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# INCREMENTO DO POTENCIAL (HEMI)CELULOLÍTICO DE *PENICILLIUM OXALICUM* APLICADO À DESCONSTRUÇÃO DE BIOMASSAS

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# INCREMENTO DO POTENCIAL (HEMI)CELULOLÍTICO DE *PENICILLIUM OXALICUM* APLICADO À DESCONSTRUÇÃO DE BIOMASSAS

*Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Mestra em Genética e Biologia Molecular, na área de Genética de Microrganismos*

*Orientador: Prof. Dr. Gonçalo Amarante Guimarães Pereira*

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**COMISSÃO EXAMINADORA**

Prof. Dr. Gonçalo Amarante Guimarães Pereira

Dr. César Rafael Fanchini Terrasan

Dra. Fernanda Mandelli

*Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.*

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa de Genética e Biologia Molecular da Unidade Instituto de Biologia.

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**RESUMO**

A obtenção de combustíveis renováveis como o etanol é altamente desejável tendo em vista a redução da emissão de gases promotores do efeito estufa na atmosfera. O etanol pode ser produzido pelo processo de primeira (1G) e segunda geração (2G). Apesar das vantagens da produção 2G, esta é ainda considerada onerosa, principalmente devido a etapa de sacarificação que requer uma alta dosagem de enzimas para a conversão dos polímeros que constituem a biomassa em açúcares fermentescíveis. Além dos custos inerentes à produção dessas enzimas, a logística e a estabilização também contribuem para o alto valor dispensado nessa etapa. Por conseguinte, o incremento da atividade de celulases, hemicelulases e atividades acessórias produzidas por microrganismos é altamente desejável. Nesse sentido, o fungo filamentoso *Penicillium oxalicum*, devido ao seu amplo espectro de CAZymes (enzimas relacionadas com a degradação de polissacarídeos) secretadas em determinadas condições de cultivo, emerge como uma alternativa promissora para produção dessas enzimas. Diante do exposto acima, o trabalho objetivou otimizar a produção de enzimas relacionadas à desconstrução da biomassa por uma linhagem selvagem de *P. oxalicum* adotando fontes alternativas de carbono e nitrogênio e a obtenção de linhagens com maior atividade de CAZymes por meio de mutagênese aleatória. A adoção da metodologia experimental *design of experiments* (DOE) permitiu observar os efeitos de diversas variáveis do meio de cultivo na produção de enzimas por *P. oxalicum*. Dentre elas, a concentração da fonte de nitrogênio (peptona) e a agitação tiveram efeito significativo na atividade em papel de filtro (FPase, U/mL) e o máximo de atividade foi obtido nas seguintes condições: peptona, 0,72 g/L e agitação, 180 rpm. Posteriormente, a fim de reduzir os custos inerentes a fontes de nitrogênio empregada para o cultivo do micro-organismo, a peptona foi total ou parcialmente substituída por farelo de soja ou casca de soja, o que não afetou os títulos de FPAse, mas impactou os custos do processo. Este foi o primeiro estudo a explorar a cana energia como substrato para promover a produção de CAZymes por linhagens fúngicas, o que contribui para o aumento do espectro de biomassas empregadas para essa finalidade. As metodologias de mutagênese física (luz UV) e química com o reagente etil metano sulfanato (EMS) também foram adotadas no presente estudo para propiciar o aumento da atividade de CAZymes produzidas por *P. oxalicum*. Nesse sentido, o mutante EMS19, obtido após duas rodadas de mutagênese, apresentou um aumento de 100 % na atividade de FPAse e secretou 50 % mais proteínas quando cultivado em meio contendo

uma mistura de avicel e xilana como fonte de carbono. A atividade de hemicelulases e β-glucanases, principalmente, também foi pronunciada em EMS19 quando comparada à linhagem parental (WT). A comparação dos secretomas de WT e EMS19 auxiliou na compreensão sobre CAZymes importantes na degradação das biomassas lignocelulósica no fungo mutante que impactam a atividade enzimática do mesmo.

**ABSTRACT**

Obtaining renewable fuels such as ethanol is highly desirable to reduce the emission of greenhouse gases into the atmosphere. Ethanol can be produced by the first (1G) and second generation (2G) technologies. Despite the advantages of 2G production, this is still considered costly, mainly due to the saccharification stage which requires a high dosage of enzymes for the conversion of biomass polymers into fermentable sugars. In addition to the costs inherent to the production of these enzymes, the logistics and stabilization also contribute to the high value dispensed at this stage. Therefore, the increase in enzymatic activities of cellulases, hemicellulases and accessory activities produced by microrganisms is highly desirable. By this way, the filamentous fungus *Penicillium oxalicum*, due to its wide spectrum of CAZymes (enzymes related to the breakdown of polysaccharides) secreted under certain growth conditions, emerges as a promising alternative to produce these enzymes. According to that describe above, the aim of this work aim was to optimize the production of enzymes related to the deconstruction of biomass by a wild-type strain of *P. oxalicum* by adopting alternative sources of carbon and nitrogen and obtain lineages with improved enzyme-activity properties by applying random mutagenesis methodologies. The adoption of the design of experiments (DOE) allowed to observe the effects of some culture medium variables in the production of enzymes by *P. oxalicum*. Among them, the concentration of the nitrogen source (peptone) and agitation had a significant effect on filter paper activity (FPase, U/mL) and the maximum activity was reached in the following conditions: peptone, 0.72 g/L and agitation, 180 rpm. Subsequently, in order to reduce the costs inherent to nitrogen sources, peptone was totally or partially replaced by soybean meal or soybean hulls, which did not affect the FPAse titles, but impact the process costs. This is the first study to explore energy cane as a carbon source to promote the production of CAZymes by fungi strains, what contributes to the spectrum of biomass to be applied in the field of biomass deconstruction. The random mutagenesis strategies such as physical (UV light) and chemical (EMS) mutagenesis was also adopted in this study to obtain *P. oxalicum* lineages with improved CAZymes activities. The mutant EMS19, obtained after two rounds of mutagenesis, resulted in 100% more FPAse activity and secreted 50% more proteins when grown in avicel and xylan as carbon sources. The xylanases and β-glucanases activities were also found improved in EMS 19 compared to parental strain (WT). The secretomes comparison of WT and EMS19 secretome allowed to understand

the pivotal CAZymes for lignocellulosic degradation in EMS19, which affected the enzymatic activity displayed by this fungus.

**SUMÁRIO**

## 1. INTRODUÇÃO

A demanda por combustíveis renováveis tem aumentado nas últimas décadas devido à instabilidade das regiões onde são encontradas as reservas de petróleo e a necessidade de reduzir a emissão de gases promotores do efeito estufa na atmosfera (Saini et al., 2015; Obeng et al., 2017). O etanol derivado da cana-de-açúcar no processo de primeira geração apresenta um índice de intensidade do carbono (IC) de 58,40 g de $CO_2$e/MJ, menor que o da gasolina (95,86g de $CO_2$e/MJ) (Air Resources Board, 2015). Desta forma, reduções na emissão de $CO_2$ podem ser alcançadas substituindo-se a gasolina ou outros combustíveis pelo etanol (Santos et al., 2016).

A principal matéria-prima empregada para a produção do etanol de primeira geração (1G) no Brasil é o melaço da cana-de-açúcar (Goldenberg, 2007). Com o advento do etanol de segunda geração (2G), a biomassa lignocelulósica passou a ser uma alternativa para a sua produção (Ripoli et al., 2010; Kim et al., 2011). Além da cana-de-açúcar, a cana energia também pode ser empregada como substrato para a produção do etanol 2G. A cana energia é um híbrido de duas variedades de cana-de-açúcar com alto potencial de produção de biomassa por apresentar maior teor de fibras (Ripoli et al., 2010; Kim et al., 2011).

A biomassa lignocelulósica é considerada a fonte de energia renovável mais abundante da Terra e apresenta grande potencial para a produção de combustíveis líquidos e outros químicos (Zhang et al., 2004). Sua estrutura é constituída por celulose (40-50%), hemicelulose (20-40%), lignina (20-30%) e compostos minoritários (Horn et al., 2012).

Devido a sua inerente recalcitrância (Oliveira et al., 2014), a biomassa lignocelulósica deve inicialmente ser submetida a um pré-tratamento que propicia o aumento da superfície do substrato e reduz a cristalinidade da celulose e a etapa de sacarificação, a qual libera açúcares monoméricos que serão posteriormente fermentados por leveduras para a produção do etanol (Obeng et al., 2017; Soccol et al., 2010; Zhao et al., 2012).

A etapa de sacarificação depende da sinergia entre celulases, hemicelulases, enzimas que atuam na lignina e atividades auxiliares (AAs) para a liberação de açúcares fermentescíveis (Obeng et al., 2017). Na natureza, essas enzimas são produzidas principalmente por micro-organismos como fungos e bactérias. Dentre as espécies de fungos filamentosos utilizados na indústria, *Trichoderma* sp. e *Aspergillus* sp. devem ser ressaltados. Entretanto, apesar do potencial biotecnológico dessas espécies, estas ainda possuem baixa produção de algumas enzimas essenciais ao processo de sacarificação (Lehmann et al., 2016). Deste modo, a busca

por novos organismos e/ou o melhoramento genético dos previamente descritos se torna necessária.

Diversos estudos de genômica e proteômica têm apontado o potencial (hemi)celulolítico do fungo filamentoso *Penicillium oxalicum* em relação a outros fungos modelo quanto a produção dessas enzimas (Liu et al., 2013; Vaishnav et al., 2018). Alguns autores o consideram uma alternativa ao *T. reesei* para aplicação industrial (Gusakov et al., 2011).

Apesar das constantes iniciativas privadas e acadêmicas quanto ao desenvolvimento do processo de sacarificação enzimática, o mesmo ainda é considerado um gargalo para a produção do etanol 2G (Klein-Marcuschamer et al., 2012). Estima-se que cerca de 15% dos custos operacionais da uma indústria de etanol 2G estejam relacionados a essa etapa (Ramos et al., 2016). Uma alternativa é o emprego do processo *On-site Manufacture*, o qual as enzimas são produzidas adjacentes às plantas de fabricação dos químicos e os custos com logística, estabilização das mesmas são reduzidos (Kovacs et al., 2009; Johnson, 2016).

Diante do exposto acima, o objetivo do presente trabalho foi a otimização de um meio de cultivo para a produção de enzimas relacionadas à desconstrução da biomassa lignocelulósica por *P. oxalicum* (Capítulo 1) e o incremento da atividade hemicelulolítica do mesmo fungo por meio de metodologias de mutagênese randômica (Capítulo 2).

## 2. REVISÃO DE LITERATURA

### 2.1. Etanol: visão geral

O etanol pode ser produzido pelas vias química ou biológica. A via química é baseada na hidratação do etileno, enquanto a biológica é realizada pela fermentação, por leveduras, dos monômeros de açúcares contidos em diferentes matérias primas, por exemplo, milho e cana-de-açúcar (Hidzir et al., 2014). Os dois maiores produtores de etanol no mundo são os Estados Unidos e o Brasil que alcançaram a produção de 15.776 e 8.570 milhões de galões, respectivamente, em 2019 (Renewable Fuels Association, 2020).

A crise petrolífera da década de 1970 impulsionou o governo brasileiro a criar o programa “Proálcool” (Programa Nacional do Álcool) em 1975 que tinha como meta a redução da dependência das importações de petróleo no país. (Amorim et al., 2005). O percentual da frota brasileira de automóveis movida a etanol chegou a 95 % em 1980, aumentando o seu consumo e reduzindo a dependência do petróleo. Entretanto, fatores como a queda nos preços

do barril de petróleo, a redução de subsídios aos produtores e o aumento do preço do açúcar resultaram no declínio do uso do etanol, desacelerando a sua produção (Potter, 2008).

O etanol voltou a ganhar destaque no cenário energético devido ao aumento do preço do petróleo no início do século XXI e ao desenvolvimento da tecnologia do motor flexível ou flex (Amorim et al., 2005). Os veículos flex podem ser movidos tanto com 100 % de etanol ou em diferentes misturas de etanol e gasolina. Dentre os benefícios das misturas de etanol, estão a substituição de aditivos tóxicos à gasolina, como por exemplo, o chumbo tetra etílico e o benzeno derivado de óleo, a melhora da qualidade do ar devido a redução das emissões de monóxido de carbono e a flexibilidade ao consumidor de escolher o combustível de menor custo. A gasolina comum contém uma mistura de 27 % de etanol anidro e 73 % de gasolina (Wang et al., 2012; Portaria N° 75, 2015).

O uso de etanol como biocombustível no Brasil foi considerado o programa de maior sucesso para substituir os combustíveis fósseis no mundo. Em 2018, de acordo com o estudo do Sindipeças 67, 1 % dos veículos em circulação possuíam motor flex. Desta forma, o Brasil e vários países investem em seus programas de produção e uso de etanol como combustível para reduzir a dependência de petróleo e as emissões dos gases do efeito estufa (Goldemberg et al., 2013; Sindipeças 2019).

## 2.2. Etanol de primeira e segunda geração

Os processos para a produção do etanol pela via biológica são conhecidos como de primeira (1G) e segunda geração (2G). Em ambos, os açúcares presentes na biomassa são fermentados por micro-organismos como leveduras do gênero *Saccharomyces* sp., produzindo o etanol (Figura 1).

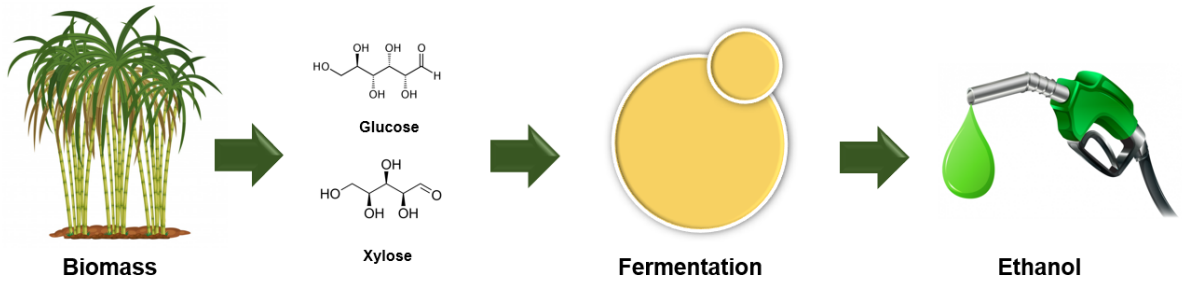


**Figura 1.** Processo de produção do etanol (Adaptado de Kang et al., 2015). Os açúcares contidos na biomassa, por exemplo, cana-de-açúcar são fermentados por micro-organismos como leveduras gerando álcool que é posteriormente destilado.

O processo brasileiro 1G gera etanol a partir da fermentação do caldo extraído da cana-de-açúcar. Entretanto, devido à demanda da cana-de-açúcar para o consumo humano, a biomassa lignocelulósica torna-se uma alternativa para a produção de etanol no processo chamado 2G , que também pode incluir o uso de outras variedades de cana, como a cana energia, para a sua produção (Geddes et al., 2011; Ojeda et al., 2011).

Devido a recalcitrância da biomassa lignocelulósica, o etanol produzido pela tecnologia 2G requer um pré-tratamento físico ou químico que promove o "afrouxamento" das fibras e a sacarificação do material lignocelulósico pela adição de coquetéis enzimáticos, anteriores a etapa de fermentação (Figura 2) (Canilha et al., 2012).

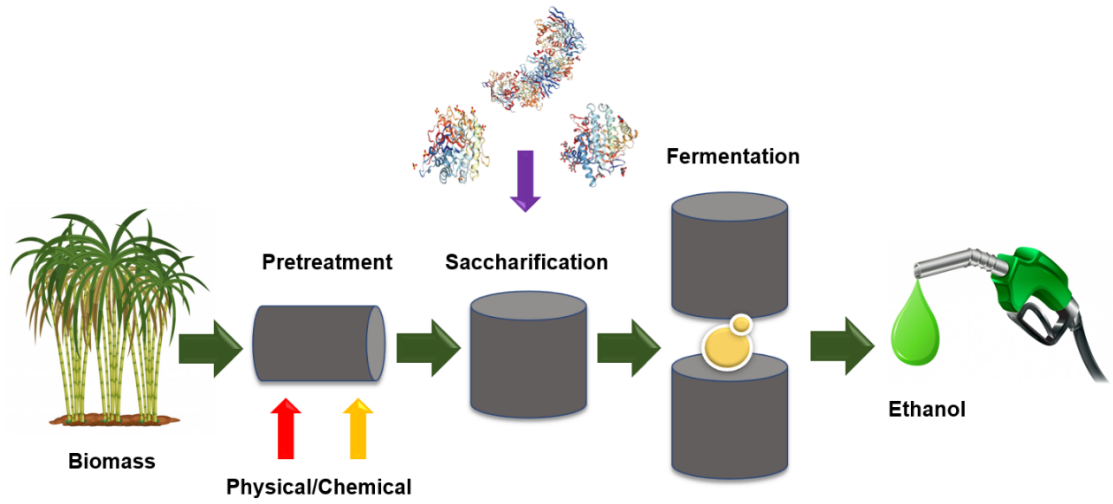


**Figura 2.** Processo de produção do etanol 2G (Adaptado de Payne et al., 2015). A biomassa lignocelulósica é submetida a um pré-tratamento, seguido de uma etapa de sacarificação, na qual diversas enzimas atuam na desconstrução da biomassa liberando monômeros de açúcares para a fermentação por micro-organismos e destilação do combustível.

Todavia, apesar do acesso facilitado à biomassa lignocelulósica, a viabilidade de produção do etanol 2G apresenta como principais gargalos a recalcitrância da biomassa lignocelulósica, devido a presença da lignina, a estrutura cristalina da celulose e a composição heterogênea da hemicelulose que requer um pré-tratamento que favoreça a acessibilidade das enzimas aos constituintes da biomassa na etapa de sacarificação; e o custo dos coquetéis enzimáticos empregados na etapa de sacarificação (Zhao et al., 2011, Scully et al, 2015)

Diversos estudos avaliaram o preço dos coquetéis enzimáticos empregados na etapa de sacarificação do etanol 2G. Alguns autores relataram que o custo das celulases pode variar de $0.10 a $0.40 por galão de etanol, o que torna a tecnologia 2G atual viável (Sassner et al., 2008;

Lynd et al., 2008). Porém, também foi demostrado que o custo enzimático pode chegar a $2.71/galão de etanol, correspondendo a 48% do preço mínimo de venda do etanol lignocelulósico (Liu et al., 2016). Uma alternativa para a redução desses valores é a produção enzimática adjacente as usinas, no processo *on-site* e a utilização da biomassa lignocelulósica como fonte de carbono no processo chamado de integrado (Merino et al., 2007). Segundo Johnson (2016) os custos inerentes a etapa de sacarificação podem ser reduzidos de $0.78 para $0.58 e $0.23/galão etanol mudando os processos de *off-site* para *on-site* e integrado, respectivamente. Dessa forma a adoção da tecnologia *on-site* e integrada na produção enzimática podem tornar economicamente factível a produção de etanol 2G.

### 2.3. Pré-tratamento da biomassa lignocelulósica

O pré-tratamento é uma das principais etapas do processo 2G devido a sua ação na alteração do complexo formado por celulose, hemicelulose e lignina, com o objetivo de expor a fração de celulose para a maior interação entre enzima-substrato na etapa de sacarificação (Mood et al., 2013; Sharma et al., 2019). Desta forma, a escolha do pré-tratamento interfere diretamente nos rendimentos do processo enzimático. (Zhang et al., 2009; Silva et al., 2013; Singh et al., 2015).

Um pré-tratamento é considerado eficaz quando possibilita a liberação de açúcares por meio da etapa de sacarificação, evita a perda e/ou degradação dos açúcares formados e apresenta baixa demanda de energia e custos operacionais reduzidos (Alvira et al. 2010; Zabed et al., 2016; Sharma et al., 2019). Além disso, a redução de compostos inibidores como ácidos fracos e furanos provenientes das frações de hemicelulose e celulose e compostos fenólicos advindos da lignina é extremamente necessária, tendo em vista, que esses compostos apresentam efeitos negativos nas etapas de sacarificação e fermentação (Palmqvist 2000; Jönsson et al. 2013)

Os pré-tratamentos são divididos em: físico, físico-químico, químico e biológico. Como exemplo de pré-tratamento físico está a moagem, responsável por reduzir o tamanho das partículas, a cristalinidade da celulose e não produzir inibidores. Entretanto, a moagem não remove a lignina e nem altera a hemicelulose. Além disso, requer alto consumo de energia para a operação dos moinhos, o que resulta no aumento de custo da produção (Taherzadeh et al., 2008; Alvira et al. 2010; Silva et al., 2013)

Dentre os físico-químicos devem ser ressaltados os pré-tratamentos com água quente, expansão da fibra por amônia (AFEX), explosão com $CO_2$, oxidação úmida e a explosão a vapor. O pré tratamento com a água quente possibilita a solubilização da hemicelulose, tornando a celulose acessível à sacarificação. Entretanto, a demanda de água e energia são altas

(Mosier et al.,2005). AFEX leva a uma redução da cristalinidade da celulose, remove hemicelulose e lignina e reduz a formação de inibidores.Entretanto, é considerado um processo oneroso devido à grande quantidade de amônia requerida (Laureano-Pérez et al., 2005).

A explosão com $CO_2$ não modifica a lignina e a hemicelulose, não forma compostos inibitórios, entretanto necessita de alta pressão. A oxidação úmida solubiliza a hemicelulose e lignina, reduz a formação de compostos inibitórios, todavia, os requerimentos quanto ao oxigênio e catalisador alcalino aumentam os custos do processo (Zheng et al. 1998).

A explosão a vapor possibilita a degradação da lignina, solubiliza a fração hemicelulósica e facilita a sacarificação da celulose. Entretanto, gera compostos inibitórios derivados das xilanas (Alvira et al. 2010; Taherzadeh et al., 2008).

Os pré-tratamentos químicos podem ser realizados pela adição de ácido concentrado, base, solvente orgânico ou ozonólise. O ácido concentrado solubiliza a hemicelulose e altera a estrutura da lignina, porém há a formação de compostos tóxicos e danos corrosivos aos equipamentos (Mosier et al. 2005).

O pré-tratamento alcalino diminui o grau de polimerização e cristalinidade da celulose, solubiliza a hemicelulose e remove a lignina. Entretanto, é considerado de alto custo devido a fatores como o tratamento dos resíduos liberados, portanto, inviável em larga escala (Taherzadeh et al., 2008). O pré-tratamento químico com adição de solvente orgânico promove a desconstrução da lignina e da hemicelulose, porém é considerado de alto custo devido a processos de recuperação do solvente. A ozonólise auxilia na remoção da lignina e hemicelulose sem produzir resíduos tóxicos, sendo a reação realizada em temperatura e pressão ambiente. Todavia, é considerada de alto custo devido a grande quantidade de ozônio requerida. Os tratamentos com solventes orgânicos como o etanol e metanol levam à despolimerização da lignina e da hemicelulose, porém os mesmos necessitam ser drenados e reciclados, desta forma, aumentando o custo de produção (Zhao et al., 2009).

O pré-tratamento biológico é realizado com o uso de micro-organismos produtores de enzimas como os fungos filamentosos. Este pré-tratamento proporciona a deslignificação da biomassa, a redução no grau de polimerização da celulose, a hidrólise parcial da hemicelulose, não requer químicos e os requisitos de energia são baixos. Porém possui baixo rendimento, o que não permite a sua aplicação comercial (Kuhar et al., 2008).

Apesar dos vários tipos de pré-tratamentos existentes, a dificuldade na desconstrução do complexo lignocelulósico específico para cada biomassa ainda é um fator limitante desse processo. Desta forma, nenhuma das tecnologias de pré-tratamento possibilita a conversão total da biomassa em açúcares fermentescíveis, o que afeta o rendimento final do etanol. Além disso,

os custos elevados envolvidos na aplicação dessas tecnologias são repassados ao preço final do biocombustível (Maurya et al., 2015).

## 2.4. Sacarificação enzimática

A biomassa lignocelulósica consiste principalmente em três polímeros: celulose (40 a 50 %), hemicelulose (20 a 40 %) e lignina (20 a 30%) que estão conectados e embebidos em uma hetero-matriz (Figura 3). Alguns componentes minoritários também são encontrados como proteínas, lipídeos, pectina, açúcares solúveis e minerais (Pauly et al., 2008). A abundância de cada um desses constituintes varia de acordo com o tipo de biomassa (Chandra et al., 2007).

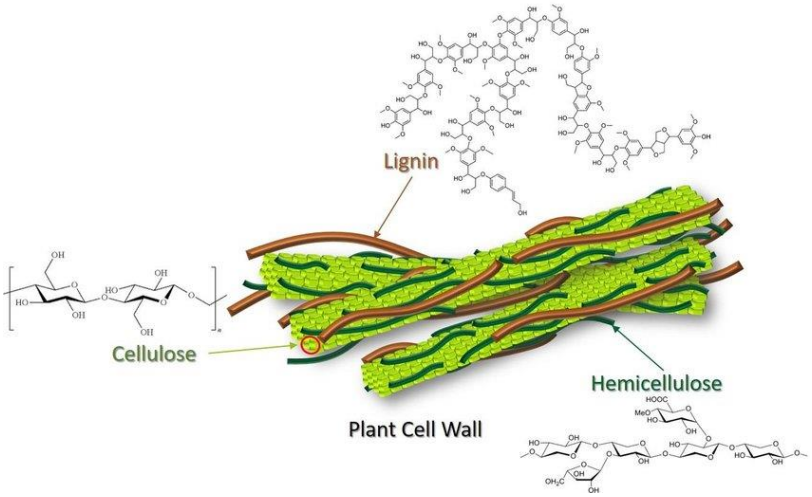


**Figura 3**. Principais componentes da biomassa lignocelulósica: celulose, hemicelulose e lignina (Jensen et al., 2017).

O processo de desconstrução da biomassa lignocelulósica requer a ação cooperativa de enzimas que atuam na celulose (endoglucanases, celobiohidrolases e β-glucosidases), hemicelulose (endoxilanases e β-xilosidases), lignina (manganês peroxidases, lignina peroxidases, peroxidase versáteis e lacases) e pectina (poligalacturonases, pectina-liases, pectina-metilesterases e pectato-liases) (Escamilla-Alvarado et al., 2016). Enzimas envolvidas na síntese, quebra ou modificação de polissacarídeos encontram-se agrupadas de acordo com a similaridade de sequências de aminoácidos, mecanismo catalítico e estrutura 3D nas famílias: glicosídeo hidrolases (GHs), glicosiltransferases (GT), polissacarídeo liases (PL), carboidrato esterases (CE), módulos de ligação a carboidratos (CBM) e atividades auxiliares (AA) no banco de dados CAZy (www.cazy.org) que elenca as enzimas ativas em carboidratos (Martinez et al., 2008; Cantarel et al., 2009).

As GHs, mais abundante entre todas as CAZymes, compreendem 167 famílias. A clivagem pela ação das GHs pode ser dois tipos: hidrólise com retenção ou inversão da configuração anomérica no ponto de clivagem e mecanismos envolvendo eliminação, hidratação ou ambos (Koshland et al., 1953; Jongkees et al., 2013).

As GTs encontram-se distribuídas em 111 famílias e são responsáveis pela formação das ligações glicosídicas por meio da transferência das porções de açúcar de moléculas doadoras ativadas para moléculas aceptoras, por exemplo, açúcares, lipídios, proteínas e ácidos nucleicos (Lairson et al., 2008).

As PLs clivam cadeias de polissacarídeos contendo ácidos urônicos por meio de um mecanismo de β-eliminação. As PLs correspondem a 40 família de CAZymes (Lombard et al., 2010). As CEs estão divididas em 18 famílias de CAZymes. Essas enzimas catalisam a remoção das modificações baseadas em éster de mono-, oligo- e polissacarídeos o que facilita o acesso das GHs na degradação do polissacarídeo (Christov et al, 1993; Cantarel et al., 2009). Em relação aos CBMs, são domínios não catalíticos conectados por um *linker* a outras CAZymes que promovem a interação do sítio ativo da enzima ao substrato, potencializando a sua ação. Os CBMs constituem 87 famílias de CAZymes (Cantarel et al., 2009).

As atividades auxiliares foram recentemente adicionadas ao banco de dados CAZyme e estão divididas em 16 famílias. As AAs atuam auxiliando outras CAZymes na degradação dos substratos, potencializando a ação das mesmas. Dentre os membros das AAs ressaltam-se lacases, celobiose desidrogenases (CDHs), monooxigenases líticas de polissacarídeos (LPMOs) e outras enzimas envolvidas com o mecanismo redox (Levasseur et al., 2013).

As características específicas das enzimas citadas acima serão abordadas nos tópicos posteriores.

### 2.4.1. Celulose

A celulose é o polímero mais abundante da Terra (Bayer et al., 1998). Trata-se de um polissacarídeo linear constituído por centenas a milhares de unidades de glicose unidas por ligações β-1,4 que são organizadas em microfibrilas por ligações de hidrogênio e interações de van der Waals (Parthasarathi et al., 2011). A maior parte da fração celulósica é cristalina e bem ordenada, sendo o restante composto por regiões desordenadas, mais amorfas. A despolimerização da fibra ocorre mais facilmente na porção amorfa comparada à porção cristalina (Yadav et al., 2019).

O modelo clássico da desconstrução da celulose envolve a ação sinérgica de três classes de hidrolases: as endo-1,4-β-glucanases, exo-1,4-β-glucanases e as β-glucosidases. As endo-

1,4-β-glucanases (EG, E.C. 3.2.1.4) iniciam o processo de hidrólise sendo responsáveis por clivar de forma aleatória as ligações internas do polímero de celulose diminuindo o seu grau de polimerização e propiciando a formação de extremidades redutoras para a ação das exo-1,4-β-glucanases. Além disso, as endoglucanes também podem ser ativas em celodextrinas, um produto intermediário da hidrólise, permitindo a liberação de celobiose e glicose (Figura 4) (Saha et al., 2004; Sajith et al., 2016).

As exo-1,4-β-glucanases ou celobiohidrolases (CBH, E.C. 3.2.1.91) possuem afinidade com as porções cristalina da fibra celulósica e clivam a partir da extremidade redutora (CBHI) ou não redutora (CBHII), liberando como produto glicose e principalmente celobiose, a qual pode causar a sua inibição (Zhang et al., 2004). Por fim, a celobiose e os celo-oligossacarídeos são convertidos em glicose, pela ação das beta-glucosidases (BG, EC 3.2.1.21) (Figura 4) (Zhang et al., 2004; Sajith et al., 2016).

As EGs e CBHs podem apresentar uma estrutura chamada de módulo de ligação de carboidratos (CBM) conectado ao domínio catalítico por um conector flexível rico em prolina, serina e treonina. CBMs favorecem a interação das enzimas a celulose facilitando a sua atividade hidrolítica (Dashtban et al., 2009)

Recentemente, as endoglucanases pertencentes a família GH61 foram reclassificadas em monooxigenases líticas de polissacarídeos (LPMOs - *lytic polysaccharide monooxygenases*) e inseridas na família das atividades auxiliares (AA) devido a elucidação do seu real mecanismo de ação e a sua sinergia com as enzimas canônicas citadas acima. As LPMOs clivam a molécula de celulose por meio da introdução de um átomo de oxigênio na posição C1, C4 ou ambas do polissacarídeo. O mecanismo catalítico envolve a redução do cobre presente em seu sítio ativo plano (o que favorece a interação com porções cristalinas da celulose) de Cu (II) para Cu (I) por um agente redutor externo, podendo este ser biológico. O cobre reduzido reage com uma molécula de $O_2$ acarretando na ligação de uma espécie de oxigênio ativado ao polissacarídeo. Desta forma, LPMOs geram extremidades oxidadas na cadeia de celulose que são reconhecidas por celulases clássicas, o que aumenta a liberação de açúcares (Figura 4) (Horn et al., 2012; Levasseur et al., 2013; Corrêa et al., 2016).

### 2.4.2. Hemicelulose

Ao contrário da celulose, a hemicelulose é um polímero heterogêneo contendo cadeias de aldopentoses (xilose e arabinose) e aldohexoses (glicose, manose e galactose) em suas cadeias principais ou decorações. A mesma encontra-se intimamente associada à lignina e a celulose (Zhang et al., 2011).

Devido a heterogeneidade da hemicelulose, diversas enzimas ativas na cadeia principal e nas cadeias laterais são recrutadas para a sua desconstrução. Em relação a despolimerização da hemicelulose em açúcares e ácidos orgânicos, a ação sinérgica das xilanases, xilosidases, arabinofuranosidases e glucuronidases se tornam necessárias (Hu et al., 2011; Steinbach et al., 2017).

As xilanases são enzimas centrais na conversão da fração hemicelulósica tendo em vista que o xilano que é o seu principal constituinte (Beg et al., 2001). O xilano é composto por unidades de D-xilose e decorações como resíduos de acetil, arabinosil e glucuronil podem estar presentes. A conversão do xilano em xilose se dá a partir da ação sinérgica entre a endo-β-1,4 xilanases, β-xilosidases e enzimas acessórias como α-l-arabinofuranosidases, acetil xilano esterases, α-glucuronidases e feruloil esterases (Figura 4) (Polizeli et al., 2005; Guais et al., 2010).

As endo-1,4-β-xilanases (EX, E.C. 3.2.1.8) clivam as ligações β-1,4-glicosídicas do xilano, liberando xilooligossacarídeos. As β-xilosidases (E.C. 3.2.1.37) por sua vez hidrolisam os xilooligossacarídeos em unidades de xilose. Assim como as celulases, as hemicelulases também podem apresentar CBMs (Conejo- Saucedo et al., 2017).

As α-l--arabinofuranosidases (E.C. 3.2.1.55) são enzimas acessórias que hidrolisam a extremidade não redutora dos grupos α-l--arabinofuranosil encontrados em arabinanos, arabinoxilanos e arabinogalactanos. As α-l--arabinofuranosidases atuam sinergicamente com outras hemicelulases e pectinases (Hu et al., 2011).

As acetil xilano esterases (E.C. 3.1.1.72) clivam os grupos laterais acetil ligados a estrutura do xilano, enquanto as α-glucuronidases (E.C. 3.2.1.131) atuam na hidrólise das ligações α-1,2 entre ácido glucurônico e resíduos de xilose do glucuronoxilano (Golan et al., 2004). Finalmente, as feruloil esterases promovem a clivagem das ligações éster entre o ácido carboxílicos associados às xilanas (Polizeli et al., 2005).

### 2.4.3. Lignina

A lignina é um polímero aromático complexo composto por unidades de fenilpropano ligadas a carbono e éter, presente na parede celular secundária das plantas (Souza 2013). As subunidades de fenilpropanos são classificadas em álcool coniferílico (guaiacil propanol), álcool cumarílico (p-hidroxifenil propanol) e álcool sinapílico (álcool siringílico) (Bajpai, 2016; Datta et al. 2017).

Dentre as funções da lignina, ressaltam-se: suporte mecânico, controle da osmolaridade e defesa vegetal ao ataque de organismos e suas enzimas (Singh et al., 2014**).**

A lignina causa a inibição da sacarificação enzimática devido a fatores como a adsorção de celulases através de interações hidrofóbicas, eletrostáticas ou ligação de hidrogênio, o que causa o bloqueio a superfície acessível de carboidratos e pela inibição de celulases por meio de seus derivados. Portanto, a remoção da lignina pelas ligninases pode aumentar a ação enzimática das celulases e hemicelulases (Singh et al., 2014).

As principais enzimas relacionadas com a desconstrução da lignina são as lacases (E.C. 1.10.3.3), lignina-peroxidases (LiP, EC 1.11.1.14), manganês peroxidases (MnP, E.C. 1.11.1.13) e peroxidases (VP, EC 1.11.1.16) (Baldrian et al., 2006). Lacases catalisam a oxidação de compostos orgânicos e inorgânicos, como difenóis, polifenóis, diaminas e aminas aromáticas (More et al. 2011) (Figura 4) (Ferraroni et al. 2007).

As lignina-peroxidases atuam na oxidação de anéis aromáticos e na degradação de compostos aromáticos como o álcool veratrílico (3,4-dimetoxibenzil) e metoxibenzenos enquanto as manganês peroxidases catalisam reações de peroxidação lipídica das unidades fenólicas e não fenólicas da lignina (Baldrian et al., 2006).

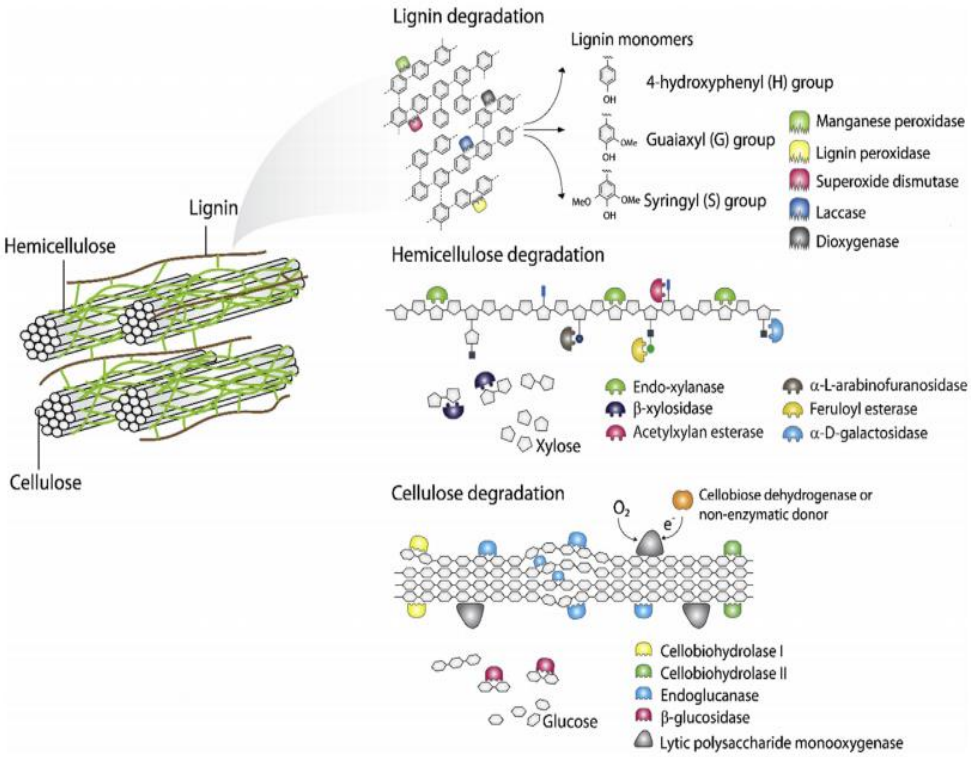


**Figura 4.** Degradação da biomassa lignocelulósica pela ação de celulases, hemicelulases, enzimas ativas em lignina e atividades auxiliares (Champreda et al., 2019).

## 2.5. Cana energia: uma alternativa à cana-de-açúcar na produção do etanol 2G

A cana-de-açúcar (*Saccharum* spp.) tem sido aplicada para a produção de etanol devido a características como alta produtividade a diminuição da liberação de gases promotores do efeito estufa na atmosfera (Macedo et al., 2008; Leal et al., 2010). Entretanto, com a crescente demanda por fontes renováveis de energia, cientistas tem buscado entender a fisiologia da planta nos processos de captura da energia solar, no acúmulo de açúcar e no uso total da biomassa (Waclawovsky et al., 2010; Souza et al., 2013). Neste sentido, estudos conduzidos por Alexander na década de 80 em Porto Rico, resultaram em uma variedade de cana denominada cana-energia, a qual possui um alto teor de fibras comparada a cana-de-açúcar. De acordo com este autor, a cana é produtor inigualável de biomassa e não somente uma planta com capacidade de estocagem de açúcar (Alexander, 1985).

Segundo Tew e Cobill (2008), variedades de cana podem ser divididas em cana-de-açúcar tradicional e dois tipos de cana energia. A cana-de-açúcar tradicional contém cerca de 75 % de água, 12 % de fibras e 13 % de açúcar e fornece o suco para a produção de açúcar e etanol 1G, e a fibra para a geração de energia. A cana energia tipo I, conceituada por Alexander em 1985, foi selecionada para maximizar a produção de açúcar e fibra e contém 65 % de água, conteúdo de fibras entre 13-17% (um aumento de 40% em relação a cana-de-açúcar tradicional). Esta variedade fornece o suco para a produção de açúcar e etanol 1G e, devido ao seu alto teor de fibras, também contribui para o etanol 2G, além da produção de energia. A cana energia do tipo II foi selecionada para maximizar o conteúdo de fibras (em torno de 30 %) e apresenta baixo teor de açúcar e menor proporção de água (60 %). Esta variedade de cana é especializada para a geração de energia por meio da biomassa no processo 2G.

Os primeiros híbridos de cana energia no Brasil, foram obtidos pelo cruzamento das espécies *Saccharum officinarum* com *Saccharum spontaneum* pela empresa de melhoramento genético vegetal Canavialis nos anos 2005-2006. O melhor clone obtido produziu 138 % mais biomassa total e 235 % mais fibra por área quando comparada a uma variedade convencional de cana-de-açúcar. Atualmente, outros programas de melhoramento de cana são conduzidos no Brasil por empresas como Vignis, CTC (Centro de Tecnologia Canavieira), IAC (Instituto Agronômico de Campinas), GranBio e RIDESA (Rede Interuniversitária para o Desenvolvimento do Setor Sucroenergético) (Matsuoka et al, 2015a).

Dentre as características vantajosas presentes na cana-energia, além da alta produção da biomassa por meio da conversão do carbono atmosférico em carbono orgânico, estão a alta resistência a estresses bióticos e abióticos. Desta forma, a cana energia pode ser cultivada com

menores doses de fertilizantes e pesticidas e em solos menos férteis que podem apresentar menor disponibilidade de água e temperaturas mais elevadas. Portanto, não competindo com as areas férteis usadas na produção de alimentos. Além disso, devido a suas raízes robustas, a cana energia pode atuar no controle da erosão do solo e auxiliar na recuperação de solos degradados. Ao contrário da cana-de-açúcar, a cana energia não necessita do processo de maturação que envolve a formação de açúcares nas folhas e o seu deslocamento e armazenamento no colmo para a colheita, portanto, a cana energia pode ser colhida quase o ano inteiro. Outra característica interessante da cana energia é o alto perfilhamento que é o processo de emissão de colmos por uma mesma planta. A cana energia possui uma taxa de multiplicação de 1:30 enquanto a cana-de-açúcar possui uma taxa menor de multiplicação de 1:10 (Figura 5) (Hill et al., 2006; Matsuoka et al, 2015; Johnson et al., 2017).

**Figura 5.** Comparação entre as culturas de cana-de-açúcar (esquerda) e cana energia (direita) (Santos et al., 2016). Devido ao seu sistema radicular, a cana energia possui maior produtividade de biomassa mesmo em áreas marginais quando comparada a cana-de-açúcar.

A produtividade da cana energia pode ser de 2 a 3 vezes maior que a da cana-de-açúcar quando cultivada em uma mesma área (Santos et al., 2016). Segundo Grassi e Pereira (2019), considerando a produtividade de 180 t/ha, a cana energia é passível de produzir cerca de 216 barris de petróleo por hectare. De acordo com os mesmos autores, o consumo mundial anual de petróleo seria sanado pelo cultivo de cana energia em 166 milhões de hectares tendo em vista os dados de consumo mundial de barris de petróleo (aproximadamente 35.733 milhões), fornecido pela Agência Internacional de Energia em 2017. Esta área não é considerada exorbitante tendo em vista que o Brasil apresenta 190 milhões de hectares dedicados a pastagens, dos quais muitos possuem baixa produtividade. Portanto, incluindo áreas

percententes a América, Ásia e África, a produção da biomassa necessária para substituir o uso total de combustiveis fosseis pode ser possivel (Grassi e Pereira, 2019).

Deste modo, a cana energia é considerada uma biomassa potencial na geração de etanol, possibilitando o aumento da competitividade brasileira na indústria bioenergética e trazendo benefícios à sociedade (Matsuoka et al, 2015). Além disso, estudos sobre a sacarificação da mesma mostraram a eficiência de enzimas lignocelulolíticas na conversão dessa biomassa em açúcares fermentescíveis. Dessa forma, a cana energia pode ser usada como uma fonte para o crescimento desses micro-organismos produtores de enzimas e portanto, contribuir para o processo integrado da produção do etanol lignocelulósico (Shields et al., 2011; Suhardi et al., 2013)

## 2.6. *Penicillium oxalicum*: um potencial para a produção de enzimas relacionadas à desconstrução da biomassa lignocelulósica

Fungos filamentosos são os principais produtores de enzimas empregadas na sacarificação da biomassa lignocelulósica (Lynd et al., 2002). Dentre os gêneros mais relevantes, *Trichoderma, Aspergillus* e *Penicillium* devem ser destacados (Gomes et al., 2015; Li et al., 2015). *T. reesei* é o fungo modelo quanto a produção de celulases e tem sido utilizado como plataforma para produção de enzimas por empresas como Novozymes e Genencor-Dupont (Novozymes, 2013; Dupont, 2016). *T. reesei* codifica várias EGs e CBHs. Entretanto, o mesmo não ocorre para BGs e hemicelulases (Sipos et al., 2010; Adav et al., 2012; Singhania et al., 2013). Em contrapartida, fungos do gênero *Aspergillus* são excelentes produtores de BGs, mas apresentam baixa atividade de EGs, sendo comum o uso combinado dos sobrenadantes das culturas de *T. reesei* e *Aspergillus* sp. (Rana et al., 2014). Entretanto, para a redução dos custos de produção, é altamente desejável que apenas um micro-organismo seja capaz de produzir todas as enzimas envolvidas com o processo de sacarificação (Hasunuma et al., 2012).

O gênero *Penicillium* é descrito como produtor de enzimas do complexo (hemi) celulolítico (Zhao et al., 2006; Shen et al., 2008). Vários estudos têm evidenciado a secreção de enzimas envolvidas com a desconstrução da biomassa lignocelulósica quando o mesmo é cultivado em diferentes fontes de carbono (Ribeiro et al., 2012; Liao et al., 2014).

Gong et al. (2015), ao comparar os secretomas de *P. oxalicum* 114-2 com *A. niger* ATCC1015 e *T. reesei* QM9414 cultivados em farelo de milho e trigo (fermentação estado sólido), observaram que *P. oxalicum* possui um grande potencial de produção de enzimas relacionadas à degradação de polissacarídeos. Foram encontradas oito EGs das famílias GH5, 7, 12 e 45, enquanto apenas três (GH5, 7, 12) e duas (GH5 e 12) foram detectadas no secretoma

de *T. reesei* e *A. niger*, respectivamente. Três BGs pertencentes à família GH3 foram identificadas no secretoma de *A. niger* e *P. oxalicum* enquanto apenas uma foi detectada em *T. reesei*. Uma BG da família GH1 era exclusiva de *P. oxalicum*.

Estudos conduzidos por Ribeiro et al. (2012) confirmaram o potencial (hemi) celulolítico desse gênero, ao observar que *P. echinulatum*, quando cultivado em bagaço de cana-de-açúcar, secreta várias enzimas pertencentes à família das glicosil hidrolases (GHs), dentre elas: endoglucanases GH5, 7, 6, 12, 17 e 61, β-glicosidases GH3, xilanases GH10 e GH11, hemicelulases GH43, GH62 e pectinases GH28.

Liao et al. (2014) observaram que *P. oxalicum* cultivado em avicel:xilana (meio composto) apresenta maiores atividades de FPase, CMCase e xilanase quando comparado aos cultivos realizados com glicose, avicel e xilana como substratos. Análises de q-PCR revelaram uma alta expressão de genes que codificam as celulases egl1, egl2, egl3, cbh2 e hemicellulases xyl3 e xyl4 no meio composto. O secretoma dos meios avicel, xilana, glicose e avicel:xilana identificaram 157, 141, 101 e 86 proteínas, respectivamente. O último apresentou a maior porcentagem de celulases (10.3%) e hemicelulases (21.8%), sendo CBH II e xilanase II as mais abundantes. Além disso, algumas proteínas importantes para a degradação da biomassa foram encontradas exclusivamente no meio avicel:xilana, por exemplo, algumas CBHs e endo-beta-1,4-xilanases. Esse trabalho sugere uma diferente regulação na produção de celulases entre *P. oxalicum* e *T. reesei*, além da alta indução do sistema (hemi)celulolítico de *P. oxalicum* em substratos mais complexos.

Análises comparativas do genoma de *P. oxalicum* com os genomas de *A. nidulans*, *A. niger*, *P. chrysogenum e T. reesei* demonstraram que o mesmo possui um arsenal de enzimas lignocelulolíticas mais diversificado dentre os fungos filamentosos citados. Ademais, o genoma de *P. oxalicum* possui maior número de CBMs e CAZymes que *T. reesei* (Liu et al., 2013). De acordo com os dados do Mycocosm são encontradas 223 GHs e 27 AAs no genoma de *P. oxalicum* 114-2, enquanto a cepa de *T. reesei* RUT-C30 possui 200 GHs e 31 AAs (Pel et al 2007; Liu et al., 2013; Jourdier et al., 2017). Alguns autores sugerem o gênero *Penicillium* como alternativa ao *Trichoderma* em preparações de coquetéis enzimáticos, principalmente, devido ao seu repertório de BGs (Vaishnav et al., 2018).

## 2.7. Otimização das condições de cultivo para a produção de enzimas por fungos filamentosos

A sacarificação enzimática é um dos principais gargalos para o desenvolvimento de tecnologias 2G tendo em vista o custo inerente a essa etapa, fato ressaltado acima. Além do

melhoramento genético, algumas alternativas como a otimização de meios de cultivo e diferentes estratégias fermentativas podem ser adotadas a fim de incrementar a produção de enzimas por micro-organismos (Riswan Ali et al., 2012).

*Design of experiments* (DOE) é uma técnica estatística empregada no planejamento e análise de dados visando projetar e modelar a relação entre fatores avaliados e respostas observadas (Papaneophytou et al., 2014). Essa abordagem reduz o número de experimentos, pois permite a avaliação de vários fatores ao mesmo tempo, ao contrário do método tradicional onde um único fator é avaliado enquanto os outros estão fixados (Zhou et al., 2017). DOE auxilia na avaliação da influência de variáveis experimentais e suas interações a fim de identificar as condições que desencadearão uma melhor resposta (Nam et al., 2017).

O delineamento Plackett-Burman elenca quais variáveis são significativas para a resposta. Em seguida, o planejamento *Central Composite Rotatable Design* (CCRD) é empregado a fim de compreender a interação entre tais variáveis e o efeito na resposta (Patil and Jena, 2015; Ungureanu et al., 2015).

Diversos estudos relatam o aumento da atividade de enzimas como celulases e xilanases empregando-se a metodologia DOE para o cultivo de micro-organismos (Biswas, 2014). Bocchini et al. (2002) incrementaram a atividade de xilanases produzidas por *Bacillus circulans* em cerca de 315% variando-se a concentração de xilano e tempo de cultivo, parâmetros tidos como estatisticamente significativos nesse estudo. Aumento na atividade de celulases foi observada também para o fungo *Penicillium waksmanii* F10-2 por meio da alteração da concentração de determinados componentes do meio de cultivo como a fonte de carbono (palha de trigo), nitrogênio (peptona) e minerais, bem como pH, agitação e temperatura (Han et al., 2009).

Após otimização das concentrações de avicel (celulose microcristalina), farelo de trigo, sulfato de amônio e do tempo de incubação, a produção de celulases por *P. oxalicum* aumentou cerca de 1,7 vezes em escala de bancada. Quando o fungo foi cultivado em fermentador nas condições otimizadas, o tempo de incubação necessário para alcançar a mesma atividade observada em bancada reduziu de oito para quatro dias (Saini et al., 2015), revelando a efetividade da estratégia estatística.

Diante do exposto acima, a adoção da metodologia DOE pode levar a um aumento da produção de enzimas por micro-organismos ao mesmo tempo em que reduz o seu custo tendo em vista que a concentração de alguns componentes do meio de cultivo pode ser reduzida e o tempo para a produção da carga enzimática pode ser alterado. Além disso, com o aumento do interesse pela produção de biorrenováveis, novos substratos podem ser utilizados como

elicitores da produção de enzimas por micro-organismos e se tornam alternativas interessantes para o desenvolvimento do *Integrated process*, uma metodologia *on-site manufacture* onde parte da biomassa lignocelulósica é destinada para o cultivo de micro-organismos/produção de enzimas (Olofsson et al., 2017).

## 2.8. Estratégias de mutagênese aleatória aplicadas a obtenção de micro-organismos com produção aumentada de enzimas

A produção de enzimas por cepas selvagens de fungos filamentosos normalmente não atende à demanda industrial. Sendo assim, algumas estratégias de mutagênese aleatória ou dirigida tem sido utilizada para incrementar a produção das mesmas por micro-organismos (Ennis, 2001).

Estratégias de mutagênese aleatória são divididas em duas classes: física e química. A mutagênese física causada pela incidência de radiação ultravioleta (UV) induz duas pirimidinas adjacentes, timina e citosina, a se ligarem e formarem um dímero de pirimidina. Se o dano ao DNA não for reparado, a DNA polimerase replicará o erro nas próximas gerações, fixando a mutação, mutação (Ennis, 2001) (Figura 7).

A mutagênese química inclui a ação de agentes químicos como brometo de etídio (EtBr), N-metil-N'-nitro-N-nitroguanidina (NTG) e etil metano sulfonato (EMS). As moléculas de EtBr podem se inserir entre as fitas de DNA, fazendo com que a DNA polimerase insira nucleotídeos extras nas fitas de DNA oposta, o que acarreta a mutação (Ennis, 2001). Agentes químicos como NTG e EMS adicionam grupos alquilas aos nucleotídeos em várias posições na fita de DNA, o que leva a substituição dos pares de base. O EMS especificamente reage com as bases guaninas formando uma base anormal: O6-etilguanina. Dessa forma, durante a replicação, as DNAs polimerases inserem timina, ao invés de citosina para parear com a O6-etilguanina. Desta forma, após uma série de rodadas de replicação, o par A:T tende a substituir o par G:C original (Dhaliwal et al., 2015) (Figura 8).

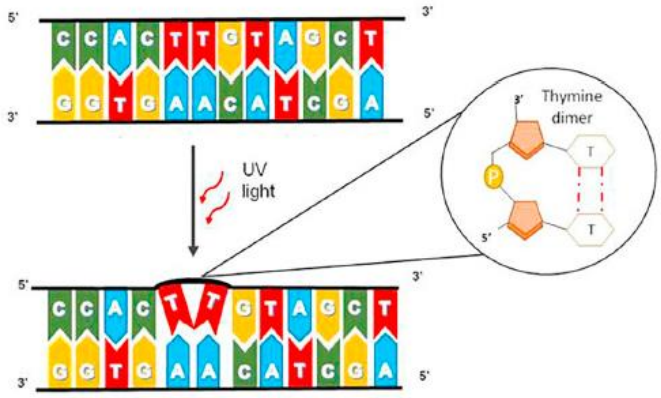


**Figura 7**. Alterações causadas ao DNA por meio da luz UV que leva a formação de dímeros de timina, que podem introduzir mutações de deslocamento ou ponto (Kun, et al., 2019).

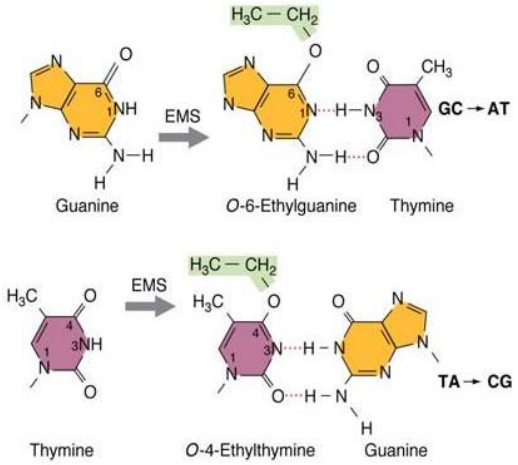


**Figura 8.** Formação do nucleotídeo anormal O6-etilguanina causada pela ação do químico etil metano sulfonato (EMS) (Schröder et al., 2008).

A mutação de nucleotídeos presentes em regiões codificantes do genoma pode levar à alteração da sequência de aminoácidos de uma proteína promovendo aumento, diminuição ou a inativação da atividade da mesma (Ennis, 2001).

O uso separado ou combinado da radiação UV e de agentes químicos tem sido eficiente na geração de cepas industriais e mutantes hipercelulolíticos de *Trichoderma* sp., *Aspergillus* sp. e *Penicillium* sp., por exemplo (Singh et al., 2017).

Todas as cepas industriais de *T. reesei* empregadas atualmente na indústria foram obtidas a partir de mutagênese aleatória da cepa parental *T. reesei* QM6a. Dentre elas, podem ser destacadas as cepas QM9414 e RUT-C30 (Peterson et al., 2012).

A cepa QM9414 foi o primeiro mutante originado a partir de *T. reesei* QM6a por meio da irradiação dos conídios do fungo. Este mutante produziu de 2 a 4 vezes mais celulase do que a cepa parental (Mandels et al., 1971). Entretanto, o processo para a obtenção da linhagem industrial *T. reesei* RUT-C30 foi realizado em três etapas com a obtenção das cepas intermediarias M7 e NG14. Primeiramente, a cepa parental T. *reesei* QM6a foi submetida à radiação UV seguida de uma triagem de linhagens com capacidades incrementadas de produção enzimática em relação à parental. Esse processo gerou a cepa M7, a qual tinha como característica o truncamento no fator de transcrição cre1 responsável pela repressão catabólica. Desta forma, o mutante produzia celulases mesmo em presença de glicose. Posteriormente, a cepa M7 foi submetida a mutagênese química utilizando N-nitroguanidina, o que proporcionou o isolamento da cepa NG14, a qual produziu o dobro de proteínas extracelulares e cinco vezes mais atividade em papel de filtro e duas vezes mais atividade de β-glucosidases e endoglucanases comparada a QM9414. A cepa NG14 foi exposta à luz UV e uma triagem foi realizada levando em consideração a atividade de celulases e a não repressão catabólica para a produção das mesmas, o que resultou na obtenção da cepa RUT-C30. Em relação a QM6a, RUT-C30 possui características que incluem o truncamento do cre1, 30 vezes mais atividade de endoglucanases e 3 vezes mais atividade de β-glicosidases e um potencial celulolítico de 15 a 2 vezes maior. Além disso, apresenta maiores taxas de crescimento de micélio e um retículo endoplasmático 6 vezes maior que a cepa parental (Montenecourt & Eveleigh, 1977a; Montenecourt and Eveleigh, 1977b; Montenecourt and Eveleigh, 1979; Peterson et al., 2012).

Estudos conduzidos por Jiang et al. (2011) demonstraram sucesso na obtenção de um mutante denominado EU2-77 a partir da cepa parental *Trichoderma viride* NP13a por meio de duas rodadas de mutagênese com o químico EMS e radiação UV. EU2-77 produziu 1,67 vezes mais proteína, 2,49 vezes mais atividades em filtro de papel, 2,16 mais atividade de β-glucosidase e 2,61 vezes mais atividade de endoglucanases (CMCase) comparado ao selvagem. A comparação da atividade hidrolítica em papel de jornal entre cepa parental, EU1-77 e *T. reesei* RUT-C30 e enzimas comerciais mostrou um melhor desempenho da cepa EU1-77 em relação à *T. reesei* RUT-C30 e resultados equivalentes aos mostrados em experimentos aplicando-se enzimas comerciais. Adicionalmente, devido à alta produção de β-glucosidase pela linhagem EU1-77 mutante, a suplementação com a mesma enzima tornou-se desnecessária para a hidrólise de papel de jornal.

A exposição de uma cepa de *Aspergillus* sp. a duas rodadas de tratamentos com radiação gama ($Co^{60}$), uma com UV , e quatro com NTG resultou no mutante XTG-4 com 2,03 mais atividade em CMC, 3,20 vezes mais FPase e 1,80 vezes mais atividade de β-glucosidase

comparada à cepa parental (Vu et al., 2009). Em um outro estudo, a exposição à luz UV durante 45 minutos, resultou em um mutante de *A. niger* com atividade três vezes maior em papel filtro (FPase) e duas vezes maior em carboximetilcelulose (CMC) comparado a cepa parental (Irfan et al., 2011).

A metodologia para a obtenção da cepa industrial de *P. oxalicum* JU-A10-T incluiu rodadas de exposição da linhagem parental *P. oxalium* 114-2 à luz UV. O mutante obtido produz 9 vezes mais atividade em papel filtro (FPase), 8 vezes mais atividade de xilanase e secreta 4 vezes mais proteínas comparado à cepa selvagem quando cultivados em farelo de trigo (Liu et al., 2013). Estudos realizados por Adsul et al. (2007) mostraram a obtenção de mutantes hipercelulolíticos pela exposição de *P. janthinellum* NCIM 1171 ao químico EMS e luz UV. A seleção de mutantes foi baseada na atividade em avicel, e o melhor mutante demonstrou 2 vezes mais atividade de FPase e CMCase que a cepa selvagem.

Desta forma, estratégias de mutagênese aleatória têm sido descritas como metodologias eficientes para a obtenção de cepas de fungos filamentosos com incremento na atividade enzimática, principalmente celulases e hemicelulases.

## 3. Capítulo 1

# Energy cane: an alternative substrate for production of (hemi)cellulases by *Penicillium oxalicum*

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## Abstract

Energy cane is a potential source for green chemicals production by 2G technologies which could be employed to replace expensive feedstocks in integrated enzyme production systems. The aim of this work was to discuss the significant variables for production of CAZymes by *P. oxalicum* using energy cane as feedstock. The concentration of nitrogen source (peptone) and agitation had significant effect in filter paper activity (FPase, U/mL) and the maximal activity was reached at peptone 0.72 g/L and 180 rpm condition. The partial or total replacement of peptone by soybean meal or soybean hulls, respectively, did not affect FPAse titters, but reduced the costs of culture medium regarding nitrogen source addition. Beyond bench scale, the best trial attained through statistic strategies was also evaluated in 1L fermenter-scale. Energy cane and soybean hulls supported the growth and secretion of CAZymes by *P. oxalicum* expanding the spectra of biomasses for production of 2G green chemicals.

**Key words:** *Penicillium oxalicum*, energy cane, soybean hulls, CAZymes

## 3.1. Introduction

In recent years, the production of green chemicals by the second generation (2G) technology has gained extensive attention in the global energy scenario given the mitigation they promote in the carbon dioxide ($CO_2$) release into the atmosphere [1].

Sugarcane bagasse and straw are the main feedstocks used for the production of 2G ethanol in Brazil [2]. However, energy cane, a specialized sugarcane hybrid (*Saccharum spontaneum* x *Saccharum officinarum*), had emerged as a potential biomass for biofuels production [3, 4] given its E high content of fibers compared to sugarcane, making it a biomass designed for 2G technology. [5]. It is estimated that 4-fold more ethanol could be obtained from energy cane compared to the same planted area of sugarcane [6].

The enzymatic conversion of cell wall polysaccharides into fermentable sugars is one of the main bottlenecks to the development of 2G ethanol industry, responding for around 15% of the production costs [7]. One alternative is the adoption of the "On-Site Manufacture (OSM)" system where enzymes are produced adjacent to the industrial plants avoiding expenses with transport and stabilization [8, 9, 10].

The OSM system includes the "Integrated Process", where part of the pretreated lignocellulosic biomass is destined to the growth of microorganisms/enzyme production [11]. This is an outstanding alternative given that the use of complex substrates allows the production of customized enzyme cocktails designed according to the feedstock employed in the industrial plant [9]. In this regard, the adoption of a robust strain for enzyme production and its optimization becomes urgent, especially concerning the carbon/nitrogen sources balance, temperature, pH, incubation time, agitation and inoculum density [12, 13].

Several works have revealed the genomic potential of *Penicillium* strains by proteomic analysis, suggesting it as an alternative to obtain enzyme cocktails aiming the release of fermentable sugars from biomass [14, 15]. For example, *Penicillium* strains grown on sugarcane bagasse secretes several glycoside hydrolases (GHs) such as exo- and endoglucanases (GH5, 6, 7, 12, 17), β-glucosidases (GH3), xylanases (GH10 and 11), other hemicellulases (GH5, 43 and 62), pectinases (GH28) and lytic polysaccharide monooxygenases (AA9) [16, 17].

This work is the first to discuss the significant variables for production of (hemi)cellulases by *P. oxalicum* using energy cane as substrate, expanding the spectrum of feedstocks for production of CAZymes by filamentous fungi.

## 3.2. Material and Methods

### Microorganism

Conidia of *P. oxalicum* (CCT2930, Coleção de Culturas Tropical André Tosello, Campinas - SP, Brazil) were maintained in glycerol 20 % at -80 $^{o}$C. The cultures were grown on Potato Dextrose Agar (PDA) medium supplemented with (g/L): peptone, 2; yeast extract, 2, for seven days at 28 °C.

### Biomass

Steam-exploded energy cane (% w/w): glucan, 51.78 $\pm$ 1.09; xylan, 10.46 $\pm$ 0.85; insoluble lignin, 30.86 $\pm$ 1.22 and soluble lignin, 2.63 $\pm$ 0.07; was kindly provided by GranBio Celere, Campinas, São Paulo, Brazil.

### Bench-scale culture conditions

After growth in PDA, a *P. oxalicum* spores solution (final concentration of $10^7$ spores/mL) was prepared in sterile Tween 80 0.01% and inoculated into a preculture containing 10 g/L glucose and 5 g/L energy cane as the carbon sources. The samples were incubated for three days at 28 °C in an orbital shaker operating at 200 rpm. After growth, 5 mL of the preculture was transferred to 45 mL of fresh Mandels and Weber medium [18] with the same composition of preculture, except glucose (g/L): urea, 0.3; $KH_2PO_4$, 2; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; CaCl2, 0.4; Tween 80, 0.2; peptone, 1; energy cane, pH 5.0. All culture medium were autoclaved at 121 °C for 20 minutes. Trace elements were filtered and added to the medium after autoclave to a final concentration of (g/L): $Fe_2SO_4.7\ H_2O$, 0.5; $MnSO_4.H_2O$, 0.2; $ZnSO_4.7\ H_2O$, 0.2 and $CoCl_2.\ 6\ H_2O$, 2. The concentration of energy cane varied with the experiment. All experiments were carried out in triplicate.

The variables of culture media evaluated by Experimental Design were the concentration of carbon source, nitrogen source, trace elements and spores. Agitation (rpm) and pH were also evaluated.

After rounds of Experimental Design, peptone was fully replaced by soybean hulls (2 g/L).

### Experimental Design

Design of experiments (DOE) technique was used as a sequential strategy for the selection of variables from the culture medium significant for fermentation and adaptation of

the system to operational parameters. Screening design (Plackett & Burman) was used for variable selection and analysis of the main effects. Central Composite Rotatable Design (CCRD) was performed to define the best conditions, according to each case [19]. The variables effects, regression coefficients and analysis of variance (ANOVA) were obtained by the online software Protimiza Experimental Design avaiable in http: // experimental-design. protimiza.com.br/.

**Enzyme assays**

FPase, CMCase, β-glucosidase and xylanase activities were determined by the 3,5-dinitrosalicylic acid (DNS) method [20]. Reactions were prepared as follows: 50 µL of crude supernatant and filter paper (Whatman number 1, 1.0 x 0.6 cm) in 100 µL of buffer (FPase); 50 µL of crude supernatant and 100 µL of 1% (w/v) carboxymethylcellulose (Sigma Aldrich) diluted in buffer (CMCase); 20 µL of crude supernatant, 80 µL of 0.5% (w/v) xylan from beechwood (Sigma Aldrich) diluted in buffer (xylanase); 20 µL of crude supernatant, 50 µL of 5 mM 4-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma Aldrich) and 30 µL of buffer (β-glucosidase). Sodium acetate 0.05 M, pH 5.0, was adopted as buffer in all reactions.

The reaction mixtures were incubated at 50 °C for 60, 30, 20 and 20 minutes for determination of FPase, CMCase, β-glucosidase and xylanase activities, respectively. Reactions with filter paper, carboxymethylcellulose and xylan were stopped by the addition of DNS reagent. All reactions were heated in boiling water for 5 min and 100 µL of each sample was transferred to a 96-well microplate and the absorbance was measured at 540 nm [21]. Reactions with *p*NPG were stopped with sodium carbonate 1 M and recorded at 405 nm [22].

One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol of products equivalent to glucose, 4-nitrophenol or xylose per minute under the standard assay conditions.

**Protein quantification**

Protein levels were determined according to Bradford [23].

**Fermenter-scale assays**

The experiments were carried out in the water-jacketed tank reactor BioFlo/CelliGen 115 with 3L total volume (New Brunswick) and 1L working volume. *P. oxalicum* was grown in 100 mL of Mandels and Weber medium [18] containing 10 g/L glucose and 5 g/L energy cane as the carbon sources (preculture). The preculture was inoculated in 900 mL of (g/L): urea,

0.3; $KH_2PO_4$, 2; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; CaCl2, 0.4; Tween 80, 0.2; soybean hulls, 2; energy cane, 5, pH 5.0. The broth was stirred at 200 rpm and a controlled flow rate of 0.7 vvm was adopted. The temperature was set to 28 $^oC$ and pH was maintained at 5.0 by the automatic addition of $H_3PO_4$ (2 M) or $NH_4OH$ (1.25 M). Foaming was controlled by the addition of Antifoam 2014 (Sigma Aldrich) at the day of inoculum. Samples were collected from bioreactor for nine days at intervals of 24 h for further analysis (enzymatic activities, protein determination and mycelium morphological assays).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

The secretomes were 5-fold concentrated and loaded into SDS-PAGE gels as described by Laemmli [24].

**Mycelium morphology**

Mycelium morphological data from *P. oxalicum* cultured in energy cane and soybean hulls at fermenter-scale experiments were obtained by immobilization of fresh mycelia into microscope slides with a coverslip. The slides were visualized in microscope Nikon eclipse 90i under 20X objective.

### 3.3. Results

Given the relevance of the integrated process to the development of 2G ethanol industry, the suitability of energy cane to elicit the production of cellulases by *P. oxalicum* was evaluated by statistical methods.

Nine variables [agitation, pH and concentration of energy cane, peptone, spores and trace metals ($FeSO_4.7H_2O$, $MnSO_4.7H_2O$, $ZnSO_4.7H_2O$ and $CoCl_2.6H_2O$)] were screened by Plackett-Burman (PB) design as 16 factorial run experiments and three central points (CPs) totalizing 19 runs. PB design is commonly used to determine which variables are statistically significant and their main effects for the response [19]. The response evaluated throughout this work was enzymatic activity (EA), accessed by total cellulases activity/filter paper assay (FPAse, U/mL). High (+1) and low (-1) conditions adopted for each variable and its respective coded levels are in Table 1.

**Table 1.** Variables in the PB16 design and the respective coded levels

| Variables | Code | -1 | 0 | +1 |
|---|---|---|---|---|
| Iron ($FeSO_4.7H_2O$, mg/L) | **x1** | 0 | 5 | 10 |
| Manganese ($MnSO_4.7H_2O$, mg/L) | **x2** | 0 | 1.6 | 3.8 |
| Zinc ($ZnSO_4.7H_2O$, mg/L) | **x3** | 0 | 1.4 | 2.8 |
| Cobalt ($CoCl_2.6H_2O$, mg/L) | **x4** | 0 | 20 | 40 |
| Agitation (rpm) | **x5** | 0 | 100 | 200 |
| Energy cane (g/L) | **x6** | 5 | 10 | 15 |
| Peptone (g/L) | **x7** | 0.5 | 1 | 1.5 |
| pH | **x8** | 4.5 | 5.0 | 5.5 |
| Spore concentration (sp/mL) | **x9** | $10^5$ | $10^6$ | $10^7$ |

According to PB design, EA varied significantly from 0 to 0.065, suggesting that the variables evaluated have effect on cellulases production by *P. oxalicum.* Agitation is an essential condition for production of cellulases by *P. oxalicum* in the conditions assayed given that no EA was found when this variable was set to zero (x5=-1, no agitation) (Table 2).

**Table 2.** Trials of PB16 in coded levels for EA

| Trials | $x_1$ | $x_2$ | $x_3$ | $x_4$ | $x_5$ | $x_6$ | $x_7$ | $x_8$ | $x_9$ | EA (FPAse, U/mL) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | -1 | -1 | -1 | 1 | -1 | -1 | 1 | 1 | 0.027 |
| 2 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | -1 | 1 | 0 |
| 3 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | -1 | 0 |
| 4 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 0 |
| 5 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 0.032 |
| 6 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | 0.035 |
| 7 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 0.059 |
| 8 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | 0.053 |
| 9 | 1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 0 |
| 10 | -1 | 1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 0.035 |
| 11 | -1 | -1 | 1 | 1 | -1 | 1 | -1 | 1 | 1 | 0 |
| 12 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | -1 | 1 | 0.049 |
| 13 | -1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | -1 | 0.035 |
| 14 | -1 | -1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 0 |
| 15 | -1 | -1 | -1 | 1 | -1 | -1 | 1 | 1 | -1 | 0 |
| 16 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 |
| 17(CP*) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.058 |
| 18(CP*) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.056 |
| 19(CP*) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.065 |

*Central points.

Data from Table 2 were used to calculate the main effects of independent variables and their impacts in EA (Table 3). Agitation ($p$-value < 0.001) and peptone concentration ($p$-value= 0.045) showed significant effect in EA taking a confidence level of 90% ($p$-value <0.10) into consideration. The Pareto chart (Additional file 1) obtained from Table 3 categorizes the variables indicating which one has the largest or smallest effect on the response. Agitation is the most pronounced variable that affects EA followed by peptone. The other variables were not significant for EA.

**Table 3.** Effect of variables on EA

| | EA (U/mL) | | | | |
|---|---|---|---|---|---|
| **Variables** | **Effect** | **Std error** | ***T*-value** | ***p*-value** | **Confidence (%)** |
| Average | 0.0203 | 0.0018 | 11.5405 | 0.0000 | |
| Curve | 0.0787 | 0.0089 | 8.8845 | 0.0000 | |
| Iron ($x_1$) | 0.0004 | 0.0035 | 0.1065 | 0.9178 | 8.2200 |
| Manganese ($x_2$) | -0.0004 | 0.0035 | -0.1065 | 0.9178 | 8.2200 |
| Zinc ($x_3$) | -0.0019 | 0.0035 | -0.5326 | 0.6088 | 39.1200 |
| Cobalt ($x_4$) | 0.0031 | 0.0035 | 0.8877 | 0.4006 | 59.9400 |
| Agitation ($x_5$) | 0.0406 | 0.0035 | 11.5405 | 0.0000* | 100.0000 |
| Carbon source ($x_6$) | 0.0049 | 0.0035 | 1.3849 | 0.2035 | 79.6500 |
| Peptone ($x_7$) | 0.0084 | 0.0035 | 2.3791 | 0.0446* | 95.5400 |
| pH ($x_8$) | -0.0031 | 0.0035 | -0.8877 | 0.4006 | 59.9400 |
| Spore concentration ($x_9$) | -0.0049 | 0.0035 | -1.3849 | 0.2035 | 79.6500 |

*$p$ < 0.10; Std, standard

From PB data, it was possible to establish variables at their lower levels, for example, pH and concentration of metals, energy cane and spores; because they were not statistically significant for EA. Therefore, iron/manganese/zinc/cobalt were removed from culture medium, probably due to its preexistence in energy cane; the amount of energy cane and spores were reduced to 5 g/L and $10^5$ sp/mL, respectively, and pH was maintained at 5.0 in order to avoid the addition of high volumes of sodium hydroxide (NaOH) to correct the medium initial pH (4.8) to 5.0.

Given that only agitation and peptone significantly affect the production of cellulases by *P. oxalicum*, the best levels of both variables were evaluated by a two levels $2^k$ central composite rotatable design (CCRD) for 2 factors (k = 2), with 4 factorial points, 4 axial points and 4 central points [19]. Twelve combinations of runs (Table 4) were carried out for further optimization of these variables in order to attain the maximal response. The superior levels (+)

for peptone (x1, 2.0 g/L) and agitation (x2, 200 rpm) were chosen based on ranges commonly adopted for filamentous fungi processes.

**Table 4.** Matrix of CCRD and EA response

| Trials | x1 (Agitation, rpm) | x2 (Peptone, g/L) | EA (FPase, U/mL) |
|---|---|---|---|
| 1 | -1 (72) | -1 (0.72) | 0.058 |
| 2 | +1 (178) | -1 (0.72) | 0.075 |
| 3 | -1 (72) | +1 (1.78) | 0.066 |
| 4 | +1 (178) | +1 (1.78) | 0.068 |
| 5 | -1.41 (50) | 0 (1.25) | 0.044 |
| 6 | +1.41 (200) | 0 (1.25) | 0.058 |
| 7 | 0 (125) | -1.41 (0.5) | 0.066 |
| 8 | 0 (125) | +1.41 (2.0) | 0.083 |
| 9 | 0 (125) | 0 (1.25) | 0.079 |
| 10 | 0 (125) | 0 (1.25) | 0.052 |
| 11 | 0 (125) | 0 (1.25) | 0.069 |
| 12 | 0 (125) | 0 (1.25) | 0.064 |

Real levels are in parenthesis

Statistical analysis revealed that the linear ($x_1$, $x_2$) and quadratic ($x_1^2$, $x_2^2$) coefficients of agitation and peptone, as well as their interactions ($x_1 \cdot x_2$), are not significant for EA at a confidence level of 90% ($p \leq 0.1$) (Additional file 2). The analysis of variance (ANOVA) (Additional file 3), used to estimate the statistical parameters, confirms that there is not a satisfactory model adjusted to the EA results since the calculated *F*-value (2.21) is lower than the tabulated one (4.39). The coefficient of variation ($R^2$ 0.65) also indicates a low correlation between the experimentally observed and the predicted values. So, it was not possible to obtain a response surface.

After CCRD, an increase of nearly 30% in FPAse was recorded (0.075 U/mL) compared to the CPs of PB design (0.059 U/mL). Although obtaining a well-fitted model and response surface were not possible, it was feasible to establish a cheaper condition for production of cellulases by *P. oxalicum* using energy cane as the sole carbon source given the reduction of peptone concentration (1.0 to 0.7 g/L). Then, the trial "2" (0.7 g/L peptone, Table 4) was adopted for further experiments.

In another round of experiments, the total replacement of peptone by lower cost nitrogen sources such as soybean meal (SM) and corn steep liquor (CSL) resulted in a statistically

significant decrease in FPAse (Table 5A). Then, a combination of peptone:SM (%, 75:25, 50:50 and 25:75) was evaluated (Table 5B).

Table 5B shows that the partial replacement of peptone by SM did not affect FPase. Alternatively, the addition of soybean hulls (SH, 2.0 g/L), source of carbon and nitrogen, led to the same FPAse as P25:SM75 (0.7 g/L) and the total replacement of peptone (Table 5C), resulting in a culture medium with reduced costs.

**Table 5.** Replacement of peptone by lower-cost sources of nitrogen (A/B) and carbon/nitrogen (C)

| **A** | |
|---|---|
| **Nitrogen Sources** | **EA (FPAse, %)** |
| Peptone (P) | 100*[a] |
| Soybean meal (SM) | 82.5 ± 11.3[b] |
| Corn steep liquor (CSL) | 66 ± 4.1[c] |
| **B** | |
| **(%:%)** | **EA (FPAse, %)** |
| P (100) | 100**[a] |
| P:SM (75:25) | 90.5 ± 11.9[a] |
| P:SM (50:50) | 92.9 ± 14.3[a] |
| P:SM (25:75) | 89.3 ± 13.1[a] |
| **C** | |
| **New Carbon/Nitrogen Source** | **EA (FPAse, %)** |
| P:SM (25:75) | 100***[a] |
| Soybean hulls (SH) | 92.4 ± 8.9[a] |

A, nitrogen sources (0.7 g/L) were evaluated. *****100% of EA was considered as 0.097 U/mL; B, mixture (%:%) of peptone and soybean meal (total of 0.7 g/L). ** 100% of EA was considered as 0.084 U/mL; C, Soybean hulls replaced P:SM (25:75). *** 100% of EA was considered as 0.079 U/mL. All the experiments were carried out with 5 g/L energy cane (EC) as carbon source. Treatments followed by the same letter do not differ at 5% probability by Tukey test.

Beyond bench scale, the activity of hydrolases produced by *P. oxalicum* grown in Mandels medium without metals, 5 g/L energy cane, 2 g/L soybean hulls, at 178 rpm/50 $^{o}$C and $10^6$ spores/mL was evaluated in 1L-fermenter for nine days.

*P. oxalicum* grows (Figure 1A) and secretes proteins (Figure 1B) under the conditions assayed. The protein concentration ranged from 0.033 to 0.204 mg/mL from the 1st to the 9th day (Figure 1C). At the 3rd day, FPase reached 0.16 U/mL, 20% higher than that observed for bench scale in the same condition and remained almost unaltered between the 4th and 9th day

of fermentation (Figure 2A). The CMCase, β-glucosidase and xylanase activities were time-dependent.

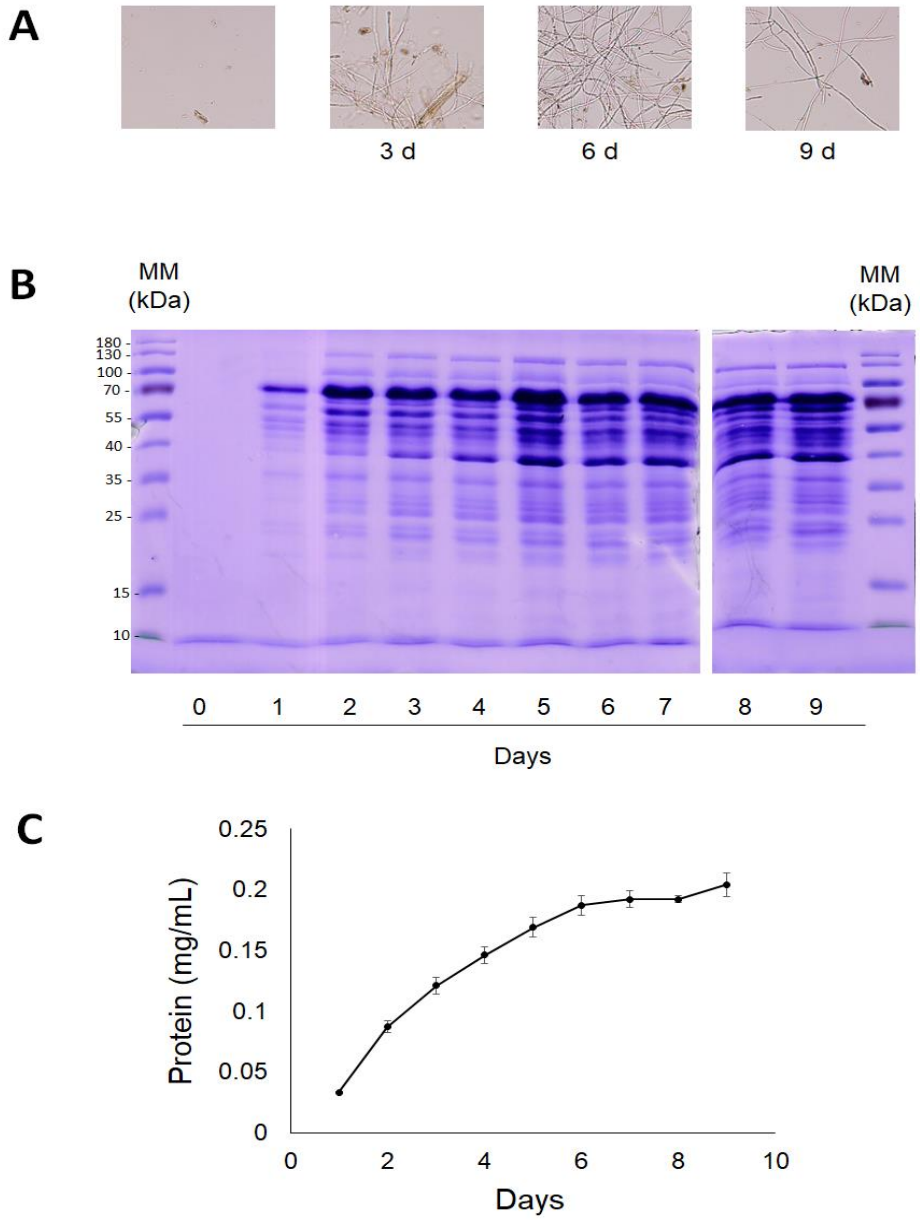


**Figure 1.** Growth (A), profile (B) and concentration of proteins secreted by *P. oxalicum* (C) in energy cane (5g/L) and soybean hulls (2 g/L) for nine days. MM, molecular marker Thermo Scientific.

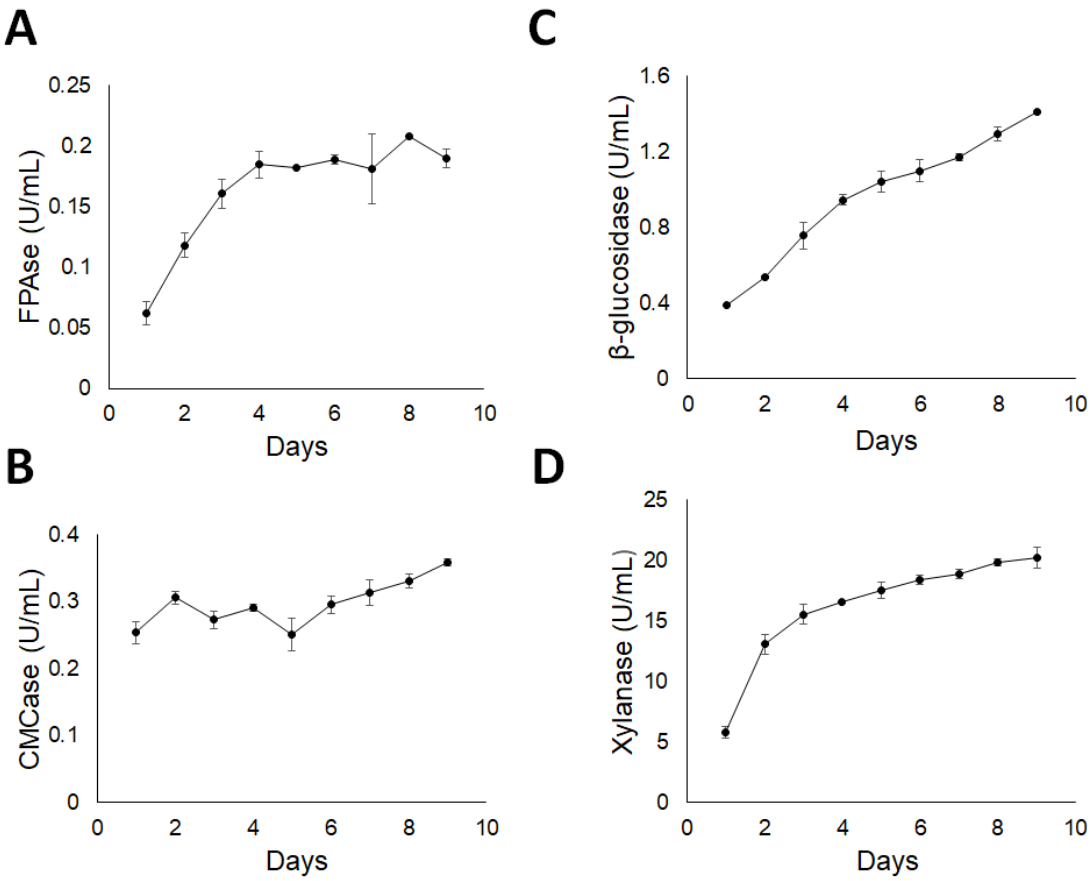


**Figure 2.** Activity of cellulases (FPAse [A], CMCase [B] and β-glucosidase [C]) and hemicellulases (xylanases [D]) secreted by *P. oxalicum* (B) grown on energy cane (5g/L) and soybean hulls (2 g/L) during nine days. Error bars are the standard deviation of three measurements.

### 3.4 Discussion

Several works highlight the potential of energy cane as starting point to obtain biorenewable materials [4]. However, researches using this biomass as inducer for carbohydrate-active enzymes (CAZymes) production by filamentous fungi are still scarce [25], being a profitable field of study.

*P. oxalicum* secretes cellulases and hemicellulases such as GH1, GH3, GH6, GH7, GH10, GH11 and CE5 when grown in energy cane as the sole carbon source (Corrêa et al., to be submitted). In this work, the composition of the culture medium and the significative variables affecting the production of hydrolases by *P. oxalicum* was evaluated by DOE statistical methods such as PB16 and CCDR.

The PB16 design pointed out agitation (rpm) and peptone concentration as statistically significant for production of cellulases by *P. oxalicum* from energy cane (Table 3). Agitation is

an essential condition for *P. oxalicum* development since no growth was observed when this parameter was set to zero (data not shown). Agitation is important for submerged cultures homogeneity, mass transfer and aeration, especially for filamentous fungi growth in lignocellulosic biomass. For example, the production of cellulases, tannases and pectinases by *Trichoderma reesei*, *Aspergillus niger* FETL FT3 and *Aspergillus niger* HFD5A-1, respectively, increases in shaken cultures compared to the static ones [26, 27, 28]. Agitation could affect the production/secretion of proteins by filamentous fungi given the mycelium morphology, the increase in fungal biomass and the up and down regulation of cellulases and cellulases-repressing coding genes [29].

Regarding peptone, it is well known that organic sources of nitrogen promote the production of enzymes by microorganisms [30, 31]. However, the excessive addition of this component leads to significant expenses in the process.

In this work, the significant effect of curvature suggests that the range of variables studied may include inflection points for the response [19]. Also, the low regression coefficient ($R^2$ 0.65) point out that the levels of agitation and peptone concentration adopted for CCRD did not cover a sufficient range to detect significative variation in FPase. The model was not predictable and obtain a response surface was not possible. However, PB and CCRD promoted an increase in FPase (0.041 U/mL [before PB] to 0.079 U/mL [after CCRD]) (Figure 3A) and, concomitantly, the decrease of peptone (1 mg/L to 0.7 mg/mL) and other variables concentration. Then, the trial "2" (CCRD) was adopted for further experiments.

Considering the costs of peptone as U$ 54.78/100g (Sigma Aldrich), the statistical design promoted a reduction of 30% regarding the addition of this variable to the culture medium (1g/L, U$ 0.54 [M1]; 0.7 g/L, U$ 0.38 [M3]) (Figure 3B). The partial replacement of peptone by SM (25:75, %) did not affect the FPase titers, but resulted in a reduction of 70% on culture medium costs (0.7 g/L, U$ 0.38 [M3]; U$ 0,09 [M4]) taking the price of SM as U$ 0.032/100g into consideration [32]. Interessantly, the total replacement of peptone by SH reduced the costs in almost 100% (U$ 0,09 [M4] to U$ 0.0002 [M5]). The price of SH was considered as U$ 0.1/100g [10]. All analyses were done taking the volume of culture medium as 1L into consideration.

SM and SH are low-cost nitrogen and carbon/nitrogen sources, respectively, obtained from soybean processing into oil and other products. They are usually added to animal feed, mainly for reproducing pigs and cattle in order to meet the nitrogen requirements for nutrition [33, 34]. SM is composed mainly by proteins (48.8%), fibers (4.5%) and oil (1.6%) [34, 35]. SH contains cellulose (29-51%), hemicellulose (10-20%), lignin (1-4%), pectin (6-15%),

proteins (9-14%) and ash (1-4%) [36, 37, 38, 39]. The partial or total replacement of peptone by SM and SH, respectively, did not altered the cellulases activity (Figure 3A, M4 and M5), even SH being also a carbon source. Then, it was hypothesized that the protein content of SM and SH were enough to support the growth and production of CAZymes by *P. oxalicum* in the conditions assayed.

Several biomasses, for example, pure cellulose (Avicel®, bacterial microcrystalline cellulose, carboxymethylcellulose), wheat bran, corn stover/straw, elephant grass, saw dust and sugarcane bagasse have been employed as substrates for CAZymes production by filamentous fungi [15, 41, 42].

The maximum FPAse activity obtained in this work was compared with those described in several reports of cellulase production by fungi using different carbon sources (Table 6). Microcrystalline cellulose is commonly used as the inducer for cellulase production by *P. oxalicum*, *Aspergillus terreus*, *Aspergillus sydowii* and *Trichoderma viride* [43]. As synthetic substrates increase the costs for enzyme's production, lignocellulosic biomass emerges as an alternative inducer. For example, using rice bran and wheat bran, FPAse of *Aspergillus niger* reached almost 3 U/mL [44]. The FPAse activity of supernatants of *P. oxalicum* grown in energy cane and soybean hulls was about 1.2-fold higher than that reported for strains of *Trichoderma sp.* grown in cellulose and glycerol [45], and 6-fold higher than that presented by *Aspergillus terreus* in CMC [46]. In addition, the use of energy cane and soybean hulls showed cellulase activity of 3-13 times higher, compared to the use of steam-explosion-pretreated sugarcane bagasse and glucose as carbon sources for strains of *Trichoderma* sp. [47].

Data from Table 6 reveal the ability of *P. oxalicum* to grow and produce enzymes in the absence of a readily assimilable inducer and severe conditions as a recalcitrant carbon source and low concentration of nitrogen.

**Table 6.** Reported cellulases activities (FPAse) obtained with different microorganisms, medium composition, time and strategy of Fermenter- scale (FS) or bench scale (BS)

| Microorganisms | Medium composition | Time (h) | Strategy | FPAse (U/mL) | Reference |
|---|---|---|---|---|---|
| ***P. oxalicum*** | Steam-exploded energy cane + soybean hulls | 72 | FS | 0.16 | This work |
| ***P. echinulatum*** | Cellulose *Celluflok E* + sucrose + soybean meal + wheat bran | 96 | BS | 1.5 | dos Reis et al (2015) |
| ***P. oxalicum*** | Avicel + wheat bran | 120 | FS | 1.3 | Saini et al (2015) |
| ***Penicillium sp.*** | Cellulose + lactose + saw dust | 168 | BS | 8.7 | Prasanna et al (2016) |
| ***T. reesei* RUT-C30** | Steam-explosion-pretreated sugarcane bagasse + glucose | 72 | BS | 0.037 | Florencio et al (2015) |
| ***T. harzianum*** | Steam-explosion-pretreated sugarcane bagasse + glucose | 72 | BS | 0.051 | Florencio et al (2015) |
| ***Trichoderma* sp.** | Steam-explosion-pretreated sugarcane bagasse + glucose | 72 | BS | 0.0125 | Florencio et al (2015) |
| ***T. longibrachiatum*** | Sugarcane bagasse + lactose | 168 | BS | 0.48 | Pachauri et al (2016) |
| ***T. harzianum*** | Raw sanitary wastewater + Avicel | 144 | FS | 1.4 | Libardi et al (2017) |
| ***Trichoderma CMIAT 054*** | Cellulose + glycerol | 168 | BS | 0.138 | Sousa et al (2018) |
| ***Trichoderma CMIAT 041*** | Cellulose + glycerol | 168 | BS | 0.0893 | Sousa et al (2018) |
| ***A. sydowii*** | Walseth celulose | 144 | BS | 1.33 | Matkar et al (2013) |
| ***A. niger*** | Rice bran + wheat bran | 168 | BS | 2.98 | Reddy et al (2015) |
| ***A. terreus*** | Carboxymethyl celulose | 96 | BS | 0.0265 | Gunny et al (2015) |

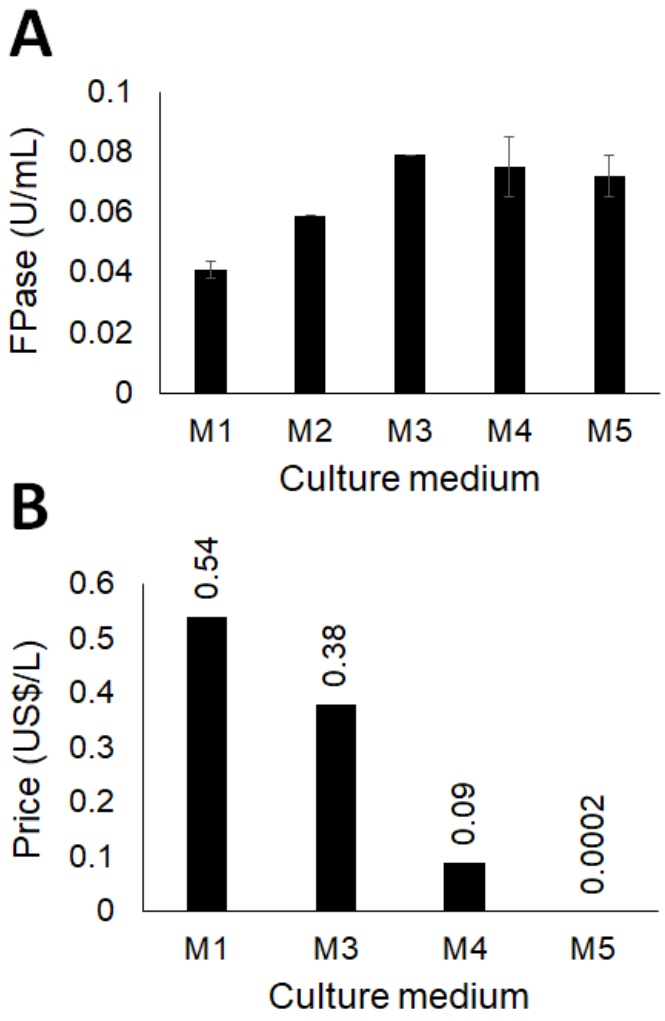


**Figure 3.** FPase (U/mL) in all treatments (A) and costs of alternative sources of nitrogen (soybean meal) and carbon/nitrogen (soybean hulls) per liter of culture. M1: culture medium before statistical methods (peptone 1g/L); M2: best hit PB16; M3: best hit CCRD (peptone 0.7 g/L); M4: partial replacement of peptone by soybean meal (%, 25:75, respectively); M5, total replacement of peptone by soybean hulls.

### 3.5 Conclusion

This is the first work highlighting the significant parameters (agitation and peptone concentration) for production of CAZymes by filamentous fungi using energy cane as feedstock. The FPase did not obey a model in the conditions evaluated by PB and CCRD in this work. However, the total replacement of peptone by soybean hulls, a carbon/nitrogen source, was possible reducing the culture medium costs in almost 100% regarding the addition of

nitrogen source. Then, this work expands the spectrum of feedstocks to produce green chemicals by 2G technology.

### 3.6 Acknowledgements

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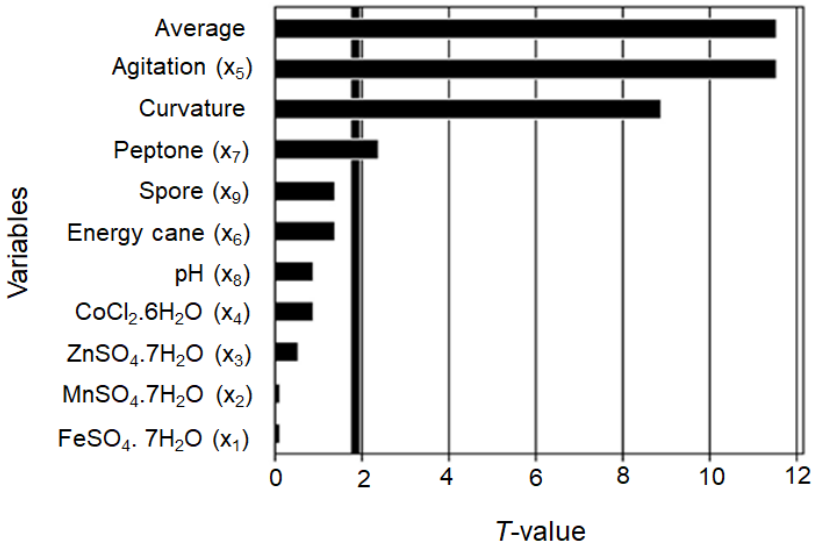
### Conflict of interests

The authors declare no conflict of interests.

### 3.7. Additional files




**Additional File 1.** Pareto chart from Plackett-Burman 16. Agitation is the most pronounced variable affecting enzymatic activity followed by peptone concentration in the conditions assayed. The other variables were not significant for enzymatic activity.

**Additional File 2.** Regression coefficients of the response EA for CCRD

| | EA (FPase, U/mL) | | | |
|---|---|---|---|---|
| **Factors** | **Regression coefficients** | **Std error** | ***T*-value** | ***p*-value** |
| Mean | 0.066 | 0.004 | 14.813 | < 0.001 |
| $x_1$ | 0.005 | 0.003 | 1.539 | 0.175 |
| $x_1^2$ | -0.006 | 0.004 | -1.845 | 0.115 |
| $x_2$ | 0.003 | 0.003 | 0.994 | 0.359 |
| $x_2^2$ | 0.005 | 0.004 | 1.490 | 0.187 |
| $x_1 \cdot x_2$ | -0.004 | 0.004 | -0.842 | 0.432 |

*$p$ < 0.10; Std, standard

**Additional File 3.** Analysis of variance (ANOVA) for CCRD

| **Source of variation** | **Sum of squares** | **Degrees of freedom** | **Mean square** | ***F*-value** | ***p*-value** | **$R^2$** |
|---|---|---|---|---|---|---|
| Regression | 0.000879222 | 5 | 0.000175844 | 2.214 | 0.180 | 0.649 |
| Residue | 0.000476444 | 6 | 7.94074E-05 | | | |
| Total | 0.001355667 | 11 | | | | |

Table *F*-value (based on degrees of freedom): 4.39

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## 4. Capítulo 2

### Deconstruction of lignocellulosic biomass by Penicillium oxalicum strains

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### Abstract

*Penicillium oxalicum* is a filamentous fungus reported as a potential alternative to produce lignocellulosic enzymes for biomass conversion into renewable compounds. In this work, the strain EMS19, with an increase of 2 and 0.5-fold on FPase activity and protein secretion, respectively, was obtained after two rounds of random mutagenesis with UV light (UV) and ethyl methylsulfonate (EMS). EMS19 also showed improved activities of beta-glucanases and xylanases compared to the parental strain. Proteomic assays revealed that the families GH20, 35, 75 and GH1, 6, 43, 45, 53, 62, AA9, CE1, PL1 were exclusively secreted by the wild-type (WT) and EMS19 strains, respectively, in glucose and avicel: xylan. An improved number of CAZymes from families GH3, 28, 30, 72 and PL3 (glucose condition) and GH3, 5, 7,10, 11, 12, 13, 16, 27, 28, 30 and 72 (avicel:xylan condition) were found in secretomes of EMS19 compared to WT. These results shed light on the deconstruction of lignocellulosic biomass by *P. oxalicum* pointing out new directions to obtain strains with desirable properties.

**Keywords:** *Penicillium oxalicum*, (hemi)cellulases, random mutagenesis, ultraviolet light, ethyl methanesulfonate

## 4.1. Introduction

The lignocellulosic biomass comprised mainly by cellulose (40-50%), hemicellulose (20-40%) and lignin (20-30%) is an abundant renewable energy source (Zhang et al., 2004; Horn et al., 2012). Due to its recalcitrance, an arsenal of enzymes is required to convert its polymers into sugars, which could be fermented by microorganisms (Sajith et al., 2016). However, the high-cost of the enzymes is one of the main bottlenecks to make the second-generation (2G) technology feasible (Klein-Marcuschamer et al., 2012; Mohanram, et al., 2013; Jaramillo et al., 2015).

Cellulases, hemicellulases, lignin-active enzymes and auxiliary activities (AAs) are recruited during the deconstruction of biomass (Obeng et al., 2017). The bioconversion of cellulose into glucose relies on the activities of endo-β-1,4-glucanases (EGs, E.C. 3.1.2.4), cellobiohydrolases (CBHs, E.C. 3.2.1.91) and β-glucosidases (BGs, EC 3.2.1.21). Recently, the importance of AAs or Lytic Polysaccharide Monooxygenases (LPMOs) was also recognized (Levasseur, 2013). LPMOs generate oxidized ends in the cellulose chain which are recognized by classical cellulases leading to an increase in sugar release (Horn et al., 2012; Levasseur et al., 2013).

Given the complexity and heterogeneity of the hemicellulosic fraction, a plethora of enzymes, such as xylanases, xylosidases, arabinofuranosidases, glucuronidases, mannanases, mannosidases, galactosidases and esterases are required. Some of these enzymes act in the main chain polysaccharides while others in the removal of decorations such as the acetil groups, feruloyl and glucoronoyl moieties (Polizeli et al., 2005; Benoit et al., 2008).

Lignin, a complex aromatic polymer composed by phenylpropane units linked by carbon and ether bonds recruits oxi-reductive enzymes for its deconstruction such as laccases (E.C. 1.10.3.3), lignin-peroxidases (E.C. 1.11.1.14), manganese peroxidases (E.C. 1.11.1.13) and versatile peroxidases (E.C. 1.11.1.16) (Baldrian et al., 2006; Souza et al, 2013).

Lignocellulolytic enzymes are secreted by bacteria and mainly by filamentous fungi such as of the genus *Trichoderma*, *Penicillium* and *Aspergillus* (Lynd et al., 2002; Gomes et al., 2015; Li et al., 2015). A comparative analysis revealed that *Penicillium oxalicum* has a more diversity of genes encoding hemicellulases and carbohydrate binding domains (CBM) than *Trichoderma reesei* and *Aspergillus niger* (Liu et al., 2013a). Moreover, higher beta-glucosidase activities than that found in *T. reesei* have been noticed in many *Penicillium* species (Gusakov et al., 2012). In addition, several works have revealed the genomic potential of *Penicillium* strains by proteomic analysis, suggesting it as an alternative to obtain enzyme

cocktails aiming the release of fermentable sugars from biomass (Liu et l., 2013a; Gong et al., 2015).

Lignocellulolytic enzymes should be produced in high titers to cope the industrial demands (Merino et al., 2007). Then, some strategies that have been adopted by academic and industrial sectors with this purpose including bioprocess improvement or strain development by genetic engineering and/or random mutagenesis (Lynd et al., 2002).

Random mutagenesis, such as those caused by the exposure of the microorganism to physical (ultraviolet light, for example) and sublethal concentrations of chemical agents (nitrosoguanidine (NTG) and ethyl methyl sulfonate (EMS), for example) has been successfully used to improve the activity of cellulases produced by *Penicillium* strains (Qu et al., 1987; Chand et al., 2005; Liu et al., 2013b). One of these mutants, called *P. oxalicum* JU-A10-T was obtained after successive rounds of UV light and NTG treatments on *P. oxalicum* 114–2 and have been applied for production of cellulases in industrial scale in China. *P. oxalicum* JU-A10-T shows high cellulase activity (3.1 FPU/mL) when compared to the wild type *P. oxalicum* 114–2 (0.8 FPU/mL). In addition, *P. oxalicum* JU-A10-T secretes cellulases in culture medium in absence of an inducer while the wild-type did not have this ability (Liu et al., 2013b). According to Liu et al.(2013b), the genetic basis responsible for these effects are mutations in the transcription regulator CreA, down-regulation of AmyR, up-regulation of cellulolytic enzymes and high transcription of genes involved in lysine and cysteine biosynthesis required for the production of lignocellulolytic enzymes. In addition, improvements in FPase and CMCase activities (2-fold) were attained in *Penicillium janthinellum* strains using EMS followed by UV light (Adsul et. al. 2007).

The aim of this work was to obtain *P. oxalicum* strains with improved hemicellulolytic activities by physical (UV light) and chemical (EMS) mutagenesis and compare both strains by enzymatic assays and secretome profiles to elucidated essential CAZymes for biomass deconstruction.

## 4.2. Materials and Methods

### Microorganism

Spores of *P. oxalicum* (CCT2930, Coleção de Culturas Tropical André Tosello, Campinas - SP, Brazil) were maintained in glycerol 20% at -80 $^{o}$C. The cultures were grown in Potato Dextrose Agar (PDA) supplemented with (g/L): peptone, 2; yeast extract, 2, for seven days at 28 °C.

### Culture conditions

A spore solution ($10^7$ spores/mL) of *P. oxalicum* was prepared in sterile Tween 80 0.01% and inoculated in a preculture containing 10 g/L glucose and 10 g/L avicel for mutant's screening or 10 g/L glucose, energy cane, avicel and avicel/ xylan (2:1), according to the final medium, for secretome analysis. The preculture was incubated for three days at 28 °C in an orbital shaker operating at 200 rpm. After growth, 5 mL of the preculture was transferred to 45 mL of fresh Mandels medium (Mandels and Weber, 1969) with the same composition of preculture, except glucose [(g/L): urea, 0.3; $KH_2PO_4$, 2; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; CaCl2, 0.4; Tween 80, 0.2; peptone, 1; carbon source , 10, pH 5.0]. All culture media were autoclaved at 121 °C for 20 minutes. Trace elements were sterile filtered and added to the medium after autoclave to a final concentration of (g/L): $Fe_2SO_4.7\ H_2O$, 0.5; $MnSO_4.H_2O$, 0.2; $ZnSO_4.7\ H_2O$, 0.2 and $CoCl_2.\ 6\ H_2O$, 2.

After six days, the cultures were centrifuged at 12,800*g* for 20 min at 4°C, filtered and the supernatant were used for enzymatic assays.

**Mutagenesis of *P. oxalicum* spores by UV light**

A solution of spores ($10^7$ spores/mL) in Tween 80 0.01% was spread in solid Mandels medium containing 10 g/L avicel as the sole carbon source. The spores were exposed to UV light (15 W, 10 cm from light source) for 3, 7 and 12 minutes. After 18 hours, the emerging colonies were isolated and cultivated in liquid Mandels medium containing 10 g/L avicel® for mutant's screening.

**Mutagenesis of *P. oxalicum* spores with EMS**

A solution of spores ($10^7$ spores/mL) of the best mutant candidate obtained by UV light treatment (strain 3.4) was exposed to EMS (400 mM) during 5, 15, 30, 60 and 180 minutes. After the EMS treatment, the spore's solution was spread in solid Mandels medium containing 10 g/L avicel.After 18 hours, the emerging colonies were isolated and cultivated in liquid Mandels medium containing 10g/L avicel for mutant's screening.

**Screening of the mutants**

The colonies obtained from UV and EMS treatments were grown in liquid Mandels medium containing 10 g/L avicel® as the sole carbon source. The FPAse activity (U/mL) were taken into consideration to select the best strains.

**Monosporic purification**

The mutant strains were submitted to monosporic purification by spreading 100 µl of a diluted spores solution on PDA plates. The isolated colonies were transferred to another PDA plate and incubated at 28°C for 16 hours. The mutant stability was assessed by FPAse activity for twelve months.

**Enzymatic assays**

The enzymatic activities of supernatants on the substrates filter paper (FP), carboxymethylcellulose (CMC), avicel, xylan from beechwood, beta-glucan, lichenan, ray arabinoxylan, xyloglucan, pectin, arabinan from sugar beet, debranched arabinoxylan, chitin, mannan and starch were evaluated by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Reactions were prepared as follows: 50 µL of crude supernatant and filter paper (Whatman number 1, 1.0x0.6 cm) in 100 µL of buffer; 50 µL of crude supernatant and 100 µL of 1% (w/v) of substrate diluted in buffer for CMC and avicel (Sigma Aldrich); 20 µL of crude supernatant, 80 µL of 0.5% (w/v) xylan from beechwood (Sigma Aldrich) diluted in buffer; 20 µL of crude supernatant, 30 µL of buffer and 50 µL of 0.5% (w/v) of substrate for beta-glucan, lichenan, ray arabinoxylan, xyloglucan, pectin, arabinan sugar beet, debranched arabinoxylan, chitin, mannan and starch substrates diluted in buffer. Sodium acetate 0.05 M, pH 5.0, was adopted as buffer in all reactions. All the mixtures were incubated at 50 °C. The reaction time for filter paper, avicel, beta-glucan, ray arabinoxylan, xyloglucan, pectin, arabinan from sugar beet and debranched arabinoxylan was 60 minutes. CMC, xylan from beechwood and lichenan were incubated for 30, 20 and 10 minutes, respectively. The time reaction for chitin, starch and mannan were 17 hours. The reactions were stopped by adding DNS reagent, heated in boiling water for 5 min and 100 µL of each sample was transferred to a 96-well microplate and the absorbance was measured at 540 nm (Camassola and Dillon, 2012).

The activity of supernatants on 4-nitrophenyl-β-D-glucopyranoside (*p*NPG), 4-nitrophenyl β-D-xylopyranoside (*p*NPX), 4-nitrophenyl β-D-cellobioside (*p*NPC), 4-nitrophenyl and α-L-arabinopyranoside (*p*NPA) substrates were accessed using 20 µL of crude supernatant, 50 µL of 5 mM the respective substrate and 30 µL of 0.05 M sodium acetate, pH 5.0. The reactions were incubated at 50 °C for 20 minutes and stopped with sodium carbonate 1 M. The absorbance was recorded at 405 nm (Gallifuoco et al., 1998).

One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol of products equivalent to glucose, 4-nitrophenol or xylose per minute under the standard assay conditions.

**Protein quantification**

Protein levels were quantified according to Bradford (1976).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

The secretomes were 5-fold concentrated and loaded into SDS-PAGE gels as described by Laemmli (1970).

**Secretome analysis**

The supernatants from fungi grown in glucose and avicel: xylan were concentrated using Vivaspin 10KDa protein concentrator spin columns (GE Healthcare Life Sciences) and submitted to denaturation by adding urea 8 M and $NH_4HCO_3$ 50 mM,pH 8.0. The samples were reduced by the addition of DTT 10 mM at 58 °C for 30 minutes and alkylated with iodoacetamide 50 mM in the dark for 30 minutes (Soares et al., 2010). The urea concentration was reduced to 1 M by dilution. A 20-μg sample of trypsin (Promega, modified sequencing grade) (1:30) was added to digest the proteins at 37 °C for 16 hours, generating peptides that were analyzed by nanoLC coupled with an Orbitrap QExactive Plus mass spectrometer (Thermo Fisher Scientific).

The peptide samples (1.8 ug) were separated in a nanoLC system (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) operating in a reverse-phase mode at a flow rate of 450 nL/min using a 90 min gradient of mobile phase A (0.1% formic acid in water) and B (80% acetonitrile and 0.1% formic acid in water). The system was connected to a nano-ESI source coupled to QExactive Plus mass spectrometer (Thermo Fisher Scientific). The spray voltage was set to 1.8 kV with a capillary temperature of 275 °C. Each MS scan was acquired in the orbitrap over a mass range of *m/z* 300-2000 at a resolution of 70 K, maximum injection time of 100 ms, automatic gain control (AGC) target 3E6 and followed by a data-dependent acquisition mode. The most intense signals (top 12) in the mass spectrum were selected for higher-energy collisional dissociation (HCD) applying a stepped normalized collision energy (sNCE) of 30-35. Fragments were detected by Orbitrap at a resolution of 17.5 K, maximum injection time 300 ms and AGC target 5E5.

All data were processed using PatternLab for Proteomics (version 4.1) (Carvalho et al., 2016). The following modifications were considered during database searches: digestion by trypsin with at most two missed cleavages, cysteine carbamidomethylation and methionine oxidation as fixed and variable modifications, respectively. Proteins sharing common peptides

were grouped according to the principle of maximum parsimony, and the relative expression of the proteins was evaluated using the NSAF (Normalized Spectral Abundance Factor) approach.

### 4.3. Results

The filamentous fungus *P. oxalicum* is described as a potential producer of lignocellulolytic enzymes. Among its unique features, the genome of *P. oxalicum* has a most diversified arsenal of genes related to lignocellulose depolymerization than *Aspergillus nidulans*, *A. niger*, *Penicillium chrysogenum* and *T. reesei* (Liu et al., 2013a).

In this work, random mutagenesis using UV light and EMS were applied in *P. oxalicum* wild-type (WT) strain to improve its native (hemi)cellulolytic system.

The first round of mutagenesis was carried out by exposing *P. oxalicum* spores to UV light for 3, 7 and 12 minutes. Nine colonies were obtained and the survival rates (colonies obtained/concentration of spores) were 0.13% (3 minutes), 0.16% (7 minutes) and 0.06% (12 minutes). One lineage, called 3.4 (from 3 minutes treatment), showed the highest FPase activity (50% more than the WT) when grown in Mandels medium containing avicel as the sole carbon source and was chosen for further experiments (Figure 1A).

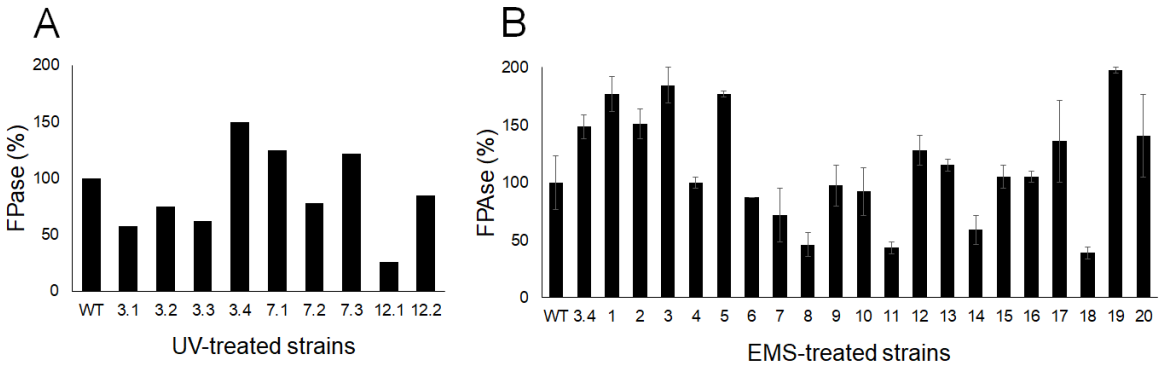


**Figure 1.** Lineages obtained upon treatment of WT spores with UV light (A) and EMS (B). 100% of enzymatic activity was considered as 0.039 U/mL. FPAse assays were performed after 3 days of growth in Mandels medium containing 10 g/L avicel. Lineages 3.4 and 19 present 50 and 100% more FPase activity compared to WT.

The strain UV3.4 was submitted to a second round of mutagenesis with EMS. A total of 20 lineages were obtained after 3 hours of treatment with EMS. Among them, the strain EMS19 was selected given the increase of 50% in FPAse activity compared to UV3.4 (Figure 1B). Concomitantly with the increase in FPase activity, the concentration of secreted proteins by EMS19 reached 74 μg/mL, 50% more than WT (Figure 2A and B).

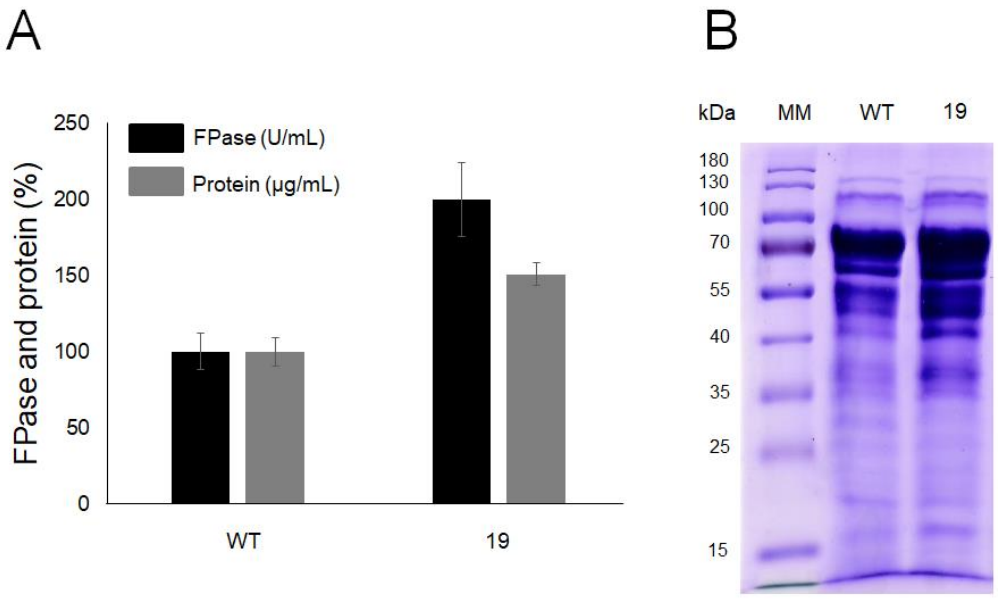


**Figure 2.** Increase in FPAse activity (100%) and protein secretion (50%) of strain EMS19 compared to WT (A). 100% of FPase activity and total protein concentration was considered as 0.042 U/mL and 49 ug/mL, respectively. Secretome profiles of WT and EMS19 after 3 days of growth in Mandels medium containing 10 g/L avicel (B). The samples were concentrated 5x and the same volume was loaded in SDS-PAGE gel. kDa, kilodaltons; MM, molecular marker.

EMS19 was submitted to a monosporic purification and one spore showing the same FPase activity and protein secretion compared to the control (data not shown) was selected for further assays.

The screening assays highlighted above were carried out taking the FPAse activity and secretion of proteins by WT, UV and EMS-treated lineages grown in avicel into consideration. The enzyme activities of secretomes of WT and EMS19 strains grown in glucose, avicel, avicel:xylan (2:1) and energy cane in the following substrates: *p*NPA, *p*NPC, *p*NPG, , *p*NPX, FP , beta-glucan,  (CMC), lichenan, xylan, rye arabinoxylan, xyloglucan, pectin, arabinan and debranched arabinan (Figure 3). Avicel, starch, chitin and mannan were also evaluated as substrates, however, no enzyme activity was detected in those substrates.

Low enzymatic activities were recorded when WT and EMS19 were grown on glucose medium (Figure 3A). When EMS19 was cultured in medium containing avicel, an activity increase of 22, 28, 30 and 157 % were seen in *p*NPC, FP, lichenan and xylan, respectively, compared to WT activities in the same substrates (Figure 3B). EMS19 grown in avicel: xylan showed improved enzyme activities for most of the substrates evaluated. An increase of 107, 36, 18, 32, 40, 30, 165 and 270 % in EMS19 supernatant activities were  detected in *p*NPA,

*p*NPC, *p*NPG, FP, beta-glucan, CMC, xylan and rye arabinan substrates, respectively, compared to WT (Figure 3C). Interestingly, when energy cane was used as the carbon source, no differences between EMS19 and WT supernatants regarding enzyme activity was detected (Figure 3D).

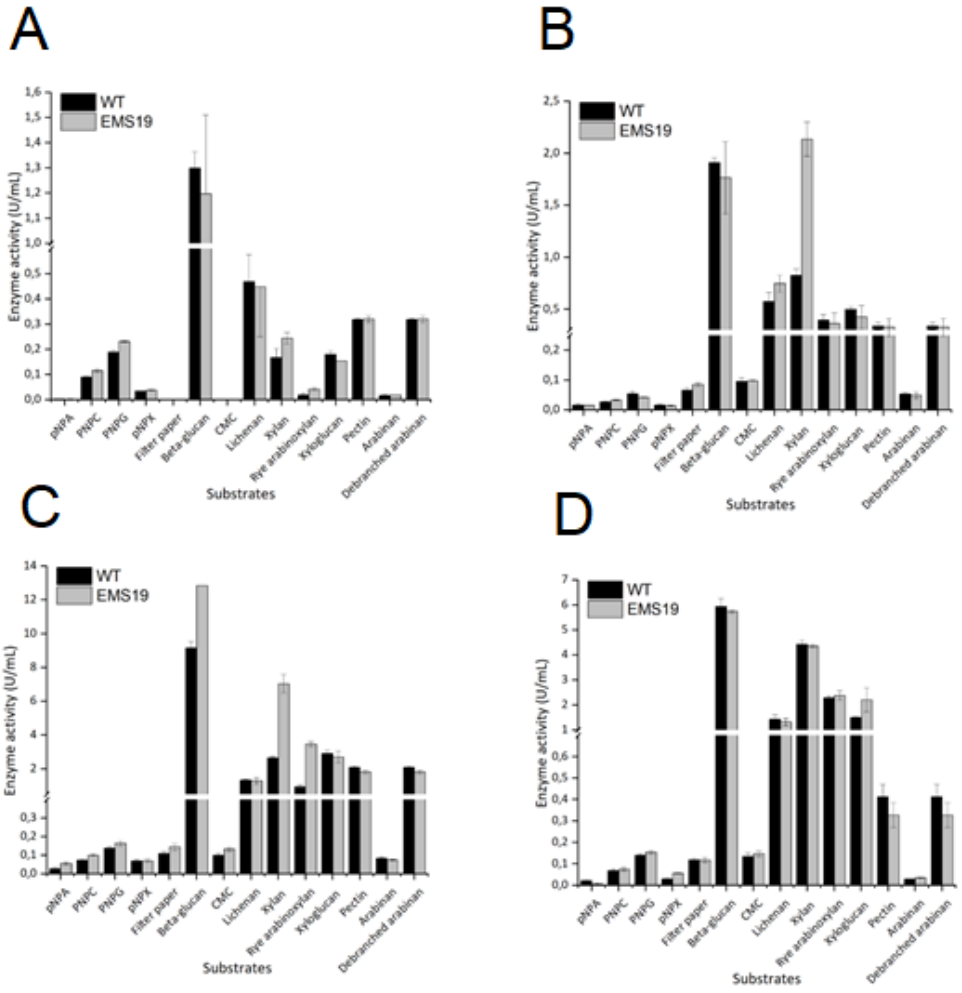


**Figure 3.** Enzyme activities (*p*NPA, *p*NPC, *p*NPG, FP, beta-glucan, (CMC), lichenan, xylan, rye arabinoxylan, xyloglucan, pectin, arabinan and debranched arabinan) of *P. oxalicum* WT and EMS19 grown on glucose (A), avicel (B), avicel:xylan (C) and energy cane (D) during three days. Each bar represents the mean and standard deviation of values from three independent experiments.

Given that the best results regarding the improvement in enzymatic activities were recorded when WT and EMS19 were grown in avicel:xylan (AVX), the secretome profiles of both fungi cultured in this condition were evaluated and compared. The secretomes of WT and EMS19 grown on glucose (GLU) was adopted as a control. A total of 196 proteins were detected in EMS19 secretome grown in GLU. These proteins were distributed in CAZymes (40 %), proteins related to cell metabolism (31 %), peptidases (8 %), lipases and proteases (1 % each)

and uncharacterized proteins (19 %) (Figure 4A). When EMS19 was cultured in AVX, 161 proteins were detected: CAZymes (50 %), proteins related to cell metabolism (26 %), peptidases (6 %), lipases and proteases (1 % each) and uncharacterized proteins (16 %) (Figure 4B).

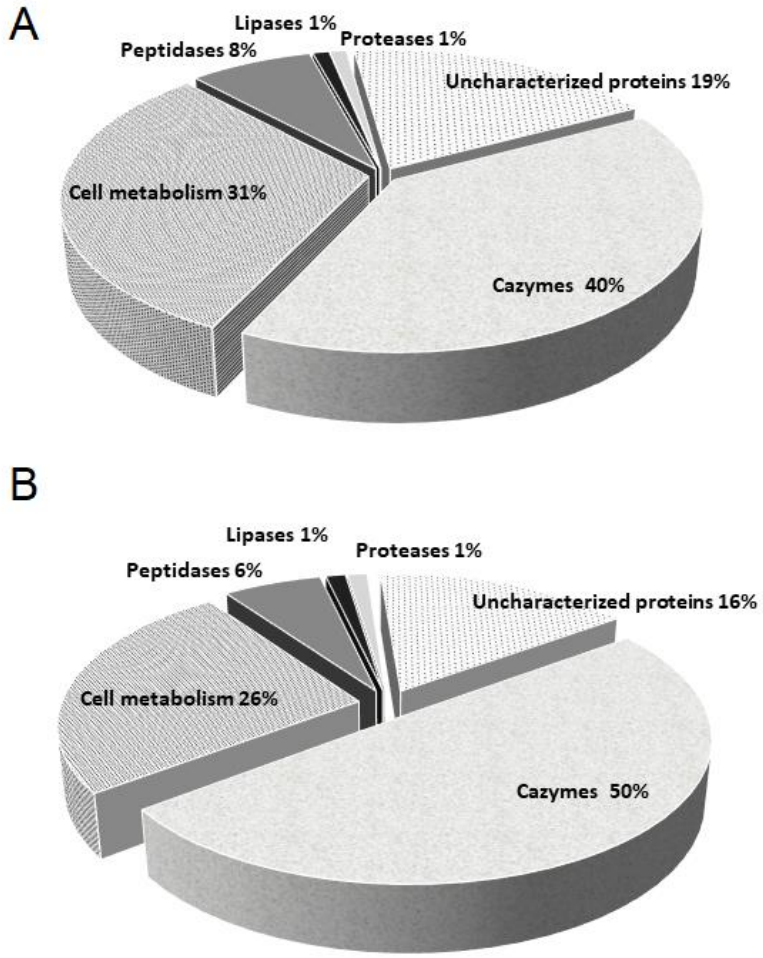


**Figure 4.** Distribution of the total proteins identified in the secretomes of EMS19 grown on GLU (A) and AVX (B) into CAZymes, peptidases, proteases, lipases, proteins related to cell metabolism and uncharacterized.

A total of 165 proteins were detected in the secretome of WT grown on GLU: CAZymes (35 %), proteins related to cell metabolism (33 %), peptidases (8 %), lipases (1 %), proteases (2 %) and uncharacterized proteins (21 %) (Figure 5A). In addition, WT cultured in AVX secreted 133 proteins classified into CAZymes (41 %), proteins related to cell metabolism (32 %), peptidases (7 %), lipases (1%), proteases (19 %) and uncharacterized proteins (25 %) (Figure 5B).

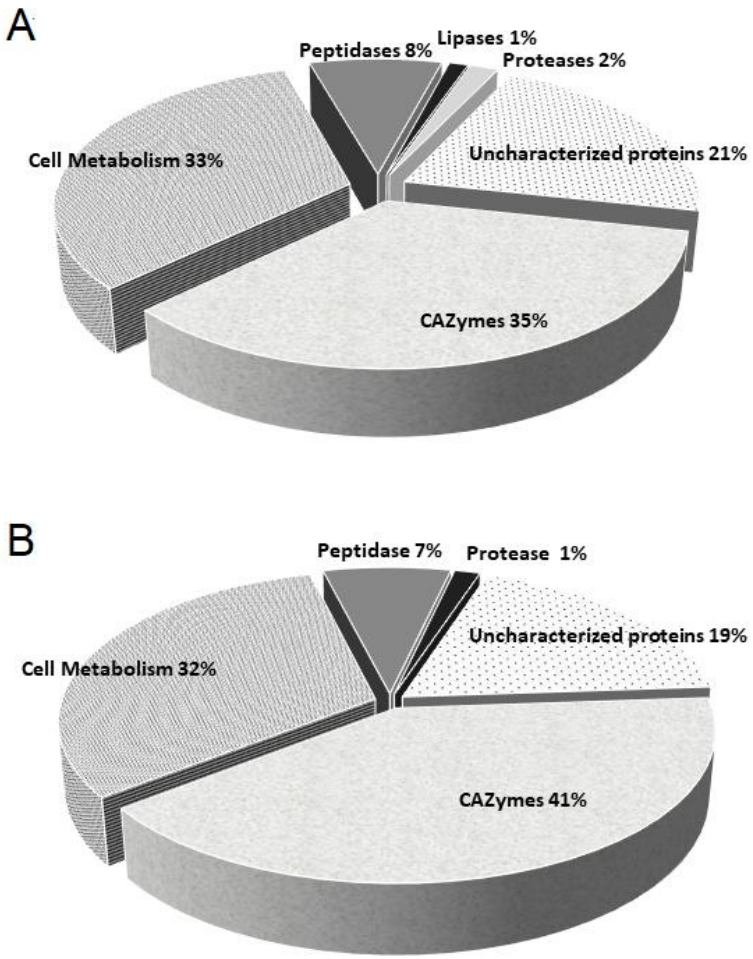


**Figure 5**. Distribution of the total proteins identified in the secretomes of WT grown on GLU (A) and AVX (B) into CAZymes, peptidases, proteases, lipases, proteins related to cell metabolism and uncharacterized.

The CAZymes detected in the secretomes of WT and EMS19 grown on GLU and AXV are highlighted in Table 1. They are distributed into cellulases, hemicellulases, pectinases, amylases and enzymes related to the degradation of fungi cell wall (Figure 6 A and B). EMS19 secreted 150, 77 and 37 % more cellulases, hemicellulases and pectinases, respectively, than WT cultured on GLU (Figure 6A). In addition, when EMS19 was grown on AVX, an increase of 114, 108 and 57 % in cellulases, hemicellulases and pectinases, respectively, was observed when compared to WT grown at the same condition (Figure 6B). Interestingly, the number of CAZymes secreted active in starch and cell wall did not show difference when EMS19 and WT were compared in both conditions (Figure 6A and B).

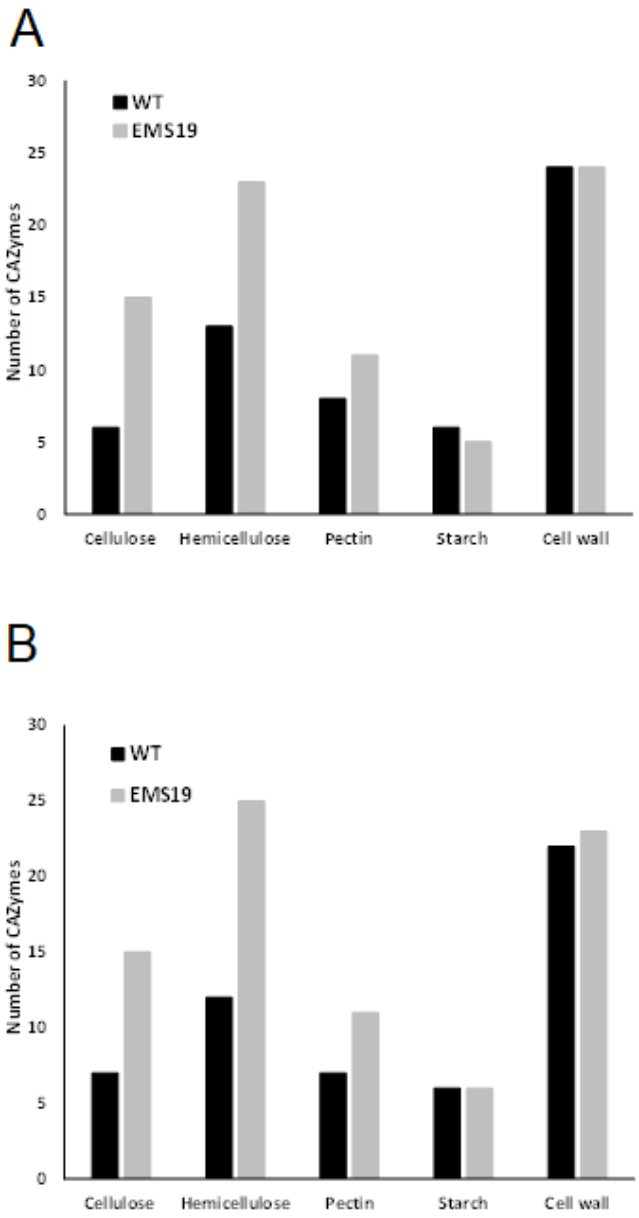


**Figure 6**. Substrates for CAZymes detected in the secretomes of WT and EMS 19 grown on GLU (A) and AVX (B).

**Table 1.** CAZymes detected in the secretomes of EMS19 and WT grown in AVX and GLU (the enzymes are divided according to the substrate)

| Uniprot ID | Protein | SP | aa | MW (kDa) | Family (CBM) | Spectrum count | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | EMS19 | | WT | |
| | | | | | | AVX | GLU | AVX | GLU |
| Cellulose | | | | | | | | | |
| S8BGM3 | Putative endo-beta-1,4-glucanase | Y | 413 | 44 | GH5 | 443 | 221 | 1 | -1 |
| S7ZDN1 | Putative endo-beta-1,4-glucanase | Y | 476 | 50.59 | GH5 (CBM1) | 22 | 14 | -1 | -1 |
| S8AVG6 | Putative endo-beta-1,4-glucanase | Y | 322 | 35.23 | GH5 | 12 | 10 | -1 | -1 |
| S7Z716 | Putative cellulose monooxygenase | Y | 355 | 36.23 | AA9 (CBM1) | 16 | 1 | -1 | -1 |
| S8AIJ2 | Endo-beta-1,4-glucanase | Y | 411 | 43.88 | GH5 (CBM1) | 100 | 27 | -1 | -1 |
| S7ZX22 | Endo-beta-1,4-glucanase Cel5C | Y | 655 | 69.34 | GH5 (CBM1) | 36 | 15 | -1 | -1 |
| S7ZP52 | Glucanase | Y | 464 | 48.50 | GH6 (CBM1) | 70 | 73 | -1 | -1 |
| S7ZPW1 | Cellulose monooxygenase Cel61A | Y | 249 | 26.23 | AA9 | 8 | 16 | -1 | -1 |
| S8B2B2 | Glucanase | Y | 474 | 49.37 | GH7 (CBM1) | 187 | 128 | -1 | -1 |
| S7ZJL3 | Glucanase | Y | 453 | 48.14 | GH7 | 94 | 79 | 2 | 3 |
| S7ZBJ9 | Putative beta-glucosidase | Y | 800 | 86.17 | GH3 | 21 | 26 | 4 | 5 |
| S7ZRD6 | Glucanase | Y | 546 | 56.88 | GH7 (CBM1) | 1145 | 743 | 12 | 8 |
| S7ZRB6 | Putative beta-fructofuranosidase | Y | 644 | 71.82 | GH32 | 7 | 7 | 10 | 8 |
| S7ZCL0 | Lysozyme | Y | 225 | 23.74 | GH25 | 36 | 36 | 53 | 86 |
| S8B0F3 | Beta-glucosidase | Y | 861 | 93.51 | GH3 | 174 | 149 | 294 | 310 |
| Hemicellulose | | | | | | | | | |
| S8B2H7 | Putative endo-beta-1,4-xylanase | Y | 480 | 52.44 | GH30 | 14 | 15 | -1 | -1 |
| S7ZTH1 | Arabinan endo-1,5-alpha-arabinosidase | Y | 324 | 35.60 | GH43 | -1 | 2 | -1 | -1 |
| S8BDN2 | Beta-xylanase | Y | 410 | 43.56 | GH10 (CBM1) | 194 | 79 | -1 | -1 |
| S7ZV78 | Putative acetyl xylan esterase | Y | 396 | 41.28 | CE1 (CBM1) | 61 | 33 | -1 | -1 |
| S7ZAV8 | Beta-xylanase | Y | 362 | 39.82 | GH10 | 2 | -1 | -1 | 3 |
| S7ZA57 | Beta-xylanase | Y | 330 | 36.03 | GH10 | 7 | -1 | -1 | -1 |
| S7Z3I8 | Putative alpha-L-arabinofuranosidase | Y | 395 | 41.48 | GH62 (CBM1) | 95 | 49 | -1 | -1 |
| S7ZW00 | Putative alpha-L-arabinofuranosidase | Y | 331 | 35.88 | GH62 | 8 | 6 | -1 | -1 |
| S8B8M7 | Putative alpha-L-arabinofuranosidase | Y | 452 | 49.37 | GH43 (CBM6) | 4 | 12 | -1 | -1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S8AH74 | Putative endo-beta-1,4-xylanase | Y | 529 | 55.37 | GH30 (CBM1) | 41 | 22 | -1 | -1 |
| S7Z4P2 | Putative alpha-L-arabinofuranosidase | Y | 582 | 62.27 | GH43 (CBM1) | 5 | 2 | -1 | -1 |
| S8AMN0 | Endo-1,4-beta-xylanase | Y | 310 | 31.28 | GH11 (CBM1) | 67 | 10 | -1 | -1 |
| S8A0T1 | Putative beta-xylosidase | Y | 778 | 84.88 | GH3 | 2 | 26 | -1 | -1 |
| S8AP92 | Endo-1,4-beta-xylanase | Y | 289 | 29.48 | GH11 (CBM1) | 15 | 2 | -1 | -1 |
| S7ZL65 | Putative beta-1,4-mannanase | Y | 442 | 47.40 | GH5 (CBM1) | 66 | 52 | -1 | -1 |
| S7ZUD9 | Putative exo-beta-1,3-galactanase | Y | 451 | 48.51 | GH43 (CBM6) | 15 | 13 | -1 | -1 |
| S7ZCW4 | Putative exo-beta-1,3-galactanase | N | 494 | 53.92 | GH43 | 28 | 23 | -1 | -1 |
| S8ATR1 | Endo-1,4-beta-xylanase | Y | 211 | 22.87 | GH11 | 24 | 28 | -1 | -1 |
| S7ZSB3 | alpha-1,2-mannosidase | N | 930 | 101.16 | GH47 | -1 | -1 | 2 | 3 |
| S7ZQR7 | Putative beta-xylosidase | Y | 900 | 97.60 | GH3 | -1 | -1 | 3 | 1 |
| S7ZCU6 | Alpha-galactosidase | Y | 471 | 51.47 | GH27 | -1 | -1 | 32 | 48 |
| S7ZFY8 | Alpha-galactosidase | Y | 524 | 55.02 | GH27 (CBM1) | 90 | 36 | 16 | 13 |
| S8B2R2 | Putative alpha-mannosidase | Y | 780 | 86.29 | GH92 | 2 | 13 | 2 | 12 |
| S8AXM3 | Putative endo-beta-1,6-galactanase | Y | 487 | 53.32 | GH30 | 41 | 30 | 14 | 18 |
| S8B0N0 | Putative beta-glucuronidase | Y | 634 | 71.34 | GH2 | 19 | 5 | 52 | 63 |
| S7ZPF5 | Putative alpha-mannosidase | Y | 807 | 88.36 | GH92 | 42 | 52 | 87 | 134 |
| S8AHA8 | Putative beta-xylosidase | Y | 796 | 87.10 | GH3 | 105 | 145 | 35 | 72 |
| S7Z5H6 | Beta-galactosidase | Y | 1,011 | 110.50 | GH35 | -1 | -1 | 16 | 19 |
| S7Z4H1 | alpha-1,2-mannosidase | Y | 510 | 56.40 | GH47 | 99 | 109 | 119 | 135 |
| S8AT05 | Putative acetyl xylan | Y | 234 | 24038 | CE5 | 131 | -1 | 146 | 9 |
| | | | | Pectin | | | | | |
| S8B1P0 | Putative polygalacturonase | Y | 380 | 38.76 | GH28 | -1 | 2 | -1 | -1 |
| S7ZWA6 | Arabinan endo-1,5-alpha-L-arabinosidase | Y | 321 | 33.95 | GH43 | 1 | -1 | -1 | -1 |
| S8B8B6 | Putative rhamnogalacturonase | Y | 446 | 47.43 | GH28 | 4 | 12 | -1 | 2 |
| S7ZBM4 | Arabinogalactan endo-beta-1,4-galactanase | Y | 354 | 38.58 | GH53 | 7 | 7 | -1 | -1 |
| S8AXN0 | Putative pectate lyase | Y | 325 | 34 | PL 1 | 17 | 22 | -1 | -1 |
| S8B0B4 | Putative endopolygalacturonase | Y | 379 | 38.66 | GH28 | 3 | 7 | 2 | 2 |
| S7ZD03 | Putative rhamnogalacturonase | Y | 435 | 47 | GH28 | 75 | 50 | 6 | 4 |
| S7ZAW1 | Putative rhamnogalacturonan alpha-L-rhamnopyranohydrolase | Y | 426 | 46.19 | GH28 | 56 | 6 | 14 | 17 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S8ASZ0 | Putative endopolygalacturonase | Y | 380 | 38.43 | GH28 | 47 | 71 | 16 | 23 |
| S8BDC2 | Putative polygalacturonase | Y | 371 | 37.62 | GH28 | 33 | 30 | 46 | 57 |
| S7ZZQ8 | Putative rhamnogalacturonan alpha-L-rhamnopyranohydrolase | Y | 465 | 51.38 | GH28 | 24 | 17 | 80 | 99 |
| S8AXD6 | Pectinesterase | Y | 327 | 34348 | CE8 | 35 | 32 | 28 | 48 |
| | | | | Starch | | | | | |
| S7ZHJ5 | Putative alpha-amylase | Y | 558 | 62.28 | GH13 | 9 | 16 | -1 | -1 |
| S8AKS0 | Starch binding domain-and chitin binding domain-containing protein | Y | 403 | 42.80 | CBM20 glucoamylase (CBM20) | 4 | 7 | 6 | 3 |
| S7ZIW0 | Glucoamylase | Y | 616 | 67.49 | GH15 (CBM20) | -1 | -1 | 192 | 191 |
| S7ZED3 | Putative alpha-glucosidase | Y | 983 | 108.06 | GH31 | 4 | -1 | 140 | 96 |
| S7Z6T2 | Alpha-amylase Amy13A | Y | 626 | 67.56 | GH13 (CBM20) | 76 | 55 | 73 | 46 |
| S7ZCN9 | Putative alpha,alpha-trehalase | Y | 1,093 | 119,708 | GH65 | 25 | 15 | 38 | 24 |
| S8B6D7 | Glucoamylase | Y | 635 | 67.21 | GH15 (CBM20) | 175 | 183 | 371 | 364 |
| | | | | Cell wall | | | | | |
| S7ZWK1 | Putative beta-1,3-glucanase | N | 641 | 68.33 | GH1 | 20 | 7 | -1 | -1 |
| S8AU36 | 1,3-beta-glucanosyltransferase | Y | 468 | 49.87 | GH72 | -1 | 2 | -1 | -1 |
| S7ZS08 | Putative beta-1,3-glucanosyltransferase | N | 577 | 58.72 | GH17 | -1 | -1 | -1 | 3 |
| S7ZMU5 | Putative beta-glucanase | Y | 659 | 67.80 | GH16 | 13 | 11 | 3 | -1 |
| S7ZMB4 | Putative endo-beta-1,4-glucanase | Y | 237 | 25.54 | GH12 | 52 | 24 | -1 | 2 |
| S8BDB6 | Endo-beta-1,4-glucanase | Y | 266 | 26.86 | GH45 (CBM1) | 30 | 11 | -1 | -1 |
| S8B590 | Putative chitin glucanosyltransferase | Y | 371 | 39.49 | GH16 | 4 | -1 | -1 | 1 |
| S8B1J2 | Putative exo-beta-1,3-glucanase | N | 1.35 | 147.87 | GH18 | -1 | -1 | 1 | -1 |
| S8BAX2 | 1,3-beta-glucanosyltransferase | Y | 539 | 57.85 | GH72 | 39 | 30 | -1 | -1 |
| S7Z850 | Putative exo-beta-glucosaminidase | Y | 895 | 100.27 | GH2 | -1 | 9 | -1 | -1 |
| S8B6N1 | Putative chitinase | Y | 965 | 96.76 | GH18 | 2 | -1 | -1 | -1 |
| S7ZRY1 | Putative UDP-Glc: sterol glucosyltransferase | N | 1,041 | 114.43 | GT28 | -1 | 1 | -1 | 2 |
| S7ZSB2 | Putative chitinase | Y | 1,246 | 133.05 | GH18 | -1 | -1 | 2 | 5 |
| S7ZAS9 | Putative endo-beta-1,3-glucanase | N | 734 | 77.44 | PL3 | -1 | 7 | 6 | 2 |
| S7Z823 | Endo-chitosanase | Y | 237 | 24.78 | GH75 | -1 | -1 | 2 | 2 |
| S8AUV1 | Putative chitinase | N | 400 | 43.73 | GH18 | 6 | -1 | 10 | 7 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S7Z7R8 | Putative chitinase | Y | 441 | 48.46 | GH18 | 12 | 3 | 7 | 7 |
| S8B0U2 | Putative endo-beta-1,4-glucanase | Y | 251 | 26.79 | GH12 | 12 | 15 | 15 | 28 |
| S7ZCP1 | Putative beta-1,3-1,4-glucanase | Y | 358 | 38.16 | GH16 | 9 | 4 | 23 | 28 |
| S8AMF6 | Putative beta-1,6-glucanase | Y | 487 | 51.45 | GH30 | 15 | 13 | 23 | 27 |
| S8AIA9 | Glycosidase | Y | 378 | 39.19 | GH16 | 51 | 41 | 75 | 82 |
| S7ZUY6 | Putative chitin glucanosyltransferase | N | 347 | 36.79 | GH16 | 25 | 16 | 59 | 71 |
| S8AWH6 | Putative chitinase | Y | 331 | 35.42 | GH18 | 19 | 11 | 69 | 56 |
| S7ZII5 | 1,3-beta-glucanosyltransferase | Y | 536 | 57.08 | GH72 | 35 | 22 | 82 | 89 |
| S7ZAG7 | Putative beta-1,3-glucanosyltransferase | Y | 315 | 34.28 | GH17 | 83 | 56 | 107 | 117 |
| S8B6N0 | Glycosidase | Y | 443 | 46.81 | GH16 | 35 | 10 | 123 | 89 |
| S8AWB1 | Putative beta-1,3-glucanase | N | 429 | 46.32 | GH64 | 126 | 167 | 163 | 217 |
| S7ZS63 | Beta-hexosaminidase | Y | 601 | 67.08 | GH20 | -1 | -1 | 120 | 108 |
| S7ZR03 | Putative chitinase | Y | 429 | 46.95 | GH18 | 91 | 17 | 163 | 86 |
| S7ZLD7 | Putative beta-N-acetylhexosaminidase | Y | 348 | 36.18 | GH3 | 67 | 56 | 133 | 142 |
| S7ZHJ4 | Putative exo-beta-1,3-glucanase | Y | 521 | 50.29 | GH17 | 66 | 52 | 201 | 233 |
| S8BDR6 | Putative exo-beta-1,3-glucanase | Y | 807 | 85.28 | PL 3 | 170 | 142 | 335 | 433 |

WT secreted 57 CAZymes belonging to 24 glycoside hydrolase (GH) families, 1 glycosyl transferase (GT), 1 polysaccharide lyase (PL), 2 carbohydrate esterase (CE) and 1 carbohydrate-binding module (CBM) in GLU medium while EMS19 secreted 78 CAZymes distributed in 28 GHs, 1 GT, 2 PLs, 1 CE, 1 CBM and 1 AA (figure 7A) in the same condition, an increase of 36.84 % of CAZymes number (Figure 7A). Fifty-four CAZymes were secreted by WT on AVX: 24 GHs, 1 PL, 2 CE and 1 CBM (Figure 7B). Eighty CAZymes were detected in the secretome of EMS19 grown on AVX, being 29 GHs, 2 PLs, 3 CEs,1 CBM and 1AA, an increase of 48% in CAZymes number compared to WT (Figure 7B).

The families GH20, 35, 75 and GH1, 6, 43, 45, 53, 62, AA9, CE1, PL1 were exclusively secreted by WT and EMS19, respectively, in the two conditions evaluated. An improved number of CAZymes from families GH3, 28, 30 and 72 (GLU condition) and GH5, 7, 10, 11, 12, 13, 16, 27, 28, 30 72 and PL3 (AVX condition) were found in secretomes of EMS19 compared to WT. Only the GLU condition promoted a higher number of GH3, 16 and 18 in WT secretome.

According to the spectrum counts, cellulases are more abundant in EMS19 AVX secretome than WT grown in the same condition. One glucanase GH6 (S7ZP52) and 2 cellulose monooxygenases AA9 (S7Z716, S7ZPW1) were found exclusively secreted by EMS19 in both conditions (GLU and AVX). In addition, an increased secretion of five GH5 (S8BGM3, S7ZDN1, S8AVG6, S8AIJ2, S7ZX22) and 3 GH7 (S8B2B2, S7ZJL3, S7ZRD6) were detected in EMS19 secretome compared to WT secretome grown on AVX (Table 1).

As observed for cellulases, hemicellulases are also found enriched in EMS19 secretomes compared to WT in both conditions of growth (Figure 6). Two alpha-L-arabinofuranosidases GH62 (S7Z3I8, S7ZW00), 2 alpha-L-arabinofuranosidases GH43 (S8B8M7, S7Z4P2) and 2 exo-beta-1,3-galactanases GH 43 (S7ZUD9, S7ZCW4) were exclusively found in EMS19 secretomes in both conditions. In addition, an increase in spectrum counts of 1 GH30 (S8B2H7) and 1 GH3 (S8A0T1) in GLU condition, and 2 GH30 (S8AH74, S8AXM3), 2GH10 (S8BDN2, S7ZA57), 2GH11 (S8AMN0, S8AP92) 1GH3 (S8AHA8), 1 GH5 (S7ZL65) and 1 GH27 (S7ZFY8) were noticed in EMS19 secretome on AVX condition. One beta-galactosidase GH35 (S7Z5H6) were only present in WT on both conditions (Table 1).

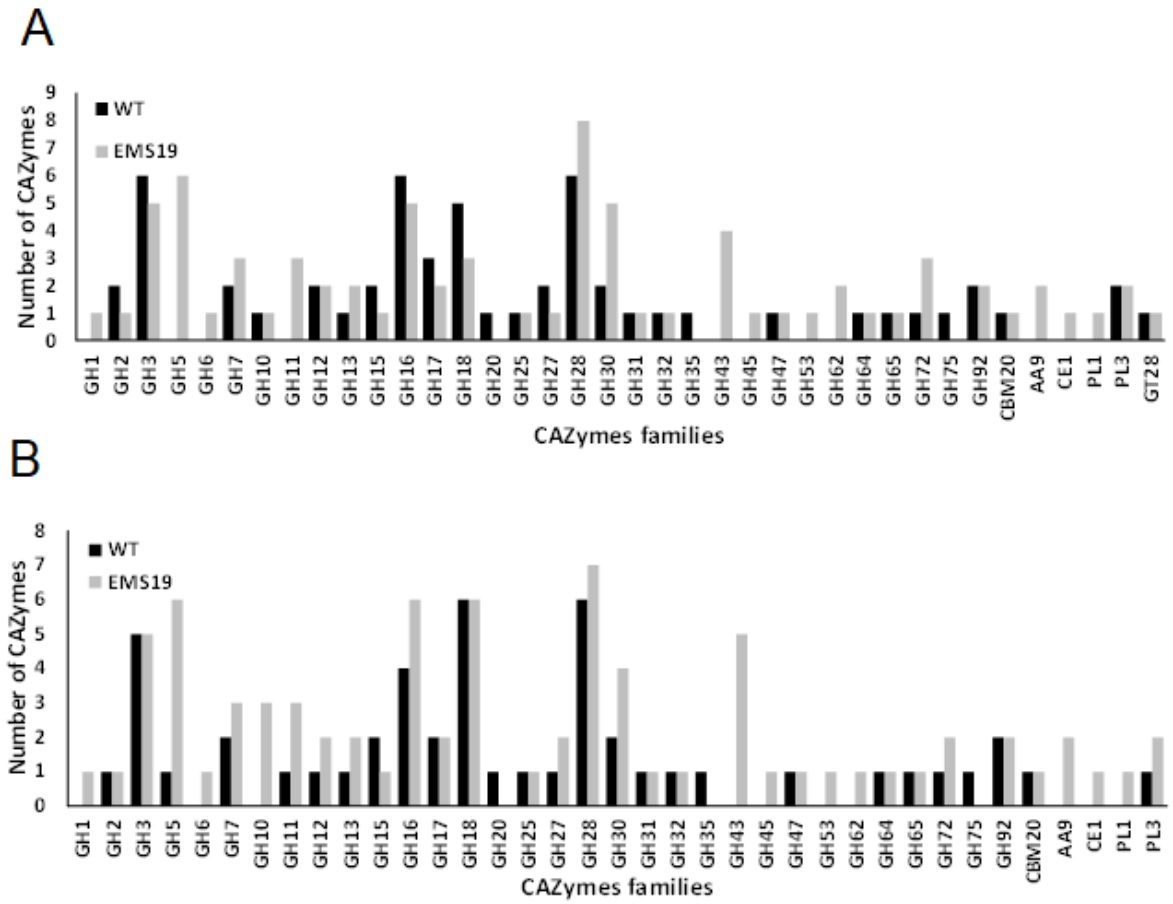


**Figure 7**. Families of CAZymes secreted by WT and EMS19 on GLU (A) and AVX (B).

Pectin-acting enzymes were noticed with larger spectrum counts in EMS19 secretomes than WT on GLU and AVX. One arabinan endo-1,5-alpha-L-arabinosidase GH43 (S7ZWA6) on AVX and 1 arabinogalactan endo-beta-1,4-galactanase GH53 (S7ZBM4) and 1 pectate lyase PL1 (S8AXN0) on both conditions were found exclusively in EMS19 secretome. Besides that, there was noticed an increase of spectral counts of 1 rhamnogalacturonase and 1 rhamnogalacturonan alpha-L-rhamnopyranohydrolase GH28 (S7ZD03, S7ZAW1) mainly on AVX condition and 1
endopolygalacturonase GH28 (S8ASZ0) on GLU.

WT secretomes in both culture conditions showed larger spectrum counts for most of the starch-acting enzymes (Table 1).

In what concerns to fungi cell wall degrading enzymes, comparing the enzymes which have action in the fungi cell wall, higher spectrum counts were detected found in WT secretome on GLU condition compared to EMS19 in the same condition. WT secretome showed the exclusive presence of One1 endo-chitosanase GH75 (S7Z823) and 1one beta-hexosaminidase GH20 (S7ZS63) were secreted exclusively by WT in both conditions. However, EMS19 secretome revealed the exclusive presence of 1 beta-1,3-glucanase GH1 (S7ZWK1) and 1 endo-beta-1,4-glucanase (GH45) on in GLU and AVX conditions. Also, there were notice an increase EMS secretome on spectral counts of 2 GH16 (S7ZMU5, S8B590), 1 GH12 (S7ZMB4), 1 GH72 (S8BAX2) and 1 GH18 (S7Z7R8) mainly were seen in EMS19 grown on AVX and 1 GH2 (S7Z850) and 1 PL3 (S7ZAS9) on GLU condition (Table 1).

An improvement in enzyme activities in cellulose, hemicellulose and cell wall substrates were detected in EMS19 compared to WT mainly on AVX condition, especially β-glucanases and xylanases. Interestingly, an increased secretion of the endo-beta-1,4-glucanases GH5 (S7ZDN1, S8AVG6, S8AIJ2, S7ZX22), endo-beta-1,4-xylanases GH30 (S8B2H7, S8AH74), beta-xylanases GH10 (S8BDN2, S7ZA57), endo-1,4-beta-xylanases GH11 (S8AMN0, S8AP92, S8ATR1) beta-xylosidases GH3 (S8A0T1, S8AHA8), beta-1,3-glucanase GH1 (S7ZWK1), beta-glucanase GH16 (S7ZMU5), endo-beta-1,4-glucanase GH12 (S7ZMB4), endo-beta-1,4-glucanase GH45 (S8BDB6) were observed in EMS19 secretome compared to WT agreeing with enzymatic assay.

Regarding cell metabolism proteins, GLU condition responds for the larger spectrum count compared to AVX for both strains. By comparing the strains, WT showed an abundance of peptidases and uncharacterized proteins cultured in GLU condition (Tables 2 and 6). Overall,

ESM19 showed the highest spectrum counts for proteins related to carbon metabolism (non-CAZymes) on GLU condition (Table 5).

### 4.4. Discussion

Random mutagenesis is a traditional tool usually applied as the first step to develop industrial fungi lineages with an improvement on CAZymes production. Filamentous fungi such as *P. oxalicum* and *T. reesei* were subject to random mutagenesis and screening until the achievement of the industrial strains JU-A10-T and RUT-30, respectively (Peterson et al., 2012).

The main aim of this work was the development of a *P. oxalicum* strain (EMS19) with an improvement in hemicellulolytic production by random mutagenesis.

The enzymatic activities of EMS19 and WT grown in glucose, avicel, avicel: xylan (1:2) and energy cane as the sole carbon sources in cellulosic, hemicellulosic and pectic substrates were evaluated in this work. Avicel and avicel:xylan conditions promoted the highest enzyme activities in EMS19 strain compared to WT. This data is in accordance with some studies which show higher lignocellulolytic activities by growing *P. oxalicum* is in culture media containing cellulose, xylan, xylose or wheat bran as carbon source in comparison with glycerol or glucose (Liu et al., 2013b; Sun et al. 2018; Qu et al. 1987). Moreover, Liao et al. (2014) noticed that the addition of xylan to culture medium containing cellulose improved the lignocellulolytic enzyme activities in *Penicillium oxalicum* GZ-2, what was confirmed in the present study. Energy cane also promoted higher activities in WT and EMS19 strains compared to glucose, however, the results were similar when both strains were compared in this condition. Due to the recalcitrance of energy cane, the diversity of enzyme arsenal (especially hemicellulases) is more important than augmented activity for its break down (Corrêa et al., 2020; to be submitted) and it is possibly an explanation why EMS19 and WT showed similar results regarding the enzymatic activity in this work.

Random mutagenesis can affect gene-regulation regions, increasing, decreasing or inhibiting protein secretion or gene regions encoding for a specific protein, altering the sequence of amino acids, promoting the increase, decrease or inactivation of its activity (Ennis, 2001). In this regard, it is important to highlight that AA9, an enzyme typically induced by lignocellulosic material, were also found in EMS19 GLU secretome, pointing out that a mutation in some transcription factor (TF) possibly occurred promoting the production and secretion of this enzyme at non-induced conditions (GLU).

Liu et al. (2013b) reported that the deletion of CreA (a cellulase repressor) and the overexpression of activator clrB (a cellulase positive regulator) could lead to the increase of cellulase production and avoid the dependence of an inducer medium for production of hemicellulolytic enzymes by *P. oxalium* strains.

EMS19 showed an increased xylanase and beta-glucanase activities compared to WT. The presence of the exclusive families GH1, GH6, GH43, GH45 and GH62 and the increase of CAZymes belonging to GH3, GH5, GH7, GH12, GH16 and GH30 in both conditions in EMS19 compared to WT agreed with enzymatic assays.

The spectrum counts for enzymes active in starch was higher in WT than EMS19. This event was also noticed in the transcriptome of JU-A10-T that showed significantly down-regulated expression of amylases and the up-regulated expression of cellulases and hemicellulases (Liu et al., 2013b).

Studies carried out by Liu et al. (2013b) showed that amino acid degradation and the secondary metabolism are reduced in the hyper cellulolytic mutant JU-A10-T of *P. oxalicum*. Consequently, there is an increase in the energy flow used for enzyme synthesis. This effect was also noticed in EMS19 strain, revealing that many peptidases and uncharacterized proteins, which could have metabolic function, were reduced in EMS19 compared to WT. However, it is important to highlight that some uncharacterized proteins are possible targets for discoveries of outstanding activities related to de deconstruction of lignocellulosic biomass.

## 4.5 Conclusion

*Penicillium* strains have been considered an alternative to *T. reesei* as a platform to produce enzymes related to the deconstruction of lignocellulosic biomass. In this work, a strain (EMS19) with improved hemicellulolytic activities compared to the wild type was obtained after two rounds of random mutagenesis. Also, the secretome profile revealed the presence of the highest spectrum count of cellulases, hemicellulases and pectinases in EMS19 compared to WT. These results provide directions for the rational design of *P. oxalicum* strains to obtain higher production of lignocellulolytic enzymes.

## 4.6. Acknowledgements

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## 4.8. Attachments

**Table 2.** Peptidases detected in EMS19 an WT in AVX and GLU secretomes

| | | | | | | Spectrum count | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | EMS19-7 | | Po | |
| **Uniprot ID** | **Identified protein** | **SP** | **aa** | **MW (kDA)** | **Family** | **Ax** | **Glu** | **Ax** | **Glu** |
| S8B8I9 | Carboxypeptidase | Y | 473 | 51,703 | Peptidase | 15 | 28 | 23 | 16 |
| S8AVX4 | Carboxypeptidase | Y | 548 | 61,111 | Peptidase | 7 | 2 | -1 | -1 |
| S7ZJA9 | Carboxypeptidase | Y | 528 | 59,751 | Peptidase | 2 | 184 | 2 | 231 |
| S7Z6B2 | Uncharacterized protein | Y | 490 | 51,797 | Peptidase | 6 | 35 | 16 | 52 |
| S8AWZ3 | Uncharacterized protein | Y | 601 | 65,147 | Peptidase | 2 | 12 | 2 | 17 |
| S7ZW27 | Uncharacterized protein | Y | 390 | 40,234 | Peptidase | 94 | 80 | 108 | 104 |
| S7ZPS7 | Uncharacterized protein | Y | 395 | 40,844 | Peptidase | 89 | 110 | 55 | 111 |
| S7ZI45 | Uncharacterized protein | N | 631 | 68,341 | Peptidase | -1 | 6 | -1 | 2 |
| S7Z5V7 | Uncharacterized protein | Y | 590 | 65,322 | Peptidase | -1 | 7 | -1 | 5 |
| S7ZJ06 | Uncharacterized protein | N | 470 | 49,721 | Peptidase | 5 | -1 | 2 | -1 |
| S7ZDF7 | Uncharacterized protein | Y | 667 | 73,322 | Peptidase | -1 | 15 | -1 | 16 |
| S8BDB9 | Uncharacterized protein | Y | 271 | 28,624 | Peptidase | 14 | 34 | 9 | 30 |
| S7Z4N3 | Carboxypeptidase | Y | 518 | 57,384 | Peptidase | -1 | 16 | 5 | 28 |
| S8AYI7 | Uncharacterized protein | Y | 594 | 63.89 | Peptidase | -1 | 3 | -1 | 2 |
| S8AV88 | Uncharacterized protein | Y | 520 | 58,003 | Peptidase | -1 | 58 | -1 | 66 |

**Table 3.** Lipases detected in EMS19 and WT in AVX and GLU secretomes.

| | | | | | | Spectrum count | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | EMS19-7 | | Po | |
| **Uniprot ID** | **Identified protein** | **SP** | **aa** | **MW (kDA)** | **Family** | **Ax** | **Glu** | **Ax** | **Glu** |
| S7ZIX0 | lipase | Y | 642 | 68,569 | Lipase | 4 | 49 | -1 | 39 |
| S7ZMJ7 | Uncharacterized protein | Y | 291 | 30,516 | Lipase | 5 | 16 | -1 | 17 |

**Table 4.** Proteases detected in EMS19 and WT in AVX and GLU secretomes

| | | | | | | Spectrum count | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | EMS19-7 | | Po | |
| **Uniprot ID** | **Identified protein** | **SP** | **aa** | **MW (kDA)** | **Family** | **Ax** | **Glu** | **Ax** | **Glu** |
| S8B3F6 | Uncharacterized protein | N | 433 | 46,815 | Protease | 6 | 19 | 11 | 16 |
| S8AUK5 | Uncharacterized protein | Y | 397 | 43,239 | Protease | -1 | -1 | -1 | 3 |
| S7ZU75 | Uncharacterized protein | Y | 588 | 63,737 | Protease | 13 | 38 | 9 | 41 |

**Table 5.** Proteins related to cell metabolim detected found in EMS19 and WT in AVX and GLU secretomes

| | | | | | | | Spectrum count | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | EMS19-7 | | Po | |
| **Uniprot ID** | **Identified protein** | **SP** | **aa** | **MW (kDA)** | **Family** | **Classification** | **Ax** | **Glu** | **Ax** | **Glu** |
| S8AXU9 | Ecm33 domain-containing protein | Y | 401 | 41968 | GPI-anchored | Growth and development | 86 | 102 | 59 | 91 |
| S7Z7A8 | Uncharacterized protein | Y | 420 | 46851 | Phosphoesterase | Growth and development | 3 | 55 | 8 | 78 |
| S8APL4 | Uncharacterized protein | Y | 453 | 49391 | Phosphoesterase | Growth and development | 15 | 49 | 16 | 66 |
| S8B2Q2 | Uncharacterized protein | Y | 288 | 27679 | CFEM domain | Growth and development | 8 | 2 | 4 | 3 |
| S8AXA3 | Histone H2B | N | 141 | 15.04 | Core histone H2A/H2B/H3/H4 | Protein related to DNA | 1 | -1 | -1 | -1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| S8AR30 | Purple acid phosphatase | Y | 607 | 66716 | Calcineurin-like phosphoesterase (Metallophos) | Growth and development | -1 | 31 | -1 | 31 |
| S8B3P5 | Uncharacterized protein | N | 146 | 15607 | Blastomyces yeast-phase-specific protein | Growth and development | -1 | 29 | -1 | 45 |
| S7ZDQ5 | Uncharacterized protein | Y | 312 | 33.74 | Pyridoxamine 5'-phosphate oxidase | Growth and development | -1 | 11 | -1 | 13 |
| S8AIW5 | Uncharacterized protein | N | 619 | 68918 | Sugar (and other) transporter | Carbon Metabolims | -1 | 2 | -1 | -1 |
| S8B1X6 | Uncharacterized protein | N | 538 | 58.67 | Sugar (and other) transporter | Carbon Metabolims | -1 | 1 | -1 | -1 |
| S8B303 | Uncharacterized protein | Y | 575 | 61643 | FAD binding domain | Growth and development | 63 | 7 | 55 | 4 |
| S8ATU2 | Uncharacterized protein | Y | 687 | 74689 | Histidine phosphatase superfamily | Growth and development | 41 | 30 | 40 | 27 |
| S8B4F0 | Uncharacterized protein | Y | 522 | 57416 | Histidine phosphatase superfamily | Growth and development | 26 | 9 | 24 | 4 |
| S7ZFL1 | Carbohydrate binding domain-containing protein | Y | 819 | 81577 | Schizosaccharomyces pombe repeat of unknown function | Carbon Metabolims | 20 | -1 | -1 | -1 |
| S8BA66 | Putative feruloyl esterase | Y | 341 | 35772 | Prolyl oligopeptidase family | Carbon Metabolims | 13 | -1 | 1 | 2 |
| S8B8C3 | Uncharacterized protein | Y | 341 | 36139 | S1/P1 Nuclease | Protein related to DNA | 8 | 1 | 27 | 2 |
| S7ZIZ2 | Putative carbohydrate acetylesterase | Y | 294 | 32643 | GDSL-like Lipase/Acylhydrolase | Carbon Metabolims | 2 | -1 | -1 | -1 |
| S7ZD15 | Amine oxidase | Y | 528 | 58988 | Flavin containing amine oxidoreductase | Growth and development | -1 | 56 | -1 | 44 |
| S8AJN6 | Carboxylic ester hydrolase | Y | 537 | 58.25 | Carboxylesterase family | Carbon Metabolims | -1 | 4 | -1 | 4 |
| S7ZNK7 | Uncharacterized protein | N | 294 | 31703 | GPR1/FUN34/yaaH family | Growth and development | -1 | 1 | -1 | -1 |
| S8ANR6 | 6,7-dimethyl-8-ribityllumazine synthase | N | 204 | 21454 | 6,7-dimethyl-8-ribityllumazine synthase | Growth and development | -1 | 1 | -1 | -1 |
| S7ZUK9 | Uncharacterized protein | N | 1322 | 146364 | Nucleoporin | Growth and development | -1 | 2 | -1 | -1 |
| S8A1J6 | Uncharacterized protein | N | 235 | 25424 | Protein of unknown function | others | -1 | -1 | 1 | -1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| S7Z887 | Uncharacterized protein | Y | 125 | 13259 | ribonuclease | Protein related to DNA | 17 | 29 | 35 | 42 |
| S8B512 | Uncharacterized protein | N | 505 | 55882 | Major Facilitator Superfamily | Carbon Metabolims | -1 | 2 | -1 | -1 |
| S7ZAB6 | Putative swollenin | Y | 499 | 51888 | CBM_1 | Carbon Metabolims | 95 | -1 | 45 | -1 |
| S7ZFY0 | Uncharacterized protein | Y | 143 | 14947 | Cupredoxins superfamily | Growth and development | 2 | -1 | 6 | -1 |
| S8B6K2 | Uncharacterized protein | Y | 604 | 67.78 | Multicopper oxidase | Growth and development | -1 | -1 | 1 | -1 |
| S7ZT19 | Uncharacterized protein | Y | 346 | 39057 | Phosphoesterase | Growth and development | 38 | 242 | 55 | 312 |
| S7Z911 | Uncharacterized protein | Y | 256 | 27981 | Ribonuclease T2 family | Protein and DNA | 36 | 52 | 46 | 59 |
| S8ALY4 | Uncharacterized protein | Y | 687 | 75267 | Domain of unknown function | others | 200 | 385 | 151 | 352 |
| S7ZLI8 | Uncharacterized protein | Y | 419 | 45106 | Lactonase, 7-bladed beta-propeller | Carbon Metabolims | -1 | 15 | -1 | 17 |
| S8ALZ0 | Thioredoxin | N | 107 | 11811 | Thioredoxin | Growth and development | -1 | 8 | -1 | 4 |
| S7ZDT5 | Uncharacterized protein | Y | 405 | 43759 | SMP-30/Gluconolactonase/LRE-like region | Carbon Metabolims | -1 | 3 | 2 | 1 |
| S7ZM05 | Uncharacterized protein | Y | 470 | 53508 | Phosphoesterase | Growth and development | -1 | 1 | -1 | 8 |
| S7ZS92 | Putative alpha-L-arabinofuranosidase | Y | 636 | 69789 | Alpha-L-arabinofuranosidase C-terminal domain | Carbon Metabolims | -1 | -1 | 3 | -1 |
| S8AX25 | Catalase | Y | 730 | 79946 | Catalase | Growth and development | 10 | 13 | 41 | 6 |
| S7ZCF1 | Putative acetyl xylan esterase | Y | 426 | 44152 | CBM1 | Carbon Metabolims | 51 | -1 | 23 | -1 |
| S7ZVP1 | Uncharacterized protein | Y | 749 | 82405 | Multicopper oxidase | Growth and development | -1 | -1 | 9 | -1 |
| S8B698 | Uncharacterized protein | N | 575 | 63418 | Sugar (and other) transporter | Carbon Metabolims | -1 | 5 | -1 | -1 |
| S7ZLZ3 | Uncharacterized protein | N | 651 | 72296 | Calcineurin-like phosphoesterase | Growth and development | -1 | 2 | -1 | 1 |
| S7ZI49 | Chitin binding domain-containing protein | Y | 271 | 27628 | CBM18 | Carbon Metabolims | -1 | 3 | -1 | 3 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| S7ZBX8 | Putative alpha-L-rhamnosidase | Y | 679 | 74855 | Bacterial alpha-L-rhamnosidase 6 hairpin glycosidase domain | Carbon Metabolims | -1 | -1 | -1 | 2 |
| S7Z876 | Purple acid phosphatase | Y | 496 | 54007 | Calcineurin-like phosphoesterase | Growth and development | -1 | -1 | -1 | 3 |
| S7ZM49 | Uncharacterized protein | N | 1878 | 210745 | Zinc finger | Growth and development | -1 | -1 | -1 | 1 |
| S8B7P9 | Putative alpha-L-arabinofuranosidase | Y | 506 | 52547 | AbfB | Carbon Metabolims | 76 | 52 | 70 | 64 |
| S7Z892 | Uncharacterized protein | Y | 172 | 18.41 | Histidine phosphatase | Growth and development | 114 | 124 | 83 | 154 |
| S7ZV59 | Uncharacterized protein | Y | 489 | 52345 | FAD binding domain | Growth and development | 28 | 38 | 21 | 44 |
| S7Z989 | Uncharacterized protein | Y | 599 | 66366 | Multicopper oxidase | Growth and development | -1 | -1 | 11 | -1 |
| S7ZA56 | Uncharacterized protein | Y | 630 | 69.59 | Phosphoesterase | Growth and development | 9 | 149 | 11 | 201 |
| S8BD27 | Uncharacterized protein | Y | 535 | 59857 | A domain of unknown function | others | 8 | 28 | 9 | 44 |
| S8AUW0 | Uncharacterized protein | Y | 310 | 32215 | Ubiquitin 3 binding protein But2 C-terminal domain | Growth and development | 12 | 127 | 4 | 164 |
| S7ZCD7 | Carboxylic ester hydrolase | Y | 543 | 59618 | Carboxylesterase family | Carbon Metabolims | 11 | 56 | 3 | 60 |
| S7ZP61 | Uncharacterized protein | N | 109 | 12031 | Dimeric alpha+beta barrel superfamily | Carbon Metabolims | -1 | -1 | 3 | 1 |
| S8AY60 | Uncharacterized protein | N | 231 | 26104 | Complex I intermediate-associated protein 30 (CIA30)/cbm11 | Growth and development | -1 | -1 | -1 | 1 |
| S7ZDH7 | Uncharacterized protein | Y | 147 | 15.04 | Cerato-platanin | Growth and development | 32 | 121 | 39 | 175 |
| S7ZQW4 | Carboxylic ester hydrolase | Y | 566 | 62179 | Carboxylesterase family | Carbon Metabolims | 18 | 85 | 15 | 98 |
| S8B127 | Uncharacterized protein | N | 287 | 30855 | PLC-like phosphodiesterases superfamily | Growth and development | 23 | 94 | 9 | 112 |
| S8B672 | Uncharacterized protein | Y | 400 | 43684 | Aldose 1-epimerase | Carbon Metabolims | 14 | 36 | 25 | 37 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| S8B8Z7 | Uncharacterized protein | Y | 535 | 56222 | PAN 1/Schizosaccharomyces pombe repeat of unknown function (DUF963) | others | 4 | 6 | -1 | 7 |
| S7ZNW1 | Uncharacterized protein | Y | 467 | 50423 | PLC-like phosphodiesterases superfamily | Growth and development | 3 | 13 | 2 | 14 |
| S7Z822 | Plasma membrane ATPase | N | 992 | 109.3 | ATPase P-type | Growth and development | -1 | 10 | -1 | -1 |
| S7ZV14 | Uncharacterized protein | N | 578 | 61139 | Major Facilitator Superfamily | Carbon Metabolims | -1 | 3 | -1 | -1 |
| S7ZDW5 | Uncharacterized protein | Y | 193 | 19872 | WSC domain | Carbon Metabolims | -1 | 3 | -1 | 2 |
| S7ZU36 | Uncharacterized protein | N | 1205 | 126627 | Cortical protein marker for cell polarity (Rax2) | Growth and development | -1 | 4 | -1 | 7 |
| S8ANT9 | Uncharacterized protein | N | 513 | 55273 | PNGaseA | Growth and development | -1 | 1 | -1 | -1 |
| S7ZMN9 | Cytidine deaminase | N | 142 | 15219 | Cytidine deaminase | Protein related to DNA | -1 | 1 | -1 | 1 |
| S8B6T3 | Uncharacterized protein | N | 258 | 27881 | Ctr copper transporter family | Growth and development | -1 | 1 | -1 | -1 |
| S8B0M4 | Uncharacterized protein | N | 581 | 64613 | CoA-transferase family III | Growth and development | -1 | 1 | -1 | -1 |
| S8ANV1 | Uncharacterized protein | Y | 197 | 18651 | CFEM domain | Growth and development | 20 | 37 | 17 | 30 |
| S7ZM04 | Uncharacterized protein | Y | 392 | 43508 | Calcineurin-like phosphoesterase (Metallophos) | Growth and development | -1 | -1 | -1 | 2 |
| S7ZQE8 | Uncharacterized protein | N | 511 | 57091 | RibD C-terminal domain | Growth and development | -1 | 2 | -1 | 2 |
| S7ZQE7 | Uncharacterized protein | Y | 217 | 20.9 | CFEM domain | Growth and development | 120 | 242 | 77 | 251 |
| S7ZBT6 | Actin | N | 375 | 41711 | Actin | Growth and development | 6 | -1 | -1 | -1 |
| S8AKV1 | Uncharacterized protein | N | 209 | 24361 | Zinc finger C-x8-C-x5-C-x3-H type (zf-CCCH ) | Growth and development | 3 | -1 | -1 | -1 |
| S7ZMT5 | Uncharacterized protein | N | 633 | 69742 | Transmembrane protein | Growth and development | -1 | 6 | -1 | -1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S7ZX42 | Uncharacterized protein | N | 537 | 59639 | Sugar (and other) transporter | Carbon Metabolims | -1 | 1 | -1 | -1 |
| S8AR94 | Uncharacterized protein | Y | 381 | 41078 | Dioxygenase_C | Growth and development | -1 | -1 | 3 | -1 |
| S7Z758 | Uncharacterized protein | Y | 223 | 22281 | GPI-anchored | Growth and development | 9 | 16 | 5 | 25 |
| S8AYY9 | Uncharacterized protein | Y | 245 | 25747 | GPI-anchored | Growth and development | 6 | 7 | 3 | 11 |
| S7ZGV3 | Uncharacterized protein | Y | 267 | 26633 | GPI-anchored | Growth and development | 6 | 4 | -1 | 1 |
| S8B585 | Uncharacterized protein | Y | 227 | 22.52 | GPI-anchored | Growth and development | 15 | 21 | 13 | 24 |

**Table 6**. Uncharacterized proteins detected in EMS19 and WT in AVX and GLU secretomes

| | | | | | | **Spectrum count** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **MW** | | **EMS19-7** | | **Po** | |
| **Uniprot ID** | **Identified protein** | **SP** | **aa** | **(kDA)** | **Family** | **Ax** | **Glu** | **Ax** | **Glu** |
| S7ZGU9 | Uncharacterized protein | Y | 320 | 33,168 | Uncharacterized proteins | 90 | 78 | 37 | 66 |
| S7ZH04 | Uncharacterized protein | Y | 189 | 19,577 | Uncharacterized proteins | 21 | 92 | 24 | 118 |
| S7ZIH0 | Uncharacterized protein | Y | 225 | 23,379 | Uncharacterized proteins | 16 | 63 | 9 | 74 |
| S7Z789 | Uncharacterized protein | Y | 161 | 16,905 | Uncharacterized proteins | 10 | 7 | 18 | 11 |
| S7ZWZ8 | Uncharacterized protein | N | 265 | 29,339 | Uncharacterized proteins | 5 | 3 | -1 | 1 |
| S8AKL5 | Uncharacterized protein | Y | 95 | 10,079 | Uncharacterized proteins | 1 | -1 | 3 | 1 |
| S8B6N4 | Uncharacterized protein | Y | 521 | 58,306 | Uncharacterized proteins | -1 | 2 | -1 | 2 |
| S7ZFF5 | Uncharacterized protein | N | 111 | 11,981 | Uncharacterized proteins | 1 | -1 | -1 | -1 |
| S7ZQH1 | Uncharacterized protein | Y | 272 | 27,613 | Uncharacterized proteins | -1 | 1 | -1 | 1 |
| S8AX52 | Uncharacterized protein | N | 126 | 13.27 | Uncharacterized proteins | -1 | 2 | -1 | 4 |
| S7Z615 | Uncharacterized protein | N | 158 | 17.43 | Uncharacterized proteins | 13 | 64 | 16 | 67 |
| S8B504 | Uncharacterized protein | N | 336 | 36,605 | Uncharacterized proteins | -1 | 7 | -1 | 7 |
| S7Z620 | Uncharacterized protein | Y | 509 | 54,683 | Uncharacterized proteins | -1 | 2 | -1 | -1 |
| S8ALV6 | Uncharacterized protein | Y | 138 | 14,973 | Uncharacterized proteins | 1 | 3 | 5 | 4 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S7Z6W8 | Uncharacterized protein | N | 492 | 53,948 | Uncharacterized proteins | -1 | 3 | -1 | -1 |
| S7ZG94 | Uncharacterized protein | Y | 89 | 9,022 | Uncharacterized proteins | 1 | -1 | 2 | 1 |
| S8AWN9 | Uncharacterized protein | N | 173 | 18.42 | Uncharacterized proteins | -1 | -1 | 2 | -1 |
| S7ZNE7 | Uncharacterized protein | Y | 240 | 25,146 | Uncharacterized proteins | 5 | 15 | 3 | 16 |
| S7ZLU9 | Uncharacterized protein | Y | 381 | 41,043 | Uncharacterized proteins | 2 | -1 | 5 | -1 |
| S7ZL93 | Uncharacterized protein | Y | 456 | 50 | Uncharacterized proteins | -1 | 24 | -1 | 29 |
| S7ZPP9 | Uncharacterized protein | Y | 301 | 31,777 | Uncharacterized proteins | -1 | 5 | -1 | 6 |
| S8B129 | Uncharacterized protein | Y | 183 | 18,368 | Uncharacterized proteins | -1 | 1 | -1 | -1 |
| S7ZPQ0 | Uncharacterized protein | Y | 404 | 43,978 | Uncharacterized proteins | -1 | 1 | -1 | -1 |
| S7ZGP5 | Uncharacterized protein | Y | 357 | 38,129 | Uncharacterized proteins | 6 | -1 | 26 | -1 |
| S8AQ73 | Uncharacterized protein | Y | 204 | 20.49 | Uncharacterized proteins | 5 | 6 | 5 | 8 |
| S8B9U0 | Uncharacterized protein | Y | 195 | 20,011 | Uncharacterized proteins | 1 | 7 | 4 | 6 |
| S8B1L6 | Uncharacterized protein | Y | 136 | 14,795 | Uncharacterized proteins | -1 | 3 | -1 | 6 |
| S7ZC90 | Uncharacterized protein | Y | 151 | 16,195 | Uncharacterized proteins | -1 | 3 | -1 | 10 |
| S8BBA3 | Uncharacterized protein | Y | 145 | 15,141 | Uncharacterized proteins | 2 | 201 | 12 | 511 |
| S8AXT8 | Uncharacterized protein | Y | 177 | 16,652 | Uncharacterized proteins | 21 | 23 | 12 | 19 |
| S8ALG7 | Uncharacterized protein | N | 798 | 85,322 | Uncharacterized proteins | 19 | 4 | 5 | -1 |
| S7ZH82 | Uncharacterized protein | Y | 180 | 18,918 | Uncharacterized proteins | 5 | 6 | 4 | 6 |
| S7ZUG0 | Uncharacterized protein | Y | 166 | 17,969 | Uncharacterized proteins | 12 | 45 | 8 | 53 |
| S8AWZ5 | Uncharacterized protein | N | 160 | 15,888 | Uncharacterized proteins | 6 | 10 | 3 | 11 |
| S7Z604 | Uncharacterized protein | Y | 210 | 21,267 | Uncharacterized proteins | 12 | 4 | -1 | 13 |
| S7ZLE5 | Uncharacterized protein | N | 837 | 85,681 | Uncharacterized proteins | 5 | -1 | -1 | -1 |
| S8ASE7 | Uncharacterized protein | Y | 147 | 15,715 | Uncharacterized proteins | -1 | 2 | 1 | 8 |
| S7ZTB9 | Uncharacterized protein | N | 293 | 28,911 | Uncharacterized proteins | -1 | 2 | -1 | 4 |
| S7ZM28 | Uncharacterized protein | Y | 216 | 23,142 | Uncharacterized proteins | 6 | 5 | 6 | 7 |
| S8BB55 | Uncharacterized protein | Y | 462 | 47,608 | Uncharacterized proteins | -1 | 1 | -1 | 2 |
| S8BA22 | Uncharacterized protein | Y | 212 | 24,186 | Uncharacterized proteins | -1 | -1 | -1 | 3 |
| S7ZLD1 | Uncharacterized protein | Y | 166 | 18,162 | Uncharacterized proteins | 14 | 57 | 4 | 82 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S7ZAL8 | Uncharacterized protein | Y | 115 | 12,377 | Uncharacterized proteins | -1 | 39 | 4 | 49 |
| S7ZBF9 | Uncharacterized protein | Y | 237 | 25,806 | Uncharacterized proteins | 12 | 43 | 20 | 55 |
| S7ZSU2 | Uncharacterized protein | Y | 460 | 50.48 | Uncharacterized proteins | -1 | 29 | -1 | 18 |
| S8A111 | Uncharacterized protein | Y | 243 | 26,247 | Uncharacterized proteins | -1 | 3 | -1 | -1 |

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# 6. ANEXOS

## 6.1 CIBio-IB/UNICAMP

UNICAMP

Of. CIBio/IB 35/2010

Cidade Universitária "Zeferino Vaz", 16 de agosto de 2011.

**Prof. Dr. MARCELO BROCCHI**
Chefe do Departamento Genética, Evolução e Bioagantes
Instituto de Biologia - UNICAMP

Prezado Professor:

Informamos que o projeto abaixo relacionado, envolvendo OGM do tipo I, sob responsabilidade do **Prof. Dr. GONÇALO A. G. PEREIRA**, protocolado sob o número **2011/03**, foi aprovado pela **CIBio-IB/UNICAMP**, em reunião sua 55ª. ordinária (15/08/2011) para ser desenvolvido nas dependências do Departamento Genética, Evolução e Bioagantes, Laboratório de Genômica e Expressão:

| No. Projeto (data da aprovação) | Data de recepção | Nome do Projeto | Prazo para envio de relatório à CIBio |
|---|---|---|---|
| 2011/03 (15/08/2011) | 19/07/2011 | Genômica e Biotecnologia, sub-projetos: 1) 2010/01 - Projeto Gene Discovery em Eucalipto; 2) Rotas Verdes para o Propeno, 3) Modificação de linhagens industriais de Saccharomyces cerevisiae para o aumento da produtividade e floculação condicional., 4) Projeto genoma de Crinipellis perniciosa, fungo causador da doença vassoura-de-bruxa do cacau, 5) Cultivo de microalgas para produção de cadeias carbônicas lipídicas, e 6) Transformação genética de cana-de-açúcar com o gene do inibidor de ripsina de inga laurina e análise da toxidade das plantas transgênicas sobre o desenvolvimento biológico de Diatraea saccharalis | Fevereiro/2012 |

Recomendamos que sejam observadas as instruções normativas referentes transporte e contenção da OGMs, disponíveis na webpage da CTNBio <www.ctnbio.gov.br>.

Atenciosamente,

Marcelo Lancellotti
Presidente da CIBio/IB-UNICAMP

C/C.: Prof. Dr. Gonçalo A. G. Pereira

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## 6.2 Direitos Autorais

**Declaração**

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Incremento do potencial (hemi)celulolítico de Penicillium oxalicum aplicado à desconstrução de biomassas**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 25 de agosto de 2020

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