UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

INFLUÊNCIA DOS COQUETÉIS ENZIMÁTICOS PRODUZIDOS POR *Trichoderma reesei* e *Aspergillus niger* PELO PROCESSO DE FERMENTAÇÃO SEQUENCIAL NA HIDRÓLISE DO BAGAÇO DE CANA-DE-AÇÚCAR

CAMILA FLORENCIO

SÃO CARLOS – SP
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Tese apresentada como parte dos requisitos para obtenção do Título de Doutor ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de São Carlos.

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Lista de Siglas e Abreviações

1G: primeira geração

2G: segunda geração

AA: Auxiliary Activities (Atividades Auxiliares)

BC: bagaço de cana-de-açúcar

BEX: bagaço de cana explodido, bagaço de cana pré-tratado por explosão a vapor

BIN: bagaço "in natura"

BSA: Bovine serum albumin (albumina sérica bovina)

CAZy: Carbohydrate-Active enzymes

CBH: celobiohidrolase

CBM: Carbohydrate-Binding Module (Módulo/Domínio de ligação a carboidrato)

CTC: Centro de Tecnologia Canavieira

CONAB: Companhia Nacional de Abastecimento

EG/EGase: endoglucanase

FES: fermentação em estado sólido

FSeq: fermentação sequencial

FSm: fermentação submersa

GH: glicosil hidrolase

HFS: hidrólise e fermentação separados

LPMO: Lytic polisaccharide mono-oxygenases (mono-oxigenases de polissacarpideos líticas)

MME: Ministério de Minas a Energia

MS: mass spectometry (espectrometria de massas)

PEG: polietileno glicol

SFS: sacarificação e fermentação simultâneas

UNCTAD: United Nations Conference on Trade And Developments

UNICA: União da Industria de Cana-de-Açúcar

Resumo

Atualmente, um dos grandes desafios para a produção de etanol de segunda geração consiste em diminuir o custo das enzimas celulolíticas. Assim, o desenvolvimento de bioprocessos para produção das enzimas on-site e estratégias para aumentar o rendimento final da hidrólise enzimática são necessários para assegurar que a conversão de biomassa seja economicamente viável. Para tanto, o objetivo deste trabalho foi estudar a produção e caracterização de coquetéis enzimáticos envolvidos na degradação da biomassa vegetal pelos fungos filamentosos *Trichoderma reesei* e *Aspegillus niger* cultivados por fermentação sequencial, bem como avaliar a aplicação dos mesmos no processo de sacarificação do bagaço de cana-de-açúcar. Primeiramente foi realizada a avaliação e validação da metodologia de cultivo de fermentação sequencial para diferentes linhagens de *Trichoderma*. Os cultivos foram feitos utilizando o bagaço de cana "in natura" e pré-tratado por explosão a vapor, como fonte de carbono. O melhor resultado foi observado para *T. reesei* Rut C30, em que a produção de endoglucanase foi 4,2 vezes maior do que os valores obtidos em cultivo convencional de fermentação submersa. Os extratos enzimáticos foram caracterizados em termos de pH e temperatura ótimos e perfil de endoglucanase. A termo-estabilidade foi diretamente influenciada pelo tipo de fonte de carbono e tipo de cultivo. Posteriormente, foram realizadas as análises proteômicas dos coquetéis enzimáticos do *T. reesei* Rut C30 e *A. niger* A12 produzidos por fermentação submersa convencional e fermentação sequencial, na presença de bagaço de cana pré-tratado. A performance dos coquetéis enzimáticos na sacarificação do bagaço de cana pré-tratado mostraram que a combinação dos coquetéis enzimáticos de *T. reesei* e *A. niger* produzidos por fermentação sequencial tiveram um rendimento 3 vezes maior do que os coquetéis da fermentação submersa. A fim de explorar melhor a ação dos coquetéis enzimáticos produzidos por *T. reesei* e *A. niger* na sacarificação do bagaço, na última etapa do trabalho foi estudo o efeito de aditivos durante a hidrólise do bagaco de cana visando à redução da adsorção improdutiva de enzimas na lignina. Os resultados de sacarificação na presença da proteína de soja foram 2 vezes maiores do que os controles (sem aditivo) para os coquetéis enzimáticos dos dois fungos estudados produzidos por fermentação em estado sólido, indicando o potencial do uso da proteína de soja como aditivo para minimizar a adsorção improdutiva das enzimas na lignina. De modo geral, o presente trabalho conseguiu uma contribuição final interessante no processo de produção das celulases e a aplicação do coquetel enzimático na hidrólise do bagaço de cana.

Palavras-chave: *Trichoderma reesei, Aspergillus niger*, enzimas hemicelulolíticas, processos fermentativos, hidrólise enzimática, bagaço de cana-de-açúcar.

Abstract

Currently, one of the major challenges for second generation ethanol is to reduce the cost of cellulolytic enzymes. Thus, the development of bioprocesses for the enzyme production on-site and strategies to increase the final yield of the enzymatic hydrolysis are required to ensure that biomass conversion to be economically feasible. Therefore, the objective of this work was to study the production and characterization of enzyme cocktails involved in the degradation of plant biomass by filamentous fungi *Trichoderma reesei* and *Aspergillus niger* grown in sequential fermentation and evaluate the application of these cocktails in the saccharification process of sugarcane bagasse. Firstly, evaluation and validation of sequential fermentation cultivation methodology to different strains of *Trichoderma*. Cultivation were made using sugarcane bagasse "in natura" and pretreated by steam explosion, as a carbon source. The result more significantly was observed for *T. reesei* Rut C30, the endoglucanase production was 4.2-fold higher than the values obtained in conventional submerged fermentation. The enzyme extracts were characterized in terms of optimum pH and temperature and endoglucanase profile. The thermostability was directly influenced by the type of carbon source and type of cultivation method. Subsequently, the proteomic analysis were performed of enzyme cocktails from *T. reesei* Rut C30 and *A. niger* A12 produced by submerged and sequential fermentation in the presence of pretreated bagasse. The performance of the enzyme cocktail in saccharification of pretreated bagasse showed that the combination of enzyme cocktails from *T. reesei* and *A. niger* produced by sequential fermentation had a yield than 3-fold higher than the enzyme cocktails of submerged fermentation. In order to evaluate the action of the enzyme cocktails produced by *T. reesei* and *A. niger* in sugarcane bagasse saccharification, the last step of the work was to study the additives effects during the sugarcane bagasse hydrolysis aiming at reducing non-productive adsorption of enzymes into lignin. The saccharification results in the presence of soybean protein were 2-fold higher than the controls (no additive) to the enzyme cocktails of two fungi studied produced by solid state fermentation, indicating the potential use of soybean protein as an additive to minimize non-productive adsorption of the enzyme into lignin. Overall, this study presents an interesting final contribution in the cellulase production process and the application of the enzyme cocktail in the hydrolysis of sugarcane bagasse.

Keywords: *Trichoderma reesei*, *Aspergillus niger*, hemicellulolític enzymes, fermentative process, enzymatic hydrolysis and sugarcane bagasse.

INTRODUÇÃO

A crescente preocupação com a escassez das reservas de petróleo e carvão mineral aliada à necessidade de preservação do meio ambiente, são os principais fatores para a busca de fontes renováveis para a produção de energia e de combustíveis sustentáveis (Pereira, Sandra Cerqueira *et al.*, 2015). Atualmente cerca 80% da energia primária consumida no mundo tem origem nos combustíveis fósseis (Morales *et al.*, 2015). Diante desse cenário, os biocombustíveis surgem como alternativa de novas fontes de energia, entre eles está o etanol de segunda geração (2G) produzido a partir de biomassas lignocelulósicas.

No entanto, a viabilidade econômica do processo de produção do etanol 2G depende de fatores como o desenvolvimento de novos processos para a produção de enzimas, visto que o alto custo desse insumo pode ser um fator limitante no processo de produção desse biocombustível (Klein-Marcuschamer *et al.*, 2012). Nesse sentido, o uso de materiais lignocelulósicos como fonte de carbono para o cultivo de microrganismos capazes de degradar os componentes desse tipo de biomassa vegetal, como o bagaço de cana-de-açúcar (BC), tem sido abordado na intenção de reduzir os custos na produção das enzimas celulases e, consequentemente, contribuir para diminuir os custos da hidrólise enzimática. (Chandel, Anuj K. *et al.*, 2012; Chandel, A. K. *et al.*, 2012).

Atualmente são usados dois métodos de cultivo convencionais para produção das enzimas celulolíticas: a fermentação em estado sólido (FES), caracterizada pela ausência de água livre, e a fermentação submersa (FSm), que ocorre em meio líquido (Singhania *et al.*, 2010). Cada um dos processos convencionais apresenta suas vantagens e desvantagens associadas a condições ambientais e operacionais. Alternativamente, outro método de cultivo, denominado de fermentação sequencial (FSeq), foi desenvolvido recentemente na tentativa de unir as vantagens dos processos de cultivo convencionais mencionados anteriormente. A FSeq é baseada na preparação do pré-cultivo inicialmente no estado sólido e posterior transição para estado submerso (Cunha, Esperanca*, et al.*, 2012; Florencio *et al.*, 2015; Vasconcellos *et al.*, 2015).

Em termos de microrganismos capazes de produzir enzimas celulolíticas capazes de degradar a biomasa, os fungos filamentosos se destacam, principalmente as linhagens *Trichoderma reesei* e *Aspergillus niger*. O fungo *T. reesei* tem sido amplamente utilizado na produção industrial de coquetéis enzimáticos celulolíticos (Gusakov, 2011; Jourdier *et al.*, 2013). Apesar de ter um número reduzido de enzimas celulolíticas em comparação

com outros fungos lignocelulósicos (Martinez *et al.*, 2008), o fungo *T. reesei* possui sistemas eficientes para o transporte de nutrientes e alta capacidade de indução/ secreção de celulases e hemicellulases (Castro *et al.*, 2014). O *A. niger* é considerado um dos mais importantes fungos para aplicações biotecnológicas, e diferentes linhagens industriais são comumente utilizadas na produção de enzimas entre outros produtos de alto valor agregado (Pandey *et al.*, 1999). Essa espécie de *Aspergillus* é capaz de produzir uma ampla gama de enzimas relacionadas a degradação de polissacarídeos vegetais tais como celulose, xilana, xiloglucano, pectina, entre outros (De Vries and Visser, 2001). Em *A. niger*, a expressão de todas as principais celulases e hemicellulases é regulada pela mesma molécula indutora, D-xilose, mas os mecanismos de indução do *T. reesei* são mais diversos (De Souza *et al.*, 2011).

A produção das principais celulases por ambos os fungos, *T. reesei* e *A. niger*, é controlada por um sofisticado sistema de regulação que evita o gasto de energia com processos desnecessários quando há fontes de carbono metabolizáveis presentes (Kang *et al.*, 2004; Schuster and Schmoll, 2010). Estudos de proteoma das linhagens *A. niger* e *T. reesei* para identificar proteínas secretadas na presença de bagaço de cana tem demonstrado que essa biomassa lignocelulósica é capaz de induzir a produção de diferentes tipos de celulases, hemicellulases, esterases e outras proteínas putativas importantes para a sacarificação do bagaço de cana, tais como proteínas acessórias não hidrolíticas que aumentam ou favorecem a eficiência enzimática (De Souza *et al.*, 2011; Borin *et al.*, 2015). O bagaço de cana-de-açúcar tem sido amplamente utilizado como uma biomassa lignocelulósica indutora, além de ser utilizado no processo de sacarificação para liberação dos açúcares fermentescíveis. Porém, há necessidade de um pré-tratamento dessa biomassa vegetal para aumentar a acessibilidade da celulose para a ação das celulases durante a etapa de hidrólise enzimática (Zhang, Zhanying *et al.*, 2012; Nasirpour *et al.*, 2014).

A hidrólise enzimática compreende uma etapa de adsorção das celulases no material lignocelulósico, porém a presença de lignina causa adsorção improdutiva dessas enzimas. As celulases de *T. reesei* possuem resíduos de aminoácidos hidrofóbicos expostos em sua superfície; assim, esses resíduos podem interagir com a superfície hidrofóbica da lignina, causando a adsorção improdutiva das celulases com a desativação das mesmas, reduzindo a eficiência do processo catalítico (Palonen *et al.*, 2004; Yang *et al.*, 2006; Börjesson, Engqvist*, et al.*, 2007). As celulases de *A. niger* também sofrem adsorção improdutiva, porém estudos demonstram um perfil diferente, por exemplo a β-

glucosidase de *A. niger* exibe menor adsorção a lignina do que a produzida pela linhagem *T. reesei* (Ko, Ja Kyong, Ximenes, Eduardo*, et al.*, 2015). Além disso, é menos afetada pela lignina de madeira pré-tratada do que outras enzimas, como celulases e hemicelulases (Sipos *et al.*, 2010).

Dessa forma, o presente estudo abordou aspectos relacionados a estratégias para a redução do custo das enzimas celulolíticas desde a etapa de produção "on-site", caracterização do coquetel e aplicação na sacarificação da biomassa vegetal. A produção enzimática foi estudada através de experimentos de validação do processo de fermentação sequencial para linhagens fúngicas do gênero *Trichoderma*. Para o entendimento da produção das celulases nesse método de cultivo foi realizada a caracterização em termos da estabilidade e atividade em função do pH e temperatura das endoglucanases produzidas pela linhagem *T. reesei*. Análises do secretoma foram usadas como ferramenta para identificação das proteínas secretadas por *T. reesei* e *A. niger* pelo método de cultivo convencional submerso e sequencial na presença de bagaço de cana pré-tratado. Além disso, aspectos relacionados ao processo de hidrólise enzimática do bagaço de cana, como a adsorção improdutiva sofrida pelas celulases na lignina foram avaliados para tentar melhorar a eficiência do processo. Foram avaliados diferentes tipos de aditivos, em especial a proteína de soja, para minimizar o efeito da adsorção improdutiva no processo de sacarificação.

Estrutura do trabalho

A Tese de doutorado aqui apresentada foi escrita na forma de Capítulos. No Capítulo I é apresentada uma revisão bibliográfica conceitual e atualizada, na qual é possível encontrar informações relevantes sobre o trabalho desenvolvido, além dos objetivos propostos na Tese de doutorado. O Capítulo II traz os resultados da etapa de validação da metodologia de produção de enzimas, através do processo de fermentação sequencial, para linhagens do gênero *Trichoderma*, em comparação ao processo convencional submerso. A caracterização dos coquetéis enzimáticos produzidos pelos dois tipos de cultivos citados acima foi realizada em termos de pH e temperatura, além da estabilidade. Esses resultados estão apresentados em forma de artigo, publicado na revista científica *Applied Biochemistry and Biotecnhology* e tem como título *"Validation*

of a Novel Sequential Cultivation Method for the Production of Enzymatic cocktails from Trichoderma strains".

No Capítulo III, são apresentados os resultados obtidos durante o período de doutorado sanduíche realizado na *Purdue University* (West Lafayette, EUA,) sob orientação do professor Dr. Michael R. Ladisch e coorientação do Dr. Eduardo Ximenes. Para este estudo foram feitas análises proteômicas dos coquetéis enzimáticos secretados pelos fungos *T. reesei* e *A. niger* em cultivo submerso e sequencial, utilizando o bagaço de cana pré-tratado por explosão a vapor (BEX) como fonte de carbono. Os coquetéis enzimáticos produzidos por cada um dos fungos foram combinados e avaliados no processo de hidrólise enzimática do bagaço de cana. Os resultados se encontram em forma de artigo, intitulado "*Secretome analysis of Trichoderma reesei and Aspergillus niger cultivated by submerged and sequential fermentation processes: enzyme production for sugarcane bagasse hydrolysis"*, submetido na revista científica *"Enzyme Microbial and Technology*", e também em um recente formato de artigo de dados, denominado *"Data in Brief"*, intitulado "*Secretome data from Trichoderma reesei and Aspergillus niger cultivated in submerged and sequential fermentation methods"*.

No capítulo IV, os coquetéis enzimáticos produzidos por *T. reesei* e *A. niger* sob três diferentes formas de cultivo (fermentação em estado sólido, submersa e sequencial) utilizando o bagaço de cana pré-tratado (BEX) como fonte de carbono, foram avaliados no processo de hidrólise enzimática do mesmo bagaço de cana, na presença de diferentes tipos de aditivos. Esses resultados são mostrados na forma de artigo, que também será submetido para revista científica. Por fim, no Capítulo V são apresentadas as conclusões e perspectivas quanto ao trabalho desenvolvido no presente doutorado.

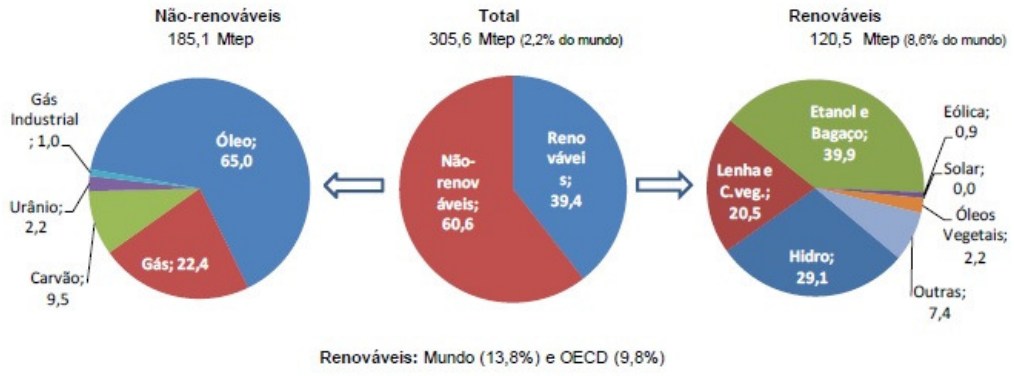
1. CAPÍTULO I

1.1. Revisão Bibliográfica

1.1.1. Etanol Segunda Geração e a Produção atual

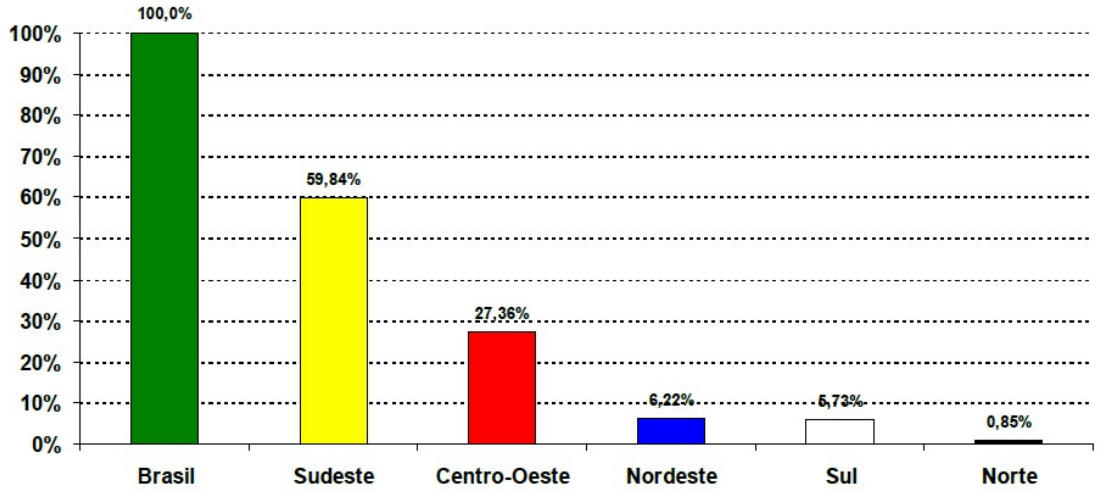
O desenvolvimento de biocombustíveis, como o etanol de primeira geração (1G), aconteceu como alternativa para a substituição dos combustíveis fósseis, na tentativa de diminuir a dependência das importações de petróleo e carvão mineral. Essa dependência coloca muitos países em situação de vulnerabilidade estratégica, devido a um possível esgotamento desses recursos, pois são fontes não renováveis de energia, além de sofrerem oscilações de preços no mercado internacional e muitos problemas ambientais. Diante desse tripé da sustentabilidade, meio ambiente, sociedade e economia, a busca por fontes renováveis e competitivas frente aos combustíveis fósseis acelerou no final do século XX (Ferraresi De Araujo *et al.*, 2013). Segundo o Ministério de Minas e Energia (2015), a matriz energética brasileira se dividiu no ano de 2014 em 60,6% para fontes não-renováveis e 39,4 % fontes renováveis. Tais valores são significativos quando comparado com os valores para os países que compõe a Organização para a Cooperação e o Desenvolvimento Econômico (OCDE), em que a oferta de fontes renováveis foi de 9,8%. A distribuição e a oferta interna de energia no Brasil, pode ser vista em detalhes na Figura 1.

Figura 1. Oferta Interna de energia no Brasil e distribuição entre fontes renováveis e não-renováveis no ano de 2014 (Ministério Minas e Energia, 2015).



O Brasil juntamente com os Estados Unidos são considerados os maiores produtores de etanol de primeira geração, e atingem 89% da produção mundial (Morales *et al.*, 2015). A produção total de etanol no Brasil chegou a 28 bilhões de litros na safra 2014/2015 (CONAB, 2014), sendo quase 60% da produção na região sudeste. A distribuição de etanol por região pode ser vista na Figura 2. No entanto, para atender à crescente demanda nacional e internacional por energia limpa e renovável, algumas medidas para aumentar a produção geram conflitos de caráter econômico, como competição direta com as matérias-primas, cana-de-açúcar e milho no mercado global de alimentos, e a concorrência direta com o uso de terra para agricultura (Ferraresi De Araujo *et al.*, 2013).

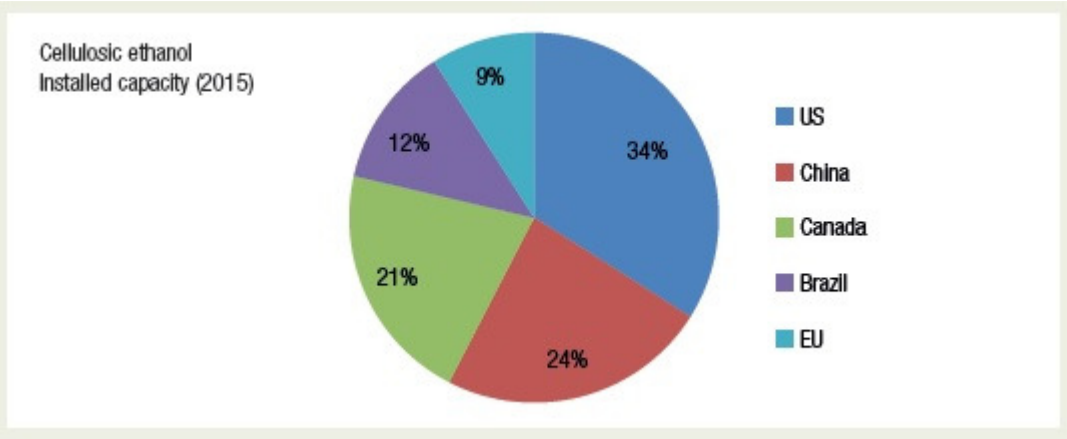
Figura 2. Produção brasileira de etanol por região (CONAB, 2014).



Para evitar a expansão desmedida das áreas de cultivo, o conflito direto com a produção de alimentos e diminuir impactos ambientais, muitos esforços têm sido realizados nas últimas décadas para desenvolver um processo comercial que permita a utilização de biomassas residuais de composição lignocelulósica para a produção de etanol de segunda geração, também chamado de etanol celulósico. Atualmente, os Estados Unidos da América (EUA) é o maior detentor da capacidade de produção do etanol 2G com instalações já implementadas, seguido por China, Canadá, União Européia (UE) e Brasil, respectivamente (Figura 3). O projeto de cada país varia significativamente em suas abordagens tecnológicas e tipo de matéria-prima usada para a produção do etanol celulósico, incluindo o uso de palha de milho, bagaço de cana,

palha de arroz, palha de trigo, resíduos sólidos urbanos e resíduos florestais, entre outros (Morales *et al.*, 2015).

Figura 3. Divisão dos países com produção de etanol celulósico implementado (UNCTAD, 2016).



A maior expectativa para o aumento do mercado de etanol celulósico vem dos Estados Unidos, uma vez que governo federal e empresas de capital de risco tem investido muito neste setor nos últimos anos. Europa e Japão são também mercados de etanol com grande possibilidade de expansão em um curto período, assim como a China (UNCTAD, 2016). Apesar do papel de destaque no mercado global de etanol celulósico, o mercado brasileiro está ainda em expansão, com aumento na capacidade de produção de unidades já existentes e novas unidades que começarão a operar em futuro próximo (Mussatto *et al.*, 2010). A Tabela 1 mostra a distribuição mundial de etanol celulósico em 2015 segundo a UNCTAD (2016).

A indústria de biocombustíveis de segunda geração no Brasil segue uma tendência de se desenvolver com base em infraestrutura e matéria-prima de logística existentes, que estão em vigor para a sua indústria estabelecida de primeira geração. Em 2014, a Usina São Manoel, associada a Copersucar, em parceria com o Centro Tecnologia Canavieira (CTC) inauguraram uma planta de escala de demonstração de etanol 2G, com capacidade para produzir 3 milhões de litros por ano. Segundo o CTC (2013) o projeto é diferenciado devido ao seu desenvolvimento ter sido especificamente para a biomassa do resíduo da cana (bagaço e palha) e ser totalmente integrado com a produção de etanol 1G já existente na usina.

A empresa GranBio, inaugurou em 2014 uma planta para geração de etanol 2G, chamada Bioflex 1, localizada em São Miguel dos Campos (AL). A capacidade inicial de produção é de 82 milhões de litros por ano de etanol e até 2020 a GranBio planeja investir R$ 4 bilhões em 10 novas unidades para alcançar a produção de 1 bilhão de litros de etanol celulósico por ano. Apesar de todo investimento a produção não será exclusiva de etanol 2G, pois irão ser instaladas unidades comerciais para a produção de bioquímicos e biorrefinarias flexíveis, que produzirão tanto bioquímicos quanto etanol celulósico. Em novembro de 2014, a empresa Raízen iniciou a operação da primeira usina exclusiva e com produção em escala comercial para a produção de etanol 2G, com capacidade de 42 milhões de litros de etanol por ano. Apesar da indefinição de metas internas para o consumo de etanol celulósico e da falta de legislação oficial no Brasil, de acordo com (Milanez *et al.*, 2015), a perspectiva é que o Brasil contará com uma participação de 10 bilhões de litros de etanol 2G em 2025. As previsões levam em conta que as políticas públicas sejam implementadas a fim de incentivar a produção e o consumo de etanol celulósico (Tabela 1). O ano de 2015 foi um marco na bioeconomia, como o momento em que a produção de segunda geração de biocombustíveis (2G) finalmente decolou em escala comercial (UNCTAD, 2016).

Tabela 1. Potencial produção de etanol 2G no Brasil (bilhões de litros (L)) por tipo de investimento.

	Potencial Produção (bilhões de L)		
Tipo de investimento	**2016-2020**	**2021-2025**	**Total**
Retrofit das Usinas existentes para etanol 2G	2,50	2,50	5,00
Expansão das Usinas com etanol 2G	0,75	0,75	1,50
Novas Usinas com etanol 2G	0	3,50	3,50
Total	3,25	6,75	10,00

Fonte: BNDES.

1.1.2. Produção Enzimas envolvidas na hidrólise enzimática

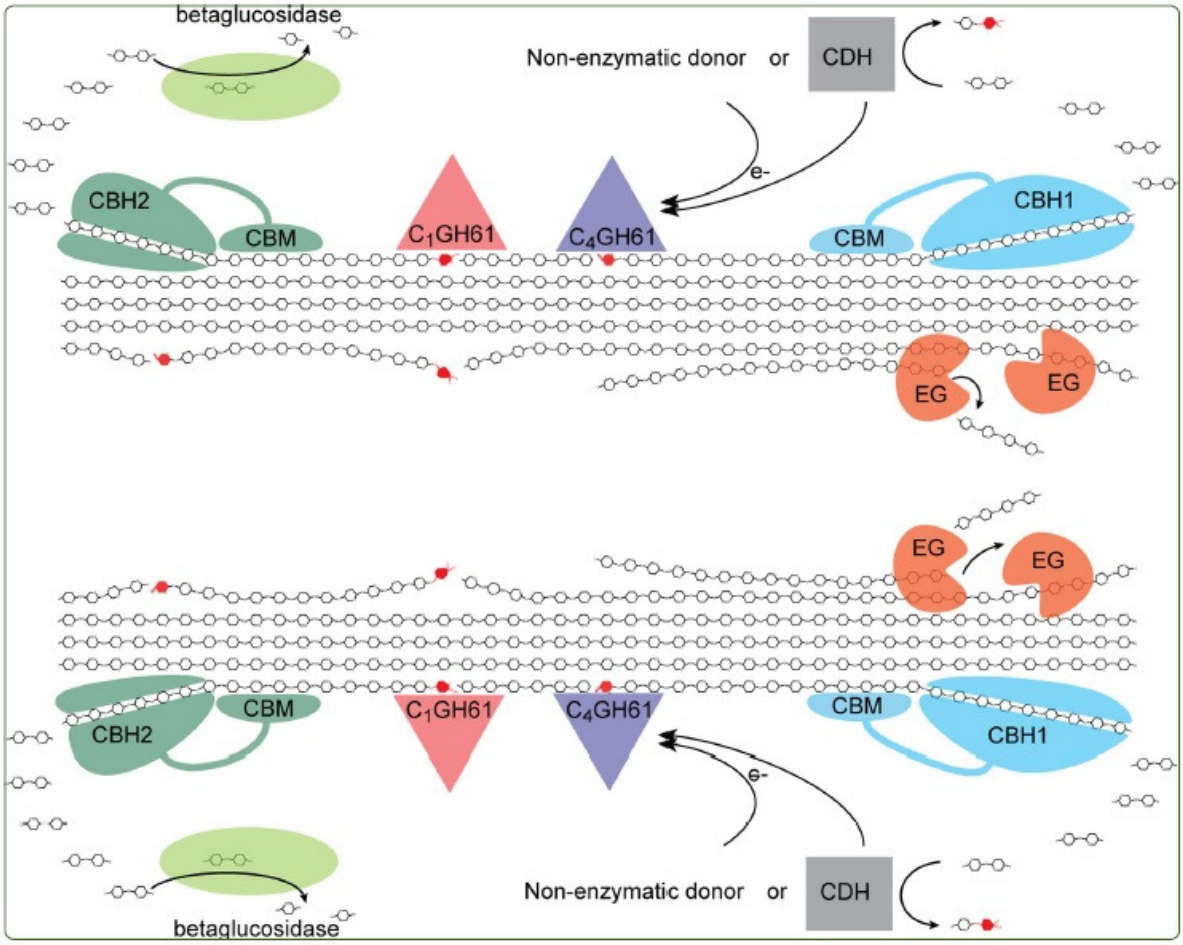
As enzimas são biocatalisadores de reações que compõem vias anabólicas e catabólicas do metabolismo celular. O poder catalítico desse grupo de proteínas está associado a conformação nativa que depende de algumas condições específicas como

pH e temperatura. Essas moléculas orgânicas possuem elevado potencial biotecnológico e o uso em processos industriais de modo geral é altamente atrativo devido a sua especificidade biocatalítica, baixa toxicidade química e redução de bioprodutos indesejáveis (Bhat and Bhat, 1997).

A degradação da parede celular dos vegetais requer um arsenal de enzimas catalíticas e não-catalíticas produzidas por microrganismos lignocelulolíticos. Dentre as classes enzimáticas responsáveis pela hidrólise da biomassa lignocelulósica, encontram-se as celulases, capazes de atuar na hidrólise do polímero de celulose através da ação sinérgica dos seus três principais grupos de enzimas: Endoglucanases (EGases/EG) clivam aleatoriamente as ligações β-1,4 das áreas amorfas da celulose e geram novos terminais redutores e não-redutores. Estas enzimas são classificadas em várias famílias CAZy (Carbohydrate-active enzymes), nomeadas como GH5, GH7, GH12 e GH45, e compartilham uma estrutura comum que inclui uma grande fenda contendo aminoácidos catalíticos (Gupta *et al.*, 2016); Celobiohidrolases (CBH) são enzimas processivas que liberam celobiose a partir de ambas as extremidades, redutoras (GH7) e não-redutoras (GH6) dos fragmentos de celulose produzidas pelas endoglucanases; e beta-glucosidases (GH1 e GH3) que hidrolisam celobiose ou celo-oligossacarídeos em glicose, estas enzimas são caracterizadas por uma topologia que permite a detecção dos terminais não-redutores e a clivagem em unidades de açúcares (Figura 4).

Os microrganismos mais estudados e responsáveis pela produção de enzimas lignocelulolíticas são as bactérias e fungos, pois são capazes de secretar as enzimas que degradam as paredes celulares vegetais sinergicamente (Dashtban *et al.*, 2009). Porém, a hidrólise enzimática é um processo complexo, a presença de hemicelulose e lignina nos substratos lignocelulósicos limita a ação das enzimas hidrolíticas, fazendo-se necessária a remoção ou modificação química desses componentes por pré-tratamentos químicos e físicos. Deste modo, outras enzimas acessórias são necessárias para degradação da biomassa lignocelulósica, como as hemicelulases, mono-oxigenases, entre outras (Shallom and Shoham, 2003).

Figura 4. Ação das celulases e mono-oxigenases no polímero de celulose (Horn *et al.*, 2012).



O custo para a obtenção de enzimas ainda limita sua utilização em vários processos industriais, inclusive para as celulases aplicadas a produção do etanol 2G (Zhang *et al.*, 2009), por isso alternativas para diminuir o custo tem sido objetivo de inúmeros trabalhos encontrados na literatura. Esses estudos buscam aumentar a produção das celulases, através da seleção da fonte de carbono (Juhasz *et al.*, 2005; Dashtban *et al.*, 2011), da seleção de microrganismos capazes de secretar uma alta quantidade de enzimas e um coquetel enzimático eficiente (Delabona, Farinas, *et al.*, 2012; Delabona, Pirota, *et al.*, 2012), do entendimento da composição do coquetel celulolítico secretado por esses microrganismos (Gomez-Mendoza *et al.*, 2014; Borin *et al.*, 2015), da complementação do coquetel enzimático com outras enzimas (Banerjee *et al.*, 2010; Pinto Braga *et al.*, 2014), entre outros estudos.

A redução de custos na utilização de enzimas celulolíticas tem sido o centro de esforços na comercialização de etanol a partir de biomassa lignocelulósica, por isso

estabelecer um método de avaliação sobre o custo das enzimas do complexo celulolítico é crucialmente importante para apoiar o desenvolvimento futuro da indústria de biorrefinaria. Atualmente, os métodos de avaliação de custos das celulases são complicados e possuem vários resultados controversos, ou mesmo conflituosos (Liu *et al.*, 2016). Em termos econômicos, no ano de 2001, a obtenção de enzimas a partir de microrganismos celulolíticos era em torno de 50% do custo global do processo de produção do etanol 2G (Wyman, 2001). Diferentes estudos relacionados ao custo das enzimas nesse processo são contabilizados em dólares/galão de etanol celulósico (Klein-Marcuschamer *et al.*, 2012). Alguns estudos reportam que o custo das celulases variou apenas de $0.1 a $0.4/gal de etanol, dando suporte a ideia de que a tecnologia atual era economicamente viável (Wingren *et al.*, 2005; Lynd *et al.*, 2008; Sassner *et al.*, 2008; Aden and Foust, 2009). Por outro lado, outros estudos pontuam o custo da enzima de $0.68 /gal etanol baseado em rendimento máximo teórico (Kazi *et al.*, 2010) e $1.47 /gal etanol se o rendimento for baseado na sacarificação e na fermentação, previamente reportados na literatura (Klein-Marcuschamer *et al.*, 2012). Com base no preço real de compra das celulases no mercado industrial de enzimas e na produção de etanol convencional, segundo estudo realizado em 2016, o custo da enzima é de até $2.71/gal etanol, sendo responsável por 48% do preço mínimo de venda do etanol celulósico (Liu *et al.*, 2016).

Tendo em vista esses valores do custo da enzima no processo de produção de etanol 2G a produção de enzimas “on-site” pode reduzir significativamente o custo da enzima, proporcionando uma alternativa promissora para a produção de etanol celulósico em larga escala. Estudos mostram uma redução significativa no custo da enzima, abaixo de $0.3 /gal de etanol, devido a sua purificação simplificada e logística, bem como a potencial utilização de fonte de carbono de baixo custo a partir de material lignocelulósico (Merino and Cherry, 2007). A comparação de custos da produção de celulases, para o rendimento de proteínas “on-site” e o retorno de investimento para produção “off-site”, segundo (Hong *et al.*, 2013), mostrou que as enzimas produzidas “on-site” reduziram até 30% do custo da enzima comparada com as enzimas comerciais. E segundo (Takimura *et al.*, 2013), a redução foi de até 70% no custo das celulases quando produzidas “on-site”, tendo como fonte de carbono a palha de arroz, em relação as celulases comerciais.

A classificação das enzimas em famílias ocorre de acordo com sua função no processo de clivagem ou construção de hidratos de carbono complexos e pode ser

encontrada na base de dados "Carbohydrate-active enzymes database" (CAZy). Descobertas recentes da função real de membros da família CBM33 (ação sobre chitina e/ou celulose) e família GH61 (Figura 4) como sendo mono-oxigenases de polissacarídeos líticas (LPMO), resultou na inserção dessas enzimas em uma nova categoria chamada de "atividades auxiliares" (AA) (Levasseur *et al.*, 2013). Essas enzimas integram um grupo de módulos catalíticos envolvidos na degradação parede celular vegetal, por isso essa nova classificação AA fornece uma visão complementar das enzimas lignocelulolíticas, concentrando-se em famílias de enzimas oxidativas.

A desconstrução completa da lignocelulose em açúcares fermentescíveis por enzimas requer a atividade combinada não apenas das celulases (endoglucanases, exoglucanases e β-glicosidases), mas também das ligninases (manganês peroxidase, lignina peroxidase e lacase) e hemicelulases (xilanases, xilosidases, mananases, arabinases, entre outras). A presença ou adição de enzimas que despolimerizam a pectina e hidrolizam substituintes da cadeia lateral podem melhorar em alguns casos, a taxa de hidrólise da biomassa (Gupta *et al.*, 2016). A descoberta dessas novas funções assim como de outras enzimas acessórias (Rogowski *et al.*, 2014) representa uma evolução no processamento enzimático da biomassa e confirma que a ação das celulases hidrolíticas clássicas é facilitada pela ação das LPMOs e de outras enzimas acessórias, melhorando o processo de hidrólise enzimática da biomassa lignocelulolítica (Horn *et al.*, 2012) e auxiliando na tentativa da redução dos custos do coquetel enzimático.

1.1.3. Processos de Cultivo para produção enzimática

Há muitos anos os processos fermentativos são de grande importância prática e econômica para a espécie humana. Diversos produtos de interesse comercial, fabricados por processos de fermentação através de microrganismos, têm sido aplicados com sucesso em diferentes setores que incluem farmacêutico, têxtil, alimentar, entre outros (Singhania *et al.*, 2009; Sanchez and Demain, 2011). Além, do grande potencial para o uso desse processo em novas aplicações, como na conversão de materiais lignocelulósicos gerados a partir da agroindústria.

No Brasil, onde a agricultura é uma das principais fontes de atividade econômica, a geração de resíduos florestais e agroindustriais é considerável. O principal resíduo agroindustrial é o bagaço de cana, gerado a partir das usinas que produzem açúcar e etanol de primeira geração. A cada tonelada de cana-de-açúcar moída na fabricação

desses dois produtos é gerado em média 250 kg de bagaço e 200 kg entre palha e ponteira (UNICA, 2012). Uma parte do excedente dos resíduos agroindustriais atualmente é utilizada para produção de bioeletricidade, enquanto outra grande fração é queimada ou deixada no campo, muitas vezes tornando-se um problema ambiental (Farinas, 2015). Portanto, a bioconversão dos resíduos lignocelulósicos em produtos de alto valor agregado poderia proporcionar ajuda econômica e contribuiria para diminuição da poluição ambiental.

Os processos fermentativos realizados por microrganismos podem dar origem a diversos bioprodutos, dentre eles as enzimas industriais, em especial as celulases. O desenvolvimento dos processos biotecnológicos tem sido foco de grande parte dos esforços para a redução nos custos das enzimas. Esses processos podem ser conduzidos em meio sólido, chamado de fermentação em estado sólido (FES), em meio líquido, fermentação submersa (FSm), ou uma combinação dos dois métodos anteriores, metodologia recentemente desenvolvida por Cunha, Esperança, et al., (2012), chamada de fermentação sequencial (FSeq) e que tem gerado resultados positivos na produção de celulases. Grande parte dos avanços na produção de celulases microbianas foi desenvolvida para FSm, no entanto, o crescimento de fungos filamentosos produtores de enzimas celulolíticas ocorre naturalmente em condições similares à FES (Singhania *et al.*, 2009). Ambos os processos apresentam características positivas e negativas, as quais devem ser consideradas de acordo com o produto desejado e o microrganismo a ser utilizado.

No processo de FES o crescimento do microrganismo ocorre em substrato sólido com umidade suficiente apenas para manutenção do metabolismo e desenvolvimento microbiano, não há liquido na forma livre. A água indispensável para o crescimento é adsorvida num suporte sólido ou complexado no interior de uma matriz sólida. Para fungos filamentosos a FES é considerada interessante, pois suas características assemelham-se as condições sob as quais a maiorias das espécies fúngicas crescem na natureza (Holker *et al.*, 2004; Thomas *et al.*, 2013). Existem outras vantagens inerentes a FES como maior produtividade dos coquetéis enzimáticos, menor susceptibilidade a inibição pelo produto e substrato e são mais estáveis em termos de efeito de pH e temperatura (Holker and Lenz, 2005; Barrios-González, 2012). Do ponto de vista ambiental, uma vantagem importante da FES é a capacidade de utilizar substratos sólidos como resíduos agroindustriais, que servem como fontes de carbono e de energia para o crescimento do microrganismo e a produção de enzima (Farinas, 2015).

Na fermentação submersa o meio essencial consiste de água contendo nutrientes dissolvidos, sendo que a água pode constituir cerca de 90 a 99% da massa total do material. O processo de cultivo submerso apresenta vantagens relacionadas à instrumentação e controle dos parâmetros físicos-químicos, como controle de temperatura e pH. A FSm é considerada uma mistura homogênea, na qual a distribuição de nutrientes e metabólitos dentro do reator é semelhante para microrganismos, além disso esse tipo de cultivo pode contribuir para uma melhor absorção de nutrientes pelo microrgansimos e facilita a recuperação de metabólitos (Mathew *et al.*, 2008; Singhania *et al.*, 2010).

Alternativamente aos meios de cultivo convencionais, FES e FSm, foi descrito por Cunha, Esperança, et al, (2012) recentemente uma nova configuração de processo fermentativo, chamado de fermentação sequencial (FSeq). O processo é caracterizado pela preparação de pré-cultivo com fase inicial de crescimento fúngico sob estado sólido seguido por uma transição para estado submerso. A FSeq apresentou resultados significativos em relação ao processo submerso convencional, com produtividade de endoglucanase 3 vezes maior, sugerindo o potencial da técnica como uma alternativa promissora para a produção de enzimas hemicelulolíticas.

A morfologia de crescimento dos fungos difere de acordo com as condições de cultivo usadas, consequentemente, afetam de modo direto na produção enzimática. Para cada forma de fermentação usada tem sido investigado a influência de pH e temperatura, tipo de reator, tipo de meio nutriente, cultivo de cultura mistas, umidade ideal para cada microrganismo (Farinas *et al.*, 2011; Delabona, P. D. S., Perpetua Buzon Pirota, R. D., *et al.*, 2013; Rodriguez-Zuniga *et al.*, 2013; Cunha *et al.*, 2015; Florencio *et al.*, 2015). Atualmente a produção de enzimas comerciais tem sido feita pelo processo de fermentação submersa, (Said and Pietro, 2004). Cada processo fermentativo descrito acima apresenta suas vantagens e desvantagens no processo de produção enzimática, o importante na utilização de cada forma de cultivo é ter o entendimento adequado acerca dos parâmetros operacionais que envolvem cada processo fermentativo.

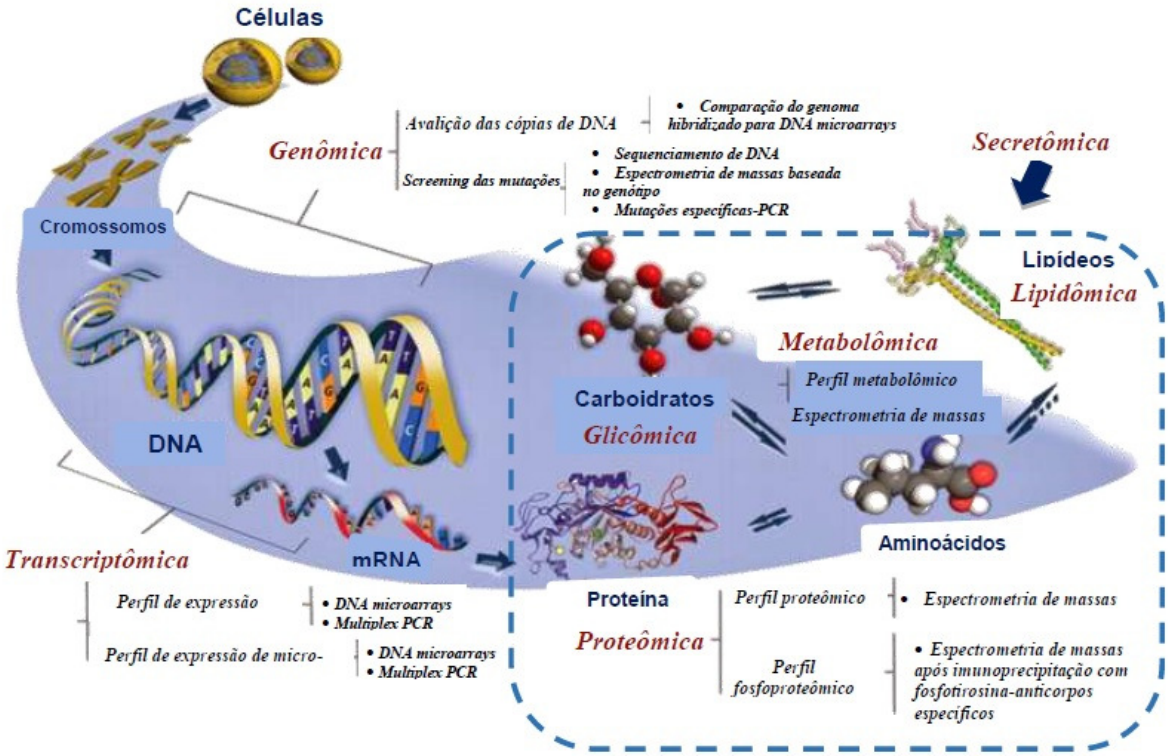
1.1.4. Enzimas fúngicas (Produção x Secretoma)

O mecanismo de produção e secreção das enzimas celulolíticas por microrganismos, em especial os fungos filamentosos, sempre foi alvo de estudos. A princípio acreditava-se na hipótese de que o mecanismo de reconhecimento molecular

de polissacarídeos vegetais consistia apenas na secreção constitutiva basal. Segundo este modelo, o microrganismo era capaz de secretar constantemente enzimas hidrolíticas em baixas concentrações. Tais enzimas degradariam os polissacarídeos em moléculas menores, as quais entrariam na célula provocando indução transcricional de determinados genes para enzimas (hemi)celulolíticas. Esta hipótese excluiria a possibilidade de existência de um receptor para o reconhecimento dos substratos na membrana plasmática. No entanto, muitos estudos têm mostrado outra via de sinalização hipotética. A sugestão da existência de uma proteína receptora (sensora) situada na membrana plasmática da linhagem *Trichoderma reesei*, possivelmente acoplada a uma via de sinalização celular específica que intensificaria a produção de algumas enzimas e induziria outras (Sternberg and Mandels, 1979; Karaffa *et al.*, 2006), vem em contraponto a primeira hipótese.

Um grande desafio da biologia está no entendimento da expressão, função, regulação do grupo de proteínas codificadas nos genomas fúngicos, o que forneceria importantes informações sobre mecanismos de colonização fúngicas, interação fungo-planta, patogênese e adaptação ecológica (Bhadauria, Popescu*, et al.*, 2007). Para melhor entendimento desses mecanismos, o uso de estratégias pós-genômicas, incluindo a proteômica faz-se necessário. A análise sistemática do proteoma, também definida como proteômica, trata-se do conjunto de proteínas expressas por um determinado genoma, célula ou tecido em uma condição específica. A proteômica permite identificar e quantificar o número de proteínas que influenciam diretamente a bioquímica celular, e prover uma análise do estado celular, ou mudanças que ocorrem durante o crescimento, desenvolvimento ou resposta a fatores ambientais, mostrando-se útil no estudo de sistemas biológicos altamente dinâmicos e complexos (Chen and Harmon, 2006; Bhadauria, Zhao*, et al.*, 2007). Devido à alta complexidade dos proteomas, uma estratégia comumente adotada é o estudo das frações específicas do proteoma total, ou seja, sub-proteomas (Figura 5). Sub-proteomas de organelas (mitocôndria e núcleo), glicoproteomas (proteínas glicosiladas), fosfoproteomas (proteínas fosforiladas) e secretoma (proteínas e/ou enzimas secretadas por um organismo) (Kim *et al.*, 2007).

Figura 5. Esquema das tecnologias ômicas, seus alvos de análise correspondente e seus respectivos métodos de estudo. DNA (genômica) é primeiramente transcrito a mRNA (transcriptômica) e traduzido a proteínas (proteômica) que catalisa reações, originando metabólitos (metabolômica), glicoproteínas e carboidratos (glicômica) e vários lipídos (lipidômica). Ao conjunto de produtos secretados por um microrganismo dá-se o nome secretômica (Sawyers, 2008).



A secreção de proteínas produzidas por fungos filamentosos é de extrema importância na nutrição dos mesmos e algumas dessas enzimas secretadas recebem atenção pelo potencial industrial que possuem, estimulando pesquisas relacionadas à genética e mecanismos de secreção dessas enzimas, como é o caso das enzimas celulolíticas. A análise do secretoma, definido como o conjunto de enzimas e demais proteínas secretadas por um determinado tipo celular, por um conjunto de células ou organismo (Tjalsma *et al.*, 2000), assim como entendimento da maquinaria responsável pela secreção destas proteínas, são indispensáveis para conhecer a identidade e função do arsenal de enzimas hidrolíticas extracelulares que participam na degradação de compostos lignocelulósicos e outros biopolímeros em resposta à adaptação a diferentes fontes de carbono e nitrogênio visando uma aplicação biotecnológica (Bouws *et al.*, 2008).

O melhoramento de técnicas de análise de separação e caracterização de proteínas combinada com avanços de espectrometria de massas (MS), tem permitido aumentar o conhecimento sobre as vias de secreção e a expressão diferencial de proteínas de fungos filamentosos com relevância biotecnológica, em relação as variáveis ambientais (Carberry and Doyle, 2007). Além disso, estudos do secretoma estão sendo direcionados não somente para o entendimento do papel dos fungos filamentosos na natureza, mas também como uma espécie de maquinaria celular capaz de secretar quantidades consideráveis de proteínas. No caso de fungos celulolíticos, o foco é na identificação de glicosil hidrolases e componentes acessórios envolvidos na degradação de polissacarídeos da parede celular de plantas (Ribeiro *et al.*, 2012).

Recentemente a correlação, produção de enzimas x fonte indutora de carbono tem sido amplamente estudada para identificar os substratos de maior efeito para a produção enzimática de celulases. Segundo a maioria dos autores a produção de diferentes tipos de enzimas celulolíticas secretada por fungos filamentosos é consequência da fonte indutora de carbono presentes no meio de cultura, dentre algumas estudadas estão lactose, soforose, D-galactose, sacarose, a própria celulose, entre outras (Jorgensen *et al.*, 2009; Verbeke *et al.*, 2009; Javier Fernandez-Acero *et al.*, 2010; Lu *et al.*, 2010; Mahajan and Master, 2010; Jun *et al.*, 2011; Jun *et al.*, 2013). Estudo realizado com *Aspergillus flavus* utilizando diferentes fontes de carbono para crescimento (glicose, batata dextrose e rutina) demonstraram proteínas secretadas diferencialmente expressas, foram encontradas proteínas comuns aos três meios, duas únicas para o meio de batata, 10 para o meio contendo apenas glicose e 18 proteínas no meio com rutina (Medina *et al.*, 2004).

As diferentes espécies de fungos filamentosos com interesse industrial têm tido o secretoma investigado nos últimos anos, como é o caso da linhagem amplamente conhecida como a mais eficiente produtora de celulases, *Trichoderma reesei*. Estudos do proteoma envolvendo esse fungo tem explorado diferentes fontes de carbono (Jun *et al.*, 2011), diferentes pHs (Adav *et al.*, 2011), diferentes características morfológicas (Chao *et al.*, 2012), entre outros. A composição proteica do secretoma produzido pela linhagem industrial *T. reesei* (CL847) cultivada em meio de cultura para a produção de celulases e hemicellulases foi explorada e posteriormente comparada com a linhagem super-produtora de celulases *T. reesei* Rut C30 (Herpoel-Gimbert *et al.*, 2008). Foram identificadas 22 proteínas potencialmente envolvidas na degradação da biomassa para o fungo CL847, enquanto estudos prévios haviam identificado apenas 10 proteínas

(Vinzant *et al.*, 2001). Uma maior diversidade no secretoma da linhagem CL847 sugere que este fungo possa ser um hiper secretor mais geral enquanto que linhagem *T. reesei* Rut C30 pode ser ter a produção mais orientada para as celulases (Herpoel-Gimbert *et al.*, 2008).

A comparação do secretoma da linhagem mutante *T. reesei* Rut C30 e a selvagem *T. reesei* QM6a crescidos em meio contendo celulose, serragem e palha de trigo foi estudada por (Adav, Chao*, et al.*, 2012), e os autores concluíram que as enzimas lignocelulolíticas no secretoma de ambas as linhagens são dependentes da fonte de carbono. A classificação funcional destas proteínas quantificadas revelou 31,3, 17,9, 13,4, 22,0, 6,3, 3,3, e 5,6% de celulases, hemicelulases, proteínas de degradação de lignina, peptidases, quitinases e fosfatases, transporte e proteínas hipotéticas, respectivamente. O sequenciamento do genoma da linhagem selvagem *T. reesei* QM6a realizado em 2008 (Martinez *et al.*, 2008), tem facilitado os estudos de sistemas biológicos deste fungo e verificou-se que *T. reesei* possui relativamente menos genes que codificam enzimas lignocelulolíticas do que muitos outros fungos sequenciados, com exceção de algumas hemicellulases (Kubicek, 2013).

A linhagem *Aspergillus niger* também tem sido alvo de diversos estudos secretômicos, pois sabe-se da sua capacidade como produtor de metabólitos primários e de enzimas lignocelulolíticas (Lu *et al.*, 2010). Estudos empregando diferentes fontes de carbono têm sido realizados para este fungo, a fim de induzir a produção de pectinases (Tsang *et al.*, 2009), celulases e xilanases (De Oliveira *et al.*, 2011). A utilização de xilose e/ou maltose como fonte de carbono tem a capacidade de afetar fortemente a composição do secretoma do *A. niger*, mas tem uma menor influência na composição do proteoma intracelular (Lu *et al.*, 2010). Esses autores observaram que a composição do proteoma extracelular foi completamente diferente para ambas as culturas realizadas em frascos agitados e biorreator. *A. niger* cultivado em xilose secretou principalmente hidrolases envolvidas na degradação de polímeros da parede celular vegetal enquanto o secretoma do *A. niger* cultivado em maltose foi dominado por glicoamilases, assim como as enzimas envolvidas na remoção das espécies reativas de oxigênio foram mais abundantes no proteoma extracelular.

Estudos de análises de secretoma utilizando resíduos agroindustriais como fonte de carbono para a produção de enzimas lignocelulolíticas são recentes, mas tem avançado rapidamente (De Souza *et al.*, 2011; Adav, Chao*, et al.*, 2012; Adav, Cheow*, et al.*, 2012; Da Silva *et al.*, 2012; Delmas *et al.*, 2012; Hakkinen *et al.*, 2012; Ribeiro

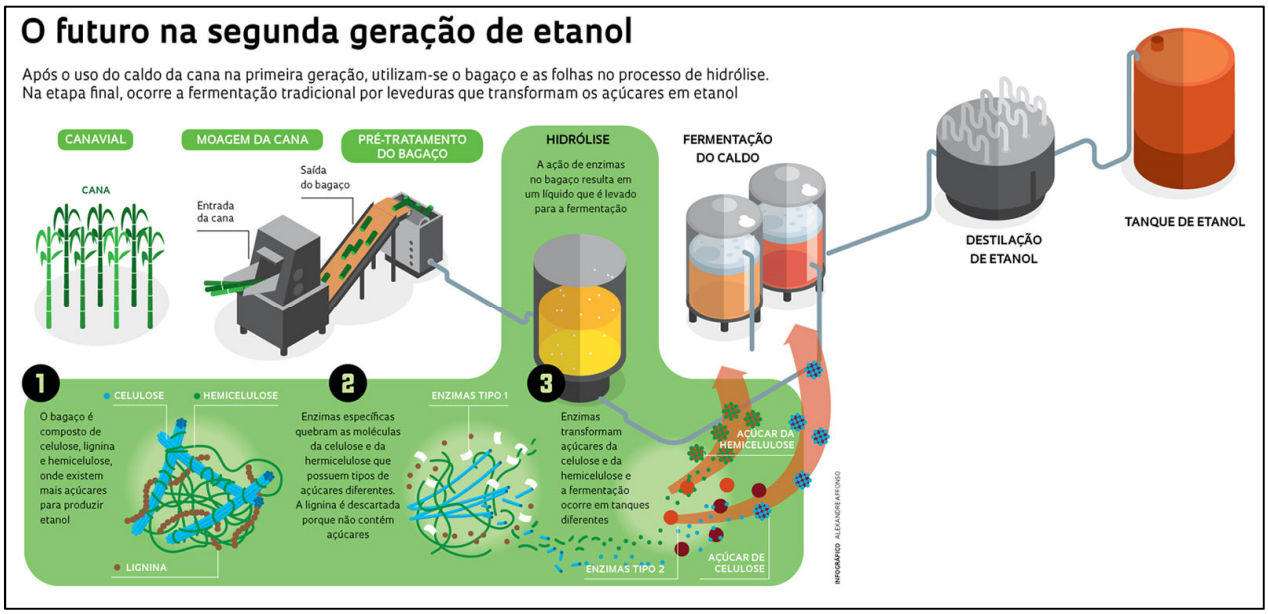
et al., 2012; Delabona, P. D. S., Cota, J.*, et al.*, 2013; Marx *et al.*, 2013; Crivelente Horta *et al.*, 2014; Gomez-Mendoza *et al.*, 2014; Borin *et al.*, 2015). A primeira análise global transcricional descrita usando bagaço de cana pré-tratado por explosão a vapor (BEX) e a linhagem *A. niger* foi em 2011 (De Souza *et al.*, 2011). Os estudos revelaram genes que são especificamente induzidos quando o BEX é usado como fonte de carbono. A degradação do BEX requer a produção de diferentes enzimas que são reguladas pelo tipo e complexidade do substrato disponível. Isto é essencial para compreender quais os genes que codificam enzimas hidrolíticas são induzidos na presença de bagaço de cana, já que a intenção foi produzir coquetéis enzimáticos para hidrolisar esta biomassa pré-tratada. Segundo este estudo foram identificadas 18 celulases e 21 hemicelulases, que representam 58% das enzimas preditas de *A. niger*.

Várias diferenças na regulação da produção de glicosil hidrolases entre os fungos *A. niger* e *T. reesei* já foram descritos (Stricker *et al.*, 2008; Glass *et al.*, 2013; Tani *et al.*, 2014), mas estudos comparativos poderiam proporcionar uma visão mais abrangente de como essas importantes espécies industriais produzem as enzimas hidrolíticas. (Borin *et al.*, 2015) realizaram uma análise comparativa do secretoma das linhagens *A. niger* e *T. reesei* cultivadas na biomassa da cana com dois níveis de complexidade diferentes, colmo *"in natura"* e bagaço pré-tratado por explosão a vapor. A produção das enzimas foi monitorada por 24 h e foi observado que ambas as linhagens são capazes de hidrolisar os polissacarídeos da parede celular da cana desde às 6 h pós-inoculação. O fungo *A. niger* produziu mais enzimas, em todos os pontos testados, do que o *T. reesei*, qualitativamente e quantitativamente. Entretanto, as enzimas mais importantes relacionadas a degradação da biomassa, incluindo celobiohidrolases, endoglucanases, β-glicosidases, β-xilosidases, endoxilanases, xiloglucanases e α-arabinofuranosidases foram identificadas em ambos os secretomas. Considerando a diferença no mecanismo de degradação da biomassa para as linhagens *A. niger* e *T. reesei* os dados obtidos por (Borin *et al.*, 2015) sugerem que uma combinação das enzimas a partir das duas espécies pode ser uma opção interessante para aumentar a eficiência da sacarificação.

1.1.5. Processo de Hidrólise Enzimática

A conversão de materiais lignocelulósicos segue algumas etapas até a produção final de etanol 2G, e as quatro principais são: pré-tratamento que tem como proposta modificar macro e microscopicamente a estrutura lignocelulósica, de maneira que a celulose fique mais acessível à ação de ácidos ou enzimas. Posteriormente, a etapa de sacarificação ou hidrólise, que irá disponibilizar os açúcares fermentescíveis. A terceira etapa é a fermentação dos açúcares liberados, realizada por microrganismos especializados. E a última etapa é a de recuperação do etanol, também conhecida como destilação (Figura 6).

Figura 6. Esquema de representação das etapas de produção do etanol 2G (Revista Fapesp, 2012).



Os processos devem ser analisados em sua totalidade, levando em consideração as várias operações e seus níveis de integração. A etapa de hidrólise da biomassa pode apresentar as configurações de hidrólise e fermentação separados (HFS) e sacarificação e fermentação simultâneas (SFS) (Olofsson *et al.*, 2008). No processo de HFS, as etapas de hidrólise e fermentação ocorrem em reatores separados, após o término da etapa de hidrólise da celulose completa a glicose é fermentada e o etanol produzido. Esse processo é vantajoso, pois é possível otimizar a operação de cada etapa, principalmente com relação aos parâmetros pH e temperatura. Porém, pode ocorrer inibição das enzimas pelas altas concentrações de produto liberado e o processo de hidrólise da

celulose não ser completo (Sun and Cheng, 2002). Na SFS as etapas são realizadas no mesmo reator e os microrganismos convertem a glicose em etanol, portanto nesse processo o efeito de inibição das enzimas pelo produto é reduzido (Cardona *et al.*, 2010). Além disso, a utilização de um único sistema para hidrólise e fermentação reduz o custo do processo. A maior desvantagem é quanto a otimização dos parâmetros. A temperatura de hidrólise da biomassa, por volta de 50 °C, é maior do que no processo de fermentação em que a levedura tem sua atuação ótima, 38 °C (Sun and Cheng, 2002).

As rotas tecnológicas empregadas na hidrólise da celulose que se destacam são: a química e a enzimática. A primeira envolve a exposição por determinado período de tempo da biomassa lignocelulolítica a um produto químico, talvez um ácido diluído, ácido concentrado (Balat, 2011) ou líquidos iônicos (Binder and Raines, 2010; Clough *et al.*, 2015), em uma determinada temperatura. As caraterísticas da rota química permitem que o processo de pré-tratamento e hidrólise do material sejam realizados em uma única etapa. A rota enzimática é uma alternativa ambientalmente favorável que ocorre pela ação sinérgica das enzimas do complexo celulolítico capazes de converter a celulose em açúcares fermentescíveis (Balat, 2011). Porém, a hidrólise enzimática é um processo complexo e outras enzimas são necessárias para que ocorra um processo completo de conversão da biomassa. A presença de outras classes enzimáticas como hemicellulases e ligninases são essenciais para a obtenção de maiores rendimentos na etapa de sacarificação, permitindo a redução da carga de celulases no processo (Hu *et al.*, 2011).

A presença da lignina no material lignocelulósico, além de ser uma barreira física para o processo de hidrólise, torna-se inconveniente, pois a lignina pode adsorver as celulases, causando perda de atividade devido a diminuição da quantidade de enzimas disponíveis, limitando a eficiência da sacarificação (Kim *et al.*, 2015; Ko, Ja Kyong, Kim, Youngmi*, et al.*, 2015). Portanto, estratégias que diminuam os efeitos inibidores da lignina são necessários para assegurar uma hidrólise enzimática eficiente da celulose (Kim *et al.*, 2015). Outro tipo de inibição sofrida pelo complexo celulolítico é a inibição pelos produtos liberados, glicose e celobiose, durante o processo de hidrólise enzimática, ocasionando a limitação da eficiência do processo (Hsieh *et al.*, 2014).

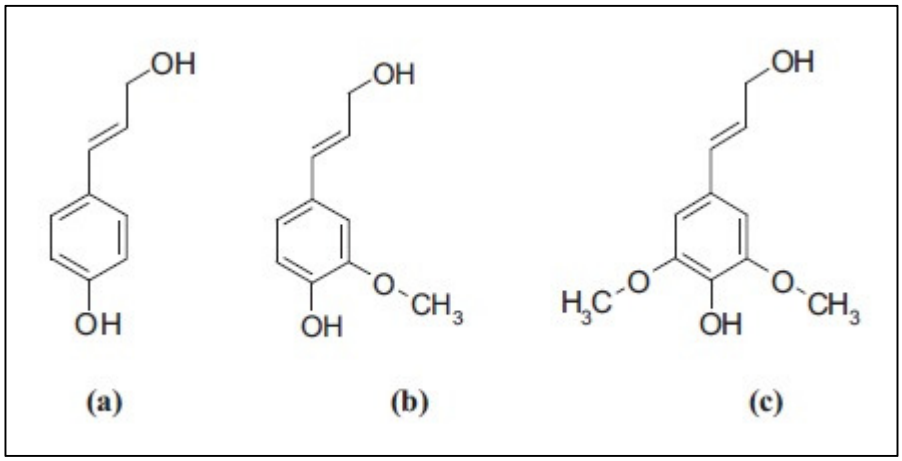
Os fatores que influenciam na etapa de hidrólise da biomassa lignocelulolítica tem sido estudados buscando a melhor condição para a obtenção do etanol 2G. Dentre esses estudos estão o tipo de substrato e o teor de sólidos (Ramos *et al.*, 2015), a atividade das enzimas e o melhoramento do coquetel enzimático (Borges *et al.*, 2014), os parâmetros

da reação, tais como temperatura e pH (Billard *et al.*, 2012; Zheng *et al.*, 2013) e o consumo de energia que proporcione as boas condições de mistura (Correa *et al.*, 2016). A escolha do coquetel enzimático, as condições globais e a estratégia do processo industrial, bem como as características da biomassa lignocelulósica devem ser investigadas buscando sempre o melhor desempenho para a produção de etanol celulósico (Cannella and Jorgensen, 2014). Todos esses fatores acerca das caraterísticas de organização e composição da biomassa, assim como os mecanismos envolvidos na degradação do material lignocelulósico, além das enzimas devem ser observados de forma global, pois todos afetam diretamente o rendimento da sacarificação (Jorgensen *et al.*, 2007).

1.1.6. Adsorção Improdutiva pela presença de Lignina

A lignina é uma biomolécula não polissacarídica, de natureza hidrofóbica, formada por componentes fenólicos e alifáticos, portanto é um material heterogêneo. Todas essas características conferem a lignina maior complexidade entre os constituintes do material lignocelulósico. A interação da lignina com a celulose e hemicelulose proporciona força e rigidez à parede celular vegetal, além da maior resistência a ataques biológicos de várias formas do que a celulose e outros polissacarídeos (Martone *et al.*, 2009; Zhang, Zisheng *et al.*, 2012). Diferentes materiais lignocelulósicos apresentam tipos distintos de lignina, sendo que a classificação ocorre de acordo com as unidades de fenilpropano e a proporção entre elas. As estruturas monoméricas precursoras da lignina diferem-se no grau de substituição de oxigênio no anel fenil, a estrutura H (4-hidroxi-fenil) apresenta um grupo hidroxi ou metoxi, a G (guaiacil) possui os dois grupos e a estrutura S (siringil) apresenta três grupos (Figura 7). Gramíneas como o bagaço de cana-de-açúcar apresentam os três grupos ρ-cumaril, guaiacil e siringil, resultando em uma proporção G:S:H de 1:2:0,8 (Fernandez *et al.*, 1990).

Figura 7. Monômeros de fenilproparo precursores da lignina. (a) álcool ρ-cumarílico (4-hidroxi-fenil, H), (b) álcool coniferílico (guaiacil, G) e (c) álcool sinapílico (siringil, S) (Doherty *et al.*, 2011).



A presença da lignina confere ao material lignocelulósico limitação física à sacarificação enzimática da celulose, diminuindo a acessibilidade das enzimas hidrolíticas às fibras celulósicas. Vários trabalhos têm buscado tratamentos que visem remover esse componente, atingindo, dessa forma, maiores valores de conversão de polissacarídeos (Mosier *et al.*, 2005; Himmel *et al.*, 2007). A remoção da lignina gera poros no material, aumentando a área acessível as enzimas celulolíticas. No entanto, o processo de remoção da lignina apresenta algumas desvantagens como o aumento de etapas no processo, alto custo de instalação da tecnologia usada, limitação da capacidade de deslignificação, entre outras (Maia and Colodette, 2003). Estratégias para a redução do teor de lignina precisam ser desenvolvidas, porém sem alterar o desenvolvimento da planta ou causar efeitos indesejáveis. Portanto, a maior limitação para o processo de redução da lignina seria a falta de especificidade do tecido exibida por abordagens como métodos de modificação clássica da lignina (Eudes *et al.*, 2014).

Alguns tipos de pré-tratamentos, com ácido diluído (Benjamin *et al.*, 2013; 2014), por explosão a vapor (Kumar *et al.*, 2012; Oliveira *et al.*, 2013), organosolv (Arantes *et al.*, 2014), hidrotérmico (Kim *et al.*, 2009; Kim *et al.*, 2015), entre outros, promovem apenas a relocalização da lignina na parede celular, garantindo também maiores valores de conversão, sem alterar, contudo, a quantidade de lignina no material (Mooney *et al.*, 1997; Chang and Holtzapple, 2000; Lee *et al.*, 2009; Varnai *et al.*, 2010). A correlação positiva entre o aumento da porosidade do bagaço com os rendimentos de hidrólise da celulose foi mostrada em estudo recente com a deslignificação do bagaço de cana não

tratado, confirmando a importância da limitação da acessibilidade causada pela lignina na ineficiência da sacarificação (Santi Junior *et al.*, 2013).

Outra dificuldade apresentada pelas enzimas celulolíticas na presença de lignina é a adsorção improdutiva, esse efeito tem origem quando as celulases são adsorvidas na lignina, principalmente através de interações hidrofóbicas, reduzindo a quantidade de enzimas disponível para atuar na hidrólise da fibra celulósica. A hidrólise enzimática da celulose acontece de forma heterogênea e as interações entre enzima/substrato são geralmente não-covalentes, as forças motrizes envolvem principalmente interações hidrofóbicas e eletrostáticas repulsivas e atrativas, com menor contribuição das interações de hidrogênio e dipolo (Claesson *et al.*, 1995; Norde, 1996). As interações hidrofóbicas frequentemente dominam a adsorção da enzima na celulose, porém as enzimas podem se ligar a outras superfícies, como a lignina (Jeoh *et al.*, 2007). Esse efeito tem sido observado em diversos materiais lignocelulolíticos, submetidos a diferentes tipos de pré-tratamentos, e tem sido considerado um importante efeito negativo da presença da lignina (Palonen *et al.*, 2004; Berlin *et al.*, 2005; Nakagame *et al.*, 2010; Rahikainen *et al.*, 2011), prejudicial para a economia do processo de sacarificação.

Por ser uma molécula poliaromática os grupos funcionais da lignina exercem influência na adsorção improdutiva, pois podem ligar-se às enzimas por pareamento de anéis e outras interações hidrofóbicas (Linder *et al.*, 1995). Durante o pré-tratamento, modificações químicas da lignina podem alterar sua afinidade por proteínas, o que resultam no aumento de hidroxilas fenólicas que podem aumentar a capacidade adsortiva da lignina residual (Rahikainen, Jenni L. *et al.*, 2013; Yu *et al.*, 2014), por outro lado, são gerados materiais menos susceptíveis a inibição quando o pré-tratamento aumenta a hidrofilicidade da lignina, principalmente pela introdução de grupos ácidos (Nakagame *et al.*, 2011; Lou *et al.*, 2013). Lignina de diferentes materiais lignocelulósicos também apresentam diferentes capacidades adsortivas, além das diferenças devido ao tipo de pré-tratamento. Segundo estudo as ligninas de algumas biomassas como a palha de milho, adsorvem menos celulases que a lignina de madeiras, tanto de coníferas quanto de folhosas (Nakagame *et al.*, 2010).

A adsorção improdutiva não depende apenas dos grupos funcionais das ligninas, mas também das propriedades das enzimas, como ponto isoelétrico e superfície hidrofóbica (Lai *et al.*, 2014; Sammond *et al.*, 2014). Estudos realizados com celulases produzidas por *T. reesei* mostraram que estas enzimas possuem resíduos de aminoácidos

hidrofóbicos expostos em sua superfície, capazes de interagir com a superfície hidrofóbica da lignina, causando adsorção improdutiva das celulases com desativação das mesmas, reduzindo a eficiência do processo catalítico (Reinikainen *et al.*, 1995; Palonen *et al.*, 2004). Os domínios de ligação a carboidratos (CBMs), encontrados em algumas celulases, provavelmente também exercem influência na adsorção improdutiva (Rahikainen, Jenni Liisa *et al.*, 2013), devido a presença de três resíduos de tirosina alinhados (Y5, Y31, Y32), importantes para o pareamento de anéis de interação CBM-celulose (Linder *et al.*, 1995), uma vez que tem sido observado aumento na adsorção improdutiva pela presença de CBM.

A maior parte dos estudos de adsorção são realizados em temperaturas baixas (0 a 10 °C), para evitar modificações estruturais nos substratos devido a hidrólise, além de evitar a inativação térmica das enzimas. A essa temperatura, as enzimas que são adsorvidas na lignina podem ser recuperadas, com perda mínima na atividade catalítica. Porém, na temperatura média do processo de hidrólise (acima de 45 °C), as interações proteínas-lignina são intensificadas e as enzimas perdem a suas estruturas nativas, sofrem desnaturação e ligam-se irreversivelmente à lignina (Rahikainen *et al.*, 2011).

Tentativas para diminuir o efeito negativo da adsorção improdutiva tem sido feitas pela adição de uma quantidade relativamente alta de enzimas ao processo de sacarificação, pois a adsorção improdutiva é um fenômeno dependente da concentração (Nakagame *et al.*, 2010; Kumar *et al.*, 2012), e a superfície disponível para a interação diminui à medida que a concentração de proteínas aumenta. Outra alternativa de contornar esse efeito negativo é pela adição de outras proteínas, como a albumina sérica bovina (BSA), antes da adição das celulases (Yang and Wyman, 2006). A proteína BSA possui uma das mais elevadas hidrofobicidades superficiais, o que contribui para a sua adsorção preferencial da lignina, pois o bloqueio dos sítios de adsorção da lignina ocorre não só pelo efeito da concentração de proteínas, mas também devido as propriedades químicas dessa proteína (Lijnzaad *et al.*, 1996; Sammond *et al.*, 2014).

O efeito da adsorção improdutiva também pode ser diminuído pela adição de polímeros, como o polietileno glicol (PEG). Existem algumas explicações possíveis desse efeito que incluem a capacidade do PEG de aumentar a estabilidade da celulase, diminuir a adsorção não-produtiva da celulase no substrato e aumentar a dessorção das enzimas a partir do substrato (Malmsten and Vanalstine, 1996; Börjesson, Engqvist*, et al.*, 2007; Börjesson, Peterson*, et al.*, 2007; Kristensen *et al.*, 2007). A adição de PEG a hidrólise enzimática de biomassas lignocelulósicas pré-tratadas por explosão a vapor

(pinho, salgueiro, palha de trigo, palha de milho, bagaço de sorgo) mostrou ser benéfica para o aumento das celulases livres no sobrenadante da hidrólise (Sipos *et al.*, 2011). Os resultados sugerem que o diferente grau de aumento na atividade das celulases livres obtidos pela adição do PEG é baseada nas várias estruturas da lignina presentes em cada substrato. Portanto, os autores concluíram que os grupos hidroxil fenólicos expostos na superfície da lignina interagem com o PEG por meio de ligações de hidrogênio, formando uma camada de PEG na superfície da lignina, o que impede a ligação improdutiva das celulases na lignina. Em contraponto, estudos recentes apresentaram resultados interessantes quanto ao efeito da adição de PEG durante o processo de hidrólise do Avicel, celulose pura e cristalina, pelas enzimas purificadas endoglucanase produzida a partir da linhagem *Talaromyces emersonii* e celobiohidrolase I de *Trichoderma longibrachiatum* (Hsieh *et al.*, 2015). Segundo os autores o efeito positivo sobre o rendimento da hidrólise parece ser específico para a enzima celobiohidrolase I (Hsieh *et al.*, 2015), visto que não há presença da lignina nesse material.

A adição de surfactantes não-iônicos também tem sido base de estudos para diminuir o efeito negativo da adsorção improdutiva consequentemente melhorar o rendimento da hidrólise. A presença de Tween 20 e 80 durante o processo de hidrólise podem eliminar a desativação enzimática atribuída à lignina, devido a exclusão das enzimas da superfície da lignina. Além da atuação na dessorção das celulases dos substratos durante a sacarificação, o que aumenta o rendimento da mesma (Park *et al.*, 1992; Eriksson *et al.*, 2002; Okino *et al.*, 2013). Um estudo avaliou o efeito da adição de Tween 80 na hidrólise da palha de milho realizada por enzimas comerciais e os resultados permitiram compreender melhor o papel da lignina na redução da adsorção das celulases sobre o substrato, em parte, devido a adsorção do surfactante na lignina, que ocupava a superfície hidrofóbica na lignina da palha de milho (Li, Yanfei *et al.*, 2016). Em resumo, a adição de aditivos (proteínas não catalíticas, polímeros e surfactantes não-iônicos, entre outros) é significativamente eficaz e reduz a adsorção improdutiva das enzimas do complexo celulolítico sobre a lignina. Porém, contornar esse efeito sem aumentar o custo do processo ainda representa um desafio.

1.2. OBJETIVOS

O objetivo deste trabalho foi estudar a produção e caracterização de coquetéis enzimáticos envolvidos na degradação da biomassa vegetal pelos fungos filamentosos *Trichoderma reesei* e *Aspergillus niger* cultivados por fermentação sequencial, bem como avaliar a aplicação dos mesmos no processo de sacarificação do bagaço de cana-de-açúcar. Para atingir o objetivo geral as seguintes etapas foram realizadas:

1. Adaptação e validação da metodologia de cultivo, fermentação sequencial para diferentes linhagens fúngicas do gênero *Trichoderma* utilizando o bagaço de cana como fonte de carbono;
2. Caracterização dos coquetéis enzimáticos obtidos pelo cultivo sequencial e submerso convencional em relação aos parâmetros pH e temperatura ótimos, além de estudos de termo-estabilidade;
3. Análise proteômica dos coquetéis enzimáticos secretados por *Trichoderma reesei* e *Aspergillus niger* cultivados por fermentação sequencial em comparação ao cultivo submerso convencional utilizando o bagaço de cana como fonte de carbono;
4. Avaliação da performance dos coquetéis enzimáticos combinados (*A. niger* + *T. reesei*) produzidos a partir dos diferentes métodos de cultivo na hidrólise do bagaço de cana pré-tratado por explosão a vapor;
5. Avaliação da adsorção improdutiva da lignina na presença de aditivos (polietilenoglicol, tween, BSA proteína de soja), durante o processo de hidrólise enzimática do bagaço de cana, com coquetéis enzimáticos produzidos por *Aspergillus niger* e *Trichoderma reesei* cultivados sob diferentes métodos de cultivo (fermentação em estado sólido, submersa e sequencial);

2. CAPÍTULO II

Validation of a Novel Sequential Fermentation Method for the Production of Enzymatic Cocktails from *Trichoderma* strains

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Neste trabalho foram realizados experimentos de cultivo para produção de enzimas celulolíticas a partir de uma nova metodologia, denominada de fermentação sequencial, no intuito de validar esse processo de cultivo para linhagens fúngicas do gênero *Trichoderma*. Foram utilizadas para o estudo a linhagem hipercelulolítica, *Trichoderma reesei* Rut C30 e duas linhagens selvagens, *Trichoderma harzianum* P49P11 e *Trichoderma* sp INPA666. Os cultivos foram feitos utilizando o bagaço de cana "in natura" e pré-tratado por explosão a vapor, como fonte de carbono. Para os coquetéis enzimáticos produzidos pelas 3 linhagens fúngicas foram quantificadas as atividades de endoglucanase e os resultados foram comparados com os valores obtidos em cultivo convencional de fermentação submersa. Os extratos enzimáticos foram caracterizados em termos de pH e temperatura ótimos e perfil de endoglucanase. Os resultados obtidos nesta etapa se referem aos objetivos 1 e 2 apresentado no item 1.2, e sugerem que esta configuração de bioprocesso, iniciada com uma etapa sólida seguida por uma submersa é promissora no desenvolvimento industrial de biocombustíveis celulósicos.

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Abstract The development of new cost-effective bioprocesses for the production of cellulolytic enzymes is needed in order to ensure that the conversion of biomass becomes economically viable. The aim of this study was to determine whether a novel sequential solid-state and submerged fermentation method (SF) could be validated for different strains of the *Trichoderma* genus. Cultivation of the *Trichoderma reesei* Rut-C30 reference strain under SF using sugarcane bagasse as substrate was shown to be favorable for endoglucanase (EGase) production, resulting in up to 4.2-fold improvement compared with conventional submerged fermentation. Characterization of the enzymes in terms of the optimum pH and temperature for EGase activity and comparison of the hydrolysis profiles obtained using a synthetic substrate did not reveal any qualitative differences among the different cultivation conditions investigated. However, the thermostability of the EGase was influenced by the type of carbon source and cultivation system. All three strains of *Trichoderma* tested (*T. reesei* Rut-C30, *Trichoderma harzianum*, and *Trichoderma* sp INPA 666) achieved higher enzymatic productivity when cultivated under SF, hence validating the proposed SF method for use with different *Trichoderma* strains. The results suggest that this bioprocess configuration is a very promising development for the cellulosic biofuels industry.

Keywords Sequential fermentation · Cellulase · Xylanase · Sugarcane bagasse · *Trichoderma* · On-site enzyme production

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Introduction

The enzymatic hydrolysis of biomass is considered one of the most sustainable technologies for the production of cellulosic ethanol. Cellulolytic enzymes are capable of breaking down cellulose polymers into soluble sugars that can be converted into ethanol by means of microbial fermentation. However, cellulosic ethanol production is still not profitable on an industrial scale. Difficulties that need to be overcome are related to the effectiveness and the high cost of the enzymatic cocktails [1]. For this reason, much remains to be done concerning the development of new cost-effective bioprocesses for cellulolytic enzymes production. In addition to the high demand for cellulolytic enzymes in the biofuels industry, such enzymes are widely applied in the pulp and paper, textile, food and beverages, detergent, and animal feed industries [2]. On-site production of enzymes is a potential strategy that could be used to reduce costs, since there is less need to stabilize the enzyme preparations, hence avoiding the expenses associated with transport and long-term storage [3–6]. The development of a microbial enzyme production bioprocess in which the carbon source is a lignocellulosic material from a stream within the ethanol process is a potential configuration that could enable on-site enzyme production. This would contribute to cost reduction as well as to the production of more specific enzymatic cocktails suitable for degrading the same type of biomass. Among the lignocellulosic materials that can be used for cellulosic ethanol production and as substrates for enzyme production, sugarcane bagasse is especially attractive in Brazil, where it is readily available in large amounts at the sugarcane mills [7].

In terms of the choice of microorganism for enzyme production, the filamentous fungus *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is widely used for the industrial production of cellulolytic enzyme cocktails due to its very high protein secretion capacity [8–12]. Different cultivation methods have been used for cellulase production by *T. reesei*, such as submerged fermentation (SmF) and solid-state fermentation (SSF) [2]. Each of these cultivation systems has its own advantages and disadvantages associated with the environmental and operational conditions. Nevertheless, SSF more closely resembles the natural environment of the fungus, because *T. reesei* grows naturally on solid cellulosic material. The application of SSF for industrial enzyme production has received increasing attention over the past 20 years, and various studies have described the use of this technique to produce enzymes involved in biomass deconstruction [2, 13]. From the environmental perspective, the benefit of SSF is related to the use of agro-industrial residues as solid substrates acting as sources of both carbon and energy. However, industrial-scale production of enzymes by SSF still faces technical limitations [14, 15]. On the other hand, SmF is the most widely used cultivation method for industrial enzyme production, because the operational techniques and the control of environmental factors (such as temperature and pH) are well established.

In recent work [16], we proposed a novel cultivation methodology that combines the advantages of the two conventional cultivation processes (SmF and SSF). The technique, called sequential fermentation (SF), is based on the use of a lignocellulosic material, such as sugarcane bagasse, as the inducer substrate for pre-culture preparation, employing an SSF step followed by a transition to SmF. The results of the study showed that the SF process was superior to conventional SmF for cultivations using both shake flasks and aerated bubble column bioreactors. The endoglucanase volumetric productivity values were threefold higher for the bubble column bioreactor using the new method for cultivation of *Aspergillus niger*. The novel SF methodology therefore offers a promising alternative bioprocess strategy for enzyme production, which should be further investigated.

In light of the earlier findings, the aim of the present work was to determine whether the new sequential fermentation method could be validated for other fungi, such as different

strains of *Trichoderma* cultivated using sugarcane bagasse as carbon source for cellulase production. In order to achieve this, comparisons were made of cultivations performed using the SF and conventional SmF methods. Evaluation was made of the influence of fungal strain, cultivation method, and sugarcane bagasse pretreatment on enzyme production. The enzymatic cocktails obtained were characterized in terms of optimum pH and temperature, as well as stability.

Methods

Fungal Strains

Three *Trichoderma* strains were used in the present study. The first was a *T. reesei* Rut-C30 strain that had been purchased from the Centre for Agricultural Bioscience International culture collection in the UK (IMI number: 345108). The second was a strain of *Trichoderma* sp (INPA 666) obtained from the Embrapa Tropical Agroindustry collection (CNPAT, Fortaleza, Brazil). The third strain was a *Trichoderma harzianum* P49P11 isolated from the Amazon forest [17] and deposited at the Embrapa Food Technology microorganism collection (Rio de Janeiro, Brazil). Plate assays showing the production of cellulolytic enzymes by the *Trichoderma* strains has been previously reported [12]. All strains were grown on plates of potato dextrose agar at 30 °C.

Culture Conditions

Submerged Fermentation (SmF)

In the conventional submerged fermentation process, the pre-culture was initiated with a conidia suspension of 10^7 spores/mL, which was transferred to Erlenmeyer flasks containing 100 mL of nutrient medium, adapted from Mandels and Sternberg [18], and enriched with 30 g/L of glucose. The composition of the medium (in % *w/v*) was as follows—0.14 % $(NH_4)_2SO_4$, 0.20 % KH_2PO_4, 0.03 % $CaCl_2$, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.50 % peptone, 0.20 % yeast extract, 0.03 % urea, 0.10 % Tween 80, and 0.10 % of salt solution (5 mg/L $FeSO_4 \cdot 7H_2O$, 1.6 mg/L $MnSO_4 \cdot H_2O$, 1.4 mg/L $ZnSO_4 \cdot 7H_2O$, and 2.0 mg/L $CoCl_2$). The incubation was carried out for 48 h at 30 °C with stirring at 200 rpm. A volume of pre-culture suspension corresponding to 10 % (*w/v*) was transferred to the culture medium and supplemented with 10 g/L of glucose and 1 % (*w/v*) of either untreated sugarcane bagasse (UB) or steam-explosion-pretreated sugarcane bagasse (PB) that had been donated by a local sugarcane mill (Usina Nardini, São Paulo, Brazil). The cultivations were performed for 72 h at 30 °C and 200 rpm. The cultivation broth was then filtered and centrifuged at 10,000 rpm for 10 min, and the crude enzymatic extract was stored at −18 °C for further analysis. All cultivation experiments were carried out in triplicate, and the data were calculated as means±standard deviations. The mean values obtained for each condition were analyzed statistically using Origin software.

Sequential Fermentation (SF)

In the sequential cultivation method (SF), the pre-culture was initiated as solid-state fermentation (SSF), using 5 g of dry sugarcane bagasse (either UB or PB) as solid substrate. In the SSF cultivation step, the moisture was adjusted to 70 % (*v/v*) by the addition of nutrient

medium. A concentration of 10^7 spores/g of dry bagasse was added, and the culture was maintained as SSF under static conditions for 24 h at 30 °C. The pre-culture cultivation was continued as SmF after addition of a volume of nutrient medium enriched with 30 g/L of glucose (40 parts of nutrient medium per gram of dry solid). The SmF step was performed in an orbital shaker for 48 h at 30 °C and 200 rpm. A volume of pre-culture suspension corresponding to 10 % (*v/v*) was transferred to the culture medium supplemented with 10 g/L of glucose and 1 % (*w/v*) of either UB or PB, and the cultivation for cellulase production was performed as described above. After 72 h, the broth was filtered and centrifuged at 10,000 rpm for 10 min, and the crude enzymatic extract was stored at −18 °C prior to further analysis. All cultivation experiments were carried out in triplicate, and the data were calculated as means± standard deviations. The mean values obtained for each condition were analyzed statistically using Origin software.

Experimental Design for pH and Temperature Characterization Study

A full factorial design followed by response surface analysis was used to evaluate the effects of two variables (pH and temperature), as well as their possible interaction, on the endoglucanase activity present in the crude enzymatic cocktail. A central composite design (CCD) was used, comprising of 11 runs, with four cube points, four axial points, and three central points. The experiments were carried out in random order. The dependent (response) variable was the endoglucanase activity. The factors and levels investigated are shown in Table 1. Statsoft v. 7.0 software was used for data analysis including analysis of variance (ANOVA) and the plotting of response surfaces.

Enzyme Assays

Endoglucanase (EGase), filter paper cellulase (FPase), and β-glucosidase activities were assayed in the presence of carboxymethylcellulose (CMC) (Sigma, USA), Whatman No. 1

Table 1 Enzymatic activity (IU per liter) of submerged and sequential fermentation extracts from *T. reesei* Rut C30 grown using different types of sugarcane bagasse (UB and PB)

			EGase activity (IU/L)			
Assay	pH	Temperature	SmF-UB	SF-UB	SmF-PB	SF-PB
1	3 (−1)	30 (−1)	80.6	58.6	146.5	229.5
2	6 (+1)	30 (−1)	19.5	87.9	339.4	498.2
3	3 (−1)	80 (+1)	9.8	26.9	87.9	109.9
4	6 (+1)	80 (+1)	14.7	24.4	173.4	173.4
5	2.5 (−1.41)	55 (0)	7.3	9.8	4.9	4.9
6	6.5 (+1.41)	55 (0)	85.5	239.3	705.7	1,050.1
7	4.5 (0)	20 (−1.41)	24.4	67.8	255.6	258.9
8	4.5 (0)	90 (+1.41)	4.9	49.0	124.8	134.3
9	4.5 (0)	55 (0)	102.6	268.7	664.5	1,245.4
10	4.5 (0)	55 (0)	100.1	232.1	635.1	1,160.0
11	4.5 (0)	55 (0)	102.6	224.8	647.4	1,050.1
12[a]	4.8	50	136.8	259.1	635.3	1,018.3

[a] Standard condition

filter paper strips, and cellobiose (Sigma, USA), respectively, using the standard method proposed by Ghose [19]. In the case of β-glucosidase activity, quantification of the glucose released was performed with an enzymatic kit for glucose measurement (Doles, Brazil). Exoglucanase activity was determined by mixing 0.1-mL volumes of the suitably diluted enzyme extract with 0.9 mL of 1 % (*w/v*) Avicel solution in sodium citrate buffer (50 mM, pH 4.8). The mixture was incubated at 50 °C for 60 min. Xylanase activity was measured using the method described by Bailey and Poutanen [20]. The reducing sugar concentration was determined by the dinitrosalicyclic acid (DNS) method [21]. All enzymatic analyses were carried out in duplicate. One unit of EGase, FPase, or xylanase corresponded to 1 μmol of reducing sugars released per minute of reaction. The total protein concentration in the crude extracts was determined by the method described by Bradford [22], using bovine serum as a standard.

In the experiments conducted to identify the optimum pH and temperature for the EGase activity of the crude extracts described in Section "Experimental Design for pH and Temperature Characterization Study," the reaction mixture was incubated at different pHs (from 2.5 to 6.5) and temperatures (from 20 to 90 °C), which were selected according to the experimental design (Table 1).

Hydrolysis of a Synthetic Substrate

The degree of hydrolysis of the synthetic substrate (low viscosity CMC at a 2 % (*m/v*) ratio) was evaluated during a period of 24 h, using the pH and temperature conditions described for the central point of the experimental design (Table 1). The reducing sugars released after incubation for 10 min and for 2, 4, 6, 12, and 24 h were measured using the DNS method [21].

Enzyme Stability and Inactivation

The thermal stability of EGase was evaluated by measuring the residual enzymatic activity after incubation of the crude supernatant at 50 °C for a total period of 24 h. Sampling was performed after time intervals of 10 min and 2, 4, 6, 12, and 24 h. At the end of the incubation period, the test tubes containing the enzymes were immediately cooled by placing them on ice. Measurement of EGase activity was performed under standard pH (4.8) and temperature (50 °C) conditions.

The single-step non-first-order model proposed by Sadana and Henley [23] was fitted to the experimental data. This model considers that a single-step inactivation leads to a final state, exhibiting a residual activity that is very stable (the protein shows no further unfolding and/or inactivation). The model can be described by the expression:

$$\frac{A}{A_0} = (1-\alpha)\cdot e^{-k\cdot t} + \alpha, \tag{1}$$

Where A/A_0 is the dimensionless activity, α is the ratio between the specific activity of the final state and the specific activity of the initial state, and k is the first-order deactivation rate constant (per time). The parameter k describes the unfolding (or inactivation) process, and the parameter α reflects the long-term level of activity [23]. The biocatalyst half-life was then calculated using the fitted model.

Results and Discussion

Validation of the Sequential Fermentation for *T. reesei* Rut-C30

In the earlier work by Cunha et al. [16], encouraging results were obtained for the production of cellulase by *A. niger* cultivated under sequential solid-state and submerged cultivation. It was therefore of great interest to investigate whether the proposed SF cultivation method could also be applied to other genera of filamentous fungi, such as the industrially important *T. reesei* Rut-C30 strain. In order to evaluate the validity of the SF method for *T. reesei* Rut-C30, a set of conventional SmFs were carried out in parallel. This enabled comparison of the efficiencies of the two methods for cellulase production under similar conditions of pH, temperature, and carbon source (Fig. 1). The effect of using UB or PB as carbon source was also investigated.

A significantly higher EGase activity was achieved when *T. reesei* was cultivated under SF, compared with conventional SmF. Furthermore, the use of steam-explosion-pretreated sugarcane bagasse (PB) also favored EGase production, compared with the use of UB as carbon source. The EGase activity values varied from 159±6 (SmF) to 670±36 IU/L (SF) using UB, and from 650±8 (SmF) to 945±66 IU/L (SF) using PB (Fig. 1). These differences in cultivation conditions corresponded to up to sixfold improvements in EGase production.

A possible explanation for the increases in EGase activity could be differences in the morphology of the *Trichoderma* fungi because, in SF, there was a predominance of filamentous dispersed mycelia while, in the conventional SmF process, there was early formation of fungal pellets. Cunha et al. [16] reported that *A. niger* also developed dispersed filamentous growth morphology under SF cultivation. This difference in fungal morphology was due to the pre-culture conditions because, in SF, the cell development began on a solid medium. However, it is not possible to draw firm conclusions concerning a relationship between fungal morphology and cellulase production, because of the existence of many other factors that might influence the cultivation process. For example, Ahamed and Vermette [24] evaluated the effects of culture medium composition on the growth, mycelia morphology, and cellulase production of *T. reesei* Rut-C30 and found a positive correlation between the total mycelia, the number of tips, and the volumetric enzyme productivity. Domingues et al. [25] also reported that, under conditions that induced pellet formation in *T. reesei* Rut-C30 cultivated under SmF,

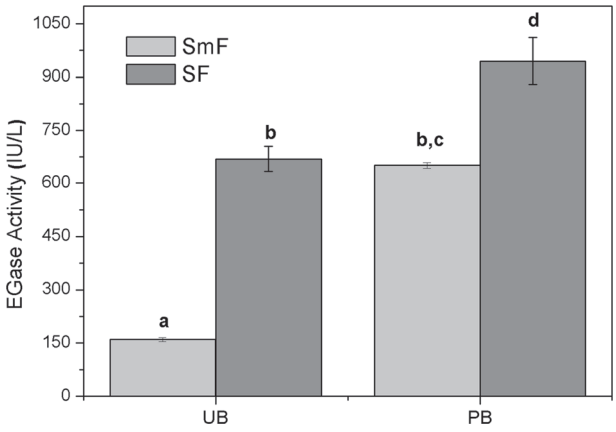

Fig. 1 EGase activity (IU per liter) for *T. reesei* Rut-C30 cultivation under SmF and SF using different types of sugarcane bagasse (UB and PB). The letters (*a*, *b*, *c*, *d*) indicate results that differ significantly, according to the Tukey's test ($p<0.1$)

there were decreases in protein concentration and cellulase activity. Another possible reason for the higher EGase activity achieved under SF is that the solid medium used in the SSF pre-culture step could act as an inducer for cellulase production during the early stage of cell development, hence contributing to enzyme production.

In experiments in which SF was carried out using steam-exploded-sugarcane bagasse (PB), EGase activities increased by up to 1.4 times, compared with use of UB, while increases of up to 4.2-fold were observed in the case of SmF. This can be explained by the fact that the pretreatment process makes a substantial contribution to the breakdown of the lignocellulosic structure [26]. Consequently, the accessibility of the cellulose components to fungal development is enhanced, compared with untreated sugarcane bagasse. Rodriguez-Zuniga et al. [27] reported that use of liquid hot water-pretreated sugarcane bagasse resulted in higher cellulase and xylanase activities, compared with untreated bagasse, when *A. niger* was cultivated under SSF. This was due to structural modifications in the pretreated bagasse that assisted the adherence and penetration of the fungal hyphae.

The findings showed that the SF cultivation method and the use of a pretreated type of sugarcane bagasse were both directly related to an increase in enzymatic activity. The sequential cultivation method was able to improve EGase production by *T. reesei* Rut-C30, which confirmed the advantages of the novel cultivation procedure and validated its use with this fungus that is considered a reference strain for cellulase production.

Effect of pH and Temperature on EGase Activity

Characterization of the enzymes produced by different cultivation methods is necessary in order to identify qualitative and quantitative differences between enzymatic cocktails. Here, the effects of pH and temperature on the EGase activity in the crude enzymatic cocktails obtained using SF and SmF were evaluated under different conditions, selected according to a central composite design. Table 1 presents the experimental conditions and the corresponding responses for EGase activity in the crude enzymatic extracts obtained during cultivation of *T. reesei* Rut C-30 under SmF and SF, using either UB or PB as carbon source. Run 12 employed the standard conditions used in EGase assays (pH 4.8 and temperature of 50 °C) and was performed for comparative purposes.

The ANOVA analysis of EGase activity for all the crude enzymatic extracts (Table 2) resulted in correlation coefficients (R) exceeding 0.80 and F-test values ($F_{measured}/F_{critical}$) that varied from 2.92 to 6.91. This enabled model prediction with a 90 % level of confidence. As an illustration, the coefficients obtained for the SF-PB enzymatic extract were used to describe the response surface plot for EGase activity as a function of pH and temperature (Fig. 2). The response surface plots for the other cultivation conditions tested showed similar shapes (data not shown).

For all enzymatic extracts, both pH and temperature (within the ranges tested) showed a statistically significant influence on EGase activity, which was not observed for the synergistic effect between temperature and pH. The response surface plots obtained for each cultivation condition indicated that higher values of EGase activity were obtained in the experiments carried out using pH values in the range from 3.5 to 5.5, and temperatures between 42 and 67 °C, for all enzymatic extracts. An advantage of using the experimental design methodology was that pH and temperature ranges were identified for optimum enzyme activity, which can enable greater flexibility during bioprocess development [28].

The results revealed no qualitative differences between the enzymatic extracts obtained using the different cultivation conditions, in terms of the optimum pH and temperature for EGase activity. However, it is important to note that the analytical assay used here for the

Table 2 Coefficient values and statistical analysis of EGase activity in the SmF and SF extracts from *T. reesei* Rut C30 grown using different types of sugarcane bagasse (UB and PB)

Factor	SmF (UB)		SF (UB)		SmF (PB)		SF (PB)	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Mean	101.75*	0.001	241.87*	0.000	648.99*	0.000	1151.81*	0.000
pH	6.80	0.515	43.93*	0.081	158.70*	0.016	226.28*	0.027
pH^2	−27.52*	0.063	−69.17*	0.034	−168.32*	0.024	−339.49*	0.011
T	−12.91	0.240	−15.23	0.484	−51.20	0.301	−77.57	0.339
T^2	−43.40*	0.013	−102.25*	0.008	−250.88*	0.005	−504.93*	0.002
T x pH	16.48	0.283	−7.93	0.792	−26.86	0.686	−51.28	0.642
R^2	0.80		0.84		0.89		0.91	
F-value	9.10		12.86		16.28		21.22	
$F_{measured}$ /$F_{critical}$	2.92		5.30		4.19		6.91	

R^2 coefficient of determination.

* p=0.1 level of significance for these values

quantification of EGase activity is unable to discriminate between the different types of endoglucanase. For instance, analytical methodologies such as zymography assays and fluorophore-assisted carbohydrate electrophoresis were used to analyze the glycoside hydrolyses secreted by *A. niger* cultured in media containing different carbon sources [29]. Regarding the *Trichoderma* genus, eight endoglucanases belonging to different hydrolase families have been identified in the *T. reesei* genome, with endoglucanases I (Cel7B) and II (Cel5A) usually secreted in higher amounts [8]. Nevertheless, Cel7B is the major endoglucanase, accounting for 6–10 % of the total cellulase proteins from *T. reesei* [11].

In recent work concerning the characterization of *T. reesei* endoglucanase II, Samanta et al. [30] observed that the recombinant EGase was most active at pH 5.0 and 55 °C, in agreement with the pH and temperature optima found here. de Castro et al. [31] reported an optimum pH

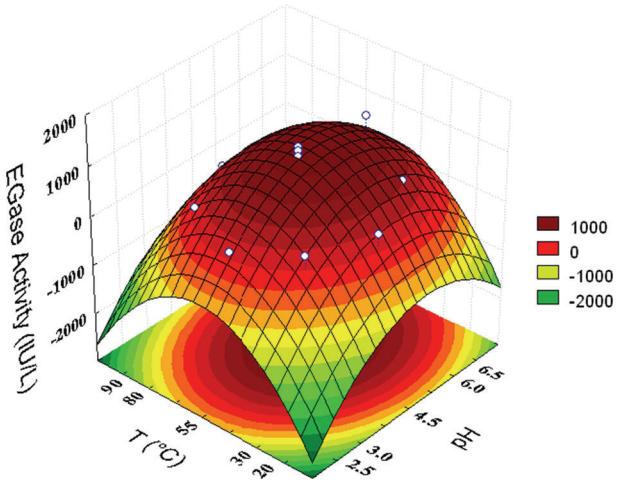

Fig. 2 Three-dimensional response surface for the effects of pH and temperature on EGase activity present in the cellulolytic enzyme complex produced by *T. reesei* Rut-C30 grown on PB under SF

of 5.1 and temperature range of 52–57 °C for cellulases from *T. reesei* Rut-C30, which is also in agreement with the present results. Nevertheless, the wider optimum ranges of pH (from 3.5 to 5.5) and temperature (from 42 to 67 °C) observed here for all the enzymatic extracts could be advantageous in hydrolysis processes.

Hydrolysis of a Synthetic Substrate

The temporal profiles of the concentrations of reducing sugars released during hydrolysis were investigated in a set of experiments performed using low-viscosity CMC as substrate. Figure 3 shows the concentration of reducing sugars (micromoles per milliliter) released during the hydrolysis of CMC carried out using the same experimental conditions (pH 4.5 and temperature of 55 °C) as the central point of the CCD used to evaluate the effects of pH and temperature on EGase activity. The concentration of products increased throughout the 24-h period, using the extracts produced by all combinations of cultivation methods (SmF and SF) and sugarcane bagasse types (UB and PB). The hydrolysis was more effective using the extracts produced from SF with PB, which resulted in the concentration of reducing sugars reaching 66±7 μmol/mL. These results confirmed the higher EGase activity observed for the enzymes from the SF–PB cultivation. The enzymatic extract from SmF–UB showed a lower maximum concentration of products (13±2 μmol/mL), although the temporal trend was similar to that of the other cultivations.

The profile of product formation during the action of the EGase enzyme was used to evaluate the effect of cultivation method (SmF or SF) on the performance of the enzymatic extracts produced using pretreated or untreated sugarcane bagasse. Despite significant differences between the values of reducing sugars, the trend of increasing product concentration was the same for all enzymatic extracts, indicating that there were no differences between the enzymes in terms of their ability to hydrolyze a synthetic substrate.

Thermostability and Deactivation Studies

Thermostability is a highly desirable quality of industrial enzymes. One way of determining thermostability is to measure the enzyme activity half-life at elevated temperatures [11]. Here,

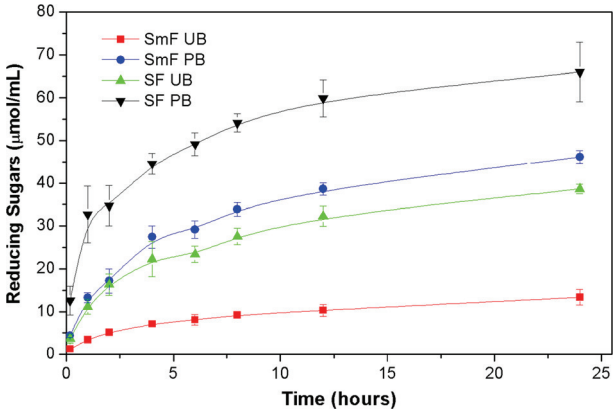

Fig. 3 Concentrations of products from hydrolysis of the synthetic substrate over a period of 24 h by *T. reesei* Rut-C30 enzymatic extracts obtained using the fermentation processes: (*closed square*) SmF-UB, (*closed upright triangle*) SF-UB, (*closed circle*) SmF-PB, and (*closed inverted triangle*) SF-PB

the crude extracts produced using SmF and SF with UB and PB as substrates were incubated at 50 °C for a period of 24 h. Figure 4 shows the residual activities as a function of time, fitted using the model of Sadana and Henley [23]. Table 3 presents the results for the model parameters k and α, together with the EGase half-life and the coefficient of determination (R^2).

The EGase enzymes of the crude extract produced under SmF using UB were up to two times more stable than those produced by SF using the same carbon source, with half-lives ($t_{1/2}$) of 17 h (SmF–UB) and 8 h (SF–UB). A smaller impact of the cultivation method on enzyme stability was observed for cultivations using the pretreated sugarcane bagasse, where $t_{1/2}$ of 6 and 4 h were obtained for EGase enzymes from the SmF–PB and SF–PB extracts, respectively. The SmF extracts were therefore 1.5 times more stable than the SF extracts.

One of the preferred sugarcane bagasse pretreatment processes is steam explosion, which causes substantial breakdown of the lignocellulosic structure. The hemicellulose fraction is hydrolyzed by the acetic and other acids released during the pretreatment [26]. In the present study, although the EGase activity was higher for SF–PB, the enzymes in this fermentation broth were less stable than those produced using untreated sugarcane bagasse. A possible explanation is that the release of inhibitors such as lignin-derived phenolics during the steam explosion process could have negatively affected the stability of the enzymes produced using this material as carbon source. It has been reported previously that cellulase enzymes can be inhibited or deactivated by phenolic compounds [32, 33].

In the thermostability studies conducted by Saqib et al. [34], comparison was made between the crude extracts obtained from SSF and SmF, which revealed higher thermostability of the EGases from SSF. It was suggested that the primary structures of the enzymes in the endoglucanase preparations could have differed, raising new questions about possible structural differences in the enzymes produced under the two fermentation processes. In the present work, despite the fact that SF included an initial solid-state step during preparation of the pre-culture, greater enzyme stability was achieved using SmF. A possible explanation is the additional amount of sugarcane bagasse used in the solid-state step of the SF pre-culture. The fact that the sugarcane was not washed before the cultivations could have had a negative effect on the stability of the enzymes produced by the SF method. However, studies of lignocellulosic biomass hydrolysis have indicated that washing and filtration of liquid hot water-pretreated biomass can improve the enzymatic digestibility of cellulose by removing inhibitors [35]. Therefore, the release of inhibitory compounds during the fungal cultivation

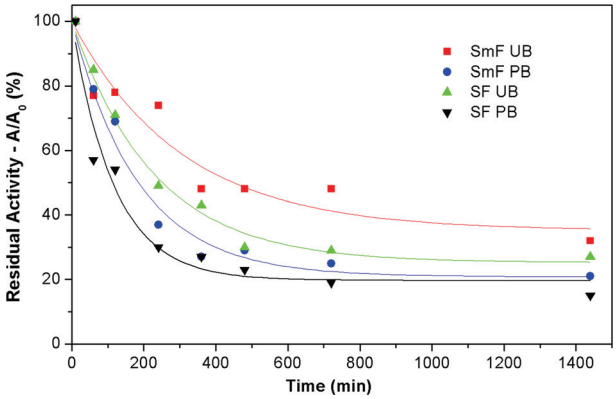

Fig. 4 Thermostability and residual enzymatic activity at 50 °C and pH 4.8 of EGase present in the cellulolytic complex produced by *T. reesei* Rut-C30: (*closed square*) SmF-UB, (*closed upright triangle*) SF-PB, (*closed circle*) SmF-PB, and (*closed inverted triangle*) SF-PB

Table 3 Half-life ($t_{1/2}$ (hours)) of the endoglucanase enzymes produced by *T. reesei* Rut-C30 under different cultivation methods

	α (h^{-1})	k	Half-life (h)[a]	Coefficient (R^2)
SmF–UB	0.352	0.003	17	0.90
SF–UB	0.253	0.004	8	0.99
SmF–PB	0.208	0.005	6	0.97
SF–PB	0.197	0.009	4	0.95

[a] Half-life ($t_{1/2}$) calculated from Eq. 1

process might be able to be avoided by including a washing step after the sugarcane bagasse pretreatment. Recently, Ximenes et al. [36] reported that the use of bio-abatement for the removal of enzyme inhibitors released during lignocellulose pretreatment yielded improved enzyme hydrolysis. Thus, further research will be necessary to select the most appropriate strategy to overcome this stability limitation observed here when using pretreated bagasse as carbon source.

The extracts produced by *T. reesei* can show varying thermostability, depending on the culture conditions and the strains employed. According to Viikari et al. [11], the *T. reesei* cellulase system is rapidly inactivated at temperatures above 45 °C. Samanta et al. [30] investigated the effect of temperature on a recombinant EGase enzyme and reported that the enzymes remained active for 60 min at 55 °C. The endoglucanase activity was mostly retained for up to 40 min at 60 °C and then diminished appreciably. Nevertheless, a hyperglycosylation effect could have interfered in this result.

Problems involving thermal stability might be able to be mitigated using alternative strategies to improve performance when these enzymes are employed with substrates requiring longer hydrolysis times. For example, Viikari et al. [11] produced individual cloned thermostable enzymes using a modified strain of *T. reesei*. Process strategies to remove inhibitors from the pretreated biomass employed in the cultivation media could also be applied.

Validation of Sequential Fermentation for Different *Trichoderma* Strains and Comparison of Multi-enzyme Production

Comparison of SF with SmF for three different *Trichoderma* strains (*T. reesei* Rut-C30, *T. harzianum*, and *T.* sp INPA 666) considered the activity values for EGase, xylanase, β-glucosidase, Avicelase, and FPase, as well as the total protein concentration, obtained after 72 h of cultivation using PB as carbon source (Table 4). The enzyme productivities were mostly much higher for the SF cultivation method, so the proposed methodology could therefore be considered validated for use with the different *Trichoderma* strains.

In the case of xylanase activity, the highest value using SF was observed for *T.* sp INPA 666 (3993±57 IU/L). The *T. reesei* Rut-C30 strain showed xylanase activity similar to that displayed by *T. harzianum* P49P11, with values of 2048±39 and 2022±3 IU/L, respectively. These results for xylanase were comparable to the findings of [16], who obtained a xylanase activity of 1961±102 IU/L for *A. niger* under SF cultivation in a bubble column reactor.

The highest EGase activity was achieved by *T. harzianum* P49P11 under SF cultivation (1,668±131 IU/L). This value was almost two times higher than achieved with

T. reesei Rut-C30 (944±66 IU/L) and *T.* sp INPA 666 (833±13 IU/L). In the preliminary study by Cunha et al. [16], EGase activity of 1,052±34 IU/L was achieved with *A. niger* under SF cultivation in shake flasks. It is important to point out that in the Cunha et al. [16] study, the relevant parameters of the SF process, such as substrate moisture content, pH, temperature, and inoculum size were optimized for the filamentous fungus *A. niger*. It is therefore believed that, in the case of the *Trichoderma* strains, even higher activity values could be achieved following further optimization of operational conditions for this fungal genus.

The other enzymatic activities evaluated (β-glucosidase, Avicelase, and FPase) showed the highest values for *T. harzianum* P49P11 cultivated under SF. In terms of total protein, the highest value was obtained for *T. reesei* Rut-C30 (738±26 mg/L). This means that a comparison made in terms of the specific activity values would show even greater superiority of the extract obtained using *T. harzianum* P49P11.

In work by de Castro et al. [31] with a *T. harzianum* IOC-4038 strain cultivated under SmF using a pretreated sugarcane bagasse, the maximum activity values obtained were 559 (EGase), 97 (FPase), and 745 IU/L (β-glucosidase). The *T. harzianum* IOC-4038 fermentation was found to be faster than for *T. reesei* Rut-C30, and the extract showed a better balance of enzymes. In another study performed with a *Trichoderma atroviride* 676 strain under SmF using sugarcane bagasse as substrate, the best results were obtained after 3–4 days fermentation, with 1,900 IU/L of EGase, 250 IU/L of FPase, and 170 IU/L of β-glucosidase [37]. It was also shown that *T. atroviride* 676 was able to produce the cellulolytic enzymes more efficiently than *T. reesei* Rut-C30. The *T. harzianum* P49P11 wild-type strain used here was previously isolated from the Amazon rainforest and also showed superior capacities for xylanase and cellulase production [3, 17]. This finding emphasizes the importance of enzyme-prospecting research to identify opportunities for enhancing the activity of enzyme preparations.

In summary, all three strains of *Trichoderma* showed the same trend of achieving higher enzymatic production when cultivated using SF, compared with SmF, hence extending the validation of sequential fermentation to different *Trichoderma* strains. An important additional consideration is that further enhancement of enzymatic activities might be achieved after full optimization of the SF process conditions for strains of the genus *Trichoderma*.

Table 4 Enzymatic activity profiles for three *Trichoderma* strains grown under different culture conditions (SmF and SF), with pretreated sugarcane bagasse as inducer substrate after 72 h of cultivation

Enzymes	Enzymatic activity (IU/L)					
	T. reesei Rut-C30		*T. harzianum* P49P11		*Trichoderma* sp INPA 666	
	SmF	SF	SmF	SF	SmF	SF
Xylanase	1,673.3±74	2,048.9±39	1,187.4±55	2,022.1±3	3,861.5±16	3,993.2±57
Endoglucanase	649.7±8	944.9±66	619.4±40	1,668.3±131	619.4±40	833.6±13
β-glucosidase	6.3±0	45.9±3	26.1±5	200.7±17	11.9±1	32.2±3
Avicelase	9.1±1	24.8±1	53.0±16	85.4±2	14.6±4	27.4±5
FPase	5.2±0	37.1±3	21.2±4	51.0±14	5.3±1	12.5±3
Total protein, mg/L	480.8±36	738.1±26	194.6±20	291.0±36	99.7±11	157.4±10

Conclusions

A new sequential cultivation methodology was validated for *T. reesei* Rut-C30, which is considered a reference strain for cellulase production. The use of steam-explosion-pretreated sugarcane bagasse (PB) was shown to be more favorable for EGase production, compared with UB. Characterization of the extracts in terms of the effects of pH and temperature on EGase activity, as well as the hydrolysis profiles obtained for a synthetic substrate, revealed no qualitative differences among the different cultivations tested. However, the enzymes produced using the SmF–UB procedure showed greater thermostability, compared with those obtained under the other cultivation conditions. This indicated the need for further optimization in relation to the removal of inhibitors. Overall, the SF cultivation method for enzyme production was superior to conventional SmF for three different *Trichoderma* strains.

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3. CAPÍTULO III

Secretome analysis of *Trichoderma reesei* and *Aspergillus niger* cultivated by submerged and sequential fermentation processes: enzyme production for sugarcane bagasse hydrolysis

Artigo publicado e reimpresso sob termos e condições estabelecidas pela editora Elsevier na revista Enzyme and Microbial Technology, volume 90, páginas 53-60, ano 2016.

Nesta etapa do trabalho foram feitas análises proteômicas para caracterizar o secretoma do *Trichoderma reesei* Rut C30 e *Aspergillus niger* A12 produzidos por fermentação submersa convencional versus fermentação sequencial, na presença de bagaço de cana pré-tratado por explosão a vapor. A partir dos resultados de caracterização do secretoma, foi avaliada a performance dos coquetéis enzimáticos na hidrólise enzimáticas do bagaço de cana pré-tratado. Os resultados dessa etapa correspondem aos objetivos 3 e 4 do item 1.2. Estes resultados fornecem uma melhor compreensão de como a diversidade de perfis de proteínas obtidas a partir de diferentes processos de cultivo afeta o desempenho de hidrólise. Os dados de toda análise secretômica, com as proteínas identificadas, estão na forma de um artigo denominado *"Data in Brief"* intitulado "*Secretome data from Trichoderma reesei and Aspergillus niger cultivated in submerged and sequential fermentation methods"*.

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Secretome analysis of *Trichoderma reesei* and *Aspergillus niger* cultivated by submerged and sequential fermentation processes: Enzyme production for sugarcane bagasse hydrolysis

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ABSTRACT

Cellulases and hemicellulases from *Trichoderma reesei* and *Aspergillus niger* have been shown to be powerful enzymes for biomass conversion to sugars, but the production costs are still relatively high for commercial application. The choice of an effective microbial cultivation process employed for enzyme production is important, since it may affect titers and the profile of protein secretion. We used proteomic analysis to characterize the secretome of *T. reesei* and *A. niger* cultivated in submerged and sequential fermentation processes. The information gained was key to understand differences in hydrolysis of steam exploded sugarcane bagasse for enzyme cocktails obtained from two different cultivation processes. The sequential process for cultivating *A. niger* gave xylanase and β-glucosidase activities 3- and 8-fold higher, respectively, than corresponding activities from the submerged process. A greater protein diversity of critical cellulolytic and hemicellulolytic enzymes were also observed through secretome analyses. These results helped to explain the 3-fold higher yield for hydrolysis of non-washed pretreated bagasse when combined *T. reesei* and *A. niger* enzyme extracts from sequential fermentation were used in place of enzymes obtained from submerged fermentation. An enzyme loading of 0.7 FPU cellulase activity/g glucan was surprisingly effective when compared to the 5–15 times more enzyme loadings commonly reported for other cellulose hydrolysis studies. Analyses showed that more than 80% consisted of proteins other than cellulases whose role is important to the hydrolysis of a lignocellulose substrate. Our work combined proteomic analyses and enzymology studies to show that sequential and submerged cultivation methods differently influence both titers and secretion profile of key enzymes required for the hydrolysis of sugarcane bagasse. The higher diversity of feruloyl esterases, xylanases and other auxiliary hemicellulolytic enzymes observed in the enzyme mixtures from the sequential fermentation could be one major reason for the more efficient enzyme hydrolysis that results when using the combined secretomes from *A. niger* and *T. reesei*.

1. Introduction

Hydrothermal, steam-explosion, and dilute acid pretreatments have been applied to enhance the digestibility of sugarcane bagasse by facilitating the access of the cellulose to enzymes or microorganisms [1,2]. However, the enzymes for hydrolysis of pretreated bagasse and other types of biomass are costly. The use of lignocellulosic substrates as both carbon source and inducer provides an option for reducing costs [3,4]. Cultivation of industrially relevant fungal strains such as *Aspergillus niger* and *Trichoderma reesei* on steam pretreated sugarcane bagasse has shown that, when solid state culture is transitioned to a submerged fermentation (i.e., sequential fermentation process), enzyme titers are increased, and results in showing cellulases and hemicellulases with relatively

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high specific activities [5–7]. Even though such studies indicate that the activity of enzymes from the sequential fermentation is higher than the conventional submerged fermentation, it remains unknown whether the profile of protein activities secreted upon a sequential fermentation process is responsible for the higher conversion of lignocellulose to monosaccharides.

T. reesei (teleomorph *Hypocrea jecorina*) is a mesophilic soft-rot ascomycete fungus that is widely used in industry as a source of cellulases and hemicellulases for the hydrolysis of plant cell wall polysaccharides. Although it has been shown to be the anamorph of the pantropical ascomycete *Hypocrea jecorina*, the organism remains most widely recognized by its former name. In addition to a long history of safe use for industrial enzyme production, *T. reesei* has also been an important model system for studying lignocellulose degradation [8–10].

Martinez et al. [10] assembled 89 scaffolds (sets of ordered and oriented contigs) to generate 34 Mbp of nearly contiguous *T. reesei* genome sequence comprising 9129 predicted gene models. Interestingly, despite the industrial utility and effectiveness of the carbohydrate-active enzymes of this fungus, its genome encodes fewer cellulases and hemicellulases than other sequenced fungus able to hydrolyze plant cell wall polysaccharides. It was also observed that many *T. reesei* genes encoding carbohydrate-active enzymes are distributed nonrandomly in clusters that lie between regions of synteny with other Sordariomycetes. It was hypothesized that numerous genes encoding biosynthetic pathways for secondary metabolites may promote survival of *T. reesei* in its competitive soil habitat, but genome analysis provided little mechanistic insight into its amazing capacity for protein secretion [10].

Aspergilli, of which *A. niger* is a member, contain a wide spectrum of enzymes for polysaccharide, protein and lipid degradation [11]. Cellulases, hemicellulases, pectinases, amylases, inulinases, lipases and proteases are used in a range of industrial applications. *A. niger* has a long tradition of safe use in the production of enzymes and organic acids. Many of these products have obtained GRAS (generally regarded as safe) status [12–15]. Pel et al. [13] sequenced the 33.9-megabase genome of *A. niger* CBS 513.88, the ancestor of currently used enzyme production strains. A high level of synteny was observed with other aspergilli sequenced, and strong function predictions were made for 6506 of the 14,165 open reading frames identified. They observed striking differences in the hydrolytic enzyme spectra of aspergilli. Some specific differences include, in contrast to the other three aspergilla studied, *A. niger* contains only one GH10 and four GH11 endoxylanases, from a larger oligosaccharide liberating xylanase family.

Recent work [16] reported a detailed secretome analysis of *A. niger* and *T. reesei* grown on sugarcane biomass under the conventional submerged fermentation method. Important enzymes related to biomass degradation, including cellobiohydrolases, endoglucanases, β-glucosidases, β-xylosidases, endoxylanases, xyloglucanases, and α-arabinofuranosidases were identified in both fungi secretomes. Additional studies on the secretome analysis of *T. reesei* [17–20] or *A. niger* [21] strains cultivated on a variety of carbon sources have also been previously reported. However, a combined evaluation on how the fermentation process employed for enzyme production affects titers and the profile of proteins secreted, and in turn how this affects performance on lignocellulose hydrolysis is still not fully understood.

This work used proteomics as a tool to map characterize and compare the secretomes from *T. reesei* Rut C30 and *A. niger* A12 cultivated for 72 h in submerged and sequential fermentation processes (summarized in Fig. 1) and helped to assess how the type of process employed for enzyme production affected the profile of proteins secreted by two relevant strains. The performance of combined *T. reesei* and *A. niger* enzymatic extracts produced under both fermentation processes were evaluated based on hydrolysis of non-washed steam-exploded sugarcane bagasse.

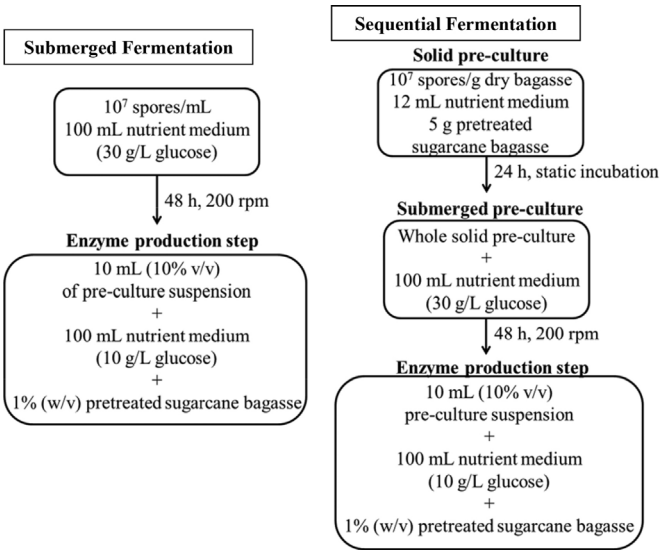

Fig. 1. Experimental procedures for submerged and sequential cultivation methods. Submerged fermentation consists of pre-culture and enzyme production steps, while sequential fermentation consists of solid phase pre-culture followed by submerged pre-culture, and then enzyme production. Conditions given in the figure are discussed in Section 2.

2. Materials and methods

2.1. Fungal strains

The *T. reesei* Rut-C30 strain was purchased from the Centre for Agricultural Bioscience International (CABI) culture collection in the United Kingdom (IMI number: 345108). The *A. niger* wild type A12 strain, originally isolated from black pepper [22], was obtained from Embrapa Food Technology collection (Rio de Janeiro, Brazil). The strains were maintained at −18 °C in a 20% (w/w) glycerol/water solution and were activated in potato dextrose agar (PDA) for 7 days at 30 °C (*T. reesei*) and for 4 days at 32 °C (*A. niger*).

2.2. Cultivation conditions

2.2.1. Submerged fermentation method

In the conventional submerged fermentation process, the pre-culture was initiated with a conidia suspension of 10^7 spores/mL in a total volume of 100 mL of nutrient medium enriched with 30 g/L of glucose (Fig. 1), as described initially from Mandels & Stenberg [23] and adapted by Cunha et al. [24]. The medium contained (% w/v): 0.14 $(NH_4)_2SO_4$, 0.20 KH_2PO_4, 0.03$CaCl_2$, 0.02 $MgSO_4{\cdot}7H_2O$, 0.50 peptone, 0.20 yeast extract, 0.03 urea, 0.10 Tween 80, and 0.10 of salt solution (5 mg/L $FeSO_4.7H_2O$, 1.6 mg/L, $MnSO_4.H_2O$, 1.4 mg/L $ZnSO_4{\cdot}7H_2O$, and 2.0 mg/L $CoCl_2$). The incubation was carried out at an agitation rate of 200 rpm for 48 h at 30 °C (for *T. reesei)* or at 32 °C (for *A. niger*). For enzyme production, a volume of pre-culture suspension corresponding to 10% (v/v) was transferred to the culture medium and supplemented with 10 g/L of glucose and 1% (w/v) of steam-exploded non-washed sugarcane bagasse (donated by the Sugarcane Research Center (CTC, Brazil)). The steam explosion was conducted at 1667 kPa after the bagasse had been held at 205 °C for 20 min. The cultivations were kept in an orbital incubator shaker at 200 rpm for 72 h at 30 °C (for *T. reesei*) or 32 °C (for *A. niger*) (Fig. 1). The cultivation broth was filtered, centrifuged at 4 °C and 10,000 rpm for 15 min, and stored at −18 °C for further analysis.

The cultivation experiments were carried out in triplicate, and the averaged data presented with standard deviations.

2.2.2. Sequential fermentation method

Sequential fermentation was performed in triplicate according to the methodology described previously [5]. Briefly, pre-culture was initiated as a two-step solid state fermentation using 5 g of dry sugarcane bagasse as solid substrate in 500 mL Erlenmeyer flasks. Substrate moisture was adjusted through the addition of 12 mL of nutrient medium (Fig. 1). First the bagasse was inoculated with 10^7 spores/g of dry bagasse, and the culture was maintained under static conditions for 24 h at 30 °C for *T. reesei* and 32 °C for *A. niger*. The pre-culture step was continued as a submerged fermentation after addition of 100 mL of nutrient medium containing 30 g/L of glucose per 5 g of dry bagasse in an orbital incubator shaker for 48 h, 200 rpm and 30 °C and 32 °C, respectively, followed by enzyme production carried out as described in Section 2.2.1 (Fig. 1). Both fermentations were carried out with non-washed sugarcane bagasse as the carbon source.

2.3. Enzyme activity assays

Endoglucanase (EGase), xylanase, and β-xylosidase activities were determined following standard methods [25] with filter paper (FPase) and β-glucosidase activities based on protocols of the International Union of Pure and Applied Chemistry (IUPAC) [26]. Release of reducing sugar was measured using the DNS method [27]. Protein concentration was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL) using bovine serum albumin (BSA) as the standard. All enzymatic analyses were carried out in duplicate (Table 1).

2.4. Secretome analysis

T. reesei and *A. niger* protein extracts were prepared for proteomic analysis by vacuum filtration followed by centrifugation at 4 °C and 10,000 rpm for 15 min. The supernatant was concentrated by about 10 times through ultrafiltration (Amicon ultrafiltration system/millipore) using a 76 mm diameter, 10 kDa cut-off membrane. Two samples from each type of fermentation were subjected to duplicate proteomic analysis using high resolution LTQ-Orbitrap XL LC–MS/MS. A detailed description of procedures for determining the secretome, together with results in Tables 1 and 2 are both given in the companion Data in Brief article submitted concomitantly to this paper [28].

2.5. Enzymatic hydrolysis of sugarcane bagasse by fungal combined extracts

Enzymatic hydrolysis was carried out in 250 mL Erlenmeyer flasks with 50 mM citrate buffer at pH 4.8, in an orbital incubator shaker operated at an agitation speed of 200 rpm at 50 °C for 96 h, using a combination of enzymes from *T. reesei* and *A. niger* that were produced by either submerged or sequential fermentations. The non-washed steam-exploded sugarcane bagasse was applied at a concentration of 1% (w/v) of total solids. Composition of the pretreated bagasse on a w/w basis was 71% glucan, 0.5% pentosan, 27.8% lignin and 3% ash [29]. The final reaction volume of 100 mL consisted of enzymes, bagasse solids and buffer.

Saccharification was performed using combined extracts from *T. reesei* and *A. niger* (enzyme composition in both Tables 2 of this manuscript and Data in Brief article [28]). Enzyme loadings of 0.7 filter paper cellulase unit (FPU) or 71 mg protein (*T. reesei* cocktails) were mixed with 3.6 cellobiase unit (CBU) or 44.3 mg protein (*A. niger* cocktails) per gram of glucan [21,30]. As control experiments, reaction blanks for both the substrate and enzymes were carried out. Samples were taken at 0, 24, 48, 72 and 96 h. All hydrolysis experiments were performed in triplicate.

2.6. Chemical analysis of hydrolyzed samples

Collected samples (0, 24, 48, 72 and 96 h) were filtered using 0.2 μm Syringe Filters with nylon membrane (Pall Corporation, Port Washington, NY) and diluted accordingly. The released glucose was measured by D-glucose assay procedure kit (Megazyme International, Ireland). HPLC analysis methodology used was previously described by Ximenes et al. [31].

3. Results and discussion

3.1. Enzymatic profile of extracts produced by T. reesei and A. niger

Cellulase and hemicellulase production was measured for *A. niger* and *T. reesei* grown through conventional submerged and the sequential solid-state cultures followed by submerged fermentations as shown in Table 1. The highest values of activity for most of the quantified enzymes were observed when the fungi were cultivated upon the sequential fermentation method. The cultivation of *A. niger* upon this method resulted in 8 and 3.4 times higher β-glucosidase and xylanase production, respectively, compared to results from submerged fermentation. For *T. reesei*, xylanase and β-xylosidase were 2.3 and 2.2-fold higher under the sequential method. Additionally, the sequential method resulted in higher values of endoglucanase activities, protein concentrations and specific activities for both strains (Table 1). These findings are in agreement with previous reports in the literature showing that the sequential solid-state and submerged cultivation method for enzyme production was superior to the conventional submerged fermentation for both *Aspergillus* and *Trichoderma* fungal strains [5–7].

3.2. Secretome analysis

A proteomic analysis of the proteins expressed and secreted by *A. niger* and *T. reesei* grown under the conventional submerged and sequential fermentation methods was carried out in order to identify their differential glycoside hydrolase profiles (Table 1, Data in Brief article [28]). These analyses revealed 97 and 79 proteins for *T. reesei* and 99 and 112 proteins for *A. niger* extracts from the submerged and sequential fermentations, respectively. In previous studies [16] 45 different enzymes from CAZy database (CAZymes) in *T. reesei* and 89 in *A. niger* were identified. A longer cultivation time in our study (72 versus 24 h) and/or differences between the medium composition, could have resulted in a higher number of proteins. Some proteins in common were only found in secretomes from the same fungus cultivated upon different methods (Table 1, Data in Brief article [28]). In a previous study was reported that secretome profiles of *T. reesei* and *A. niger* grown in steam exploded bagasse and culm (the stem of sugarcane) *in natura* upon submerged fermentation indicated that they differed considerable, but the pattern of *A. niger* enzyme production was similar in the presence of both substrates. Even though there were no differences in fungi growth between these 2 substrates, it was observed that *A. niger* and *T. reesei* secreted more enzymes when grown on culm, which was argued could be related to differences in the recalcitrance of substrates.

The *T. reesei* secretomes from submerged and sequential fermentations showed 27% of the total 176 identified proteins to be in common to both cultivation methods. They included 3 endoglucanases, 2 cellobiohydrolases (CBH), 8 hemicellulases, 10 families of glycoside hydrolases, 4 proteases, 6 predicted proteins, 3 hypothetical proteins and 10 other proteins, including some esterases and

Table 1
Comparison of enzyme activity profiles for *T. reesei* Rut C30 and *A. niger* A12 under submerged (A) and sequential fermentation (B).

Enzymes	Substrate	Method	Enzyme Activity (IU mL^{-1})			
			T. reesei Rut C30		*A. niger* A12	
			A	B	A	B
Endoglucanase	carboximethylcellulose	Dien et al, 2008	1.6	2.5 ± 0.6	0.6	0.9
β-glucosidase	cellobiose	Ghose, 1977	0.02	0.02	0.1	0.8
FPase	filter paper	Ghose, 1977	0.02	0.02	0.01	0.01
Xylanase	xylan birchwood	Dien et al, 2008	7.9 ± 0.6	18.3 ± 0.3	7.8 ± 0.6	26.4 ± 2.1
β-xylosidase	ρ-nitrophenol	Dien et al, 2008	0.6	1.3	24.6	31.5
Total protein[a]	bovine serum albumin	Pierce BCA protein assay kit	3.0 ± 0.2	3.7 ± 0.1	2.9 ± 0.1	3.2

[a] Expressed in mg mL^{-1}.

cell-wall proteins, resulting in a total of 46 common proteins in both cultivation methods (Table 1, Data in Brief article) [28]. Hypothetical proteins are those which may have the same profile of another protein, but no assigned function. Together with the predicted proteins, these are representative of the secretomes of different fungi [21,32], including *T. reesei* [20]. It is noteworthy that some relevant biomass-degrading enzymes such as α-glucuronidases (GH67) were only present in the sequential cultivation extract. Such enzymes remove uronic acid from the non-reducing end of glucurono-xylooligosaccharides, and thus contribute to the complete hydrolysis of xylan chains [33].

The secretomes from *A. niger* grown under submerged and sequential fermentations showed 29% of the total 211 proteins to be common between the two methods. These common proteins included 2 endoglucanases, 1CBH, 3 β-glucosidases, 14 hemicellulases, 5 proteases, 6 hypothetical proteins, 14 unnamed proteins, 3 proteins associated with fungal cell-wall, and 14 additional proteins including amylases, esterases and others (Table 1, Data in Brief article) [28]. It is interesting to note that, even though the sequential fermentation method also has a submerged fermentation step, many enzymes secreted in submerged fermentation were not found in the sequential fermentation cocktails. These results may indicate that, in the sequential process, the germination of the spores in the solid-state fermentation step in the presence of sugarcane bagasse as inducer substrate could direct the fungus metabolism towards the secretion of some distinct proteins from those produced when the fungal spores germinated during the submerged fermentation process. A more detailed comparative analysis on how the cultivation method affected the profile of proteins in the individual secretomes from each of these two relevant industrial strains is presented as follows.

3.2.1. T. reesei *secretome*

The enzymatic proteins produced by *T. reesei* under sequential fermentation contained the highest levels of cellulolytic and hemicellulolytic activities, while those from submerged fermentation showed higher protein diversity according to the LC–MS/MS data (Table 1, Data in Brief article) [28]. The percentages of the secreted proteins involved in cellulose and hemicellulose degradation were similar for submerged (20.6%) and sequential (19%) fermentation methods. In both cultivation methods, expansion-like proteins (such as swollenin, which plays a non-hydrolytic role in the disruption of lignocellulose) [18], and CIP1 protein (which acts as cellulase enhancing protein) were also identified.

The α- and β-glycoside hydrolase (GH) families in the secretome of *T. reesei* made up 25.7% of the 97 proteins in submerged fermentation and 17.7% of the 79 proteins in the sequential fermentation, according to the carbohydrate-active enzyme database (CAZy, www.cazy.org) (Fig. 2). Grouping and distribution analysis of the secreted proteins identified 46 different GH families in the total pool of proteins from secretome extracts of both cultivation methods. Potential representatives

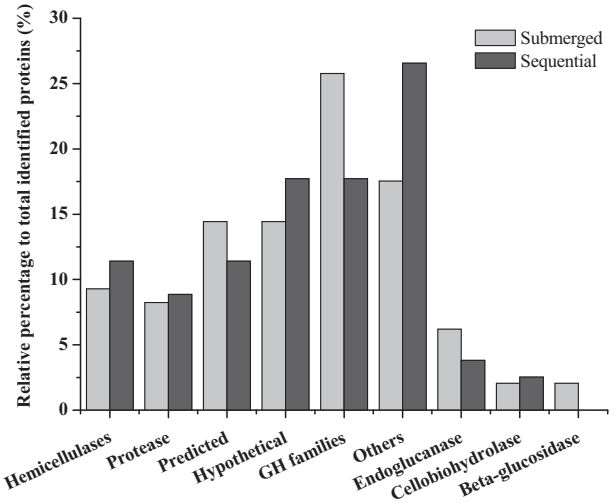

Fig. 2. Functional classification of proteins identified from *T. reesei* Rut C30 secretome. The proteins identified by LC–MS/MS analysis of the secretome produced from growth in submerged and sequential fermentation were classified according their biological function.

of the GH6 and GH7 (cellobiohydrolases), GH11 (endoxylanases), GH54 (α-L/N-arabinofuranosidases), GH74 (xyloglucanases), GH61 (cooper-dependent lytic polysaccharide monooxygenases now reclassified in family AA9) families were detected in extracts from both cultivation methods. However, similar to what has been reported in other studies [18], not all of the predicted GHs were identified.

Proteins belonging to GH families 5 (endo-β-1,4-glucanase, β-mannosidase, among others), 16 (glucanosyltransferase, endoglucanase, galactosidase, among others), 28 (polygalacturonase), 72 (glucanosyltransglycosylase), 92 (mannosidase) were detected in extracts from the submerged fermentations. Representatives of GH families 17 (glucanosyltransglycosylase), 55 (endoglucanase), 74 (endoglucanase and xyloglucanase) were unique to submerged fermentations while only representative of GH families 3 (β-glucosidases and β-xylosidases), 38 (mannosidase), 67 (glucuronidase) were found for extracts produced upon sequential fermentation.

The presence of GH families 16 (endo-1,4-β-D-glucanase) and 55 (endo-1,3-β-D-glucanase) in the submerged fermentation secretome is not commonly reported in the literature. A comparative secretome analyses study of two *T. reesei* strains (Rut-C30 and CL847) using lactose as carbon source and inducer of cellulases has shown the expression of a new putative endoglucanase, in addition to an arabinofuranosidase, by the reference map of the secreted proteins and experimental evidence [34]. The more diversified secretome of CL847 (an industrial mutant strain) suggests a more general hypersecretory strain, while Rut-C30 may be more

cellulase production oriented. This difference in the secretome of these 2 strains may have occurred due to the completely different "mutation route" followed towards becoming efficient enzyme producers.

The extracellular cellulolytic system of *T. reesei* is composed of 60–80% cellobiohydrolases or exoglucanases (EC3.2.1.74), 20–36% endoglucanases (EC3.2.1.4) and 1% β-glucosidases (EC3.2.1.21), which act synergistically in the conversion of cellulose into glucose [35,36]. The cellulolytic system includes eight *endo*-1,4-β-D-glucanase (Cel7B, Cel12, Cel45, two Cel5 and three Cel61) responsible for primary hydrolysis that cleaves accessible intramolecular β-1,4-glucosidic bonds of cellulose chains randomly in a non-proccessive manner with formation of new chain ends [37]. Our data showed that only endoglucanase Cel45 was not secreted by *T. reesei* upon submerged fermentation conditions. Three endoglucanases (Cel7, Cel12 and Cel45) were secreted during the sequential fermentation. Cellobiohydrolases I and II, belonging to the GH7 and GH6 families, respectively [20], were identified in secretomes of both cultivation methods. Only two of the seven β-glucosidases that are part of the cellulolytic system of *T. reesei* strain [37] were observed for the submerged fermentation. None accumulated in the sequential fermentation secretome, although GH3 proteins [38,39] were present.

The predicted proteins corresponded to 14.4% and 11.4% in the submerged and sequential fermentation methods, respectively; while hypothetical proteins were 14.4% and 17.7% (Fig. 2). Similar levels of proteases and peptidases (8%) and lignocellulolytic-related enzymes (9%) were identified in secretomes of both cultivation methods. Other proteins in the submerged (16.5%) and sequential fermentation (25.3%) secretomes included those involved in lipid transportation and metabolism, pectin degradation, cell-wall biosynthesis and morphogenesis, and chitin degradation. The *T. reesei* submerged fermentation secretome also contained one bifunctional catalase-peroxidase involved in the removal of H_2O_2 from the cells, which may also contribute to lignin degradation [17,40].

According to some studies, the enzyme production by *T. reesei* is transcriptionally regulated and dependent on the carbon source [41–44]. The range of potential substrates is still limited because most carbon sources are too expensive for industrial scale fermentations [19,45]. The effective use of sugarcane bagasse as carbon source for fungal growth may address this limitation. Our results revealed that the enzymes induced in the presence of sugarcane bagasse under different cultivation methods display a distinctive profile, suggesting that the cultivation conditions (reported in this work) enable production of tailored enzymatic compositions that efficiently hydrolyze pretreated biomass.

3.2.2. A. niger *secretome*

In the submerged and sequential fermentation secretomes obtained from *A. niger* (Fig. 3) the proteins involved in cellulose and hemicellulose degradation corresponded to 26% and 29%, respectively, of the total 99 secreted proteins in submerged and 112 in sequential fermentation. The number of proteins acting in the cellulose and hemicellulose degradation pathways was almost 1.5-fold higher in the extract from the sequential fermentation. This is in agreement with the higher enzymatic activity (cellulases and hemicellulases) observed in the enzyme mixture produced under these conditions (Table 1).

GH families acting in the cellulose degradation were observed in both cultivation methods, including GH3, 12 and 16 (β-glucosidase), GH5 (endoglucanase), and GH7 (cellobiohydrolase). Some identified proteins and one GH protein were exclusive to sequential fermentation extracts (β-glucosidase M4, endo-β-1,4-glucanase A and GH31 (β-glucosidase)). GH proteins that degrade hemicellulose were also observed in both cultivation methods, including GH2 (mannosidase), GH10 and GH11 (endoxylanase), and GH62 (α-arabirofuranosidase). GH38 (mannosidase) and GH43 (endo-arabinase) were observed only in the submerged and sequential fermentation secretomes, respectively.

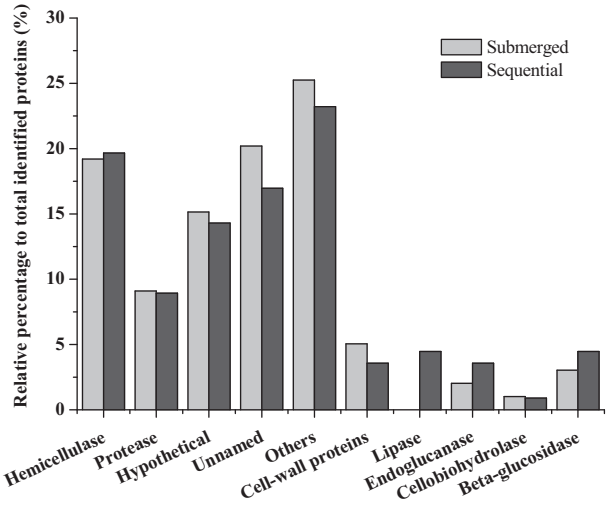

Fig. 3. Functional classification of proteins identified from *A. niger* A12 secretome. The proteins identified by LC–MS/MS analysis of the secretome produced from growth in submerged and sequential fermentation were classified according their biological function.

Table 2
Activity profile for enzyme extracts combined for hydrolysis of non-washed sugarcane bagasse. Enzyme extracts were produced by *T. reesei* Rut C30 and *A. niger* A12 under (A) submerged and (B) sequential fermentation.

	Enzymes (g of glucan)	*T. reesei*	*A. niger*	Total
A	FPase (FPU g^{-1})	0.7	0	0.7
	Endoglucanase (IU g^{-1})	57.1	2.1	59.3
	Cellobiase (CBU g^{-1})	1.0	3.6	4.6
	Xylanase (IU g^{-1})	506.4	26.4	532.9
	Total protein (mg g^{-1})	69.1	1.9	71.0
B	FPase (FPU g^{-1})	0.7	0	0.7
	Endoglucanase (IU g^{-1})	61.9	0.7	62.6
	Cellobiase (CBU g^{-1})	0.7	3.6	4.3
	Xylanase (IU g^{-1})	662.6	65.7	728.3
	Total protein (mg g^{-1})	43.6	0.7	44.3

The number of identified proteins in hypothetical and unnamed categories represents 35% and 31% for submerged and sequential fermentation methods, respectively. Some hypothetical proteins belonging to GH families were observed in secretome of both cultivation methods, including GH72 (glucanosyltransglycosylase) and 75 (chitosanase). The sequential fermentation secretome showed 2 GH families as hypothetical proteins: GH3 (β-glucosidase and β-xylosidase) and GH43 (arabinofuranosidase and β-xylosidase). Hypothetical proteins belonging to GH family 1 (β-glucosidase) and 31 (β-glucosidase and β-xylosidase) were also observed, as well as proteins of unknown function. Some of them may encode putative proteins important for sugarcane bagasse hydrolysis, such as non-hydrolytic accessory proteins that increase or favor enzymatic efficiency [46]. The percentage of the identified peptidases and proteases in the *A. niger* secretome under both cultivation methods were similar (9%).

A. niger also produces cell-wall proteins including chitinases. This study shows that these proteins represent 5% and 3.5% of the total secreted proteins in submerged and sequential fermentations, respectively. Two (B and C) and three (A, B1 and C) feruloyl esterases were observed in submerged and sequential fermentation secretomes, respectively. Feruloyl esterases hydrolyze diferulate cross-links in plant cell-walls and facilitate access to the

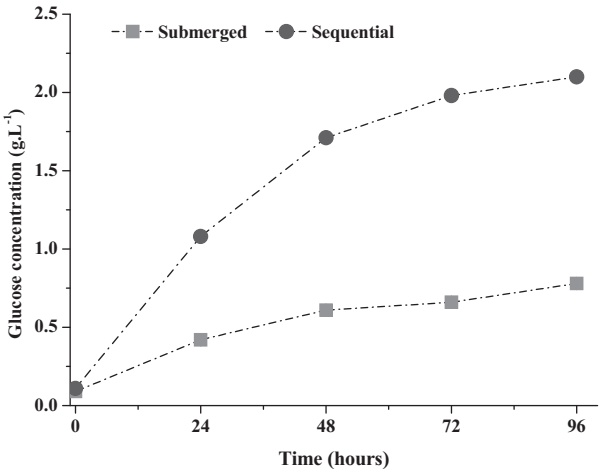

Fig. 4. Comparison of performance of submerged and sequential fermentation enzyme extracts based on glucose released during enzymatic hydrolysis (96 h) of sugarcane bagasse using the equivalent loading of both enzymatic extracts. Conditions for hydrolysis of sugarcane bagasse: 0.7 FPU/g glucan or 71 mg protein/g glucan (for *T. reesei*): 3.6CBU/g glucan or 44.3 mg protein/g glucan (from *A. niger*) at a bagasse solids loading of 1% (w/v) based steam exploded sugarcane bagasse with composition given in Section 2.6. Hydrolysis carried out at pH 4.8 and 50 °C in an orbital shaker at 200 rpm for 96 h.

polysaccharide backbone [11]. Five lipases were observed only in the sequential fermentation secretome.

Lignocellulolytic-related proteins (in the combined hemicellulase and cellulase fraction) corresponded to 24.2% and 23.2% in the submerged and sequential fermentation secretomes, respectively, and other enzymes components, in addition to the hydrolases, were identified as dehydrogenases with a suggested role to dehydrogenate or oxidize cellobiose and other cellodextrins [47,48]. Oxygenases, belonging to the class of copper-dependent metalloenzymes, which oxidatively cleave glycosidic bonds on the surface of cellulose without requiring a separation of the glucan chain, were also identified [49,50].

Comparison of the *T. reesei* and *A. niger* proteomic results shows that *A. niger* expresses a more versatile range of cellulases, hemicellulases and esterases than *T. reesei*. The latter two groups of enzymes become even more important for pretreatment steps that remove xylans [46]. The observation that the secretome of the 2 fungal strains studied here do not share proteins with the same amino acid sequence is consistent with their effective complementary use in lignocellulose hydrolysis, as pointed out by the work of Borin et al. [16] and the results presented in this paper. In the work of Borin et al. [16], it was observed GHs involved in hemicellulose degradation that were exclusively found in *A. niger* secretome. *T. reesei* produces more efficient cellulases, while *A. niger* produces more effective hemicellulases and β-glucosidases [51], with enzymes from these two sources showing different patterns of resistance to lignin-derived inhibitors and deactivators [31,52,53]. Hence, formulations involving enzymes from both microorganisms are expected to result in more effective enzyme hydrolysis.

3.3. *Enzymatic hydrolysis of sugarcane bagasse using the combined* T. reesei *and* A. niger *enzymes*

Enzymes from the sequential fermentation at 0.7 FPU/g glucan gave 30% conversion to glucose while enzyme extracts from the submerged fermentation gave 10% for hydrolysis of steam exploded sugarcane bagasse (Fig. 4). The better performance of enzymes produced by the sequential fermentation may be explained by the higher xylanase titers (Table 2) and a balance of cellulolytic/hemicellulolytic enzymes for the hydrolysis of the pretreated sugarcane bagasse (Table 2, Data in Brief article) [28]. Table 2 showed that fixing the enzyme loadings for the steam-exploded sugarcane bagasse hydrolysis experiments at 0.7 FPU and 3.6CBU for *T. reesei* and *A. niger* extracts, respectively, did not result in major differences in the final enzyme activities measured as filter paper units, endoglucanase and cellobiase activities for the submerged and sequential fermentation enzyme extracts. However, higher levels of xylanase activities were present in the latter (Table 1). Additionally, the overall amount of proteins identified by LC–MS/MS as hemicellulases were higher for the sequential fermentation compared to the submerged fermentation (52% versus 41%, respectively, Table 2, Data in Brief article) [28].

The proteomic analysis shown in Table 2 (Data in Brief article) [28] is for extracts of *T. reesei* and *A. niger* that were combined in a ratio of 1:5 filter paper to cellobiase activities giving the resulting enzyme profiles. Even though these runs were performed with combined extracts at relatively low enzyme loading to hydrolyze non-washed steam exploded pretreated bagasse, which is known to contain enzyme inhibitors [31,54], significant hydrolysis of 30% occurred. This indicates important role of the non-cellulolytic proteins in the combined extract. While the cellulase loading of 0.7 FPU/g glucan was low, the total protein was still 70 mg/g which is consistent with lignin blocking role of protein [55].

Wang et al. [56] and Yu et al. [57], respectively, reported 60% and 52% cellulose conversion of liquid hot water pretreated sugarcane bagasse (5% w/v) using commercial enzyme preparations at loadings of 20 and 15 FPU/g of cellulose. We used 0.7 FPU/g glucan, or about 20–30 times lower loading for hydrolysis of 1% w/v non-washed steam exploded sugarcane bagasse. While the hydrolysis conditions are not directly comparable, the significantly lower cellulolytic enzyme loadings of extracts, where only 15–20% of the protein was cellulase, was notable, and may reflect the diversity of other proteins in the secretome.

A higher diversity of feruloyl esterases, xylanases and other auxiliary hemicellulolytic enzymes was observed in the enzyme mixtures from the sequential fermentation (Data in Brief article, Table 2) [28]. In lignocellulose hydrolysis, xylanases and β-xylosidases play a key role in minimizing inhibitory effects of xylo-oligosaccharides on cellulases [31,58]. The auxiliary hemicellulolytic enzymes including ferulic acid esterases, acetylxylan esterase and α-arabinofuranosidase further enhance the hydrolysis of lignocellulose by cleaving ester bonds on xylan, removing α-arabinose and O-acetyl groups, and allowing the xylan/xylooligosaccharides to be hydrolyzed to monomeric sugars that are much less inhibitory [59].

The higher saccharification efficiency obtained by using a combination of enzymes from *T. reesei* and *A. niger* has been previously observed [16,60]. The proteins identified in the secretomes of the two fermentation methods studied here represented a broad range of hydrolase activities, where endoglucanase, cellobiohydrolase, and β-glucosidase activities accounted for 15–20% of the total protein. The additional proteins secreted by *A. niger and T. reesei* may have the potential to enable a low cellulase loading through interactions with the lignocellulosic biomass, by blocking adsorption of cellulases on lignin, and providing enzyme activities that cleave inhibitory oligosaccharides. This is consistent with prior reports that document the effects of added, non-cellulolytic enzyme proteins in blocking cellulase adsorption on lignin, removing product inhibitors, and reducing the amount of cellulases and β-glucosidase needed for hydrolysis of pretreated biomass [31,52,53,55,61–65].

4. Conclusion

A remarkable variation in the secretome of *T. reesei* and *A. niger* occurred as function of cultivation methods. Higher

cellulolytic and hemicellulolytic production occurred for sequential (solid state followed by submerged) fermentation compared to submerged fermentation for extracts of both fungi when non-washed steam-exploded sugarcane bagasse was used as carbon source. Proteomic analysis for the *A. niger* strain showed that sequential fermentation gave the secretome with highest number of identified proteins and titers of enzyme activity. Higher enzymatic activities and/or a better balance of secretome composition from the sequential fermentation in key cellulolytic and hemicellulolytic activities was reflected by 3-fold higher saccharification of the pretreated sugarcane bagasse for the combined enzyme mixtures from the sequential fermentations of *A. niger* and *T. reesei*. This new knowledge about the secretomes provides additional rationale for combining enzyme extracts [16,66,67] or culture broths from *T. reesei* and *A. niger* to achieve enhanced lignocellulose hydrolysis as well as demonstrating the potential benefits of extended fermentation times in producing these fungal enzymes.

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4. CAPÍTULO IV

Secretome data from *Trichoderma reesei* and *Aspergillus niger* cultivated in submerged and sequential fermentation methods

Artigo de dados publicado e reimpresso sob termos e condições estabelecidas pela editora Elsevier na Data in Brief, volume 8, páginas 588-598, ano 2016.

Nesta etapa do trabalho os dados de toda análise secretômica realizada no artigo apresentado no capítulo 3 foram reunidos e colocados na forma de um artigo de dados, todas as proteínas identificadas estão dispostas em tabelas no *"Data in Brief"* intitulado "*Secretome data from Trichoderma reesei and Aspergillus niger cultivated in submerged and sequential fermentation methods"*.

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Data Article

Secretome data from *Trichoderma reesei* and *Aspergillus niger* cultivated in submerged and sequential fermentation methods

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ABSTRACT

The cultivation procedure and the fungal strain applied for enzyme production may influence levels and profile of the proteins produced. The proteomic analysis data presented here provide critical information to compare proteins secreted by *Trichoderma reesei* and *Aspergillus niger* when cultivated through submerged and sequential fermentation processes, using steam-explosion sugarcane bagasse as inducer for enzyme production. The proteins were organized according to the families described in CAZy database as cellulases, hemicellulases, proteases/peptidases, cell-wall-protein, lipases, others (catalase, esterase, etc.), glycoside hydrolases families, predicted and hypothetical proteins. Further detailed analysis of this data is provided in "Secretome analysis of *Trichoderma reesei* and *Aspergillus niger* cultivated by submerged and sequential fermentation process: enzyme production for sugarcane bagasse hydrolysis" C. Florencio, F.M. Cunha, A.C Badino, C.S. Farinas, E. Ximenes, M.R. Ladisch (2016) [1].

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Specifications Table

Subject area	Biochemistry
More specific subject area	Proteomic
Type of data	Table
How data was acquired	LC MS/MS analysis using Mascot Daemon version 2.4.0 (Matrix Science)
Data format	Analyzed
Experimental factors	Concentrated enzymatic cocktail from *A. niger* A12 and *T. reesei* Rut C30 produced by submerged fermentation and sequential fermentation
Experimental features	Peptides from enzyme cocktail of *A. niger* A12 and *T. reesei* Rut C30 were analyzed by LC-MS/MS
Data source location	Purdue University, West Lafayette, USA.
Data accessibility	Data is with this article

Value of the data

- This data set will be of value to the scientific community aiming to analyze the identified proteins secreted by *T. reesei* and *A. niger* under different cultivation methods.
- The data can be a useful tool to effectively select fungal strain and cultivation procedure for the production of proteins of interest.
- The data provided here identify key enzymes from *T. reesei* and *A. niger* for combined use to effectively degrade lignocellulose substrates, and therefore provide an opportunity to help researchers in the field to formulate enzyme cocktails in according to characteristics of lignocellulose biomass and enzyme activities found in the secretome.

1. Data

In Table 1, the proteins identified by proteomic analysis of enzymatic cocktails from *Trichoderma reesei* and *Aspergillus niger*, cultivated on pretreated sugarcane bagasse by either submerged or sequential fermentation processes, are presented according to the families classification from CAZy database.

The enzymatic hydrolysis of pretreated sugarcane bagasse was performed with combined extracts from *T. reesei* Rut C30 and *A. niger* A12, and the data of proteomic analysis of this combination of identified proteins is shown in Table 2. The indicated enzyme loadings were applied for steam-explosion sugarcane bagasse saccharification as described by Florencio et al. [1].

2. Experimental design, materials and methods

2.1. Fungal strains

The strains used for enzyme production were *T. reesei* Rut-C30 and *A. niger* wild type A12 obtained from Centre for Agricultural Bioscience International (CABI) culture collection (United Kingdom) and Embrapa Food Technology collection (Rio de Janeiro, Brazil), respectively. The conditions in which strains were maintained are described in Florencio et al. [1].

2.2. Cultivation conditions

Submerged and sequential fermentations carried out to obtain the enzymatic cocktails from *T. reesei* and *A. niger* are described in detail in Florencio et al. [1]. Briefly, the submerged fermentation was initiated with a 48 h pre-culture that contained a final conidia concentration of 10^7 spores/mL in

Table 1
Major proteins identified in the secretome of *Trichoderma reesei* and *Aspergillus niger* cultivated under submerged (A) and sequential (B) fermentation methods.

Gene ID	Enzyme		Family	*T. reesei*		*A. niger*	
				A	B	A	B
21842121	Cellulases	Endoglucanase	GH12	x	x		
3757552		Endoglucanase A	GH12			x	x
145235569		Endo-beta-1,4-glucanase A	GH12				x
145228915		Endo-beta-1,4-glucanase A	GH12				x
2833231		Endoglucanase I	GH7	x	x		
121794		Endoglucanase II	GH5	x			
201066457		Endoglucanase IV (AA9)	GH61	x	x		
145235523		Glucan endo-1,3-beta-glucosidase eglC	–			x	x
320592482		Beta-glucanase	–	x			
403314396		Endoglucanase VI	GH61	x			
145229151		Endo-1,3(4)-beta-glucanase	GH16			x	x
202072834		Cellobiohydrolase I	GH7	x	x		
95115828		Cellobiohydrolase II	GH6	x	x		
74698499		1,4-beta-D-glucan cellobiohydrolase	GH7			x	x
201066459		Glucosidase	GH3	x			
126046487		β-glucosidase	GH3			x	x
145242946		β-glucosidase M 4	GH3				x
145255120		Glucan 1,3-beta-glucosidase A	GH5			x	x
400602153		Glucan 1,3-β-glucosidase	GH17	x			
257187		Alpha-glucosidase P2 subunit 5	GH31				x
317035725	Hemicellulases	Endo-arabinase	GH43				x
145234699		Alpha-L-arabinofuranosidase axhA	GH62			x	x
358375978		Arabinoxylan arabinofuranohydrolase	GH62			x	x
145233623		Endo-1,5-alpha-L-arabinosidase C	GH43			x	x
145250511		Alpha-N-arabinofuranosidase B	–			x	x
78101601		Anfaea-ferulic Acid Complex	–			x	
23821545		Feruloyl esterase B	–			x	
145246174		Feruloyl esterase C	–			x	x
48425840		Ferulic acid esterase	–				x
145247672		Feruloyl esterase B-1	–				x
145230716		Beta-galactosidase E	GH35			x	x
350630290		Alpha-galactosidase extracellular	–				x
74626383		Alpha-galactosidase B	–			x	x
317034650		Alpha-galactosidase D	–				x
307776646		Beta-mannanase	GH5	x	x		
358367813		Alpha-mannosidase	GH38			x	
145233855		Alpha-mannosidase	GH38			x	
572273984		Beta-mannosidase A	GH2	x			
572273001		Putative beta-mannosidase A	GH2	x	x		
317032967		Beta-mannosidase A	GH2			x	X
358369379		Beta-mannosidase (MndA)	GH2				x
145230794		Alpha-1,2-mannosidase 1B	GH47			x	x
145256261		Pectate lyase plyB	–			x	
572278177		Pectin lyase-like protein	–	x	x		
165906534		Endoxylanase	GH10	x	x		
11513450		Acetyl Xylan Esterase	–	x	x		
292495278		Endo-1,4-beta-xylanase C	GH10			x	x
549461	Hemicellulases	Endo-1,4-beta-xylanase 2	GH11	x	x		
145250044		Endo-1,4-beta-xylanase 5	GH11			x	x
157488002		Swollenin	CBM1	x	x		
9858848		Xylanase	GH11			x	
42716406		Xylanase	GH11	x	x		
13242071		Xylanase	GH11				x
26514830		Xylanase	GH11		x		
83638302		Xylanase	GH11				x
380293098		Xylanase II	GH11	x	x		

Table 1 (*continued*)

Gene ID	Enzyme		Family	*T. reesei*		*A. niger*	
				A	B	A	B
145242002		Alpha-xylosidase	GH31			x	x
145230215		Exo-1,4-beta-xylosidase xlnD	GH3			x	x
145243586		Xylosidase/arabinosidase	–			x	x
145228611	Proteases/ Peptidases	Aorsin	–				x
530795		Pepsinogen	–			x	x
589101183		Aminopeptidase	–	x			
145257498		Aminopeptidase 2	–			x	
145242728		Vaculolar aspartyl aminopeptidase Lap4	–			x	x
145583569		Aspartic endopeptidase	–	x			
145254317		Aspartic-type endopeptidase opsB	–				x
145248205		Aspartic-type endopeptidase opsB	–				x
145256471		Dipeptidyl peptidase III	–			x	
145249068		Tripeptidyl-peptidase sed2	–				x
629687989		Tripeptidyl peptidase precursor	–	x			
145246822		Extracellular serine carboxypeptidase	–			x	
1093596		Ser carboxypeptidase	–				x
145235505		Serine carboxypeptidase	–			x	x
317026828		Serine-type carboxypeptidase	–			x	x
134077081		Endoprotease Endo-Pro-A. niger	–			x	x
62002221		Subtilase protease	–		x		
115111226		Subtilisin-like protease	–	x	x		
589111601		Serine protease	–		x		
29421423		Extracellular serine protease	–	x			
124295071		SprT - serine protease	–	x	x		
464359		Subtilisin-like serine protease pepC	–			x	
589099267		Trypsin-like serine protease	–	x	x		
193735605		Vacuolar protease A	–		x		
387772861		Aspartic proteinase	–	x	x		
38256986	Cell-wall protein	Cell wall protein	–			x	
47028077		Cell-wall protein - CwpA	–				x
145252266		GPI anchored cell wall protein	GH64			x	x
589109601		Ceramidase family protein	–		x		
145255556		Alkaline nonlysosomal ceramidase	–			x	
387772865		Cerato-platanin	–	x	x		
270160616		Chitinase	GH18	x	x		
145232927		Endochitinase 1	GH18			x	x
1839391		Exochitinase	GH20	x			
145256696		Protein ecm33	–			x	x
145241592	Lipases	Lysophospholipase 1	–				x
145234164		Lysophospholipase 1	–				x
145231236		Phospholipase C PLC-C	–				x
109677003		Triacylglycerol lipase precursor	–				x
110431975		Triacyglycerol lipase B	–				x
589114715	Others	Amidase	–	x	x		
145239143		Aminotransferase, class V	–			x	
145241960		Alpha-amylase	–			x	
350631148		Alpha-amylase A	CBM20				x
145243632		Alpha-amylase a type-1/2	–			x	x
224027		Glucoamylase G1	GH15			x	x
145241784		N-acetylglucosaminidase	GH20			x	
113206519		Acetyl esterase	–		x		
589098125		Carbohydrate esterase	–	x	x		
358388255		Carbohydrate esterase family 15 protein	CBM15	x	x		
572279065		Carboxylesterase	–		x		
145233451		Cholinesterase	–			x	x
1705640		Catalase R	–				x
589115621		Catalase/peroxidase	–	x			

Table 1 (*continued*)

Gene ID	Enzyme		Family	*T. reesei*		*A. niger*	
				A	B	A	B
145228625		Catalase R	–			x	
119474019		Mycelial catalase Cat1	–			x	
404312830		Cellulose Induced Protein, CIP1	–	x	x		
589107171		Oxalate decarboxylase	–	x	x		
380482942		Oxalate decarboxylase family bicupin	–	x			
1169291		Aldehyde dehydrogenase	–			x	
572279542		Dihydrolipoyl dehydrogenase	–	x	x		
350631179		FAD/FMN-containing dehydrogenase	–			x	
589113573		Malate dehydrogenase	–	x			
19702487		Malate dehydrogenase			x		
145257405		Short-chain dehydrogenase	–			x	x
145230419		Glycosidase crf1	–				x
145256130		1,3-beta-glucanosyltransferase gel1	GH72			x	x
145240407		1,3-beta-glucanosyltransferase gel2	GH72			x	x
145241490		1,3-beta-glucanosyltransferase gel3	GH72				x
145234270		Glutaminase GtaA	–			x	x
145247260		Inulinase	GH32			x	x
145242650		Nucleoside diphosphate kinase	–			x	
589102565		Acid phosphatase-like protein	–	x	x		
130734		Phosphate-repressible acid phosphatase	–				x
145232002		Phosphatidylglycerol	–			x	x
145251519		Phosphoglycerate mutase family protein	–				x
572278887	Glycoside Hydrolases families	Glycoside Hydrolase (GH)	GH	x			
572275960		GH, partial	GH		x		
358381827		GH family 2 protein	GH2	x			
589104105		GH family 3	GH3		x		
358388254		GH family 5 protein	GH5	x			
589100793		GH family 10	GH10	x	x		
261825113		GH family 15 protein (glucoamylase)	GH15	x	x		
589113453		GH family 16	GH16	x			
358382969		GH family 16 protein	GH17	x			
589111611		GH family 17	GH17	x			
589113629		GH 18 protein (chitinase)	GH18	x	x		
317028062		GH, family 18	GH18			x	
589109851		GH family 28	GH28	x			
358380963		GH family 28 protein	GH28	x			
572273805		Family 31 GH	GH31	x	x		
589103027		GH family 38 protein	GH38		x		
358387943		GH family 43 protein	GH43	x			
589101105		GH family 47	GH47	x	x		
631371154		GH family 47 protein	GH47	x	x		
589100379		GH family 54 (lignin-degrading)	GH57	x	x		
589115645		GH family 55	GH55	x			
589114155		GH familiy 67	GH67		x		
358384989		GH family 71 protein	GH71	x			
589103161		GH family 71 protein	GH71	x			
589109155		GH family 71 protein	GH71	x	x		
589111135		GH family 72 (lignin-degrading)	GH72	x			
589108435		GH 74	GH74	x	x		
358380926		GH family 74 protein	GH74	x			
589098631		GH 92	GH92	x	x		
589100807		GH family 92	GH92	x			
255722211	Predicted proteins	Predicted protein	–				x
589105897		Predicted protein	–	x	x		
589101909		Predicted protein	–	x	x		
589110563		Predicted protein	GH16	x	x		
589113917		Predicted protein	–	x			
589109549		Predicted protein	GH67	x	x		

Table 1 (*continued*)

Gene ID	Enzyme		Family	*T. reesei*		*A. niger*	
				A	B	A	B
589108581		Pr Predicted protein	GH16	x			
403411875		Predicted protein	–	x			
589105505		Predicted protein	–	x			
589107107		Predicted protein	–	x	x		
589100041		Predicted protein	–	x	x		
589115849		Predicted protein	–	x			
589099057		Predicted protein	–	x			
589112857		Predicted protein	–	x			
589116001		Predicted protein	–	x			
589113291		Predicted protein	–		x		
589115927		Predicted protein	–		x		
154322591		Predicted protein	–		x		
358390109	Hypothetical proteins	Hypothetical protein TRIATDRAFT_129231	–	x			
358386311		Hypothetical protein TRIVIDRAFT_45439	–	x			
358390537		Hypothetical protein TRIATDRAFT_302472	–	x	x		
572280833		Hypothetical protein M419DRAFT_97005	–	x			
116199677		Conserved hypothetical protein	–	x			
589112113		Hypothetical protein TRIREDRAFT_66935	–	x	x		
358386247		Hypothetical protein TRIVIDRAFT_179276	–	x			
572280092		Hypothetical protein M419DRAFT_62371	–	x			
572273052		Hypothetical protein M419DRAFT_125562	–	x			
358380920		Hypothetical protein TRIVIDRAFT_118319	–	x			
572284103		Hypothetical protein M419DRAFT_94877	GH71	x	x		
589108875		Hypothetical protein TRIREDRAFT_122487	–	x			
380490319		Hypothetical protein CH063_07742	–	x			
358394718		Hypothetical protein TRIATDRAFT_300431	–	x			
345562011		Hypothetical protein AOL_s00173g184	CBM1		x		
440640361		Hypothetical protein GMDG_04666	–		x		
358381566		Hypothetical protein TRIVIDRAFT_49497	–		x		
358385331		Hypothetical protein TRIVIDRAFT_60255	–		x		
358388440		Hypothetical protein TRIVIDRAFT_141673	–		x		
358381654		Hypothetical protein TRIVIDRAFT_4609	–		x		
46127631		Hypothetical protein FG08193.1	–		x		
310800235		Hypothetical protein GLRG_10272	–		x		
598027367		Hypothetical protein AURDEDRAFT_162084	–		x		
646290693		Hypothetical protein BOTBODRAFT_162340	–		x		
598062595		Hypothetical protein SPAPADRAFT_57777	–		x		
350636308		Hypothetical protein ASPNIDRAFT_182100	GH43			x	
350629486		Hypothetical protein ASPNIDRAFT_47677	GH43			x	
350632025		Hypothetical protein ASPNIDRAFT_128537	–			x	x
145246196		Hypothetical protein ANI_1_1560104	–			x	
350635020		Hypothetical protein ASPNIDRAFT_197780	–			x	
568447829		Hypothetical protein AGABI2DRAFT_199975	GH3			x	
350631594		Hypothetical protein ASPNIDRAFT_53033	GH72			x	x
46122475		Hypothetical protein FG05615.1	–			x	
134082115		Hypothetical protein An15g00620	–			x	
350637823		Hypothetical protein ASPNIDRAFT_52061	GH75			x	x
145258972		Hypothetical protein ANI_1_2174184	–			x	x
145254751		Hypothetical protein ANI_1_1218164	–			x	x
145233749		Hypothetical protein ANI_1_1558024	–			x	x
350633910		Hypothetical protein ASPNIDRAFT_54865	–			x	x
350639816		Hypothetical protein ASPNIDRAFT_124700	–			x	
350638529		Hypothetical protein ASPNIDRAFT_119858	GH31				x
350638823		Hypothetical protein ASPNIDRAFT_205361	–				x
350636991		Hypothetical protein ASPNIDRAFT_56689	–				x
350633205		Hypothetical protein ASPNIDRAFT_55058	–				x
350629696		Hypothetical protein ASPNIDRAFT_126535	–				x
145243362		Hypothetical protein ANI_1_1704094	GH1				x
563290941		Hypothetical protein SBOR_8115	–				x
398407925		Hypothetical protein MYCGRDRAFT_30155	–				x
350636557		Hypothetical protein ASPNIDRAFT_53540	–				x

Table 2
Major proteins identified in the submerged (A) and sequential (B) fermentation enzymatic extracts from *Trichoderma reesei* + *Aspergillus niger*, which were used in the hydrolysis process of the pretreated sugarcane bagasse at a 1:5 ratio, respectively.

Gene ID	Enzyme		Family	*T. reesei* + *A. niger* (1:5)	
				A	B
21842121	Cellulases	Endoglucanase	GH12	x	x
3757552		Endoglucanase A	GH12	x	x
145235569		Endo-beta-1,4-glucanase A	GH12		x
145228915		Endo-beta-1,4-glucanase A	GH12		x
2833231		Endoglucanase I	GH7	x	x
121794		Endoglucanase II	GH5	x	
201066457		Endoglucanase IV (AA9)	GH61	x	x
145235523		Glucan endo-1,3-beta-glucosidase eglC	–	x	x
320592482		Beta-glucanase	–	x	
403314396		Endoglucanase VI (AA9)	GH61	x	
145229151		Endo-1,3(4)-beta-glucanase	GH16	x	x
202072834		Cellobiohydrolase I	GH7	x	x
95115828		Cellobiohydrolase II	GH6	x	x
74698499		1,4-beta-D-glucan cellobiohydrolase	GH7	x	x
201066459		Glucosidase	GH3	x	
126046487		β-glucosidase	GH3	x	x
145242946		β-glucosidase M 4	GH3		x
145255120		Glucan 1,3-beta-glucosidase A	GH5	x	x
400602153		Glucan 1,3-β-glucosidase	GH17	x	
257187		Alpha-glucosidase P2 subunit 5	GH31		x
317035725	Hemicellulases	Endo-arabinase	GH43		x
145234699		Alpha-L-arabinofuranosidase axhA	GH62	x	x
358375978		Arabinoxylan arabinofuranohydrolase	GH62	x	x
145233623		Endo-1,5-alpha-L-arabinosidase C	GH43	x	x
145250511		Alpha-N-arabinofuranosidase B	–	x	x
78101601		Anfaea-ferulic Acid Complex	–	x	
23821545		Feruloyl esterase B	–	x	
145246174		Feruloyl esterase C	–	x	x
48425840		Ferulic acid esterase	–		x
145247672		Feruloyl esterase B-1	–		x
145230716		Beta-galactosidase E	GH35	x	x
350630290		Alpha-galactosidase extracellular	–		x
74626383		Alpha-galactosidase B	–	x	x
317034650		Alpha-galactosidase D	–		x
307776646		Beta-mannanase	GH5	x	x
358367813		Alpha-mannosidase	GH38	x	
145233855		Alpha-mannosidase	GH38	x	
572273984		Beta-mannosidase A	GH2	x	
572273001		Putative beta-mannosidase A	GH2	x	x
317032967		Beta-mannosidase A	GH2	x	x
358369379		Beta-mannosidase (MndA)	GH2		x
145230794		Alpha-1,2-mannosidase 1B	GH47	x	x
145256261		Pectate lyase plyB	–		x
572278177		Pectin lyase-like protein	–	x	x
165906534		Endoxylanase	GH10	x	x
11513450		Acetyl Xylan Esterase	–	x	x
292495278		Endo-1,4-beta-xylanase C	GH10	x	x
549461		Endo-1,4-beta-xylanase 2	GH11	x	x
145250044		Endo-1,4-beta-xylanase 5	GH11	x	x
157488002	Hemicellulases	Swollenin	CBM1	x	x
9858848		Xylanase	GH11	x	
42716406		Xylanase	GH11	x	x
13242071		Xylanase	GH11		x
26514830		Xylanase	GH11		x
83638302		Xylanase	GH11		x

Table 2 (*continued*)

Gene ID	Enzyme		Family	*T. reesei* + *A. niger* (1:5)	
				A	B
380293098		Xylanase II	GH11	x	x
145242002		Alpha-xylosidase	GH31	x	x
145230215		Exo-1,4-beta-xylosidase xlnD	GH3	x	x
145243586		Xylosidase/arabinosidase	–	x	x
572278887	Glycoside Hydrolases families	Glycoside Hydrolase (GH)	GH	x	
572275960		GH, partial	GH		x
358381827		GH family 2 protein	GH2	x	
589104105		GH family 3	GH3		x
358388254		GH family 5 protein	GH5	x	
589100793		GH family 10	GH10	x	x
261825113		GH family 15 protein (glucoamylase)	GH15	x	x
589113453		GH family 16	GH16	x	
358382969		GH family 16 protein	GH17	x	
589111611		GH family 17	GH17	x	
589113629		GH 18 protein (chitinase)	GH18	x	x
317028062		GH, family 18	GH18	x	
589109851		GH family 28	GH28	x	
358380963		GH family 28 protein	GH28	x	
572273805		Family 31 GH	GH31	x	x
589103027		GH family 38 protein	GH38		x
358387943		GH family 43 protein	GH43	x	
589101105		GH family 47	GH47	x	x
631371154		GH family 47 protein	GH47	x	x
589100379		GH family 54 (lignin-degrading)	GH57	x	x
589115645		GH family 55	GH55	x	
589114155		GH familiy 67	GH67		x
358384989		GH family 71 protein	GH71	x	
589103161		GH family 71 protein	GH71	x	
589109155	GH families	GH family 71 protein	GH71	x	x
589111135		GH family 72 (lignin-degrading)	GH72	x	
589108435		GH 74	GH74	x	x
358380926		GH family 74 protein	GH74	x	
589098631		GH 92	GH92	x	x
589100807		GH family 92	GH92	x	
255722211	Predicted proteins	Predicted protein	–		x
589105897		Predicted protein	–	x	x
589101909		Predicted protein	–	x	x
589110563		Predicted protein	GH16	x	x
589113917		Predicted protein	–	x	
589109549		Predicted protein	GH67	x	x
589108581		Predicted protein	GH16	x	
403411875		Predicted protein	–	x	
589105505		Predicted protein	–	x	
589107107		Predicted protein	–	x	x
589100041		Predicted protein	–	x	x
589115849		Predicted protein	–	x	
589099057		Predicted protein	–	x	
589112857		Predicted protein	–	x	
589116001		Predicted protein	–	x	
589113291		Predicted protein	–		x
589115927		Predicted protein	–		x
154322591		Predicted protein	–		x
358390109	Hypothetical proteins	Hypothetical protein TRIATDRAFT_129231	–	x	
358386311		Hypothetical protein TRIVIDRAFT_45439	–	x	
358390537		Hypothetical protein TRIATDRAFT_302472	–	x	x
572280833		Hypothetical protein M419DRAFT_97005	–	x	

Table 2 (*continued*)

Gene ID	Enzyme		Family	*T. reesei* + *A. niger* (1:5)	
				A	B
116199677		Conserved hypothetical protein	–	x	
589112113		Hypothetical protein TRIREDRAFT_66935	–	x	x
358386247		Hypothetical protein TRIVIDRAFT_179276	–	x	
572280092		Hypothetical protein M419DRAFT_62371	–	x	
572273052		Hypothetical protein M419DRAFT_125562	–	x	
358380920		Hypothetical protein TRIVIDRAFT_118319	–	x	
572284103		Hypothetical protein M419DRAFT_94877	GH71	x	x
589108875		Hypothetical protein TRIREDRAFT_122487	–	x	
380490319		Hypothetical protein CH063_07742	–	x	
358394718		Hypothetical protein TRIATDRAFT_300431	–	x	
345562011		Hypothetical protein AOL_s00173g184	CBM1		x
440640361		Hypothetical protein GMDG_04666	–		x
358381566		Hypothetical protein TRIVIDRAFT_49497	–		x
358385331		Hypothetical protein TRIVIDRAFT_60255	–		x
358388440		Hypothetical protein TRIVIDRAFT_141673	–		x
358381654		Hypothetical protein TRIVIDRAFT_4609	–		x
46127631		Hypothetical protein FG08193.1	–		x
310800235		Hypothetical protein GLRG_10272	–		x
598027367		Hypothetical protein AURDEDRAFT_162084	–		x
646290693		Hypothetical protein BOTBODRAFT_162340	–		x
598062595		Hypothetical protein SPAPADRAFT_57777	–		x
350636308		Hypothetical protein ASPNIDRAFT_182100	GH43	x	
350629486		Hypothetical protein ASPNIDRAFT_47677	GH43	x	
350632025		Hypothetical protein ASPNIDRAFT_128537	–	x	x
145246196		Hypothetical protein ANI_1_1560104	–	x	
350635020		Hypothetical protein ASPNIDRAFT_197780	–	x	
568447829	Hypothetical proteins	Hypothetical protein AGABI2DRAFT_199975	GH3	x	
350631594		Hypothetical protein ASPNIDRAFT_53033	GH72	x	x
46122475		Hypothetical protein FG05615.1	–	x	
134082115		Hypothetical protein An15g00620	–	x	
350637823		Hypothetical protein ASPNIDRAFT_52061	GH75	x	x
145258972		Hypothetical protein ANI_1_2174184	–	x	x
145254751		Hypothetical protein ANI_1_1218164	–	x	x
145233749		Hypothetical protein ANI_1_1558024	–	x	x
350633910		Hypothetical protein ASPNIDRAFT_54865	–	x	x
350639816		Hypothetical protein ASPNIDRAFT_124700	–	x	
350638529		Hypothetical protein ASPNIDRAFT_119858	GH31		x
350638823		Hypothetical protein ASPNIDRAFT_205361	–		x
350636991		Hypothetical protein ASPNIDRAFT_56689	–		x
350633205		Hypothetical protein ASPNIDRAFT_55058	–		x
350629696		Hypothetical protein ASPNIDRAFT_126535	–		x
145243362		Hypothetical protein ANI_1_1704094	GH1		x
563290941		Hypothetical protein SBOR_8115	–		x
398407925		Hypothetical protein MYCGRDRAFT_30155	–		x
350636557		Hypothetical protein ASPNIDRAFT_53540	–		x

100 mL of nutrient medium with 30 g/L of glucose, as described initially from Mandels and Stenberg [2] and adapted by Cunha et al. [3].

In the sequential fermentation, solid state fermentation was initiated using 5 g of dry sugarcane bagasse as solid substrate, and substrate moisture was adjusted through the addition of 12 mL of nutrient medium. The inoculum was added for a final concentration of 10^7 spores/g of dry bagasse in the pre-culture, which was maintained under static conditions for 24 h. Then, the pre-culture step was continued as a submerged fermentation after the addition of 100 mL of nutrient medium enriched with 30 g/L of glucose per 5 g of dry bagasse. After 48 h for both submerged and sequential fermentation, a volume of pre-culture suspension corresponding to 10% (v/v) was transferred to

100 mL of culture medium for enzyme production, which was supplemented with 10 g/L of glucose and 1% (w/v) of steam-exploded non-washed sugarcane bagasse. All cultivation experiments were carried out in triplicate, and the averaged data presented with standard deviations.

3. Secretome analysis

3.1. Sample preparation

Sequence grade Lys–C/Trypsin (Promega) was used to enzymatically digest the samples. Acetone precipitation was performed prior to sample digestion. The protein samples were reduced with a 10 mM dithiothreitol (DTT)/25 mM ammonium bicarbonate solution at 37 °C for 1 h and alkylated at 37 °C also for 1 h using a solution of 97% acetonitrile (ACN), 2% iodoethanol, and 0.5% triethylphosphine (v/v). Samples were dried before adding Lys–C/trypsin to them in a 25:1 ratio of protease to protein. Digestions were carried out in a barocycler NEP2320 (PBI) at 50 °C and 20 kpsi for 2 h. The samples were cleaned over C18 columns (MicroSpin, Nest Group), dried and resuspended in 97% purified water/3% ACN/0.1% formic acid (FA). A volume of 1 μL was used for LC-MS/MS analysis.

3.2. LC-MS/MS analysis

A nanoLC system (1100 Series LC, Agilent Technologies, Santa Clara, CA) was used to separate the peptides for downstream MS analysis using a C18 reversed phase ZORBAX 300SB-C18 analytical column (0.75 μm × 150 mm, 3.5 um) from Agilent. The column was directly connected to New Objective's emission tip coupled to the nano-electrospray ionization (ESI) source of the high resolution hybrid ion trap mass spectrometer LTQ-Orbitrap XL (Thermo Scientific). Elution was conducted using an ACN/0.1% FA (mobile phase B) linear gradient. The column was equilibrated initially for 5 min with 95% H_2O /0.1% FA (mobile phase A) followed by the linear gradient of 5–40% B for 85 min at 0.3uL/min, then from 40–95% B for 12 min. Blank injections were performed in between experimental runs. The resulting eluents were analyzed by a data-dependent positive acquisition mode at full MS scan (30,000 resolution) where the eight most abundant molecular ions were selected and fragmented by collision induced dissociation (CID) using a normalized collision energy of 35% to acquire the data for the LTQ-Orbitrap XL.

3.3. Data analysis

Database search analyses were done using Mascot Daemon version 2.4.0 (Matrix Science) against an all fungal protein database from the NCBI database. Peptide and spectral count data were performed on the searches. For protein identification, at least two peptides detected were considered, and the false discovery rate (FDR) was set to 1%.

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Transparency document. Supplementary material

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dib.2016.05.080.

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5. CAPÍTULO V

Reduction of non-productive adsorption of enzymes by using soybean protein as a cost-effective lignin-blocking additive in the saccharification of sugarcane bagasse

Artigo em fase de preparação

Nesta etapa do trabalho, foi estudado o efeito de diferentes tipos de aditivos durante a hidrólise do bagaço de cana visando à redução da adsorção improdutiva de enzimas na lignina. Os coquetéis enzimáticos dos fungos *A. niger* A12 e *T. reesei* Rut C30, produzidos em cultivos isolados por fermentação em estado sólido (FES), submerso (FSm) e sequencial (FSeq), foram utilizados para a sacarificação do bagaço de cana pré-tratado por explosão a vapor por um período de 24 h. Os primeiros experimentos foram realizados utilizando os coquetéis enzimáticos do cultivo sólido para a hidrólise enzimática do bagaço de cana lavado (BEX_L) e não lavado (BEX) com os aditivos: Tween 20 e 80, polietilenoglicol 1500, 6000 e 8000, além da proteína de soja. Os resultados de sacarificação utilizando o BEX na presença da proteína de soja foram positivos para os coquetéis enzimáticos dos dois fungos estudados produzidos por FES. Para os experimentos de hidrólise enzimática com coquetéis produzidos através dos cultivos submerso e sequencial utilizou-se BEX na presença da proteína de soja, em diferentes concentrações. Os resultados destacam o uso da proteína de soja como aditivo para um melhor processo de sacarificação do bagaço, além de fornecer dados para uma melhor compreensão do processo de adsorção improdutiva que ocorre das enzimas na lignina presente. Os resultados desta etapa se referem ao objetivo 5, do item 1.2.

Reduction of non-productive adsorption of enzymes by using soybean protein as a cost-effective lignin-blocking additive in the saccharification of sugarcane bagasse

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Abstract

Addition of non-ionic surfactants, polymers and non-catalytic proteins have been reported to improve the enzymatic hydrolysis of lignocellulosic materials. However, the use of an additive introduces an additional cost to the biomass conversion process. In this work, soybean protein was evaluated as an alternative cost-effective additive in the enzymatic hydrolysis of pretreated sugarcane bagasse. The effect of the enzyme source used in saccharification was also investigated by cultivation of *A. niger* and *T. reesei* under solid-state, submerged and sequential fermentation. Different additives such as Tween, Polyethylene glycol and bovine serum albumin were used for comparison purpose. The soybean protein was the additive that showed the highest positive effect in the hydrolysis of pretreated sugarcane bagasse with the enzyme cocktails from solid-state fermentation. About 2-fold increase in sugar release in relation to the control (without additive) was obtained for both *A. niger* and *T. reesei*. Moreover, the combined (1:1) enzyme cocktails from *A. niger* and *T. reesei* in the presence of soybean protein resulted in glucose released values 54% higher than the control. These findings demonstrated that soybean protein is a cost-effetive alternative additive for use in the enzymatic hydrolysis process and open opportunity for further studies to understand the relationship cellulases-lignin-soybean protein.

Keywords sugarcane bagasse, soybean protein, adsorption, lignin, saccharification

1. Introduction

Although the first industrial plants of cellulosic ethanol have already started operation at commercial scale, several technological challenges still needs to be addressed in order to obtain a competitive product on the market. As an example of these challenges it can be highlight the requirement of solid processing at high loads. In addition to the challenges from the process engineering point of view, such as the difficulties of pumping and agitation at high solids load, the presence of inhibitors of the biochemical reactions may negatively impact both the efficiency of enzymatic hydrolysis and alcoholic fermentation (Ximenes *et al.*, 2010; Kim *et al.*, 2011; Ximenes *et al.*, 2011; Michelin *et al.*, 2016). Such inhibitors, together with the residual lignin will influence a very critical point on the final cost of the cellulosic ethanol: the quantity of enzyme required to convert cellulose into glucose. Despite the significant progress already achieved in this regard, studies indicate that the cost of enzymes is much more significant than what has been commonly reported (Klein-Marcuschamer *et al.*, 2012).

A potential strategy to address these issues and contribute to improve the efficiency of the enzymatic hydrolysis step would be the development of cost-effective technologies to reduce the amount of enzyme lost in the process due to non-productive adsorption onto lignin. One approach that has been described for such application is the addition of lignin blocking agents to the enzymatic reaction medium (Kim *et al.*, 2015; Ko, J. K. *et al.*, 2015). The use of additives such as surfactants agents Tween 20 or 80, polyethylene glycol (PEG) and bovine serum albumin (BSA) increases yield and the rate of enzymatic hydrolysis (Yang and Wyman, 2006; Kumar and Wyman, 2009b; Okino *et al.*, 2013; Cannella and Jorgensen, 2014; Kim *et al.*, 2015). Although the use of an additive introduces an additional cost to the cellulosic ethanol production process, clear benefits have been shown. Reduction of unproductive binding to lignin will make a more effective

use of the added enzymes or even contribute to decrease the enzyme loading (Kim *et al.*, 2015). In some studies, the addition of PEG improved wheat straw hydrolysis conversion rate by up to 45% (Cannella and Jorgensen, 2014; Hsieh *et al.*, 2015) and the presence of Tween 80 in spruce hydrolysis increased the conversation rate up to 58% (Kristensen *et al.*, 2007). However, there is a demand to find additives more cost-effective to use in enzymatic hydrolysis process. (Hui *et al.*, 2015) studied the effect of non-enzymatic protein additives (corn steep liquor (CSL), peptone and yeast extract) on enzymatic hydrolysis and simultaneous saccharification and fermentation of rice straw. The findings showed that the glucose concentration after 72h increased up to 13.7%with the use of such additives. The positive effect exerted by the non-enzymatic proteins was clearly better than that exerted by model protein BSA, showing that utilization of inexpensive non-enzymatic proteins is promising for future applications in glucose and ethanol production from lignocellulosic materials (Hui *et al.*, 2015).

A second approach to address the limitation related to non-productive enzymes adsorption could be to tailor enzymatic preparations less susceptible to adsorb onto lignin. It has been previously reported that enzymes from *T. reesei* and *A. niger* responds differently to lignin adsorption during hydrolysis of liquid hot water pretreated hardwoods (Ko, Ja Kyong, Ximenes, Eduardo*, et al.*, 2015). Therefore, the production of enzymes on-site using different cultivations methods can be a promising strategy to obtain enzymatic cocktails with different characteristics towards lignin adsorption. Among the cultivations methods to produce enzymes, the traditional cultivations systems such as solid-state fermentation (SSF) and submerged fermentation (SmF) has been widely described to obtain cellulases and hemicellulases (Singhania *et al.*, 2010; Farinas, 2015; Gasparotto *et al.*, 2015). More recently, a sequential fermentation (SF) method was developed with the proposal to combine the advantages of both conventional process

(SSF and SmF) for cellulase production (Cunha, Esperanca*, et al.*, 2012; Cunha *et al.*, 2015; Florencio *et al.*, 2015). This SF methodology was shown to result in enzymatic cocktails with different stability characteristics when comparing to ones from SSF and SmF (Vasconcellos *et al.*, 2015).

This paper reports a study on the effects of different additives on the enzymatic hydrolysis of pretreated sugarcane bagasse. For that the differential effect of additives Tween, PEG, BSA and soybean protein) on the performance of enzymatic cocktails produced in-house by *T. reesei* and *A. niger* under different cultivations systems (SSF, SmF, and SF) was evaluated in order to reduce the non-productive adsorption of enzymes onto lignin.

2. Materials and methods

2.1 Fungal Strains

The *T. reesei* Rut-C30 strain was purchased from the Centre for Agricultural Bioscience International (CABI) culture collection in the United Kingdom (IMI number: 345108). The *A. niger* wild-type A12 strain was obtained from Embrapa Food Technology collection (Rio de Janeiro, Brazil). The strains were maintained at -18ºC in a 20% (w/w) glycerol/water solution and were activated in potato dextrose agar (PDA) for 5 days at 30ºC (*T. reesei*) and for 3 days at 32ºC (*A. niger*).

2.2 Substrate

The inducer substrate used to cultivation from *T. reesei* and *A. niger* was washed steam-exploded sugarcane bagasse (SEB_w) donated by a local sugarcane mill (Usina Nardini, Vista Alegre do Alto, São Paulo, Brazil). The wash was carried out according to (Vasconcellos *et al.*, 2015). The sugarcane bagasse samples were subsequently milled and sieved, and the particle size selected was $1.0 \leq dp \leq 2.0$ mm. Chemical characterization

was performed as described previously (Gouveia *et al.*, 2009). Composition of the pretreated bagasse was (w/w) 41 % glucan, 12.1 % pentosan, 34 % lignin and 12.9 % ash. The substrate to enzymatic hydrolysis was washed and non-washed steam-exploded sugarcane bagasse (SEB_w and SEB, respectively) with the particle size dp≤1.0.

2.3 Cultivation Conditions

2.2.1 Solid State Fermentation (SSF)

The SSF was performed using 5 g of dry SEB as solid substrate in 500 mL Erlenmeyer flaks. Substrate moisture was adjusted through the addition of 12 mL of nutrient medium described by (Mandels and Sternberg, 1976) and adapted by (Cunha, Bacchin*, et al.*, 2012). An inoculum suspension of 10^7 spores/g of dry bagasse was added, and the culture was maintained under static conditions for 72 h at 30°C for *T. reesei* and 32ºC for *A. niger*. The enzymes were extracted by the addition of 1:10 (w/v) 50 mM sodium citrate buffer solution (pH 4.8), with 120 rpm agitation at 30 °C and 32 °C to *T. reesei* and *A. niger*, respectively, for 40 min. The final enzymatic extracts were vacuum-filtered, centrifuged at 10,000 rpm for 15 min at 4◦C, and kept frozen at -18 °C prior to the analytical assays. All cultivation experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

2.2.2 Submerged Fermentation (SmF)

In the conventional submerged fermentation process, the pre-culture was initiated with a conidia suspension of 10^7 spores/mL, which was transferred to Erlenmeyer flasks containing 100 mL of nutrient medium. The incubation was carried out with agitation rate of 200 rpm for 48 h at 30°C (for *T. reesei)* or at 32ºC (for *A. niger*). For enzyme production, a volume of pre-culture suspension corresponding to 10% (v/v) was transferred to the culture medium and supplemented with 10 g/L of glucose and 1% (w/v) of SEB. The cultivations were kept in an orbital incubator shaker at 200 rpm for 72 h at

30°C (for *T. reesei*) or 32ºC (for *A. niger*). The cultivation broth was filtered, centrifuged at 4ºC and 10,000 rpm for 15 min, and stored at -18°C for further analysis. The cultivation experiments were carried out in triplicate, and the averaged data presented with standard deviations.

2.2.3 Sequential Fermentation (SF)

Sequential fermentation was performed according to the methodology described previously (Cunha, Esperanca*, et al.*, 2012). Briefly, pre-culture was initiated as solid-state fermentation using an inoculum of 10^7 spores/g of dry bagasse, and the culture was maintained under static conditions for 24 h at 30°C for *T. reesei* and 32ºC for *A. niger*. The pre-culture step was continued as a submerged fermentation after addition of 100 mL of nutrient medium (Mandels and Sternberg, 1976) enriched with 30 g/L of glucose. This step was performed in an orbital incubator shaker for 48 h, 200 rpm and under appropriate temperatures for each fungal strain. For enzyme production step under SF, the process was performed as described in section 2.2.1.

2.2. Enzyme Activity Assays

The endoglucanase (EGase), filter paper (FPase) and β-glucosidase activities were determined according to protocols of the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987), in the presence of carboxymethylcellulose (CMC) (Sigma, USA), filter paper whatman n°1 and celobiose, respectively. Xylanase activity was determined following standard methods (Bailey and Poutanen, 1989), in the presence of beechwood xylan (Sigma, USA). The release of the reducing sugar was measured using the DNS method (Miller, 1959). Protein concentration was determined by Bradford method using bovine serum albumin (BSA) as standard (Bradford, 1976). All enzymatic analyses were carried out in duplicate.

2.3 Sugarcane bagasse enzymatic hydrolysis

Enzymatic hydrolysis experiments were carried out in 5 mL tubes in a hybridization incubator operated at an agitation speed of 30 rpm at 50ºC for 24 h. A volume ±1 mL was used of 50 mM citrate buffer at pH 4.8, 3 mL of cocktail of enzymes from each filamentous fungi, *T. reesei* and *A. niger*, that were produced by either solid-state, submerged or sequential fermentations, respectively. The SEB_w and SEB was applied at a concentration of 5% (w/v) or 0.2 g of total solids. The final reaction volume of 4 mL included enzymes, solids and buffer. An additional set of enzymatic hydrolysis experiment with the final volume of 100 mL was performed to validate the above hydrolysis. The proportions to total solids, buffer volume and enzyme loadings were maintained. Orbital shaker was used and operated at an agitation speed of 200 rpm at 50 °C for 24 h.

Saccharification was performed with crude extracts (3 mL) from *T. reesei* and *A. niger* produced by solid-state, submerged and sequential fermentation methods. Enzyme loadings in mg protein per gram of glucan were of 0.13 (SSF), 0.09 (SmF) and 0.13 (SF) mg protein by *T. reesei* cocktails and 0.17 (SSF), 0.09 (SmF) and 0.13 (SF) mg protein by *A. niger* cocktails. An experiment of saccharification was performed with combined extracts from *A. niger* and *T. reesei* in the ratio 1:1. As control experiments, reaction blanks for both the substrate and enzymes were carried out. Samples were taken at 24 h. The released glucose was measured by D-glucose assay procedure kit (Labtest, Brazil). All hydrolysis experiments were performed in triplicate.

2.3 Additives

The additives soybean protein (SP), polyethylene glycol (PEG) with different molecular weight 1500, 6000 and 8000 (Sigma Aldrich, USA) were used at a

concentration of 4% and Tween 20 and 80 (polyoxyethylene sorbitan monooleate, Dinamica, Brazil) at 0.1%. The concentrations used were based in previous studies (Okino *et al.*, 2013; Cannella and Jorgensen, 2014). The BSA was used to compare the results of additive effect selected among cited above.

3. Results and discussion

3.1 Profile of enzymes produced by A. niger and T. reesei

Cellulase and hemicellulase production was measured for *A. niger* and *T. reesei* cultivated under solid-state, submerged and the sequential fermentation methods (Table 1). The highest values of activity for most of the quantified enzymes were observed when the fungi were cultivated under the solid-state fermentation followed by the sequential fermentation method. The cultivation of *A. niger* under SSF resulted in higher enzymatic activites, the xylanase and endoglucanase production was about 7-fold and 6-fold higher, respectively, when compared to results from SmF. The SF method also resulted in higher activity values than SmF for *A. niger*, about 3-fold and 2-fold to xylanase and endoglucanase, respectively. For *T. reesei* the enzymatic cocktail produced in SSF also showed the higher enzymatic production, both β-glucosidase and endoglucanase activities were about 4-fold higher than SmF. The enzymatic cocktail produced by SF from *T. reesei* also presented highest activities than SmF about 2-fold to β-glucosidase and endoglucanase enzymes (Table 1).

A comparison in terms of enzyme production from each fungal strain revealed that enzymatic activities produced in SSF from *A. niger* were superior than from *T. reesei* for all four activities quantified (endoglucanase, xylanase, β-glucosidase and FPase). On the other hand, the endoglucanase activity was higher in the *T. reesei* enzymatic cokctail than in the *A. niger* obtained under SmF and SF cultivations. However, xylanase and β-

glucosidase enzymes showed higher activities in enzyme cocktail from *A. niger* than *T. reesei*, highlighting β-glucosidase that was about 6-fold and 5-fold higher in enzyme cocktail from *A. niger* in SmF and SF, respectively.

Table 1. Comparison of enzyme activity profiles for *A. niger* A12 and *T. reesei* Rut C30 under solid-state (SSF), submerged (SmF) and sequential fermentation (SF).

Enzyme Activity ($IU.mL^{-1}$)						
	A. niger A12			*T. reesei* Rut C30		
Enzymes	SSF	SmF	SF	SSF	SmF	SF
Endoglucanase	2.3	0.4	0.9	1.8	0.5	1.2
β-glucosidase	5.3	1.8	3.2	1.2	0.3	0.6
FPase	0.04	0.02	0.02	0.02	0.01	0.01
Xylanase	29.5	4.2	12.4	10.7	3.8	8.3
Total protein*	0.04	0.02	0.03	0.03	0.02	0.03

*expressed in $mg.mL^{-1}$

Previous reports in literature showed that cellulases, hemicellulases, lipase, protease produced from solid-state (Bhargav *et al.*, 2008; Coradi *et al.*, 2013) and cellulases and hemicellulases from sequential fermentation (Cunha, Esperanca*, et al.*, 2012; Cunha *et al.*, 2015; Florencio *et al.*, 2015; Vasconcellos *et al.*, 2015) methods were superior to submerged fermentation to both fungi, *A. niger* and *T. reesei*, among others fungal species. The values showed here are in according to literature, the xylanase and endoglucanase activities in SSF enzyme cocktail from *A. niger* in this present study were higher than the production by SmF and SF. However, the β-glucosidase production in SmF and SF from *A. niger* was higher than SSF (Vasconcellos *et al.*, 2015). In SF enzyme cocktail, the xylanase, endoglucanase and β-glucosidase activities was highest here, than SmF, whereas the findings presented by (Vasconcellos *et al.*, 2015) showed the xylanase

activity higher in SmF than in SF, and endoglucanase and β-glucosidase activities were similar.

3.2 Effect of additives on enzymatic hydrolysis of sugarcane bagasse

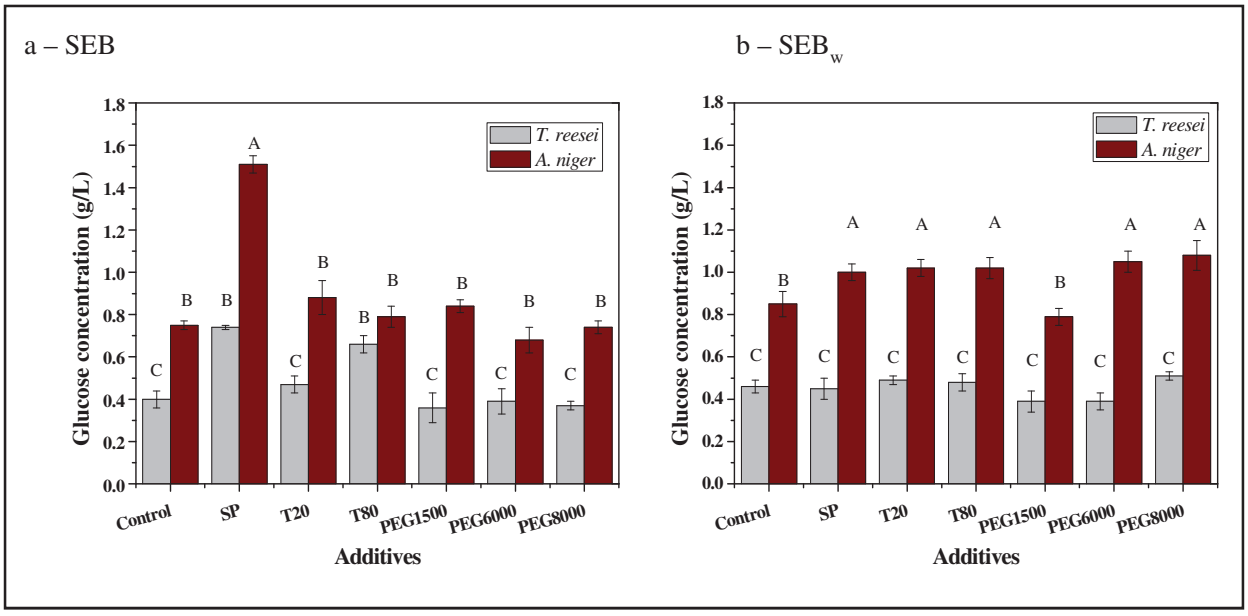
Enzymes produced by *A. niger* and *T. reesei* under SSF were used to evaluate the effect of additives during the enzymatic hydrolysis of SEB and SEB_w (Figure 1). The most interesting positive effect was noted during the enzymatic hydrolysis of SEB using soybean protein (SP) as additive and enzyme cocktail from both *A. niger and T. reesei*, in which the results for glucose releaded were about 2-fold higher than the control without additives (Figure 1a). Furthermore, the amount of released glucose was 2.0 × higher in the enzymatic hydrolysis using enzyme cocktail from *A. niger* (1.51 g/L with SP) than *T. reesei* (0.75 g/L with SP).

Soybean proteins are among the most investigated natural compounds for nonfood applications including wood adhesives, films for food packaging, composites, biobased plastics, and paper coatings. Nevertheless these proteins have amphiphilic nature, because they possess both hydrophilic and hydrophobic domains that from a physicochemical perspective, have the potential to interact strongly with lignin (Salas *et al.*, 2013). However, despite their industrial relevance, the nature of the adsorption and interfacial interactions need to be elucidated in order to benefit from soy protein and lignin unique functionalities in composite materials.

As soy protein was tested as an additive in the present work, the addition of non-catalytic proteins or non-enzymatic proteins, such as BSA, yeast extract, peptone, corn steep liquor, among others, have been investigated the enzymatic hydrolysis of lignocellulosic materials in order to reduce non-productive adsorption of enzymes onto lignin (Wang *et al.*, 2013; Hui *et al.*, 2015; Wang, H. *et al.*, 2015).

SEB_w hydrolysis experiments from enzyme cocktail of *A. niger* produced in SSF showed that the use of SP, Tween 20, Tween 80, PEG 6000 and PEG 8000 additives presented significant improvement in glucose release relative to the control sample without additive (Figure 1b). As for enzymes from *T. reesei* produced in SSF none of the additives showed significant results to enzymatic hydrolysis of SEB_w (Figure 1b). It was also observed in these hydrolysis experiments that the majority of glucose released values were lower than the ones from non-washed SEB hydrolysis using either *A. niger* or *T. reesei* enzymatic complexes.

Figure 1. Effect of additives in enzymatic hydrolysis of non-washed (a) and washed (b) pretreated sugarcane bagasse (SEB and SEB_w), respectively, using enzyme cocktails from *A. niger* and *T. reesei* produced by solid-state fermentation (SSF). The letters (A, B, C) indicate results that differ significantly, according to Tukey's test (p<0.05).



The SEB washing process is an alternative to remove soluble materials as soluble lignin degradation products may inhibit/deactivate enzymes (Berlin *et al.*, 2006; Ximenes *et al.*, 2010) to the experiments performed here (presence soluble lignin) (Ximenes *et al.*, 2011). Our findings showed that in the presence of additives, glucose released were higher

for the enzymatic hydrolysis of non-washed SEB than the SEB_w, indicating that the additives reduced inhibition of enzyme by soluble lignin. Here the soluble lignin decreased after washing 3.44% to 0.64% of the total lignin content in SEB and SEB_w, respectively, according to characterization performed.

The mechanism of the positive effect of non-ionic surfactant and non-catalytic protein on the enzymatic hydrolysis of pretreated lignocellulosic biomass is generally believed to be the prevention of the non-productive adsorption of cellulases onto the lignin fraction, which increases the amount of free enzyme that would be beneficial for the hydrolysis of cellulose substrate (Alkasrawi *et al.*, 2003; Sipos *et al.*, 2011; Zhou *et al.*, 2015). For example, PEG was shown only to have an effect on hydrolysis when lignin was present in the substrate. The adsorption of PEG on lignocellulose is proposed to be due to hydrophobic and hydrogen bonding interactions between PEG and the lignin part in the lignocellulose (Börjesson, Engqvist*, et al.*, 2007).

Based on results by (Berlin *et al.*, 2006) that soluble lignin components may inhibit enzyme activity, the effect of adding soluble lignin prior to Avicel hydrolysis was studied to determine the impact of soluble lignin on cellulase effectiveness and whether any resulting inhibition could be overcome by additives (Kumar and Wyman, 2009b). The fact that adding just sugars in an amount equal to that present with the acid soluble lignin gave the same inhibition as the acid soluble lignin solution indicates that the sugars in the acid soluble lignin mixture were primarily responsible for cellulase inhibition. This suggests that the lignin itself had a limited effect, if any (Kumar and Wyman, 2009b).

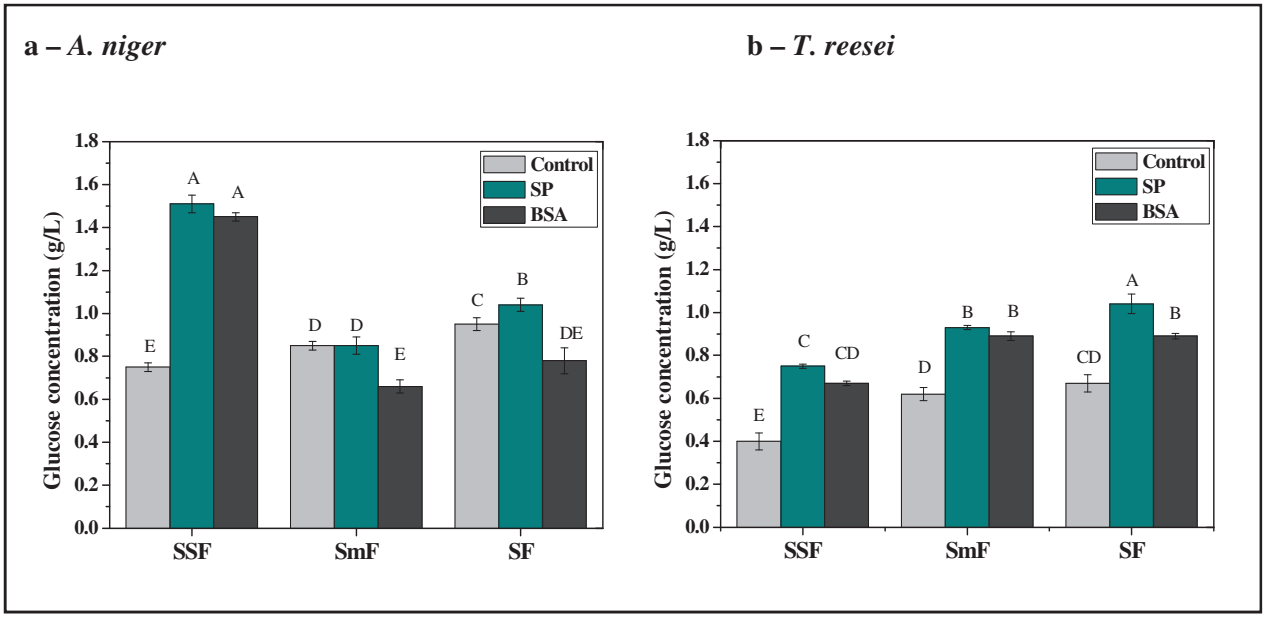
3.3 Enzymatic hydrolysis using different enzyme cocktails (SSF, SmF and SF)

The efficiency of using soybean protein as a lignin-blocking additive in the hydrolysis of SEB using enzymatic cocktails obtained under different cultivation methods was evaluated. For that, the enzyme cocktails produced by *A. niger* and *T. reesei*, under solid

state, submerged and sequential fermentation (SSF, SmF and SF) were compared in terms of saccharification performance without additives (control) and with soybean protein at 4% (Figure 2). For this set of experiments, the use of the protein BSA as a lignin-blocking agent was also investigated for comparison purposes, as many studies have been done demonstrating the efficacy of adsorption of BSA onto different lignins (Kawamoto *et al.*, 1992; Yang and Wyman, 2006; Salas *et al.*, 2013; Kim *et al.*, 2015).

The greatest positive effect of the addition of soybean protein was observed in the saccharification of SEB by the *A. niger* enzyme cocktail produced under SSF (Figure 2a). For this condition, the values of glucose released adding the soybean protein were 2-fold higher than control without additives and similar to adding BSA. An improvement in the enzymatic hydrolysis was also observed using the *A. niger* cocktail from the SF cultivation method, considering the Tukey test, nevertheless the increased was only slightly higher than enzymatic hydrolysis without additives. SEB hydrolysis using the *A. niger* enzyme cocktail from SmF, no improvements was observed by adding soybean protein or BSA. As for the enzymatic hydrolysis experiments carried out using the enzyme cocktail of *T. reesei* from the three cultivation methods (SSF, SmF and SF), a significant improvement in the presence of soybean protein was observed, with glucose released values about 2 fold higher than the hydrolysis without additive. The addition of BSA also showed an increase in the glucose released compared to control hydrolysis experiments using the enzyme cocktail of *T. reesei* (Figure 2b).

Figure 2. Effect of soybean protein (SP) and bovine serum albumin (BSA) in enzymatic hydrolysis of non-washed pretreated sugarcane bagasse (SEB), using enzyme cocktails from *A. niger* (a) and *T. reesei* (b) produced by solid-state (SSF), submerged (SmF) and sequential fermentations. The letters (A, B, C, D, E) indicate results that differ significantly, according to Tukey's test (p<0.05).

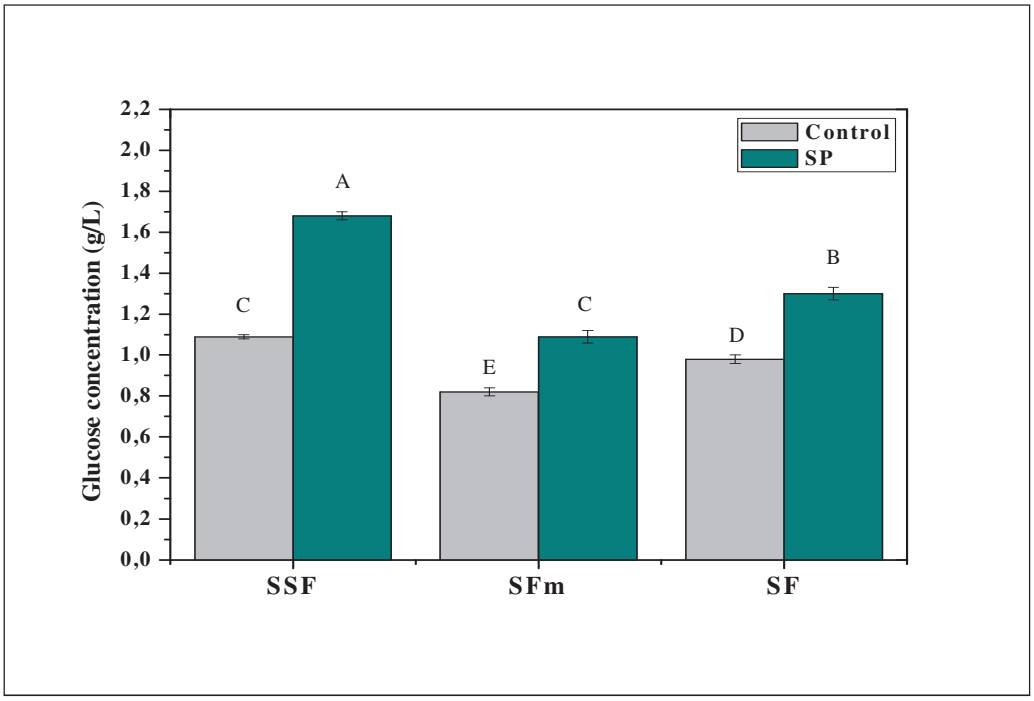


The enzyme cocktail of each filamentous fungus has a different composition of cellulases that depends on its genome and external actions such as inducing carbon source. Thus the intensity of adsorption interaction depends on the type of enzyme and lignin characteristics (Li, Yun *et al.*, 2016). Since the enzyme components which are required to synergistically hydrolyze cellulose have different profiles (molecular weight, hydrophobicity and isoeletric point), they exhibit different adsorption behaviors with lignin, and thereby change the ratio of enzyme activities needed for synergism during cellulose hydrolysis. Studies performed to examine the enzyme-lignin interactions showed that among the enzyme components of *Trichoderma reesei* cellulase cocktail, β-glucosidase showed the strongest adsorption onto lignin (Ko, Ja Kyong, Ximenes, Eduardo*, et al.*, 2015). Only 2–18% of the initial β-glucosidase activity remained in the supernatant while 50–60% of cellobiohydrolase and endoglucanase activities were

recovered after incubation with lignin. However, β-glucosidase from *Aspergillus niger* exhibits less adsorption than β-glucosidase from *T. reesei*. These results further confirm the β-glucosidase adsorption is related to the microorganism from which it is derived as suggested by (Ximenes *et al.*, 2010) and indicates protein engineering with directed evolution could result in β-glucosidase having lower affinity for lignin. (Ko, Ja Kyong, Ximenes, Eduardo*, et al.*, 2015). The difference between secreted proteins by each fungi and each cultivation method used to cellulase production may have contributed greatly to enzymatic hydrolysis results in the presence of additive soybean protein.

Figure 3 shows the results of enzymatic hydrolysis with and without soybean protein performed with a combination of enzymes from *A. niger* and *T. reesei* that were produced by either solid-state, submerged or sequential fermentations, respectively. The results showed the enzyme cocktails when combined have a higher glucose released than when the cocktails used for each fungus alone. The enzymatic hydrolysis from cocktail of SSF was more was positively influenced by the presence of the additive (SP). The glucose released values reached 1.7 g/L in 24 h and it was 70% higher than control experiment (without soybean protein). Subsequently the hydrolysis from enzymes of SF with 1.3 g/L and 30% compared to the control experiment. Finally, enzyme cocktail from SmF reached 1.0 g/L of released glucose and it was 25% higher than control. All hydrolysis experiments that combined of enzymes from *A. niger* and *T. reesei* in the presence of soybean protein showed results higher than the enzyme cocktails from *A. niger* or *T. reesei* alone. These findings corroborate those found in the literature that the combination of extracts can help increase the yield of the enzymatic hydrolysis (Borin *et al.*, 2015; Florencio C. *et al.*, in press).

Figure 3. Effect of soybean protein (SP) in enzymatic hydrolysis of non-washed pretreated sugarcane bagasse (SEB), using enzyme cocktails combined (1:1) from *A. niger* and *T. reesei* produced by solid-state (SSF), submerged (SmF) and sequential fermentations. The letters (A, B, C, D, E) indicate results that differ significantly, according to Tukey's test (p<0.05).



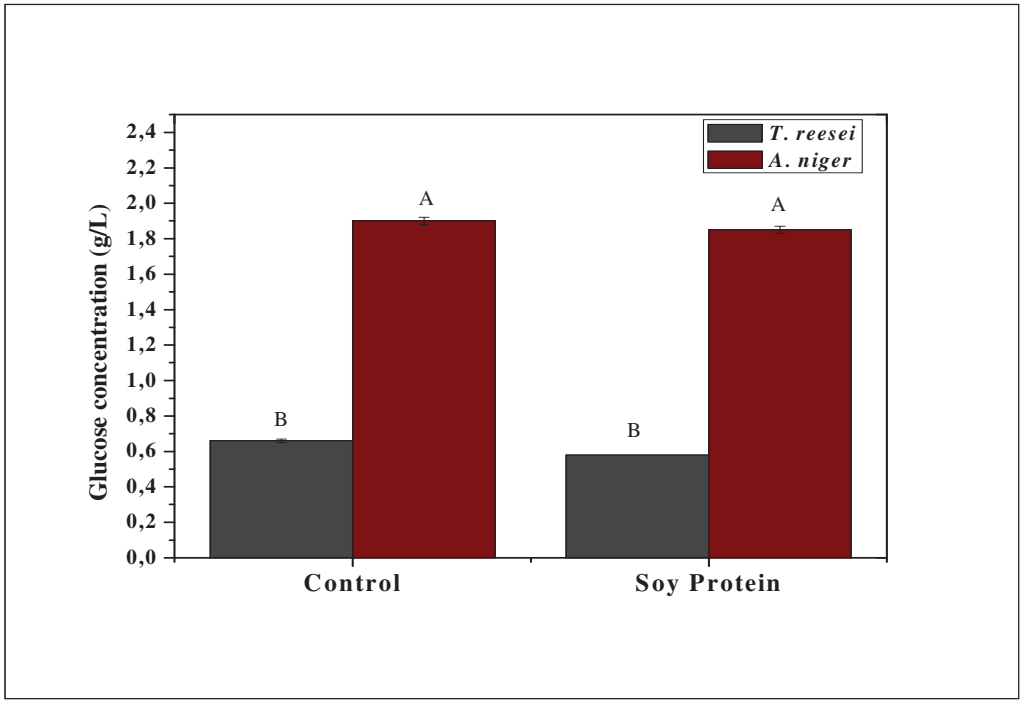
3.4 Enzymatic hydrolysis of pure cellulose

Lignin presence in lignocellulolytic biomass as sugarcane bagasse limits the saccharification of this type of agroindustrial residue as well as the unproductive enzyme adsorption. The removal of part of lignin by pretreatments as steam-exploded facilitates the accessibility of cellulose, but the residual lignin can competitively adsorb the enzyme (Zheng *et al.*, 2013). Thus, enzymatic hydrolysis of pure cellulose was carried out to investigate the role of lignin in enzymatic hydrolysis using soybean protein as additive.

Figure 4 showed the findings to pure cellulose hydrolysis experiments from enzyme cocktails of *A. niger* and *T. reesei* produced by SSF with and without (control) SP. The released glucose values were similar for the fungus *A. niger*, both in the absence and

presence of soybean protein (1.87±0.02 g/L). For *T. reesei* the values was lower than *A. niger*, but it was also noted that the presence of soybean protein did not affect the results of enzymatic hydrolysis of pure cellulose.

Figure 4. Effect of soybean protein (SP) in enzymatic hydrolysis of pure cellulose, using enzyme cocktails from *A. niger* and *T. reesei* produced by solid-state fermentation (SSF). The letters (A, B) indicate results that differ significantly, according to Tukey's test (p<0.05).



The results of the enzymatic hydrolysis of pure cellulose with soybean protein suggest that the presence of this additive in the hydrolysis experiments with SEB would be avoiding unproductive enzyme adsorption and increased the cellulose conversion. Most studies performed to evaluate the effects of lignin and surfactants on adsorption of cellulases make use of commercial enzymes, pure cellulose and lignin isolated which would facilitate the understanding and the control of the adsorption process (Yang *et al.*, 2011; Zhang *et al.*, 2013; Ko, Ja Kyong, Ximenes, Eduardo*, et al.*, 2015). However, some recent studies have been conducted to available the influence of lignin on the adsorption

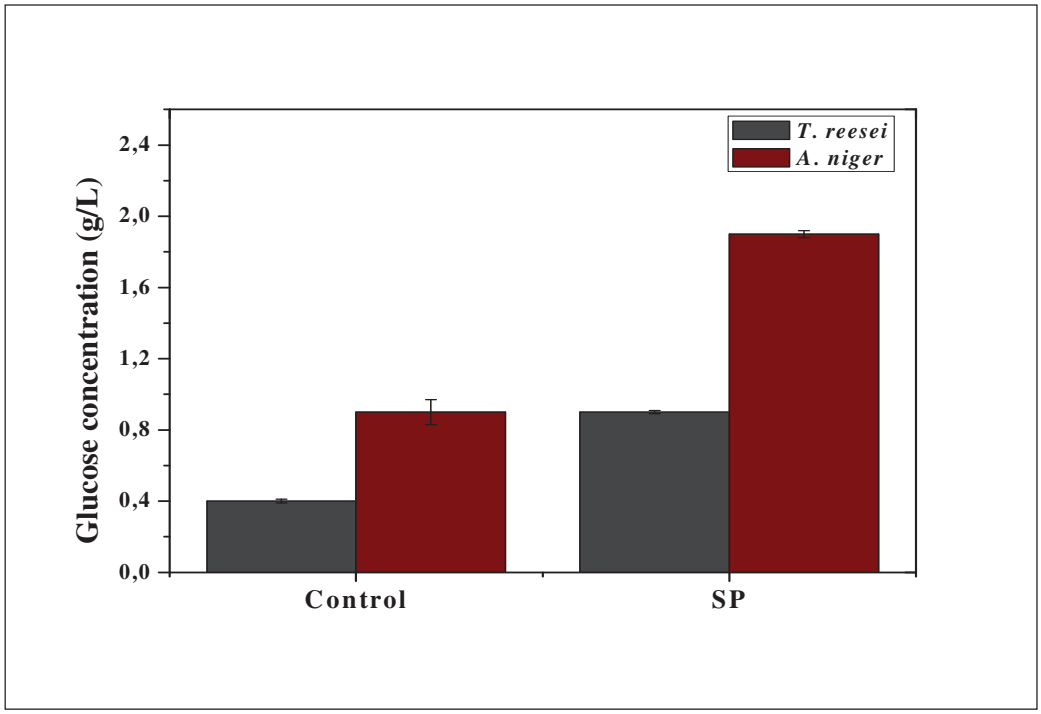
of cellulases on pretreated raw biomass, as corn stover (Kumar and Wyman, 2009b; Li, Yanfei *et al.*, 2016). In our study was observed an increase in enzymatic hydrolysis of SEB with the additive use, whereas in pure cellulose saccharification the SP addition no influenced in the process, showing that the presence of lignin in SEB interacts with the soybean protein. For all experiments it is known that the presence of lignin, added to pure cellulose or existing in lignocellulosic biomass, decreased the maximum adsorption capacity of cellulases on cellulose and the addition of surfactants/non-catalytic proteins in enzymatic hydrolysis process could alleviate the adsorption of cellulases and enhanced desorption of cellulases on/from lignin and biomass (Kumar and Wyman, 2009a; Ouyang *et al.*, 2010; Sipos *et al.*, 2010; Li, Yanfei *et al.*, 2016).

3.5 Enzymatic hydrolysis of sugarcane bagasse in shake flasks

An enzymatic hydrolysis experiment in shake flasks with a final volume of 100 mL was carried out to further investigate the use of the additive (soybean protein) which was the most interesting result in the adsorption process with lignin. For that, the enzyme cocktails of SSF produced by *A. niger* and *T. reesei* were used individually, since the SSF enzyme cocktails provided the most significant results as regards the released glucose in the presence of soybean protein (Figure 5). Enzyme from *A. niger* were able of hydrolyzing SEB releasing 1.9 g/L of glucose in the presence of soybean protein corresponding to a 41% cellulose conversion, while control experiment resulted in 20% conversion without soybean protein as additive. As for the SEB hydrolysis using the enzyme cocktail from *T. reesei,* the maximum of glucose released was 0.9 g/L, which corresponds to a 20% cellulose conversion, a 2-fold higher than control experiment. In comparison with previous experiments, these findings showed an increase of 26% and 20% in relation the enzymatic hydrolysis with previous results using a final volume of 4 mL to *A. niger* and *T. reesei*, respectively (Figure 2ab). Some hypotheses could explain

the increase in conversion in the enzymatic hydrolysis carried out in Erlenmeyer flasks, one possibility would be free physical space available within the flasks, which would facilitate agitation. Another possibility to increase observed could be higher agitation in flasks experiments, in flasks the agitation was 200 rpm while in tubes was 30 rpm.

Figure 5. Effect of soybean protein (SP) in enzymatic hydrolysis of SEB in shake flasks, using enzyme cocktails from *A. niger* and *T. reesei* produced by solid-state fermentation (SSF).



The application of two soybean protein by (Salas *et al.*, 2013) onto lignin surface revealed that different adsorption behaviors for each protein, which highlights the complexity of protein adsorption onto solid surfaces, even as a higher adsorption onto lignin films was observed compared to cellulose. Overall, favorable interactions with hydrophobic substrates were measured, which could be used as rationale for the application of soybean proteins in surface modification. To these experiments is believed that the better performance of enzymatic hydrolysis in the presence of soybean protein may be explained by principle that the additives could adsorb on the exposed lignin by

pretreatment (steam explosion) to prevent unspecific adsorption of cellulases, thereby producing better recycles of enzymes and higher hydrolysis yields.

4. Conclusions

The effect of different additive in the enzymatic hydrolysis of steam-exploded sugarcane bagasse was evaluated using crude enzyme cocktails from *A. niger* and *T. reesei* produced by solid-state, submerged and sequential cultivation methods. The results showed that the addition of soybean protein significantly increased the enzymatic hydrolysis to both enzyme cocktails from *A. niger* and *T. reesei* by SSF, SmF and SF. The. Pure cellulose enzymatic hydrolysis confirmed that soybean protein acting as a lignin-blocking in the pretreated sugarcane bagasse, therefore more cellulases would be free to cellulose hydrolysis. These findings demonstrated that soybean protein is a cost-effetive alternative additive for use in the enzymatic hydrolysis process and open opportunity for further studies to understand the relationship cellulases-lignin-soybean protein.

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6. CAPÍTULO V

6.1. Considerações Finais

A produção de coquetéis enzimáticos eficientes a um custo viável para a sacarificação da biomassa lignocelulósica é uma das chaves para ultrapassar os desafios no processo de conversão do material lignocelulósico em açúcares fermentescíveis para a produção de etanol 2G. Os resultados obtidos no presente trabalho contribuem para superar esse desafio, permitindo concluir que:

- É possível se utilizar de novas metodologias de cultivo para aumentar a produção enzimática. O processo de fermentação sequencial para linhagens de *Trichoderma* frente ao processo submerso convencional mostrou ser favorável a produção de endoglucanase, com um aumento de 4,2 vezes.
- A caracterização das endoglucanases produzidas por *T. reesei* em termos de pH e temperatura, assim como o perfil de hidrólise de substrato sintético não revelaram diferenças qualitativas entre as condições de cultivo investigadas. Entretanto, a termoestabilidade sofreu influência tanto pelo sistema de cultivo empregado quanto pelo tipo de fonte de carbono.
- A análise do secretoma dos coquetéis enzimáticos produzidos por fermentação submersa e sequencial a partir das linhagens *A. niger* e *T. reesei* permitiram a identificação de proteínas importantes no processo de sacarificação do bagaço de cana.
- A hidrólise do bagaço de cana pré-tratado por explosão a vapor foi 3 vezes mais eficiente quando usado a combinação de coquetéis enzimáticos de *A. niger* e *T. reesei* produzidos por fermentação sequencial quando comparado com os resultados da sacarificação pelo coquetel combinado a partir da fermentação submersa. A presença de uma quantidade maior de xilanases no coquetel foi o diferencial para o resultado de hidrólise apresentado.
- O uso da proteína de soja durante a hidrólise do bagaço de cana pré-tratado por explosão a vapor teve efeito positivo no processo, levando a um aumento na liberação de glicose em 2 vezes para o coquetel enzimático produzido por FES pela linhagem *A. niger*, 1,2 vezes para FSeq e para a FSm não houve aumento com relação ao controle (sem aditivo). Para os coquetéis enzimáticos da linhagem *T.*

reesei o aumento foi de aproximadamente 2 vezes para FES, 1,5 vezes para FSeq e FSm quando comparadas ao controle.

6.2.Perspectivas

Para trabalhos futuros sugere-se:

- Trabalhar com mais exclusividade em algumas proteínas identificadas nos secretomas produzidos pelos fungos filamentosos sob diferentes condições de cultivo (indução).
- Aprofundar os estudos de adsorção da lignina aplicando diferentes técnicas para um melhor entendimento do processo (exemplo: Ressonância Magnética Nuclear – RMN).

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