasilia, Brazil (genetic heritage number 010237/2015-1). The strain was also deposited in the bank of microorganisms for control of plant pathogens and weeds of the Brazilian Agricultural Research Corporation (EMBRAPA). The collection is registered at the World Data Centre for Microorganisms (WDCM), under the code MCPPW 1128. It was preserved in 0.9% NaCl (*w/v*), 50% glycerol (*v/v*), and 0.01% Tween 80 (*v/v*) solution at − 80 °C and propagated on potato dextrose agar plates at 28 °C. The isolate was initially subjected to morphological identification according to Klich [23] and Samson et al. [24]. The internal transcribed spacer region (ITS) and genes for β-tubulin (BT) and calmodulin (CMD) were used as molecular markers to confirm fungal identity. Primers employed in gene amplification are shown in Online Resource 1.

Enzyme Production Using Liquors as a Carbon Source

Liquors were supplemented with KH_2PO_4 (7.0 g l^{-1}), K_2HPO_4 (2.0 g l^{-1}), $MgSO_4$ (0.5 g l^{-1}), $(NH_4)_2SO_4$ (1.0 g l^{-1}), and yeast extract (0.6 g l^{-1}), adjusted to pH 7.0, and used as media for *A. niger* DCFS11 growth. Supplemented liquors (75 ml) were inoculated with 7.5×10^6 spores in 250-ml conical flasks. As a reference, raw SCB (10 g l^{-1}) was employed as an insoluble carbon source in submerged fermentations using liquid media containing the nutrients cited above dissolved in distilled water. Aliquots were periodically removed, centrifuged (5368*g*, 10 min), and subjected to enzymatic assays. *A. niger* DCFS11 was also cultivated on a variety of synthetic media containing defined carbohydrate concentrations, as further described in the "Enzyme Production Using Synthetic Media" section.

Enzyme production was scaled-up to a stirred tank bioreactor (BioFlo 415, Eppendorf, Hamburg, Germany) with a working volume of 3.8 l, employing liquor 16 as a carbon source. Fermentations were set to 200 rpm, 28 °C, 1 vvm of aeration, and pH 7.0 ± 0.2 (maintained using 0.2 M H_2SO_4 and 0.5 M NaOH). Foam was controlled with automatic addition of sterile 5% (*w/v*) silicone solution.

Enzymatic Assays

Xylanase and β-1,3-glucanase activities were determined by mixing 5 μl enzyme solution with 10 μl oat spelt xylan or laminarin (10 mg ml^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 50 °C for 30 min. Released reducing sugars were measured using the DNS method [25]. ABFase activity was determined by mixing 5 μl enzyme solution with 45 μl *p*-nitrophenyl-α-L-arabinofuranoside (5 mM) in 100 mM sodium acetate buffer, pH 5.0, at 50 °C for 30 min. Then, 100 μl of 1 M sodium carbonate was added and the released *p*-nitrophenol was measured at 430 nm. Activities were expressed as micromole of product formed per minute per milliliter of enzyme solution, i.e., as IU ml^{-1}.

Results and Discussion

Feedstock Composition

Raw SCB was composed of 2.55% arabinan, 0.68% galactosan, 22.43% xylan, 43.46% glucan, 19.79% acid-insoluble lignin, 5.62% acid-soluble lignin, and 0.9% ashes, based on dry weight. These results are in agreement with other reports [13]; moreover, the high arabinoxylan content makes SCB a good model feedstock for hemicellulose valorization.

Characterization of Pretreatment Liquors

The volume of liquor recovered after pretreatment by vacuum filtration was negatively affected ($p < 0.0001$) by increases in SCB loading owing to high water retention by the lignocellulosic structure, but was not significantly affected by pretreatment temperature and incubation period (Fig. 1a and Online Resource 2). Liquor recovery is an important factor when pretreatment is employed for subsequent use of the liquid fraction as a substrate for enzyme production. Low SCB loadings would be preferable if high pretreatment liquor recovery is desired. In contrast, liquor pH was not significantly affected by SCB loading but was inversely proportional to pretreatment severity, i.e., liquor became significantly ($p < 0.05$) more acidic as pretreatment temperature and incubation period increased (Fig. 1b and Online Resource 2). Decreases in liquor final pH are caused by the release of weak acids during pretreatment, mainly acetic and uronic acids from xylan and pectin [2].

The three pretreatment variables exerted statistically significant positive effect ($p < 0.05$), within the studied range, on the total carbohydrate content of liquors as quantified by HPAEC-PAD (Online Resource 2). The highest carbohydrate content (12.225 g l^{-1}) was observed in liquor 8 (180 °C, 45 min, 9% SCB, SF = 4.01), whereas the lowest content (0.262 g l^{-1}) was found in liquor 14 (170 °C, 5 min, 6% SCB, SF = 3.04) (Fig. 1c). An empirical model was built to predict the carbohydrate content of liquors as a function of pretreatment variables (Fig. 2a–c). The statistical reliability of the model was validated by ANOVA (Online Resource 2). The equation used was [total carbohydrates (g l^{-1})] = 5.55 + 1.54 T + 2.25 IP − 0.97 IP^2 + 2.17 SCB + 0.9 IP × SCB, ($R^2 = 0.93$, $-1.68 < T$, IP and SCB $< +1.68$). Overall, incubation period caused the greatest influence on the total carbohydrate content of liquors, followed by SCB loading and pretreatment temperature.

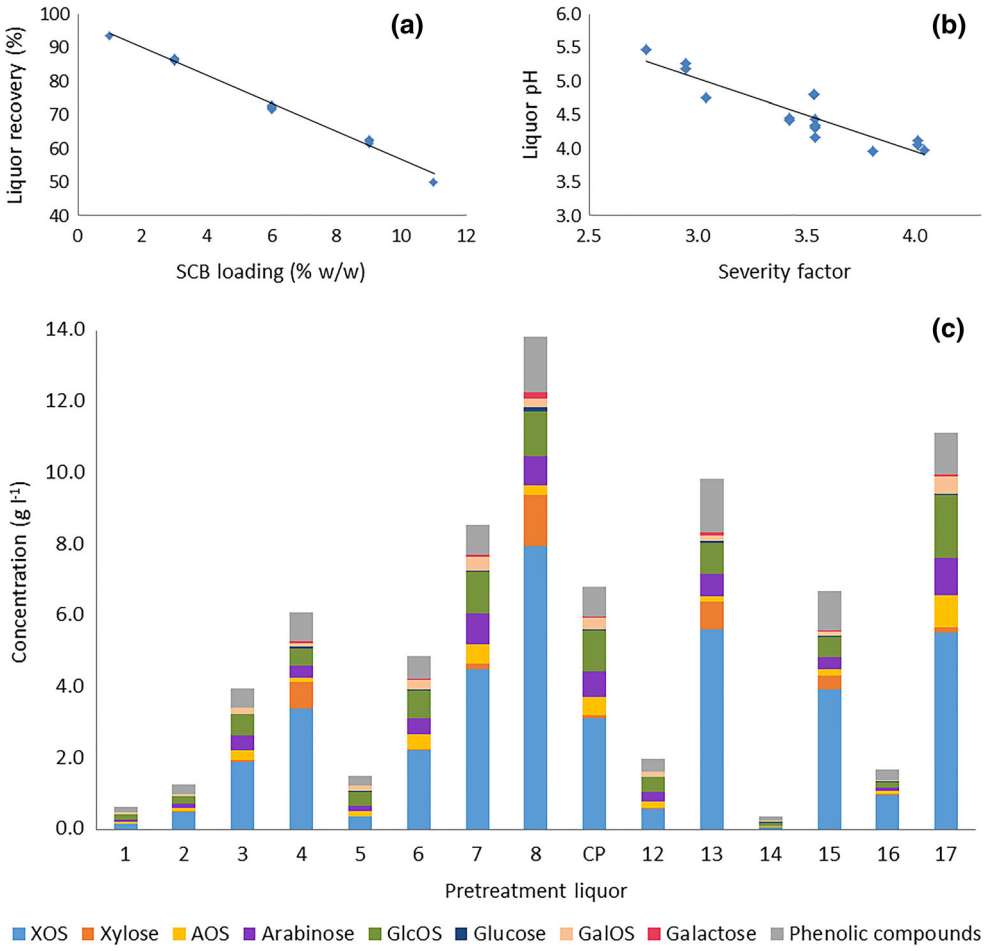


Fig. 1 Physicochemical characterization of SCB pretreatment liquors. Liquor recovery as a percentage of the initial volume of water employed in pretreatment (**a**), liquor pH versus pretreatment severity factor plot (**b**), and compound concentration in liquors (**c**)

Carbohydrates were found mainly in oligomeric form, as expected for hydrothermal pretreatment (Fig. 1c). Xylooligosaccharides (XOS) comprised the main components of liquors, followed by glucooligosaccharides (GlcOS), arabinose, arabinooligosaccharides (AOS), galactooligosaccharides (GalOS), monomeric xylose, glucose, and galactose. Mannose was detected at trace levels (results not shown), reflecting the low mannan content in raw SCB.

Mass spectrometry (MS) analyses provided further insight into the composition of liquors (Online Resource 3). Notably, ions in MS were detected in the form of sodiated adducts ($[M + Na]^+$) and/or in deprotonated form ($[M - H]^-$) in positive and negative ionization modes, respectively. A myriad of arabinoxylan autohydrolysis products were detected in liquors including xylose/arabinose ($C_5H_{10}O_5$, *m/z* 173.04 $[M + Na]^+$) and XOS with a degree of polymerization (DP) between 2 ($C_{10}H_{18}O_9$, *m/z* 282.09 $[M + Na]^+$) and 7 ($C_{35}H_{58}O_{29}$, *m/z* 942.30 $[M + Na]^+$). XOS with DP ≥ 8 ($C_{40}H_{66}O_{33}$, *m/z* 1097.92 $[M + Na]^+$) were not detected owing to the detection range of the instrument, but were likely also present as in previous studies, broader MS detection ranges allowed the identification of XOS with DP 3–13 [26] and 5–16 [27] in liquors arising from wheat and barley straws and rice husks, respectively. Xylose and arabinose, being isomers, could not be discriminated by direct infusion MS, nor could linear XOS, arabinosylated XOS, and AOS (side chains from rhamnogalacturonan I). The presence of feruloyl-arabinofuranose ($C_{15}H_{18}O_8$, *m/z* 349.08 $[M + Na]^+$), *p*-coumaroyl-arabinofuranose ($C_{14}H_{16}O_7$, *m/z* 295.08 $[M - H]^-$), and various feruloylated and *p*-coumaroylated pentose oligomers, however, suggested the presence of arabinosylated XOS, given that these phenolic moieties are attached to xylan by means of arabinofuranosyl branches. Diferulic acid ($C_{20}H_{18}O_8$, *m/z* 385.09 $[M - H]^-$) and diferuloyl-arabinofuranose ($C_{25}H_{26}O_{12}$, *m/z* 517.13 $[M - H]^-$) were also detected, indicating that hydrothermal pretreatment disrupted a portion of the cross-links that tie xylan to other cell wall polysaccharides and lignin [28, 29].

Hydrothermal pretreatment did not promote total xylan deacetylation, as XOS with various degrees of acetylation,

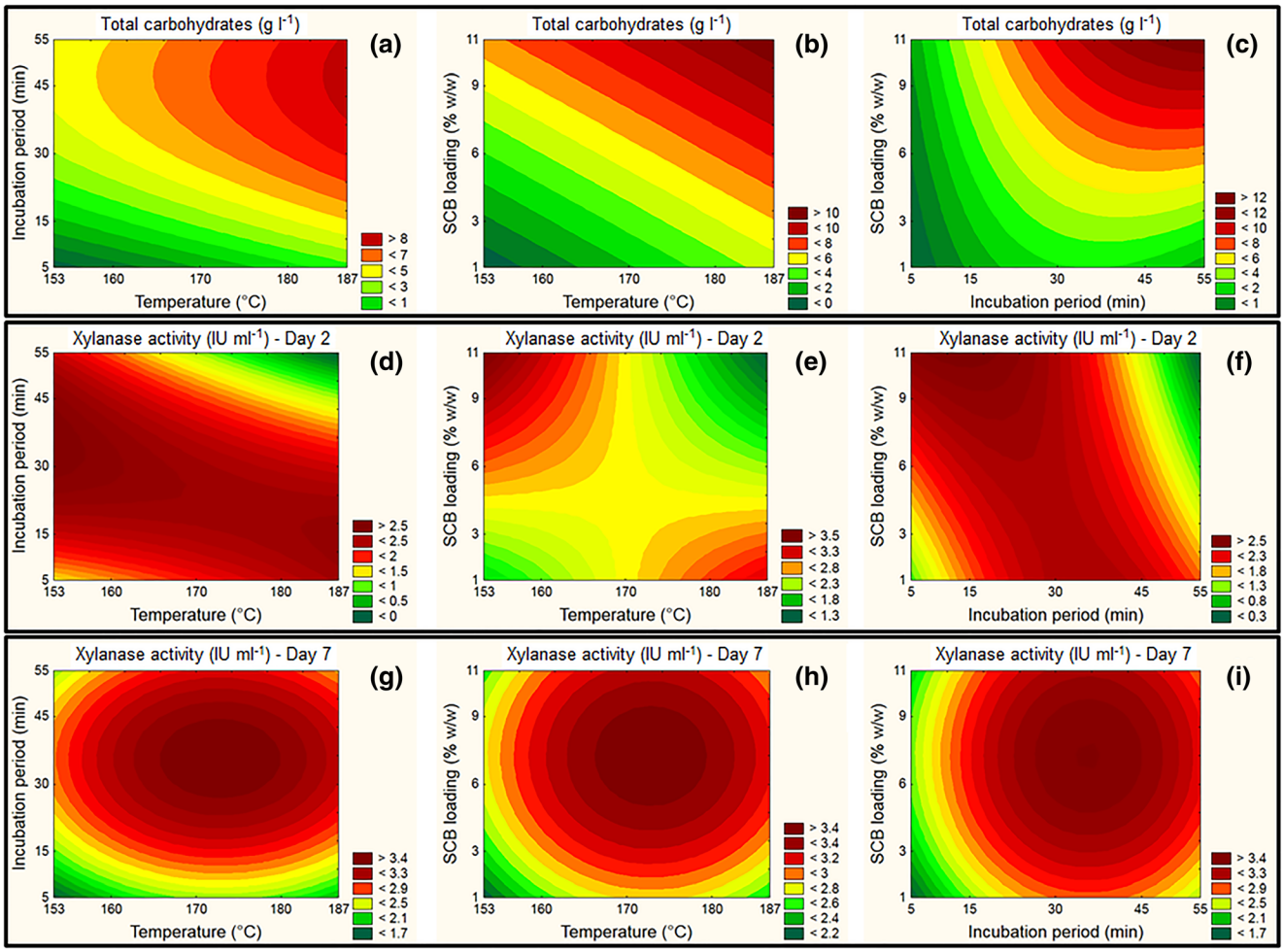


Fig. 2 Prediction plots of the total carbohydrate content of SCB pretreatment liquors (**a**–**c**) and the xylanase activity levels produced by *A. niger* DCFS11 after 2 (**d**–**f**) or 7 (**g**–**i**) days of growth on liquors, according to pretreatment variables. In all prediction plots, the third pretreatment variable was fixed at the coded level of 0

e.g., monoacetylated xylohexaose ($C_{32}H_{52}O_{26}$, *m/z* 875.26 $[M + Na]^+$) and pentaacetylated xylopentaose ($C_{35}H_{52}O_{26}$, *m/z* 911.26 $[M + Na]^+$), were detected in liquors. According to Morais de Carvalho et al. [16], approximately 30% of xylosyl residues in xylan from SCB are acetylated, of which 10% are doubly acetylated (at O2 and O3 positions). Acetylated XOS were also identified in pretreatment liquors from rice husks and wheat straw, albeit with lower acetylation degrees [27, 30]. In general, the intensity of acetylated XOS peaks was higher than that of non-acetylated XOS, whether owing to their higher abundance in liquors, higher solubility, or higher ionization efficiency. Acetyl-xylobiose ($C_{12}H_{20}O_{10}$, *m/z* 347.09 $[M + Na]^+$) was the most intense peak in the mass spectra (obtained in the positive ionization mode) of liquors from high-severity pretreatments. According to Chen et al. [31], acetylated portions of xylan are more easily solubilized during hydrothermal pretreatment of biomass than unsubstituted portions, which are more tightly bound to the cellulose surface.

Hexose monosaccharide ($C_6H_{12}O_6$, *m/z* 203.05 $[M + Na]^+$) and oligosaccharides with DP between 2 ($C_{12}H_{22}O_{11}$, *m/z* 365.10 $[M + Na]^+$) and 5 ($C_{30}H_{52}O_{26}$, *m/z* 851.27 $[M + Na]^+$) were also detected by MS, corresponding to the GlcOS and GalOS categories quantified by HPAEC-PAD. Glucose and GlcOS in liquors are most likely derived from the hydrolysis of mixed-linkage β-glucans, an important hemicellulosic component in sugarcane cell walls with a substantial fraction being quite soluble, loosely bound to the wall [14, 15]. The interspaced β-1,3 and β-1,4 linkages prevent packing of β-glucan chains into highly ordered structures [32], making it more easily extracted by hydrothermal pretreatment than crystalline cellulose. Glucose and GlcOS may also be derived from amorphous cellulose and xyloglucan. Galactose and GalOS are most likely derived from the hydrolysis of galactosan side-chains from rhamnogalacturonan I, the presence of which is also revealed by the detection of rhamnose ($C_6H_{12}O_5$, *m/z* 187.05 $[M + Na]^+$) in liquors. Considering the high intensities of hexose disaccharide in liquors 5 and 14 (mild pretreatments, high SCB loadings), this molecule possibly corresponds to residual sucrose from sugarcane juice, although it might also correspond to laminaribiose, cellobiose, and galactobiose. Hexose-pentose disaccharide ($C_{11}H_{20}O_{10}$, *m/z* 335.09 $[M + Na]^+$) was also detected, possibly derived from the hydrolysis of xyloglucan (glucopyranosyl-xylose) or pectin (galactopyranosyl-arabinose).

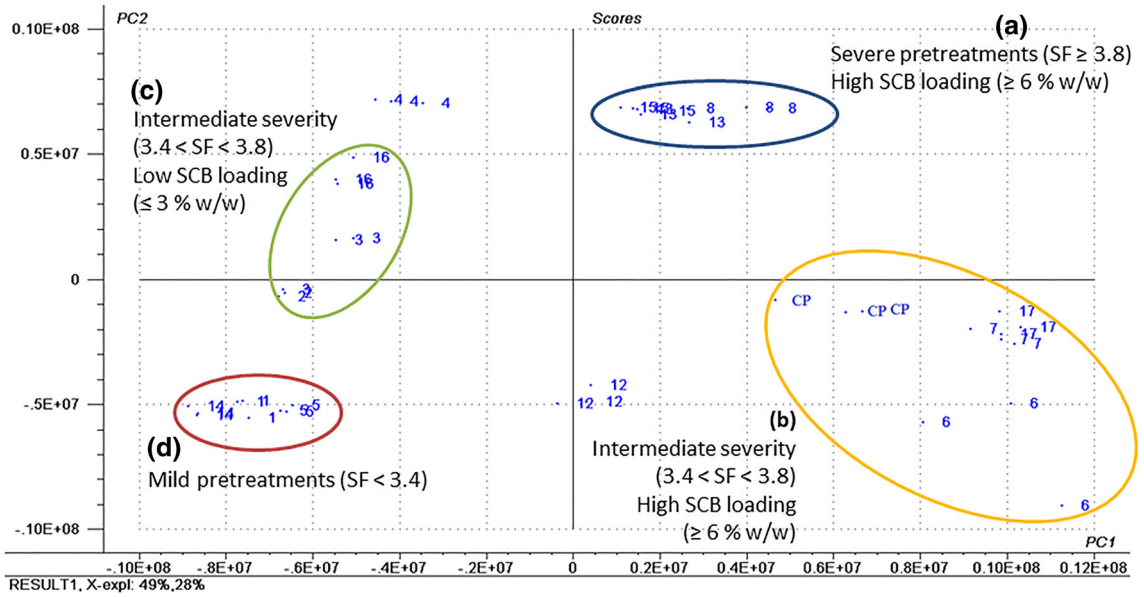
Although not expected, C5 and C6 sugar alcohols corresponding to xylitol/arabitol ($C_5H_{12}O_5$, *m/z* 175.05 $[M + Na]^+$) and sorbitol/galactitol/mannitol ($C_6H_{14}O_6$, *m/z* 205.06 $[M + Na]^+$) were detected in all liquors, especially in those arising from mild pretreatments (1, 5, and 14). These polyols may have been generated by fermentation of bagasse piles in the industrial yard where the feedstock was collected, and are probably not products of pretreatment.

Several low molecular weight lignin-derived aromatic compounds were identified by MS. *p*-Coumaric acid ($C_9H_8O_3$, *m/z* 163.03 $[M - H]^-$), benzoic acid ($C_7H_6O_2$, *m/z* 121.02 $[M - H]^-$), phenylacetaldehyde (C_8H_8O, *m/z* 119.04 $[M - H]^-$), and vanillin ($C_8H_8O_3$, *m/z* 151.03 $[M - H]^-$) were the most intensely detected, especially in liquors from high-severity pretreatments. The concentration of total phenolic compounds was positively influenced ($p < 0.0005$) by the increases in temperature, incubation period, and SCB loading employed in pretreatment (Fig. 1c and Online Resource 2). Sugar dehydration products including furfural ($C_5H_4O_2$, *m/z* 95.01 $[M - H]^-$), HMF ($C_6H_6O_3$, *m/z* 149.02 $[M + Na]^+$), furfuryl alcohol ($C_5H_6O_2$, *m/z* 121.02 $[M + Na]^+$), furoic acid ($C_5H_4O_3$, *m/z* 111.00 $[M - H]^-$), and levulinic acid ($C_5H_8O_3$, *m/z* 139.03 $[M + Na]^+$) were also identified, with detection intensities increasing according to pretreatment severity and biomass loading. Sugar dehydration products and phenolic compounds are primarily responsible for the inhibition of microbial growth and enzyme production in high-severity pretreatment liquors.

Owing to the immense quantity of compounds detected in each liquor mass spectra, chemometric analysis was required for the interpretation of the data set. PCA clustered liquors according to pretreatment severity and SCB loading employed in the process (Fig. 3). According to PCA score plots from spectral data obtained with positive ionization mode, the ESI(+)-MS, group (a) was characterized by the highest intensities of xylose/arabinose and various acetylated, arabinosylated, feruloylated, and *p*-coumaroylated XOS, as well as sugar dehydration products. Liquors from group (b) were indicated by the high intensities of peaks assigned as synapyl alcohol, hydroxybenzoic acid, and guaiacol, in addition to several non-identified ions with low values of *m/z*. Liquors in group (c) were not differentiated by the presence of any particular compound, showing low peak intensities. Group (d) members were characterized by high intensities of C5 and C6 sugar alcohols and hexose disaccharide. Regarding spectra from negative ionization mode, liquors from group (e) featured high intensity of arabinoxylan hydrolysis products, a variety of aromatic compounds, and sugar dehydration products, whereas group (f) presented lower intensities of such compounds.

Microorganism

The isolated fungal strain was initially identified as *A. foetidus* based on morphology in accordance with Klich [23]. According to Houbraken et al. [33], however, *A. foetidus* is currently considered an invalid classification and any strain previously assigned as such should be reclassified based on a detailed molecular analysis, which usually requires sequencing of at least two genome regions [34]. According to Peterson [35] and Varga et al. [34], *A. foetidus* is synonymous with *A. niger* for having identical sequences of the ITS region and genes for BT, CMD, large subunit rDNA, and RNA polymerase II. BLAST analysis of ITS, BT, and CMD genomic sequences of the strain used in this study resulted in a robust match with *A. niger*, showing 100% identity with dozens of *A. niger* strains. Lower identity levels with other closely related species within the Nigri section were also



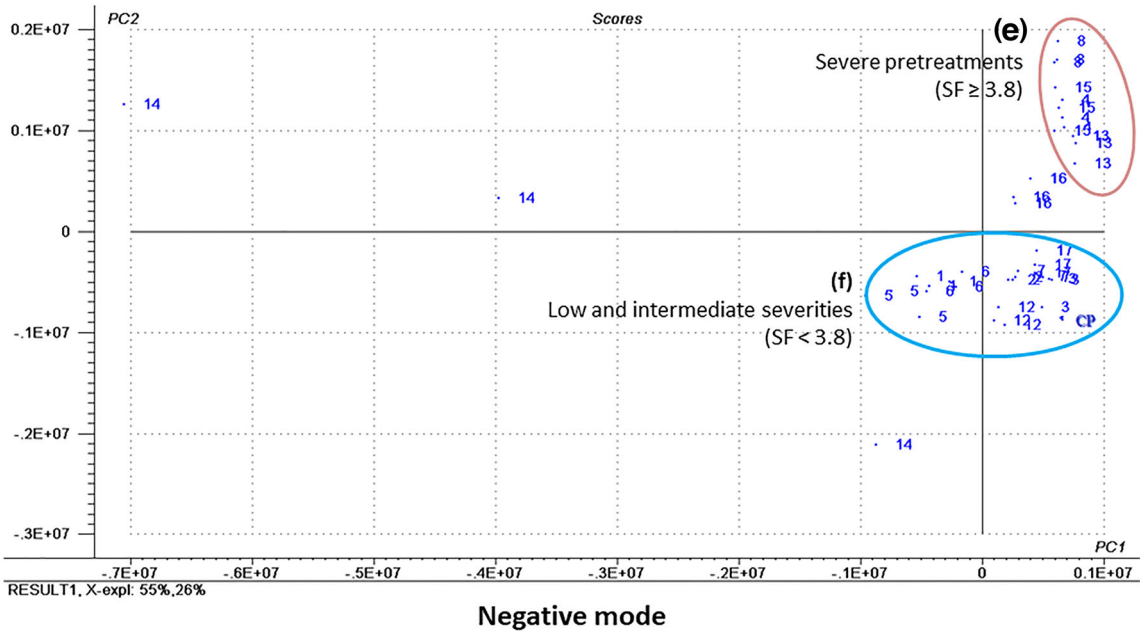


Fig. 3 Principal component analysis (PCA) score plots of liquors mass spectrum data obtained at positive and negative ionization modes. Liquors were clustered according to pretreatment severity and SCB loading (circles)

observed including *A. luchuensis*, *A. awamori*, and *A. welwitschiae*. Thus, the fungal isolate DCFS11 was finally classified as *A. niger*.

Enzyme Production Using Liquors as a Carbon Source

The application of pretreatment upon SCB and the use of the resulting liquors as a carbon source for *A. niger* DCFS11 markedly increased xylanase and ABFase production, in comparison to that obtained using raw SCB substrate (Table 1). Xylanase activities produced by *A. niger* grown on liquors varied between 2.27 and 3.53 IU ml^{-1} after 7 days of cultivation. The highest activities were produced on liquors 9, 10, and 11 (intermediate severity, SF = 3.54), whereas the lowest were obtained on liquors 1 and 14 (arising from the lowest severity pretreatments, SF ≤ 3.04). Liquor 6 (SF = 3.42) induced the highest ABFase activity (0.172 IU ml^{-1}), whereas liquor 14 induced the lowest titer (0.023 IU ml^{-1}). When used as a substrate, raw SCB (10 g l^{-1}) did not induce as high a quantity of these enzymes as liquor-based media, even though it contains higher carbohydrate content (6.91 g l^{-1}) than the majority of liquors. Maximum xylanase and ABFase levels

produced with raw SCB substrate were 1.79 and 0.02 IU ml^{-1}, respectively. Despite the lower concentration of total carbohydrates, liquors offer more accessible and readily available sugars (mainly in the form of soluble oligosaccharides) that favor fungal growth and enzyme production. In contrast, untreated biomass provides only insoluble, less accessible polysaccharides that need to be hydrolyzed before utilization by the fungus. In a previous study by Milagres and Prade [36], SCB steam pretreatment liquor was also a strong inducer of xylanases from *Penicillium janthinellum*. These authors demonstrated that size exclusion chromatographic fractions of SCB liquors containing high molecular weight XOS induced higher xylanase production than fractions containing xylose, evidencing the induction potential of XOS. The content of arabinose in sugarcane bagasse liquors, both as free monosaccharide or linked to XOS, is likely responsible for ABFase induction. L-arabitol is also a known inducer of this enzyme in *A. niger* [37] and likely takes part in the overall ABFase induction by liquors.

Although not tested, we hypothesize that the presence of acetylated-, feruloylated-, and *p*-coumaroylated XOS would also induce acetyl xylan esterase, feruloyl-esterase, and *p*-coumaroyl-esterase production, which would create new sites for enzymatic hydrolysis of oligosaccharides while also making acetic acid and phenolic compounds available for consumption. Lichenases were also expected to be induced by mixed-linkage β-glucans solubilized during pretreatment. This hypothesis was tested and β-1,3-glucanase activity was detected in all enzyme broths, with liquors also inducing higher titers than untreated SCB (Table 1).

Empirical models were built for the prediction of xylanase titers produced by *A. niger* DCFS11 at initial or advanced growth stages, represented by the 2nd and 7th days of cultivation, respectively (Fig. 2d–i). Model equations were [xylanase activity (IU ml^{-1}) at day 2] = 2.54–0.19 T − 0.27 IP − 0.36 IP^2 − 0.31 $T \times IP$ − 0.35 $T \times SCB$ − 0.31 IP × SCB ($R^2 = 0.82$) and [xylanase activity (IU ml^{-1}) at day 7] = 3.44 + 0.10 T − 0.19 T^2 + 0.21 IP − 0.27 IP^2 + 0.12 SCB − 0.15 SCB^2 ($R^2 = 0.95$). Xylanase prediction models (Fig. 2d–i) did not coincide with that of total carbohydrates (Fig. 2a–c). The increased carbohydrate content in liquors was not indefinitely accompanied by increases in xylanase production. This may be explained by the inhibition of enzyme synthesis caused by furans, phenolic compounds, and monosaccharides (via a carbon catabolite repression mechanism, as described in the "Enzyme Production in a Stirred Tank Bioreactor" section), the concentrations of which are increased along with the total carbohydrates with increased pretreatment severity and SCB loadings. Indeed, sugar dehydration products, specially HMF, and lignin-derived compounds such as *p*-coumaric acid, benzoic acid, phenylacetaldehyde, and vanillin, had the highest MS intensities in high-severity and high-gravity (high solids loadings) pretreatment liquors (Online Resource 3).

Distinct optimal pretreatment configurations are thus required if maximum activities are expected in initial vs. advanced cultivation phases. With respect to early growth stage (Fig. 2d–f), setting the pretreatment parameters at + 1 levels (180 °C, 45 min, and 9% *w*/*w*) or above simultaneously causes a significant negative effect on xylanase induction, meaning that longer lag phases are observed in liquors arising from high-severity and high-gravity pretreatments. In fact, mycelia was only observed 4 days after spore inoculation on liquor 8 (SF = 4.01). If xylanase production is expected to achieve its maximum level at the initial growth stages, either low SCB loading (≤ 6% *w*/*w*) or low pretreatment severity (≤ 170 °C or ≤ 30 min) are required. This is evidenced in the two red areas displayed in Fig. 2e. Conversely, setting pretreatment variables at − 1.68 levels (153 °C, 5 min, and 1% *w*/*w*) simultaneously also causes negative impact on xylanase production, possibly because of the low carbohydrate content in liquor available for fungal growth. When xylanase levels obtained at advanced growth stages are considered (Fig. 2g–i), optimal pretreatment configurations are shifted toward higher severity. Despite an initial lag phase, the higher carbohydrate content in liquors from high-severity and high-gravity pretreatments possibly sustained more vigorous fungal growth and enzyme induction. These liquors were enriched in all XOS ions described in Online Resource 3, known as inducers of xylanase. The highest xylanase titers empirically observed in liquors 9, 10, and 11 (intermediate severity, SF = 3.54) are probably correlated with a fine balance between the concentrations of inducing molecules (XOS) and inhibitory molecules (furans and phenolic compounds). Liquors 9, 10, and 11, however, were particularly enriched by synapyl alcohol, hydroxybenzoic acid, and guaiacol, which indicate that these molecules are likely less inhibitory than other phenolic compounds intensely detected in liquors from higher severity pretreatments. From mathematical models, maximum xylanase activity was predicted to be produced in liquor arising from pretreatments employing 7.25% *w*/*w* SCB loading at 172.8 °C for 35.7 min. A validation experiment of *A. niger* DCFS11 cultivation on theoretical optimal liquor was performed but could not fully meet the predicted activity value (3.52 IU ml^{-1}), reaching an observed activity of 3.23 IU ml^{-1}.

In contrast, reliable prediction models could not be built for ABFase. The effect of each pretreatment variable was nevertheless calculated (Online Resource 2). SCB loading employed in pretreatment had a significant positive influence on enzyme induction regardless of the cultivation stage, whereas high values of temperature and incubation period played a negative role on ABFase production during fungus initial growth stage (day 2), i.e., a lag phase was also observed for ABFase production in high-severity pretreatment liquors.

Even though increases in pretreatment biomass loading were beneficial for enzyme production at advanced growth stages, it was clear that low SCB loadings employed in pretreatment were sufficient for the induction of xylanases and ABFase. The use of 1% *w*/*w* biomass (liquor 16) yielded the highest xylanase and ABFase

activities per SCB mass employed in pretreatment (Online Resource 4). Furthermore, low biomass loadings allowed high liquor recovery, which was beneficial for enzyme production scale-up in large volumes. Considering this, pretreatments employing 1% SCB were further investigated, using temperatures and incubation periods varying between 160 and 180 °C and 15 and 30 min. Liquor composition and hemicellulase induction in response to pretreatment severity are shown in Fig. 4. The highest and most rapid xylanase and ABFase production was achieved with liquor obtained at 170 °C and 30 min (SF = 3.54), confirming liquor 16 as a source of carbon suitable for hemicellulase production scale-up. When pretreatments of similar severity are compared, such as 170 °C/30 min (SF = 3.54) and 180 °C/15 min (SF = 3.53), lower temperature accompanied by longer incubation period are preferred if hemicellulase induction is a target of optimization (Fig. 4d). Severity factor of 3.24 or lower resulted in low enzyme induction possibly due to low carbohydrate solubilization while severity factor of 3.83 was beneficial for hemicellulose solubilization but detrimental to enzyme induction probably due to the higher concentration of phenolic compounds and furans in the resulting liquor.

Enzyme Production in a Stirred Tank Bioreactor

Figure 5 summarizes the results of *A. niger* DCSF11 batch fermentation in a stirred tank bioreactor using liquor 16 as a carbon source. Despite the constant air feed (1 vvm), dissolved oxygen (DO) saturation dropped below 10% value in the first 24 h of fermentation, likely caused by the high oxygen uptake and intense consumption of monosaccharides during initial hours of growth. Total reducing sugars (TRS) experienced an increase after 24 h, probably owing to the hydrolysis of oligosaccharides by the produced hemicellulases. Respiration decelerated after 32 h, which coincides with a

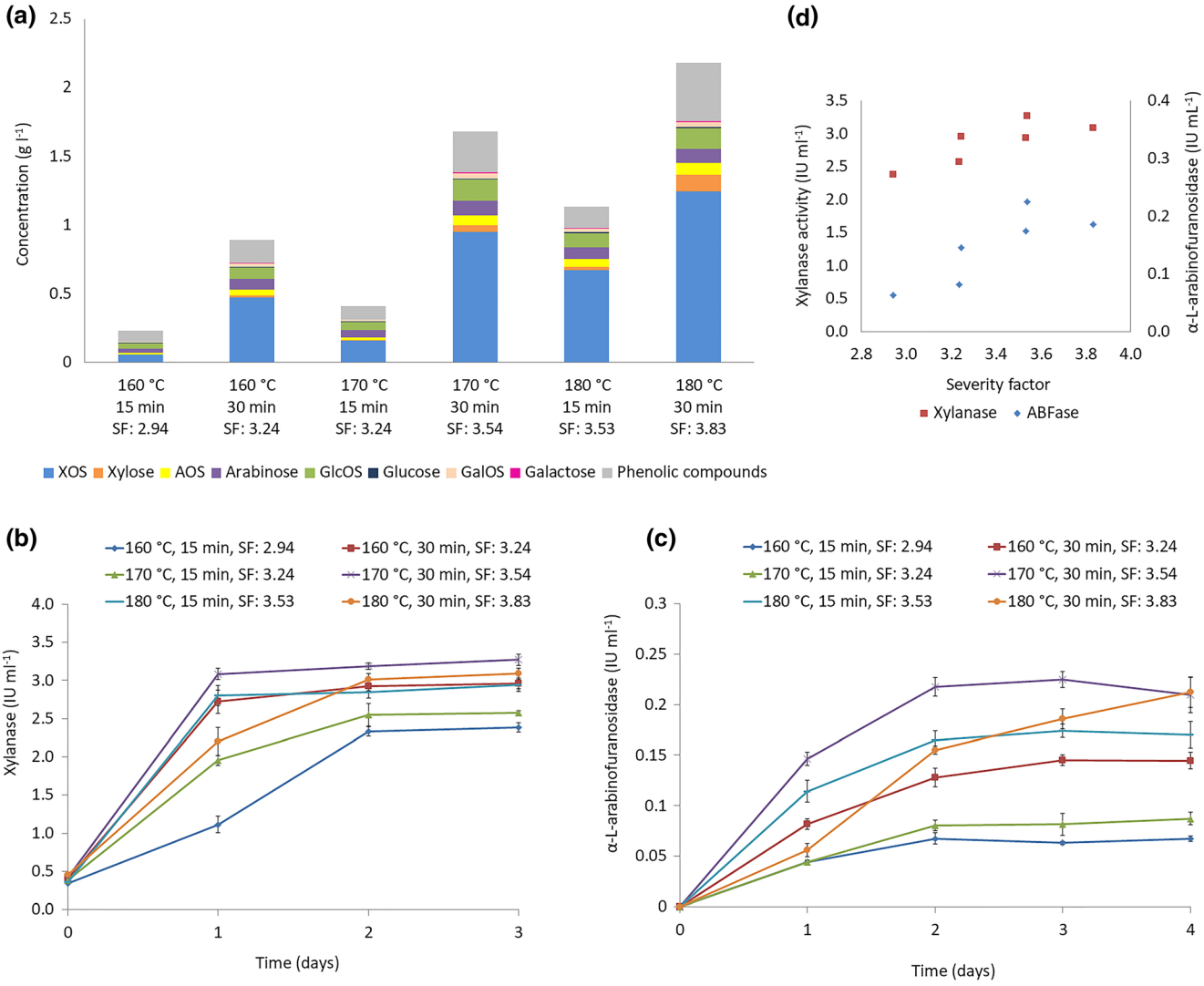


Fig. 4 Composition of liquors obtained from pretreatments employing 1% *w/w* SCB loading (**a**) and the production of xylanase (**b**) and α-L-arabinofuranosidase (**c**) by *A. niger* DCFS11 when using liquors as the carbon sources. A correlation plot between pretreatment severity and enzyme production is shown (**d**)

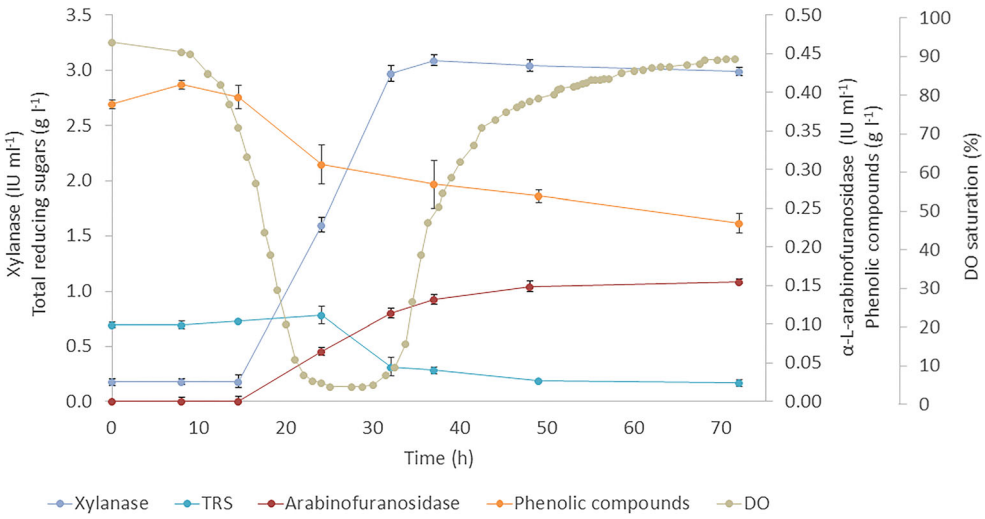


Fig. 5 *A. niger* DCFS11 fermentation in a stirred tank bioreactor using liquor 16 as the carbon source

significant decrease in TRS concentration in fermentation medium, indicating carbohydrate exhaustion. Xylanase and ABFase production initiated during the high oxygen uptake phase and achieved their highest activity values only after TRS concentration was reduced. This indicates that monosaccharides likely resulted in some level of repression of enzyme synthesis until that time point. The level of xylanase and ABFase gene expression in *A. niger* is the result of a balance between induction by XlnR (master regulator) and AraR transcription activators and repression by the CreA protein. At low concentrations, xylose and arabinose induce transcription activators to enhance the expression of these enzymes. At high concentrations, however, CreA protein inhibits the expression of such transcription activators, blocking the expression of regulated hemicellulases in a mechanism referred to as carbon catabolite repression (CCR) [38, 39]. In a similar study by Robl et al. [7], xylanase production by *A. niger* DR02 was also associated with the exhaustion of xylose and XOS from SCB hydrothermal pretreatment liquor used as the carbon source under batch fermentation. Aiming at the mitigation of the CCR effect on *A. niger* DR02, fed-batch fermentations were designed to maintain monosaccharide concentrations at low levels throughout the entire cultivation, which allowed xylanase titers to achieve a twofold increase [7]. These results highlight carbon-limited regimes as potential fermentation strategies for enzyme production by catabolite-repressed organisms, such as the wild strain of *A. niger* used in the present study, when using biomass pretreatment liquors as a substrate.

In addition to producing hemicellulases, *A. niger* DCFS11 could also perform partial liquor detoxification. XOS (including acetylated XOS) and mixed-linkage GlcOS constitute strong inhibitors of cellulase activity [40]. The detection of xylanase, ABFase, and β-1,3-glucanase activities in enzyme broths indicates that XOS and GlcOS are hydrolyzed and consumed, at least in part, during liquor fermentation. Previous studies have shown that the hydrolysis of inhibitory oligosaccharides in pretreated lignocellulose slurries by hemicellulases alleviates cellulase inhibition [40, 41]. Total phenolic compound concentration, also known as inhibitors of holocellulases and microbial growth, was also reduced during fungal growth (Fig. 5). Enzymatic and microbial inhibitor consumption or conversion into less repressing compounds is important for the further application of the resulting enzymatic broth in subsequent saccharification and fermentation of pretreated solids. The potential of filamentous fungi for biodetoxification of pretreatment liquors with simultaneous production of cellulases and hemicellulases has been shown in previous studies with *Trichoderma reesei* RUT C30, *A. nidulans* FLZ10, *A. awamori*, and *A. niger* DR02 [42, 43, 6, 7].

Enzyme Production Using Synthetic Media

To evaluate the effect of individual carbohydrates on enzyme induction, *A. niger* DCFS11 was also cultivated on a variety of synthetic media containing the same total carbohydrate amount as in liquor 16 (Fig. 6). Media A and F, containing xylan as a substituent of total carbohydrates or XOS content, yielded the same xylanase activities as liquor 16, indicating that the presence of oligomeric or polymeric xylose is important for xylanase induction, rather than xylose in monomeric form only, such as in media B and E. According to the manufacturer, oat spelt xylan corresponds to arabinoxylan, being composed of ≤ 10% arabinose, which partially explains why media A and F also induce ABFase. Small amounts of monosaccharides in medium F promoted faster xylanase and ABFase production than those in medium A. Monosaccharides may support initial mycelial growth and induce hemicellulases once they achieve non-repressive levels. Arabinose as the sole carbon source (medium C) induced the highest ABFase activities, apparently after 1 day of carbon catabolite repression phase. The results suggest that XOS in liquors are chiefly responsible for xylanase induction, whereas free arabinose is likely the main inducer of ABFase, followed by arabinosylated XOS.

(a)

Medium	Xylan (g l^{-1})	Xylose (g l^{-1})	Arabinose (g l^{-1})	Glucose (g l^{-1})	Galactose (g l^{-1})	Total (g l^{-1})
A	1.388	-	-	-	-	1.388
B	-	1.388	-	-	-	1.388
C	-	-	1.388	-	-	1.388
D	-	-	-	1.388	-	1.388
E	-	0.998	0.181	0.160	0.049	1.388
F	0.949	0.049	0.181	0.160	0.049	1.388
Liquor 16	0.949[1]	0.049	0.181[2]	0.160[2]	0.049[2]	1.388

[1] corresponding to XOS

[2] corresponding to the sum of mono and oligosaccharides

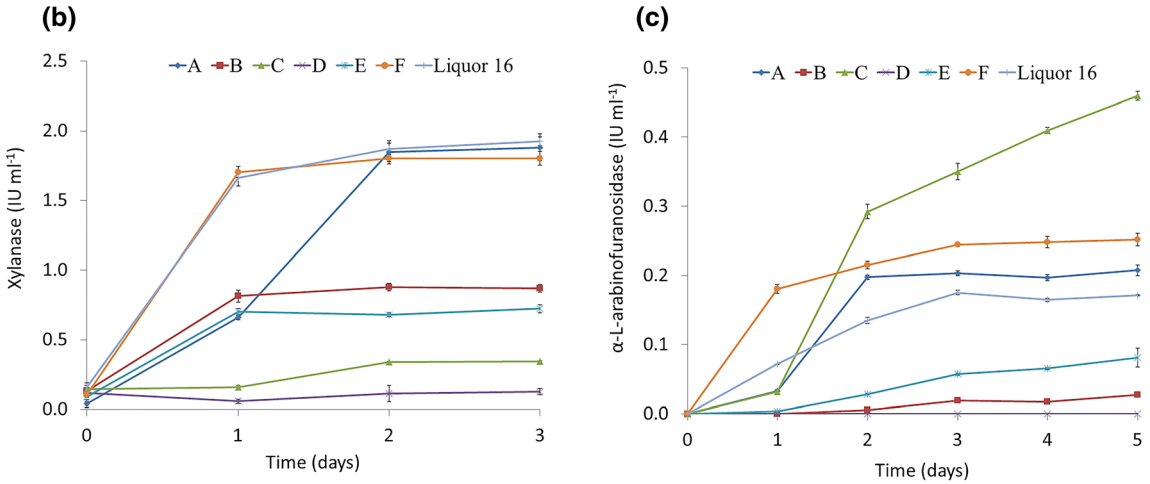


Fig. 6 Composition of synthetic media in comparison to liquor 16 (**a**) and the production of xylanase (**b**) and α-L-arabinofuranosidase (**c**) by *A. niger* DCFS11

Conclusions

As expected, xylanase production by wild-type *A. niger* DCFS11 strain was lower than those observed in patented xylanase production platforms, which correspond to expression hosts (bacteria, yeasts, or filamentous fungi) bearing heterologous genes under over-expression conditions that may produce xylanase titers reaching up to 71,686 U ml^{-1} [44]. In spite of that, rather than focusing on strain improvement or heterologous expression, this work concentrated on the proposal of an alternative bioprocess for integrated enzyme production in lignocellulosic biorefineries by exploiting biomass pretreatment liquors as a carbon source and *A. niger* as a model hemicellulolytic organism.

The hydrothermal pretreatment parameters presented here for hemicellulase production using a liquid stream differ from those usually applied when maximum cellulose saccharification and ethanol yields are desired, which are high-severity and high-gravity processes. Conversely, low severity is required if enzyme production from liquor is desired with no additional detoxification steps. In a biorefinery application scenario, a fraction of the incoming biomass would have to be treated in mild conditions. In an alternative strategy, a two-step biomass pretreatment would also enable the generation of non-inhibitory liquors in a first step and maximum reduction in cellulose recalcitrance in a second stage.

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Electronic supplementary material 1. Primers employed in amplification of molecular markers.

Region	Primer	Direction	Sequence 5' - 3'	Reference
ITS	ITS1F	Forward	CTTGGTCATTTAGAGGAAGTAA	[45]
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	[46]
β-tubulin	Bt2a	Forward	GGTAACCAAATCGGTGCTGCTTTC	[47]
	Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC	[47]
Calmodulin	Cmd5	Forward	CCGAGTACAAGGAGGCCTTC	[48]
	Cmd6	Reverse	CCGAGTACAAGGAGGCCTTC	[48]

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Electronic supplementary material 2. Effect estimates of pretreatment variables on dependent responses and validation of prediction models by analysis of variance (ANOVA). Significant *p* values are marked with asterisks.

Effects

Response	Pretreatment variable	Effect	Std.Err.	t(7)	*p*-value	-90.%	+90.%
Liquor recovery (% of initial water volume)							
	Mean/Interaction	**71.943**	**0.808**	**89.076**	**< 0.0001***	**70.412**	**73.473**
	Temperature (T)	0.373	0.759	0.491	0.6382	-1.064	1.810
	T^2	0.878	0.835	1.052	0.3279	-0.704	2.460
	Incubation period (IP)	0.259	0.759	0.341	0.7428	-1.178	1.696
	IP^2	1.408	0.835	1.687	0.1355	-0.174	2.990
	SCB loading (SCB)	**-25.090**	**0.759**	**-33.076**	**< 0.0001***	**-26.527**	**-23.653**
	SCB^2	0.595	0.835	0.713	0.4990	-0.987	2.177
	T*IP	0.100	0.991	0.101	0.9225	-1.778	1.978
	T*SCB	-0.300	0.991	-0.303	0.7709	-2.178	1.578
	IP*SCB	0.200	0.991	0.202	0.8458	-1.678	2.078
Liquor pH							
	Mean/Interaction	**4.348**	**0.085**	**51.340**	**< 0.0001***	**4.187**	**4.508**
	Temperature (T)	**-0.417**	**0.080**	**-5.242**	**0.0012***	**-0.568**	**-0.266**
	T^2	0.086	0.088	0.981	0.3592	-0.080	0.252
	Incubation period (IP)	**-0.810**	**0.080**	**-10.180**	**< 0.0001***	**-0.960**	**-0.659**
	IP^2	**0.333**	**0.088**	**3.808**	**0.0066***	**0.168**	**0.499**
	SCB loading (SCB)	0.038	0.080	0.475	0.6490	-0.113	0.189
	SCB^2	0.003	0.088	0.039	0.9701	-0.162	0.169
	T*IP	0.033	0.104	0.321	0.7578	-0.164	0.230

ANOVA

Response	Source	Sum of Squares	Degrees of freedom	Mean Square	F Value	*p*-value
Liquor recovery (% of initial water volume)						
$R^2 = 0.99$	Model	**2149.298**	**1**	**2149.298**	**1535.100**	**< 0.0001***
	SCB loading (SCB)	**2149.298**	**1**	**2149.298**	**1535.100**	**< 0.0001***
	Residual	21.002	15	1.400		
	Lack of Fit	**20.875**	**13**	**1.606**	**25.354**	**0.0386***
	Pure Error	0.127	2	0.063		
	Cor Total	2170.300	16			
Liquor pH						
$R^2 = 0.94$	Model	**3.151**	**3**	**1.050**	**73.970**	**< 0.0001***
	Temperature (T)	**0.594**	**1**	**0.594**	**41.799**	**< 0.0001***
	Incubation period (IP)	**2.239**	**1**	**2.239**	**157.639**	**< 0.0001***
	IP^2	**0.319**	**1**	**0.319**	**22.472**	**0.0004***
	Residual	0.185	13	0.014		
	Lack of Fit	0.178	11	0.016	4.598	0.1921
	Pure Error	0.007	2	0.004		
	Cor Total	3.336	16			

Effects

Response	Pretreatment variable	Effect	Std.Err.	t(7)	*p*-value	-90.%	+90.%
Total carbohydrates (g/L)	T*SCB	-0.023	0.104	-0.225	0.8288	-0.220	0.174
	IP*SCB	-0.038	0.104	-0.369	0.7231	-0.235	0.159
	Mean/Interaction	**5.982**	**0.613**	**9.763**	**< 0.0001***	**4.821**	**7.143**
	Temperature (T)	**3.082**	**0.576**	**5.356**	**0.0011***	**1.992**	**4.173**
	T^2	-0.676	0.633	-1.067	0.3212	-1.876	0.524
	Incubation period (IP)	**4.494**	**0.576**	**7.808**	**0.0001***	**3.403**	**5.584**
	IP^2	**-2.149**	**0.633**	**-3.393**	**0.0116***	**-3.349**	**-0.949**
	SCB loading (SCB)	**4.359**	**0.576**	**7.574**	**0.0001***	**3.268**	**5.449**
	SCB^2	-0.207	0.633	-0.327	0.7530	-1.407	0.993
	T*IP	0.674	0.752	0.897	0.3996	-0.750	2.099
	T*SCB	1.293	0.752	1.719	0.1292	-0.132	2.717
	IP*SCB	**1.800**	**0.752**	**2.394**	**0.0479***	**0.376**	**3.225**
Phenolic compounds (g/L)	Mean/Interaction	**0.837**	**0.078**	**10.702**	**< 0.0001***	**0.689**	**0.986**
	Temperature (T)	**0.498**	**0.073**	**6.774**	**0.0003***	**0.359**	**0.637**
	T^2	0.006	0.081	0.078	0.9397	-0.147	0.160
	Incubation period (IP)	**0.602**	**0.073**	**8.199**	**< 0.0001***	**0.463**	**0.742**
	IP^2	**-0.209**	**0.081**	**-2.578**	**0.0366***	**-0.362**	**-0.055**
	SCB loading (SCB)	**0.449**	**0.073**	**6.110**	**0.0005***	**0.310**	**0.588**
	SCB^2	-0.115	0.081	-1.427	0.1966	-0.269	0.038
	T*IP	0.124	0.096	1.297	0.2359	-0.057	0.306
	T*SCB	0.171	0.096	1.781	0.1181	-0.011	0.353

ANOVA

Response	Source	Sum of Squares	Degrees of freedom	Mean Square	F Value	*p*-value
Liquor total carbohydrates (g/L)						
$R^2 = 0.93$	Model	**185.080**	**5**	**37.016**	**30.258**	**< 0.0001***
	Temperature (T)	**32.438**	**1**	**32.438**	**26.516**	**0.0003***
	Incubation period (IP)	**68.939**	**1**	**68.939**	**56.354**	**< 0.0001***
	SCB loading (SCB)	**64.866**	**1**	**64.866**	**53.024**	**< 0.0001***
	IP*SCB	**6.481**	**1**	**6.481**	**5.298**	**0.0419***
	IP^2	**12.356**	**1**	**12.356**	**10.100**	**0.0088***
	Residual	13.457	11	1.223		
	Lack of Fit	13.019	9	1.447	6.613	0.1382
	Pure Error	0.437	2	0.219		
	Cor Total	198.537	16			
Phenolic compounds (g/L)						
$R^2 = 0.90$	Model	**2.883**	**4**	**0.721**	**29.053**	**< 0.0001***
	A-Temperature	**0.846**	**1**	**0.846**	**34.094**	**< 0.0001***
	B-Incubation period	**1.239**	**1**	**1.239**	**49.952**	**< 0.0001***
	C-SCB loading	**0.688**	**1**	**0.688**	**27.741**	**0.0002***
	B^2	**0.110**	**1**	**0.110**	**4.428**	**0.0571***
	Residual	0.298	12	0.025		
	Lack of Fit	**0.296**	**10**	**0.030**	**38.846**	**0.0253***
	Pure Error	0.002	2	0.001		
	Cor Total	3.181	16			

Effects

Response	Pretreatment variable	Effect	Std.Err.	t(7)	*p*-value	-90.%	+90.%
Xylanase activity at 2nd day (IU/mL)	IP*SCB	0.135	0.096	1.409	0.2016	-0.047	0.317
	Mean/Interaction	**2.655**	**0.212**	**12.501**	**< 0.0001***	**2.252**	**3.057**
	Temperature (T)	-0.378	0.199	-1.893	0.1002	-0.755	0.000
	T^2	-0.174	0.220	-0.791	0.4548	-0.590	0.242
	Incubation period (IP)	**-0.532**	**0.199**	**-2.667**	**0.0322***	**-0.910**	**-0.154**
	IP^2	**-0.763**	**0.220**	**-3.475**	**0.0103***	**-1.179**	**-0.347**
	SCB loading (SCB)	-0.236	0.199	-1.186	0.2744	-0.614	0.141
	SCB^2	-0.054	0.220	-0.244	0.8143	-0.469	0.362
	T*IP	**-0.611**	**0.261**	**-2.344**	**0.0516***	**-1.104**	**-0.117**
	T*SCB	**-0.705**	**0.261**	**-2.706**	**0.0304***	**-1.199**	**-0.212**
	IP*SCB	**-0.614**	**0.261**	**-2.357**	**0.0506***	**-1.108**	**-0.120**
Xylanase activity at 7th day (IU/mL)							
	Mean/Interaction	**3.441**	**0.041**	**84.321**	**< 0.0001***	**3.364**	**3.519**
	Temperature (T)	**0.210**	**0.038**	**5.476**	**0.0009***	**0.137**	**0.283**
	T^2	**-0.371**	**0.042**	**-8.798**	**< 0.0001***	**-0.451**	**-0.291**
	Incubation period (IP)	**0.415**	**0.038**	**10.815**	**< 0.0001***	**0.342**	**0.487**
	IP^2	**-0.546**	**0.042**	**-12.945**	**< 0.0001***	**-0.626**	**-0.466**
	SCB loading (SCB)	**0.247**	**0.038**	**6.446**	**0.0004***	**0.174**	**0.320**
	SCB^2	**-0.296**	**0.042**	**-7.017**	**0.0002***	**-0.376**	**-0.216**
	T*IP	-0.024	0.050	-0.487	0.6409	-0.119	0.070
	T*SCB	-0.044	0.050	-0.873	0.4116	-0.139	0.051

ANOVA

Response	Source	Sum of Squares	Degrees of freedom	Mean Square	F Value	*p*-value
Xylanase activity at 2nd day (IU/mL)						
$R^2 = 0.82$	Model	**5.108**	**5**	**1.022**	**6.558**	**0.0046***
	Temperature (T)	**0.487**	**1**	**0.487**	**24.017**	**0.0392***
	Incubation period (IP)	**0.966**	**1**	**0.966**	**47.637**	**0.0204***
	T*IP	**0.746**	**1**	**0.746**	**36.797**	**0.0261***
	T*SCB	**0.995**	**1**	**0.995**	**49.070**	**0.0198***
	IP*SCB	**0.754**	**1**	**0.754**	**37.215**	**0.0258***
	IP^2	**1.647**	**1**	**1.647**	**81.243**	**0.0121***
	Residual	1.713	11	0.156		
	Lack of Fit	1.186	8	0.148	7.313	0.1258
	Pure Error	0.041	2	0.020		
	Cor Total	6.822	16			
Xylanase activity at 7th day (IU/mL)						
$R^2 = 0.95$	Model	**1.947**	**6**	**0.324**	**30.416**	**< 0.0001***
	Temperature (T)	**0.150**	**1**	**0.150**	**14.102**	**0.0038***
	Incubation period (IP)	**0.587**	**1**	**0.587**	**55.004**	**< 0.0001***
	SCB loading (SCB)	**0.208**	**1**	**0.208**	**19.541**	**0.0013***
	T^2	**0.388**	**1**	**0.388**	**36.399**	**0.0001***
	IP^2	**0.841**	**1**	**0.841**	**78.799**	**< 0.0001***
	SCB^2	**0.247**	**1**	**0.247**	**23.155**	**0.0007***
	Residual	0.107	10	0.011		
	Lack of Fit	0.087	8	0.011	1.112	0.5558

Effects

Response	Pretreatment variable	Effect	Std.Err.	t(7)	*p*-value	-90.%	+90.%
	IP*SCB	**-0.182**	**0.050**	**-3.642**	**0.0083***	**-0.277**	**-0.088**
Arabinofuranosidase activity at 2nd day (IU/mL)							
	Mean/Interaction	**0.033**	**0.010**	**3.395**	**0.0115***	**0.015**	**0.052**
	Temperature (T)	**-0.026**	**0.009**	**-2.852**	**0.0246***	**-0.043**	**-0.009**
	T^2	0.013	0.010	1.255	0.2496	-0.006	0.032
	Incubation period (IP)	**-0.023**	**0.009**	**-2.530**	**0.0392***	**-0.041**	**-0.006**
	IP^2	-0.012	0.010	-1.162	0.2834	-0.031	0.007
	SCB loading (SCB)	**0.020**	**0.009**	**2.166**	**0.0670***	**0.002**	**0.037**
	SCB^2	0.001	0.010	0.073	0.9438	-0.018	0.020
	T*IP	-0.018	0.012	-1.527	0.1705	-0.041	0.004
	T*SCB	-0.010	0.012	-0.856	0.4205	-0.033	0.012
	IP*SCB	-0.014	0.012	-1.144	0.2901	-0.036	0.009
Arabinofuranosidase activity at 7th day (IU/mL)							
	Mean/Interaction	**0.129**	**0.017**	**7.641**	**0.0001***	**0.097**	**0.161**
	Temperature (T)	-0.009	0.016	-0.560	0.5928	-0.039	0.021
	T^2	-0.004	0.017	-0.244	0.8139	-0.037	0.029
	Incubation period (IP)	0.016	0.016	0.997	0.3520	-0.014	0.046
	IP^2	**-0.042**	**0.017**	**-2.427**	**0.0456***	**-0.075**	**-0.009**
	SCB loading (SCB)	**0.055**	**0.016**	**3.484**	**0.0102***	**0.025**	**0.085**
	SCB^2	-0.028	0.017	-1.598	0.1541	-0.061	0.005
	T*IP	**-0.053**	**0.021**	**-2.536**	**0.0389***	**-0.092**	**-0.013**

ANOVA

Response	Source	Sum of Squares	Degrees of freedom	Mean Square	F Value	*p*-value
	Pure Error	0.020	2	0.010		
	Cor Total	2.054	16			
Arabinofuranosidase activity at 2nd day (IU/mL)						
$R^2 = 0.55$	Model	**0.006**	**3**	**0.002**	**5.366**	**0.0126***
	Temperature (T)	**0.002**	**1**	**0.002**	**6.812**	**0.0216***
	Incubation period (IP)	**0.002**	**1**	**0.002**	**5.359**	**0.0376***
	SCB loading (SCB)	**0.001**	**1**	**0.001**	**3.927**	**0.0691***
	Residual	0.004	13	0.000		
	Lack of Fit	0.004	11	0.000	6.841	0.1343
	Pure Error	0.000	2	0.000		
	Cor Total	0.010	16			
Arabinofuranosidase activity at 7th day (IU/mL)						
$R^2 = 0.61$	Model	**0.020**	**3**	**0.007**	**6.705**	**0.0057***
	SCB loading (SCB)	**0.010**	**1**	**0.010**	**10.512**	**0.0064***
	T*IP	**0.006**	**1**	**0.006**	**5.570**	**0.0346***
	IP^2	**0.004**	**1**	**0.004**	**4.032**	**0.0659***
	Residual	0.013	13	0.001		
	Lack of Fit	**0.013**	**11**	**0.001**	**12.348**	**0.0772***
	Pure Error	0.000	2	0.000		
	Cor Total	0.033	16			

Effects

Response	Pretreatment variable	Effect	Std.Err.	t(7)	*p*-value	-90.%	+90.%
	T*SCB	-0.022	0.021	-1.068	0.3209	-0.061	0.017
	IP*SCB	-0.036	0.021	-1.718	0.1295	-0.075	0.004

ANOVA

Response	Source	Sum of Squares	Degrees of freedom	Mean Square	F Value	*p*-value

Electronic supplementary material 3, Part 1. List of compounds identified in sugarcane bagasse pretreatment liquors by direct infusion mass spectrometry analyses in positive ionization mode and their respective chemical formula, *m/z* ratio and peak intensity. All ions were detected in the form of sodiated adducts in the positive ionization mode. Empty cells correspond to inconclusive results.

Annotated compounds	Formula	*m/z*	POSITIVE MODE														
		$[M+Na]^+$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Carbohydrates																	
Xylose, arabinose	$C_5H_{10}O_5$	173.0419	38305	84000	98645	153688	42877	43070	45416	139286	42123	43407	137383	6944	138038	74942	41097
Xylobiose, arabinofuranosyl-xylose	$C_{10}H_{18}O_9$	305.0849	8054	19370	29796	92633	11903	13474	13380	76868	11226	11554	77154	4372	77033	38245	12967
Xylotriose, arabinofuranosyl-xylobiose	$C_{15}H_{26}O_{13}$	437.127	4535	7993	13419	29157	8758	5471	4891	24705	4424	4110	25792	7127	26117	17149	5247
Xylotetraose, arabinofuranosyl-xylotriose	$C_{20}H_{34}O_{17}$	569.1752	3558	5211	8101	12411	7104	3701	3532	12500	3039	3005	13030	5423	12164	511	3621
Xylopentaose, arabinofuranosyl-xylotetraose	$C_{25}H_{42}O_{21}$	701.2191	1414	3070	4429	6262	3803	2964	3334	7897	2515	2522	7517	2656	7063	4605	3717
Xylohexaose, arabinofuranosyl-xylopentaose	$C_{30}H_{50}O_{25}$	833.2533	1094	1755	3102	4820	2289	2078	2115	7386	1800	1492	5800	1384	5650	2600	2598
Xyloheptaose, arabinofuranosyl-xylohexaose	$C_{35}H_{58}O_{29}$	965.2956	434	797	1429	3073	1029	1168	1588	6481	1225	692	4362	595	4077	1209	1922
Acetyl-xylose	$C_7H_{12}O_6$	215.0534	2774	7850	12617	59845	3978	7654	8358	60785	8755	5979	52134	908	46784	16045	8483
Acetyl-xylobiose	$C_{12}H_{20}O_{10}$	347.096	1967	6178	20102	267343	3445	8848	10450	228690	10485	2997	184326	897	175140	51559	11248
Diacetylated xylobiose	$C_{14}H_{22}O_{11}$	389.1017	1378	2787	6886	57102	5197	12925	17599	78025	11945	8715	56442	933	55247	10337	21514
Acetyl-xylotriose	$C_{17}H_{28}O_{14}$	479.1378	2483	6443	15074	89034	5534	6445	5779	74650	5671	3088	68611	1476	69543	31656	6682
Diacetylated xylotriose	$C_{19}H_{30}O_{15}$	521.1504	1511	3676	9005	67396	2210	4433	576	70054	4936	1735	58085	735	1559	19925	5870
Triacetylated xylotriose	$C_{21}H_{32}O_{16}$	563.158	1409	2439	3129	28007	1399	3152	3944	42589	3339	1520	29236	626	29064	5600	4677
Acetyl-xylotetraose	$C_{22}H_{36}O_{18}$	611.1897	2351	5195	10647	32244	5855	4618	4102	29290	3778	2732	28930	1869	29399	17585	4681
Diacetylated xylotetraose	$C_{24}H_{38}O_{19}$	653.19	2788	4537	11852	54606	3474	5092	4965	51934	5104	3346	47347	1804	47311	22653	5570

Annotated compounds	Formula	*m/z*	POSITIVE MODE														
		[M+Na]$^+$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Triacetylated xylotetraose	$C_{26}H_{40}O_{20}$	695.2117	859	2257	6031	35914	1470	3177	4070	43904	3507	1390	35897	618	36067	11541	4698
Acetyl-xylopentaose	$C_{27}H_{44}O_{22}$	743.2267	1144	2825	6309	13077	3189	2709	2783	13760	2505	1636	14015	1088	13581	8242	3564
Diacetylated xylopentaose	$C_{29}H_{46}O_{23}$	785.239	1074	3172	8399	28282	2361	3698	3692	27436	3496	1723	27798	628	27329	13857	3975
Triacetylated xylopentaose	$C_{31}H_{48}O_{24}$	827.2458	807	2163	6338	27564	1504	3153	3548	31243	3148	1286	27291	564	28234	10701	3973
Tetraacetylated xylopentaose	$C_{33}H_{50}O_{25}$	869.2554	501	1207	3204	15909	1311	2499	2893	25460	2549	1169	19178	0	19677	4607	3580
Pentaacetylated xylopentaose	$C_{35}H_{52}O_{26}$	911.2663	416	713	1363	5600	1001	1287	1822	10851	1361	803	7603	465	7248	1582	2199
Acetyl-xylohexaose	$C_{32}H_{52}O_{26}$	875.267	741	1666	3646	5956	1677	1849	2024	7672	1618	1269	7276	649	6988	3826	2529
Diacetylated xylohexaose	$C_{34}H_{54}O_{27}$	917.2788	679	1858	4345	11735	1552	2246	2118	12552	1865	1001	12240	486	11805	6261	2534
Triacetylated xylohexaose	$C_{36}H_{56}O_{28}$	959.285	444	1550	4827	15502	1023	2192	2380	16492	1963	926	16108	410	16006	7087	2806
Feruloyl-arabinofuranose	$C_{15}H_{18}O_8$	349.0904	6740	19331	33701	52462	4802	7546	10687	48385	12592	8810	51792	1630	52610	33053	9933
Diferuloyl-arabinofuranose	$C_{25}H_{26}O_{12}$	541.1316	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p-Coumaroyl-arabinofuranose	$C_{14}H_{16}O_7$	319.0813	1951	5126	9207	11790	2141	3773	3947	11515	3745	2693	11024	544	12114	9146	4502
Feruloyl-arabinofuranosyl-xylose	$C_{20}H_{26}O_{12}$	481.1413	1111	1935	3143	9442	1178	2216	2670	11438	2263	1398	10377	459	9545	3793	2952
Feruloyl-arabinofuranosyl-xylobiose	$C_{25}H_{34}O_{16}$	613.1782	1752	2462	3140	6078	3206	2697	3115	8153	2437	2399	7708	2107	7035	3266	3715
Feruloyl-arabinofuranosyl-xylotriose	$C_{30}H_{42}O_{20}$	745.224	1448	2124	2317	4576	2533	1813	2047	5970	1467	1521	5954	1925	5167	2741	2490
Feruloyl-arabinofuranosyl-xylotetraose	$C_{35}H_{50}O_{24}$	877.2584	683	1230	1491	3314	1135	1375	1732	5188	1254	967	4241	922	3812	1486	2277
p-Coumaroyl-arabinofuranosyl-xylose	$C_{19}H_{24}O_{11}$	451.1211	540	828	1117	3369	739	1710	1801	4819	1466	1043	4508	376	3531	768	2201
p-Coumaroyl-arabinofuranosyl-xylobiose	$C_{24}H_{32}O_{15}$	583.1682	998	1295	1726	3133	1694	2036	2618	5055	1678	1670	4395	1540	4046	1504	2849
p-Coumaroyl-arabinofuranosyl-xylotriose	$C_{29}H_{40}O_{19}$	715.2056	796	1366	1630	4838	1310	1824	2106	6183	1434	1283	5072	751	4719	1595	2676

Annotated compounds	Formula	*m/z*	POSITIVE MODE														
		[M+Na]$^+$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
p-Coumaroyl-arabinofuranosyl-xylotetraose	$C_{34}H_{48}O_{23}$	847.2495	759	1059	1406	6357	980	1647	1930	7221	1350	931	6119	574	5615	1546	2624
p-Coumaroyl-arabinofuranosyl-xylopentaose	$C_{39}H_{56}O_{27}$	979.2914	0	507	1151	4653	624	1254	1548	5871	831	604	4902	291	4678	989	1853
Acetyl-feruloyl-arabinofuranosyl-xylotriose	$C_{37}H_{52}O_{25}$	919.269	587	1489	2864	5062	1538	1634	1894	6738	1522	973	6298	777	6283	3341	2126
4-O-methyl-glucuronosyl-xylose, galacturonyl-rhamnose	$C_{12}H_{20}O_{11}$	363.0898	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-O-methyl-glucuronosyl-xylobiose	$C_{17}H_{28}O_{15}$	495.1320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-O-methyl-glucuronosyl-xylotriose	$C_{22}H_{36}O_{19}$	627.1743	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-O-methyl-glucuronosyl-xylotetraose	$C_{27}H_{44}O_{23}$	759.2166	601	1082	1550	5648	1506	3218	3850	11852	2318	1389	9210	706	8240	1139	4355
Glucuronosyl-xylotriose	$C_{11}H_{18}O_{11}$	613.1587	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexose (glucose, mannose, galactose, fructose)	$C_6H_{12}O_6$	203.053	11227	15834	17146	24696	17161	9053	8558	26013	13960	10008	26275	7756	26226	14234	8392
Hexose disaccharide (sucrose, laminaribiose, cellobiose, galactopyranosyl-glucose, mannopyranosyl-glucose)	$C_{12}H_{22}O_{11}$	365.1057	27674	22242	14178	11309	47080	8869	7138	-	5287	13725	12549	49366	12264	8497	-
Hexose trisaccharide (mixed-linkage glucose trisaccharide, cellotriose, mannotriose, galactotriose)	$C_{18}H_{32}O_{16}$	527.1597	2064	3329	3560	6940	5030	2230	2918	8334	2340	2021	7336	7521	6812	3820	3435
Hexose tetrasaccharide (mixed-linkage glucose tetrasaccharide, cellotetraose, mannotetraose, galactotetraose)	$C_{24}H_{42}O_{21}$	689.2156	1656	2698	3268	5887	3528	2109	2721	7380	2011	1718	6467	2695	6185	4103	3389
Hexose pentasaccharode (mixed-linkage glucose pentasaccharide, cellopentaose, mannopentaose, galactopentaose)	$C_{30}H_{52}O_{26}$	851.2703	827	1337	2142	5561	2370	1889	2005	6911	1389	1488	5940	1473	5914	1989	2492
Hexose-pentose (glucopyranosyl-xylose, galactopyranosyl-arabinose, xylopyranosyl-galactose)	$C_{11}H_{20}O_{10}$	335.0955	4669	9154	10935	13988	5793	7916	9736	37386	7672	7212	26190	1603	24237	6986	10301
Rhamnose, fucose	$C_6H_{12}O_5$	187.0574	3363	12926	13427	37394	5868	8427	7979	35768	7336	5363	43922	1199	29279	11312	8410
Acetyl-galactose	$C_8H_{14}O_7$	245.0632	1146	1811	3158	13316	941	9776	19076	23467	13002	5545	16826	1033	17137	2838	18881

Annotated compounds	Formula	*m/z*	POSITIVE MODE														
		$[M+Na]^+$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Acetyl-galacturonic acid	$C_8H_{12}O_8$	259.0583	720	958	1165	4409	772	2940	3800	6670	2835	1339	5234	535	5219	724	4349
Sugar alcohols																	
Xylitol, arabitol	$C_5H_{12}O_5$	175.0576	43631	37271	18735	13716	69896	17428	16285	24551	10118	19184	19305	58585	19075	8928	17905
Sorbitol, mannitol, galactitol	$C_6H_{14}O_6$	205.0682	26906	23771	15878	13223	45974	10048	6950	12167	4493	15575	12012	48005	13090	10431	6997
Lignin-derived compounds																	
p-Coumaric acid	$C_9H_8O_3$	187.0366	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Benzoic acid, hydroxybenzaldehyde	$C_7H_6O_2$	145.026	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Syringaldehyde	$C_9H_{10}O_4$	205.0471	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenylacetaldehyde	C_8H_8O	143.0467	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vanillin	$C_8H_8O_3$	175.0366	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroxybenzoic acid	$C_7H_6O_3$	161.0209	417	477	1160	5524	607	8588	18991	17778	10663	2633	12226	492	11317	178	19959
Coniferyl aldehyde	$C_{10}H_{10}O_3$	201.0527	1034	2509	2691	3341	955	2279	2835	4524	1995	1575	4097	339	3662	2276	3065
Vanillic acid, 2,6-dimethoxybenzoquinone	$C_8H_8O_4$	191.0315	18758	16850	9713	10542	35219	17100	5958	23024	3160	5656	12703	33460	14400	4049	10473
Syringic acid	$C_9H_{10}O_5$	221.0423	2678	1977	1773	2190	10364	5504	5174	4447	2098	5572	3762	9397	3723	615	6147
Guaiacylglycerol-beta-guaiacyl ether	$C_{17}H_{20}O_6$	343.1155	7911	9440	7850	6149	7847	4785	4444	7264	5603	4986	9039	2976	7034	5285	4954
Benzenediol	$C_6H_6O_2$	109.0284	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Guaiacol	$C_7H_8O_2$	147.0416	0	337	1516	7507	1556	15380	23556	17439	19334	10035	14728	0	12532	163	26832
Diferulic acid	$C_{20}H_{18}O_8$	409.0894	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Syringol	$C_8H_{10}O_3$	177.0522	632	938	1978	10331	1250	5240	10980	20895	8011	2654	17055	918	15310	1232	11627
Benzoquinone	$C_6H_4O_2$	131.0104	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Annotated compounds	Formula	*m/z*	POSITIVE MODE														
		$[M+Na]^+$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Synapyl alcohol	$C_{11}H_{14}O_4$	233.0783	3808	7900	7068	8316	4057	25928	26139	9168	56222	8439	21392	2225	17370	28534	19240
Synapyl aldehyde	$C_{11}H_{12}O_4$	231.0627	1468	3315	4671	6461	1292	3182	4114	8220	3973	4721	8060	648	7121	4054	4679
Benzaldehyde	C_7H_6O	129.031	0	119	163	489	359	1464	2127	1733	638	478	1512	145	1261	0	2541
Cinnamic acid	$C_9H_8O_2$	171.0417	128	450	503	1826	1132	6776	7237	6121	2457	2510	3986	111	4012	0	9544
Methylguaiacol	$C_9H_{12}O_3$	161.0573	133	168	353	1848	301	5557	8954	6518	12661	1609	4819	119	3683	294	8621
Sugar dehydration products																	
Furfural	$C_5H_4O_2$	119.0104	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Furfuryl alcohol	$C_5H_6O_2$	121.026	0	0	166	1961	119	961	1472	4895	1123	341	3624	0	3156	0	1809
Furoic acid	$C_5H_4O_3$	135.0053	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Levulinic acid	$C_5H_8O_3$	139.0366	0	199	557	2239	623	2675	3306	6163	3283	1095	4864	0	3908	194	4229
Hydroxymethylfurfural (HMF)	$C_6H_6O_3$	149.021	1452	1607	1553	6446	2167	1668	1282	7733	1571	743	6872	1555	5928	1176	1405

Electronic supplementary material 3, Part 2. List of compounds identified in sugarcane bagasse pretreatment liquors by direct infusion mass spectrometry analyses in negative ionization mode and their respective chemical formula, *m/z* ratio and peak intensity. All ions were detected in deprotonated formin the negtaive ionization mode. Empty cells correspond to inconclusive results.

Annotated compounds	Formula	*m/z*	NEGATIVE MODE														
		[M-H]$^-$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Carbohydrates																	
Xylose, arabinose	$C_5H_{10}O_5$	149.0445	3642	9570	15205	45475	9008	10802	19563	83531	10376	6698	59878	1637	54425	10951	23776
Xylobiose, arabinofuranosyl-xylose	$C_{10}H_{18}O_9$	281.0867	741	1732	3223	12964	2280	2648	3778	24597	2047	1463	17078	1065	18131	3687	4967
Xylotriose, arabinofuranosyl-xylobiose	$C_{15}H_{26}O_{13}$	413.129	692	1018	1828	6446	1840	1579	1868	11039	1304	844	7920	998	7886	2216	2626
Xylotetraose, arabinofuranosyl-xylotriose	$C_{20}H_{34}O_{17}$	545.1712	552	828	1326	2936	1466	1320	1603	5682	937	834	4473	920	4387	1193	2062
Xylopentaose, arabinofuranosyl-xylotetraose	$C_{25}H_{42}O_{21}$	677.2135	259	411	745	1110	805	883	1100	2279	810	299	1885	354	1947	489	1458
Xylohexaose, arabinofuranosyl-xylopentaose	$C_{30}H_{50}O_{25}$	809.2557	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Xyloheptaose, arabinofuranosyl-xylohexaose	$C_{35}H_{58}O_{29}$	941.298	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetyl-xylose	$C_7H_{12}O_6$	191.055	1329	2552	3324	6963	5688	6849	8846	13126	6363	6731	9924	1985	9636	1516	9465
Acetyl-xylobiose	$C_{12}H_{20}O_{10}$	323.0972	242	561	1293	11029	735	957	2089	17913	576	208	12277	379	12716	2151	1925
Diacetylated xylobiose	$C_{14}H_{22}O_{11}$	366.1157	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetyl-xylotriose	$C_{17}H_{28}O_{14}$	455.1385	385	648	1149	5661	836	1140	1176	10170	840	356	7202	235	6875	1711	1647
Diacetylated xylotriose	$C_{19}H_{30}O_{15}$	497.1501	258	472	687	2384	661	596	812	4112	124	242	2987	395	3060	850	337
Triacetylated xylotriose	$C_{21}H_{32}O_{16}$	540.1685	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetyl-xylotetraose	$C_{22}H_{36}O_{18}$	587.1818	413	653	1222	4337	1258	1221	1379	8001	855	670	6221	366	5936	1520	2021
Diacetylated xylotetraose	$C_{24}H_{38}O_{19}$	629.1924	222	564	1082	3862	797	1032	1261	6916	809	429	5185	143	5484	1365	1672

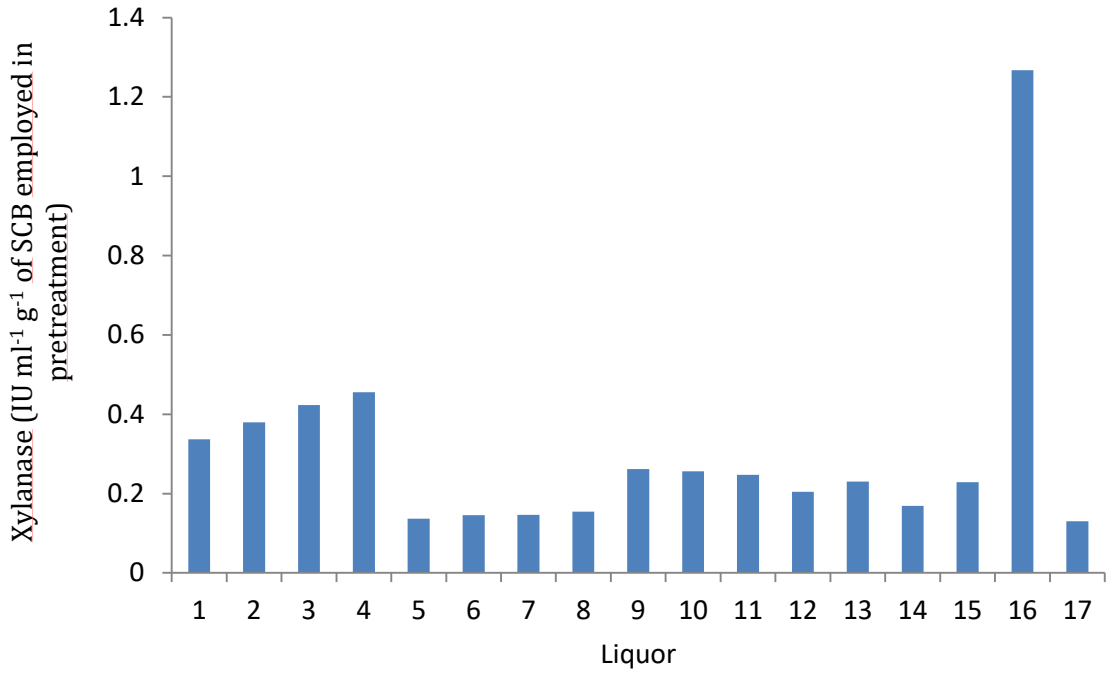
Annotated compounds	Formula	*m/z*	NEGATIVE MODE														
		[M-H]$^-$	**Liquors**														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Triacetylated xylotetraose	$C_{26}H_{40}O_{20}$	672.211	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetyl-xylopentaose	$C_{27}H_{44}O_{22}$	719.224	147	372	686	1461	564	545	804	3096	121	109	2578	217	2641	560	951
Diacetylated xylopentaose	$C_{29}H_{46}O_{23}$	761.2346	0	313	637	1505	456	605	636	3155	501	276	2421	104	2554	608	951
Triacetylated xylopentaose	$C_{31}H_{48}O_{24}$	803.2452	0	158	356	774	258	399	496	1926	444	108	1403	0	1476	417	713
Tetraacetylated xylopentaose	$C_{33}H_{50}O_{25}$	845.2557	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pentaacetylated xylopentaose	$C_{35}H_{52}O_{26}$	887.2663	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetyl-xylohexaose	$C_{32}H_{52}O_{26}$	851.2663	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diacetylated xylohexaose	$C_{34}H_{54}O_{27}$	893.2768	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triacetylated xylohexaose	$C_{36}H_{56}O_{28}$	935.2874	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Feruloyl-arabinofuranose	$C_{15}H_{18}O_8$	325.0918	2418	7750	17101	33708	3405	7708	15732	56013	11345	4472	47430	675	48571	14748	17439
Diferuloyl-arabinofuranose	$C_{25}H_{26}O_{12}$	517.1341	429	743	1245	2113	1188	1561	2034	2759	1241	1034	2454	640	1854	721	2095
p-Coumaroyl-arabinofuranose	$C_{14}H_{16}O_7$	295.0812	1088	3127	7357	13685	151	459	6827	23267	4774	2180	18270	442	20144	6025	7967
Feruloyl-arabinofuranosyl-xylose	$C_{20}H_{26}O_{12}$	457.1341	224	696	1175	2693	1128	1247	1663	4447	1082	748	3977	591	3743	997	1901
Feruloyl-arabinofuranosyl-xylobiose	$C_{25}H_{34}O_{16}$	589.1763	519	670	1025	1718	1265	1176	1401	3205	955	644	2569	876	2585	736	1741
Feruloyl-arabinofuranosyl-xylotriose	$C_{30}H_{42}O_{20}$	721.2186	246	352	598	770	796	712	850	1663	521	361	1384	430	1424	544	1123
Feruloyl-arabinofuranosyl-xylotetraose	$C_{35}H_{50}O_{24}$	853.2608	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p-Coumaroyl-arabinofuranosyl-xylose	$C_{19}H_{24}O_{11}$	427.1235	129	455	837	1440	1018	1130	997	2194	111	433	1967	505	1880	566	1183
p-Coumaroyl-arabinofuranosyl-xylobiose	$C_{24}H_{32}0_{15}$	559.1657	415	372	838	1294	1218	1029	1044	1916	744	505	1652	558	1709	496	1174
p-Coumaroyl-arabinofuranosyl-xylotriose	$C_{29}H_{40}O_{19}$	691.208	147	243	499	898	443	613	733	1642	557	402	1510	283	1431	290	937

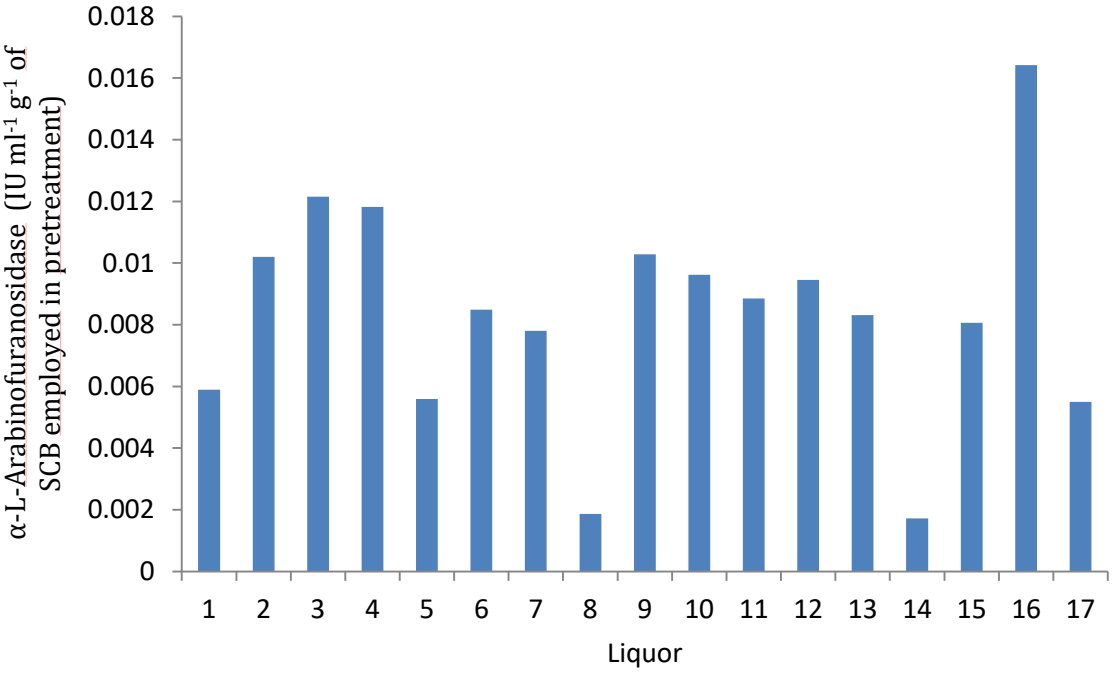
Annotated compounds	Formula	*m/z*	NEGATIVE MODE														
		[M-H]$^-$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
p-Coumaroyl-arabinofuranosyl-xylotetraose	$C_{34}H_{48}O_{23}$	823.2503	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p-Coumaroyl-arabinofuranosyl-xylopentaose	$C_{39}H_{56}O_{27}$	955.2925	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetyl-feruloyl-arabinofuranosyl-xylotriose	$C_{37}H_{52}O_{25}$	895.2714	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-O-methyl-glucuronosyl-xylose, galacturonyl-rhamnose	$C_{12}H_{20}O_{11}$	339.0922	473	1082	2133	2478	1626	3371	4359	5422	3203	1276	4149	682	5484	1469	5074
4-O-methyl-glucuronosyl-xylobiose	$C_{17}H_{28}O_{15}$	471.1344	403	802	1847	2344	1360	2478	3285	4941	2393	892	3679	758	4823	1164	3428
4-O-methyl-glucuronosyl-xylotriose	$C_{22}H_{36}O_{19}$	603.1767	365	663	1184	1414	1423	1644	1899	2826	1473	790	2190	554	2757	668	2619
4-O-methyl-glucuronosyl-xylotetraose	$C_{27}H_{44}O_{23}$	735.2190	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glucuronosyl-xylotriose	$C_{11}H_{18}O_{11}$	589.1611	519	670	1025	1718	1265	1176	1401	3205	955	644	2569	876	2585	736	1741
Hexose (glucose, mannose, galactose, fructose)	$C_6H_{12}O_6$	179.055	5625	11916	824	5588	4537	1476	4086	10753	4189	890	9229	2543	7944	5908	5908
Hexose disaccharide (sucrose, laminaribiose, cellobiose, galactopyranosyl-glucose, mannopyranosyl-glucose)	$C_{12}H_{22}O_{11}$	341.1078	3180	2699	2594	6146	9305	2829	3327	11583	1718	2480	8546	9360	8655	2029	4344
Hexose trisaccharide (mixed-linkage glucose trisaccharide, cellotriose, mannotriose, galactotriose)	$C_{18}H_{32}O_{16}$	503.1607	633	801	1098	2473	1609	1150	1330	3968	897	732	3342	1792	3030	1011	1734
Hexose tetrasaccharide (mixed-linkage glucose tetrasaccharide, cellotetraose, mannotetraose, galactotetraose)	$C_{24}H_{42}O_{21}$	665.2135	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexose pentasaccharode (mixed-linkage glucose pentasaccharide, cellopentaose, mannopentaose, galactopentaose)	$C_{30}H_{52}O_{26}$	827.2663	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexose-pentose (glucopyranosyl-xylose, galactopyranosyl-arabinose, xylopyranosyl-galactose)	$C_{11}H_{20}O_{10}$	311.0973	515	962	1187	418	1130	808	1719	1553	790	709	1522	593	1624	793	2042
Rhamnose, fucose	$C_6H_{12}O_5$	163.0601	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetyl-galactose	$C_8H_{14}O_7$	221.0656	174	507	767	1703	729	694	1342	3268	626	450	2552	268	2439	556	1481

Annotated compounds	Formula	*m/z*	NEGATIVE MODE														
		[M-H]$^-$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Acetyl-galacturonic acid	$C_8H_{12}O_8$	235.0448	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sugar alcohols																	
Xylitol, arabitol	$C_5H_{12}O_5$	151.0601	5216	4097	3128	1887	13303	3761	3775	4257	885	3320	3828	13307	4318	1669	5798
Sorbitol, mannitol, galactitol	$C_6H_{14}O_6$	181.0707	4932	4136	2732	1931	12632	3351	2929	4324	554	2215	2853	12337	4030	1359	4679
Lignin-derived compounds																	
p-Coumaric acid	$C_9H_8O_3$	163.039	38107	67766	93160	96103	94147	84803	127313	141006	106131	79807	148206	22468	130180	44957	159300
Benzoic acid, hydroxybenzaldehyde	$C_7H_6O_2$	121.0284	24536	47999	55237	74207	42062	56572	86734	134736	54941	44803	125028	12995	122427	38248	113962
Syringaldehyde	$C_9H_{10}O_4$	181.0495	1593	3546	5231	7767	3860	3286	4640	11730	3162	2306	10727	302	11570	5991	5681
Phenylacetaldehyde	C_8H_8O	119.0491	6300	11432	18414	28977	15032	16201	25427	44047	20264	14996	43529	683	39204	10148	30122
Vanillin	$C_8H_8O_3$	151.039	3412	5857	8713	14501	5368	5411	8714	22481	5450	4063	20309	2584	20775	8724	10440
Hydroxybenzoic acid	$C_7H_6O_3$	137.0233	1009	1785	2835	5956	2974	3508	4144	10082	3180	2514	8941	1280	7162	1952	5333
Coniferyl aldehyde	$C_{10}H_{10}O_3$	177.0546	2436	4421	5985	7825	2610	2754	4603	10270	3368	2088	10115	1301	10326	6109	5369
Vanillic acid, 2,6-dimethoxybenzoquinone	$C_8H_8O_4$	167.0339	1349	2210	2636	4797	4592	4868	4584	7670	3136	2987	6193	2045	6112	1625	6189
Syringic acid	$C_9H_{10}O_5$	197.0454	1474	2023	2809	4511	4750	4404	4479	7465	3168	3054	5961	1842	6534	1354	5921
Guaiacylglycerol-beta-guaiacyl ether	$C_{17}H_{20}O_6$	319.1176	1183	1260	1139	698	2029	1089	1143	1161	877	870	1005	776	982	654	1553
Benzenediol	$C_6H_6O_2$	133.026	323	433	471	865	824	1041	965	1945	809	560	1604	454	1506	361	1289
Guaiacol	$C_7H_8O_2$	123.044	216	257	204	687	699	875	875	1297	643	446	958	385	870	172	1261
Diferulic acid	$C_{20}H_{18}O_8$	385.0918	366	432	600	887	791	937	1157	1171	759	719	1202	625	1075	405	1274
Syringol	$C_8H_{10}O_3$	153.0546	222	306	511	1192	805	912	1134	2081	566	541	1653	336	1595	351	1156
Benzoquinone	$C_6H_4O_2$	108.0206	323	494	537	835	658	665	784	1348	518	507	1054	0	1090	462	1080

Annotated compounds	Formula	*m/z*	NEGATIVE MODE														
		$[M-H]^-$	Liquors														
			1	2	3	4	5	6	7	8	9-11 (CP)	12	13	14	15	16	17
Synapyl alcohol	$C_{11}H_{14}O_4$	209.0808	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Synapyl aldehyde	$C_{11}H_{12}O_4$	207.0652	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Benzaldehyde	C_7H_6O	105.0335	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cinnamic acid	$C_9H_8O_2$	147.044	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methylguaiacol	$C_9H_{12}O_3$	108.0206	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sugar dehydration products																	
Furfural	$C_5H_4O_2$	95.0128	281	324	572	634	726	992	1100	1273	708	599	1018	308	1083	304	1190
Furfuryl alcohol	$C_5H_6O_2$	97.0284	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Furoic acid	$C_5H_4O_3$	111.0077	526	1050	1420	2427	2103	3364	4307	5850	2536	2258	4191	1082	4780	693	4443
Levulinic acid	$C_5H_8O_3$	115.039	628	821	871	1398	2159	1728	1249	3160	590	843	2058	916	2126	434	2178
Hydroxymethylfurfural (HMF)	$C_6H_6O_3$	125.0233	283	460	528	765	1093	1507	1589	1493	1627	860	1267	624	1326	284	1584

Electronic supplementary material 4. Enzymatic activities per mass of sugarcane bagasse (SCB) employed in hydrothermal pretreatment, produced by *A. niger* DSCF11 after 7 days of growth using liquors arising from pretreatment as a carbon source.





Capítulo IV

Caracterização bioquímica e proteômica de secretomas de *Aspergillus niger* produzidos em biomassas pré-tratadas

1. Introdução

Análises transcriptômicas e proteômicas de fungos filamentosos cultivados em substratos lignocelulósicos são importantes metodologias para a identificação e quantificação de proteínas envolvidas no processo de degradação de biomassa, contribuindo para o entendimento dos mecanismos de utilização da parede celular vegetal e gerando informações valiosas para o desenvolvimento de coquetéis enzimáticos eficientes (Di Cologna *et al.*, 2017; Rosnow *et al.*, 2017). Na última década, diversos grupos de pesquisa têm feito uso destas abordagens para investigar a produção de enzimas por fungos filamentosos. Boa parte dos trabalhos disponíveis compara o uso de substratos complexos (materiais lignocelulósicos) com carboidratos simples (ex: glicose, frutose, lactose, xilose, arabinose) ou polissacarídeos purificados (ex: celulose cristalina, carboximetil celulose, xilana) como fontes de carbono. Alguns trabalhos também fazem comparação de secretomas fúngicos produzidos em diferentes substratos lignocelulósicos (Mahajan e Master, 2010; De Souza *et al.*, 2011; Adav *et al.*, 2012; Delmas *et al.*, 2012; Ravalason *et al.*, 2012; GóMez-Mendoza *et al.*, 2014; Horta *et al.*, 2014; Brown *et al.*, 2016; Sharma Ghimire *et al.*, 2016; Shi *et al.*, 2016). Poucos trabalhos, entretanto, fazem uso de análises "ômicas" para investigar o efeito de etapas de pré-tratamento de substratos lignocelulósicos sobre a produção de holocelulases por fungos filamentosos.

Etapas de pré-tratamento são tradicionalmente aplicadas à biomassa com objetivo de diminuir sua recalcitrância à degradação enzimática e microbiana e representam importantes ferramentas para geração de substratos mais acessíveis para produção de enzimas por organismos lignocelulolíticos (Silva e Ferreira Filho, 2017). Como mostrado no capítulo II, diversos autores já investigaram o efeito de diferentes tecnologias de pré-tratamento sobre a produção de holocelulases por fungos filamentosos através da comparação de biomassas *in natura* ou pré-tratadas como fontes de carbono. A maioria destes trabalhos foca na comparação dos secretomas em termos

de níveis de atividade enzimática e capacidade para sacarificação de biomassa. Poucos fazem uso de métodos transcriptômicos ou proteômicos para investigar mais detalhadamente as mudanças de metabolismo e secreção de enzimas provocadas pelo pré-tratamento do substrato lignocelulósico empregado.

Ribeiro *et al.* (2012), Borin *et al.* (2015) e Daly *et al.* (2017) utilizaram abordagens "ômicas" para estudar o efeito de pré-tratamentos de biomassa (hidrotérmicos, ácidos ou com líquidos iônicos) sobre a produção de enzimas por *A. niger*, *Trichoderma reesei* e *Penicillium echinullatum*, cujos resultados serão apresentados ao longo deste texto como base de comparação ao presente trabalho. Estes estudos, entretanto, focaram apenas no uso das frações sólidas pré-tratadas como substrato para o cultivo microbiano. O uso de licores de tratamento hidrotérmico para produção de enzimas já foi estudado por abordagem proteômica e transcriptômica por outros autores (Ottenheim *et al.*, 2014; Robl *et al.*, 2015), mas uma comparação com o uso de biomassa *in natura* ou sólidos pré-tratados nunca foi realizada através de método proteômico.

A proposta do presente trabalho foi avaliar o efeito do pré-tratamento hidrotérmico do bagaço de cana-de-açúcar sobre a produção de holocelulases por *A. niger* quando utilizando as frações sólida e líquida (licor) resultantes do processo como fontes de carbono, em comparação à biomassa *in natura*. Análise proteômica quantitativa *label-free* foi utilizada para comparar os secretomas produzidos em cada condição e obter entendimento detalhado da resposta de *A. niger* às diferenças de composição e acessibilidade dos substratos utilizados. Análises de atividade enzimática, zimografia e ensaios de sacarificação enzimática de biomassa também foram realizados.

A técnica de análise proteômica quantitativa *label-free* é cada vez mais utilizada para estudar a expressão diferencial de proteínas em amostras complexas. A quantificação relativa de proteínas pela estratégia *label-free* normalmente segue as seguintes etapas: (1) preparo de amostra, que envolve extração, redução, alquilação e digestão tríptica das proteínas; (2) análise das amostras por cromatografia líquida (LC) acoplada a espectrometria de massas (MS/MS); (3) análise de dados, incluindo identificação de proteínas, quantificação relativa e análise estatística. Um esquema da metodologia proteômica quantitativa *label-free* é ilustrado na Figura 1. Cada amostra é preparada e analisada separadamente e a quantificação relativa de cada proteína identificada é realizada através de duas possíveis estratégias: (1) contagem espectral (baseia-se no número de vezes que um peptídeo é detectado); (2) comparação direta da intensidade dos picos nos cromatogramas gerados para cada amostra/condição

(abordagem utilizada neste trabalho). Esta metodologia nos permite comparar a abundância de uma proteína em diferentes amostras, mas não é capaz de comparar a abundância de diferentes proteínas em uma mesma amostra (Zhu *et al.*, 2009).

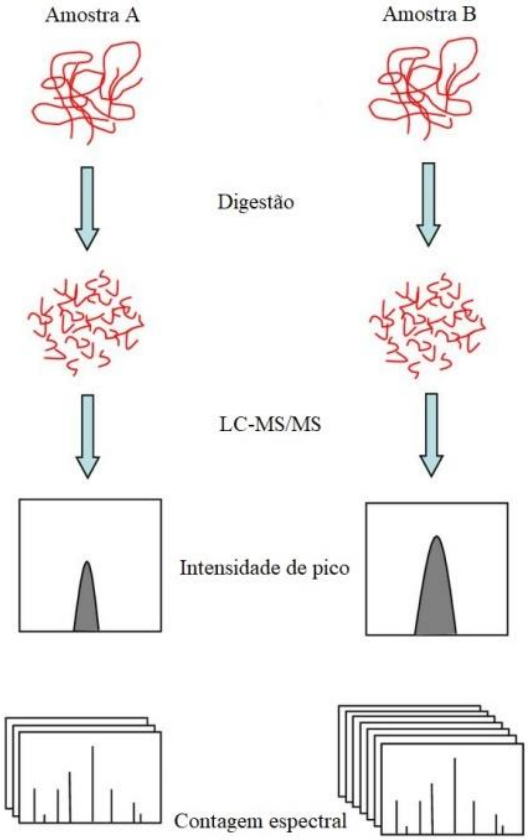


Figura 1. Esquema de quantificação relativa de proteínas por análise proteômica LC-MS/MS *label-free*. Extraído e modificado de Zhu *et al.* (2009).

Tendo em vista que a redução no custo de produção de enzimas envolvidas na sacarificação de biomassa é um dos maiores gargalos para a viabilização econômica de biorrefinarias de lignocelulose, este estudo visa contribuir para o desenvolvimento do esquema de produção local e integrado de enzimas, situação em que estas são produzidas na própria usina onde e biomassa é hidrolisada e fermentada e utilizando a mesma matéria prima que será posteriormente hidrolisada (Johnson, 2016).

2. Materiais e métodos

2.1. Tratamento hidrotérmico do bagaço de cana-de-açúcar

Com base nos resultados obtidos no capítulo III desta tese, as condições de tratamento hidrotérmico do bagaço de cana-de-açúcar selecionadas para prosseguir com os estudos de produção de holocelulases por *A. niger* foram 170 °C, 30 minutos e 1 % (m/m) de bagaço de cana-de-açúcar (Figura 2). Os parâmetros acima foram

selecionados devido à alta e rápida produção de atividade xilanolítica por *A. niger* quanto utilizando a fração líquida (licor) resultante do tratamento. O uso de baixa carga de biomassa no tratamento hidrotérmico (1 % m/m, ou 10 g/L) é compatível com a concentração de biomassa comumente utilizada (10 g/L) na literatura como fonte de carbono em cultivos de fungos filamentosos (Gomes *et al.*, 2017). A metodologia do processo de tratamento hidrotérmico está descrita no capítulo III. A Figura 2 representa um esquema do tratamento hidrotérmico aplicado ao bagaço de cana-de-açúcar.

Figura 2. Esquema do tratamento hidrotérmico do bagaço de cana-de-açúcar *in natura* para obtenção de frações menos recalcitrantes para cultivo de *A. niger*. O destino dos principais componentes da biomassa *in natura* é mostrado.

2.2. Análise de composição dos substratos lignocelulósicos

Para a análise de composição das biomassas sólidas (bagaço de cana-de-açúcar *in natura* e bagaço tratado), estas foram primeiramente submetidas a processo de remoção de extrativos hidrossolúveis e lipossolúveis utilizando água e etanol 95% como solventes, respectivamente, realizado em extrator de Soxhlet durante 24 horas (Sluiter, A, Ruiz, R, *et al.*, 2008). Depois de secas a 65°C, as biomassas livres de extrativos foram submetidas a hidrólise com ácido sulfúrico em duas etapas, como descrito por Sluiter, Amie *et al.* (2008). Em uma primeira etapa, as biomassas foram hidrolisadas

com ácido sulfúrico 72 % (m/m) a 30°C por 1 hora. Em seguida, o ácido foi diluído para concentração final de 4 % (m/m) e aquecido a 121°C por 2 horas. Os sólidos remanescentes foram secos e utilizados para quantificação de lignina ácido-insolúvel e teor de cinzas por muflagem (575 °C, *overnight*). A fração de lignina ácido-solúvel foi determinada por espectrofotometria (Sluiter, Amie *et al.*, 2008). Os carboidratos solubilizados durante a hidrólise ácida foram quantificados por cromatografia líquida de troca aniônica de alto desempenho acoplada ao detector de pulso amperométrico (HPAEC-PAD) em um sistema de cromatografia Dionex ICS3000 Ion Chromatography DC System (Dionex Co., Sunnyvale, CA, EUA), utilizando coluna e pré-coluna CarboPac PA-1. As corridas foram realizadas a 20°C e fluxo de 0,2 mL/min. As amostras foram eluídas com gradiente linear de 15 – 22 mM NaOH (Sigma-Aldrich Co., St. Louis, MO, EUA) durante 30 minutos. A coluna foi lavada com 200 mM NaOH por 5 minutos seguido de uma etapa de reequilíbrio com 15 mM NaOH por 15 minutos durante as injeções sucessivas. Xilose, arabinose, glicose, galactose e manose (Sigma Chemical Co., St. Louis, MO, EUA) a 1.25 – 60.0 µg/mL foram utilizados como padrões para construção da curva de calibração do equipamento.

Considerando a análise de composição dos licores, os monossacarídeos foram quantificados diretamente por HPAEC-PAD, como mencionado acima. Para a quantificação de oligossacarídeos, o licor foi submetido à hidrólise ácida (4 % ácido sulfúrico, 121 °C por 2 horas) para clivagem de ligações glicosídicas e então submetido à análise por HPAEC-PAD. Os monossacarídeos previamente quantificados foram subtraídos e um fator de correção de 0.88 para pentoses e 0.9 para hexoses foi aplicado para o cálculo de concentração de oligossacarídeos (Sluiter, A, Hames, B, *et al.*, 2008). Estes fatores de correção correspondem ao ganho de molécula de água durante hidrólise ácida dos oligossacarídeos.

2.3. Microscopia eletrônica de varredura

A microscopia eletrônica de varredura (MEV) foi utilizada com o objetivo de visualizar as mudanças estruturais na superfície das fibras de bagaço de cana-de-açúcar causadas pelo tratamento hidrotérmico. Amostras de bagaço de cana-de-açúcar *in-natura* e tratado hidrotermicamente foram secas a 65°C, metalizadas em equipamento Sputter Coater SCD 050 (Balzers, Alemanha) e visualizadas em microscópio eletrônico

de varredura modelo JMS 7001F (JEOL, Japão) utilizando voltagem de 15 kV, de acordo com protocolo adaptado de Li *et al.* (2014).

2.4. Cultivos

Quatro fontes de carbono foram utilizadas para cultivo submerso de *A. niger*, sendo elas: bagaço de cana-de-açúcar *in-natura*/não tratado (BNT), sólidos obtidos após tratamento hidrotérmico do bagaço (BPT), licor obtido após tratamento hidrotérmico do bagaço (LIC) e sólidos tratados em conjunto com licor (BPT+LIC), como esquematizado na Figura 3. Um cultivo sem adição de fonte de carbono também foi realizado como controle negativo.

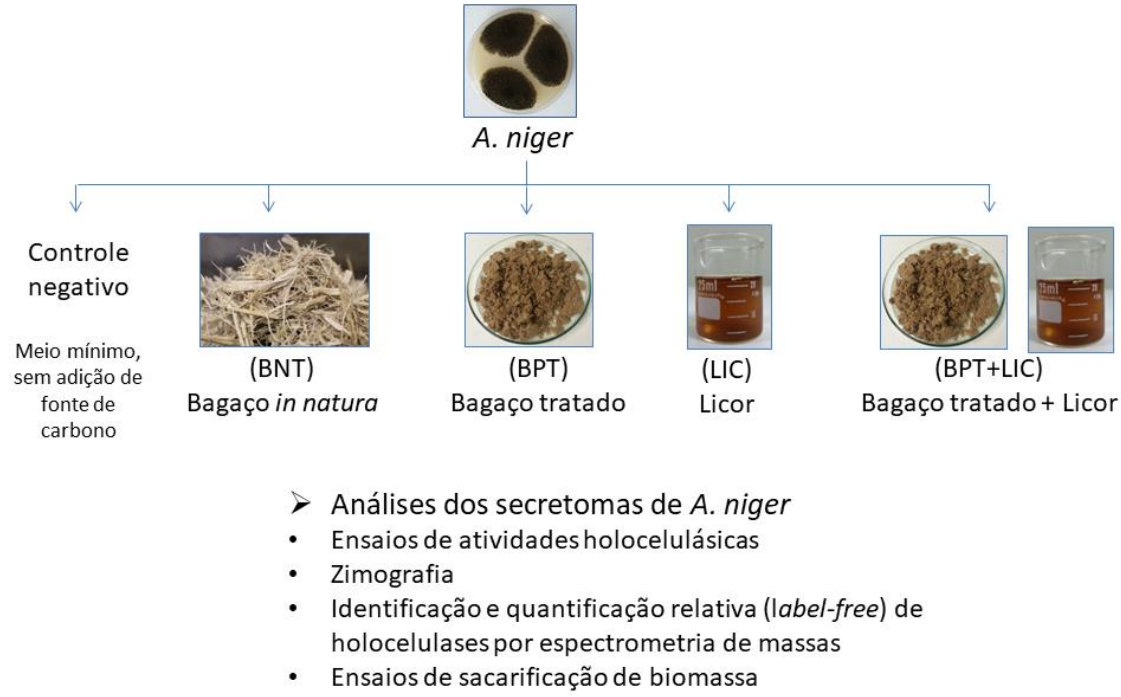


Figura 3. Esquema de produção de enzimas por *A. niger* utilizando biomassas *in natura* ou obtidas após tratamento hidrotérmico do bagaço de cana-de-açúcar. Os secretomas foram então caracterizados de acordo com os itens mostrados.

No caso das condições BNT e BPT, as biomassas foram adicionadas a meio mínimo preparado com água destilada contendo KH_2PO_4 (7,0 g/L), K_2HPO_4 (2,0 g/L), $MgSO_4.7H_2O$ (0,5 g/L), $(NH_4)_2SO_4$ (1,0 g/L) e extrato de levedura (0,6 g/L), pH 7,0. No caso das condições LIC e BPT+LIC, os mesmos nutrientes citados para o meio mínimo foram diluídos no licor resultante do tratamento hidrotérmico do bagaço, o qual foi

usado sozinho (LIC) ou em conjunto com os sólidos tratados (BPT+LIC) como fonte de carbono. No caso do controle negativo, *A. niger* foi inoculado em meio mínimo preparado em água destilada, sem adição de nenhuma fonte de carbono. Os cultivos foram realizados em frascos erlenmeyer de 250 mL com volume de trabalho de 75 mL, a 28 °C e 120 rpm por 5 dias. A quantidade de carboidratos e lignina presente em cada condição de cultivo está mostrada na Figura 4. A quantidade de carboidratos totais presentes nas diferentes condições não foi normalizada, uma vez que o objetivo do experimento foi avaliar a utilização das frações sólida e líquida da maneira como foram geradas a partir do tratamento hidrotérmico do bagaço de cana-de-açúcar *in natura*. Todos os cultivos foram feitos em triplicata.

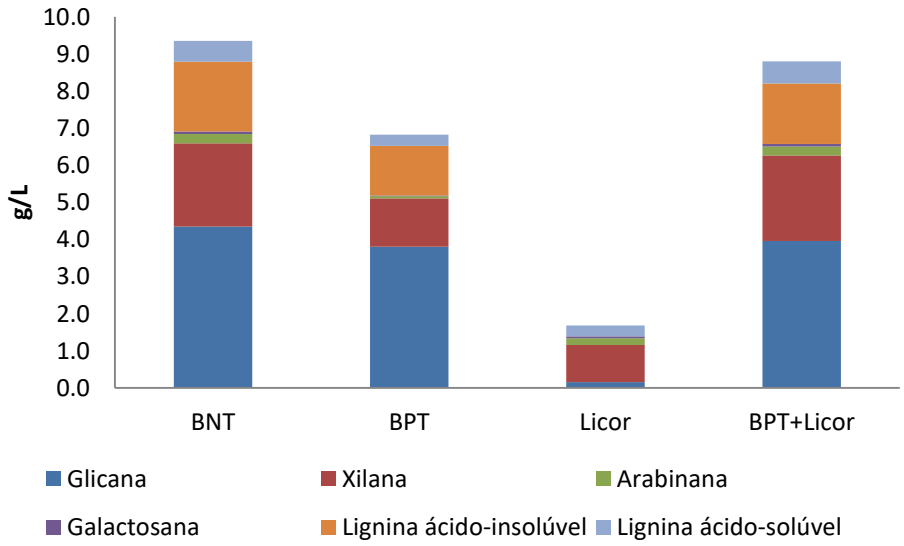


Figura 4. Teores de carboidratos e lignina presente nas condições de cultivo de *A. niger*. No caso das condições BNT (bagaço de cana-de-açúcar *in natura* como fonte de carbono) e BPT (bagaço de cana-de-açúcar tratado como fonte de carbono), os carboidratos correspondem a polissacarídeos. No caso da condição LIC (licor como fonte de carbono), a concentração de carboidratos corresponde à soma de oligossacarídeos e monossacarídeos. No caso da condição BPT+LIC (bagaço de cana-de-açúcar tratado em conjunto com o licor como fontes de carbono), a concentração de carboidratos corresponde à soma de polissacarídeos, oligossacarídeos e monossacarídeos.

2.5. Caracterização das atividades enzimáticas

Os secretomas foram caracterizados quanto à presença de atividades holocelulolíticas. Para os ensaios de atividade de xilanase, endoglicanase (CMCase), mananase e pectinase, 5 µL de secretoma foram incubados com 10 µL de solução de xilana de aveia, carboximetilcelulose (CMC), manana (*locust bean gum*) ou pectina de

maçã (todas na concentração de 1 % (m/v)), respectivamente, a 50°C por 30 minutos. Para ensaio de celulases totais em papel de filtro (FPase), 100 µL de secretoma foram incubados com 200 µL de tampão acetato de sódio 100 mM, pH 5,0, e 10 mg de papel de filtro Whatman nº1 a 50°C por 60 minutos. Os açúcares redutores liberados foram quantificados por ácido dinitrosalicílico (DNS) (Miller, 1959). Para ensaios de β-glicosidase, β-xilosidase, α-L-arabinofuranosidase e α-galactosidase, 5 µL de secretoma foram incubados com 45 µL de solução 5 mM de *p*-nitrofenil(PNP)-β-D-glicopiranosídeo, PNP-β-xilopiranosídeo, PNP-α-L-arabinofuranosídeo e PNP-α-galactopiranosídeo, respectivamente, a 50 °C por 30 minutos. A reação foi interrompida pela adição de carbonato de sódio 1M (50 µL) e a liberação de *p*-nitrofenol foi quantificada espectrofotometricamente a 430 nm. As atividades enzimáticas foram expressas como µmol de produto formado por minuto (UI) e por mL de solução enzimática. Todos os substratos naturais e sintéticos foram obtidos da Sigma-Aldrich (St Louis, Missouri, EUA).

2.6. SDS-PAGE e zimografia

As amostras de secretoma foram submetidas a eletroforese em géis de poliacrilamida 12 % sob condições desnaturantes (SDS-PAGE) como descrito por Laemmli (1970) utilizando sistema Mini-Protean III Cell (BioRad Laboratories, Hercules, CA, EUA). Alíquotas de secretoma contendo 10 µg de proteína total foram precipitadas com solução 10 % (m/v) de ácido tricloroacético (TCA), ressuspendidas em 20 µL de tampão de amostra contendo Tris-HCl (125 mM), 2,0 % (m/v) de SDS, 0,05% (m/v) de azul de bromofenol, 0,05% (m/v) de glicerol e 5,0 % (v/v) de β-mercaptoetanol, e, posteriormente, fervidas por 3 minutos antes de serem aplicadas no gel. O tampão de corrida utilizado consistiu de Tris base (12,5 mM), glicina (96 mM) e SDS 0,05 % (m/v). Os géis foram corados com nitrato de prata.

Os secretomas também foram submetidos a zimografia para detecção de atividades de xilanase, celulase, mananase e pectinase em géis de SDS-PAGE. Para tal, os géis de acrilamida foram preparados como descrito acima e co-polimerizados com xilana, CMC, manana e pectina a uma concentração final de 0,1 % (m/v). Alíquotas contendo 50 µg de proteína foram preparadas e aplicadas conforme descrito acima. Após a corrida, as proteínas presentes no gel foram renaturadas em solução Triton X-100 2,5% (v/v) por 1 h, sob agitação e em seguida o gel foi incubado a 50° C em solução 100 mM

de tampão acetato de sódio pH 5,0 por 1h30. Os géis co-polimerizados com xilana, CMC e manana foram corados com solução 0,1% (m/v) de vermelho Congo por 30 min, descorados em solução 1M de NaCl e contrastados com solução de ácido acético 0,5% (v/v). O gel co-polimerizado com pectina foi corado com solução de 0,02 % (m/v) de vermelho de rutênio e descorado com água destilada.

2.7. Caracterização dos secretomas por análise proteômica quantitativa *label-free*

Alíquotas de cada secretoma foram submetidas à precipitação de proteínas totais pelo método acetona-NaCl (Crowell *et al.*, 2013), ressuspendidas em tampão acetato de sódio 100 mM pH 5,0 (mesmo volume inicial) e quantificadas pelo método de Bradford (1976). Esta etapa de precipitação foi realizada com objetivo de remover pigmentos, principalmente compostos fenólicos solubilizados a partir da lignina durante tratamento hidrotérmico do bagaço de cana-de-açúcar, que inferem na quantificação de proteínas. Uma vez quantificados os teores de proteína, alíquotas de cada secretoma contendo 50 µg de proteína total foram precipitadas pelo método acetona-NaCl (Crowell *et al.*, 2013) e secas em *Speed-Vac* durante 2 horas a 30°C. Os pellets foram ressuspendidos em 150 µL de solução contendo 8 M de uréia, 7,5 mM de NaCl, 50 mM de bicarbonato de trietilamônio (TEAB) e 5 mM de ditiotreitol (DTT), pH 8.2, e incubados por 25 minutos a 55°C. Esta etapa promove a quebra de ligações dissulfeto presente na proteínas (processo chamado de redução de proteínas). Em seguida, solução estoque de iodacetamida foi adicionada às misturas de modo a atingir uma concentração final de 14 mM (etapa de alquilação), e as amostras foram incubadas a temperatura ambiente, no escuro, por 40 minutos. Após a incubação, DTT foi adicionado a uma concentração final de 10 mM para interromper a reação de alquilação, e a mistura foi diluída na proporção 1:5 com soluções 25 mM de TEAB e 1 mM de $CaCl_2$, pH 7.9. Em seguida, tripsina (1µg/50µg de proteína total) foi adicionada à mistura e a digestão de proteínas foi realizada a 37°C durante a noite. Para pausar a reação de tripsinização, ácido trifluoroacético (TFA) foi adicionado a uma concentração final de 0,5 % (v/v). A amostra foi seca em *Speed-vac* e ressuspendida em solução de TFA 0.1 % (v/v). As soluções contendo peptídeos trípticos foram dessalinizadas utilizando membrana hidrofóbica Empore C18 montadas em ponteiras "*low-binding*" de 200µL (*Stage tips*) e eluídas por lavagens sequenciais com as seguintes soluções: (a) acetonitrila (ACN) 25 % (v/v) e ácido acético 0,5% (v/v); (b) ACN 50 % (v/v) e ácido acético 0,5 % (v/v); (c)

ACN 80 % (v/v) e ácido acético 0,5 % (v/v); (d) ACN 100 % (v/v). Os peptídeos foram então quantificados por fluorometria (Qubit, ThermoFischer Scientific).

Alíquotas contendo 1 µg de peptídeos trípticos foram então injetadas em um sistema LC-ESI-MS/MS. A etapa de cromatografia líquida foi realizada em equipamento Nano LC Ultimate 3000 (Dionex, Amsterdã, Holanda) equipado com pré-coluna (tamanho de partícula de 5 µm, 5 cm de comprimento, diâmetros interno e externo de 100 µm e 360 µm, respectivamente) e coluna (tamanho de partícula 3 µm, 15 cm de comprimento, diâmetros interno e externo de 75 µm e 360 µm, respectivamente) empacotadas com resina Reprosil-Pur 120 C18-AQ (Dr. Maish, Ammerbuch, Alemanha), previamente equilibradas com solvente A [acetonitrila (ACN) 5% (v/v), ácido fórmico (AF) 0,1% (v/v)]. As amostras foram eluídas em gradiente de 5-20 % solvente B [ACN 95% (v/v), AF 0,1% (v/v)] durante 30 minutos, 20-50 % solvente B durante 15 minutos; 50-98 % solvente B durante 5 minutos e 98 % solvente B durante 10 minutos, em um fluxo de 250 nL/min. Os peptídeos foram eluídos da cromatografia de fase reversa para um espectrômetro de massas modelo LTQ-Orbitrap Elite (ThermoFisher Scientific, Bremen, Alemanha) através de sonda nanospray (ThermoFisher Scientific) com voltagem de spray de 3,02 kV e temperatura de transferência capilar de 275ºC. O espectrômetro foi operado em modo *"Data-Dependent Aquisition"* através do software Xcalibur versão 2.2 (ThermoFisher Scientific), utilizando uma faixa de detecção de 350-1500 m/z a uma resolução de 120000 em 400 m/z. Os 15 íons precursores mais intensos em um ciclo de aquisição foram fragmentados por dissociação induzida por colisão (HCD) sob energia normalizada de colisão de 35 % para análise MS/MS e o limiar de seleção de íons foi configurado para 1500 contagens usando uma janela de isolamento de precursor de 2 amu. A ativação do parâmetro "q" e o tempo de ativação foram programados para 0,25 e 0,10 ms, respectivamente. Os precursores previamente fragmentados foram dinamicamente excluídos durante o ciclo por mais 30 segundos.

A identificação das proteínas foi realizada através do software Peaks (versão 7, Bioinformatics Solutions Inc., Waterloo, Ontário, Canadá), baseando-se no proteoma predito de três linhagens de *A. niger* cujos genomas estão disponíveis publicamente na plataforma Uniprot (uniprot.org). As linhagens CBS 513.88 (designada ASPN_A), ATCC 1015 (designada ASPN_C) e An76 (designada ASPN_G) foram utilizadas. A identificação de proteínas foi realizada sob os seguintes critérios: taxa de falsos-positivos (FDR) de 1 % e considerando a detecção de pelo menos 1 peptídeo único.

Foram empregados filtros de tolerância de 0,5 Da para o espectro parental e de 10 ppm para espectros tandem. Carbamidometilação de cisteína e acetilação da extremidade amino-terminal foram consideradas modificações fixas.

A quantificação relativa das proteínas foi realizada através do software Progenesis QI for Proteomics (NonLinear Dynamics, Durham, EUA). Os valores de abundância normalizada das proteínas presentes nas condições BPT, LIC e BPT+LIC foram comparados com a condição controle (bagaço *in-natura*, BNT). A significância estatística da diferença de abundância das proteínas presentes nos diferentes secretomas foi avaliada por análise de variância (ANOVA, $p<0,05$) e teste T de Student ($p<0,05$). A quantificação relativa foi realizada apenas para as proteínas presentes em comum nas quatro condições de cultivo.

As proteínas identificadas foram analisadas quanto à presença de domínios conservados de enzimas ativas sobre carboidratos (CAZymes) através do software hmmscan (HMMER3, versão 3.1) utilizando a base de dados dbCAN (Yin *et al.*, 2012). As proteínas foram analisadas quanto à presença de peptídeos sinal para secreção utilizando SignalP 4.1 Server (Nielsen, 2017) e Phobius web server (Käll *et al.*, 2007). A função molecular e o processo biológico no qual cada proteína está envolvida foram preditos através de Gene Ontology (Ashburner *et al.*, 2000; Consortium, 2017) e análise Blast2Go (Conesa *et al.*, 2005).

2.8. Sacarificação de bagaço de cana-de-açúcar

Para os ensaios de sacarificação, a termoestabilidade de xilanases e celulases dos secretomas foram previamente testadas. Para tal, os secretomas foram incubados a 40 e 50°C sob agitação de 120 rpm e alíquotas foram coletadas periodicamente para testes de atividade enzimática. Uma vez determinada a termoestabilidade das amostras, as condições de sacarificação foram fixadas.

Os ensaios de sacarificação de bagaço de cana-de-açúcar *in natura* e tratado (gerado a 170°C, 30 min e biomassa 1 % m/m) foram realizados em frascos erlenmeyer de 250 mL com volume de trabalho de 50 mL, a 40°C, 120 rpm, em tampão acetato de sódio (50 mM) pH 5.0 e 0.1 % (m/v) de azida sódica, com uma concentração de biomassa de 10 g/L (peso seco) e carga de proteínas de 5 mg de proteína total por g de substrato. Para atingir a carga de proteína total necessária, os secretomas foram liofilizados e

ressuspendidos no tampão citado acima. Alíquotas foram coletadas periodicamente (fervidas por 5 minutos para interrupção do processo de hidrólise) e submetidas à quantificação de açúcares redutores totais por DNS e glicose pelo kit enzimático de glicose-oxidase de acordo com as instruções do fabricante (Doles, Goiânia, Brasil).

3. Resultados e discussão

3.1. Caracterização dos secretomas quanto às atividades de holocelulases e os teores de proteínas totais

A Tabela 1 apresenta um painel de atividades de holocelulases e teor de proteínas totais produzidas por *A. niger* durante cultivo submerso utilizando como fonte de carbono a biomassa *in natura* ou as biomassas advindas do tratamento hidrotérmico. O secretoma gerado na ausência de fonte de carbono lignocelulósica também foi testado para teor de proteínas totais e algumas atividades de holocelulases.

Tabela 1. Painel de atividades de holocelulases e teor de proteínas totais produzidos por *A. niger* em diferentes condições de cultivo. As fontes de carbono utilizadas foram: BNT = bagaço de cana-de-açúcar *in natura*; BPT = sólidos obtidos após tratamento hidrotérmico do bagaço; LIC = licor; BPT+LIC = sólidos tratados + licor. O controle negativo corresponde a cultivos sem adição de fonte de carbono.

	Contr. neg.	BNT	BPT	LIC	BPT+LIC
			UI/mL		
FPase		0.030 ± 0.003	0.092 ± 0.008	0.078 ± 0.007	0.107 ± 0.007
CMCase	0.020 ± 0.019	0.066 ± 0.009	0.211 ± 0.006	0.168 ± 0.022	0.225 ± 0.003
β-glicosidase		0.209 ± 0.011	0.150 ± 0.007	0.242 ± 0.02	0.355 ± 0.112
β-xilanase	0.131 ± 0.003	1.808 ± 0.081	4.233 ± 0.121	3.700 ± 0.030	4.244 ± 0.153
β-xilosidase		0.018 ± 0.004	0.100 ± 0.017	0.193 ± 0.010	0.245 ± 0.010
α-L-arabinofuranosidase		0.008 ± 0.002	0.034 ± 0.001	0.101 ± 0.018	0.148 ± 0.013
β-mananase	0.000 ± 0.000	0.031 ± 0.003	0.218 ± 0.011	0.074 ± 0.012	0.200 ± 0.002
β-manosidase		0.000 ± 0.004	0.001 ± 0.001	0.000 ± 0.002	0.000 ± 0.001
α-galactosidase		0.114 ± 0.003	0.103 ± 0.017	0.267 ± 0.06	0.258 ± 0.015
Pectinase	0.000 ± 0.000	0.000 ± 0.008	0.065 ± 0.025	0.012 ± 0.008	0.100 ± 0.014
			µg/mL		
Proteínas totais	7.482 ± 0.738	15.946 ± 2.300	28.623 ± 0.281	22.993 ± 0.493	31.539 ± 0.345

Pouco micélio foi observado na condição controle negativo. Apesar de não apresentar uma fonte de carbono lignocelulósica, o controle negativo apresenta extrato de levedura (0,6 g/L) em sua composição que atua como uma fonte de carbono e nitrogênio, ainda que limitada. Uma baixa quantidade de proteínas totais e atividades basais de xilanase e CMCase foram observadas no secretoma do controle negativo.

Atividades basais de holocelulases são comumente observadas em secretomas de fungos filamentosos enfrentando situações de limitação de carbono, o que é geralmente interpretado como uma estratégia empregada pelo fungo para sondar o ambiente em busca de substratos disponíveis. Na presença de polissacarídeos, as enzimas "escoteiras" liberariam moléculas indutoras necessárias para a indução de um arsenal enzimático apropriado para a hidrólise do substrato detectado (van Munster *et al.*, 2014).

O teor de proteínas totais nos secretomas de *A. niger* foi superior quando os substratos gerados no tratamento hidrotérmico do bagaço de cana-de-açúcar foram utilizados como fonte de carbono (condições BPT, LIC e BPT+LIC), em comparação com bagaço *in natura* (BNT). Seguindo a mesma tendência, as atividades de holocelulases produzidas foram superiores utilizando fontes de carbono pré-tratadas, apesar de estes substratos apresentarem teor de carboidratos totais menores que o bagaço *in natura* (Figura 4). Em especial, o licor apresentou teor de carboidratos totais (1,38 g/L) correspondente à apenas 20 % do teor presente na condição BNT (6,91 g/L), e mesmo assim foi capaz de induzir maiores níveis de proteínas e holocelulases. Além disso, no caso de β-glicosidase, β-xilosidase e α-L-arabinofuranosidase, o licor induziu atividades maiores que os sólidos tratados (BPT) (contendo 5,18 g/L de carboidratos totais). Da mesma forma, o bagaço tratado (condição BPT), mesmo contendo teor de carboidratos menor que o bagaço *in natura*, induziu atividades superiores. Como únicas exceções, as atividades de β-glicosidase e α-galactosidase foram menores no secretoma BPT em comparação ao secretoma BNT. A condição BPT+LIC, de modo geral, induziu os maiores níveis de proteínas totais e atividades de holocelulases provavelmente devido à presença combinada de carboidratos solúveis e sólidos mais acessíveis. Esses resultados indicam que a acessibilidade dos carboidratos presentes na fonte de carbono, e não só o teor de carboidratos totais, é um importante fator para a indução de holocelulases.

A maior parte da arabinoxilana presente no bagaço *in natura* (originalmente 25,85 % do peso seco) ficou retida nos sólidos tratados (18,42 % do peso seco), provavelmente em forma mais acessível, sendo este um importante fator para a indução de hemicelulases na condição BPT. Entretanto, apesar de o tratamento hidrotérmico ter solubilizado apenas 47,1 % da arabinoxilana para a fase aquosa (licor), a porção solubilizada no licor teve alto poder indutor e foi suficiente para induzir atividades

superiores àquelas induzidas pelo bagaço *in natura*. Como discutido no capítulo III, o licor é rico em oligossacarídeos solúveis, prontamente acessíveis ao fungo.

Os maiores níveis de proteínas totais e maiores atividades enzimáticas no secretoma de *A. niger* cultivado no licor (LIC) em comparação ao bagaço *in natura* (BNT) faz-nos levantar a hipótese de que isso se deve simplesmente ao fato de não haver perda de proteínas no sobrenadante da condição LIC por adsorção à biomassa sólida (adsorção específica à celulose cristalina por meio de módulos de ligação ao carboidrato e/ou adsorção inespecífica à lignina presente no bagaço por interação hidrofóbica), como ocorreria na condição BNT. Esta hipótese, entretanto, pode ser descartada pelo fato de haver também maior teor de proteínas totais e atividades enzimáticas nos secretomas produzidos nas condições BPT e BPT+LIC, que também apresentam celulose cristalina e lignina em suas composições e representam situações nas quais também haveria adsorção de enzimas ao material sólido. Outra característica que refuta esta hipótese é o fato de a lignina submetida a tratamento hidrotérmico geralmente adquirir capacidade de adsorver enzimas superior àquela observada na biomassa *in natura* (Ko *et al.*, 2015; Lu *et al.*, 2016). O aumento na condensação e hidrofobicidade da lignina após o pré-tratamento favorece a adsorção das enzimas por meio de interação hidrofóbica (Lu *et al.*, 2016). Mesmo sob estas circunstâncias, os secretomas BPT e BPT+LIC mostraram níveis de atividade enzimáticas maiores que BNT e LIC. Estes resultados indicam que as atividades de holocelulases e abundância de enzimas em LIC são devido ao alto potencial indutor dos carboidratos ali presentes.

Não apenas a composição química, mas as alterações físicas e estruturais causadas pelo tratamento hidrotérmico da biomassa tem importante papel na indução de holocelulases em fungos filamentosos. As imagens de MEV (Figura 5) evidenciam uma mudança estrutural na superfície das fibras do bagaço de cana-de-açúcar causada pelo tratamento hidrotérmico. O substrato tratado tem aspecto mais irregular e com maior área superficial que o bagaço *in natura* (estrutura mais ordenada), que possivelmente permite maior acesso do micélio de *A. niger* e maior quantidade de enzimas secretadas. Esse resultado está de acordo com a literatura. Nos trabalhos de Reddy *et al.* (2015) e Yu, Zhuang, Yuan, *et al.* (2013), amostras bagaço de cana-de-açúcar submetidas a tratamento hidrotérmico também apresentaram superfície mais irregular e enrugada que a biomassa *in natura*. Além de aumentar a área superficial específica, o tratamento hidrotérmico geralmente também promove diminuição no tamanho de partículas e

aumento no diâmetro e volume de poros, permitindo a acomodação de enzimas em locais previamente inacessíveis (Li *et al.*, 2014). Desta forma, os materiais lignocelulósicos ficam menos recalcitrantes e mais sujeitos à sacarificação, permitindo que o fungo fique exposto a uma maior concentração de monossacarídeos derivados da hidrólise da biomassa durante o cultivo em materiais pré-tratados quando comparado ao crescimento em biomassa *in natura* (Daly *et al.*, 2017), afetando o crescimento do micélio e consequentemente a produção de holocelulases.

Uma característica comum em biomassas submetidas ao tratamento hidrotérmico é o surgimento de esferas de lignina na superfície das fibras, geradas pela redistribuição deste componente durante o tratamento com água quente e que geralmente aumentam de tamanho com o aumento da severidade do tratamento (Yu, Zhuang, Yuan, *et al.*, 2013; Reddy *et al.*, 2015). Estes artefatos não puderam ser observados no presente trabalho, uma vez que um aumento de 10000x é necessário para visualizá-las, o que não foi realizado neste experimento.

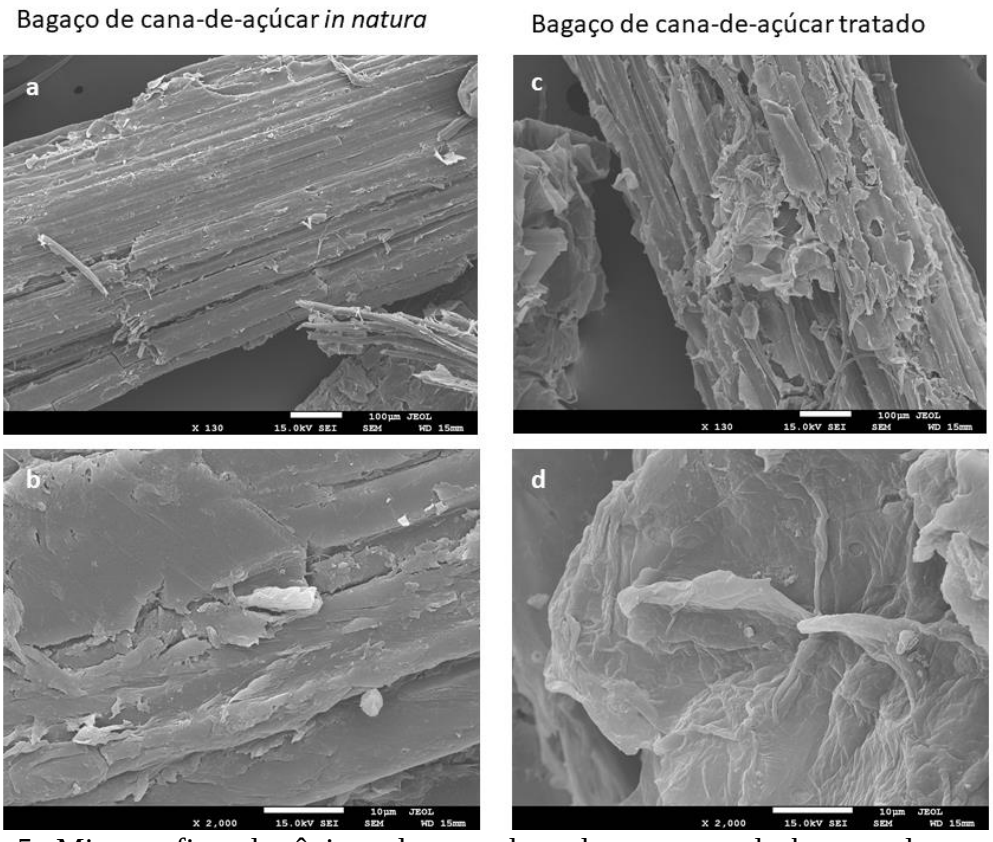


Figura 5. Micrografias eletrônicas de varredura de amostras de bagaço de cana-de-açúcar *in natura* (a e b) e tratadas hidrotermicamente a 170°C por 30 minutos (c e d). As imagens foram obtidas com aumento de 130x (a e c) e 2000x (b e d).

Como revisado no capítulo II (Silva e Ferreira Filho, 2017), outros trabalhos evidenciam o efeito positivo do tratamento hidrotérmico de substratos lignocelulósicos para a produção de holocelulases por fungos filamentosos. No trabalho de Souza *et al.* (2011), as atividades de xilanase e endoglicanase produzidas por *A. niger* N402 foram superiores quando bagaço de cana-de-açúcar pré-tratado por explosão à vapor foi utilizado como fonte de carbono, em comparação ao bagaço *in natura*. O uso de bagaço de cana-de-açúcar pré-tratado por explosão à vapor também foi mais favorável à produção de endoglicanase por *T. reesei* RUT-C30, quando comparado com bagaço *in natura* (Florencio *et al.*, 2015). No trabalho de Pereira *et al.* (2013), *P. echinulatum* produziu maiores atividades de celulase e xilanase quando cultivado na presença de bagaço de cana-de-açúcar pré-tratado hidrotermicamente do que em bagaço *in natura*, e isso foi parcialmente atribuído ao aumento da área superficial específica (de 0,65 para 1,07 m^2/g). No trabalho de Brijwani e Vadlani (2011), a aplicação de tratamento hidrotérmico brando sobre cascas do grão de soja também promoveu aumento na produção de FPase, endoglicanase e β-glicosidase por *T. reesei* e de endoglicanase, β-glicosidase e xilanase por *A. oryzae* em fermentações em estado sólido. Apesar de causar pouca variação na composição química das cascas da soja, o pré-tratamento causou aumento na porosidade do material, permitindo maior oxigenação do sistema de fermentação em estado sólido e levando ao maior crescimento fúngico e produção enzimática (Brijwani e Vadlani, 2011).

Os secretomas de *A. niger* foram analisados em gel SDS-PAGE e os resultados são mostrados na Figura 6. As condições BPT, LIC e BPT+LIC têm perfis de bandas de SDS-PAGE similares entre si e apresentam bandas mais intensas que aquelas observadas na condição BNT. A Figura 6 também mostra resultados de zimografia para detecção de atividades de xilanase, CMCase, pectinase e mananase em gel. Nos zimogramas, as condições BPT, LIC e BPT+LIC apresentam maior número e maior intensidade de bandas com atividade enzimática que a condição BNT. Apesar do arraste observado no zimograma de xilanase, é possível visualizar múltiplas bandas com atividade xilanolítica nas condições BPT, LIC e BPT+LIC, havendo apenas duas bandas evidentes em BNT (Figura 6). No zimograma de CMCase também houve arraste, mas foi possível observar três ou quatro bandas com atividade em todas as condições de cultivo (Figura 6). A intensidade de bandas com atividade de CMCase seguiu o mesmo padrão de atividade de CMCase observado no painel de atividades (Tabela 1), ou seja,

foram superiores nas condições BPT, LIC e BPT+LIC em relação à condição BNT. Considerando o zimograma de pectinase (Figura 6), uma banda de atividade foi observada nas condições de cultivo contendo sólidos tratados como fonte de carbono (BPT e BPT+LIC). Este resultado zimográfico corrobora os resultados observados no painel de atividades (Tabela 1), que mostra atividades de pectina mais expressivas nas condições BPT e BPT+LIC. No caso do zimograma de mananases, três bandas são observadas nas condições BPT, LIC e BPT+LIC, não sendo possível visualizar bandas na condição BNT (Figura 6). Estes resultados também estão de acordo com aqueles observados na Tabela 1, onde o secretoma obtido na condição BNT apresentou atividade de mananase inferior às outras condições. Em conjunto, os resultados de ensaio enzimático e zimografia confirmam o efeito positivo do tratamento hidrotérmico do substrato bagaço de cana-de-açúcar sobre a produção de holocelulases.

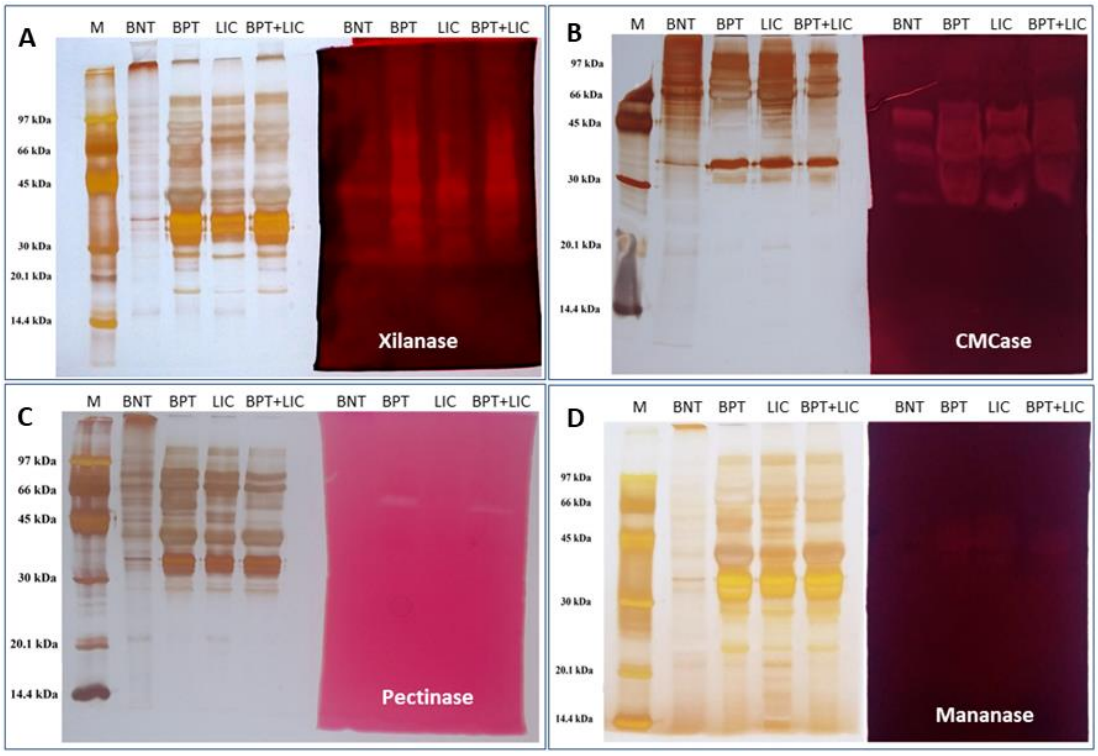


Figura 6. SDS-PAGE e zimogramas para detecção de atividade de xilanase (quadro A), CMCase (quadro B), pectinase (quadro C) e mananase (quadro D) em secretomas de *A. niger* produzidos em diferentes fontes de carbono. M = marcador molecular; BNT = secretoma produzido por *A. niger* quando cultivado em bagaço de cana-de-açúcar *in natura*; BPT = secretoma produzido por *A. niger* quando cultivado em sólidos obtidos após tratamento hidrotérmico do bagaço; LIC = secretoma produzido por *A. niger* quando cultivado em licor obtido após tratamento hidrotérmico do bagaço; BPT+LIC = secretoma produzido por *A. niger* quando cultivado em sólidos tratados em conjunto com o licor.

3.2. Análise proteômica

A Tabela 2 apresenta a lista de proteínas identificadas em todas as condições de cultivo de *A. niger*. A Tabela 2 também apresenta a quantificação relativa das proteínas nas condições BPT, LIC e BPT+LIC em relação à condição controle (bagaço de cana-de-açúcar *in natura*, BNT) na forma de Log_2(abundância relativa), bem como a presença de peptídeo sinal para secreção e de domínios conservados de enzimas ativas sobre carboidratos.

Tabela 2. Proteínas identificadas nos diferentes secretomas de *A. niger*. "Y" e "N" representam presença e ausência de peptídeo sinal para secreção, respectivamente. A coluna dbCAN indica a família de enzimas ativas sobre carboidratos à qual a proteína pertence. Abundâncias significativamente diferentes em relação à condição controle (BNT) são marcadas com asterisco e negrito. Log_2(abundância relativa) > zero: proteínas mais abundantes. Log_2(abundância relativa) < zero: proteínas menos abundantes.

Número de acesso	Peptídeo sinal	dbCAN	Nome da proteína	Descrição	Polissacarídeo em que atua	Quantificação relativa		
						Log_2 (BPT/BNT)	Log_2 (LIC/BNT)	Log_2 (BPT+LIC/BNT)
Holocelulases								
A2QVN9_ASPNC	Y	GH1		β-glicosidase putativa	Celulose	4.133	3.639	4.955
G3Y786_ASPNA	N	GH1		β-glicosidase	Celulose	1.324	-9.620	2.559
A0A100IJJ3_ASPNG	N	GH3		β-glicosidase	Celulose	-0.212	1.818	1.207
C7C4Z9_ASPNG	Y	GH3	Bgl	β-glicosidase	Celulose	**0.610***	**0.959***	**2.126***
A0A1V1FQ75_ASPNG	Y	GH3	XlnD	β-xilosidase	Xilana	**2.722***	**3.248***	**3.069***
A0A100IKN9_ASPNG	Y	GH3		α-galactosidase	Galactomanana	**3.913***	1.820	**3.919***
A0A1V1G264_ASPNG	Y	GH3	XlsV	β-xilosidase putativa	Xilana	1.076	0.252	**1.653***
G3Y4E8_ASPNA	N	GH3	BglI	β-glicosidase	Celulose	**3.022***	1.117	2.383
A2R2S3_ASPNC	Y	GH3		β-glicosidase putativa	Celulose	**-2.806***	**-4.150***	**-3.369***
G3YBE0_ASPNA	Y	GH3		β-glicosidase	Celulose	-1.125	-0.014	0.708
A0A023UH08_ASPNG	Y	GH5	Eg1	Endo-β-1, 4-glicanase	Celulose	**11.226***	6.836	**9.979***
H2E6Y8_ASPNG	Y	GH5	ManA	Endo-β-1,4-mananase	Manana	**1.644***	4.951	1.536
A2QAI8_ASPNC	Y	GH5, CBM1		Endo-glicanase	Celulose	**7.143***	**3.467***	**5.969***
G3Y873_ASPNA	Y	GH5		Endo-β-1,6-galactanase	Pectina	-1.886	9.460	-0.792
G3XZI3_ASPNA	Y	GH5	EglB	Endoglicanase	Celulose	**5.098***	1.926	**4.134***
A2QQ99_ASPNC	Y	GH6		Glicanase	Celulose	**6.098***	**2.849***	**4.249***
A2QYR9_ASPNC	Y	GH6, CBM1	CbhC	Celobiohidrolase	Celulose	**2.932***	**2.493***	**2.728***
A0A100IHS6_ASPNG	Y	GH6		Glicanase	Celulose	**2.959***	3.775	1.072
Q9UVS9_ASPNG	Y	GH7	CbhA	Celobiohidrolase	Celulose	**4.299***	**3.076***	**2.858***

Número de acesso	Peptídeo sinal	dbCAN	Nome da proteína	Descrição	Polissacarídeo em que atua	Quantificação relativa		
						Log_2 (BPT/BNT)	Log_2 (LIC/BNT)	Log_2 (BPT+LIC/BNT)
A2QAI7_ASPNC	Y	GH7, CBM1	CbhB	Celobiohidrolase	Celulose	**3.904***	**2.473***	**2.690***
G3Y866_ASPNA	Y	GH10	XynC	Endo-β-1,4-xilanase	Xilana	**3.813***	**2.639***	**3.763***
Q6QA21_9EURO	Y	GH11, CBM60	XynB	Endo-β-1,4-xilanase	Xilana	**3.303***	**2.852***	**3.653***
U6C3R6_ASPNG	Y	GH11	XynV	Endo-β-1,4-xilanase	Xilana	0.966	3.852	**2.325***
E3UN71_ASPNG	Y	GH11, CBM60	XynA	Endo-β-1,4-xilanase	Xilana	**1.387***	**2.016***	**2.007***
O74705_ASPNG	Y	GH12	EglA	Endoglicanase	Celulose	**4.710***	2.624	**4.016***
G3XRM3_ASPNA	Y	GH12	XgeA	Endo-1,4-β-glicanase específica para xiloglicana	Xiloglicano	1.025	**2.897***	**2.455***
G5D7B5_ASPNG	N	GH27, CBM13	AglA	α-galactosidase	Galactomanana	**2.134***	-0.166	**2.364***
A0A0U5AE32_ASPNG	Y	GH27	AglB	α-galactosidase	Galactomanana	1.370	-1.150	**2.716***
G3XQY4_ASPNA	Y	GH28	Pgal	Endo-poligalacturonase I	Pectina	5.988	-0.645	**6.924***
A2QK83_ASPNC	Y	GH28	XghA	Endo-xilogalacturonana hidrolase	Pectina	**3.906***	-0.945	**2.448***
A2QTU5_ASPNC	Y	GH31	AxlA	α-xilosidase A	Xiloglicano	**1.474***	**4.071***	**3.671***
B6HYI9_ASPNG	Y	GH35, CBM37	LacZ	β-galactosidase	Pectina	0.519	2.056	**2.494***
A0A1D8MQA0_ASPNG	Y	GH35, CBM67	LacB	β-galactosidase	Pectina	1.114	**2.328***	**3.323***
A0A1D8MQF6_ASPNG	Y	GH35, CBM67	LacB (isoforma)	β-galactosidase	Pectina	**2.897***	2.599	**4.262***
G3XM01_ASPNA	Y	GH36	AglC	α-galactosidase	Galactomanana	0.819	1.434	**2.101***
A0A0S2CVZ9_ASPNG	Y	GH43		Xilosidase:arabinofuranosidase	Xilana	**4.076***	**2.516***	**4.161***
G3XY38_ASPNA	Y	GH43		Proteína não caracterizada		**-1.920***	1.176	0.674
U6C191_ASPNG	N	GH43		Xilanase putativa	Xilana	-2.256	1.530	0.059
A2R511_ASPNC	Y	GH54, CBM42	AbfB	α-L-arabinofuranosidase	Xilana, pectina	**3.099***	**2.750***	**4.872***
A0A100I6G0_ASPNG	Y	GH62		α-L-arabinofuranosidase	Xilana, pectina	2.762	2.001	**2.524***
A2QFV9_ASPNC	Y	GH62	AxhA	α-L-arabinofuranosidase	Xilana, pectina	**2.893***	**1.861***	**2.865***
Q96WX9_ASPNG	Y	GH67	AguA	α-glicuronidase	Xilana	**2.522***	**2.699***	**3.253***

Número de acesso	Peptídeo sinal	dbCAN	Nome da proteína	Descrição	Polissacarídeo em que atua	Quantificação relativa		
						Log_2 (BPT/BNT)	Log_2 (LIC/BNT)	Log_2 (BPT+LIC/BNT)
Q8TFP1_ASPNG	Y	GH74, CBM1	EglC	Endoglicanase ativa sobre xiloglicana	Xiloglicano	**5.085***	**1.040***	**3.387***
G3XSS5_ASPNA	Y	GH78		α-L-rhamnosidase	Pectina	**-3.347***	0.197	0.259
G3XVM1_ASPNA	Y	CE1	AceA	Acetilxilana esterase A	Xilana	**4.921***	3.329	**4.718***
G3Y471_ASPNA	Y	CE3		Acetilxilana esterase	Xilana	**5.611***	1.631	6.521
A2QSY5_ASPNC	Y	CE5	FeaA	Provável Feruloil esterase A	Xilana, pectina	**4.697***	**4.201***	**5.153***
G3YAH8_ASPNA	Y	CE12	RgaeB	Rhamnogalacturonana acetil esterase	Pectina	**3.367***	3.874	**5.002***
G3XZI2_ASPNA	N	CE16		Proteína não caracterizada		**3.457***	**4.059***	**4.041***
A2QPC2_ASPNC	N	CE16	PaeB	Pectina acetil esterase putativa	Pectina	**4.150***	**2.663***	**3.697***
G3Y497_ASPNA	Y	CE16		Proteína não caracterizada		**3.633***	**1.488***	**3.647***
G3Y478_ASPNA	Y	PL1	PlyA	Pectato liase	Pectina	**2.252***	-0.217	**2.558***
A5ABH4_ASPNC	Y	PL4	RglB	Rhamnogalacturonato liase B	Pectina	0.757	**3.788***	**5.843***
Q8WZI8_ASPNG	Y	-	FaeB	Feruloil esterase B	Xilana, pectina	**4.896***	3.841	**5.270***
A2QFR3_ASPNC	Y	AA7		Glicooligossacarídeo oxidase		**2.022***	**1.351***	**2.170***
A2QW39_ASPNC	Y	AA7		Glicooligossacarídeo oxidase		**3.224***	**2.182***	**3.808***
A2QMJ7_ASPNC	Y	AA7		Glicooligossacarídeo oxidase		**4.772***	3.284	**7.253***
A2QHB3_ASPNC	Y	AA7		Glicooligossacarídeo oxidase		**3.113***	**2.482***	**2.947***
A0A117E071_ASPNG	Y	AA9, CBM1		LPMO	Celulose	**2.755***	**2.602***	**4.512***
G3XY89_ASPNA	Y	AA9, CBM1		LPMO	Celulose	1.634	1.533	**2.978***
G3XPC9_ASPNA	Y	AA9, CBM1		LPMO	Celulose	**4.962***	3.118	**5.772***
G3YHB3_ASPNA	Y	AA9		LPMO	Celulose	2.544	0.578	6.559
A2QZE1_ASPNC	Y	AA9		LPMO	Celulose	**1.558***	**1.367***	**1.989***
Outras enzimas ativas sobre carboidratos								

Número de acesso	Peptídeo sinal	dbCAN	Nome da proteína	Descrição	Polissacarídeo em que atua	Quantificação relativa		
						Log_2 (BPT/BNT)	Log_2 (LIC/BNT)	Log_2 (BPT+LIC/BNT)
A0A100INA6_ASPNG	Y	GH5	ExgA	Exo-β-1,3-glicanase	β-1,3-glicano	**2.954***	2.896	**4.826***
G3YCJ0_ASPNA	Y	GH13, CBM20		α-amilase A	Amido	-0.330	2.441	1.116
G3Y7U0_ASPNA	Y	GH15, CBM20	GlaA	Glicoamilase	Amido	0.498	**0.893***	**2.149***
A2Q8J5_ASPNC	Y	GH16		Proteína não caracterizada		**-3.815***	1.044	**-1.033***
G3XQP9_ASPNA	Y	GH16		Proteína não caracterizada	β-1,3-glicano	**3.395***	2.895	**3.804***
EGLC_ASPNC	Y	GH17		Endo-β-1,3-glicanase	β-1,3-glicano	**2.046***	1.693	**3.131***
A2QQS9_ASPNC	Y	GH17		Proteína não caracterizada	β-1,3-glicano	**2.780***	1.860	**3.920***
G3Y3I7_ASPNA	N	GH18		Proteína não caracterizada	Quitina	**-4.634***	**-0.905***	**-2.184***
G3Y2N5_ASPNA	Y	GH18, CBM18		Proteína não caracterizada	Quitina	3.889	**4.801***	**7.124***
A2QTI6_ASPNC	Y	GH20		β-hexosaminidase	Quitina	-0.177	0.575	**2.438***
G3Y837_ASPNA	Y	GH30		Proteína não caracterizada		**4.081***	**1.660***	**4.208***
A2QAC1_ASPNC	Y	GH31		Proteína não caracterizada		-1.353	0.599	-0.964
A0PCH8_ASPNG	Y	GH31		α-glicosidase		**-3.298***	-0.499	**-1.677***
Q76HP6_ASPNG	Y	GH32	InuE	Exo-inulinase	Inulina	**-1.854***	-1.235	**-0.553***
G3XRF8_ASPNA	Y	GH47	Mns1B	α-1,2-Mannosidase		**2.943***	2.061	**2.719***
A2QAR3_ASPNC	Y	GH55		Proteína não caracterizada		**1.124***	**1.932***	**2.610***
A2QF38_ASPNC	N	GH55		Proteína não caracterizada		4.288	1.000	**5.568***
A0A100INU2_ASPNG	Y	GH72, CBM43		1,3-β-glicanosiltransferase	β-1,3-glicano	**3.419***	**1.971***	**3.935***
G3Y439_ASPNA	Y	GH72		1,3-β-glicanosiltransferase	β-1,3-glicano	**3.397***	**2.152***	**4.187***
G3Y7R0_ASPNA	Y	GH72, CBM43		1,3-β-glicanosiltransferase	β-1,3-glicano	**-0.612***	-1.026	0.129
GUS79_ASPNC	Y	GH79		β-glicuronidase		**0.922***	**2.501***	**2.887***
A2Q852_ASPNC	Y	GH 81		Proteína não caracterizada		0.644	3.211	**3.178***
A2QJY8_ASPNC	Y	GH 125		exo-α-1,6-mannosidase		0.539	0.378	**1.527***
Q6ED33_ASPNG	Y	CE10	EstA	Carboxil-esterase		**4.017***	**2.217***	**3.563***
A2QE77_ASPNC	Y	CE10		Carboxil-esterase		**1.922***	2.038	**3.210***

Número de acesso	Peptídeo sinal	dbCAN	Nome da proteína	Descrição	Polissacarídeo em que atua	Quantificação relativa		
						Log_2 (BPT/BNT)	Log_2 (LIC/BNT)	Log_2 (BPT+LIC/BNT)
A0A100IAD1_ASPNG	N	CE10		Aminopeptidase C		-5.474	0.427	1.302
G3Y6X4_ASPNA	Y	CE10		Carboxil-esterase		1.112	**1.966***	**2.863***
Proteases e peptidases								
A2QR21_ASPNC	Y	-		Endoprotease		-8.250	-8.454	-9.411
G3Y6Y3_ASPNA	Y	-		Carboxipeptidase		**2.600***	**3.571***	**4.878***
G3Y8B3_ASPNA	Y	-		Peptídeo hidrolase		0.030	0.310	**1.009***
G3YB87_ASPNA	N	-		Protease aspártica		3.734	**-1.203***	**6.048***
A2QMZ7_ASPNC	Y	-		Serina proteinase		-3.415	-0.646	-1.670
G3XLQ6_ASPNA	N	-		Aminopeptidase		-0.686	0.123	**0.643***
G3Y926_ASPNA	N	-		Aminopeptidase		**2.520***	3.061	1.416
Outras proteínas								
G3XR06_ASPNA	Y	-		Catalase		0.954	**4.051***	4.075
G3XSP9_ASPNA	Y	-		Proteína não caracterizada		0.277	0.729	**1.522***
A2QBC2_ASPNC	Y	-		Proteína não caracterizada		**1.123***	0.048	**1.877***
A2QPX1_ASPNC	N	-		Proteína não caracterizada		2.361	2.993	**4.021***
A0A100INJ0_ASPNG	N	-		Proteína não caracterizada		-0.983	-5.539	-0.699
A2QF94_ASPNC	Y	-		Proteína não caracterizada		-2.112	0.512	-0.920
G3Y2G0_ASPNA	Y	-		Proteína não caracterizada		**2.182***	-5.354	**1.995***
G3XZA0_ASPNA	N	-		Proteína não caracterizada		2.413	0.752	8.825
G3XTE9_ASPNA	N	-		Proteína não caracterizada		-0.503	**3.905***	-0.478
G3XNW4_ASPNA	Y	-		Proteína não caracterizada		**2.465***	3.734	**3.999***
G3XZT4_ASPNA	N	-		Proteína não caracterizada		-2.060	0.866	-6.133
G3XPF7_ASPNA	Y	-		Proteína não caracterizada		0.333	0.906	**4.207***
G3XPQ3_ASPNA	Y	-		Proteína não caracterizada		2.251	1.835	**4.270***
A2QWT0_ASPNC	Y	-		Proteína não caracterizada		2.193	2.883	3.613

Número de acesso	Peptídeo sinal	dbCAN	Nome da proteína	Descrição	Polissacarídeo em que atua	Quantificação relativa		
						Log_2 (BPT/BNT)	Log_2 (LIC/BNT)	Log_2 (BPT+LIC/BNT)
A2QM91_ASPNC	Y	-		Proteína não caracterizada		**2.557***	-10.726	**3.810***
A2QAF5_ASPNC	Y	-		Proteína não caracterizada		**7.299***	-0.200	**10.494***
G3XQT2_ASPNA	N	-		Proteína não caracterizada		**-5.006***	**-2.519***	**-2.764***
G3Y1S6_ASPNA	N	-		Proteína não caracterizada		**-2.770***	**-1.478***	**-1.635***
A2R1I0_ASPNC	Y	-		Proteína não caracterizada		**-2.889***	**-2.426***	**-1.512***
A2R7J0_ASPNC	Y	-		Proteína não caracterizada		1.542	2.991	**3.081***
A2QJT0_ASPNC	Y	-		Proteína não caracterizada		**4.520***	0.800	**3.753***
G3YBC9_ASPNA	Y	-		Proteína não caracterizada		**2.146***	1.058	**2.468***
A2QIQ4_ASPNC	N	-		Proteína não caracterizada		-1.084	7.331	**3.924***
A2R2Z3_ASPNC	Y	-		Proteína não caracterizada		**2.872***	2.081	**3.065***
A2QPE5_ASPNC	Y	-		Proteína não caracterizada		-3.403	**3.382***	1.470
G3Y8K0_ASPNA	N	-		Proteína não caracterizada		-3.540	0.045	-1.916
A2QM98_ASPNC	Y	-		Proteína não caracterizada		1.577	0.299	**5.433***
A5AC00_ASPNC	N	-		Proteína não caracterizada		3.581	-3.030	**3.902***
A2QT26_ASPNC	Y	-		Proteína não caracterizada		1.792	5.690	3.734
A2QWX4_ASPNC	N	-		Proteína não caracterizada		-4.716	-4.953	-5.074
A2R1K1_ASPNC	Y	-		Proteína não caracterizada		3.845	4.369	2.690
G3XSH5_ASPNA	N	-		Proteína não caracterizada		-1.533	3.056	-2.226
G3Y6Q6_ASPNA	N	-		Proteína não caracterizada		3.622	0.800	7.627
G3YGH1_ASPNA	N	-		Proteína não caracterizada		-6.279	-0.779	-4.814
G3YGD8_ASPNA	Y	-		Proteína não caracterizada		-1.459	-1.054	5.185
A0A100IRM3_ASPNG	Y	-	GtaA	Glutaminase		-12.406	-3.000	-8.104
Q5DR86_ASPNG	Y	-	CwpA	Proteína de parede celular		-0.021	**-3.473***	**-0.885***
A0A100IJJ2_ASPNG	N	-		Aldeído redutase 1		-2.103	**6.239***	2.796
Q49LQ8_ASPNG	N	-	ActA	Actina (Fragmento)		**4.845***	0.408	5.243

Número de acesso	Peptídeo sinal	dbCAN	Nome da proteína	Descrição	Polissacarídeo em que atua	Quantificação relativa		
						Log_2 (BPT/BNT)	**Log_2 (LIC/BNT)**	**Log_2 (BPT+LIC/BNT)**
A0A117DX26_ASPNG	Y	-	Ecm3	Proteína de organização da parede celular		**6.417***	**3.658***	**6.895***
A2R2S8_ASPNC	Y	-	PhiA	Proteína de parede celular		**6.183***	**4.645***	**8.047***
A2RAZ0_ASPNC	N	-		Fator de elongação 1-alfa		2.955	-4.022	**2.615***
A2QJA6_ASPNC	N	-		Aspartato aminotransferase		-1.363	3.746	**-4.798***
A0A117E041_ASPNG	N	-		Ribose 5-fosfato isomerase A		**1.367***	-0.115	**2.063***
G3YG20_ASPNA	N	-		Adenosilhomocisteinase		**2.442***	2.348	-0.062
G3YCF6_ASPNA	N	-		Lactoilglutationa liase		-4.630	-1.565	-4.787
A0A100I978_ASPNG	N	-		Proteína rica em serina		-4.197	-1.016	-3.188
A0A124BWL8_ASPNG	N	-		Proteína de morfogênese celular		**2.937***	**3.009***	**4.067***
G3XRD3_ASPNA	N	-		Fator de iniciação de transcrição 5A		0.907	**1.488***	**2.526***
G3XXE5_ASPNA	N	-		Formato desidrogenase		**3.170***	1.730	**2.817***
A0A117E3G2_ASPNG	Y	-		Proteína da via Tat		**4.746***	-0.782	**2.734***
A0A100IT82_ASPNG	Y	-		Proteína não caracterizada		**5.762***	3.377	**6.157***
A5AAV2_ASPNC	N	-		Fosfatase alcalina		1.688	-2.340	**3.242***
P41751_ASPNG	N	-	AldA	Aldeído desidrogenase		-2.128	**2.942***	1.704
A0A100ILW0_ASPNG	N	-		Glicose-6-fosfato isomerase		1.841	2.276	3.699
A0A100INU6_ASPNG	N	-		NADH-ubiquinona oxidoredutase		4.838	8.622	3.212
A2QUK3_ASPNC	Y	-	Sox	Sulphidril oxidase		**3.665***	**2.583***	**3.021***

No total, 153 proteínas foram identificadas em comum nos secretomas de *A. niger* nas 4 diferentes condições de cultivo. Um total de 89 enzimas ativas sobre carboidratos foram identificadas, dentre as quais 63 são enzimas relacionadas com a degradação de polissacarídeos estruturais presentes na parede celular vegetal (holocelulases) (Figura 7), 56 delas contendo peptídeo sinal para secreção. Além das 56 holocelulases secretadas, outras 56 proteínas com peptídeo sinal para secreção (incluindo outras enzimas ativas sobre carboidratos, enzimas com outras atividades e proteínas desconhecidas) foram identificadas, totalizando 112 proteínas contendo sinal para secreção. As 41 proteínas sem peptídeo sinal identificadas podem corresponder a proteínas intracelulares extravasadas para o meio extracelular devido à morte celular, devido à lise celular causada pela extração dos secretomas por filtração a vácuo (Adav *et al.*, 2012), ou podem corresponder a proteínas secretadas por via de secreção alternativa àquelas preditas por SignalP e Phobius (Ribeiro *et al.*, 2012).

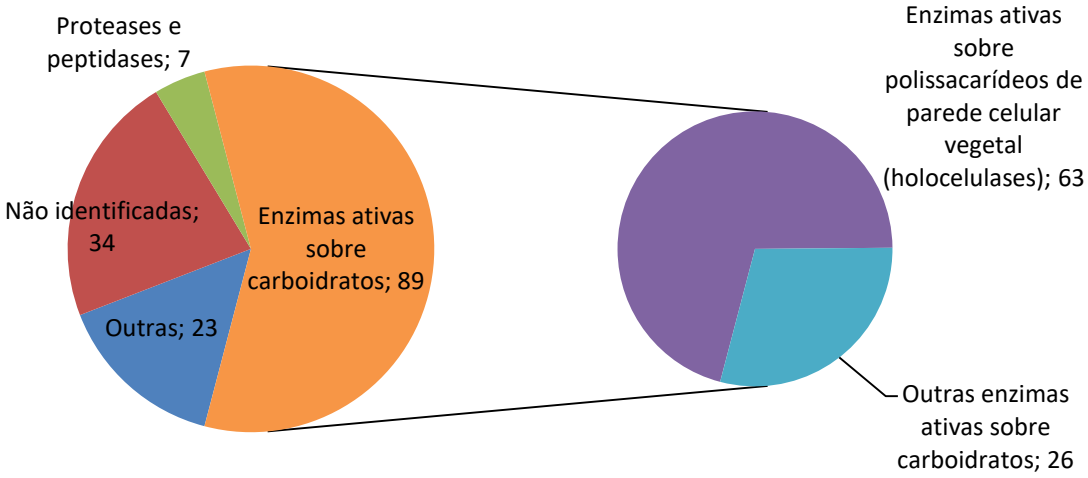


Figura 7. Classificação das proteínas identificadas nos secretomas de *A. niger* produzidos nas diferentes condições de cultivo.

As holocelulases corresponderam a 50 % das proteínas com peptídeo sinal, evidenciando o grande aporte de energia empregado por *A. niger* para degradar os polissacarídeos do substrato lignocelulósico. As holocelulases identificadas incluíram enzimas envolvidas na degradação dos três grandes grupos de polissacarídeos da parede celular vegetal, sendo eles celulose, hemiceluloses (glicuronoarabinoxilana acetilada, galactomanana, xiloglicano) e pectina. Uma parte das holocelulases identificadas, apesar de serem reconhecidas como holocelulases devido à presença de domínios conservados, não são ainda caracterizadas bioquimicamente e são potenciais alvos para futuros estudos. Um percentual de 17% das proteínas identificadas com peptídeo sinal

não são caracterizadas e não apresentam domínio conservado de enzimas ativas sobre carboidratos, podendo potencialmente corresponder a proteínas envolvidas na degradação da parede celular vegetal cujo papel é ainda desconhecido. Estas enzimas também são potenciais alvos para futuros estudos.

Diversas holocelulases identificadas nos secretomas (Tabela 2) apresentam estrutura modular, ou seja, são compostas por um módulo de ligação a carboidrato (CBM) anexado a um módulo catalítico (GH ou AA). Como exemplo, sete enzimas identificadas, dentre elas endoglicanases (GH5 e GH74), celobiohidrolases (GH6 e GH7) e LPMOs (AA9), apresentam um módulo de ligação a carboidrato pertencente à família CBM1, cujos membros são conhecidos por exibirem alta afinidade por celulose. Duas xilanases identificadas (XynA e XynB) apresentam CBM pertencente à família 60, exclusivamente encontrados em xilanases e que exibem função de ligação à xilana (Tabela 2). Os CBMs apresentam importante papel na degradação de substratos lignocelulósicos, pois atuam no reconhecimento do substrato alvo, na aproximação da enzima ao substrato, na concentração das enzimas sobre a superfície do substrato e, em alguns casos, também atuam na ruptura não catalítica da celulose cristalina, promovendo amorfogênese da celulose (Boraston *et al.*, 2004; Arantes e Saddler, 2010).

As enzimas ativas sobre carboidratos que não foram classificadas como holocelulases incluem enzimas ativas sobre polissacarídeos e oligossacarídeos não estruturais (amilases e inulinases), enzimas ativas sobre polissacarídeos da parede celular fúngica (quitinases e β-1,3-glicanases), e enzimas envolvidas na modificação de glicosilação de proteínas (α-manosidases).

Considerando a condição BPT, 74 proteínas tiveram a produção significativamente aumentada (teste T de Student, $p < 0,05$) em comparação com a condição BNT, dentre elas 54 enzimas ativas sobre carboidratos (sendo 42 holocelulases). Em contrapartida, 11 proteínas foram detectadas em níveis menores àqueles observados em BNT, dentre elas 8 enzimas ativas sobre carboidratos (sendo 3 holocelulases). Quando o licor foi utilizado como fonte de carbono (condição LIC), 46 proteínas foram detectadas em maior quantidade (37 enzimas ativas sobre carboidratos, sendo 28 holocelulases) e 7 em menor quantidade (2 enzimas ativas sobre carboidratos, sendo 1 holocelulase). Na condição BPT+LIC, 99 proteínas foram positivamente reguladas (68 enzimas ativas sobre carboidratos, sendo 49 holocelulases) e 10 negativamente reguladas (5 enzimas

ativas sobre carboidratos, sendo 1 holocelulase). De modo geral, a produção de holocelulases por *A. niger* foi positivamente influenciada pelo pré-tratamento hidrotérmico aplicado sobre o bagaço utilizado como fonte de carbono, considerando tanto a fração sólida quanto a líquida (licor). As figuras 8, 9, 10 e 11 mostram a abundância normalizada das holoceluloses secretadas que tiveram diferença de abundância significativamente diferente (teste T de Student, $p < 0,05$) em pelo menos uma das condições de cultivo em comparação à condição controle (BNT).

3.2.1. Enzimas celulolíticas

Dentre as enzimas celulolíticas secretadas (contendo peptídeo sinal) em comum nas diferentes condições de cultivo, foram identificadas quatro β-glicosidases das famílias GH1 e GH3, três endo-glicanases da família GH5, três exo-glicanases (celobiohidrolases) das famílias GH6 e GH7, duas glicanases (sem informação sobre mecanismo endo ou exo) da família GH6 e cinco monooxigenases líticas de polissacarídeos cobre-dependentes (LPMOs) pertencentes à família AA9 (Tabela 2). Estes 4 grupos de enzimas atuam em concerto para a degradação completa da celulose até glicose através de mecanismos hidrolíticos (enzimas das famílias de glicosil hidrolases - GH) ou oxidativos (enzimas das famílias de atividades auxiliares - AA). As β-glicosidases atuam na degradação de celooligossacarídeos e principalmente celobiose, produtos finais da ação hidrolítica conjunta de endo- e exo-glicanases sobre a celulose, enquanto que as LPMOs promovem clivagens nas cadeias de celulose por mecanismo oxidativo, gerando novos sítios para atuação de enzimas hidrolíticas (Monclaro e Ferreira Filho, 2017). As LPMOs de *A. niger* identificadas, entretanto, não são caracterizadas bioquimicamente e podem ser ativas também sobre hemiceluloses, como já foi descrito para outras enzimas fúngicas da família AA9 (Agger *et al.*, 2014; Kojima *et al.*, 2016).

A enzima celobiose desidrogenase (CDH) ou outras oxiredutases (por exemplo, glicose-metanol-colina oxiredutases) da família AA3 não foram detectadas nos secretomas. Estas enzimas são consideradas parceiras das AA9, atuando como doadoras de elétrons para o núcleo de cobre das LPMOs. Apesar da presença das CDHs (ou outras AA3) aumentar a atividade das LPMOs, a atividade destas não é dependente daquelas, uma vez que os elétrons necessários para a atividade das AA9 podem ser providos por outras moléculas presentes no sistema fungo-biomassa, como compostos

fenólicos advindos da lignina ou secretados pelo próprio fungo (Kracher *et al.*, 2016; Monclaro e Ferreira Filho, 2017).

β-glicosidases intracelulares também foram identificadas. Estas enzimas podem ser responsáveis pela degradação intracelular de celobiose. Apesar de ainda não haver registros na literatura sobre a importação de celobiose em *A. niger*, a espécie *A. nidulans* é capaz de importar celobiose através de um transportador específico chamado CltA (Reis *et al.*, 2016).

Quatro enzimas não caracterizadas da família AA7 com peptídeo sinal também foram identificadas nos secretomas de *A. niger*. Os membros desta família correspondem a glicooligossacarídeo-oxidases (www.cazy.org), enzimas que contém grupo FAD e que atuam na despolimerização oxidativa de oligossacarídeos de glicose conectados por ligações do tipo α(1-4) ou β(1-4). Desta forma, estas enzimas possivelmente atuam sobre celooligossacarídeos gerados pela ação de celulases. No trabalho de Borin *et al.* (2017), a expressão de várias enzimas AA7 por *A. niger* N402 cultivado na presença de bagaço de cana-de-açúcar pré-tratado por explosão a vapor também foi observada por abordagem transcriptômica, e em níveis muito superiores àqueles observados em cultivos utilizando frutose como fonte de carbono. Estes resultados indicam que estas enzimas oxidativas possivelmente desempenham um importante papel na degradação do bagaço de cana-de-açúcar.

A figura 8 mostra a abundância normalizada das enzimas celulolíticas que tiveram diferença de abundância significativa (teste T de Student, $p < 0,05$) em pelo menos uma das condições de cultivo em comparação à condição controle (BNT). Dentre elas, duas β-glicosidases (GH3) tiveram produção significativamente diferente nas condições BPT, LIC e BPT+LIC em comparação à condição BNT (Figura 8). A β-glicosidase Bgl (GH3) teve a produção significativamente aumentada na presença de biomassa pré-tratada, principalmente na condição BPT+LIC. Tanto os sólidos tratados (BPT) quanto o licor (LIC) promoveram um aumento na produção da enzima Bgl, e a presença de ambos (BPT+LIC) teve efeito indutor somatório. Já a β-glicosidase A2R2S3 (GH3) teve a produção fortemente reduzida na presença dos sólidos ou licor gerados no tratamento do bagaço, indicando que a presença de algum polissacarídeo ou molécula indutora presente principalmente na biomassa *in natura* é necessária para a indução de altos níveis desta enzima.

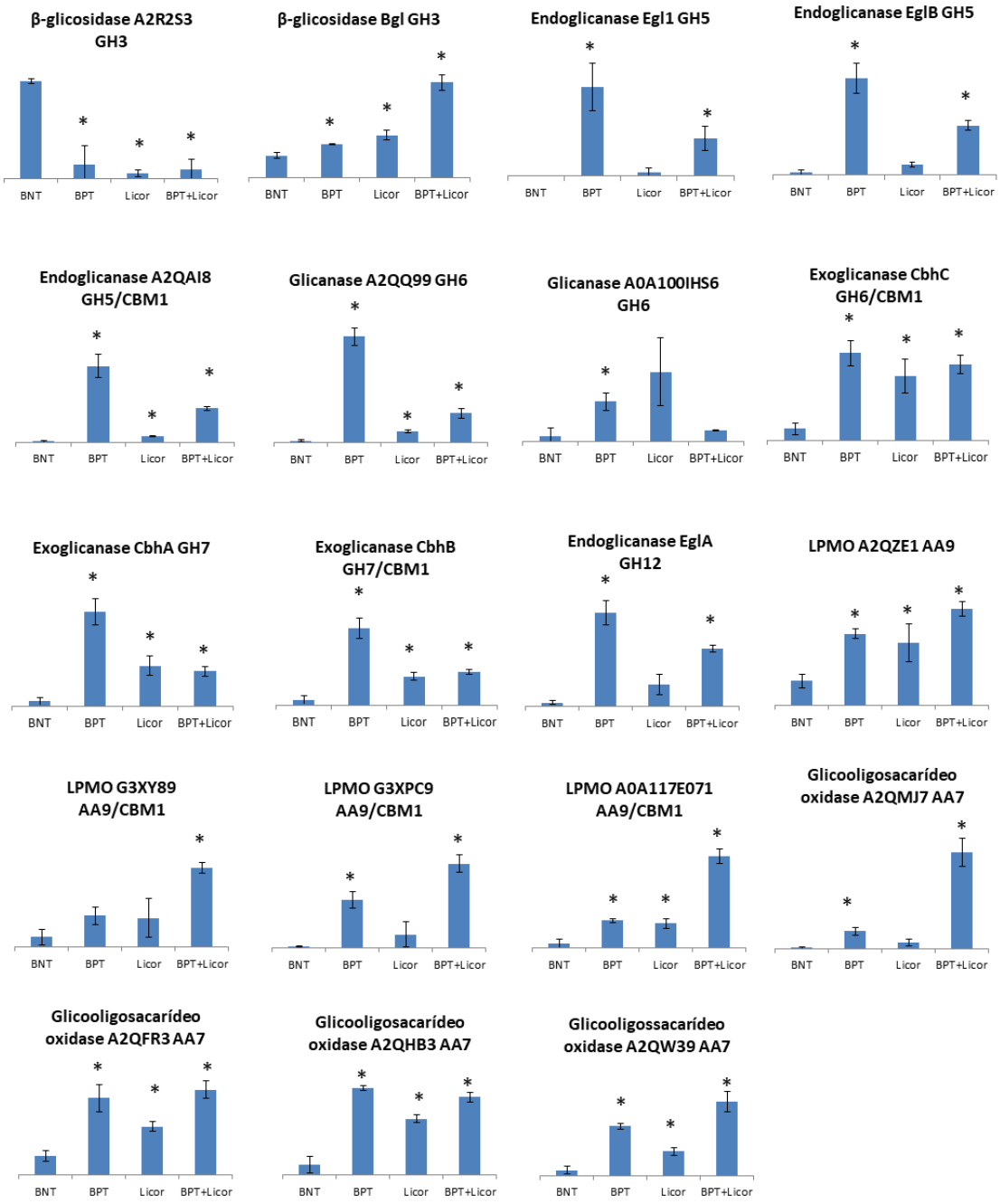


Figura 8. Abundância relativa normalizada de enzimas celulolíticas presentes nos secretomas de *A. niger* cultivado na presença de bagaço de cana-de-açúcar *in natura* (BNT), bagaço tratado (BPT), licor (LIC) ou bagaço tratado + licor (BPT+Licor). Barras marcadas com asterisco (*) são abundâncias significativamente diferentes (teste T de Student, $p < 0,05$) em comparação com a condição BNT. Eixos das ordenadas (y) correspondem a valores de abundância normalizada e foram omitidos por representarem unidade arbitrária.

A abundância das endoglicanases Eg1 (GH5), EglB (GH5), A2QA18 (GH5/CBM1) e EglA (GH12) e da glicanase A2QQ99 (GH6, sem mecanismo de ação descrito) seguiram um padrão nas diferentes condições de cultivo, com abundâncias crescentes nas condições BNT < LIC < BPT+LIC < BPT (Figura 8). A similaridade no padrão de

abundância indica que a expressão destas enzimas é possivelmente regulada pelo mesmo mecanismo e em resposta aos mesmos fatores indutores. Estas celulases foram induzidas principalmente pela fração dos sólidos tratados (BPT). O potencial indutor dos sólidos tratados para estas enzimas, entretanto, foi reduzido na presença do licor (condição BPT+LIC). O licor como única fonte de carbono (condição LIC) induziu baixos níveis destas enzimas, mas em alguns casos (enzimas A2QA18 e A2QQ99) resultou em abundância significativamente superior à indução causada pelo bagaço *in natura* (BNT). O maior acesso do micélio e das enzimas secretadas por *A. niger* à celulose remanescente nos sólidos tratados pode ter causado maior liberação de moléculas responsáveis pela indução destas celulases (como celobiose ou celooligossacarídeos). A menor indução destas celulases pelo licor (em comparação às condições BPT e BPT+LIC) pode ser devido à ausência de celulose, enquanto que o efeito negativo da presença do licor na condição BPT+LIC pode ser resultado da presença de monossacarídeos em concentrações inibitórias (por repressão catabólica) ou da presença de inibidores fenólicos e furfurais.

Não só o maior acesso à celulose, mas também o maior acesso do fungo e enzimas secretadas à hemicelulose e à pectina podem ter contribuído para a maior indução de celulases nas condições BPT, LIC e BPT+LIC. Isto porque genes de diversas β-glicosidases, endoglicanases e exoglicanases são positivamente regulados na presença de xilose e arabinose advindos da hemicelulose e pectina através de mecanismo intermediado pelos ativadores de transcrição XlnR e AraR sensíveis à presença destes monossacarídeos (Souza *et al.*, 2013).

As três exoglicanases identificadas nos secretomas (CbhA GH7, CbhB GH7/CBM1 e CbhC GH6/CBM1) foram significativamente mais abundantes nas condições BPT, LIC e BPT+LIC em comparação a BNT (Figura 8). As enzimas CbhA e CbhB tiveram padrão de abundância semelhantes, sendo mais expressas na presença dos sólidos tratados. O licor foi capaz de induzir mais que o bagaço *in natura*, porém em níveis inferiores a BPT, causando também uma diminuição do potencial indutor dos sólidos na condição BPT+LIC, como mencionado para as endoglicanases. A enzima CbhC foi expressa em níveis semelhantes nas condições BPT, LIC e BPT+LIC.

As LPMOs da família AA9 (A2QZE1, G3XY89, G3XPC9 e A0A117E071) tiveram maior abundância na condição BPT+LIC, indicando que o uso conjunto de ambas as

frações (sólida e líquida) resultantes do tratamento do bagaço foi necessário para a indução de altos níveis destas proteínas.

Dentre as holocelulases produzidas na condição BPT, as celulases Eg1 (GH5), A2QA18 (GH5/CBM1) e A2QQ99 (GH6) foram as enzimas mais positivamente afetadas pelo tratamento hidrotérmico do bagaço de cana-de-açúcar, com níveis de abundância aumentados em 68 – 2394 vezes (Tabela 2). Isto indica que o pré-tratamento hidrotérmico aplicado sobre o substrato causou uma mudança no arsenal enzimático secretado por *A. niger,* priorizando a hidrólise e utilização da celulose. Um resultado similar foi observado por Daly *et al.* (2017). Estes autores investigaram a expressão de holocelulases por *A. niger* N402 a nível transcriptômico utilizando como fontes de carbono palha de trigo *in natura* (passada em moinho de facas) ou submetida a pré-tratamento hidrotérmico. Diversas enzimas celulolíticas (endoglicanases, celobiohidrolases, β-glicosidases e LPMOs) de *A. niger* N402 tiveram a expressão gênica aumentada na presença de palha de trigo pré-tratada (Daly *et al.*, 2017). Um fenômeno semelhante foi observado quando *P. echinulatum* foi cultivado na presença de bagaço de cana-de-açúcar integral ou submetido a pré-tratamentos hidrotérmicos (Ribeiro *et al.*, 2012). Através de análise proteômica qualitativa, Ribeiro *et al.* (2012) observaram que o uso de bagaço pré-tratado hidrotermicamente como fonte de carbono induziu a produção de um número maior de enzimas celulolíticas por *P. echinulatum* em comparação ao bagaço integral, secretando um arsenal enzimático orientado para a hidrólise de celulose. A menor complexidade do substrato tratado, com menor teor de hemicelulose e a maior exposição da celulose foram consideradas possíveis causas para esse fenômeno.

3.2.2. Hemicelulases

Diversas enzimas ativas sobre glicuronoarabinoxilana acetilada (principal hemicelulose presente no bagaço de cana-de-açúcar) com peptídeo sinal foram identificadas nos secretomas, sendo elas: quatro endo-β-1,4-xilanases (GH10 e GH11), duas β-xilosidases (GH3), uma xilosidase:arabinofuranosidase bifuncional (GH43), três arabinofuranosidases (GH62 e GH54/CBM42), uma α-glicuronidase (GH 67), duas acetilxilana esterases (CE1 e CE3) e duas feruloil esterases (uma delas pertencente à família CE5). A enzima feruloil esterase B (FaeB), apesar de não apresentar domínio conservado de enzimas ativas sobre carboidrato, tem ação hidrolítica sobre

arabinoxilana e pectina experimentalmente comprovada (De Vries *et al.*, 2002) e por isso foi incluída no grupo de holocelulases. As enzimas identificadas atuam em sinergismo na hidrólise da cadeia principal da glicuronoarabinoxilana e na remoção dos principais substituintes laterais, sendo eles grupos acetil, arabinofuranose, ácido-4-O-metil-glicorônico e ácido ferúlico. A Figura 9 mostra a abundância normalizada das enzimas xilanolíticas que tiveram diferença de abundância significativa (teste T de Student, $p < 0,05$) em pelo menos uma das condições de cultivo em comparação à condição controle (BNT).

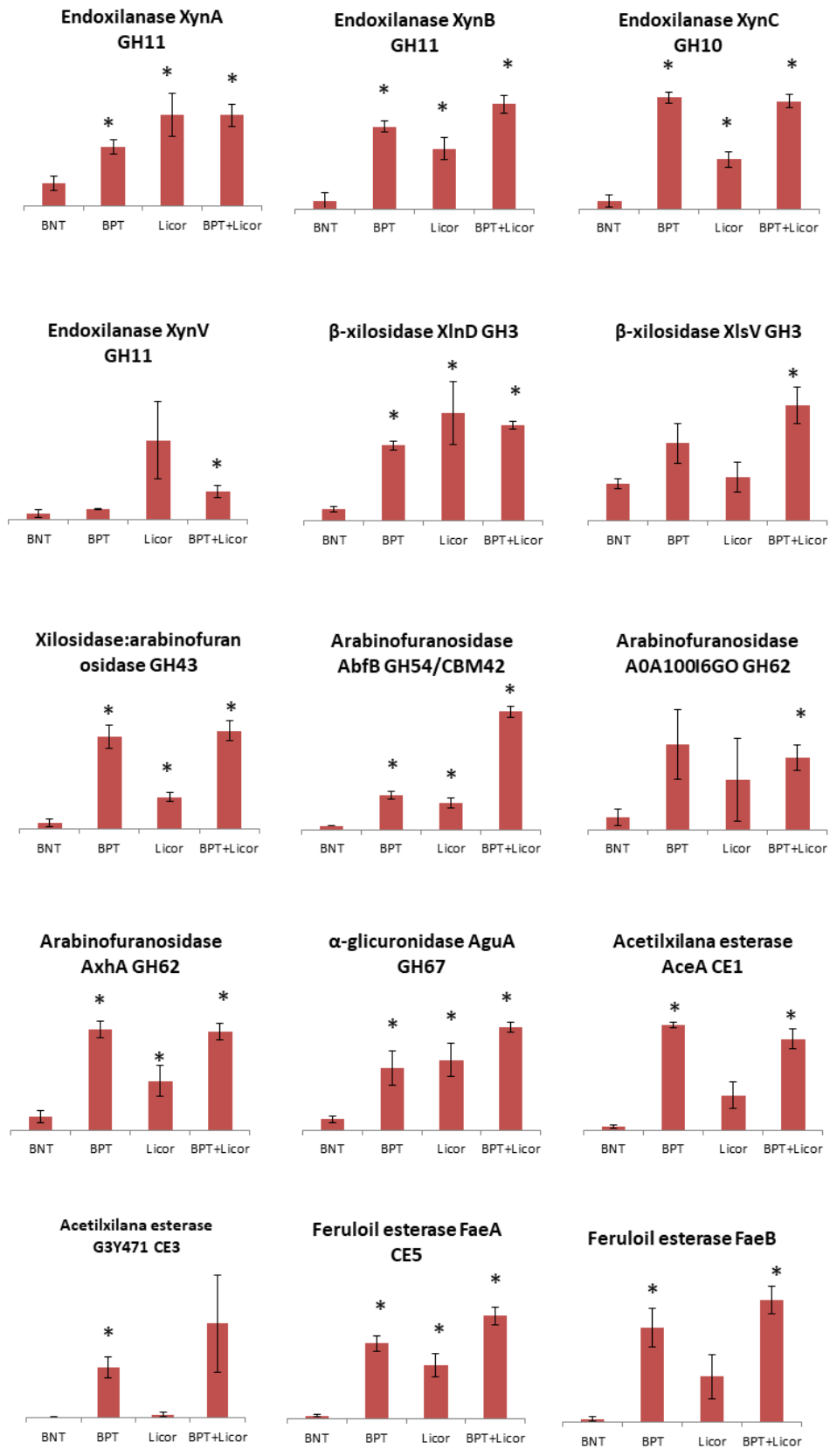


Figura 9. Abundância relativa normalizada de enzimas xilanolíticas presentes nos secretomas de *A. niger* cultivado na presença de bagaço de cana-de-açúcar *in natura* (BNT), bagaço tratado (BPT), licor ou bagaço tratado + licor (BPT+Licor). Barras marcadas com asterisco (*) são abundâncias significativamente diferentes (teste T de Student, $p < 0,05$) em comparação com bagaço de cana *in natura* (BNT). Eixos das ordenadas (y) correspondem a valores de abundância normalizada e foram omitidos por representarem unidade arbitrária.

As principais enzimas necessárias para a desconstrução da glicuronoarabinoxilana acetilada (xilanases, xilosidases, arabinofuranosidases, glicuronidases, acetilxilana esterases e feruloil esterases) foram mais abundantes nas condições BPT, LIC e/ou BPT+LIC em comparação à condição BNT, corroborando o efeito positivo do pré-tratamento hidrotérmico brando do substrato para a produção de hemicelulases.

De modo geral, as enzimas xilanolíticas foram mais abundantes nas condições de cultivo contendo sólidos tratados com substrato (condições BPT e BPT+LIC). Como discutido anteriormente, uma parte da arabinoxilana ficou retida nos sólidos tratados (retenção de 57,8 % de xilose e 25 % de arabinose) devido à baixa severidade do tratamento hidrotérmico, sendo este um importante fator para alta indução destas enzimas pelos sólidos.

Estes resultados diferem daqueles observados por Daly *et al.* (2017), que investigou a produção de holocelulases por *A. niger* N402 cultivado em palha de trigo *in natura* e pré-tratada hidrotermicamente. A palha de trigo é um resíduo agroindustrial semelhante ao bagaço de cana-de-açúcar em termos de composição química, contendo teores similares de celulose, arabinoxilana, pectina e lignina. No trabalho de Daly *et al.* (2017), o pré-tratamento hidrotérmico aplicado sobre a biomassa foi drasticamente mais severo (200 °C por 2 horas) do que o aplicado no presente trabalho (170 °C por 30 min), causando um impacto distinto sobre a produção de hemicelulases. Os teores de xilose, arabinose e galactose (indicativos da presença de hemicelulose e pectina) na palha de trigo foram drasticamente reduzidos pelo pré-tratamento severo (redução na ordem de 6 a 10 vezes). Apesar de dezenas de hemicelulases terem sido expressas por *A. niger* N402 na presença tanto de biomassa *in natura* quanto de biomassa pré-tratada, os níveis de expressão das hemicelulases na presença de palha de trigo pré-tratada foram, de modo geral, iguais ou inferiores àqueles observados na presença de biomassa *in natura.* Este resultado evidencia a importância da severidade do tratamento hidrotérmico aplicado ao substrato lignocelulósico indutor de holocelulases. Em contraste ao tratamento severo, a aplicação de um tratamento brando sobre o bagaço de cana-de-açúcar foi capaz de aumentar a secreção não só de enzimas celulolíticas, como também de hemicelulases por *A. niger*.

Apesar da abundância da maioria das enzimas xilanolíticas terem atingido os maiores níveis na presença dos sólidos tratados (condições BPT ou BPT+LIC), boa parte destas enzimas foi positivamente regulada no cultivo em licor (condição LIC) (Figura 9). Dentre as enzimas significativamente mais expressas na condição LIC estão: três endoxilanases (XynA, XynB e XynC), β-xilosidase XlnD, a enzima bifuncional xilosidase:arabinofuranosidase, duas arabinofuranosidases (AbfB e AxhA), α-glicuronidase (AguA) e feruloil esterase (FaeA). Isso reflete a composição do licor, rico em xilooligossacarídeos ramificados com arabinose e acido ferúlico. Apesar de o licor apresentar xilooligossacarídeos acetilados em sua composição (mostrado no capítulo III), a indução das acetilxilana esterases AceA (CE1) e G3Y471 (CE3) na condição LIC não foi significativamente maior do que os níveis detectados na condição BNT.

Apesar dos baixos teores de galactomanana presentes no bagaço de cana-de-açúcar, enzimas que atuam na hidrólise desta hemicelulose foram detectadas nos secretomas de *A. niger*, incluindo três α-galactosidases (GH3, GH27 e GH 36) e uma endo-β-1,4-mananase (GH5). β-manosidases não foram detectadas na análise proteômica, corroborando a ausência de atividade de β-manosidase nos secretomas (Tabela 1). As enzimas mananolíticas foram significativamente mais abundantes nas condições BPT e/ou BPT+LIC (Figura 10).

Duas endo-1,4-β-glicanases específicas para xiloglicano (GH12 e GH74/CBM1) e uma α-xilosidase (GH31) também foram identificadas. Estas enzimas são responsáveis pela hidrólise da cadeia principal e das ramificações de xilose presentes no xiloglicano, respectivamente. A figura 10 mostra a abundância relativa destas enzimas nas diferentes condições de cultivo.

No trabalho de Daly *et al.* (2017) citado acima, a xiloglicanase EglC (GH74/CBM1) foi a única hemicelulase que teve a expressão aumentada de forma acentuada como consequência do tratamento hidrotérmico severo da palha de trigo. De acordo com De Souza *et al.* (2013), o xiloglicano presente nas paredes celulares do colmo de cana-de-açúcar é intimamente aderido às fibras de celulose e permanece na parede celular mesmo após extrações severas com álcali. Estas informações, em conjunto com os resultados obtidos, são um indicativo de que tratamentos hidrotérmicos brandos ou severos são capazes de expor o xiloglicano presente na biomassa sem ocasionar a sua remoção completa, permitindo o aumento na produção de xiloglicanases por *A. niger*.

A enzima A2Q8J5 (família GH16) apresenta função molecular predita de β-1,3 e β-1,4-glicanase, de acordo com Gene Ontology. Esta enzima pode, portanto, corresponder a uma lichenase. As liquenases (ou β-1,3-1,4-glicanases) atuam na hidrólise de β-glicanos de ligações mistas (β-1,3-1,4-glicanos), classe de polissacarídeos que correspondem a um importante componente hemicelulósico presente na parede das células da cana-de-açúcar (De Souza *et al.*, 2013; Costa *et al.*, 2016). A abundância da enzima A2Q8J5 foi significativamente menor nas condições BPT e BPT+LIC em relação à condição BNT (Figura 10). O β-glicano de ligações mistas presente na cana-de-açúcar é altamente solúvel (De Souza *et al.*, 2013), sendo possivelmente solubilizado de forma extensiva durante o tratamento hidrotérmico do bagaço de cana-de-açúcar. A indução desta enzima foi superior na condição LIC, mas não de forma estatisticamente significativa (Figura 10).

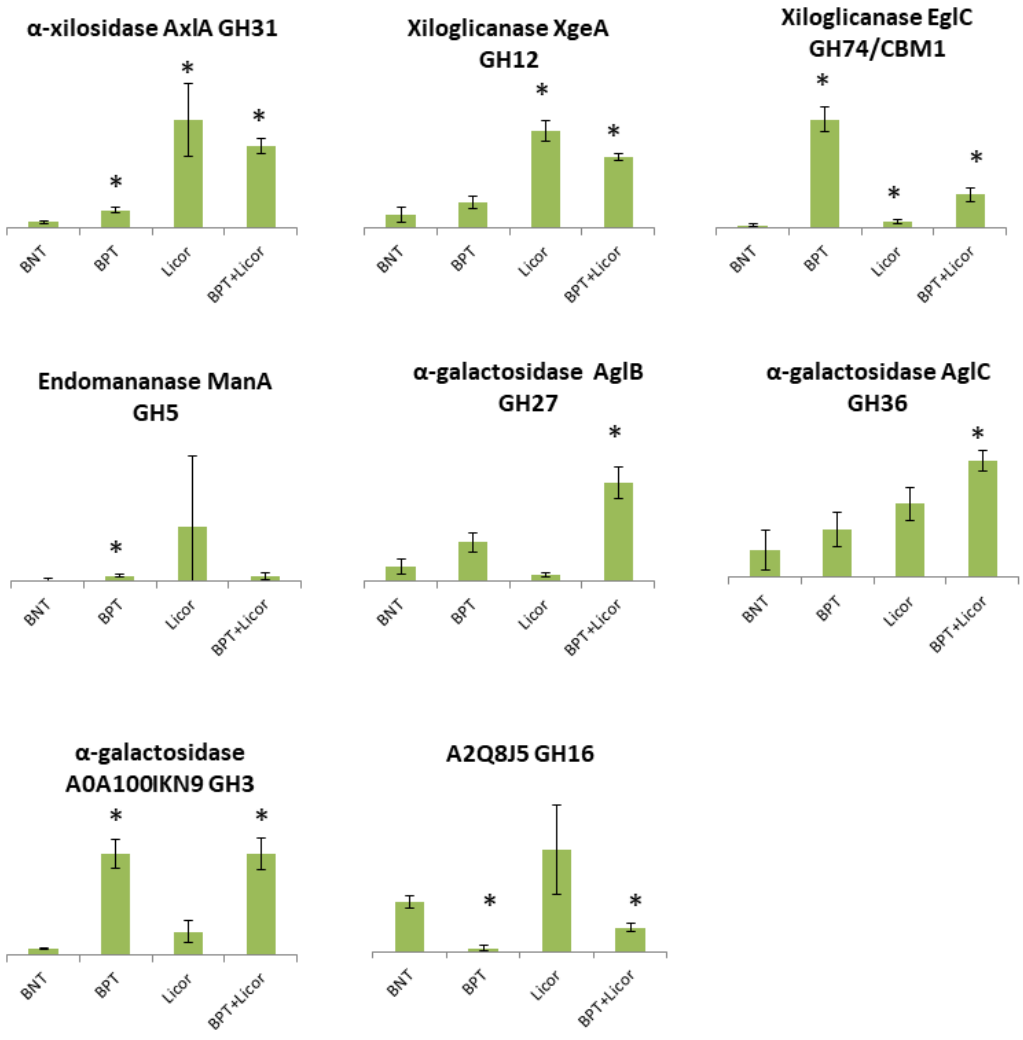


Figura 10. Abundância relativa normalizada de enzimas ativas sobre xiloglicanos, (galacto)manana e β-1,3-1,4-glicanos presentes nos secretomas de *A. niger* cultivado na presença de bagaço de cana-de-açúcar *in natura* (BNT), bagaço tratado (BPT), licor ou bagaço tratado + licor (BPT+Licor). Barras marcadas com asterisco (*) são abundâncias significativamente diferentes (teste T de Student, $p < 0,05$) em comparação com bagaço de cana *in natura* (BNT). Eixos das ordenadas (y) correspondem a valores de abundância normalizada e foram omitidos por representarem unidade arbitrária.

3.2.3. Pectinases

Em relação às enzimas pectinolíticas, foram identificadas uma endo-β-1,6-galactanase (GH6), uma endopoligalacturonase (GH28), uma endo-xilogalacturonana hidrolase (GH 28), três β-galactosidases (GH35), uma α-L-rhamnosidase (GH78), uma pectato liase (PL1), uma rhamnogalacturonato liase (PL4) e uma rhamnogalacturonana acetil esterase (CE12) (Tabela 2). As enzimas α-L-arabinofuranosidases e feruloil esterases categorizadas anteriormente como hemicelulases têm ação tanto sobre arabinoxilana quanto sobre a pectina. As enzimas identificadas atuam em conjunto na desconstrução de homopoligalacturonana e rhamnogalacturonana I, polissacarídeos presentes na parede celular da cana-de-açúcar (De Souza *et al.*, 2013). A Figura 11 mostra a abundância normalizada das enzimas pectinolíticas que tiveram diferença de abundância significativa (teste T de Student, $p < 0,05$) em pelo menos uma das condições de cultivo em comparação à condição controle (BNT).

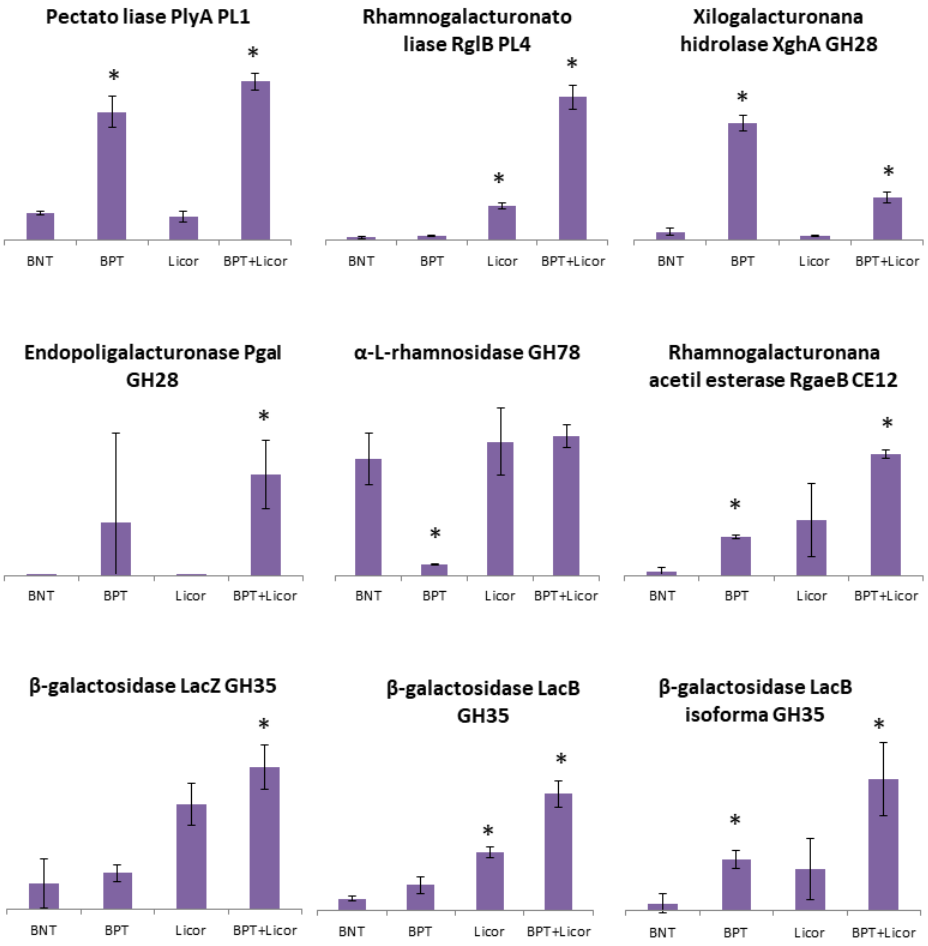


Figura 11. Abundância relativa normalizada de enzimas pectinolíticas presentes nos secretomas de *A. niger* cultivado na presença de bagaço de cana-de-açúcar *in natura* (BNT), bagaço tratado (BPT), licor ou bagaço tratado + licor (BPT+Licor). Barras marcadas com asterisco (*) são abundâncias significativamente diferentes (teste T de Student, $p < 0,05$) em comparação com bagaço de cana *in natura* (BNT). Eixos das ordenadas (y) correspondem a valores de abundância normalizada e foram omitidos por representarem unidade arbitrária.

A maior parte das enzimas pectinolíticas detectadas tiveram a secreção aumentada em resposta ao pré-tratamento hidrotérmico do bagaço de cana-de-açúcar, seja na condição BPT, LIC ou BPT+LIC. Algumas enzimas (PlyA PL1, XghA GH28 e PgaI GH28) foram induzidas principalmente pela fração dos sólidos tratados (condições BPT e BPT+LIC), enquanto outras (RglB PL4, RgaeB CE12, LacZ GH35 e LacB GH35) tiveram secreção positivamente influenciada por ambas as frações (sólidos e licor). A única exceção foi a enzima α-L-rhamnosidase (GH78), que foi induzida principalmente pelo bagaço *in natura* (condição BNT) e pela presença do licor (condições LIC e BPT+LIC), tendo a secreção fortemente reduzida quando o cultivo foi realizado na presença dos sólidos tratados como única fonte de carbono (condição BPT). Isto indica que algum componente da pectina presente na biomassa *in natura* e solubilizada para o licor é importante para a indução desta enzima.

O aumento na produção de pectinases vai de encontro ao que é descrito na literatura. Ribeiro *et al.* (2012), através de análise proteômica qualitativa, observaram que o número de enzimas pectinolíticas (principalmente das famílias CE8, CE12, CE16, GH28) produzidas por *P. echinulatum* na presença de colmo de cana-de-açúcar *in natura* foi superior àquele observado em cultivo na presença de bagaço de cana-de-açúcar pré-tratado por explosão à vapor. Apesar de 7 pectinases terem sido identificadas em comum nas duas condições, 12 pectinases foram secretadas exclusivamente na presença do colmo *in natura* (Ribeiro *et al.*, 2012). De forma similar, uma característica marcante no trabalho de Daly *et al.* (2017) foi a redução na expressão de genes de enzimas pectinolíticas – tanto no número de genes expressos quanto nos níveis de expressão gênica – por *A. niger* N402 causada pelo pré-tratamento hidrotérmico da palha de trigo utilizada com fonte de carbono para seu crescimento. A redução na abundância de pectinases foi confirmada por análise proteômica quantitativa dos secretomas de *A. niger* N402 e por ensaios de atividade enzimática (Daly *et al.*, 2017). Os autores atribuíram este efeito negativo à extensa solubilização dos componentes pécticos durante os tratamentos hidrotérmicos severos empregados.

3.2.4. Outras enzimas

Uma catalase foi identificada nos secretomas de *A. niger*, cuja abundância foi significativamente aumentada na condição LIC. Diversas catalases também foram expressas por *A. niger* N402 e *T. reesei* RUT-C30 durante degradação de bagaço de cana-de-açúcar pré-tratado por explosão a vapor, identificadas através de análise transcriptômica por Borin *et al.* (2017). A enzima catalase é enquadrada na categoria PAD (enzimas Prooxidantes, Antioxidantes e Detoxificantes), grupo de enzimas cuja atuação tem sido associada à degradação de lignocelulose devido ao aumento de expressão observado durante cultivo de fungos filamentosos em biomassas lignocelulósicas (Borin *et al.*, 2017). A enzima catalase promove a decomposição do peróxido de hidrogênio em água e oxigênio molecular. Esta enzima pode desempenhar papel na neutralização de espécies reativas de oxigênio geradas pela ação oxidativa de enzimas das famílias AA7 e AA9, também encontradas nos secretomas de *A. niger*. De acordo com Scott *et al.* (2016), a adição de catalases a coquetéis enzimáticos celulolíticos aumenta a eficiência de hidrólise de biomassa lignocelulósica através da proteção das enzimas contra inativação oxidativa por espécies reativas de oxigênio.

Diversas enzimas ativas sobre β-1,3-glicanos (componentes da parede celular fúngica) foram identificadas, incluindo β-1,3-glicanases das famílias GH5, GH16 e GH17 e 1,3-β-glicanosiltransferases da família GH72. De acordo com a classificação do Gene Ontology, estas enzimas são envolvidas no processo biológico de organização da parede celular fúngica. Enzimas das famílias GH18 e GH20 possivelmente envolvidas na hidrólise da quitina (importante polissacarídeo cristalino presente na parede celular fúngica) também foram identificadas nos secretomas. As proteínas CwpA, Ecm3 e PhiA, envolvidas na organização da parede celular fúngica, também foram detectadas. A maioria destas enzimas apresenta peptídeo sinal para secreção e/ou destinação para a membrana celular. Boa parte destas enzimas teve abundância aumentada nas condições BPT, LIC e/ou BPT+LIC, o que pode ser resultado de uma possível aceleração do crescimento micelial nas biomassas advindas do tratamento hidrotérmico do bagaço (mais facilmente sacarificáveis). Enzimas envolvidas na modelagem de parede celular fúngica são comumente identificadas nos transcriptomas e secretomas de fungos filamentosos cultivados em biomassa lignocelulósica (Ribeiro *et al.*, 2012; Borin *et al.*, 2015; Florencio *et al.*, 2016). Diversas proteínas intracelulares (sem peptídeo sinal) provavelmente extravasadas para o meio extracelular também foram mais abundantes nas condições BPT, LIC e/ou BPT+LIC, possivelmente devido ao maior crescimento micelial nas biomassas tratadas em comparação à biomassa *in natura*.

3.2.5. O potencial do licor como fonte de carbono alternativa

A produção de holocelulases por *A. niger* em licor de pré-tratamento hidrotérmico de bagaço de cana-de-açúcar já havia sido previamente investigada sob o prisma proteômico por Robl *et al.* (2015). Estes autores evidenciaram o potencial do licor de induzir a produção de endoxilanases, β-xilosidases, α-L-arabinofuranosidases, endoglicanases, celobiohidrolases e β-glicosidases. Todavia, o número de holocelulases identificadas no secretoma LIC do presente trabalho (63 no total) foi superior àquele identificado por Robl *et al.* (2015) (22 no total) e incluiu uma maior variedade de enzimas que atuam nas cadeias laterais da xilana, sobre outras hemiceluloses, celulose e pectina. O licor utilizado como fonte de carbono no trabalho de Robl *et al.* (2015) apresentou alta toxicidade devido à alta severidade e alta carga de biomassa empregados no tratamento (190 °C, 10 min, 10 %), sendo necessário diluí-lo para que o cultivo de *A. niger* DR02 fosse possível. A maior severidade pode ter sido o motivo do menor

número de enzimas detectadas. Diferenças na metodologia proteômica aplicada também podem ser a causa.

3.3. Sacarificação enzimática de bagaço de cana-de-açúcar

Testes de termoestabilidade dos secretomas foram realizados com o objetivo de determinar a temperatura ideal para os experimentos subsequentes de sacarificação do bagaço de cana-de-açúcar. As atividades de xilanase e endoglicanase, duas das principais enzimas envolvidas na degradação do bagaço, foram acompanhadas a 50 ou 40°C durante 4 dias (Figura 12 e Figura 13).

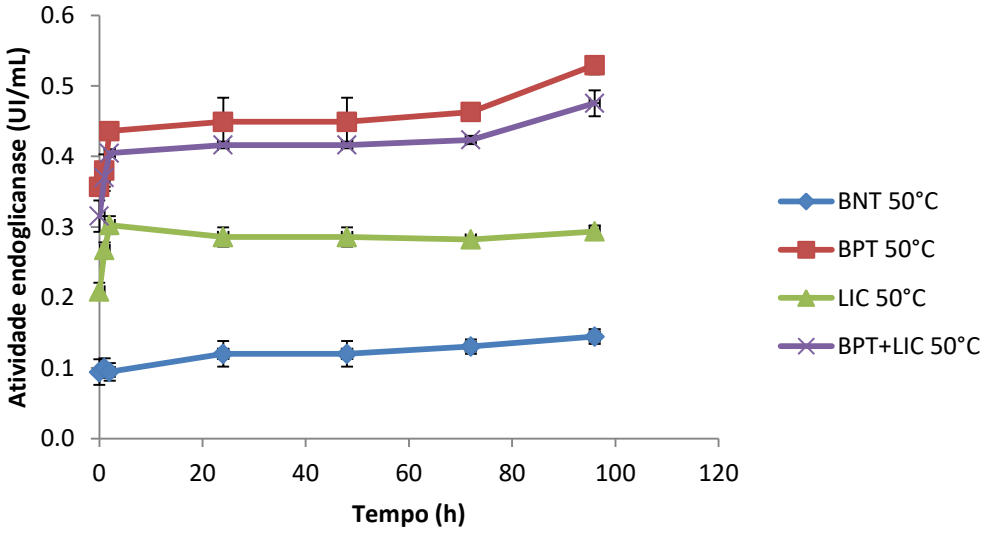


Figura 12. Termoestabilidade da atividade de endoglicanase (CMCase) dos secretomas de *A. niger*. BNT: secretoma produzido por *A. niger* quando cultivado na presença de bagaço de cana-de-açúcar *in natura*; BPT: secretoma produzido por *A. niger* quando cultivado na presença de bagaço de cana-de-açúcar tratado; LIC: secretoma produzido por *A. niger* quando cultivado na presença de licor; BPT+LIC: secretoma produzido por *A. niger* quando cultivado na presença de bagaço de cana-de-açúcar tratado em conjunto com o licor.

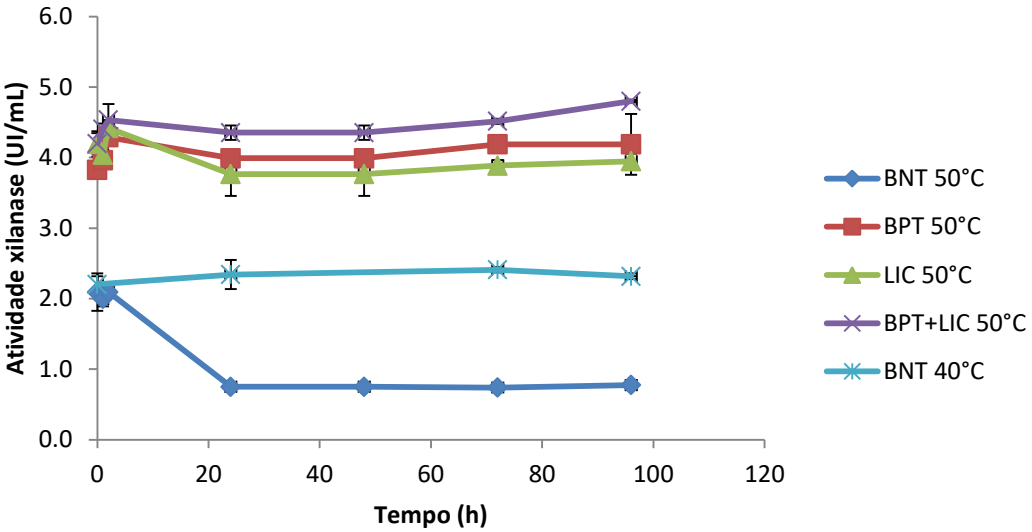


Figura 13. Termoestabilidade da atividade de xilanase dos secretomas de *A. niger*. BNT: secretoma produzido por *A. niger* quando cultivado na presença de bagaço de cana-de-açúcar *in natura*; BPT: secretoma produzido por *A. niger* quando cultivado na presença de bagaço de cana-de-açúcar tratado; LIC: secretoma produzido por *A. niger* quando cultivado na presença de licor; BPT+LIC: secretoma produzido por *A. niger* quando cultivado na presença de bagaço de cana-de-açúcar tratado em conjunto com o licor.

As atividades de endoglicanase e xilanase dos diferentes secretomas foram estáveis a 50°C, com exceção da atividade de xilanase do secretoma BNT. A termoestabilidade do secretoma BNT foi então testada a 40°C, situação em que a atividade de xilanase se mostrou estável. Foi observado, portanto, que as enzimas produzidas por *A. niger* quando cultivado na presença de biomassas pré-tratadas (BPT, LIC ou BPT+LIC) foram mais termoestáveis, revelando maior potencial para aplicação na hidrólise de biomassa do que as enzimas produzidas a partir de bagaço *in natura* (BNT). O mesmo resultado, entretanto, não foi observado no trabalho de Florencio *et al.* (2015), no qual foi mostrado que secretomas de *T. reesei* RUT-C30 cultivado em bagaço de cana-de-açúcar *in natura* apresentaram atividades de endoglicanase mais termoestáveis à 50°C do que aquelas produzidas na presença de bagaço de cana-de-açúcar pré-tratado por explosão à vapor. Isso foi atribuído à presença de compostos fenólicos liberados a partir da lignina durante a explosão à vapor, uma vez que estes compostos já foram reportados por diminuir a termoestabilidade de celulases (Florencio *et al.*, 2015). A baixa severidade do tratamento aplicado no presente trabalho pode ter evitado este efeito negativo sobre a termoestabilidade dos secretomas BPT, LIC e BPT+LIC de *A. niger*.

Os experimentos de sacarificação de bagaço de cana-de-açúcar foram realizados a 40°C de modo que todos os secretomas mantivessem atividade xilanolítica e celulolítica

durante a hidrólise. A liberação de açúcares redutores totais (quantificados por DNS) e glicose (quantificada por ensaio de glicose oxidase) durante a sacarificação enzimática do bagaço de cana-de-açúcar *in natura* ou pré-tratado hidrotermicamente pelos diferentes secretomas de *A. niger* são mostrados nas figuras 14, 15, 16 e 17.

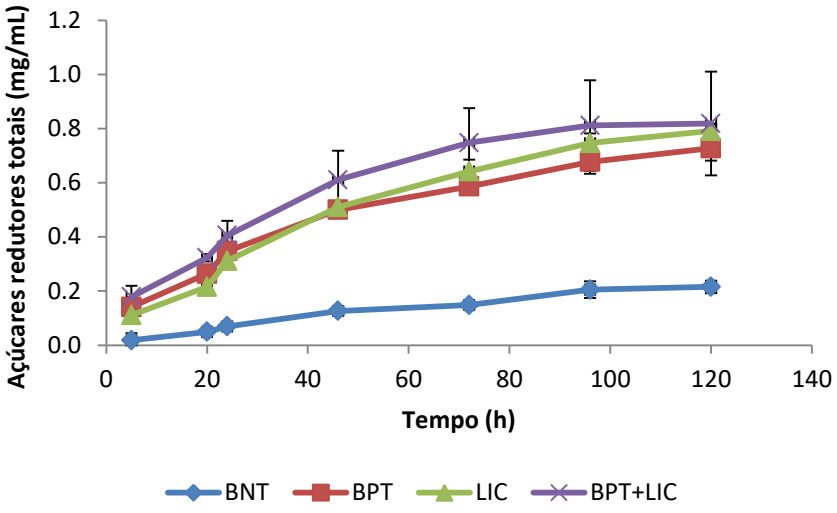


Figura 14. Liberação de açúcares redutores totais durante sacarificação enzimática do bagaço de cana-de-açúcar *in natura*.

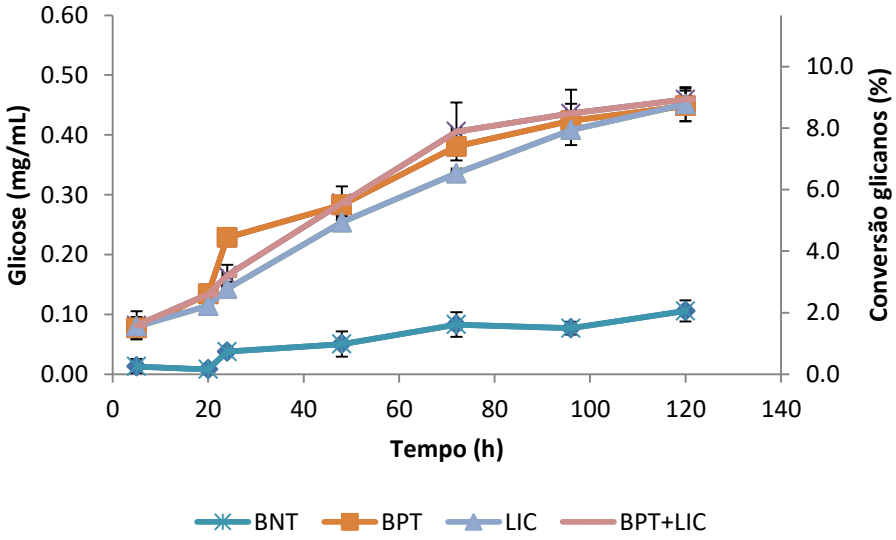


Figura 15. Liberação de glicose durante sacarificação enzimática do bagaço de cana-de-açúcar *in natura*.

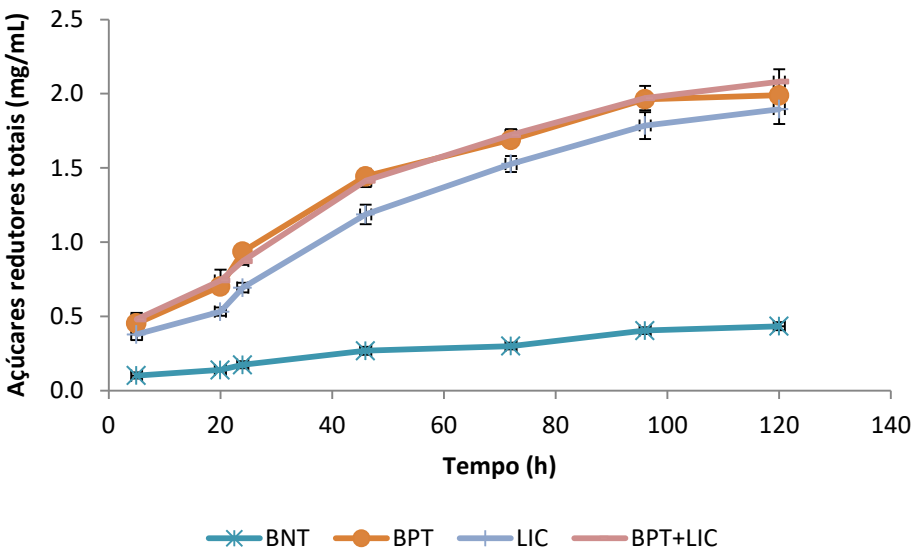


Figura 16. Liberação de açúcares redutores totais durante sacarificação enzimática do bagaço de cana-de-açúcar pré-tratado hidrotermicamente.

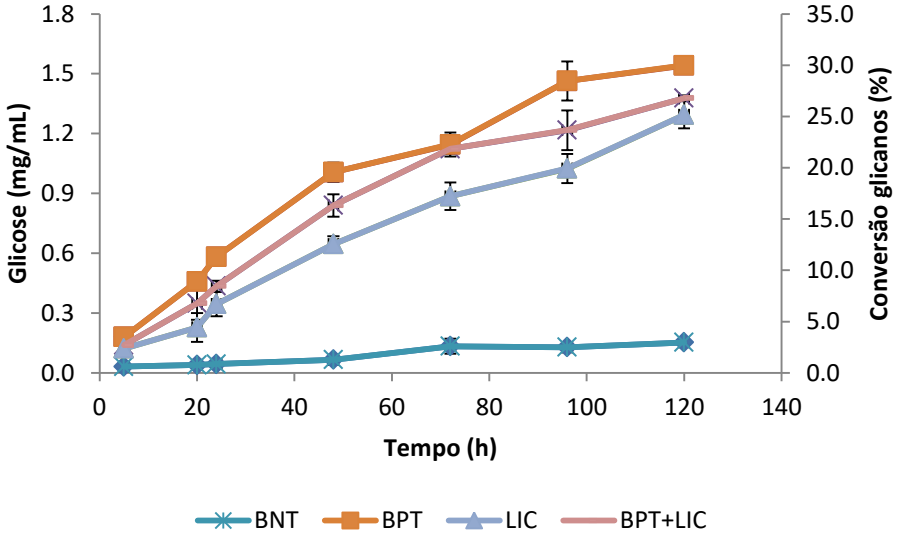


Figura 17. Liberação de glicose durante sacarificação enzimática do bagaço de cana-de-açúcar pré-tratado hidrotermicamente.

Os resultados de sacarificação enzimática consolidaram dois pontos: a redução da recalcitrância do bagaço de cana-de-açúcar causada pelo tratamento hidrotérmico foi positiva tanto para produção de holocelulases por *A. niger* quando cultivado neste substrato quanto para a sacarificação deste material pelas enzimas secretadas.

A sacarificação enzimática do bagaço pré-tratado resultou em maior liberação de açúcares redutores totais e glicose do que a sacarificação do bagaço *in natura*, como

esperado e já descrito na literatura (Florencio *et al.*, 2015). O maior rendimento de glicose foi alcançado pelo secretoma BPT na sacarificação de bagaço pré-tratado (30% de conversão de glicanos). A superioridade do secretoma BPT na liberação de glicose pode ser consequência da maior abundância de endoglicanases e exoglicanases neste secretoma, como mostrado na Figura 8. Outra possível causa seria o fato do secretoma BPT ter sido produzido por *A. niger* na presença da mesma biomassa que foi submetida ao ensaio de sacarificação, gerando um arsenal enzimático mais apropriado para sua hidrólise do que aqueles produzidos na presença do licor (condições LIC e BPT+LIC).

Os rendimentos de glicose ficaram aquém do almejado para um cenário de aplicação industrial. Isso se deve, em parte, ao fato de o tratamento hidrotérmico aplicado sobre o bagaço de cana-de-açúcar ter sido mais brando do que o necessário para que a conversão da celulose atinja níveis mais próximos a 100%, o que normalmente requer temperaturas acima de 180°C (Hongdan *et al.*, 2013; Yu, Zhuang, Lv, *et al.*, 2013; Kim *et al.*, 2014). Além disso, a carga de proteínas utilizada na sacarificação (5 mg de proteínas/g de biomassa seca inicial) foi inferior à carga necessária para hidrólise completa do substrato (normalmente acima de 10 mg/g de biomassa, podendo chegar a até 100mg/g de biomassa) (Yu, Zhuang, Lv, *et al.*, 2013; Kim *et al.*, 2014).

Os secretomas produzidos por *A. niger* quando cultivado na presença de biomassas pré-tratadas (condições BPT, LIC e BPT+LIC) foram mais eficientes na liberação de açúcares totais e glicose, confirmando a superioridade destes secretomas sobre aquele produzido na presença de bagaço *in natura* (BNT). O secretoma BNT, apesar das menores concentrações de produtos de hidrólise, apresentou uma tendência de hidrólise ao longo do tempo similar à dos demais secretomas. Os resultados ficaram de acordo com os resultados de atividades enzimáticas, zimografias e analise proteômica quantitativa mostrados anteriormente. Florencio *et al.* (2015) encontraram resultado similar, em que a sacarificação de bagaço de cana-de-açúcar explodido à vapor foi mais efetivo utilizando extratos enzimáticos de *T. reesei* RUT-C30 produzidos com bagaço pré-tratado do que com bagaço *in natura* como fonte de carbono.

3.4. Conclusões

O tratamento hidrotérmico brando do bagaço de cana-de-açúcar (170 °C, 30 min, 1 % biomassa m/m) revelou-se uma ferramenta para aumentar a produção de holocelulases por *A. niger* quando cultivado na presença das frações sólida e líquida

resultantes. Os secretomas produzidos por *A. niger* na presença de biomassas pré-tratadas (sólidos tratados e/ou licor) tiveram maior atividade de holocelulases, maior termoestabilidade, maior eficiência na sacarificação enzimática do bagaço de cana-de-açúcar e maior abundância de holocelulases do que o secretoma produzido na presença de bagaço *in natura*.

Em contraste a tratamentos de alta severidade, a aplicação de um tratamento hidrotérmico brando sobre a biomassa possibilitou a utilização da fração líquida (licor) como fonte de carbono, por si só ou em conjunto com a fração sólida, para produção de holocelulases por *A. niger* sem que houvesse necessidade de aplicação de métodos de destoxificação do licor. O licor gerado em baixa severidade se mostrou uma potencial fonte de carbono solúvel para indução de extenso arsenal enzimático holocelulolítico, comparável ao observado com o uso dos sólidos tratados. A aplicação de um tratamento brando foi capaz de aumentar a secreção de celulases, hemicelulases e pectinases por *A. niger*. Ambas as frações (líquida e sólida) apresentaram potencial indutor de holocelulases, uma vez que uma parte da hemicelulose e pectina foi solubilizada para o licor e outra parte ficou retida nos sólidos tratados.

O tratamento hidrotérmico do bagaço de cana-de-açúcar gerou substratos mais acessíveis para crescimento microbiano, tanto na forma de açúcares solúveis no licor quanto na forma de polissacarídeos mais acessíveis nos sólidos tratados. A biomassa adquiriu aspecto superficial mais irregular após o tratamento hidrotérmico, levando à exposição de polissacarídeos previamente inacessíveis dentro da estrutura lignocelulósica *in natura*. Observou-se que a composição dos secretomas é dependente não só da composição química, mas também da acessibilidade do substrato.

Este trabalho foi inédito na comparação de secretomas de *A. niger* produzidos em bagaço de cana-de-açúcar *in natura*, bagaço de cana-de-açúcar pré-tratado e/ou licor de pré-tratamento por abordagem proteômica quantitativa. Observou-se ser possível modular a composição do secretoma de *A. niger* usando o tratamento hidrotérmico do substrato como ferramenta, uma vez que o fungo fez ajuste na abundância de enzimas de acordo com a fração da biomassa utilizada.

Os parâmetros do tratamento hidrotérmico de biomassa (severidade e carga de sólidos) utilizados para gerar licores adequados para indução de holocelulases não são compatíveis com os parâmetros necessários para maximizar a sacarificação enzimática

da celulose. Em um contexto industrial de biorrefinaria em que a produção de enzimas é feita na mesma instalação em que a hidrólise e fermentação de biomassa são realizadas (esquema de produção local e integrada de enzimas), propõe-se que sejam aplicados pré-tratamentos diferentes à biomassa, um para cada aplicação (Figura 18). No caso do uso do licor de pré-tratamento brando como única fonte de carbono para produção de enzimas, os sólidos podem ser submetidos a uma segunda etapa de tratamento hidrotérmico mais severo, visando sua preparação para posterior sacarificação enzimática.

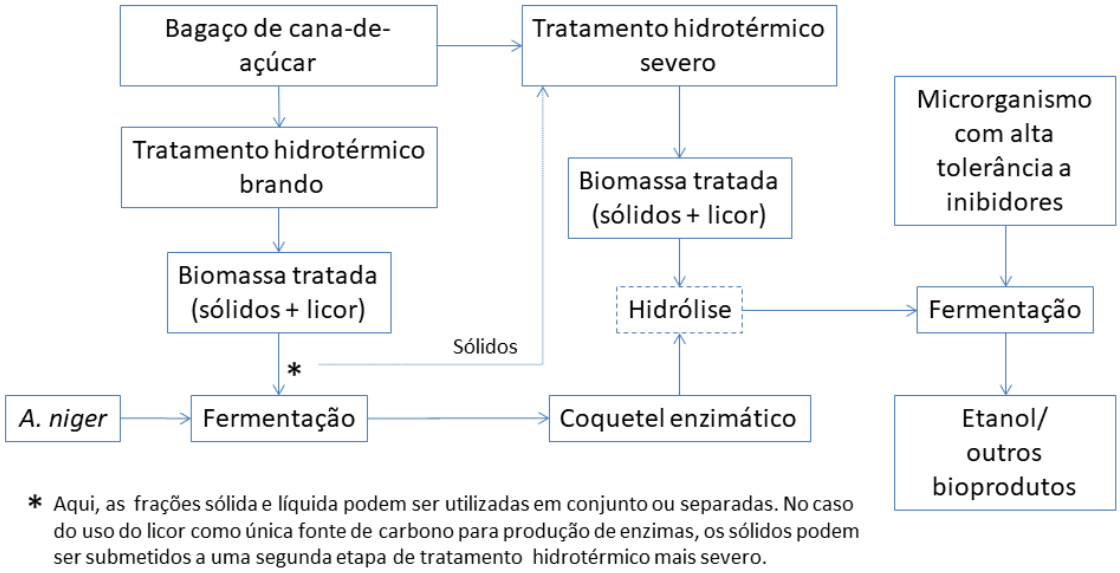


Figura 18. Esquema de aplicação de tratamento hidrotérmico brando para produção de coquetel enzimático por *A. niger* em biorrefinarias de bagaço de cana-de-açúcar.

3.5. Perspectivas

Identificação e quantificação de mono e oligossacarídeos resultantes da sacarificação enzimática do bagaço de cana-de-açúcar por cromatografia líquida (HPAEC-PAD).

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Conclusões

A proposta inicial deste trabalho foi avaliar a utilização do pré-tratamento hidrotérmico do bagaço de cana-de-açúcar como ferramenta para gerar substratos menos recalcitrantes para cultivo de *A. niger* e aumentar a produção de holocelulases, com o objetivo de reduzir os custos associados à produção destas enzimas.

Uma revisão detalhada sobre o efeito de diferentes tecnologias de pré-tratamento de biomassa lignocelulósica sobre a produção de holocelulases por fungos filamentosos quando cultivados nestes substratos foi realizada no Capítulo II. A partir desta revisão concluímos que, em um contexto de produção integrada de enzimas em biorrefinarias, o pré-tratamento hidrotérmico da biomassa pode aumentar a produção de holocelulases desde que aplicado em severidades adequadas.

Nos capítulos III e IV, foi observado experimentalmente que, quando aplicado em uma faixa de severidade adequada, ambas as frações (líquida e sólida) geradas a partir do tratamento hidrotérmico do bagaço de cana-de-açúcar puderam ser utilizadas como fontes de carbono eficientes para produção de holocelulases por *A. niger*, sem que houvesse necessidade de etapas de destoxificação. Licores obtidos em tratamentos de severidade entre 2.76 e 4.01 e empregando concentrações de biomassa entre 1 e 11 % (m/m) foram capazes de induzir maiores quantidades de xilanases, α-L-arabinofuranosidases e β-1,3-glucanases que o bagaço de cana-de-açúcar *in natura*. Diversos mono e oligossacarídeos responsáveis pela indução de hemicelulases foram identificados nos licores por métodos analíticos. O tratamento hidrotérmico de baixa severidade e baixa concentração de sólidos (170°C, 30 min, 1 % m/m bagaço de cana-de-açúcar) foi selecionado como condição ótima devido à alta e rápida indução enzimática pelo licor resultante. A utilização do licor também facilitou o cultivo de *A. niger* em fermentador, permitindo o escalonamento da produção de enzimas.

No capítulo IV, foi observado que os secretomas produzidos por *A. niger* na presença de biomassas pré-tratadas (as frações sólida e líquida utilizadas em conjunto ou separadas como fontes de carbono) mostraram maior atividade de holocelulases, maior abundância de holocelulases, maior termoestabilidade e maior eficiência na sacarificação enzimática do bagaço de cana-de-açúcar do que o secretoma produzido na presença de bagaço *in natura*. Apesar do licor por si só induzir um arsenal

holocelulolítico superior àquele induzido por bagaço *in natura*, a presença dos sólidos tratados aumentou os níveis de secreção da maioria das holocelulases identificadas.

Este trabalho confirmou o potencial do tratamento hidrotérmico do bagaço de cana-de-açúcar como ferramenta para aumentar a produção de holocelulases por *A. niger*, uma vez que gerou substratos menos recalcitrantes para crescimento microbiano na forma de carboidratos solúveis (licor) ou polissacarídeos mais acessíveis nos sólidos tratados.

Adicionalmente ao objetivo inicial desta tese, um artigo de revisão sobre biorrefinarias (capítulo V) e um capítulo de livro sobre produção de etanol (capítulo VI) foram publicados. No capítulo V, um panorama global de biorrefinarias de lignocelulose foi apresentado e o Brasil foi proposto como potencial centro mundial de biorrefinarias. Além disso, a importância de tecnologias "ômicas", engenharia de proteínas, estudos de sinergismo enzimático e imobilização de enzimas para o desenvolvimento de plataformas enzimáticas eficientes para conversão de biomassa foi demonstrada por meio de exemplos. No capítulo VI, vantagens da substituição da gasolina por etanol combustível foram apresentadas e os processos de produção de etanol de primeira, segunda terceira e quarta gerações foram descritos.

Anexo I

Bringing plant cell wall-degrading enzymes into the lignocellulosic biorefinery concept

Artigo de revisão publicado na revista Biofuels, Bioproducts and Biorefining (Editora Wiley), disponível on-line (DOI: 10.1002/bbb.1832). Fator de impacto 3,694. Conceito Qualis A2 (Ciências Biológicas I).

Neste artigo, abordagens técnicas e sistemáticas são discutidas para superar os principais desafios enfrentados pela indústria de biorrefino de biomassa para atingir sua viabilidade econômica. Um panorama nacional e global das biorrefinarias de lignocelulose atualmente em operação é apresentado. O artigo aborda como tecnologias “ômicas", engenharia genética, estudos de sinergismo enzimático e imobilização de enzimas podem ser utilizados para o desenvolvimento de plataformas enzimáticas mais eficientes para conversão de biomassa. Um modelo ideal de biorrefinaria é sugerido e o Brasil é proposto como um potencial centro mundial, ou *hub*, de produção e fornecimento de produtos de biorrefino de biomassa.

Review

Biofpr Biofuels Bioproducts & Biorefining

Bringing plant cell wall-degrading enzymes into the lignocellulosic biorefinery concept

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Abstract: Recent decades have seen the growth of immense interest in lignocellulosic biomass conversion technologies. This interest is motivated by their huge potential for energy and bioproduct generation and reduced dependency on non-renewable feedstocks, leading to improved air quality and reduced emission of greenhouse gases. It is in this context that the lignocellulose biorefinery concept arises. Among the lignocellulose conversion technologies available, enzymatic conversion has emerged as a promising candidate, since it represents a biomass management approach that integrates recycling and remediation in an environmentally friendly manner. Although already in existence, biorefineries employing enzymatic conversion of lignocellulose are at an incipient stage. There remain many operational difficulties, resulting in a very costly overall process that is reflected in product price, reducing market competitiveness. Therefore, much research is still needed to improve the operational and financial feasibility of this process. This paper covers general biorefinery concepts, as well as new and associated concepts, such as the circular economy, bioeconomy, and waste biorefinery. Subsequently, the global outlook, including examples of currently existing enzyme-based lignocellulose biorefineries and their status, is described. The main technical and economic challenges are also discussed, and various potential tools for the optimization of biomass degradation in enzyme-based biorefineries are presented. Finally, the future perspectives for the sector are considered, and models of the ideal biorefinery and globally integrated biorefinery hubs are proposed. These models may contribute to the future establishment of such biorefineries as competitive industries, consistent with the sustainable bioelectro economy paradigm. © 2017 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: circular economy; bioeconomy; biorefinery hubs; lignocellulolytic enzymes; omics approaches; enzyme cocktails

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Introduction

The lignocellulosic biorefinery concept is a viable alternative approach for the effective conversion of biomass feedstock.[1] Enzyme action is essential in this process, and the enzymatic conversion of renewable lignocellulosic biomass to biofuel and value-added products entails a number of challenges, including the improvement of enzymatic hydrolysis via enzyme efficiency, the reduction of enzyme production cost, and the use of novel substrate handling technology.[1-3] A consortium of enzymes with different specificities (lytic polysaccharide monooxygenases [LPMOs], ligninases, cellobiose dehydrogenases [CDHs], hemicellulases, and cellulases) and other proteins (swollenins and expansins) is required to completely break down the lignocellulosic structure of the plant cell wall.[4]

This enzymatic method is also consistent with the new paradigm of a sustainable bioelectro economy.[5] Analogous to the waste biorefinery concept, enzymes can be employed in a holistic approach to lignocellulosic biomass management, integrating remediation and resource recovery (bioproducts and biofuels) through a closed-loop bioprocess cascade, enabling the shift towards a circular, low-carbon bioeconomy. In this circular economy model, lignocellulosic materials are recovered and recycled for repeated use.[6] This holistic perspective necessitates the management of several parameters, such as feedstock storage and handling; pre-treatment; saccharification and fermentation; recovery of ethanol, water, and solids; and waste-water treatment. According to Cheng *et al*.,[7] the optimal design of the lignocellulose conversion process is achieved by choosing an effective technology for each step based on a specific objective. Within this context, several factors affect the enzymatic step of lignocellulose conversion, including enzyme ratios, synergistic cooperation between enzymes, substrate loading, enzyme loading, inhibitors, adsorption, and surfactants. [4] In addition, pre-treatment of lignocellulose is key to the enhancement of substrate accessibility and enzyme hydrolytic action during saccharification.[8]

Interests in the conversion of lignocellulosic biomass have been triggered by the demand for sustainable and environmentally sound sources of fuel and energy. In this review, we describe the technical and systematic approaches by which enzyme-based lignocellulose biorefining can overcome the challenges it faces, including making it economically viable for optimization at small and large scales. Our coverage of this subject incorporates several potential tools for optimizing biomass degradation in enzyme-based biorefineries and the overall prospects for this industry. Finally, a model of biorefinery is proposed, along with an outline of the global biorefinery hub.

The biorefinery

Although the term 'biorefinery' has only begun to be used in recent decades, it is not an entirely new concept. Many traditional biomass conversion technologies can be considered biorefineries, at least in part.[9] However, environmental and economic factors (e.g. global warming, scarcity of fossil resources, and energy crises) have led to the more specific use of this term, in a manner emphasizing the sustainability aspect.

Although the biorefinery concept seems broad, it is in fact relatively simple. According to IEA Bioenergy,[10] biorefining is the sustainable processing of biomass into a wide spectrum of marketable products and energy. Thus, a biorefinery can be an installation, a process, a plant, or a set of facilities; however, all must take a holistic approach to environmental, economic, and social sustainability.[11]

Lignocellulosic feedstock biorefinery

The lignocellulosic feedstock biorefinery refines lignocellulosic biomass into intermediates (cellulose, hemicellulose, and lignin) and subsequently processes these into value-added products and energy.[12-14] The lignocellulosic biomass conversion process involves five important steps: choice of appropriate biomass, choice of pre-treatment approach, biomass conversion into pentoses and hexoses, sugar fermentation, and downstream processing.[3] All steps should be planned and optimized according to the specific process goal.

Essentially, biomass is pre-treated with agents that result in the release of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are then converted primarily into glucose (C6), mannose (C6), xylose (C5), arabinose (C5), and galactose (C6). These sugars may be the end product or can be used as raw material in fermentation for the synthesis of biofuels (e.g. ethanol, butanol, and hydrogen) and chemicals (e.g. alcohol, fatty and organic acids, and amino acids). Lignin and its by-products can be utilized for the production of heat, electricity, and chemicals, including phenolic compounds and polymers, among others.[14-16] Thus, lignocellulosic feedstock biorefining has the potential to integrate several bioprocesses for simultaneous production of energy and various products. This optimized process is therefore socially, economically, and environmentally sustainable.

Enzyme-based lignocellulose biorefineries

Enzymatic hydrolysis of lignocellulose into sugar monomers has emerged as the most efficient biomass conversion technology. Synergistic degradation of lignocellulosic biomass components is achieved using an enzyme consortium incorporating different specificities (i.e., cellulases, hemicellulases, LPMOs, ligninases, and CDHs), in combination with other proteins (swollenins and expansins).[4]

The results of enzymatic hydrolysis can serve as value-added products or may be subjected to subsequent microbial fermentation. [12,14] This stage of the process fits within the concept of white biotechnology, which employs micro-organisms and enzymes to convert bioresources into industrial products with minimal energy expenditure and waste.[17] Several processes can be used for lignocellulose bioconversion and fermentation, including consolidated bioprocessing (CBP), separate hydrolysis and fermentation, simultaneous saccharification and fermentation. CBP is considered the most efficient technique and economically viable, since it avoids the substantial costs due to the addition of enzymes. However, for CBP to be a cost-competitive process, it is necessary to develop optimized micro-organisms, which represents a challenge for metabolic engineering.[18-20]

Global overview of lignocellulose enzymatic conversion biorefineries

Biorefineries demonstrate great potential to increase the competitiveness and wealth of industrialized and developing countries, while meeting the need for sustainable energy and product supply. New job opportunities, skills, markets, policies, and initiatives may result from the development of this industry.[10]

Table 1 displays several global examples of biorefineries based on enzymatic conversion of lignocellulose, showing feedstock type, products, and scale. The majority of these data have been published in IEA Bioenergy country reports.[21] From the information in Table 1 and these sources, it is possible to make some pertinent observations concerning the current worldwide array of biorefineries. Although many biorefineries are in operation, most are situated within Europe. A large number of developing countries are potentially highly suited to the installation and operation of biorefineries due to the large availability of lignocellulose feedstocks, but they do not have technology and investments sufficient for development of such projects. It is also noteworthy that the majority of biorefineries in operation focus on the generation of a single product, bioethanol, with few creating other bioproducts or exploiting the full energy potential of lignocellulose. In addition, most biorefineries still operate far from their maximum production capacity. Concerning conversion technology, all of the biorefineries under discussion employ enzymatic conversion, and the majority are supplied with enzymes by Novozymes.

Biorefineries employing enzymatic conversion of lignocellulose are thus already a reality and demonstrate great potential. One successful example is the Beta Renewables commercial-scale cellulosic ethanol plant in Crescentino, Italy, which is currently the largest cellulosic ethanol refinery in the world, producing 75 million liters of cellulosic ethanol per year. This plant is based on the patented PROESA™ process and uses enzymes provided by Novozymes for conversion of wheat straw, rice straw, and *Arundo donax* L. to ethanol. The process also involves the use of lignin in a connected electric power plant, that generates energy for biorefinery installations, with any excess being sold to the local grid (the circular economy concept).

The current status of biorefineries in operation in Brazil is exemplified by two large facilities, the GranBio and Raizen/Iogen cellulosic ethanol plants in Alagoas and São Paulo, respectively. The GranBio plant was the first Brazilian cellulosic ethanol plant to start operations, in September 2014. It uses bagasse and sugarcane straw as feedstock and employs Beta Renewables/Biochemtex PROESA™ technology and Novozymes enzymes, for a current production capacity of 83.3 million liters of ethanol per year. A few months after the launch of GranBio's operation, the Raizen/Iogen plant began production, in December 2014. This plant uses Iogen Energy technology to convert sugarcane bagasse into ethanol and the enzymes also being supplied by Novozymes. Raizen plans to produce up to 1 billion liters of cellulosic biofuel from bagasse and straw by 2024. [22]

Challenges in lignocellulose enzymatic conversion biorefining

Although lignocellulosic biomass has immense potential, the reality of lignocellulosic biorefinery operation involves many challenges. Recently, several technologies have been developed to improve conversion processes; however, the current goal is to make this system cost-competitive. [25]

The first major challenge is overcoming biomass recalcitrance, a consequence of the properties of plant tissue that represents the major cost of conversion process. Obstacles related to biomass recalcitrance primarily concern the separation and removal of lignin, breakdown of lignin's structure and its strong bond with other biomass compo-

Table 1. Examples of lignocellulose biorefineries based on enzymatic conversion.

Company/Organization	Feedstock	Products	Location	Scale	Reference
BioGasol and Estibio	Straw and agricultural residues	Bioethanol, biomethane	Ballerup, Denmark	Pilot plant	IEA[21]
Dong Energy (Inbicon)	Straw, corn stover, bagasse	Bioethanol, animal feed, electricity, heat	Kalundborg, Denmark	Demonstration plant	IEA[21]
REnescience	Municipal waste	Biogas, heat, power, fertilizer	Copenhagen, Denmark	Pilot plant	IEA[21]
Cometha Project	Lignocellulose	Bioethanol	Porto Marghera, Italy	Industrial-scale pre-commercial plant	Biofuels[22]
DuPont	Corn stover	Bioethanol	Iowa, USA	Commercial scale and pilot plant	Biofuels[22]
Clariant	Wheat straw	Bioethanol, energy	Switzerland	Demonstration plant	Biofuels[22]
Futurol (Pomacle Bazancourt Biorefinery)	Agricultural and forest residues, straws	Bioethanol	France	Pre-industrial pilot plant	Biofuels[22]
Raizen	Sugarcane bagasse and straw	Bioethanol	Piracicaba, Brazil	Commercial scale	Biofuels[22]
Beta Renewables (Crescentino Project)	*Arundo donax*, agricultural residues, wood biomass, bagasse	Bioethanol, energy	Crescentino, Italy	Industrial demonstration plant	IEA[21]
NEDO	Sugarcane bagasse	Bioethanol	Thailand	Demonstration and pilot plant	IEA[21]
GranBio (Bioflex 1)	Sugarcane straw	Bioethanol, biochemicals	Alagoas, Brazil	Commercial scale	Biofuels[22]
POET-DSM Advanced Biofuels (Project Liberty)	Corn stover	Bioethanol	Iowa, USA	Commercial scale	IEA[21]
Suomen Bioetanoli Oy	Lignocellulose	Bioethanol	Myllykoski, Finland	-	Biofuels[22]
Chempolis Biorefinery	Straw and bagasse	Bioethanol	Finland	Commercial scale	Biofuels[22]
NREL Biochemical Pilot Plant	Wide range of lignocellulosic biomass	Biofuels, chemicals, materials	Colorado, USA	Pilot plant	National Renewable Energy Laboratory[23]
Stan Mayfield Biorefinery Pilot Plant	Wide range of lignocellulosic biomass	Biofuels, organic acids	Florida, USA	Pilot plant	Florida Center for Renewable Chemicals and Fuels [24]

nents, removal and breakdown of hemicellulose, reduction of cellulose crystallinity and polymerization, and reduction of particle size. [4,12] All such barriers can be minimized with the use of pre-treatment processes. However, in order to select the most appropriate approach, it is necessary to consider feedstock type, economic viability, and environmental impact. Pre-treatment itself is highly costly, and attendant pre- and post-pre-treatment operations (such as treatment of solid waste and wastewater, and handling and disposal of by-products) exponentially increase cost.[3]

The second key challenge in lignocellulose biorefining is the need for more efficient, robust, and low-cost enzymatic conversion processes.[12] It is estimated that in the case of second-generation ethanol biorefineries, enzyme represents approximately 28% of the overall cost of generating cellulosic ethanol.[26] This expense is extremely high and compromises market competitiveness; therefore, natural enzyme properties need to be modulated in order to increase productivity and reduce costs.[27] The development of mechanisms that allow low loading and reuse

of enzymes should also be considered, always with cost reduction in mind.[28]

Omics approaches for the screening of lignocellulolytic micro-organisms and enzymes

The economic feasibility of enzyme-based lignocellulose biorefineries requires, among other things, the development of more efficient and optimized enzyme cocktails, diminishing reliance on expensive biomass pre-treatment and the high protein loadings currently used for biomass hydrolysis. A key strategy in the development of effective enzyme cocktails involves deeper exploration of natural biomass utilization systems, comprising individual micro-organisms and microbial consortia that have evolved the ability to overcome lignocellulose recalcitrance through cooperative enzyme action. [29]

Lignocellulolytic microbial communities, both from aerobic and anaerobic environments, such as those found in soil, compost, rumen, manure, insect guts, mammal guts, and biogas reactors, represent an inter-taxonomic effort to degrade lignocellulose, and harbor a vast set of genes responsible for biomass utilization. Micro-organisms are regarded as reservoirs of plant cell wall-degrading enzymes with great potential in biorefining applications and possible sources of novel strategies and mechanisms for efficient biomass conversion.[30] Multi-omics approaches, including genomics, transcriptomics, and proteomics, enable the high-throughput characterization of lignocellulolytic systems, whether isolated species or complex environmental microbial communities, and increase our understanding of the diverse molecular mechanisms underlying lignocellulose breakdown, which is fundamental for the development of enzyme platforms for biorefineries. [29,32]

Genomics and metagenomics

High-throughput technologies have enabled sequencing of the whole genomes of individual species and, more interestingly, environmental microbial DNA in a method known as metagenomics.[33] Genomes and metagenomes reveal the potential of individual species, taxonomic groups, and microbial communities to perform particular physiological functions of interest to biorefining, such as holocellulose and lignin breakdown, detoxification of pre-treatment-derived by-products, uptake and metabolization of carbohydrates other than glucose (such as pentoses and uronic acids), and biosynthesis of value-added products.[33,34] Once correctly annotated using bioinformatic tools, genomic and metagenomic sequence data include a vast catalog of genes involved in lignocellulose breakdown, along with their taxonomic origins, available for the development of enzyme cocktails.[29] In addition, adapting a microbial consortium isolated from the environment to a biorefinery setting, for example, by using culture media containing raw or pre-treated lignocellulosic feedstocks, may be used to identify taxons and enzymes better suited for the breakdown of such materials.

For instance, a metagenomic approach was used to assess the taxonomic structure of a corn-stover-adapted microbial consortium isolated from a compost ecosystem. Members of the bacterial phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were found to be prevalent, and each was associated with a metabolic niche within the consortium. Due to greater assignment of (hemi)cellulolytic GHs to members of *Firmicutes* and *Bacteroidetes*, it was concluded that such bacteria act mainly on corn stover holocellulose, while *Proteobacteria* genes were primarily associated with lignin degradation.[35] Similarly, an anaerobic biogas fermenter fed with agricultural wastes was also shown to be populated by *Firmicutes* and *Bacteroidetes*, with the former predominating.[36] As demonstrated in several previous reports, microbial consortia associated with biogas digesters often exhibit a lower lignocellulolytic capacity than natural biomass utilization communities, such as those found in rumen or manure. It was proposed that this reduced hydrolytic potential is due to the relatively low abundance of *Bacteroidetes* bacteria, which are outcompeted by *Firmicutes* species in biogas fermenters, in contrast to the more balanced ratio present in environmental samples. [36]

A new strategy for polysaccharide breakdown and utilization employed by anaerobic gram-negative bacteria of the phylum *Bacteroidetes* has recently come to light from (meta)genome sequencing. Polysaccharide utilization loci (PULs) are clusters of tandem, co-regulated, functionally related genes encoding enzymatic systems that act synergistically in the recognition, binding, hydrolysis, and utilization of specific polysaccharides. In this proposed mechanism, glycoside hydrolases (GHs) lipo-anchored to the outer bacterial membrane bind and hydrolyze polysaccharides into oligomers, which are imported into the periplasmic space by TonB-dependent transporters, where final enzymatic saccharification takes place. Monosaccharides are then recognized by regulatory PUL components that modulate PUL expression.[37] An interesting example are the three PUL systems

found in the gut bacterium *Bacteroides thetaiotaomicron*, which enables this organism to degrade nature's most complex polysaccharide, rhamnogalacturonan-II, by encoding co-regulated enzymes that non-redundantly cleave 20 of its 21 main-chain and side-chain distinct types of glycosidic linkages. Novel glycoside hydrolase and esterase families with catalytic functions never reported before were unraveled during the dissection of this elaborate pectin catabolic system.[38] These new findings open new possibilities for biorefineries employing feedstocks with high rhamnogalacturonan-II content. Even though PULs targeting pectins, starch, xyloglucan, and algae-derived polysaccharides have been described, PUL-catalyzed cellulose conversion is yet to be confirmed. [39,40]

Comparative genomics approach has also recently shed light into the exciting topic of cellulosomes from anaerobic fungi. For decades, fungal cellulosomes could not be studied as thoroughly as their bacterial counterparts because sequencing of anaerobic fungi genomes was precluded by their low GC content. Advances in DNA sequencing enabled the complete genome sequencing of three anaerobic fungi derived from the intestines of herbivores belonging to the early branching Neocallimastigomycota group. Large fungal scaffoldin proteins were identified, displaying various repeated motifs that likely function as cohesins, showing conserved amino acid sequence between the anaerobic fungi species. Moreover, hundreds of CAZymes bearing non-catalytic dockerin domains (NCDDs) have also been identified. Biologically significant interaction between dockerin modules and cohesion motifs within scaffoldin protein was experimentally confirmed, endorsing their role in tethering enzymatic complexes for the biomass degradation. Although several of the enzymes catalytic domains apparently originated from prokaryotes by horizontal gene transfer, fungal dockerins and scaffoldins show no sequence similarity with their bacterial homologues, indicating that cellulosome-based strategy evolved independently in these two groups of microorganisms.[41] New insights into this novel lignocellulose utilization system open the way for the exploration of the modular structure of fungal cellulosomes in the degradation of biomass, like is being made for bacterial cellulosomes, as discussed in the section, Enzyme synergy and cocktail design.

Multimodular enzymes from the thermophilic anaerobic gram-positive bacterium *Caldicellulosiruptor bescii* bear multiple tandemly linked catalytic and carbohydrate-binding domains and are regarded as a new paradigm among the plant cell wall-degrading enzymes. They represent an intermediate strategy between free enzymes showing single catalytic domain and large multi-enzymatic assemblies (cellulosomes). Cellulase CelA, the main enzyme from *C. bescii* secretome, comprises two catalytic domains (GH9 endoglucanase and GH48 exoglucanase) in a single peptide chain, interspaced by three CBM3b modules. The synergism conferred by the proximity of endoglucanase and exoglucanase modules in CelA makes the hydrolysis of crystalline cellulose more efficient than standard mixtures of free endoglucanases and exoglucanases. Moreover, CelA shows a distinctive, previously unknown mode of action. While fungal exoglucanases and cellulosomes seem to act only on the surface of cellulose microfibrils by an ablation mechanism and by spreading apart cellulose microfibrils at fiber edges, respectively, CelA hydrolyses internal layers of cellulose microfibrils by an excavation mechanism, forming cavities that can be observed by electron microscopy and whose dimensions are compatible with the enzyme size. These three distinct cellulose hydrolysis mechanisms can possibly synergize if applied in conjunction with the saccharification of biomass.[42,43]

Transcriptomics and metatranscriptomics

As a complement to genomic studies, investigating the transcriptomes of biomass-degrading organisms and communities reveals the smaller set of genes that are actively expressed during lignocellulose decomposition. The nature of the pool of genes utilized for degradation, along with their transcription levels and expression in response to substrate replacement or modifications over time, may be obtained from transcriptomic and metatranscriptomic studies, as opposed to the static data gleaned from genomic surveys. This valuable information can guide the design of enzyme cocktails, enabling estimation of the enzymes required and their concentrations for the hydrolysis of specific lignocellulosic substrates. [33]

A DNA microarray platform called the CAZyChip was developed by Abot *et al.*[31] to profile the expression of CAZymes in microbial systems. This technology enables the investigation of biomass degradation at transcriptomic level without directly depending on RNA sequencing technologies. This biochip allows the simultaneous identification and quantification of transcripts of up to 55 220 bacterial GH genes deposited in the CAZy database. Currently, the CAZyChip does not allow detection of fungal genes; however, the authors forecast their inclusion in

the platform, given the role of fungal enzymes in biomass degradation and their synergistic action with prokaryotic enzymes.[30,43] The development of the CAZyChip exemplifies the current trend of valorizing bacteria as key actors in lignocellulose degradation with biorefining potential. Filamentous fungi have traditionally received more attention as biomass decomposers, but recent studies have exposed the capacity of bacteria to produce plant cell wall-degrading enzymes and metabolize biomass degradation products, including lignin.[34,44] Within this context, the ligninolytic bacterium *Sphingobium sp.* SYK-6 has been considered as a potential lignin-valorization platform or source of lignin catabolism genes for its ability to use lignin-derived monomers and oligomers as sole carbon source, being simultaneously unable to metabolize carbohydrates from lignocellulose.[45]

Proteomics

Due to differences in protein turnover and mRNA translation rates, transcript expression does not necessarily correlate with enzyme level. Therefore, proteomic methods, via mass spectrometry-based analysis, are of great importance in quantifying the ultimate products of gene expression (*i.e.,* enzymes) involved in lignocellulose breakdown. Thus, proteomic analyses are frequently used to validate genomic and transcriptomic data. With proteomics, it is possible to identify and quantify (in relative or absolute terms) in a high-throughput manner a large number of the proteins comprising microbial secretomes during lignocellulose breakdown, and detect changes in the levels of particular proteins during biomass utilization. Quantitative proteomics offers important insights applicable in the design of ideal enzyme mixtures for biomass hydrolysis.[33,34]

Proteomics can also be employed for the identification of the major 'active' enzymes in a secretome, as proposed by Ma *et al.* [46] The activity-correlated quantitative proteomics platform (ACPP) may be used to correlate the activity levels of a complex microbial secretome on a particular plant cell wall polysaccharide with the abundance of specific enzymes in the secretome. This method begins with fractionation of complex secretomes by high-performance chromatography under native conditions to preserve enzyme activity. The chromatographic fractions are then subjected to multiple enzyme activity assays and analyzed in parallel with a label-free quantitative proteomics approach. Based on the notion that enzyme concentrations are proportional to their activities, pattern-matching algorithms are used to cross-correlate the detected activity patterns with the identified and quantified proteins from the eluted chromatographic fractions. For validation of the method, the ACPP was employed to detect biomass-degrading enzymes in the *Aspergillus niger* secretome. Among the 25 GHs identified in fractions exhibiting starch hydrolysis activity, 1,4-α-glucosidase elution profile correlated most closely with the pattern of amylolytic activity. The ACPP enables high-throughput measurement of the activity of each secretome protein identified. Its application to the vast set of biomass polysaccharide substrates will facilitate the discovery and evaluation of candidate lignocellulolytic enzymes from complex microbial secretomes.

Enzyme engineering

In addition to investigating natural biomass utilization systems in search of new and more efficient lignocellulose degradation mechanisms, enzyme engineering can also contribute to the development of enhanced enzymes and complexes for use in biorefining. Enzyme engineering aims to improve the performance of natural enzymes, such as increasing their activity level, thermal stability, or tolerance to inhibitors, with the aid of recombinant DNA techniques, such as point mutations in substrate-docking or active sites.[47]

For example, directed evolution (insertion of random mutations in genes coupled with functional screening for desired traits) was successfully used to increase the hydrolytic activity of β-glucosidase BGL1 from *A. niger* by reducing the rate of the transglycosidation side-reaction and increasing the enzyme's tolerance of glucose.[48] Directed evolution has also been applied to create ionic liquid (IL)-tolerant enzymes, with the aim of making IL pre-treatment feasible. Wolski *et al.*[49] isolated several randomly-mutated *Talaromyces emersonii* Stolk Cel7A cellulase variants exhibiting increased activity and thermal stability in the presence of high concentrations of imidazolium-based ILs, which are generally regarded as being incompatible with lignocellulolytic enzymes.

The construction of designer bacterial cellulosomes also highlights the relevance of protein engineering to biomass degradation efficiency. Bacterial cellulosomes are highly organized enzyme complexes that self-assemble onto structural proteins (termed scaffoldins) via specific, non-covalent cohesin-dockerin pairing. All cohesin motifs within a scaffoldin protein are able to bind any catalytic subunit bearing the dockerin domain of the same specificity. Protein engineering has been applied to precisely

control enzyme subunit assembly onto scaffoldin, enabling creation of designer cellulosomes and minicellulosomes (smaller and simpler assemblies) with increased performance tailored to specific lignocellulosic materials. The construction of chimeric scaffoldins containing cohesin motifs from various cellulosome-producing bacterial species enables the controlled attachment of different types of CAZymes, provided that they are fused with a corresponding dockerin module. Enhanced cellulosome activity has been achieved by incorporating accessory enzymes and proteins such as β-glucosidases, LPMOs, and expansins.[50,51] Moreover, the inclusion of a laccase in a designer cellulosome bearing endoglucanase, exoglucanase, and xylanase subunits has been shown to have a synergistic effect on wheat straw decomposition, combining holocellulose and lignin breakdown.[52]

Enzyme synergy and cocktail design

Ideally, enzyme cocktails comprise a minimal number of enzyme types at the lowest possible concentrations and in optimal proportions. The rational design of minimal enzyme cocktails requires precise knowledge of the synergy between each member of the enzyme interactome.[4,53,54] Bioprocess conditions should also be configured to optimize enzyme synergism, especially after the inclusion of LPMOs in enzymatic preparations.[55]

The discovery and use of LPMOs have substantially increased biomass saccharification yields due to synergism with canonical cellulases (endoglucanases and cellobiohydrolases), allowing reductions in the total enzyme loadings required for cellulose breakdown.[55] Despite the current debate on the identity of the co-substrate – molecular oxygen or hydrogen peroxide – for LPMO activity, previous discussions on the application of these enzymes in biorefineries assumed that LPMOs are only active in the presence of molecular oxygen (O_2). The requirement of aerobic hydrolysis steps makes unfeasible the use of Simultaneous Saccharification and Fermentation (SSF) strategy due to oxygen consumption by yeast, making Separate Hydrolysis and Fermentation (SHF) or pre-saccharification SSF schemes more efficient if cocktails containing LPMOs are used.[56] Excessive oxygen during saccharification, however, led to unwanted enzyme inactivation by oxidation, and the addition of catalases in enzyme preparations increase sugar yields by protecting cellulolytic enzymes from oxidation. In addition to the co-substrate, LPMOs also require electron donors, which may be enzymes (cellobiose dehydrogenases, glucose dehydrogenases, and aryl-alcohol quinone oxireductases), plant-derived molecules (such as lignin, phenolic compounds, and light-harvesting pigments), among others. In contrast to the inhibitory effect on holocellulases, lignin and its pre-treatment-derived soluble fragments can actually act as activators of LPMOs, avoiding the need for the addition of external electron donors.[57-59]

Other non-hydrolytic, disruptive proteins such as swollenins, loosenins, and expansins also act in synergy with cellulases by promoting cellulose amorphogenesis through swelling, loosening, or dispersing cellulose microfibrils, and have important role in cocktail design.[60,61] Due to the complexity of hemicellulose, a broader range of enzymes is required for its complete breakdown; however, efficient cocktail development is limited by the lack of a detailed understanding of the synergy between main-chain-cleaving and debranching enzymes and their interaction with cellulases.[54] The role of hemicellulose-active LPMOs in enzyme consortia should also be investigated in this respect.[62-64] Furthermore, enzyme cocktails should be designed with the chemical composition of the substrate and the chosen pre-treatment method in mind, enabling customized consortia for each biorefinery application. This would avoid dependence on current commercial enzyme preparations, which are able to hydrolyze a broad spectrum of substrates of varying compositions, but achieve suboptimal yields. [4,26]

Enzyme immobilization

Currently, the main goals in biocatalysis-based industries are to increase enzyme productivity, stability, and half-life.[65] Such improvements are essential to the realization of large-scale production and cost-effective enzyme formulation. In addition, enzymes reuse is extremely relevant to the widespread use of enzymatic conversion, and represents a great technical and economic challenge. [66-69]

Enzymatic immobilization is one method by which the above goals may be achieved. Immobilization is the confinement of an enzyme to a phase (a matrix or carrier) other than its substrate.[70] Immobilized enzymes demonstrate greater stability, sensitivity, and catalytic activity than free enzymes, and can be recycled and reused.[65] Many previous studies have described the use of different CAZymes and supports for immobilization. Some have reported an optimum temperature increase of 5°C,[71] an 80% increase in catalytic activity,[72] a 22% increase in product yield,[73] a 4-fold increase in enzyme half-life, and a retention of 92% of activity after 17 cycles of use.[74] Therefore, enzyme immobilization may prove to be of

some significance in biorefining, given its implications for reduced enzyme loading, making for a more efficient, robust, inexpensive, and market-competitive process.

Future perspectives

From the Kyoto Protocol (1997) to the Paris Agreement (2015), nations have substantially increased their commitments to reduce greenhouse gas emissions and restrict global warming to below the proposed limit of 2°C above pre-industrial levels. Pledges involving 185 countries are expected to be translated into bioeconomy strategies in the form of national policies and legislation, to support a shift from fossil- to bio-based economies, in which biorefineries will play an important role.[75] Such strategies are expected to drive biorefining as an industry by creating markets for bioproducts and enabling competitive pricing in relation to products of fossil fuel origin. Mandates and subsidies for biofuels are a reality in Brazil, the USA, and EU member states, among other countries, but bio-based chemicals and materials are not typically included in government incentives, slowing the pace of their introduction to the market.[12,76]

Considering these conditions, the World Economic Forum foresees strong growth in the demand for biomass, biofuels, and bio-based chemicals in future decades. This represents a great opportunity for those regions of the world producing substantial quantities of biomass, including developing countries with large agricultural industries, such as Brazil, to become centers for the supply of biomass and valuable associated bioproducts. The free availability of raw lignocellulosic wastes in such countries would make them important players in this emerging market. Surplus biorefinery products could be supplied to meet increasing global demand, particularly that from countries of minimal biomass production, such as the Netherlands.[12] Rotterdam Bioport exemplifies this new demand. It has become a large European hub of bio-based activities, encompassing biomass importation and storage and its processing into bioelectricity, heat, biofuels, and bio-based chemicals. This facility provided the foundation on which a bio-based industrial cluster was established, which currently includes five biofuel plants and two biochemical companies.[77]

Despite their potential to act as suppliers of affordable raw lignocellulosic biomass for the international market, it would be of much greater interest to countries such as Brazil to refine biomass domestically, develop their own biorefinery system, attract bio-based companies, and consolidate themselves as biorefinery hubs (Fig. 1), as has

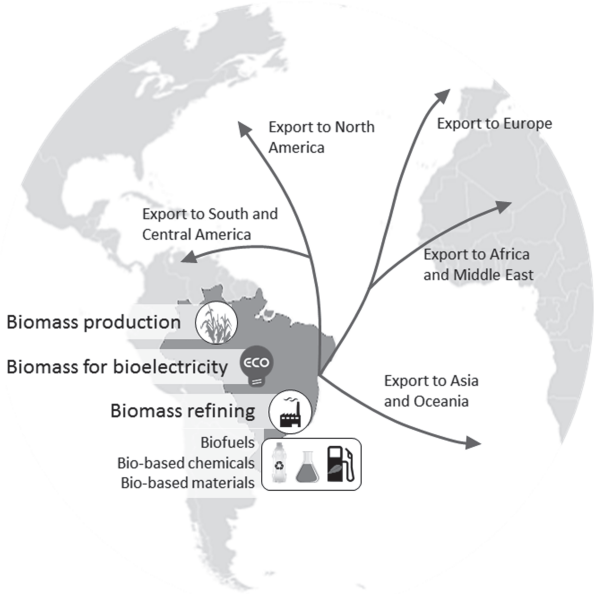

Figure 1. Proposition of Brazil as a biorefinery hub.

the Netherlands. Brazil is already regarded as a center of biofuel and bioplastic production and exportation, a status attained after decades of research dedicated to sugarcane breeding and yeast fermentation. Brazil produced 28.2 billion liters of fuel ethanol from the 2015/2016 sugarcane harvest,[78] of which approximately 7.5% was exported to countries having to comply with biofuel mandates.[79] Green polyethylene produced from sugarcane ethanol, the first commodity chemical made from a renewable resource, has also been exported from Brazil to South and North America, Europe, Asia, and Oceania.[80] In addition, Brazil is a potential exporter of bioelectricity to neighboring countries, given its established system of electricity generation from several biomass residues, principally sugarcane bagasse in ethanol/sugar plants. Brazil stands out for traditionally having had an energy matrix largely based on renewable resources. Advances in enzyme-based lignocellulose biorefinery technologies and their implementation could firmly establish this country as a center and driver of the global bioeconomy, reducing the dependence of other countries on fossil-based energy, fuels, chemicals, and materials.

However, Brazil's emergence as a major bioeconomy depends on progress in biorefinery design and paradigm change. Despite recent advances, enzymatic conversion-based lignocellulose biorefining is far from being an economically viable and sustainable model. Given the many remaining difficulties discussed above, further research into the development of integrated biorefinery systems is needed.

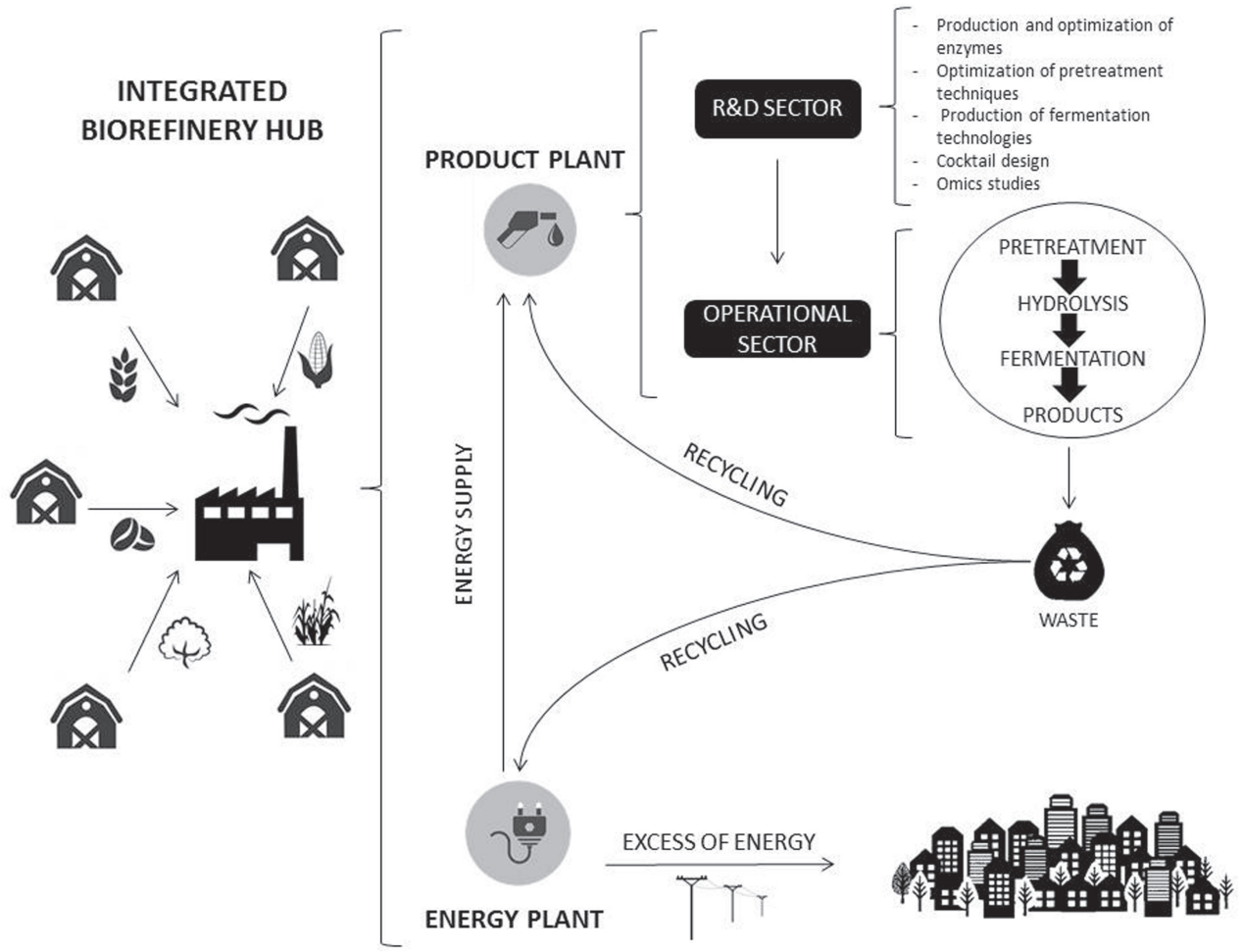

Figure 2. Biorefinery model.

In this context, it is possible to propose a model of the ideal biorefinery (Fig. 2). In this model, biorefineries would be hubs strategically located in regions of intense agricultural activity and integrated with the largest agricultural producers, minimizing the cost of acquiring, transporting, and storing lignocellulosic waste.[81] Supplying a broad spectrum of marketable products and energy is also key. Use and recovery of all lignocellulose components permits a self-sufficient and economical system. For example, sugars from cellulose and hemicellulose components can be used to generate biofuels and bioproducts (such as chemicals, fatty acids, and amino acids), while lignin components can be employed in the synthesis of other bioproducts (such as phenolic components and polymers) and heat and energy generation. The green energy produced can be used in the biorefinery itself (energetic sustainability) and any surplus can be provided to the local community at affordable prices. In addition, any waste generated in the process may serve as a source of new products and energy. Waste may be recovered and residual energy content extracted using technologies based on acidogenesis, bioelectrogenesis, photosynthesis, and photofermentation.[5] The full exploitation of this system's bioprocessing capacity will make it productive and environmentally sound, encompassing bioelectro and circular economy concepts.

Considering the obstacles faced in the biochemical conversion process, it is interesting to imagine a biorefinery model that includes specific sectors responsible for enzymatic conversion/fermentation technologies, including micro-organism screening, production of enzymes and enzyme cocktails, and optimization of enzyme and micro-organism properties, aimed at fostering a more robust and productive system. There are three principal routes by which enzymes are obtained for a production plant. The first, and most common, is by purchasing them from a supplier (off-site production). Currently, the largest such supplier in the world is Novozymes, which enjoys a market

monopoly and whose enzymes are highly costly, leading to the non-competitiveness of biorefinery products. The second comprises the construction of an enzyme factory in the biorefinery plant, using conventional carbohydrates (glucose) for on-site enzyme production. The third and most promising option is to integrate enzyme manufacture into the production process, using lignocellulosic biomass as a carbon source.[26] The main advantage of this approach is that glucose, an expensive raw material, may be replaced with a cheaper substrate (i.e., biomass).

Recent investigations[26] have shown that enzymes account for a significant percentage of the overall cost of cellulosic ethanol production. This figure is 28% using off-site strategies, falls to 22% with on-site approaches, and, surprisingly, is only 10% when enzyme production is integrated into the process. This represents a 64% reduction in costs related to enzyme use.

In conclusion, it is believed that the proposed model, comprising the development of highly integrated hubs, a wide range of end products, aspects of the circular economy, waste recovery and recycling, and integrated enzyme production, represents a promising future for lignocellulosic biorefineries, enhancing their competitiveness and allowing their establishment in the market.

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Anexo II

Microbial biofuel production: An overview on recent developments

Capítulo publicado no livro “Microbial Applications: Recent Advancements and Future Developments” (Editores: V. K. Gupta; S. Zeilinger-Migsich; E. X. F. Filho; C. Duran; D. Purchase), De Gruyter, Berlin, Boston, 2016.

Este capítulo de livro faz uma revisão sobre o uso do bioetanol como combustível substituinte da gasolina. Os processos de produção de etanol de primeira e segunda gerações são descritos, bem como os balanços de energia e emissão de gases de efeito estufa envolvidos. O capítulo também traz uma abordagem histórica sobre a produção de etanol de primeira geração no Brasil e nos EUA ao longo dos séculos XX e XXI. Os novos conceitos de etanol de terceira e quarta gerações também são apresentados.

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11 Microbial biofuel production: An overview on recent developments

11.1 Abstract

Currently, the growing energy demand together with the claiming for reduction in environmental pollution has led scientist to search for new energy sources. Ethanol presents advantages of reduction of greenhouse gases and be a renewable fuel. First generation bioethanol is a well-stabilized pathway for this fuel production which is produced mainly in Brazil and USA, from sugarcane and corn, respectively. In past decades the concern about reduction of food feedstock prompted the development of second generation bioethanol that is produced from lignocellulosic feedstock. This production utilizes microbial enzymes to hydrolysate the polysaccharides contained in biomass for subsequent fermentation. Third and four generation bioethanol is the fuel produced from aquatic microbial oxygenic photoautotrophs (AMOPs) such as algaes and cyanobacterias and engineered AMOPs, respectively. They can be cultivated in areas that do not present the competition with the production of feed and food. This chapter focuses on reviewing the main advantages and challenges of bioethanol from first to fourth generation.

11.2 Introduction

The world's economy today is highly dependent on fossil energy sources (coal, oil, natural gas) which are used to produce fuels, electricity, chemicals, and other goods. As a result of increasing population, demand of energy is escalating throughout the world. Utilization of fossil energy sources in the long run is not considered to be sustainable. In this scenario, use of renewable resources is an alternative [1]. Although estimates vary, the economically recoverable fossil fuel reserves include almost 1 trillion metric tons of coal, more than 1 trillion barrels of petroleum, and over 150 trillion cubic meters of natural gas [2].

Extreme consumption of fossil fuels, especially in large urban areas has caused more pollution due to release of greenhouse gases (GHGs) especially during the last few decades. The concentration of GHGs in the biosphere has hugely increased [3–5]. According to the World Energy Council, approximately 82 % of global energy demand is provided by fossil resources such as petroleum, natural gas and coal. Transportation sector alone consumes about 60 % of the petroleum based fuel produced globally and is responsible for production of one fifth of total CO_2 emissions [6].

Incentives for mitigating global climate change further stimulate international communities to invest in development and utilization of renewable energy [5]. To reduce our dependency on fossil fuels, except for nuclear energy, the most suitable alternative to fossil fuels is renewable sources such as hydroelectric, biomass, wind, solar, geothermal heat, and marine

tidal for the energy industry [7, 8]. Renewable energy is a basic ingredient for sustainable development. Such sources can supply the energy we need for indefinite periods of time polluting far less overall than fossil or nuclear fuels [9]. Renewable energy is one of the most efficient ways to achieve sustainable development. Increasing its share in the world matrix will help prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply on a global scale [8]. Biofuels refer to plant biomass and the refined products to be combusted for energy (heat and light). Similar to fossil fuels, biofuels exist in solid, liquid, and gaseous forms [5]. Microbial biofuel synthesis is a cost effective and environmentally sustainable way of producing replacements for gasoline, diesel, and jet fuel from lignocellulosic biomass [10]. Microorganisms are a rich source of enzyme systems displaying glycoside hydrolase and other activities, involved in the breakdown of plant cell wall polysaccharides [11]. Microbial enzymes are known to play a crucial role as metabolic catalysts, leading to their use in various industries and applications. Polymer-degrading activities are suitable for saccharification of lignocellulosic biomass for the production of second generation biofuels [12]. Fungi belonging to the genus *Trichoderma* have high levels of production of holocellulases. Particularly, cellulolytic system of *T. reesei* has been focus of research for more than 50 years [13]. Cellulolytic fungi (*T. viride*, *T. longibrachiatum, T. reesei*) have long been considered the most productive and powerful destroyers of crystalline cellulose [14]. Beyond the fungi belonging to the genus *Trichoderma*, *Aspergillus niger* (and other species of *Aspergillus*) are also commonly used in the industrial production of enzymes. In addition to *Aspergillus* species, various *Penicillium* species are been considered as an alternative to *T. reesei* for production of biomass-degrading enzymes [14].

11.3 Ethanol

Ethanol has been known as a potential alternative fuel for well over one hundred years [15]. Ethanol's unique properties and characteristics make it an extremely valuable component of gasoline. For more than three decades, refiners and blenders have used low levels of ethanol to boost the octane rating and oxygen content of finished gasoline [16]. Ethanol contains 35 % oxygen, which results in a more efficient combustion of fuel and reduced levels of harmful gases. Moreover, ethanol production uses mainly energy from renewable sources, reducing greenhouse gas emissions and the net carbon dioxide added to the environment. Pure ethanol can be burned with greater efficiency, and is thought to produce smaller amounts of ozone precursors, thus decreasing urban air pollution. It is particularly beneficial with respect to low net CO_2 put into the atmosphere [17].

11.4 First generation ethanol

First generation ethanol corresponds to ethanol produced from food or feed feedstocks, namely sucrose containing crops (such as sugarcane, sugar beets and sweet sorghum) and starch containing crops (such as corn, wheat, barley, rice and cassava) [18]. Although produced from several sources, this discussion will focus on ethanol from sugarcane sucrose

and corn starch, which are quite well established technologies used in Brazil and the US, respectively.

In Brazil, most part of sugarcane mills able to produce both ethanol and sugar, consisting of two annexed plants in the same production facility, one specific for each product. Mills with annexed plants usually address 50 % of the sugarcane to sugar production and 50 % to ethanol production, and may change the sugarcane input direction according to market situation. Autonomous distilleries, where all sugarcane is diverted for ethanol production, are minority [19, 20]. Ethanol production from sugarcane is much simpler than that from corn starch and does not require enzymatic steps. After washing and milling the sugarcane stalks, extracted sugarcane juice is screened to clean off sand and fiber debris and goes through a clarification process, where impurities are removed with the addition of a flocculant agent and the pH is adjusted. Juice, initially with 15 wt % solids, is concentrated to around 22 wt % solids prior to the addition of yeast for the fermentation step. Molasses (containing high concentrated sucrose) obtained as residue from the crystallization of sugarcane juice for sugar production in annexed plants are also sent to fermentation.

Brazilian sugarcane mills follow the Melle-Boinot process, where fed batch is employed, yeasts are recycled and nitrogen content in fermentation substrate is low. Fermentation fed batch process in Brazilian mills is characterized by a gradual change in yeast composition along the production season. When ethanol production season begins, barker's yeast (*Saccharomyces cerevisiae*) is traditionally employed in fermentations as the starter fermenting microorganism. Industrial fermenting conditions are not aseptic and contaminations with wild yeasts and bacteria are inevitable. After every fermentation cycle, fermented wine is centrifuged and the separated yeast cells are recycled for the next cycles. Centrifuged yeast cream is treated with sulphuric acid (pH 1.8–2.5 for 1–3 h) prior to new cycle in order to reduce contaminations with bacteria, and this process is repeated during all production season. Yeasts are recycled up to 600 times during the 200–250 days of the harvest seasons. As cells are recycled every fermenting cycle, wild *S. cerevisiae* strains contaminate and gradually take over as the dominant fermenting microorganism, while original starter yeast becomes less abundant. Fermentation is actually performed by a mixture of different wild strains of *S. cerevisiae*. Starter barker's yeast strains cannot compete with wild ones, which are more adapted to the stressful conditions, have higher ethanol yields and do not cause flocculation or foaming. Robust wild strains tolerate harsh conditions that include high glucose osmotic pressure, high temperatures, pH variations and high concentrations of ethanol. Several wild *S. cerevisiae* strains have been isolated from ethanol plants and some (PE-2, CAT-1, SA-1, BG-1, FT858 and JP-1) have already become commercially available. Nevertheless, introducing wild strains as starter and dominant microorganisms in industrial fermentation is difficult, since strain composition changes along time and even dominant strains may disappear [21]. Fed batches in Brazilian mills are usually short, lasting from 6 to 12 hours (a very competitive characteristic of the process) and yielding a final ethanol titer of 7–12 % w/v. Fermentation yield has improved from 75–80 % in 1975–1980 to current 92–93 % of the maximum theoretical ethanol yield from sucrose. The remaining 7–8 % of sucrose is used mainly for yeast cell growth, metabolism and glycerol side production [22]. Fermented wine is then sent to distillation. After the distillation, the remaining material, named vinasse, is considered a valuable co-product used as soil fertilizer. Sugarcane bagasse generated after milling is used

to co-generate heat and power by combustion, providing steam and electricity to support the whole production process. Surplus electricity, generated in approximately 12 % of Brazilian mills is sold as valuable co-product [19, 23–25].

In US, corn ethanol may be produced by dry-grind or wet-grind processes, being the former the most widely method used for that purpose [26]. Wet-grind mills have a much larger portfolio of products besides ethanol, including corn oil, corn gluten feed and corn gluten meal (both used as animal feed), sweeteners (such as high fructose corn syrup) and solvents [27, 28]. Meanwhile, dry-grind mills are focused on ethanol production, generating the so called distiller's grains residue with soluble (DGS), a valuable co-product used as animal feed that can be sold in wet (WDGS) or dried (DDGS) forms [28, 29]. Recently, dry-mills incorporated technologies to extract corn oil before fermentation [29] and, in 2014, 85 % of dry-mills also extracted corn oil [16]. Corn distillers oil may also be used as animal feed ingredient or for the production of biodiesel [16].

Considering starch cannot be readily fermented, ethanol production from corn starch involves enzymatic hydrolysis steps. In dry-grind mills, cleaned corn kernels are milled and mixed with water. The resulting mash containing starch is usually treated first with α-amilase (EC 3.2.1.1, glycoside hydrolase family 13), which has an endo-type mechanism and cleaves α-1,4 glucosidic bonds of starch, generating α-dextrins (glucose oligossacharides) of different degrees of polymerization. This step, called liquefaction, rapidly reduces the viscosity of the starch solution and is carried out at high temperatures (70–90 °C) and pH 6.5 using thermostable enzymes. α-Amylases are usually obtained from the thermophilic organisms *Bacillus liqueniformis* or *Bacillus stearothermophilus*, that produce more thermostable amylases then the originally used *Bacillus amyloliquefaciens*. *Aspergillus oryzae* and *Aspergillus niger* are also sources of less thermostable α-amylases. *Bacillus* enzymes, although stable at temperatures as high as 105 °C, do not tolerate low pH, requiring pH adjustment of the starch mash prior to liquefaction. α-Amylases with higher stability at low pH and low Ca^{2+} levels (stabilizing agent) are preferable, as they reduce costs by avoiding pH adjustments, calcium addition and posterior desalting steps. Bacillus α-amylases have been engineered to fit those requirements [27,30]. The second enzymatic step is called saccharification and consists of converting soluble oligosaccharides to glucose through treatment with glucoamylase (EC 3.2.1.3, glycoside hydrolase family 15), an exo-type acting enzyme that cleaves α-1,4 bonds and α-1,6 bonds from chain ends, producing glucose from branched and unbranched dextrins [28, 30, 31]. Glucoamylases are usually prepared from *A. niger, A. awamori* and *A. oryzae* and are stable at 60 °C and low pH (4.2–4.5) [27]. Pullulanases, isopullulanases and isoamylases, which cleaves α-1,6 bonds, are also important enzymes that may be used to speed the debranching reaction and improve the yield of glucose [27] Saccharification step is usually carried out at pH 4.5 and 60 °C. Yeast, usually *Saccharomyces cerevisiae*, is added to the hydrolysate after saccharification or in a simultaneous saccharification and fermentation (SSF) process. In separate hydrolysis and fermentation (SHF) processes, saccharified starch is cooled to 32 °C prior to addition of yeast. Fermentation decreases pH due to liberation of CO_2, causing increase in glucoamylase activity, which continues saccharification, and inhibits contamination. SHF reduces the amount of glucoamylase required to full saccharification. On the other hand, in SSF processes, saccharification and fermentation occurs in the same temperature (32 °C). Ethanol inhibits contamination of the saccharified starch, and the lower

glucose concentrations reduce osmotic stress in yeast. SSF is generally more energy-efficient. Fermentation usually lasts 48–72 hours, yielding final ethanol concentrations of 10–12 % [28].

After fermentation, in both sugarcane and corn mills, fermented liquor containing 10–12 % ethanol concentration is distilled to 95.4 % purity ethanol (containing 4.6 % water). Ethanol and water form an azeotrope, meaning that water cannot be further separated from ethanol at 95.4 % by conventional distillation. Further purification of ethanol (dehydration) is then achieved by either using solvents, usually benzene, to break the azeotrope and allow purification of ethanol by distillation, or using molecular sieves that absorb water [28].

11.4.1 Energy balance and greenhouse emissions

Ethanol, although a renewable biofuel, requires fossil fuels in its production process, directly or indirectly. Fossil fuels are required in the agriculture, industrial and transportation stages of the production process. Considering that, a positive energy balance and net reductions in GHG emissions can only be achieved if the amount of fossil fuels employed during production of ethanol are smaller than the amount of those avoided by the use of ethanol [32]. Ethanol production from sugarcane is much less GHG intensive than ethanol from corn. Combustion of sugarcane bagasse to provide energy for ethanol mills is the main factor contributing the lower GHG emission, in contrast to American corn ethanol mills that use mostly natural gas [29, 32]. The use of pesticides and fertilizers in both crops is an important limiting factor in energy balance of these processes and in GHG emission reduction [37]. It is noteworthy that the use of corn ethanol co-products as animal feed also offers CO_2 credits to the corn ethanol production process. Although 36.8 % of corn production in US is currently addressed to ethanol production [33], the co-products DGS, corn gluten feed, corn gluten meal and corn distillers oil are able to partially compensate the large amount of corn addressed to ethanol as they are used as animal feed [29].

According to Crago et al. [25], Brazilian ethanol production from sugarcane emits 470 kg of CO_2 per m^3 anhydrous ethanol produced, which sums up to 550 kg/m^3 when transportation to the US is included. Meanwhile, American corn ethanol production, including agriculture and industrial stages, reaches 1173 kg CO_2 per m^3 of ethanol produced, 113 % higher than sugarcane ethanol emissions. Macedo et al. [32] estimated that anhydrous sugarcane ethanol production in Brazil emitted 436 kg of CO_2 per m^3 in the 2005/2006 harvest, which represented net avoided emissions of 1886 kg of CO_2 per m^3 of anhydrous ethanol blended with gasoline.

Bitte die Hoch- + Tiefstellungen prüfen, waren nicht vorhanden.

According to Goldemberg [8] the best example of a large growth in the use of renewables is given by the sugarcane ethanol program in Brazil. The production of ethanol from sugarcane can be replicated in other countries without serious damage to natural ecosystems. Still according to Goldemgerg [8] expanding the Brazilian ethanol program by a factor of 10 would supply enough ethanol to replace 10 % of the gasoline used in the world. This land area is a small fraction compared to the crops already harvested on the planet [8]. Today the production of ethanol in Brazil is 27.96 billion of liters in the crop of 2013/2014 [34]. In the same period, the United States production was 14.3 billion of gallons of ethanol (approximately

54.34 billion liters) [16]. These 2 nations represent almost the totality of global production of 87,2 billion liters [4].

Much debate on whether US corn ethanol results in negative or positive energy balance has been taking place for decades. Although many studies from the 1980's and before estimated negative energy balances for the production of ethanol, more recent research, from 90's until today, show positive energy balances. That may be due to current improved corn productivity and industrial efficiency [29] or due to incorrectly ignored co-products and obsolete data [35].

According to Crago et al [25], on an energy-equivalent basis, use of US corn ethanol decreases GHG emissions by 44 % when compared to gasoline, while Wang et al [29] estimated 24 % GHG emission reductions. With better results, Brazilian sugarcane ethanol reduces emission of GHG by 74 %, according to Crago et al [25] or 63 % as estimated by EPA [36]. Considering sugarcane ethanol reduces emission of GHG by more than 50 %, it fits the concept of advanced biofuels of the Renewable Fuel Standard (RFS) program (discussed below). Thus, Brazilian sugarcane ethanol can compete with cellulosic ethanol in the US, representing a great opportunity for Brazil to become, once again, an exporter of ethanol do the US [25].

11.4.2 First generation ethanol in Brazil

Brazil's interest in replacing imported fossil fuels by domestically produced ethanol for powering cars, motors and lamps dates back to the beginning of the 20th century. Increase in oil prices during World War I, coupled with low sugar prices due to Brazilian overproduction, influenced the government to consider producing ethanol from sugarcane as a way to increase profitability of the sugarcane industry and having a domestic alternative fuel to oil. In 1931 federal government established for the first time at national level that gasoline should contain 5 % of ethanol [37, 38] and this governmental influence stimulated the sugarcane industry. Sugar and Alcohol Institute (Instituto do Açúcar e do Álcool – IAA) was created in 1933 to regulate this growing sugarcane sector. This public institution executed the government interventions on the sector for six decades, until its deactivation in 1990 [39]. In the 1930's, the IAA supported the construction of new ethanol mills so the sector could meet national demands. Northeastern Brazilian states, especially Alagoas and Pernambuco, were the main sugarcane producers, which headquartered important ethanol brands of the time such as Usina Serra Grande and Azulina [37]. Difficult transportation of sugar and ethanol from northeast to southeast region, especially during World War II, led the implantation of sugarcane sector in São Paulo state. Superior transportation and energy infrastructure, land of good quality and proximity to industrial complexes and to research centers consolidated sugarcane in the region, overtaking northeastern production in the 1950's [40]. Creation of the Brazilian state oil company Petrobras in 1953 and pressures from the oil and car industries halted the plan of a domestic ethanol fuel for the following decades [37].

Brazilian robust and well established sugarcane ethanol industry existing today is a consequence of the national interest on ethanol that re-emerged during the 1970's. A timeline of recent progress of ethanol in Brazil is illustrated in figure 11.1. In November 1975, the National Alcohol Program (Programa Nacional do Álcool), or ProAlcohol, was launched in response

to the first oil shock caused by the 1973 OPEC oil embargo, which prompted oil price to quadruplicate in 1973/74. Brazil imported more than 80 % of the gasoline national demand at that time, and elevated oil prices contributed to inflation, gasoline shortages and rationing [39]. Meanwhile, the international sugar prices in the second half of 70's were low due to world overproduction. ProAlcohol was a strategy to overcome Brazil's high dependency on expensive imported oil and replace gasoline by domestically produced sugarcane ethanol. Originally, other biomass sources such as cassava were also considered, but sugarcane was chosen by political and economic reasons [41]. ProAlcohol was also a means to guarantee profitability of the sugarcane sector during low sugar price period. The government associated the potential of growth of the sugarcane industry with the opportunity to lessen the dependence on imported oil after the oil shock [37,40,42].

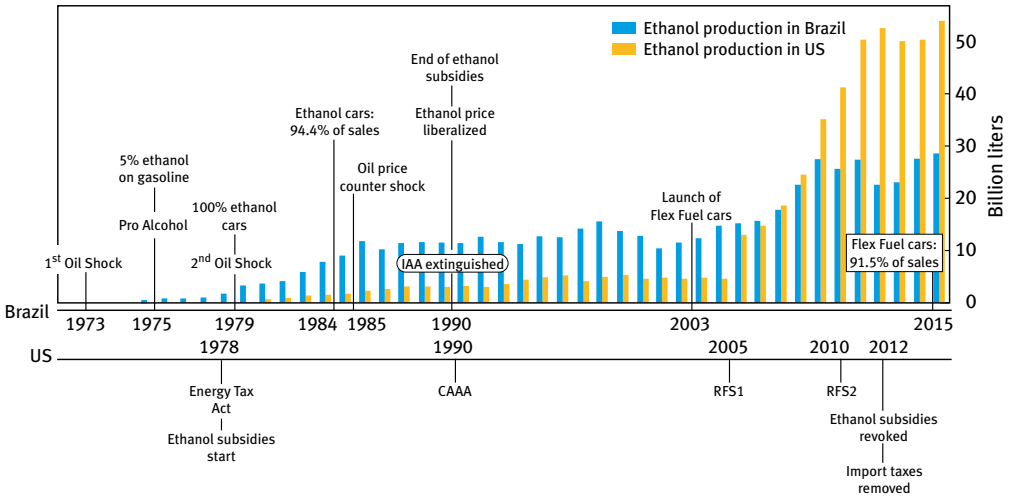


Fig. 11.1: Timeline of ethanol production in Brazil and USA.

Among ProAlcohol initial measures was the offering of financial incentives at low interest rates for the construction and expansion of ethanol mills. From 1975 to 1979, a mandate to blend 5 % of ethanol into gasoline was established. During ProAlcohol's first phase, ethanol production went from 664 million liters in 1976/77 to 3.7 billion liters in the 1980/81 crop, largely contributed by governmental incentives [37, 40, 42]

From 1979 to 1985, boosted by the second oil crisis after Iranian Revolution, the ProAlcohol entered a new phase, focusing on the use of neat ethanol as a full substituent of gasoline. The government, by reducing taxes on alcohol cars, encouraged automobile manufacturers to develop and commercialize vehicles powered by 100 % hydrated ethanol [37]. During that period, the sales of cars fueled exclusively by gasoline dropped, accompanied by sharp increase in ethanol fueled cars sales, which came to represent 94.4 % of automobile production in 1984 [43]. Ethanol production in Brazil reached 11.9 billion liters in 1985. Petrobras was responsible for purchasing and distributing ethanol and regulating prices to keep ethanol more cost favorable than gasoline, even if artificially [39, 42].

Success of the ProAlcohol program and substitution of gasoline by ethanol was heavily dependent on government subsidies and tax incentives, necessary for the construction and expansion of mills, sales of ethanol fueled cars and keeping the price of ethanol fix and advantageous in relation to gasoline, among others. Decrease in the international oil price in 1985, the so-called oil counter-shock, and the severe Brazilian economic crisis in the mid and late 1980's jeopardized the government's financial capacity to support the program. Recovery of international sugar prices and liberalization of the sugar export market re-directed sugarcane to sugar production. Ethanol production no longer could meet national demand and Brazil faced shortages during final 1980's. The 1990's was a transition phase for the ethanol sector. IAA was extinguished, subsidies were revoked, fixed ethanol prices were abolished and ethanol market was liberalized. Hydrated ethanol was not available at the pumps during the 1990's [39, 40, 42, 44].

A re-emergence of global interest on biofuels, especially ethanol, has been occurring since the 2000's driven by increase in oil prices and concerns about greenhouse gases emission, global warming and sustainability. In 2003, the development and commercialization of flex fuel vehicles that can run on ethanol, gasoline or any volumetric combination of these two fuels, was a breakthrough and encouraged consumption of ethanol once again in Brazil. In 2014, 91.5 % of the cars produced in Brazil were flex-fuel, and only 8.3 % were gasoline fueled [43].

Although Brazilian ethanol is not subsidized since early 1990's, mandatory blending still guarantees internal consumption of ethanol. Since 1993, law 8.723 determines that gasoline must contain ethanol [45]. Today, the government is allowed to change the ethanol concentration between 18 and 27.5 % is accordance to ethanol supply, which may vary due to climate variations. Poor sugarcane harvests usually leads to temporary decrease in ethanol percentage [44]. Today ethanol blend is fixed at 27 % [46].

The existing project of an ethanol pipeline for transportation of ethanol from the main producing areas to distribution centers and the port of Santos (Brazil's largest port) in expected to increase Brazilian ethanol competitiveness by reducing its GHG intensity and its cost for both domestic and international consumers.

Various research centers have played key role in the improvement of the sugarcane sector in Brazil, which has been achieved through incremental cumulative non-disruptive contributions. Agronomic Institute of Campinas (IAC) pioneered sugarcane breeding before ProAlcohol. Planalsucar, public national sugarcane breeding program, and Copersucar Sugarcane Technology Center (CTC), a private research center funded by mills, brought significant improvements in sugarcane yields during ProAlcohol. Planalsucar and CTC's varieties covered large proportions of sugarcane fields in Brazil, as well as they contributed to industrial process melioration. Dedini is the main manufacturer of industrial equipment for sugarcane mills and also added contributions. Today, Ridesa (former Planalsucar), CTC, CTBE (Brazilian Bioethanol Science and Techonology Laboratory) and EMBRAPA (Brazilian agricultural research company) Agroenergia are the major research centers focusing on the sugarcane and ethanol sector [40].

11.4.3 First generation ethanol in USA

The idea to use ethanol as fuel is dated to before the discovery of petroleum. The first internal combustion engines created independently by Samuel Morey in the US and by Nicholas Otto in Germany were designed to run on ethanol containing fuels [47]. Henry Ford's first automobile of 1896 was powered by ethanol and the Ford Model T of 1908, the first popular automobile marketed in the US, was designed as a flexible fuel vehicle that could run on gasoline, ethanol or a combination of both. Sustainable production of ethanol was already discussed in the beginning of the twentieth century in US. Henry Ford, a great supporter of ethanol fuel, predicted the development of cellulosic ethanol: "The fuel of the future is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust — almost anything". In1917, Alexander Graham Bell also pointed the feasibility of using several types of feedstock, such as crop residues, farm waste, grasses and city garbage for ethanol production, and declared, "We need never fear the exhaustion of our present fuel supplies so long as we can produce an annual crop of alcohol to any extent desired" [47–49].

Government policies, taxes and subsidies have played key roles in determining the price of ethanol and curbing or launching the use of ethanol as alternative fuel in the US. In 1861, a tax of 0.55 cents per liter of ethanol ($2.08 per gallon – equivalent to approximately $11 per liter) in 2014 was established to collect funds for the Civil War. Although originally directed to alcoholic beverages, fuel ethanol was not spared. Ethanol tax was not revoked until 1906, drastically reducing ethanol competitiveness with fossil fuels such as kerosene during that period. By the time ethanol tax was removed, new ethanol plants were built but gasoline industry was better established and gas had become the favored fuel for automobiles [50]. Moreover, discovery of new oil spots in Texas made ethanol prices soar. Ethanol demand increased to 227 million liters (60 million gallons) during World War I due to rationing of gasoline. In the mid 1930's, the emergence of the Farm Chermugy movement encouraged the production of bio-based materials from renewable agricultural sources. Ethanol production increased and blends of 5–17 % ethanol in gasoline under the names of Agrol or Alcolene were commercialized in the Midwest during that period. During World War II, ethanol demand also increased, but primarily for the production of synthetic rubber and not for use as fuel. Ethanol consumption reached 2.27 billion liters (600 million gallons) per year. However, after World War II, the use of tetraethyl lead as an anti-knock agent and octane booster contributed to the prevalence of leaded gasoline over ethanol, considering the former was cheaper and easier to produce [47–49].

The ethanol fuel production in the US was effectively set in motion by the Energy Tax Act (ETA) of 1978, which implemented a subsidy of 10.6 cents per liter (40 cents per gallon) of ethanol produced [51]. The world was facing an oil crisis scenario, when the oil prices rose sharply in 1973 after the OPEC Arab oil embargo [51]. Subsidy on ethanol, which was modified a few times in terms of values and mechanisms, lasted until 2012 [52] and was of major importance for prompting ethanol industry. Major milestones of ethanol progess in US are shown in figure 11.1.

The Clean Air Act Amendments (CAAA) of 1990 created new market perspectives for ethanol. The CAAA acknowledged the contribution of vehicle fuels to air pollution problems, namely high levels of carbon monoxide and ground-level ozone, and established the use of

oxygenated fuels blended to gasoline in many areas of the US that did not attain federal air quality standards. Higher content of oxygenate fuels improves the complete burning of gasoline, reducing the release of carbon monoxide. Furthermore, evaporated compounds from gasoline may increase formation of ground-level ozone, or smog, which is harmful to the human respiratory system. The amount of evaporative emissions of gasoline was also regulated by the US government, imposing a limit to its Reid Vapor Pressure (RVP). Ethanol fitted the RVP requirements for reducing the volatility of the blended fuel. Although ethanol was already a viable option as a gasoline substitute, methyl tertiary butyl ether (MTBE), an oxygenated fuel produced from natural gas, was the fuel additive of choice during the 90's until it was found to be toxic and associated to contamination of groundwater supplies, posing risks to public health and the environment. MTBE use was reduced and eventually banned in many states and replaced mainly by ethanol or the ethanol derived ETBE (ethyl tertiary butyl ether). Corn ethanol, for being renewable and more environmentally friendly than MTBE, reducing the volatility of gasoline and having minimum health concerns finally gained popularity and took off as the main oxygenate fuel to be blended into gasoline [53].

The Energy Policy Act (EPAct) of 2005 eliminated the requirements of blending oxygenate fuels to gasoline, but on the other hand implemented the Renewable Fuel Standard (RFS) program, created to ensure a minimum value of renewable fuels added to gasoline. Originally, the RFS1 required 28.4 billion liters of ethanol to be blended into gasoline by 2012, which was increased to 136 billion liters by 2022 under the Energy Independence and Security Act (EISA) of 2007 and reinforced by the EISA of 2010 [54]. The so called RFS2 also establishes that conventional biofuels (e.g. corn starch ethanol) should make up to 56.7 billion liters (15 billion gallons) from 2015 on, and that advanced biofuels should make up to 79.5 billion liters (21 billion gallons) by 2022. Advanced biofuels are those with life-cycle GHG emissions reduced to at least 50 % in comparison to gasoline, in which cellulosic ethanol and Brazilian sugarcane first generation ethanol are included. RFS2 also requires that corn starch ethanol should achieve a GHG emission reduction of 20 % relative to gasoline [55].

Until 2012, domestic ethanol in the US was protected from competing with lower-cost Brazilian ethanol by importation tariffs [51]. Tariffs summed up to 30 % of the imported ethanol, while fossil fuels had very low import tariffs [56]. In 2012, the import tariffs were withdrawn, providing free commercialization of ethanol between Brazil and US. This was after Brazil removed taxes on imported American ethanol in 2010. American import tax removal in 2012 was accompanied by the elimination of the $0.45 per gallon tax credit to blenders. That alleviated around $6 billion per year on subsidies for the American government [52]. Guaranteed demand on ethanol was maintained by RFS [57], though. Free commercialization of ethanol between Brazil and US is a step forward for ethanol becoming a global energy commodity, which is an interest of both parts [56].

11.5 Second generation bioethanol

Fossil fuels have always been the main energy source for transportation and industry. Current scarcity of petroleum reserves, together with necessity of reducing environmental impact caused by fossil fuels, leads to utilization of renewable fuels sources, like ethanol

from. However, ethanol produced from sugarcane, sugar beet and corn has a problem since they have value as food and feed. In this context arises the need for utilization of new sources to produce ethanol, like lignocellulosic feedstock, which does not hold debate between food and fuel. Considering that plant biomass is an attractive source for production of 2nd generation bioethanol owing to its large availability, low cost and it does not bring about the problematic of competition with food supplying.

Plant biomass presents a promising renewable energy that could suppress at least in part our demand for fossil fuels. In addition to reducing our dependence on fossil fuels, biomass utilization will also impact positively on many environmental issues and help to minimize the net production of greenhouse gases [58]. Plant biomass corresponds to the majority of cheap and abundant nonfood materials available from plants. Employment of biomass is a cost effective and sustainable source for commercial production of bioenergy as bioethanol. Second generation biofuels are produced from biomass in a more ecologically friendly way, which is truly carbon neutral or even carbon negative in terms of its impact on CO_2 concentrations [4, 59–61]. For large-scale bioethanol, it is worthy to use cheaper and abundant feedstocks in order to lower its high price which has hindered the use of fuel ethanol in the energy industries [62].

Agricultural residues are used as animal feed, domestic fuel and as fuel to run boilers. Utilization fraction of each feedstock is low and varies according to the geographic region [63]. Generally, only a small portion of the produced agricultural residues is used as animal feed, the rest is commonly burned, a common practice all over the world that increases the air pollution [64]. A large amount of agricultural feedstock is available for bioethanol production. In 2004, approximately 1.55×109 tons of agricultural residues was produced around the world. The four major agrowastes were rice straw (7.3×108 tons), wheat straw (3.5×108 tons), corn stover (2.0×108 tons) and sugarcane bagasse (1.8×108 tons) [63].

The plant cell wall is composed of three major components, cellulose, hemicellulose and lignin. Typically, most of the agricultural lignocellulosic biomass is comprised of about 10–25 % lignin, 20–30 % hemicellulose, and 40–50% cellulose [65]. Cellulose is the major structural component of plant cell wall, important for mechanical strength, it is a linear polymer of β-1,4-linked glucose units. Hemicelluloses are often composed of pentoses and hexoses, which include a variety of polysaccharides with linear or branched polymers derived from sugars such as D-xylose, D-galactose, D-mannose, D-glucose and L-arabinose. Pectin is a linear polymer of α-1,4 galacturonic acid units, some of which are methylated at C-6, some acetylated at C-2 and some with more extensive substitution [11,65]. Lignin contains three aromatic alcohols (coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol) and it forms a physical barrier against the attack on cellulose and hemicellulose. Lignocellulosic feedstock includes agricultural wastes, forestry residues, grasses and woody materials [65]. From a biotechnological point of view, a broad diversity of lignocellulosic feedstock is available to be converted into high value bioproducts such as bioethanol/biofuels. Hypothetically, all plant materials can be used to generate bioethanol. Nevertheless, it is fairly challenging to deconstruct these compound polymers into simple sugars [5, 65].

11.5.1 The role of pretreatment

One of the greatest obstacles for the production of 2nd generation bioethanol is lignin.

Many reasons have been presented as responsible for inhibition of hydrolysis by lignin, among them:

1. It acts as a physical barrier that prevents the access of cellulases and hemicellulases to their substrate [66,67];
2. It is covalently linked to hemicellulose, conferring mechanical strength to the cell wall [67];
3. Not only the presence of lignin, but the distribution and the kind of lignin also affect the enzymatic hydrolysis [68];
4. Lignin-derivable phenolic compounds have an inhibitory effect on the enzymatic activity [69,70];
5. Lignin inhibits the action of cellulases by non-specific adsorption [71].

Due to these obstacles, pretreatment is an important tool for achieving effective hydrolysis of biomass. Pretreatment is one of the most expensive steps in the biochemical conversion of biomass, accounting for up to 40 % of the total processing cost [72], however, non-pretreated lignocellulosic biomass has low yield, which increases the total fixed capital per annual gallon of capacity. Pretreated lignocellulosic biomass has high yield and lower capital requirements per annual gallon of capacity as compared to the no pretreatment case [73]. Generally, pretreatment aims to break down the lignin barrier, allowing a greater access to cellulose, which will be subjected to enzymatic hydrolysis to convert into fermentable sugars [65]. Different lignocellulosic biomass subjected to different pretreatment will have a variety of types and concentrations of sugars [74]. A variety of results will be achieved according to the pretreatment used, like the interruption of the structure of lignin and its connections to the rest of the biomass [75]; removal of a part or all of the lignin which leads to a greater porosity in the substrate [76]; removal of hemicellulose to increase the access of cellulases to cellulose [75]; disruption in the hemicellulose structure [75]; reducing the degree of polymerization and the crystallinity of cellulose [75]; reducing the size of the particle [66].

11.5.2 Enzymatic hydrolysis of the plant cell wall

Enzymatic hydrolysis is an advantageous method to achieve fermentable sugars in a eco-friendly reaction from the pretreated biomass [65], so the majority of strategies for converting lignocellulosic biomass to ethanol or other liquid fuels involve the enzyme-catalyzed depolymerization of polysaccharides [77]. Enzymes have benefits over the traditional chemical treatments due to the higher conversion efficiency, no substrate loss because of chemical modifications and the use of moderate physical-chemical conditions (lower reaction temperatures, almost neutral pH, and the use of biodegradable and nontoxic reagents), which makes the process more environmentally friendly [78]. Nevertheless, enzymes are costly since they must be produced by living organism. In view of the recalcitrance of lignocellulose, high enzymes loadings are necessary to obtain valid degradation rates. The price of production of

enzymes and the pretreatment costs are hindering the development of an economically possible second generation bioethanol industry [77]. The rate and the productivity of conversion of biomass to fermentable sugars depends on the starting biomass material, the pretreatment and action of a wide amount of enzymes [74].

A great variety of enzymes are required to deconstruct the cell wall structure because of its recalcitrant nature and heterogeneity. These enzymes are produced by a wide range of fungi and bacteria, *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase bioethanol production [79]. Among fungi, the most used species are Trichoderma reesei, Aspergillus niger, Trichoderma longibrachiatum [80], *Sclerotium rolfisii, Phanerochaete chrysosporium* beyond species from *Schizophyllum* and *Penicilium* [79]. Mutant strains of *Trichoderma sp.* (*T. viride, T. reesei, T. longibrachiatum*) have long been considered to be the most productive and powerful destroyers of crystalline cellulose [81].

Hydrolytic enzymes like cellulases, xylanases, manannases contributes to the degradation of holocelulose structure by hydrolysis of glyosidic bounds [82]. Swollenins facilitate the hydrolysis by breaking down hydrogen bonds between cellulose microfibrils or cellulose and other polysaccharides [82]. Oxidative enzymes like lytic polysaccharide monooxygenases helps in the degradation of cellulose chains by an oxidative mechanism of intrachain cellobiosyl moiety [83]. For the complete hydrolysis of holocellulase, the synergistic action of a broad range of endo and exo enzymes with different specificities is necessary. Xylan degrading enzymes are produced by a variety of fungi and bacteria, like *Trichoderma* spp., *Penicillium* spp., *Talaromices* spp., *Aspergillus* spp., *Bacillus* spp. [18].

11.5.2.1 A brief overview on enzymatic degradation of the plant cell wall

As mentioned in the last section, the complete degradation of lignocellulosic biomass requires the coordinate action of enzymes with different specificities.

As mentioned before, lignocellulose contains significant amount of cellulose which can be used as a valuable carbon source for production of value-added chemicals [84]. Cellulose is degraded by the synergistic action of endoglucanases (EC 3.2.1.4), that randomly act on soluble and insoluble cellulose chains; cellobiohydrolases (EC 3.2.1.91) which release cellobiose from the nonreducing (CBH I) and reducing (CBH II) ends of the cellulose chain; and β-glucosidase (EC 3.2.1.21) release glucose from cellobiose [82].

Hemicellulases are frequently classified according to their action on distinct substrates. Considering the heterogeneous structure and complex chemical nature of xylan, its fully hydrolysis requires a large amount of enzymes, including endo-β-1,4 xylanase (EC 3.2.1.8), which hydrolyze the glycosidic bonds in the xylan backbone releasing oligosaccharides with small degree of polymerization. β-xylosidases (EC 3.2.1.37) that catalyze the hydrolysis of small xylooligosaccharides and xylobiose, releasing xylose from the non-reducing terminus [85]. Enzymes that acts on side chain include acetylxylan esterases (EC 3.1.1.6) that remove the O-acetyl groups of acetyl xylan [85], α-L-arabinofuranosidases (EC 3.2.1.55) that cleave the terminal α -L-1,2-, α -L-1,3- and α -L-1,5-arabinofuranosyl residues [82], α-glucuronidases (EC 3.2.1.-) cleave the α-1,2 bonds between glucuronic acid residues and the main chain in glucuronoxylan [85]. Moreover, esterases activities are observed in feruloyl esterases (E.C. 3.1.1.73) and p-coumaloyl esterases (E.C. 3.1.1.73) which are capable of hydrolysate the ester-

link between arabinose and monomeric or dimeric ferulic acid and the linkages between arabinose and p-coumaric acid, respectively [82, 85]. Other enzymes are also involved in the modification of xyloglucans, like endo-transglycosylase/hydrolases (2.4.1.2070), a xyloglucan-active β-D-galactosidase (3.2.1.23), a xyloglucan specific α-L-fucosidase (3.2.1.51/3.2.1.63) and a xyloglucan oligosaccharide-specific α-D-xylosidase (3.2.1.37) [86].

The mannan-degrading enzymes system is composed of several enzymes including β-mannanase (EC 3.2.178), that randomly hydrolyses the β-1,4 linkages of the mannan backbone, β-mannosidase (EC 3.2.1.25), an exo-type enzyme that hydrolyzes the β-1,4-linked mannosides, releasing mannose from the nonreducing end of mannans and mannooligosaccharides. β-glucosidase (EC 3.2.1.21) an exo-type enzyme, cleaves the β-D- 1,4 glucopyranose at the nonreducing end of the oligosaccharides released from glucomannan and galactoglucomannan by β-mannanase [87]. Other additional enzymes are necessary, such as acetyl mannan esterase (EC 3.1.1.6) and α-galactosidase (EC 3.2.1.22), which are debranching enzymes. The first one cleaves the linkage in the acetyl groups from galactoglucomannan, while the second one cleaves the α-1,6 linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan [87].

With regards to the degradation of pectic substances, the enzymes involved in this process may be divided in three major groups. The hydrolytic enzymes, which includes polygalaturonases, like endopolygalaturonase (EC 3.2.1.15) and exopolygalacturonase (3.2.1.67) cleaves polygalacturonic acid chain forming oligogalacturonates and monogalacturonates, respectively. Pectinesterases (EC 3.1.1.11) catalyze the deesterification of methyl ester linkages of galacturonan backbone of pectic substances releasing acidic pectins and methanol. Lyases carry out the breakdown of pectinases through trans-elimination. Some exemples of these third group include endopolygalacturonase lyase (EC 4.2.2.2) and exopolygalacturonase lyase (EC 4.2.2.9) that act on pectic acids releasing unsaturated oligogalacturonates and digalacturonates, respectively [88].

Beyond the holocellulolytic enzymes, laccases, lignin peroxidase and manganese peroxidase are fundamental for delignification process. The presence of laccase, alone or together with lignin peroxidase and manganese peroxidase, has been detected in a wide variety of white rot fungi, making clear its fundamental role during delignification [82]. Due to the complex and random phenylpropanoic polymer structure of lignin, enzymes involved in this process must have broad substrate specificity [89]. Laccases (EC 1.10.3.2) oxidize phenolic compounds and aromatic amines using the molecular oxygen as a terminal electron acceptor. Manganese peroxidases (EC 1.11.1.13) and lignin peroxidases (EC 1.11.1.14) are low substrate specificity enzymes capable of oxidizing lignin at the phenolic and non-phenolic aryl-ether positions, respectively [90].

Besides these hydrolytic enzymes, another kind of enzymes also has important roles in lignocellulosic biomass degradation. Oxidative enzymes, classified as auxiliary activities such as cellobiose dehydrogenase (EC 1.1.99.18) are secreted by cellulose degrading fungi. This enzyme is capable of oxidizing a variety of sugars including cellobiose, lactose and in some cases, glucose. During substrate oxidation the cofactor FAD is fully reduced [91, 92]. Other suggested roles for that enzyme includes the activation of hydrolytic cellulases by relief of cellobiose product inhibition [93]. Currently, cellobiose dehydrogenase are classified into the AA3 family [92]. Lytic polysaccharide monooxygenases (LPMO) are classified into the AA9

family (formerly known as GH61) and are mononuclear type II copper enzymes that require an external electron donor, a small molecule, or a partner redox- protein for activity. The breakdown of the glucosidic bounds occurs with the oxidation of C-1, C-4 and C-6. LPMO degrades cellulose using C-H activation followed by O_2-dependent chain cleavage. [83, 92].

Other proteins are also associated with the plant cell wall degradation. Swollenins, proteins with sequence similarity to the plant expansins, break down hydrogen bonds between cellulose microfibrils [82]. Swollenins contain carbohydrate binding domains (CBM) and are thought to interrupt cellulose structure via non-hydrolytic mechanisms, however, the biochemichal action of these proteins needs to be completely elucidated [94].

11.5.2.2 Second generation bioethanol global production

Commercial bioethanol is currently produced from starch/sugar-based crops like sugarcane, sugar beet, sweet sorghum, corn, wheat, barley, potato, yam and cassava. A huge increase in ethanol production was observed since 1990 when the commercial bioethanol production was 4.0 billion gallons in that year. It had a small increase in 2000 (4.5 billion gallons) and then rapidly increased to 23.3 billion gallons in 2010 [5]. In 2014 the global production of bioethanol was 24,565 million of gallons, United States and Brazil were the largest producers of bioethanol with 14,300 (58 %) and 6,190 (25 %) millions of gallons, respectively. Europe had a share of 6 %, China and Canada 3 and 2 % respectively, while Thailand, Argentina and India produced 1 % each. The rest of the world together represented 3 % of the total [16].

Developing new industrial technologies requires several test stages proportional to the production perspectives: the first step in bioethanol is assessing technology through lab-scale processes, in which reactor volumes are usually less than 1000 mL and involves manual intervention at varying degrees, and experiments a large range of parameters; pilot-scale then incorporates continuous processing under distinct operating conditions, with reactors extending between 1 and 100 L; demonstration-scale is implemented when most variables are established and the technology is substantially developed, in order to mitigate risks and prove to investors that the process is efficient and satisfies market expectations using up to 40,000 L capacity. This course of action is decisive towards accomplishing success in commercial-scale technologies considering factors such as solids handling, particles size and nature, flowability of feedstock delivering, among all things that interfere on plant engineering [95, 96]. A recent production of second-generation ethanol targeting the general public has been possible as a result of decades of research in several stages. Pilot and demonstration plants have produced smaller amounts with testing goals since early 2000's. Government support and subsides were vital for every commercial-scale plant established thus far; USA and Brazil are yet again leader countries in this subject, along with Italy [97]. The first commercial-scale cellulosic ethanol plant (Crescentino Bio-refinery, Crescentino, Vercelli, Italy) entered into full operation on October, 2013. The plant is running by the Italian company Beta Renewables to annually produce 20 million gallons of ethanol from wheat straw and giant reed using the patented "Proesa" technology in which plant biomass is pre-treated with steam (high temperature and pressure), followed by enzymatic hydrolysis [5].

Studies indicate that the total international production of ethanol from lignocellulosic biomass could reach 442 billion liters [63] based on agricultural waste data. This corresponds

to 5 times current 1st generation ethanol production of 87,2 billions [4]. Nevertheless, this production is only starting to emerge and 2014 has been a fruitful year, with at least three plants inaugurated and other four under construction (Tab. 11.1). Projects involve multiple companies from different countries uniting for technology development, process optimization and providing resources such as enzymes, area, and funding, physical structure and biomass material. This is a crucial moment to analyze results, strategies and reassess perspectives for the next few years.

Tab. 11.1: Examples of second generation bioethanol producing plants.

Location		Company	Scale	Ethanol production (Kl/year)	Feedstocks used	Ref
USA	City of Nevada Iowa	Dupont	C	114,000	corn stover	[98–100]
USA	Vonore Tenessee	Dupont, Genecor International	D	950	stalks, switchgrass,	[98, 99, 101]
USA	Hugoton City, Texas	Abengoa	C	95,000	corn stover, wheat straw, milo stubble and prairie grasses	[99, 102]
USA	Emmetsburg, Iowa	POET-DSM Advanced Biofuels	C	95	corncobs, leaves and husks	[99, 103]
USA	Vero Beach, Florida	INEOS Bio and New Plant Energy Florida LLC	C	30,400	yard, wood and vegetative wastes	[104, 105]
USA	Fayetteville, Ark	Bioenergy Resources Inc and INEOS	P	ND	wide variety	[106]
BRA	São Miguel dos Campos, Alagoas	Granbio	C	311,600	sugarcane bagasse and straw	[107]
BRA	Piracicaba, São Paulo	Raízen and Iogen Corporation	C	152,000	sugarcane bagasse and straw	[108–110]
CAN	Ottawa, Ontario	Iogen Corporation	D	700	cereal straws, corn stover, sugarcane bagasse, sugarcane tops and leaves	[109]
ITA	Crescentino	Beta Renewables	C	285,000	wheat straw, rice straw, giant cane	[111–113]
SWE	Örnsköldsvik	SEKAB	D	ND	ND	[114]

Tab. 11.1: (continued)

Location		Company	Scale	Ethanol production (Kl/year)	Feedstocks used	Ref
DNK	Kalundborg	DONG Energy	D	5,400	wheat straw	[115–117]
ESP	Babilafuente, Salamanca	Abengoa	D	5,000	grain, urban solid waste	[118]
RUS	Kirov	Biochemical Plant	C	40,000	wood waste	[119]

11.5.3 Fermenting microorganisms

Unlike the saccharification of starch and sugar-based feedstock that results mostly in hexoses, lignocellulosic feedstock is composed of cellulose and hemicellulose which results in hexoses (C6) and pentoses (C5). So, beyond an efficient pretreatment, is necessary the fermentation of C6 and C5 sugars to bring second generation bioethanol to commercial reality [120]. Principal choice for second generation ethanol production from lignocellulosic biomass by biological pathways is enzymatic hydrolysis followed by fermentation – separate hydrolysis and fermentation or simultaneous saccharification and fermentation (SSF). SSF may include the conversion of C5 and C6 sugars [121].

Generally, a microorganism to be used in bioethanol production should give a high ethanol yield, high productivity and be capable of withstand elevated ethanol concentrations. Moreover, the capacity of using different sugars are essential for SSF [121].

Commonly, the most used yeast for industrial ethanol fermentation is *Saccharomyces cerevisiae*, a facultative anaerobic yeast, only capable of glucose fermentation, which is used in starch or sucrose based ethanol production [120, 121]. *Zymomonas mobilis*, a gram-negative bacterium are also commonly used to convert C6 sugars. Both, *S. cerevisiae* and Z. mobilis, are adapted to ethanol fermentation, with a high ethanol tolerance, and amenability to genetic modifications. However, both are not capable of fermenting C5 sugars [122]. Since C5 sugars are not naturally fermentable by the most common used yeast (*S. cerevisiae*), a genetic engineered microorganisms are used to convert xylose, beyond glucose [123]. Other options of engineered microorganisms include *Escherichia coli* [124] and *Zymomonas mobilis* [125]. Comparative performance trials on glucose showed that *Z. mobilis* can achieve 5 % higher bioethanol yields and up to 5-fold higher bioethanol volumetric productivity compared to traditional *S. cerevisiae* yeast [126].

Naturally fermenting yeasts are also an option for fermentation of pentoses, Pichia stipitis is able to ferment a wide range of sugars like glucose, xylose, mannose, galacose and cellobiose, has a high ethanol yield and relatively little xylitol [127, 128]. Candida shehatae- and also offers a good potential, despite a low tolerance to ethanol, a low ethanol yield and inactivity at low pH. Thermophilic bateria, e.g. *Thermoanaerobacterium sacchaarolyticum, Thermoanaerobacter ethanolicus, Clostridium thermocellum*, are extreme anaerobic bac-

teria, resistant to an extremely high temperature. These bacteria can ferment a variety of sugars, display cellulolytic activity, but exhibit a low tolerance to ethanol [122]. Bacteria, like *Z. mobilis, E. coli, Klebsiella oxytoca* have attracted attention due to their rapid fermentation capacity, which can be minutes, compared to hours for yeasts [129].

11.6 Third generation bioethanol

The major discussion over biofuels production has been focused in the problems associated to the use of higher plants as its source, like the reduction of ecosystems and the increase in the food prices [130]. Moreover, the recalcitrant nature of plant biomass imposes some challenges to the utilization of this material to the production of bioethanol [131]. In this context, another potential source of production of biofuel to replace petroleum is using aquatic microbial oxygenic photoautotrophs (AMOPs), like cyanobacteria, algae and diatoms for this propose [132, 133]. The application of marine biomass as an energy source was investigate in USA and Japan in 1970s after the oil crises, but when the prices stabilized, the studies were discontinued [134].

Marine biomass significantly differs from terrestrial biomass in terms of their chemical composition. Some features of algaes and cyanobacterias have been attracting attention for the production of third generation bioethanol, among them:

1. AMOPs are buoyant aquatic microcells and for this reason structural biopolymers are not necessary. So, they present low percentage of lignin and hemicellulose compared to lignocellulosic plants, which reduces their recalcitrance [132, 133]. The reduced amount of lignin and holocellulases do not require pretreatment and bypass the expensive pretreatment processes required for hydrolysis of higher plants [133];
2. This kind of microorganism presents rapid growth [131] and have high productivity (in comparison to typical terrestrial crops), therefore the biomass cultivation for commercial production is efficient [130];
3. They can be cultivated in areas that do not present the competition with the production of feed and food [131];
4. They do not need freshwater for their cultivation [135];
5. They use a broad variety of water sources (fresh, seawater, wastewater, brackish) [130, 134], which allows the selection of species adapted for cultivation in locally available aquifers, or may even possess anaerobic metabolism aiming the production of hydrogen, ethanol and/or organic acids by autofermentation [133];
6. They are capable of execute photosynthesis using water as electron donor [130];
7. AMOPs are non-food based feedstocks [130]. The only exception is macroalgae which are used in a few East Asia countries for food, hydrocolloids, fertilizer, and animal feed, so it presents a low risk of competition for food in this countries [135];
8. They produce co-products of high value beyond biofuels [130];
9. Seaweeds do not require an internal transport system of nutrient and water, which saves energy and enhances the mass productivity in relation to land plants [136].

11.6.1 Algae

Based on morphology and size, algae can be classified as microalgae and macroalgae. Microalgae are microscopic organisms and macroalgae are composed of multi-cellular plant like structure. Macroalgae is composed of high amount of sugars (at least 50 %) that can be used in fermentation for bioethanol production [136]. Chemical composition beyond pigment and growth are affected by their habitats conditions such as light, temperature, salinity, nutrient, pollution, and even water motion [135].

The main carbohydrate-containing macroalgae are mannan; ulvan a polysaccharide mainly built on disaccharides repeating sequences composed of sulfated rhamnose and glucuronic acid, iduronic acid or xylose [137]; agar, which is made up of alternating β-D-galactose and α-L-galactose with scarce sulfations [135]; carrageenan, that consists of repeating D-galactose unit and anhydrogalactose, which may or may not be sulfated [135] and starch [135]. This carbohydrates are not present in microalgae [135]. As mentioned before, macroalgae almost do not contain lignin because they float in the water, so the cell wall of this organisms are structurally flexible [132, 135]. Microalgae are composed of starch and monosaccharides [135]. The residual algal biomass generated in the lipid extraction for biodiesel can be appropriately utilized for the production of bioethanol or biomethane [138].

Green algae such as *Spirogyra* and *Chlorococum* are microalgaes that have being shown to accumulate high amounts of polysaccharides which make them good candidates for bioethanol production [132]. Moreover, saccharification of red algae *Gelidium elegans* consisting of acid and enzymatic hydrolysis that converted galactan and glucan to galactonse and glucose, increased the ethanol concentration (5.5 %) which is higher than economically feasible concentration (4–5 %) for distillation (Lobban and Wynne, 1981, cited by Jung et al [135]).

11.6.2 Cyanobacteria

Concerning to biomass utilization, cyanobacteria presents some convenience in relation to eukaryotic algae. Cyanobacterial cell wall contains peptidoglycan layer that more closely resembles that of Gram-positive bacteria, consequently it can be degraded by lysozyme [131, 139]. Its cell wall is less complex and less diverse than that of algaes. Moreover, cyanobacteria possess glycogen as a storage carbohydrate which is preferred over starch as a fermentation feedstock due to the fact that in vivo glycogen mobilization by heating and enzymatic treatment requires less energy than starch mobilization [131]. Furthermore, cyanobacteria presents superior photosynthesis ability, converting up to 10 % of the sun's energy into biomass, which is much more than 5 % converted by algaes and 1 % archived by conventional crops like corn and sugarcane [130].

11.6.3 Bioethanol production

Polysaccharides can be converted to simple sugars and then fermented to produce bioethanol. Marine biomass contains unique different carbohydrates from those observed in terrestrial biomass, so the technology applied for terrestrial biomass cannot be applied to marine

biomass. Thus, the conversion to sugars and the choice of the appropriate microorganism is important for successful sugar fermentation [135].

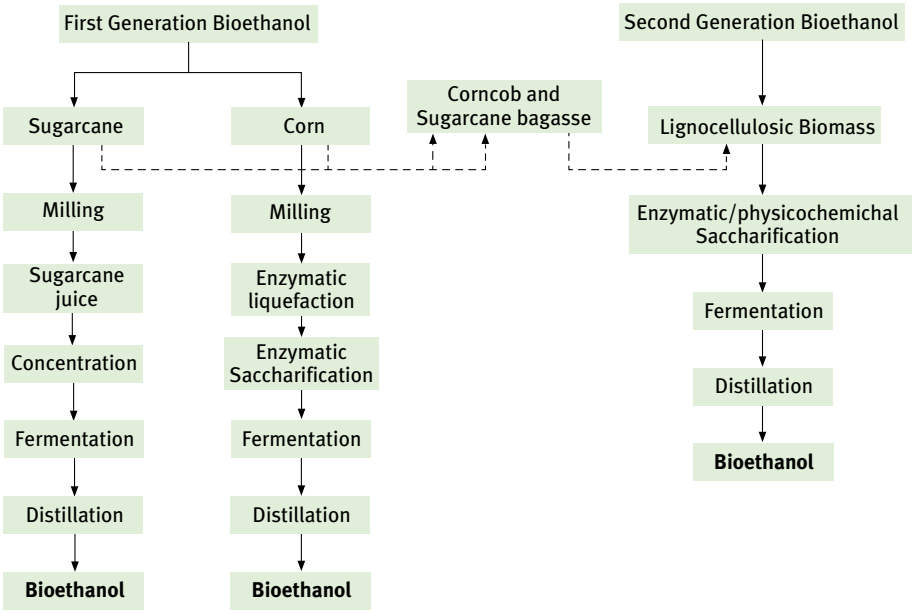
Hydrolysis of marine biomass has been performed either with physicochemical methods or enzymatic methods. In the acid hydrolysis, acid concentration and hydrolysis time influence the total yield of reducing sugars and consequently the ethanol production. Enzymatic hydrolysis is a soft approach to the saccharification of marine biomass. However, since marine organisms contain several types of polysaccharides, enzymatic hydrolysis of such materials for ethanol production has still been underdeveloped [135]. Chemical and enzymatic hydrolyses have been combined to more effectively obtain mono-sugars from seaweeds [140, 141]. Ge et al., [140] demonstrated that acid hydrolysis (dilute sulfuric acid pretreatment) expanded the reaction surface area and for this reason, the enzymatic hydrolysis (cellulases and cellobiases) were increased. The fermentation by *S. cerevisiae* resulted in a 41.2 % of ethanol conversion which corresponds to 80.8 % of the theoretical yield.

Pretreatment is used in an attempt to solubilize some polysaccharides, as laminarin. However pretreatment can inhibit microbial fermentation and for this reason a detoxification step is required to effective utilization of this biomass [135]. Heat and pH pretreatments reduced bioethanol yields from the brown *algae Saccharina latissima* [142].

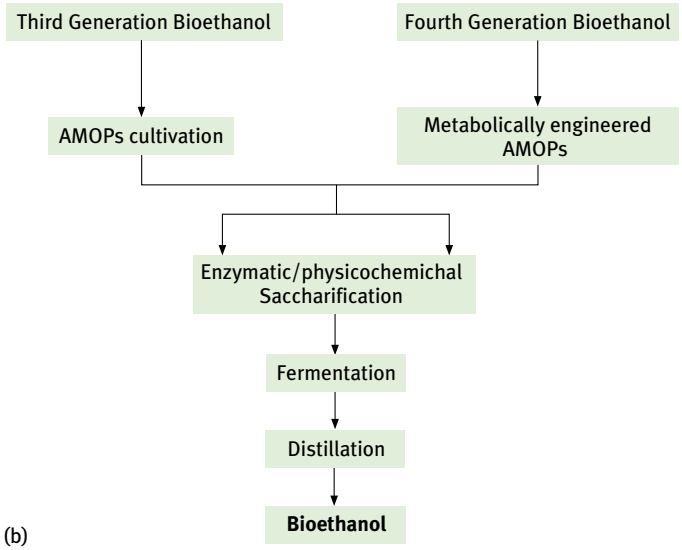
Besides pretreatment, contamination is also a problem for the cultivation of AMOPs. NREL (National Renewable Energy Laboratory) showed that non-native AMOPs species were contaminated by native AMOP species, creating a serious problem facing large-scale cultivation in open reactors [143]. Some improvements suggested include the selection of robust AMOPs that can be cultivated in environments were contamination is lower (high salt concentrations and high pH) [133].

Algal and cyanobacterial presents advantages over traditional energy crops. Nevertheless, to make its production economically feasible, environmental and economical obstacles must be overcome, with the advent of new technologies [130]. Improvements in efficiency, cost structure and ability to scale up AMOPs growth and biofuels production must be done to produce economically viable biofuels. For this purpose a defined set of new technology breakthroughs will be necessary to develop the optimum utilization of algal biomass for the commercial production of biofuel [138]. The main goal is to enhance the productivity of AMOPs to supply the demand of a rapid growing market. The inherent capacity of those organisms can be improved by genetic and metabolic engineering. The sequencing of AMOPs genomes brings facilities to cloning and manipulation of genes [144].

Metabolic engineered algae for production of biofuel is considered as fourth generation of biofuel and has great potential in providing sustainable and clean energy [145]. This new technology increases carbon entrapment ability, cultivation, harvesting, fermentation or oil extraction transesterification, or thermochemical process. This technique presents the advantages of improved yield with high lipid containing algae and more CO_2 capture ability, beyond high production rate. These crops are genetically engineered to consume more amounts of CO_2 from the atmosphere than they will produce later during combustion. Some fourth generation technology pathways include: pyrolysis, gasification, upgrading, solar-to-fuel, and genetic manipulation of organisms to secrete hydrocarbons [146]. However, the initial investment for four generation bioethanol is still high [147]. Figure 11.2 (a, b) summarizes the main differences and similarities among first to fourth generation bioethanol.



(a)



(b)

Fig. 11.2: Summary of the pathways for ethanol production from first and second generation (a), and third and fourth generation (b).

11.7 Conclusion and perspectives

Over the past one hundred years with the world's population growth and industrial development, the annual global primary energy consumption has increased nearly 10 times [5]. A wide range of microorganisms are currently used for biofuel production. Utilization of biofuels reduces the GHG emission which minimizes the greenhouse effect. Furthermore, crops used for production of bioethanol of second, third and fourth generations do not have the issue of competition with food crops.

Currently, a broad range of fungi and bacteria such as *Trichoderma* spp., *Aspergillus* spp., *Penicillium* spp., *Bacillus* spp., *Thermoanaerobacterium* spp., *Thermoanaerobacter* spp., and *Clostridium* spp., among others, have been used to enzymes production aiming to degrade lignocellulosic feedstock to bioethanol production. Traditionally, fermentation has been performed by *S. cerevisiae*, however, new possibilities have arisen for fermentation of second and third bioethanol like *Z. mobilis, P. stipilis, C. shehatae*.

Over the next years biofuel technologies are expected to change dramatically, with increasingly sophisticated industrial processing and a much more biotechnology-based agronomy [58]. In future, biofuels will present an important part in fulfilling the world's energy demands. To meet the expanding energy requirement, an uninterrupted energy supply is necessary [147]. In this context, lignocellulosic raw materials play a significant role, since they are cheap and abundant.

In terms of economic performance, today, first generation biofuel is the most cost effective fuel, however its production is limited to certain countries only as its production is highly land intensive. On the other hand, second and third generation biofuel presents elevated costs, mainly because of the high investment prices and low conversion efficiencies of feedstock into biofuel [147]. In this scenario, is necessary to improve the processes involving the production of second and third generation biofuels, aiming to reduce their costs and enhance their conversion efficiency.

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Anexo III

GH11 xylanase from *Emericella nidulans* with low sensitivity to inhibition by ethanol and lignocellulose-derived phenolic compounds

Artigo publicado na revista FEMS Microbiology Letters (Editora Oxford Academic), volume 362, fvn094, ano 2015. Fator de impacto: 1,765. Qualis B2 (Ciências Biológicas I).

Este artigo corresponde à publicação de resultados obtidos anteriormente ao início do doutorado. O resumo do trabalho foi incluído aqui em razão de ter sido escrito e publicado durante o doutorado e estar inserido no contexto geral da tese.

Este trabalho foca no tema relevante de inibição enzimática no contexto de hidrólise de biomassa lignocelulósica. O efeito de compostos fenólicos e etanol sobre a hidrólise de xilana pela endo-xilanase X_{22} purificada de extrato bruto de *E. nidulans* cultivado em bagaço de cana-de-açúcar foi testado, evidenciando que X_{22} tem baixa sensibilidade à inibição por alguns compostos fenólicos derivados de lignina e que o etanol aumenta a atividade e termoestabilidade da enzima. Os resultados indicam que X_{22} apresenta potencial para aplicação em processos de sacarificação e fermentação simultânea de biomassa pré-tratada.

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Research Letter

RESEARCH LETTER – Physiology & Biochemistry

GH11 xylanase from *Emericella nidulans* with low sensitivity to inhibition by ethanol and lignocellulose-derived phenolic compounds

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One sentence summary: The authors tested the effect of phenolic compounds and ethanol on xylanase X_{22} from *Emericella nidulans*, finding that some phenolic compounds do not inhibit X_{22} and that ethanol increases enzyme activity and thermostability.

Editor: Dieter Jahn

ABSTRACT

An endo-β-1,4-xylanase (X_{22}) was purified from crude extract of *Emericella nidulans* when cultivated on submerged fermentation using sugarcane bagasse as the carbon source. The purified protein was identified by mass spectrometry and was most active at pH and temperature intervals of 5.0–6.5 and 50–60°C, respectively. The enzyme showed half-lives of 40, 10 and 7 min at 28, 50 and 55°C, respectively, and pH 5.0. Apparent K_m and V_{max} values on soluble oat spelt xylan were 3.39 mg/mL and 230.8 IU/mg, respectively, while K_{cat} and K_{cat}/K_m were 84.6 s^{-1} and 25.0 s^{-1} mg^{-1} mL. Incubation with phenolic compounds showed that tannic acid and cinnamic acid had an inhibitory effect on X_{22} but no time-dependent deactivation. On the other hand, ferulic acid, 4-hydroxybenzoic acid, vanillin and *p*-coumaric acid did not show any inhibitory effect on X_{22} activity, although they changed X_{22} apparent kinetic parameters. Ethanol remarkably increased enzyme thermostability and apparent V_{max} and K_{cat} values, even though the affinity and catalytic efficiency for xylan were lowered.

Keywords: lignocellulosic biomass; purification; characterization; enzymatic inhibition; pre-treatment-derived compounds

INTRODUCTION

Agroindustrial residues, such as sugarcane bagasse (SCB), are abundant renewable sources of lignocellulose available for conversion into value-added products. The use of such materials as feedstock for biofuels, chemicals and materials production, within the scope of biorefineries, requires the development of enzyme blends that efficiently deconstruct plant cell wall structure (Siqueira and Filho 2010). Biomass pre-treatment steps are important to increase the susceptibility of lignocellulose to enzymatic hydrolysis (Hu and Ragauskas 2012). As a drawback, a variety of molecules are generated during biomass pre-treatment that may inhibit holocellulose-degrading enzymes (cellulases, hemicellulases and pectinases),

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Anexo IV

Certificação de trabalhos apresentados em eventos acadêmicos

- Gordon Research Conference/ Gordon Research Seminar: Cellulases & Other Carbohydrate-Active Enzymes. Apresentação de pôster: Hydrothermal pretreatment of sugarcane bagasse enhances holocellulases production by *Aspergillus foetidus*. Andover, New Hampshire, Estados Unidos da América, 2017.
- VII Simpósio do Programa de Pós-graduação em Biologia Molecular da Universidade de Brasília. Apresentação oral: Tratamento hidrotérmico de bagaço de cana-de-açúcar: ferramenta para aumentar produção de holocelulases com aplicação industrial por fungos filamentosos. Universidade de Brasília, Brasília, DF, 2017.
- XII Seminário Brasileiro de Tecnologia Enzimática. Apresentação de pôster. Hydrothermal pretreatment of sugarcane bagasse: a tool for obtaining hemicellulose rich liquors and its use for xylanase production by *Aspergillus foetidus*. Caxias do Sul, RS, 2016.
- VI Simpósio do Programa de Pós-graduação em Biologia Molecular da Universidade de Brasília. Apresentação de pôster: Tratamento hidrotérmico de bagaço de cana-de-açúcar: uma ferramenta para obtenção de licores ricos em hemicelulose e sua utilização na produção de hemicelulases por *Aspergillus foetidus*. Universidade de Brasília, Brasília, DF, 2016.
- V Simpósio do Programa de Pós-graduação em Biologia Molecular da Universidade de Brasília. Apresentação de pôster: Tratamento hidrotérmico do bagaço de cana-de-açúcar: uma ferramenta para produção de licores e sua utilização na produção de xilanases por *Aspergillus foetidus*. Universidade de Brasília, Brasília, DF, 2015.

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Brasilia, DF 70910-900
Brazil

This is a certificate of participation for:
Conference: Cellulases & Other Carbohydrate-Active Enzymes
Dates: 07/23/2017 - 07/28/2017
Location: Proctor Academy in Andover NH United States

This letter certifies your participation as a Poster Presenter at the Gordon Research Conference on Cellulases & Other Carbohydrate-Active Enzymes held 07/23/2017 - 07/28/2017 at Proctor Academy in Andover NH United States.

Presented poster titled: Hydrothermal pretreatment of sugarcane bagasse enhances holocellulases production by Aspergillus foetidus

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Seminar: Cellulases & Other Carbohydrate-Active Enzymes (GRS)
Dates: 07/22/2017 - 07/23/2017
Location: Proctor Academy in Andover NH United States

This letter certifies your participation as a Poster Presenter at the Gordon Research Seminar on Cellulases & Other Carbohydrate-Active Enzymes (GRS) held 07/22/2017 - 07/23/2017 at Proctor Academy in Andover NH United States.

Presented poster titled: Hydrothermal pretreatment of sugarcane bagasse enhances holocellulases production by Aspergillus foetidus

Dr. Nancy Ryan Gray, President and Chief Executive Officer
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Pós-Graduação Biologia Molecular

VII

VII Simpósio do Programa de Pós-Graduação em Biologia Molecular

Brasília - DF 08 a 10 Novembro 2017

CERTIFICADO

Certificamos que Caio de Oliveira Gorgulho Silva apresentou o trabalho na forma de pôster intitulado “Tratamento hidrotérmico de bagaço de cana-de-açúcar: ferramenta para aumentar produção de holocelulases com aplicação industrial por fungos filamentosos” no VII Simpósio do Programa de Pós-Graduação em Ciências Biológicas (Biologia Molecular), realizado na Universidade de Brasília, de 08 de novembro a 10 de novembro de 2017.

Sonia M. de Freitas

Profª. Dra. Sonia Maria de Freitas
Coordenadora do PPG-BioMol-UnB

ENZITEC 2016

XII SEMINÁRIO BRASILEIRO DE TECNOLOGIA ENZIMÁTICA

Certificamos que o trabalho

Hydrothermal pretreatment of sugarcane bagasse: a tool for obtaining hemicellulose rich liquors and its use for xylanase production by *Aspergillus foetidus*

de autoria de

Caio de Oliveira Gorgulho Silva, Bárbara C. Newmann, José Antônio de Aquino Ribeiro, Augusto Lopes Souto, Patrícia V. Abdelnur, Edivaldo Ximenes Ferreira Filho

foi apresentado no XII Seminário Brasileiro de Tecnologia Enzimática, realizado em Caxias do Sul/RS, no período de 17 a 20 de julho de 2016, na forma de pôster.

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VI Simpósio do Programa de Pós-Graduação em Biologia Molecular

Brasília - DF 30 Novembro a 02 Dezembro 2016

CERTIFICADO

Certificamos que o trabalho "Tratamento hidrotérmico de bagaço de cana-de-açúcar: uma ferramenta para obtenção de licores ricos em hemicelulose e sua utilização na produção de hemicelulases por Aspergillus foetidus" foi apresentado durante o VI Simpósio do Programa de Pós-graduação em Biologia Molecular: Doenças Tropicais Negligenciadas, realizado na Universidade de Brasília, de 30 de novembro a 02 de dezembro de 2016.

Autores: Caio de Oliveira Gorgulho Silva; Edivaldo Ximenes Ferreira Filho.

Sonia M de Freitas

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Biologia Molecular

V Simpósio do Programa de Pós-Graduação em Biologia Molecular

Brasília - DF 02 a 04 Dezembro 2015

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Certificamos que Caio de Oliveira Gorgulho Silva apresentou o trabalho "Tratamento hidrotérmico de bagaço de cana-de-açúcar: uma ferramenta para produção de licores e sua utilização na produção de xilanases por Aspergillus foetidus" no V Simpósio da Pós-Graduação em Biologia Molecular da Universidade de Brasília

Prof. Robert Neil Gerard Miller, Ph.D.
Coordenador do PPG-BioMol-UnB