MAÍRA NICOLAU DE ALMEIDA

CELULASES E HEMICELULASES DE ESPÉCIES DE *Acremonium* ENDOFÍTICOS

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

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APROVADA: 27 de janeiro de 2009

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Profª. Valéria Monteze Guimarães	Prof. Olinto Liparini Pereira
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(Orientador)

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BIOGRAFIA

MAÍRA NICOLAU DE ALMEIDA, filha de Marcos Belmiro de Almeida e Myriam Ruth Nicolau de Almeida, nasceu na cidade de São João Del Rei, Minas Gerais, em 16 de agosto de 1983.

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RESUMO

ALMEIDA, Maíra Nicolau, M. Sc., Universidade Federal de Viçosa, janeiro de 2009. C**elulases e hemicelulases de espécies endofíticas de *Acremonium*.** Orientador: Sebastião Tavares de Rezende. Coorientadores: Valéria Monteze Guimarães e Olinto Liparini Pereira.

Duas espécies do fungo *Acremonium* (*Acremonium zeae* EA0802 e *Acremonium* sp. EA 0810) foram estudadas quanto ao potencial de produção de celulases com atividade de hidrólise de papel de filtro (FPases), endoglicanases, β-glicosidases, xilanases, α-galactosidases, α-arabinofuranosidases e β-xilosidases. Os fungos foram cultivados em cultura submersa (CS) contendo L-arabinose, D-xilose, xilana oat spelt, bagaço de cana ou palha de milho como fonte de carbono e alíquotas diárias foram analisadas em relação à produção enzimática durante 23 dias. Também foi verificada a produção de enzimas por estes fungos em fermentação no estado sólido (FES) utilizando como fonte de carbono e suporte bagaço de cana ou palha de milho sendo as análises realizadas em intervalos de 3 dias durante 39 dias. As maiores quantidades de FPase, endoglicanase e xilanase foram produzidas quando

Acremonium sp. EA0810 e *Acremonium zeae* EA0802 foram cultivados em cultura submersa com bagaço de cana e palha de milho como fonte de carbono. *Acremonium* sp. EA0810 destacou-se na produção de β-glicosidase quando cultivado em cultura submersa contendo D-xilose como fonte de carbono. *Acremonium zeae* EA0802 apresentou maiores atividades das enzimas α-arabinofuranosidase e α-galactosidase quando cultivado em cultura submersa contendo xilana como fonte de carbono. FPase, endoglicanase, β-glicosidase e xilanase do fungo *Acremonium* sp. EA0810 apresentaram pH e temperatura ótimos de 6.0, 55 °C; 5.0, 70 ºC; 4.5, 60 °C e 6.5, 50 °C respectivamente. α-Arabinofuranosidase e α-galactosidase de *Acremonium zeae* EA0802 apresentaram pH e temperatura ótimos de 5.0, 60 °C e 4.5, 45 °C, respectivamente. Extratos enzimáticos contendo atividade de endoglicanase e xilanase foram analisados por zimogramas revelando a presença de apenas uma forma de cada uma destas enzimas. As características bioquímicas da enzima endoglicanase foram adequadas para uso industrial.

ABSTRACT

ALMEIDA, Maíra Nicolau, M. Sc., Universidade Federal de Viçosa, january 2009. C**ellulases and hemicellulases from endophytic *Acremonium* species.** Adviser: Sebastião Tavares de Rezende. Co-advisers: Valéria Monteze Guimarães and Olinto Liparini Pereira.

Two *Acremonium* species (*Acremonium zeae* EA0802 and *Acremonium* sp. EA0810) were examined in relation to their ability to produce cellulases (FPases), endoglucanases, β-glucosidases, xylanases, α-galactosidases, α-arabinofuranosidases and β-xylosidases in different carbon sources. The fungi were cultivated in submerged culture containing L-arabinose, D-xylose, oat spelt xylan, sugar cane bagasse or corn straw as a carbon source and aliquots were analyzed daily for a period of 23 days to verify enzyme production. Enzyme production in solid state fermentation utilizing sugar cane bagasse or corn straw as a carbon source and support was also tested. In this case, enzyme production was analyzed at 3 day intervals for a period of 39 days. The highest FPase, endoglucanase and xylanase activities were obtained when *Acremonium* sp. EA0810 and *Acremonium zeae* EA0802 were cultivated in submerged culture containing sugar cane bagasse and

corn straw as a carbon source. *Acremonium* sp. EA0810 show the highest β-glucosidase activity when it was cultivated in submerged culture using D-xylose as carbon source. *Acremonium zeae* EA0802 exhibit greatest α-arabinofuranosidase and α-galactosidase activities when it was cultivated in submerged culture utilizing xylan as a carbon source. FPase, endoglucanase, β-glucosidase and xylanase from *Acremonium* sp. EA0810 were found to have optimum pH and temperatures of 6.0, 55 °C; 5.0, 70 ºC; 4.5, 60 °C and 6.5, 50 °C, respectively. α-Arabinofuranosidase and α-galactosidase from *Acremonium zeae* EA0802 have optimum pH and temperatures of 5.0, 60 °C and 4.5, 45 °C, respectively. An enzymatic extract containing endoglucanase and xylanase activities was submitted to a zymogram analyze and one form of each enzyme was detected. Endoglucanase biochemical characteristics are appropriate for industrial application.

CAPÍTULO 1

1.1 - INTRODUÇÃO

Um dos maiores desafios do século XXI é atender à grande demanda por energia principalmente para transporte e processos industriais que utilizam energia proveniente de combustíveis fósseis. A crescente preocupação com a segurança do abastecimento de óleo tem sido evidente pelo aumento do preço do petróleo. Mais importante do que estes fatores econômicos é a preocupação de um desenvolvimento sustentável e para isso, o abastecimento de energia futuro deve vir acompanhado de uma substancial redução da emissão de gases causadores do efeito estufa.

Dados estes fatores, os biocombustíveis de fontes renováveis, como o etanol, têm sido apontados como solução viável. A produção de etanol, já foi introduzida em escala industrial principalmente no Brasil e nos Estados Unidos. Este biocombustível já é comercializado em preços competitivos, além disso, tecnologias como as dos carros bicombustíveis tendem a aumentar ainda mais a sua utilização.

Entretanto, as fontes atuais de etanol como cana-de-açúcar e grãos de milho apresentam a desvantagem de serem itens utilizados para alimentação humana e de

animais, além de necessitarem de terras férteis e de uso de agrotóxicos para seu cultivo.

Neste contexto, a biomassa ligninocelulósica apresenta enorme potencial para contribuir com esta tecnologia devido à grande disponibilidade e baixo custo da matéria prima. A produção de etanol a partir de resíduos desta natureza pode aumentar consideravelmente a produção do combustível sem, contudo, aumentar a área plantada. Porém, apesar de oferecer muitas vantagens, a utilização de biomassas ligninocelulósicas não é uma realidade porque a tecnologia para sua utilização ainda não está totalmente desenvolvida.

Para produção de etanol a partir de biomassa ligninocelulósicas é necessário primeiramente disponibilizar seus açúcares para que possam ser fermentados por microorganismos. A degradação dessa biomassa tem sido feita principalmente por processos físicos e químicos que podem gerar inibidores da fermentação. Como solução para este problema tem sido estudada a hidrólise da biomassa por enzimas celulases e hemicelulases, que juntamente com tratamento químicos adequados disponibilizam a maior parte dos açúcares para fermentação e produção de álcool. Um fator limitante para a ampliação desta tecnologia é o alto custo das enzimas, portanto, a busca por novas fontes de enzimas capazes de contribuir para este processo é de grande interesse e tem muito apelo sócio-econômico.

As enzimas celulases e hemicelulases que atuam na hidrólise da biomassa são utilizadas também em processos de diversos setores industriais. Entre estes, destacam-se a indústria têxtil, de papel e celulose, alimentícia e de alimentação animal.

O objetivo deste trabalho é a busca por enzimas fúngicas com alto poder hidrolítico utilizando como fonte duas espécies do gênero *Acremonium.* Também é

nosso objetivo a caracterização bioquímica destas enzimas para avaliar os seus potenciais para utilização industrial.

1.2 - REVISÃO BIBLIOGRÁFICA

1.2.1 - A Biomassa Ligninocelulósica

A biomassa ligninocelulósica é o principal recurso renovável disponível no meio ambiente. É composto por microfibrilas de celulose inseridas em uma matriz de polissacarídeos. Essa matriz é composta por dois tipos de polissacarídeos, chamados de hemiceluloses e pectina, e uma pequena quantidade de proteínas estruturais e lignina (Taiz & Zeiger, 2004, Sánchez & Cardona, 2008).

As hemiceluloses, também chamadas de glicanas de ligação cruzada, fazem ligações de hidrogênio com as microfibrilas de celulose envolvendo-as em uma rede. Os polímeros de carboidrato que fazem parte das hemiceluloses são compostos principalmente pelos açúcares D-glicose, D-manose, D-galactose, D-xilose, L-arabinose e D-ácido glicurônico (Rowell, et al., 2005).

A lignina é um composto formado por fenilpropanóides depositado durante a maturação da parede celular. Este composto faz ligações cruzadas com os

polissacarídeos da parede auxiliando no seu empacotamento. O complexo formado por lignina, celulose e hemicelulose é denominado ligninocelulose (Jeffries, 1994; Buchanan et al., 2000). Uma representação das estruturas é apresentada na figura 1.

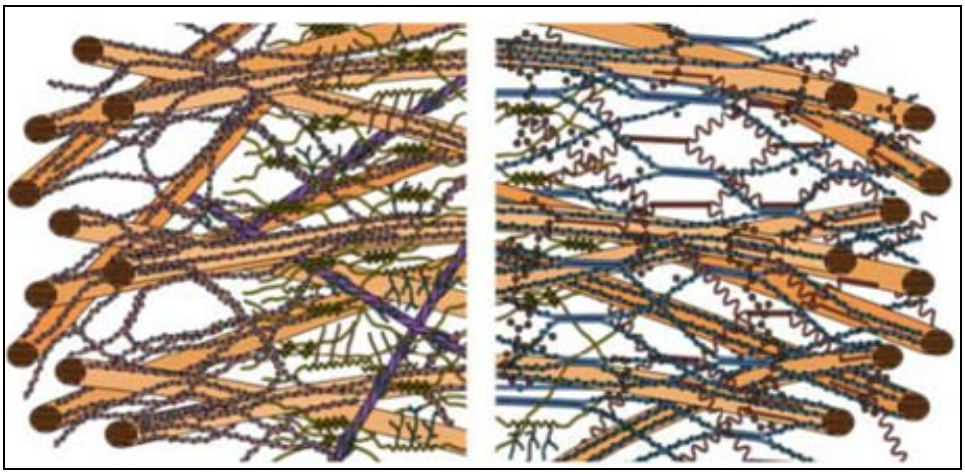

Figura 1: Ilustração das estruturas que compõem a parede celular. Os cilindros alaranjados representam as microfibrilas de celulose, azuis as hemiceluloses e os pequenos círculos vermelhos escuro representam a lignina

Fonte: Buchanan et al., 2000

Nos tópicos subseqüentes será enfatizada a composição e a degradação enzimática dos componentes da biomassa ligninocelulósica.

1.2.1.1 - Celulose

Celulose é o composto mais abundante nos lignocelulósicos, sendo um polímero linear formado exclusivamente por moléculas D-glicopiranose unidas por ligações glicosídicas β- (1 → 4) (de Azevedo & Esposito, 2004). Estudos de dispersão de luz determinaram o peso molecular da celulose, indicando que a celulose possui um grau de polimerização médio de 9000 - 10000 podendo em alguns casos atingir números muito maiores do que este. Moléculas de celulose são orientadas ao acaso e têm a tendência de formar ligações de hidrogênio inter e

intramolecular (Rowell, et al.,2005). Os modelos atuais da organização microfibrilar sugerem que ela tem uma subestrutura constituída de domínios altamente cristalinos unidos por ligações amorfas (Taiz & Zeiger, 2004). Regiões cristalinas são formadas a partir do aumento da densidade de empacotamento.

Existem celuloses acessíveis e não acessíveis. A acessibilidade refere-se a interações de hidrogênio intra e intermoleculares. Quanto mais acessíveis, mais interações podem acontecer entre a celulose e fatores diversos como água, microorganismos, etc. As superfícies da celulose cristalina são muito acessíveis, porém o resto da estrutura não é. A maioria das celuloses não cristalinas seria acessível, porém como essa estrutura é coberta por hemicelulose e lignina, sua acessibilidade passa a ser limitada (Rowell, et al., 2005).

1.2.1.2 - Degradação Enzimática da Celulose

Para a degradação completa da celulose são necessárias as atuações sinergísticas de quatro tipos de celulases. As endoglucanases (1,4- β -D- glicana-4-glicanohidrolase; EC 3.2.1.4) hidrolisam ligações β- (1 $\rightarrow$ 4) internas da celulose (Lynd, et al., 2002). Essas enzimas são inativas sobre a celulose cristalina como algodão, mas elas hidrolisam celulose amorfa e substratos solúveis como carboximetilcelulose e hidroxietilcelulose. A atividade de endoglucanases é caracterizada por hidrólise aleatória de ligações β-glicosídicas, resultando em uma rápida diminuição da viscosidase acompanhada da taxa de aumento de grupos redutores. Os produtos incluem glicose, celobiose e celodextrinas de vários tamanhos.

O segundo grupo de enzimas são as celobiohidrolases (1,4-β-D-glicana celobiohidrolase; EC 3.2.1.91), que são exocelulases. Este grupo degrada celulose amorfa por remoção consecutiva de celobiose dos terminais não redutores do polissacarídeo. Quando puras podem degradar Avicel, porém o mesmo não acontece com algodão. Em relação às endoglicanases, as celobiohidrolases apresentam uma maior capacidade de redução da viscosidade. Celobiohidrolases e endoglicanases atuam sinergísticamente para degradação completa da celulose cristalina.

O terceiro grupo de enzimas são as exoglicohidrolases (1,4-β-D-glicana glicobiohidrolase, EC 3.2.1.74), que hidrolisam consecutivamente a remoção de unidades de glicose de terminais não redutores de celodextrinas. A taxa de hidrólise diminui proporcionalmente à diminuição do tamanho da cadeia.

O quarto grupo de enzimas são as β-glicosidases (β-D-glicosideoglicohidrolase; EC 3.2.1.21) que clivam celobiose em glicose e removem glicose de terminais não redutores de pequenas celodextrinas. Ao contrário das exoglicosidases, a taxa de hidrólise das celobiases aumenta proporcionalmente à diminuição do tamanho do substrato. As β-glicosidases e as exoglicohidrolases têm em comum os substratos de cadeias de glicose de 2 até 6 unidades. Elas podem ser distinguidas baseadas na suas atividades relativas sobre os dois substratos celobiose e celohexose. As β-glicosidases hidrolisam muito mais rapidamente a celobiose do que as celobiohexoses, enquanto o oposto ocorre com as exoglicohidrolases (Whitaker, 1994).

As β-glicosidases têm um papel fundamental para a degradação de materiais lignocelulósicos. A celobiose, principal substrato para a β-glicosidase é um potente inibidor de exocelulases, portanto a ausência dessa enzima diminui o potencial do processo de sacarificação de biomassas. Han & Chen (2008) mostraram que a adição

de β-glicosidase de sabugo de milho durante a sacarificação e fermentação desta mesma biomassa diminuiu a concentração de celulose em 28 % e a produção de etanol aumentou cerca de 22 %.

Os sistemas de celulases mais eficientes não são secretados livremente e sim são encontrados acoplados à superfície de microorganismos anaeróbicos em estruturas macromoleculares chamadas celulossomos. Essas estruturas são constituídas por enzimas hidrolíticas assim como proteínas estruturais, que ancoram o celulossomo fora da célula. Essa estrutura é complexa e dinâmica e auxilia a interação sinergística entre as enzimas. Entretanto, celulases livres podem ser produzidas por fungos e bactérias aeróbicos. Apesar de enzimas bacterianas serem mais eficientes, as enzimas fúngicas como as de *Trichoderma reesei* dominam as aplicações industriais devido ao seu alto nível de expressão e secreção. Investimentos têm sido realizados visando o desenvolvimento de coquetéis de celulases especializadas e de baixo custo (Gaudin, et al., 2000; Chang, 2007).

1.2.1.3 - Lignina

Lignina é um composto que está covalentemente ligado à celulose e outros polissacarídeos da parede celular (Taiz & Zeiger, 2004). Essa estrutura empacota as microfibrilas de polissacarídeos protegendo-os contra a atividade hidrolítica de enzimas e outros fatores externos e estabiliza o complexo lignocelulose (Leonowicz, et al., 1999). A lignina é composta basicamente de unidade de fenilpropanóides ramificados dispostas aleatoriamente formando uma macromolécula tridimensional e amorfa. (de Azevedo & Esposito, 2004). Em geral é composta por três diferentes alcoóis de fenilpropanóides: coniferil, cumaril, e sinapil, que são sintetizados a partir

de fenilalaninas através de vários derivados de ácido cinâmico (figura 2). Esses alcoóis formam um polímero por ação enzimática (Taiz & Zeiger, 2004).

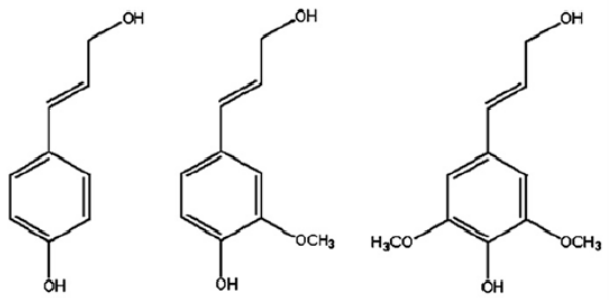

Figura 2: estruturas dos compostos fenólicos precursores de lignina: cumaril, coniferil e sinapil
Fonte: Suhas, et al., 2007

A estrutura precisa da lignina não é conhecida devido à dificuldade para sua extração das plantas, mas algumas estruturas hipotéticas são propostas. Uma destas estruturas é proposta na figura 3.

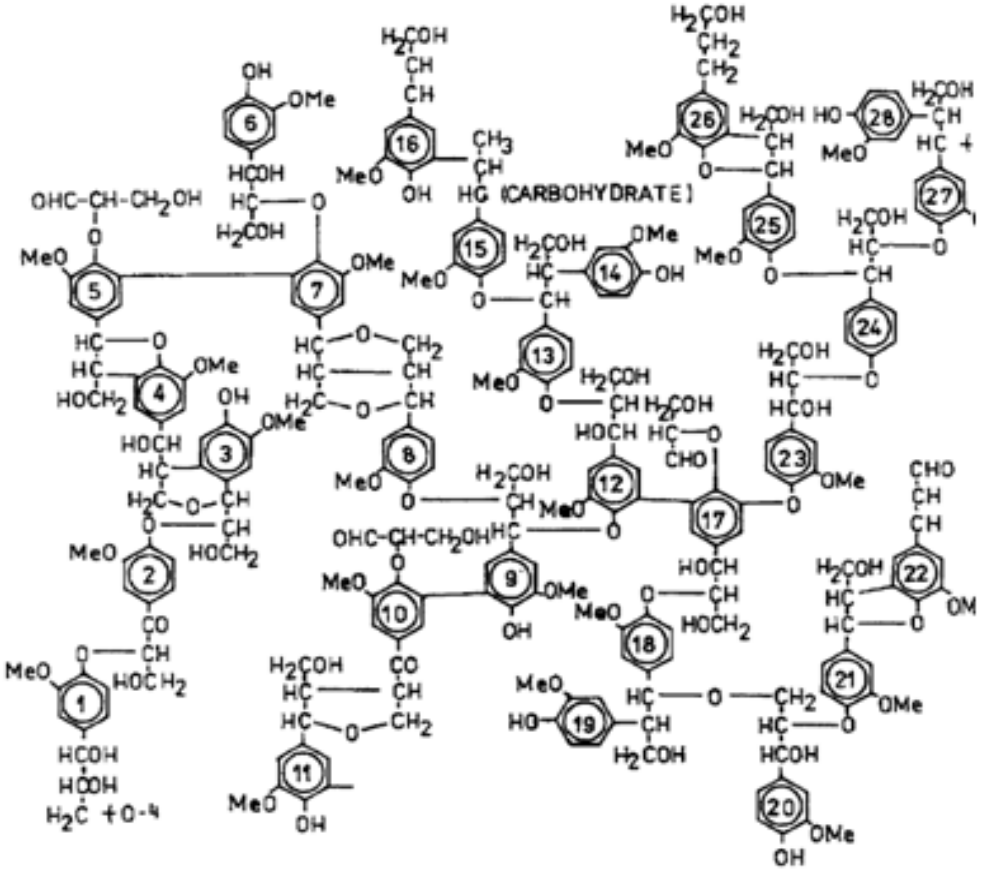

Figura 3: Estrutura hipotética para a lignina
Fonte: Leonowicz, et al., 1999

1.2.1.4 - Degradação Enzimática da Lignina

A lignina é degradada na natureza por fungos de podridão branca, na sua maioria Basidiomicetos e alguns Ascomicetos. Estes microorganismos produzem as enzimas lacases, manganês peroxidase e lignina peroxidase. Essas são as três enzimas mais estudadas na degradação da lignina e o processo completo realizado por estes fungos depende de suas ações sinergísticas (Tuomela, et al., 2000).

1.2.1.5 - Hemiceluloses

Hemiceluloses compreendem um grupo de polissacarídeos compostos principalmente pelos carboidratos D-xilopiranose, D-glicopiranose, D-galactopiranose, L-arabinofuranose, D-manopiranose, D-acído glicurônico e D-galacturônico e quantidades menores de outros açúcares (Rowell, et al., 2005). As unidades de pentoses ou hexoses são unidas por ligações β-(1 $\rightarrow$ 4) com pontos de ramificações (1 $\rightarrow$ 2), (1 $\rightarrow$ 3) e/ou (1 $\rightarrow$ 6) (Leonowicz, et al., 1999). No geral, as hemiceluloses apresentam um baixo grau de polimerização (média de 100 - 200) e a qualidade e quantidade de açúcares presentes nas hemiceluloses depende do tipo de parede celular e do organismo analisado (Rowell, et al., 2005).

Estes polímeros são classificados de acordo com sua composição, por exemplo, galactoglicomanana (heteropolímero de galactose, glicose e manose) arabinoglicuronoxilana (polímero de xilose com ramificações de ácido D-glucurônico ou arabinose), arabinogalactana (heteropolímero de arabinose e galactose), glicomanana (heteropolímero de glicose e manose), etc. As hemiceluloses também contêm como substituintes grupos acetil, ácido ferúlico e metil (Collins et al., 2005; Rowell, et al.,2005).

1.2.1.6 - Degradação Enzimática da Hemicelulose

Devido à grande complexidade e heterogeneidade da rede hemicelulolítica a sua hidrólise completa requer a atuação de várias enzimas que atuam cooperativamente. Quais enzimas serão necessárias irá depender da composição da biomassa a ser degradada. Para degradação de xilanas em geral são necessárias basicamente seis enzimas diferentes. Endo-1,4-β-D-Xilanases (EC 3.2.1.8) são enzimas que clivam aleatoriamente o esqueleto de arabinoxilana produzindo principalmente oligossacarídeos de xilose. É uma das principais enzimas envolvidas na degradação deste polímero. β-xilosidases (EC 3.2.1.37) catalisam a hidrólise de xilooligossacarídeos e xilobiose a partir de terminais não redutores liberando xilose. Esta enzima é essencial para a degradação completa da arabinoxilana em xilose. Têm uma importante atuação porque diminui a concentração de xilobiose que é um inibidor das endoxilanases. É atualmente classificada nas famílias 39, 43 e 52 da glicosilhidrolases, baseada nas suas seqüências de aminoácidos (Saha, 2003; Collins, 2005).

A remoção das cadeias laterais deste polímero requer a enzima específica para o grupo a ser hidrolisado. α-L-Arabinofuranosidase, E.C. 3.2.1.55, é uma exoenzima que hidrolisa terminais não redutores de polissacarídeos contendo resíduos L-arabinosil de cadeias laterais ou de arabinoxilanas, arabinana, goma arábica e arabinogalactana. Essas enzimas podem hidrolisar as ligações arabinosil α- (1 → 3) e (1 → 5) destes substratos (Saha, 2000). α-Galactosidases (EC 3.2.1.22) removem resíduos ligados por α (1 → 6) e α (1 → 3) além de atuar juntamente com manosidases, mananases e outras enzimas para degradação de polímeros como arabinogalactana e galactoglicomanana (Moreira & Filho, 2008). Outras enzimas

acessórias são α-D-glicuronidases (EC 3.2.1.139), que hidrolisam resíduos laterais de ácido glicurônico, acetilxilana esterases (EC 3.1.1.72), que hidrolisam grupos acetil e ácido ferúlico esterases (EC 3.1.1.73), hidrolisam resíduos de ácido ferúlico (Jeffries, 1994). A figura 4 ilustra um polímero e as enzimas que atuam para sua degradação.

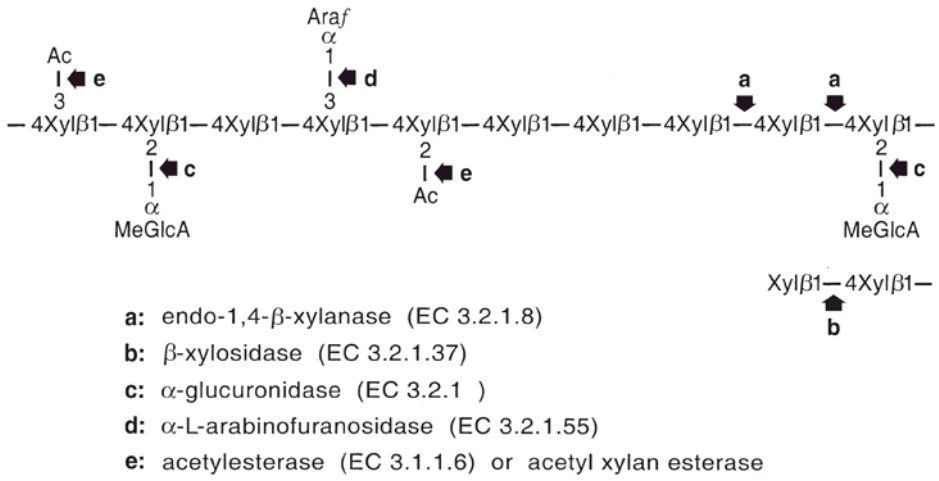

Figura 4: Polímero de xilana com indicações de pontos de clivagem enzimática

Fonte: Saha, 2000

1.2.2 - Aplicações Industriais das Celulases e Hemicelulases

1.2.2.1 - Produção de Etanol de Segunda Geração

O aumento global do uso de energia, juntamente com o aumento nos preços do óleo e do gás natural e a alta instabilidade do mercado de energia, tem destacado a importância do desenvolvimento de tecnologias para o aproveitamento de fontes de energia renováveis (Chang, 2007, Champagne, 2007).

A única fonte renovável de carbono grande o suficiente para substituir os combustíveis fósseis é a biomassa. Materiais lignocelulósicos são particularmente atraentes neste contexto devido ao seu relativo baixo custo. O impedimento

tecnológico central para a não utilização dessa importante fonte é a falta de tecnologia de baixo custo para sua degradação (Lynd, et al., 2002).

Muitas investidas têm sido realizadas para possibilitar a degradação deste material complexo em açúcares simples e fermentáveis para produção de etanol. A degradação da celulose pelas celulases é um processo muito bem estudado e a atuação destas enzimas é afetada pela porosidade e cristalinidade da celulose além do conteúdo de lignina e hemicelulose da biomassa (Balat, 2008).

Para possibilitar a remoção da lignina e hemicelulose, e também aumentar a superfície de contato da biomassa para atuação das celulases foram desenvolvidos diversos pré-tratamentos mecânicos, físico-químicos e químicos.

A trituração da biomassa é um tratamento mecânico que reduz a cristalinidade da celulose. Após este processo o tamanho da partícula reduz de 10 - 30 mm até a 0,2 -2 mm, aumentando assim a superfície de contato do material. A pirólise, um tratamento que consiste em submeter a biomassa a altas temperaturas (300 °C) converte entre 80 - 85 % da celulose em açúcares redutores (Sun & Cheng, 2002). Estes processos podem ser aliados a outros tratamentos para a degradação completa da biomassa.

Dentre os tratamentos químicos e físico-químicos estão a hidrólise com ácido diluído e a explosão da fibra com amônia. O tratamento com ácido tem mostrado resultados satisfatórios para degradação de materiais ligninocelulolíticos, entretanto são produzidos compostos tóxicos, como ácidos orgânicos, furfural e hidroximetilfurfural, que podem inibir a atuação de microorganismos fermentadores, além de necessitar de um ajuste de pH anterior à fermentação (Saha, 2000). A explosão da fibra por amônia (AFEX - ammonia fiber explosion) consiste na exposição da biomassa à amônia líquida em altas temperatura e pressão (Sun &

Cheng, 2002). Esta técnica solubiliza hemiceluloses produzindo oligossacarídeos e não produz subprodutos tóxicos para microorganismos fermentadores, podendo ser utilizada anterior a um tratamento enzimático para aumentar sua eficiência (Saha, 2000).

A sacarificação, realizada após o pré tratamento, consiste na conversão enzimática dos polissacarídeos em açúcares fermentáveis. A celulose é hidrolisada inicialmente pelas endoglicanases, que clivam aleatoriamente o polissacarídeo criando terminais livres para a atuação das exocelulases. Essas enzimas atuam sobre as cadeias de celulose liberando glicoses e celobioses. A última enzima necessária é a β-glicosidase que cliva celobioses em duas moléculas de glicose (Hahn-Hägerdal, 2006). Esta enzima é essencial para a sacarificação, pois celobiose é um inibidor das atividades de exocelulases. Para aumentar o rendimento do processo, evitando inibições por glicoses e celobioses presentes no meio, o processo de sacarificação e fermentação simultâneas tem sido utilizado. Nesta técnica os açúcares liberados pela sacarificação são prontamente fermentados por microorganismos, e assim não acumulam no meio evitando a inibição das atividades enzimáticas (Balat, 2008).

Para otimizar o processo foi estudada a expressão heteróloga dessas enzimas em microorganismos etanologênicos selvagens ou engenheirados, como *Saccharomyces cerevisiae*, *Escherichia coli* e *Klebsiella oxytoca*. O objetivo deste trabalho foi possibilitar a hidrólise da biomassa pelo mesmo organismo utilizado na fermentação, favorecendo assim a sacarificação e fermentação simultânea (Lynd, et al., 2002).

A expressão das celulases dentro da própria biomassa também é uma alternativa para facilitar o processo de produção de etanol. Neste sentido foi realizada a expressão de genes codificantes do domínio catalítico da endoglucanase

termoestável de *Acidothermus cellulolyticus* em *Arabidopsis* (Del Campillo, 1999). Porém algumas considerações devem ser feitas sobre esses estudos. A primeira é se as enzimas dentro da biomassa suportariam as condições severas de pH e temperatura necessárias no pré tratamento, a segunda é se o nível de expressão é suficiente para a hidrólise de toda a biomassa e a terceira é se a produção de enzimas heterólogas pode causar prejuízos para o desenvolvimento da planta (Lynd, et al., 2002).

Uma alternativa estudada é a redução dos teores de lignina da biomassa. Nestes casos devem ser analisados se essa modificação não alteraria o sistema de defesa da planta contra patógenos e insetos. O aumento do teor de carboidratos da biomassa também é uma alternativa genética para aumentar a eficiência dos processos fermentativos (Sticklen, 2006).

As hemicelulases são boas promessas para sacarificação de vários resíduos agroindustriais pré-tratados. Elas podem ser utilizadas posteriormente à pré-tratamentos para possibilitar a degradação da hemicelulose em monossacarídeos fermentáveis, possibilitando o aumento do rendimento da produção de etanol. Entretanto, para que pentoses, como xilose e arabinose, liberadas das hemiceluloses sejam fermentadas é necessário o desenvolvimento de microorganismo que fermentem estes açúcares com eficiência através de técnicas de engenharia metabólica (Hahn-Hägerdal, et al., 2006) Várias tentativas têm sido realizadas para superar este obstáculo, como introduzir genes para o metabolismo fermentador de pentoses em organismos etanologênicos, ou minimizar a suscetibilidade de organismos naturalmente fermentadores de pentose a inibidores gerados nos pré tratamentos, além de outras tentativas por métodos de engenharia metabólica (Balat, 2008).

1.2.2.2 - Indústria Têxtil

Os tecidos jeans e brim alcançaram uma popularidade marcante durante as últimas décadas. Nas décadas de 70 e 80 as indústrias desenvolveram a técnica de lavagem do tecido com pedra pomes para conferir ao jeans maciez proporcionando maior conforto aos consumidores. Este técnica, chamada *stone-washing*, apesar de ser eficiente para os objetivos almejados causa um rápido desgaste das máquinas industriais. Neste contexto, começaram a ser utilizados coquetéis de celulases microbianas, ricos em endoglucanase, em substituição às pedras, processo denominado *bio-stoning*.

Durante o processo de *bio-stoning*, as celulases atuam hidrolisando as pequenas fibras da superfície do tecido, proporcionando a perda do índigo. As vantagens de substituir as pedras pelas enzimas são: redução do desgaste das máquinas; aumento da produtividade das máquinas devido à maior quantidade de tecido tratado em menos tempo; é um processo não poluente e gera efluentes tratáveis; flexibilidade para criar e reproduzir novos produtos (Bhat, 2000; Belghith, et al., 2001).

Uma desvantagem do *bio-stoning* é a redeposição do índigo, que gera um desbotamento ineficiente. Este processo acontece quando as celulases utilizadas no processo possuem afinidade pelo índigo, logo este não é excluído durante a lavagem. Normalmente as enzimas de caráter ácido possuem maior afinidade pelo índigo e por isso as enzimas neutras e básicas são preferidas para utilização da indústria têxtil. Para diminuir o efeito de redeposição, proteases têm sido utilizadas após a lavagem com as celulases (Miettinen-Oinonen, 2004).

1.2.2.3 - Indústria de Papel e Celulose

Nas indústrias de papel e celulose, xilanase é a hemicelulase que tem maior importância. Esta enzima pode atuar no processo de branqueamento do papel auxiliando no desligamento da lignina da fibra. Isso faz com que o material fique mais suscetível ao tratamento químico, diminuindo assim, a utilização de cloro durante o tratamento. Estudos apontam que a utilização de extratos contendo atividade de mananase juntamente com a de xilanase são mais eficientes para o biobranqueamento do papel (Bhat, 2000). Devido ao pH alcalino e às altas temperaturas (55 - 70 °C) aos quais a polpa é submetida, é necessário que as enzimas utilizadas neste processo sejam resistentes em tais condições (Collins, et al., 2005).

Hemicelulases podem ser utilizadas também no processo de polpação biomecânica, onde as lascas de madeira são tratadas enzimaticamente e a polpação mecânica subseqüente resulta na utilização reduzida de energia, resistência melhorada, e tempo de cozimento reduzido (Bhat, 2000).

1.2.2.4 - Indústria Alimentícia

Celulases e hemicelulases têm uma ampla aplicação nas indústrias alimentícias. Essas enzimas podem ser utilizadas na extração e clarificação de sucos, melhora na textura, qualidade e vida útil de produtos de padaria, entre outras.

As celulases e hemicelulases (xilosidases, xilanases, mananases e arabinases), juntamente com as pectinases são denominadas enzimas de maceração, utilizadas para a extração e clarificação de sucos de frutas e vegetais. Durante a produção de sucos, as frutas são totalmente maceradas em polpa, que depois de processos físicos (prensamento, centrifugação e filtração) resulta em uma fase líquida de suco e uma

fase sólida que não é aproveitada. O uso das enzimas de maceração permite a utilização desta fase sólida aumentando o rendimento do processo e o valor nutricional do suco (Bhat, 2000; Haros, 2002)

As xilanases têm sido utilizadas para o aperfeiçoamento da qualidade de massas, pães, biscoitos, tortas e outros produtos de padaria. A capacidade das xilanases em hidrolisar a arabinoxilana, presente nos compostos utilizados para confecção dos produtos, facilita a redistribuição de água nas massas e nos pães. Esta alteração seria responsável pelos efeitos favoráveis na manipulação das massas, volume dos pães, textura e estabilidade. Além disso, a produção de xilooligossacarídeos nos pães acarreta benefícios à saúde humana (Flander, 2008).

As α-arabinofuranosidases e as β-glicosidases podem ser utilizadas na produção de melhores vinhos. Alguns monoterpenos de uvas contribuem significativamente para o sabor desta bebida. Estes compostos estão presentes na forma volátil e na forma não volátil (ligada glicosidicamente). Esta última fração é composta por dissacarídeos, denominados α-L-arabinofuranosil-β-D-glicopiranosídeo, e por uma fração não glicosídica que pode ser terpenol, terpenediol, 2-feniletanol ou álcool benzílico. Para a hidrólise deste composto a ligação glicosídica é hidrolisada por α-arabinofuranosidases, α-L-ramnosidase ou β-D-apiosidase e posteriormente uma β-glicosidase libera o monoterpenol, fazendo com que vinho apresente um aroma e sabor melhorados (Le Clinche, et al 1997).

1.2.3 - O gênero Acremonium

O gênero *Acremonium* consiste de aproximadamente 100 espécies distribuídas mundialmente (Kirk, et al., 2001). Este gênero é encontrado em nichos diversos

como solo, patógeno humano, patógeno de insetos e endofíticos (Domsch, et al., 2007).

Acremonium zeae, também denominado *Acremonium strictum*, *Cephalosporium* sp. ou *Cephalosporium Acremonium*, juntamente com *Fusarium verticillioides* são colonizadores de milho (*Zea maydis*) recém colhidos. A infecção por este fungo é assintomática e normalmente ocorre no embrião e no endosperma. Estudos demonstraram que *Acremonium zeae* pode atuar como um protetor para o milho devido à produção do antibiótico pirrocidina que inibe o crescimento do fungo *Aspergillus flavus* e conseqüentemente da aflatoxina produzida por este fungo. A presença do fungo endofítico representa uma defesa imediata contra ataques de patógenos à semente e à plântula (Wicklow, et al., 2005). Além da pirrocidina a produção do antibiótico cephalosporina C já foi estudada e atualmente o fungo é utilizado para este fim em processos industriais. Além do *Acremoniun zeae*, *Acremonium chrysogenum* também é muito utilizado para produção deste antibiótico (Weil, et al., 1995; El-Sabbagh, et al., 2008, Cabri, 2009,).

Espécies de *Acremonium* foram estudadas para produção de celulase e hemicelulases. O fungo *Acremonium cellulolyticus* produz celobiohidrolase, β-glicosidase, endoglucanase e xilanase. O meio de cultura para produção de celulases foi otimizado. O sobrenadante da cultura apresentou maior rendimento na sacarificação de lascas de madeira e de resíduos de papel, comparado com enzimas comerciais provenientes de *Trichoderma*. Este maior rendimento foi atribuído à presença de β-glicosidases na cultura de *Acremonium*, já que esta enzima degrada um inibidor da celobiohidrolase (Ikeda, et al., 2007).

Diversas espécies de *Acremonium*, incluindo *Acremonium persicinum* e *Cephalosporium Acremonium*, foram explorados quanto a produção de β-glucanases e β-glicosidases (Pitson, et al., 1997, McDougall & Seviour, 2002)

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CAPÍTULO 2

2.1 - ABSTRACT

Two *Acremonium* species (*Acremonium zeae* EA0802 and *Acremonium* sp. EA0810) were examined in relation to their ability to produce cellulases (FPases), endoglucanases, β-glucosidases, xylanases, α-galactosidases, α-arabinofuranosidases and β-xylosidases in different carbon sources. The fungi were cultivated in submerged culture containing L-arabinose, D-xylose, oat spelt xylan, sugar cane bagasse or corn straw as a carbon source. Enzyme production in solid state fermentation utilizing sugar cane bagasse or corn straw as a carbon source and support was also tested.

The highest FPase, endoglucanase and xylanase activities were obtained when *Acremonium* sp. EA0810 and *Acremonium zeae* EA0802 were cultivated in submerged culture containing sugar cane bagasse and corn straw as a carbon source. *Acremonium* sp. EA0810 presented the highest β-glucosidase activity when it was cultivated in submerged culture using D-xylose as carbon source. *Acremonium zeae* EA0802 show greatest α-arabinofuranosidase and α-galactosidase activities when it was cultivated in submerged culture utilizing xylan as a carbon source.

FPase, endoglucanase, β-glucosidase and xylanase from *Acremonium* sp. EA0810 were found to have optimum pH and temperatures of 6.0, 55 °C; 5.0, 70 ºC; 4.5, 60 °C and 6.5, 50 °C, respectively. α-Arabinofuranosidase and α-galactosidase from *Acremonium zeae* EA0802 have optimum pH and temperatures of 5.0, 60 °C and 4.5, 45 °C, respectively. An enzymatic extract containing endoglucanase and xylanase activities was submitted to a zymogram analyze and one form of each enzyme was detected. Endoglucanase and xylanase biochemical characteristics are appropriate for industrial application.

2.2 - INTRODUCTION

Lignocellulose is one of the most common biopolymers in nature and is composed mainly of cellulose, hemicellulose and lignin. Cellulose is a linear polymer of glucose units which can be hydrolyzed by the action of endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), exoglucohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21) (Whitaker, 1994). Hemicellulose is a heterogeneous and branched polymer of pentoses, hexoses and uronic acids. Xylans are the major sugar founded in hemicelluloses (Rowell, et al., 2005; Juhász, et al., 2005). Complete enzymatic hydrolysis of xylan requires endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), and several accessory enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetylxylan esterase (3.1.1.72) and ferulic acid esterase (EC 3.1.1.73) which are necessary for hydrolyzing various substituted xylans (Saha, 2000).

Degradation of lignocellulosic material has great importance for many industrial processes and enzymatic hydrolysis has received attention due to its

potential as an environmentally friendly process besides its enormous hydrolysis specificity (Olsson, et al., 2004). Cellulases can be used in the textile industry for bio-stoning and bio-finishing of cellulosic fibers and in the pulp and paper industry, hemicellulases can be applied for the bio-bleaching of kraft pulps. Furthermore, in the food industry, cellulases and hemicellulases can be used to extraction and clarification of fruit and vegetable juices and in the animal feed industry they can promote an increasing in the nutritive quality of feed (Bhat, 2000; Csiszar, et al., 2001; Oinonen, et al., 2003; Savitha et al., 2009). In recent decades, the interest in cellulases and hemicellulases has increased due to the inevitable depletion of world energy supplies and the needing for alternative energy sources. Ethanol derived from lignocellulosic biomass is being explored as potential low cost gasoline and diesel substitutes and is seen as an interesting alternative because it can contribute to sustainable development as well as offset fossil fuel greenhouse emissions (Lin and Tanaka, 2006; Chang, 2007). However, this technology is not completely developed and is still expensive, mainly because of the high cost of these enzymes which are essential for the hydrolysis of raw material.

There is a general interest in obtaining new, more specific, stable enzymes and using cheap inducer sources, such as sugar cane bagasse (Camassola & Dillon, 2007; Gao, et al., 2008). Enzymes for the mentioned process are produced by microorganisms and the majority of research has been on the *Trichoderma* and *Aspergillus* genera (Kang, et al., 2004; Wen, et al., 2005; Juhász et al., 2005). However, little is understood about enzymes from the *Acremonium* genus (Fang, et al., 2008; Jayus & Seviour, 2002).

The genus *Acremonium* Link comprises anamorphic Hypocreales, consisting of about 100 species with a worldwide distribution (Kirk et al., 2001). The genus is

reported from a variety of soil-borne, human pathogens, entomopathogenic and endophytes ecological niches (Domsch, et al., 2007). *Acremonium zeae*, also reported as *Acremonium strictum*, is the most prevalent colonist in preharvest maize (*Zea maydis*), typically producing symptomless kernel infections. *Acremonium zeae* is an extensively studied endophyte due to its production of the cephalosporin C antibiotic (Weil, et al., 1995; Araujo, et al., 1996; Cabri, 2009). Recently it was reported that this fungus can produce another type of antibiotic called pyrrocidines A and B that have antagonist effect against *Aspergillus flavus* and *Fusarium verticillioides* in cultural tests and interferes with *A. flavus* infection and aflatoxin contamination in preharvest maize kernels (Wicklow, et al., 2005). However, *A. zeae* cell-wall degrading enzymes have not been exploited, although it appears to be promising since the fungus grows naturally in a cellulose-rich environment.

The purpose of this study was to investigate the ability of two species of *Acremonium* (*Acremonium zeae* EA0802 and *Acremonium* sp. EA0810) to produce cellulases and hemicellulases in submerged culture and in solid state fermentation. Low cost carbon source substrates, such as sugar cane bagasse and corn straw among others substrates like oat spelt xylan, D-xylose and L-arabinose were tested for enzyme production. The objective was to evaluate the production of the cellulases: FPase (filter paper activity), endoglucanase and β-glucosidase and the hemicellulases: xylanase, α-galactosidase, α-L-arabinofuranosidase and β-xylosidase, as well as the characterization of the principal enzymes produced by *A.* zeae EA00802 and *Acremonium* sp. EA0810.

2.3 - MATERIALS AND METHODS

2.3.1 - Materials

The substrates ρ-nitrophenyl-α-D-galactopyranoside (ρNPGal), ρ-nitrophenyl-β-D-glucopyranoside (ρNPGlc), ρ-nitrophenyl-α-L-arabinofuranoside (ρNPAra) and ρ-nitrophenyl-β-D-xylopyranoside (ρNPXyl) were obtained from Sigma Chemical Co. (St. Louis, MO). Xylan from birch wood and D (+) xylose were purchased from Sigma Chemical Co. (Germany and USA, respectively). The sugar cane bagasse and corn straw were acquired from the local market. All other chemicals used were of analytical grade.

2.3.2 - Fungi isolation

Acremonium zeae EA0802 and *Acremonium* sp. EA0810 were isolated by the Blotter method (Dhingra & Sinclair, 1985) and direct culture isolation, respectively.

Commercial corn seeds, originally treated with fungicide were maintained at -5 °C for 48 h to inhibit germination. After this period of time the seeds were placed in an equidistant matrix on autoclaved filter paper, humidified in a plastic Gerbox (50 seeds per gerbox). Seeds were incubated in a growth chamber for 7 days at 27 °C to induce sporulation. After incubation, each seed was observed under a stereoscopic microscope and the typical *Acremonium* colonies were collected with an autoclaved needle under the stereoscopic microscope and transferred to a Petri dish containing 20 mL of 2 % malt extract agar with rifamycins.

The isolated were incubated at 27 °C until fungal colonies appeared. Identification of the isolates was carried out by the observation of fungal structure under an optical microscope (Motic BA 300). The isolates were maintained in Petri dishes containing a mixture of 383 mL distilled water, 108.67 mL tomato sauce, 9.58 g agar, 1.44 g calcium carbonate and 0.02 $mg.mL^{-1}$ ampicillin (Sanglard, et al., 2009). The strains were kept on Laboratory of Biochemical Technology, Department of Biochemistry and Molecular Biology at Federal University of Viçosa, Minas Gerais, Brazil.

2.2.3 - Cultivation conditions and medium

Acremonium species were cultivated in submerged culture (SC) and in solid state fermentation (SSF). The mineral medium in submerged culture contained ($g.L^{-1}$) 6 $NaNO_3$, 1.5 KH_2PO_4, 0.5 KCl, 0.5 $MgSO_4$, 0.01 $FeSO_4$, 0.01 $ZnSO_4$ and 1 % (w/v) of a carbon source. Oat spelt xylan, L-arabinose, D-xylose, milled sugar cane bagasse or milled corn straw were tested as carbon sources. Erlenmeyer flasks (500 mL) with 250 mL of mineral medium were inoculated with 50 discs (8 mm diameter)

of an actively growing 7 day old culture. The flasks were shaken at 180 rpm, 28-30 °C and aliquots were taken every day from the same flask for 23 days when the growth was finished. After the aliquots had been centrifuged for 30 min at 3,500 g, the supernatant was used as the crude enzyme.

SSF was tested using sugar cane bagasse or corn straw for support and as a carbon source. Milled bagasse was mixed with splinter of bagasse in a proportion of 60/40 (g/g). Milled corn straw was mixed with splinters of corn straw in a proportion of 60/27 (g/g). The mixture of milled and splinted biomass increased aeration of the fungi during growth. The biomasses were humidified with a mineral medium composed of ($g.L^{-1}$) 1.5 NaH_2PO_4, 0.5 $MgSO_4$ and 1.0 $(NH_4)_2SO_4$, and the initial moistures content for the corn straw and bagasse were 72 % and 76 %, respectively. Seven grams of humidified biomass was placed in a 125 mL erlenmeyer flask and autoclaved for 30 min at 121 °C. After cooling, each flask was inoculated with 3 discs (8 mm diameter) of an actively growing, 7 day old culture. Growth was conducted at 25 - 28 °C under static conditions and samples were taken every 3 days from different flasks during 39 when the growth was finished. The enzymes were extracted by adding 50 mL of 0.05 M sodium acetate buffer pH 5.0 in each flask and agitated in shaker at 100 rpm at room temperature for 30 min. The mixture was passed through nylon filters and the filtrate was used as the crude enzyme. The experiment was duplicate and the results represent the mean obtained values.

2.2.4 - Enzymes Assays

The assays for α-L-arabinofuranosidase, α-galactosidase, β-xylosidase and β-glucosidase consisted of 300 µL 0.05 M sodium acetate buffer, pH 5.0, 75 µL

enzyme solution and 125 µL of 2 mM ρNPAra, ρNPGal, ρNPXyl, ρNPGlc, respectively. The reaction was carried out for 15 - 30 min at 40 °C, terminated by the addition of 500 µL 0.5 M sodium carbonate. The activity was determined at 410 nm using a standard curve with ρNP. Xylanase was assayed using 60 µL sodium acetate buffer 0.05 M, pH 5.0, 20 µL enzyme solution and 420 µL xylan from birch wood, 1.25 % (w/v), diluted in the same buffer. The reaction was carried out for 10 - 20 min at 40 °C, stopped by the addition of 500 µL DNS reagent (Miller, 1956) and incubated in boiling water for 5 min for color development. The activity was determined at 540 nm using a standard curve with glucose. The endoglucanase assay consisted of 100 µL enzyme solution and 400 µL carboxymetilcellulose, 0.625 % (w/v), diluted in the same buffer as cited above. The reaction was conducted for 30 - 60 min at 50 °C and stopped by the addition of 500 µL DNS reagent as explained above. Filter paper activity (FPase) was determined by using Whatman n° 1 filter paper (1 x 5 cm, 40 mg) coiled in the bottom of a test tube to which 900 µL 0.05 M sodium acetate buffer, pH 5.0 was added. Enzyme solution (50 µL) was added and incubated at 50 °C for 30 - 60 min. The reaction was concluded by taking an aliquot of 500 µL from the mixture after incubation and added to a test tube containing 500 µL of DNS reagent.

One enzyme unit was defined as the amount of enzyme that released 1 µmol of product per minute under the assay conditions

2.3.5 - Biochemical characterization of enzymes

The influence of the pH and temperature on α-galactosidase, α-arabinofuranosidase, xylanase, endoglucanase, FPase and β-glucosidase activities

were evaluated using the assay system described above, but different buffer solutions (pH 3.0-8.0) using McIlvaine buffer (citric acid/sodium phosphate) (McIlvaine, 1921) or different incubation temperatures (15 - 80 °C). For determination of thermal stability, the enzyme fractions were preincubated with buffer solution at the optimum temperature for each enzyme for several time periods at pH 5.0. The residual activity was determined using the standard assay. Results of the analyses are presented as mean ±SD for three measurements.

2.3.6 - Zymogram analysis

Enzyme samples were applied to a 10 % SDS-PAGE gel (Laemmli, 1970) containing 0.2 % (w/v) oat spelt xylan or CMC polymerized within the gel matrix. SDS concentrations were altered from 0.1 % to 0.08 % (w/v) facilitating enzyme refolding. Following electrophoresis, the gel was washed twice for 20 min in 20 % isopropanol disolved in the sodium acetate buffer 100 mM, pH 5.5, followed by two washes (20 min each) in the same buffer. The gel was incubated at 50 °C in sodium acetate buffer for 30 min, stained with congo red 0.1 % (w/v) for 15 min, and destained with 2 washes (15 min each) with 1 M NaCl. Xylanase activity was visible as a clear band and to increase the contrast with the background, the gel was washed with acetic acid 5 % (w/v) (Bischoff, et al., 1998)

2.4 - RESULTS AND DISCUSSION

2.4.1 - Enzyme production

2.4.1.1 - Filter paper activity - FPase

Almost all carbon sources tested (complex and simple substrates) could induce FPase activity in the two fungi and these enzyme activity could be detected during practically all time analyzed (table 1 and 2). Although reported before for *Thermoascus aurantiacus* and *Aspergillus fumigatus* it is not common that an organism produces cellulolytic enzymes on so wide substrate range (Ximenes, et al., 1996, Feldman, et al, 1988).

Highest FPase and endoglucanase activities were obtained with sugar cane bagasse or corn straw in SC (table 1 and table 2) for *Acremonium zeae* EA0802 and *Acremonium* sp. EA0810, which can be explained by the high cellulose content in these materials (Sánchez, 2009; Sun & Cheng, 2002). In general, cellulosic materials act as inducers, and readily metabolized carbon compounds as repressors (Ximenes,

et al., 1996), however this phenomenon was not observed. The highest FPase activity was reached by *Acremonium* sp. EA0810 in SC utilizing bagasse as a carbon source after 4 days of growth with a maximum value of 0.55 $U.mL^{-1}$ (table 2). *Acremonium zeae* EA0802 also produced similar activity of FPase, 0.51 $U.mL^{-1}$ (table 1) in SC with the same carbon source, however, after 8 days. This results show the effectiveness of this carbon source as an FPase inducer for the two fungi, specially, for *Acremonium* sp. EA0810 that could produce this enzyme faster than *A. zeae* EA0802.

Oat spelt xylan, another complex carbon source, also induced FPase activity in *Acremonium zeae* EA0802, 0.17 $U.mL^{-1}$, (table 1) and *Acremonium* sp. EA0810, 0.18 $U.mL^{-1}$ (table 2). Xylose induced 0.11 $U.mL^{-1}$ of FPase in *A. zeae* EA0802 (table 1) and 0.12 $U.mL^{-1}$ in *Acremonium* sp. EA0810 (table 2), similar to *Aspergillus Fumigatus*, which was also induced to produce FPase with this monosaccharide as substrate (Ximenes, et al, 1996). The *Acremonium* species studied show to be poor FPase producers on SC with arabinose as substrate. No FPase activity could be detected in *A.* zeae EA0802 culture (table 1) and *Acremonium* sp. EA0810 produced 0.03 $U.mL^{-1}$ (table 2). This effect was also observed in *Aspergillus niger* when it was cultivated with arabinose as carbon source (Hanif, et al., 2004).

In SSF with sugar cane bagasse, it was detect an FPase activity of 0.14 $U.mL^{-1}$ in *A. zeae* EA0802, lower than when this fungus was cultivated with the same carbon source in SC, when it was verified an activity of 0.51 $U.mL^{-1}$ (table 1). When corn straw was utilized in SSF the same behavior was verified (table 1). *Acremonium* sp. EA0810 shows similar results. In SSF with corn straw, an FPase activity of 0.06 $U.mL^{-1}$ was detect while in SC this value was 0.18 $U.mL^{-1}$ (table 2). When bagasse was used in SSF the FPase activity was 0.02 $U.mL^{-1}$ and in SC it was 0.55 $U.mL^{-1}$

(table 2). These results prove that SC was more efficient for FPase induction than SSF.

Yamanobe et al (1986) reported a production of 5 $U.mL^{-1}$ of FPase by the *A. cellulolyticus* strain grown in a liquid medium with 4 % of cellulose powder as a carbon source. A hyper producer mutant of this original strain produced 15 $FPU.mL^{-1}$ after 8 days growing in an optimized medium containing Solka Floc, pure cellulose, as a carbon source (Ikeda, et al., 2007). *Neurospora crassa* produced 0.23 $U.mL^{-1}$ of FPase when it was cultivate in liquid medium with 1 % wheat straw as a carbon source (Romero, et al., 1999). Liming & Xueliang (2004) optimized the production of cellulase by a mutant strain of *Trichoderma reesei* in submerged culture using corn cobs as a carbon source. The maximum FPase yield was obtained when the fungi was cultivated with 4 % carbon source after 168 h. They obtained 5.2 $U.mL^{-1}$ of FPase with a yield of 213.4 $U.g^{-1}$, and productivity of 31.3 $U.L^{-1}.h^{-1}$. *Acremonium* sp. EA0810 in non-optimized conditions produced 0.55 $U.mL^{-1}$ (table 2) and a yield of 54.6 $U.g^{-1}$ (units of enzyme for gram of dry carbon source) in 1 % carbon source after 48 h, resulting in the productivity of 11.45 $U.L^{-1}.h$.

Acremonium sp. EA0810 it is a promising fungi to produce FPAse, since it culture medium was not optimized and the carbon source was tested in a low concentration, unlike the references cited. In addiction, the FPase induction tested in this work was done with a low cost agroindustrial residue contributing, in this way, with an appropriate utilization of it.

2.4.1.2 - Endoglucanase

Endoglucanase activity was detected in all *Acremonium* sp. EA0810 tested cultures, except in SSF with sugar cane bagasse. Highest endoglucanase activity of this strain, 0.18 $U.mL^{-1}$, was observed after 9 days of growth in the SC with corn straw as carbon source (table 2). When bagasse was used, the endoglucanase production was similar, 0.15 $U.mL^{-1}$ (table 2), after 4 days of cultivation. When arabinose, oat spelt xylan or xylose were used as a carbon source endoglucanase activities were, respectively, 0.02 $U.mL^{-1}$, 0.06 $U.mL^{-1}$ and 0.07 $U.mL^{-1}$, lower than the activities cited above (table 2).

Acremonium zeae EA0802 do not exhibit endoglucanase activity on so wide substrate range. The highest activity value was 0.09 $U.mL^{-1}$, reached on SC with corn straw after 6 days or sugar cane bagasse after 3 days (table 1). In SSF on bagasse it was detected 0.08 $U.mL^{-1}$, similar to that activity on SC, however, after 33 days of growth. When arabinose, oat spelt xylan and xylose was used as substrates in SC, none or a very low activity was detected (table 1). *Acremonium cellulolyticus* produced 82 $U.mL^{-1}$ on 4 % of pure cellulose powder (Yamanobe, et al., 1986). *Mucor circinelloides* grown on lactose secreted 0.17 - 0.25 $U.mL^{-1}$, a similar value to that obtained in this work (Saha, 2004). The higher activities on complex carbon source as corn straw and sugar cane bagasse can be due the presence of amorphous cellulose in these substrate, since endoglucanases are more active on this kind of polymer (Whitaker, 1994).

2.4.1.3 - β-Glucosidase

Acremonium zeae EA0802 showed to be poor β-glucosidase producer in SC. Highest activity, 0.08 $U.mL^{-1}$, or 2.88 $U.g^{-1}$, was achieved by this fungus in SSF on sugar cane bagasse after 33 days of growth and when corn straw was used this fungus produced 0.01 $U.mL^{-1}$ or 0.22 $U.g^{-1}$ (table 1). These values were similar to those obtained from *Acremonium* sp. EA0810 which produced 0.02 $U.ml^{-1}$, or 0.61 $U.g^{-1}$, in SSF wit sugar cane bagasse and the same values was obtained with corn straw (table 2). *Fusarium oxysporum*, an endophyte microorganism like *Acremonium*, cultivated on corn stover in SSF produced 0.14 $U.g^{-1}$ of β-glucosidase (Panagiotou, et al., 2003). The carbon sources tested in SC were not effective to induce considerable β-glucosidase activity in *A. zeae* EA0802 (table 1).

Acremonium sp. EA0810 exhibit higher β-glucosidase activities than *A. zeae* EA0802 in SC (table 2). Highest β-glucosidase activity (0.17 $U.mL^{-1}$) was obtained by *Acremonium* sp. EA0810 using D-xylose as a carbon source in SC (table 2). *Aspergillus fumigatus* induced with xylose also secreted β-glucosidase (Ximenes, et al., 1996). About 0.09 $U.mL^{-1}$ it was detected after 20 days of growth in SC with arabinose as substrate and 0.06 $U.mL^{-1}$ was produced in SC with oat spelt xylan after 9 days (table 2). The β-glucosidase induction with arabinose was also observed in *Clostridium papyrosolvens* (Thirumale, et al., 2001).

Acremonium cellulolyticus, which is already known to produce high quantities of β-glucosidase, produced 25 $U.mL^{-1}$ in an optimized medium with cellulose powder as a carbon source (Yamanobe, et al., 1986). *Acremonium persicinum* produced approximately 0.16 $U.mL^{-1}$ of β-glucosidase when grown on laminarin after 240 h (Pitson, et al., 1997).

2.4.1.4 - Xylanase

For all carbon sources, significant xylanase activities were detected. This can be explained by endophyte nature strains, since xylan is one of the major components of the plant cell wall (Suto, et al., 2002).

When *Acremonium zeae* EA0802 was cultivated in SSF with sugar cane bagasse it was detected a xylanase activity of 0.78 $U.mL^{-1}$ or 29.4 $U.g^{-1}$ and in SSF with corn straw, it was observed 0.26 $U.mL^{-1}$ or 6.5 $U.g^{-1}$ (table 1). *Penicillium echinulatum* grow on wheat bran and pretreated sugar cane bagasse produced 10 $U.g^{-1}$ (Camassola & Dillon, 2007). The *Acremonium zeae* EA0802 results encourages more studies to optimize it cultivation in SSF.

The highest xylanase activity, 2.46 $U.mL^{-1}$, was found when *Acremonium* sp. EA0810 was cultivated in SC with bagasse after 10 days (table 2). Using corn straw as the carbon source it was obtained 2.08 $U.mL^{-1}$ (table 2), a similar value to that observed with sugar cane bagasse but after 16 days of cultivation. *Acremonium zeae* EA0802 produced 2.16 $U.mL^{-1}$ with bagasse as a carbon source in SC after 14 days and with corn straw xylanase activity was 0.76 $U.mL^{-1}$, after 9 days (table 1).

Oat spelt xylan was a good xylanase inducer for *A. zeae* EA0802 and for *Acremonium* sp. EA0810. Xylanase activity of *A. zeae* EA0802 was 1.56 $U.mL^{-1}$ after 15 days, however, after 3 days this activity it already was 1.38 $U.mL^{-1}$ (table 1). In *Acremonium* sp. EA0810 culture with oat spelt xylan it was detected 2.02 $U.mL^{-1}$ after 3 days of growth (table 2). *Hymenoscyphus ericae*, an endophyte fungus, produced two xylanase forms, with a total activity of 5.8 $U.mL^{-1}$, in liquid medium with oat spelt xylan (Burke and Cairney, 1997). *Rhizopus oryzae* produced 0.39 $U.mL^{-1}$ using 1 % xylan as carbon source after 6 days and when 2 % corn cob was used the fungi produced 2.8 $U.mL^{-1}$ after 5 days (Bakir, et al, 2001).

2.4.1.5 - α-Galactosidase

Levels of α-galactosidase activity were higher in *A. zeae* EA0802. The maximum activity, 0.09 $U.mL^{-1}$, was in SC with oat spelt xylan after 4 days of growth (table 1). *Penicillium brasilianum* grown in 2.5 % of a mixture of purified cellulose, birchwood xylan and oat spelt xylan was found to produce 1.5 $U.mL^{-1}$ (Jorgensen & Olsson, 2005). In SSF with corn straw, 0.01 $U.mL^{-1}$ was produced by *A. zeae* EA0802 (table 1). This represents 0.27 $U.g^{-1}$, similar to *Aspergillus oryzae* in non-optimized SSF with bagasse which produced 0.19 $U.g^{-1}$ and 3.02 $U.g^{-1}$ in an optimized medium (Szendefy, et al., 2006). *Acremonium* sp. EA0810 did not exhibit any considerable activity of this enzyme in the tested carbon sources.

2.4.1.6 - α-Arabinofuranosidase

Acremonium zeae EA0802 showed to be a better α-arabinofuranosidase producer than the *Acremonium* sp. EA0810 strain. The highest enzyme level, 0.045 $U.mL^{-1}$, was achieved by *A. zeae* EA0802 in SC with oat spelt xylan as a carbon source (table 1). When this strain was grown on SSF with corn straw it was detected 0.01 $U.mL^{-1}$ or 0.29 $U.g^{-1}$ and with bagasse 0.02 $U.mL^{-1}$ or 0.66 $U.g^{-1}$ of this enzyme (Table 1). In SC using bagasse as carbon source no significant value was detected. A *Thermomyces lanuginosus* strain produced a maximum of 0.11 $U.mL^{-1}$ when grow on oat spelt xylan during 7 days (Singh, et al., 2000) and *Trichoderma reesei* grown on sugar beet pulp showed an arabinofuranosidase activity maximum activity of 0.02 $U.mL^{-1}$ (Olsson, et al., 2003). *Acremonium* sp. EA0810 exhibit 0.01 $U.mL^{-1}$ (0.21 $U.g^{-1}$) of arabinofuranosidase activity in SSF on bagasse, and in SC with oat spelt xylan 0.01 $U.mL^{-1}$ (table 2).

Table 1: *Acremonium zeae* EA0802 enzyme activity ($U.mL^{-1}$) in solid state fermentation (SSF) and in submerged culture (SC) with different carbons source

Carbon source	ABF	α-Gal	β-Xyl	β-Glc	Endo	FPase	Xyl
SSF-Corn straw	$0^{3}/0.01^{32}/0.01^{39}$	$0^{3}/0.01^{6}/0.01^{39}$	$0^{3}/0.01^{39}/0.01^{39}$	$0^{3}/0.01^{39}/0.01^{39}$	$0.02^{3}/0.03^{39}/0.03^{39}$	$0.01^{3}/0.06^{9}/0.05^{39}$	$0.08^{3}/0.26^{12}/0.23^{39}$
SSF-Sugar cane bagasse	$0^{3}/0.02^{36}/0.01^{39}$	nd	$0^{3}/0.01^{30}/0.01^{39}$	$0^{3}/0.08^{36}/0.04^{39}$	$0.02^{3}/0.08^{33}/0.07^{39}$	$0.08^{3}/0.14^{33}/0.14^{39}$	$0.52^{3}/0.78^{15}/0.71^{39}$
SC-Corn straw	$0^{3}/0.01^{23}/0.01^{23}$	$0.01^{3}/0.03^{5}/0.02^{23}$	nd	$0.01^{3}/0.02^{6}/0.01^{23}$	$0.06^{3}/0.09^{6}/0.05^{23}$	$0.11^{3}/0.14^{18}/0.14^{23}$	$0.71^{3}/0.76^{9}/0.70^{23}$
SC-Sugar cane bagasse	nd	$0^{3}/0.02^{7}/0.02^{23}$	nd	$0.02^{3}/0.03^{7}/0.01^{23}$	$0.09^{3}/0.09^{3}/0.05^{23}$	$0.21^{3}/0.51^{8}/0.35^{23}$	$0.94^{3}/2.16^{14}/1.72^{23}$
SC-L-Arabinose	$0^{3}/0.02^{4}/0^{23}$	0	0	$0^{3}/0.02^{6}/0^{23}$	0	0	$0^{3}/0.08^{15}/0.07^{23}$
SC-Oat spelt xylan	$0.03^{3}/0.04^{18}/0.04^{23}$	$0.09^{3}/0.09^{4}/0.07^{23}$	nd	$0.01^{3}/0.03^{9}/0.01^{23}$	nd	$0.15^{3}/0.17^{2}/0.13^{23}$	$1.38^{3}/1.56^{15}/1.54^{23}$
SC- D-xylose	nd	0	nd	$0^{3}/0.01^{4}/0.01^{23}$	nd	$0.05^{3}/0.11^{8}/0.07^{23}$	$0.11^{3}/0.32^{9}/0.14^{23}$

a- **ABF**: α-L-arabinofuranosidase activity; **α-Gal**: α-galactosidase activity; **β-Xyl**: β-xylosidase activity; **β-Glc**: β-glucosidase activity; **Endo**: endoglucanase activity; **FPase**: FPase activity; **Xyl**: xylanase activity
b- All standard deviation were less than 10 %
c- nd: not determinable
d- Superscript numbers stand for the day of growth referring to the initial activity (3 days), the day of greatest activity (variable number of days) and last day analyzed (23 or 39)

Table 2: *Acremonium* sp. EA0810 enzyme activity ($U.mL^{-1}$) in solid state fermentation (SSF) and in submerged culture (SC) with different carbons source

Carbon source	ABF	α-Gal	β-Xyl	β-Glc	Endo	FPase	Xyl
SSF-Corn straw	nd	nd	nd	$0.01^{3}/0.02^{39}/0.01^{39}$	$0.05^{3}/0.07^{6}/0.03^{39}$	$0.03^{3}/0.06^{21}/0.04^{39}$	$0.1^{3}/0.2^{15}/0.1^{39}$
SSF-Sugar cane bagasse	$0^{3}/0.01^{25}/0^{39}$	nd	nd	$0.01^{3}/0.02^{15}/0.01^{39}$	0	$0.01^{3}/0.02^{30}/0.02^{39}$	$0.07^{3}/0.07^{3}/0.07^{39}$
SC-Corn straw	nd	nd	nd	$0.01^{3}/0.02^{7}/0.01^{23}$	$0.15^{3}/0.18^{9}/0.17^{23}$	$0.18^{3}/0.18^{3}/0.17^{23}$	$1.94^{3}/2.08^{16}/1.97^{23}$
SC-Sugar cane bagasse	nd	nd	0	$0.03^{3}/0.03^{7}/0.01^{23}$	$0.13^{3}/0.15^{4}/0.05^{23}$	$0.22^{3}/0.55^{4}/0.43^{23}$	$0.93^{3}/2.46^{10}/2.46^{23}$
SC-L-Arabinose	0	nd	nd	$0.01^{3}/0.09^{20}/0.09^{23}$	$0.02^{3}/0.02^{3}/0.02^{23}$	$0^{3}/0.03^{20}/0.03^{23}$	$0.09^{3}/0.14^{17}/0.11^{23}$
SC-Oat spelt xylan	$0^{3}/0.01^{9}/0^{23}$	0	nd	$0.02^{3}/0.06^{9}/0.01^{23}$	$0.05^{3}/0.06^{5}/0.03^{23}$	$0.16^{3}/0.18^{2}/0.10^{23}$	$2.02^{3}/2.02^{3}/1.5^{23}$
SC- D-xylose	0	nd	nd	$0.01^{3}/0.17^{20}/0.17^{23}$	$0^{3}/0.07^{9}/0.03^{23}$	$0.05^{3}/0.12^{14}/0.08^{23}$	$0.11^{3}/0.15^{7}/0.09^{23}$

a-**ABF**: α-L-arabinofuranosidase activity; **α-Gal**: α-galactosidase activity; **β-Xyl**: β-xylosidase activity; **β-Glc**: β-glucosidase activity; **Endo**: endoglucanase activity; **FPase**: FPase activity; **Xyl**: xylanase activity
b- All standard deviation were less than 10 %
c- nd: not determinable
d-Superscript numbers stand for the day of growth referring to the initial activity (3 days), the day of greatest activity (variable number of days) and last day analyzed (23 or 39)

2.4.1.7 - β-xylosidase

The two *Acremonium* species analyzed in this studied proved to be poor producers of β-xylosidase. *Acremonium zeae* EA0802 produced 0.01 $U.mL^{-1}$ or 0.4 $U.g^{-1}$, when cultivated in SSF utilizing corn straw and sugar cane bagasse as carbon source and support (table 1). *Acremonium* sp. EA0810 did not produce any significant value of β-xylosidase with any of the carbon sources tested.

2.4.2 - Selection of the main enzymes activities

With the objective to evaluate the potential of the main enzymes detected in *Acremonium* cultures, specific growth condition were chosen to obtaining the maximum activity of each enzyme, considering all results showing at first part of this work. FPase, xylanase and endoglucanase from *Acremonium* sp. EA0810 cultivated in SC with sugar cane bagasse as carbon source and β-glucosidase when this fungi was cultivated in SC with xylose as substrate were analyzed. The temperature and pH effects were also analyzed on α-arabinofuranosidase and α-galactosidase activity from *A. zeae* EA0802 cultivated in SC with oat spelt xylan as a carbon source.

Acremonium sp. EA0810 in SC with bagasse as carbon source produced two FPase activity peak, one after 4 days and the other after 12 days of growth. Similar profile was observed for endoglucanase activity however, the second peak was after 11 days of growth (figure 1). The enzymatic preparation obtained after 4 days was chosen to analyze FPase and endoglucanase activities. High xylanase activity was observed after 11 days of growth (figure 1) and this enzymatic preparation was utilized to characterize xylanase biochemically. High

β-Glucosidase activity from *Acremonium* sp. EA0810 grown in SC with xylose as carbon source was obtained after 20 days of growth, however, after 14 days this activity it was already high and then this enzymatic preparation was utilized to β-glucosidase characterization (figure 2).

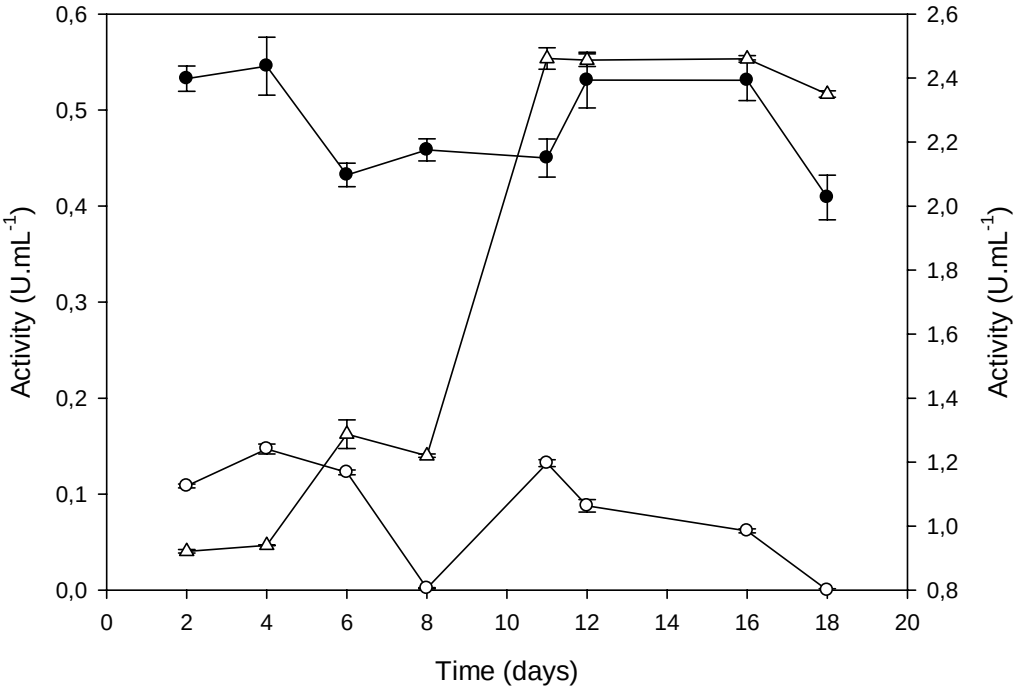

Figure 1: (●) FPase, (○) endoglucanase and (△) xylanase activities in the culture medium of *Acremonium* sp. EA0810 grown on SC with sugar cane bagasse as carbon source at 28 °C. Left axis correspond to FPase and endoglucanase activities and right axis correspond to xylanase activity. $U.mL^{-1}$ = units per ml of culture

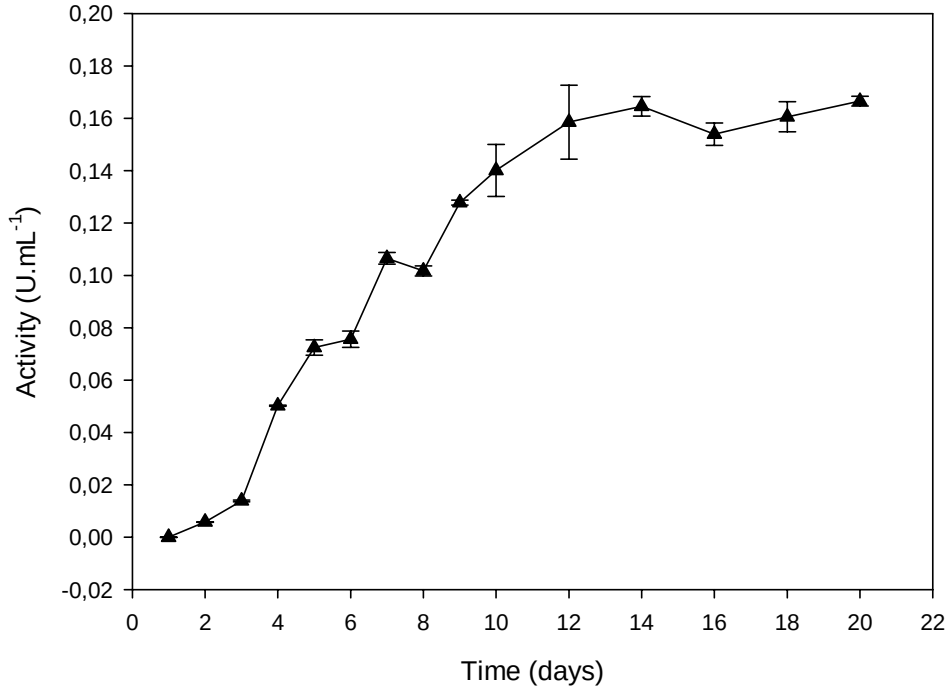

Figure 2: (▲) β-Glucosidase activity in the culture medium of *Acremonium* sp. EA0810 grown on SC with xylose as carbon source at 28 °C. $U.mL^{-1}$ = units per ml of culture

α-Arabinofuranosidase and α-galactosidase from *Acremonium zeae* EA0802, grown for 10 days, were characterized. Although 10 days it is not the best time of growth to obtain each enzyme separately, it is a good time to detected substantial activities of them together (figure 3).

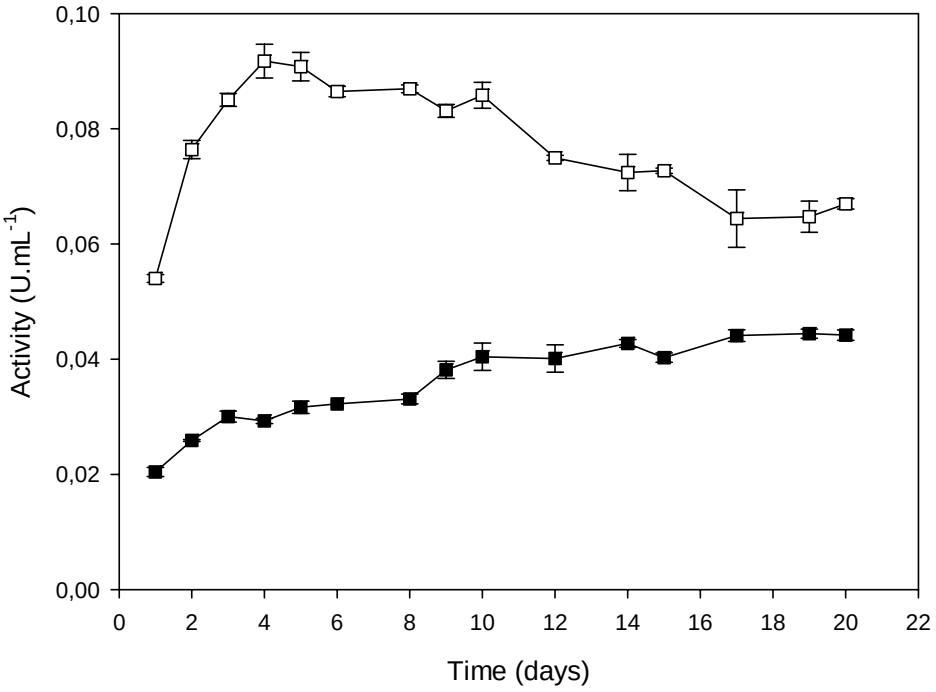

Figure 3: (□) α-Galactosidase and (■) α-arabinofuranosidase activities in the culture medium of *Acremonium zeae*. EA0802 grown on SC with oat spelt xylan as carbon source at 28 °C. $U.mL^{-1}$ = units per ml of culture

2.4.3 - Biochemical characterization of selected enzymes activities

2.4.3.1 - Effect of pH and temperature on hemicellulolytic enzyme activities

The effects of pH and temperature on α-galactosidase, α-arabinofuranosidase and xylanase are shown in Figure 4. The highest α-galactosidase activity was observed at pH 4.5 and in temperature range of 45 - 50 °C (figures 4 A and 4 B). The enzyme acid character was similar to others fungal α-galactosidases (Falkoski, et al., 2006; de Rezende, et al., 2005). The enzyme retained 99 % of its original activity after pre- incubation for 30 min at 45 °C and 36 % after 150 min (table 3).

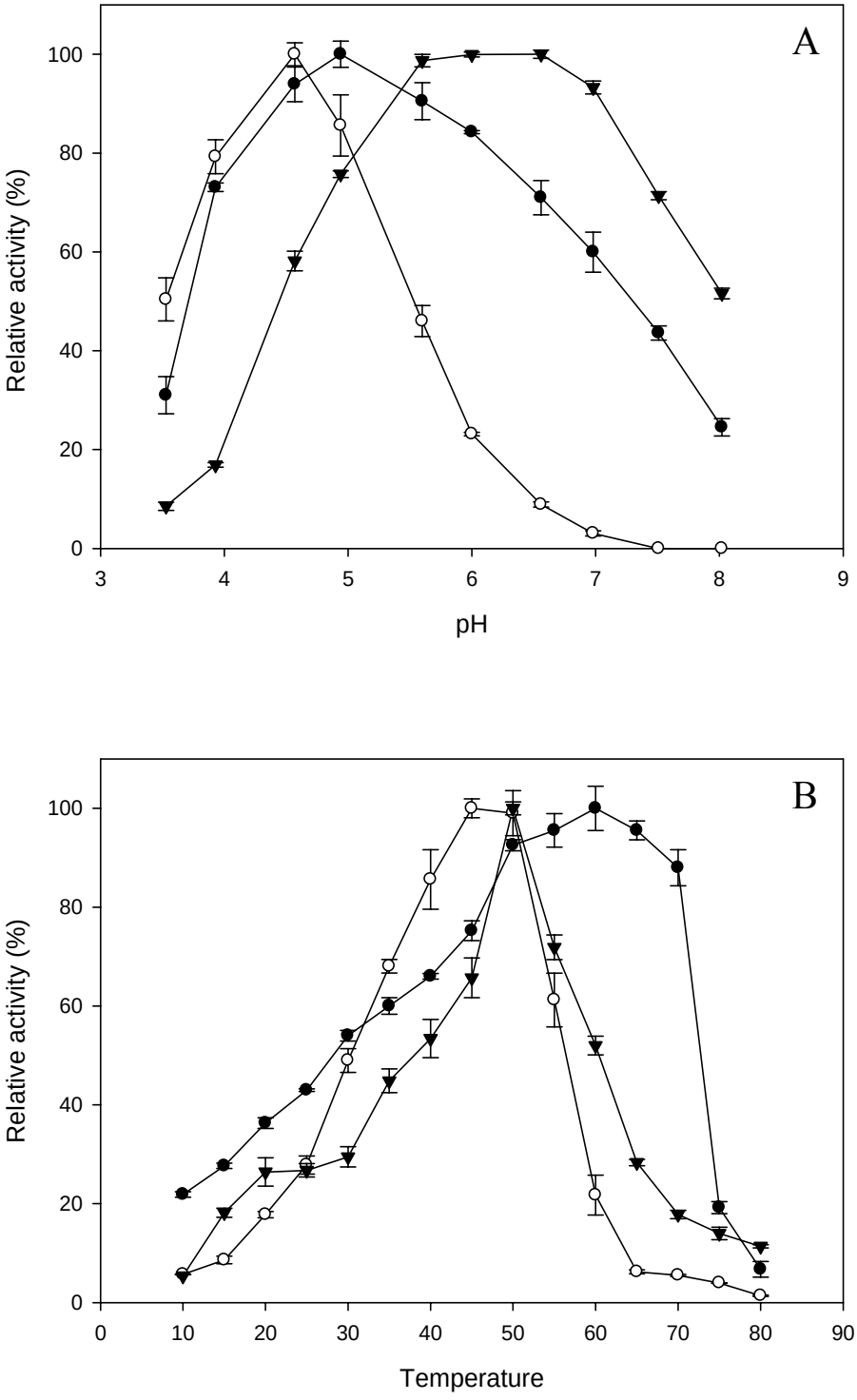

Figure 4: Effects of pH (A) and temperature (B) on hemicellulolytic enzyme activities. (●) α-arabinofuranosidase activity; (○) α-galactosidase activity; (▼) xylanase activity. The highest enzyme activity value was considered 100%.

Pre- incubation time (min)	α-Arabinofuranosidase	α-Galactosidase	Xylanase
0	100 %	100 %	100 %
15	3.8 %	99.6 %	78.5 %
30	2.4 %	99.2 %	63.8 %
60	0	77.3 %	35.6 %
90	0	56.9 %	29.4 %
150	0	36.2 %	0

Table 3: Thermal stability of the hemicellulolytic activities of *Acremonium* species. *Acremonium zeae* EA0802 α-galactosidase sample was pre-incubated for several times at 45 °C and *A. zeae* EA0802 α-arabinofuranosidase sample was pre-incubated at 60 °C. *Acremonium* sp. EA0810 xylanase sample was pre-incubated at 50 °C for several times. After pre-incubation the samples were assayed as described in the text. All standard deviation were less than 10 %

Substantial α-arabinofuranosidase activity was determined for the *A. zeae* EA0802 crude extract within the temperature range of 50 - 70 °C and at the pH range of 4.0 - 7.0. Maximal substrate hydrolysis was achieved at 60 °C and pH 5.0 (figures 4 A and 4 B). These optimum pH and temperature values are close to those determined for α-arabinofuranosidases from *Aspergillus terreus* (Le Clinche, et al., 1997) and *Aureobasidium pullulans* (Saha & Bothast, 1998). Pre-incubation of the enzyme for 15 min at 60 °C promoted a drastic loss of activity (table 3).

Xylanase from *Acremonium sp.* EA0810 was shown to be an alkaline protein, with substantial activity at the pH range of 4.5 - 8.0. The maximum activity was observed at pH 6.5 and a temperature of 50 °C (figures 4 A and 4 B). Similar results of pH and temperature were obtained for xylanase from *Aspergillus oryzae* (Chipeta et al., 2005), *Thermomyces lanuginosus* (Singh, et al., 2000) and *Aspergillus carneus* (Fang, et al., 2008). The enzyme retained more than 63 % of its maximal activity after pre-incubation at the optimum temperature (50 °C) after 30 min (table 3).

2.4.3.2 - Effect of pH and temperature on cellulolytic enzyme activities

The effect of pH and temperature on β-glucosidase, endoglucanase and FPase are shown in figure 5. Maximum β-glucosidase activity was detected at pH 4.5 to 5.0 and 60 °C. After pre-incubation of the enzyme for 30 min at 60 °C, all enzyme activity was lost (table 4). These optimum pH and temperature values are similar to those determined for the same enzyme of *Penicillium funiculosum* (Karboune, et al., 2008) and *Acremonium persicinum* (Pitson, et al., 1997).

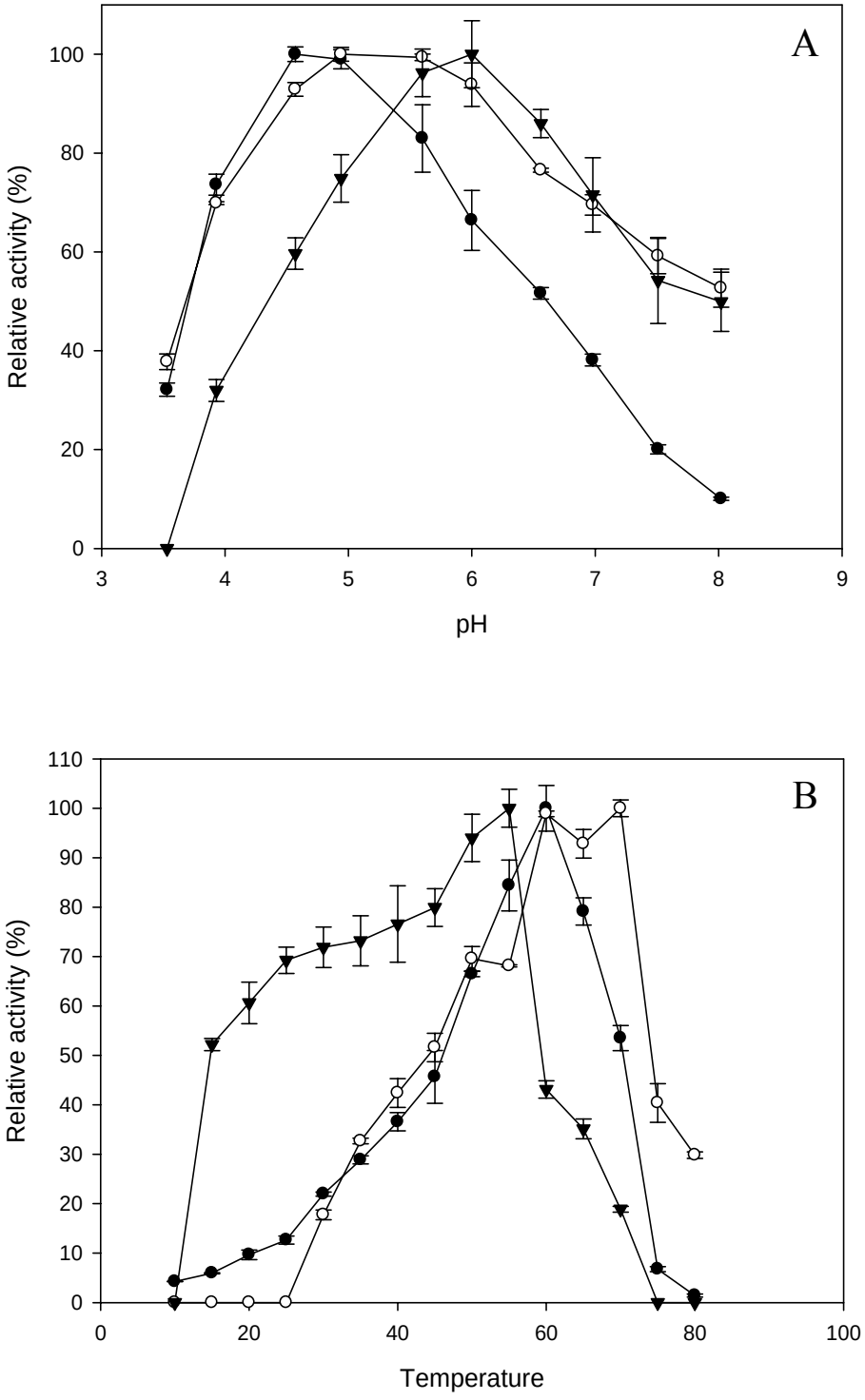

Figure 5: Effects of pH (A) and temperature (B) on the cellulolytic activities. (●) β-glucosidase activity; (○) endoglucanase activity; (▼) FPase activity. The highest enzyme activity value was considered 100%.

Pre- incubation time (min)	β-Glucosidase	Endoglucanase	FPase
0	100 %	100 %	100 %
15	3.0 %	91.6 %	98,4 %
30	0	87.9 %	29.5 %
60	0	75.9 %	0
90	0	41.6 %	0
150	0	36.9 %	0

Table 4: Thermal stability of *Acremonium* sp. EA0810 β-glucosidase, endoglucanase and FPase. The samples were pre-incubated at 60, 70 and 55 °C, respectively, for several times and then assayed as described in the text. All standard deviation were less than 10 %

Endoglucanase displayed a wide pH range since it is highly active at pH 4.5 to 6.0 and maintain 37 and 52 % at pH 3.5 and 8.0, respectively. The greatest activity was detected at pH 5.0 and 70 °C (figures 5 A and 5 B). *Acremonium* sp. EA0810 endoglucanase was thermostable and retained more than 75 % of its original activity after pre incubation for 60 min at 70 °C (table 4). Endoglucanase from *Mucor circinelloides* and *Melanocarpus albomyces* exhibited a similar pH profile (Saha, 2004; Oinonen, et al., 2004). Temperature optima is close to that determined for endoglucanase from *Daldinia eschscholzii*, however, EA0810 enzymes show to be more thermostable than endoglucanase from *D. eschscholzii* (Karnchanatat, et al., 2008). Endoglucanase thermostability and high pH stability suggested that it has potential for industrial use, mainly in the biostoning process. In the textile industry there is a need for novel cellulases that are active at neutral and alkaline pH values, because they can promote low backstaining (Miettinen-Oinonen, et al., 2004).

The greatest FPase activity was detected at pH 6.0 and 55 °C (figures 5 A and 5 B). The enzyme maintains a relatively high activity in alkaline pH retaining about 50 % of its maximum activity at pH 8.0. This is in agreement with the value reported by Dutta, et al., 2008 for *Penicillium citrinum*. A loss of 70 % in initial activity was observed after pre incubation at 55 °C for 30 min (table 4)

2.4.4 - Zymogram analysis

Zymogram analysis of the crude enzyme preparation used for characterization revealed the presence of one xylanase of about 27,8 KDa and one endoglucanase of approximately 61,6 KDa (figures 7 A and 7 B). This indicates that xylanase and endoglucanase from *Acremonium sp.* EA0810 may exist in only one form, similar to *Fusarium proliferatum* that produce one xylanase of about 22.4 kDa and to the endoglucanase from *Daldinia eschscholzii* (Saha, 2002; Karnchanatat, et al., 2007).

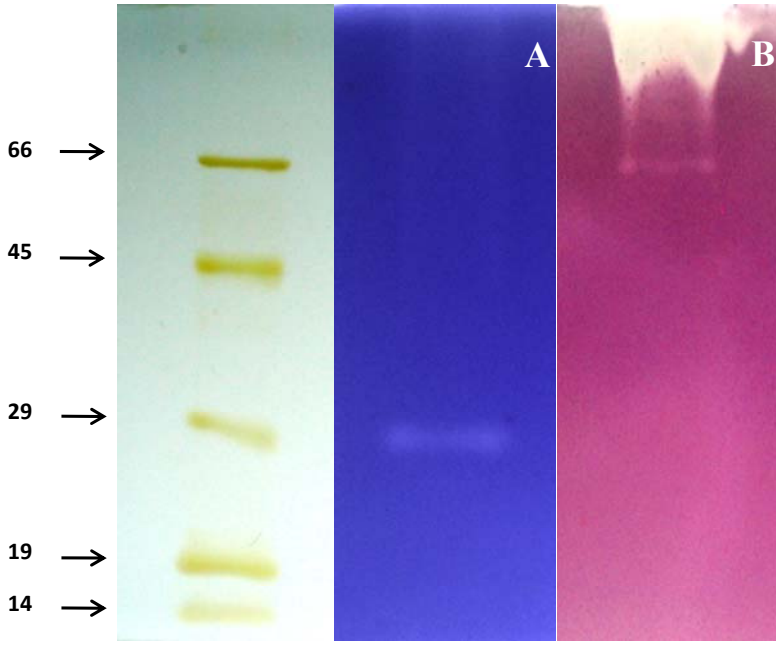

Figure 6: Zymogram analysis of crude *Acremonium* sp. EA0810 xylanase (A) and endoglucanase (B) stained with congo red

2.5 - CONCLUSION

- Submerged culture is more efficient to induced enzymes production than solid state fermentation

- Sugar cane bagasse and corn straw efficiently induce the xylanase, FPase and endoglucanase production, in *Acremonium zeae* EA0802 and in *Acremonium* sp. EA0810.

- The maximal substrate hydrolysis for xylanase iss achieved at pH 6.5 and a temperature of 50 ºC. The greatest endoglucanase activity is detected at pH 5.0 and 70 °C and for FPase the optimum activity was at pH 6.0 and 55 °C

- The best carbon source tested to induce *Acremonium* sp. EA0810 β-Glucosidase activity is D-xylose and this enzyme achieved it maximal activity at pH 4.5 to 5.0 and 60 °C.

- High α-arabinofuranosidase and α-galactosidase activity is obtained from *Acremonium* zeae EA0802 using oat spelt xylan as a carbon source after cultivation for 18 and 4 days, respectively. Substantial α-arabinofuranosidase activity is determined within the temperature range of 50 - 70 °C and at the pH range of 4.0 -7.0. The highest α-Galactosidase activity level is observed in temperature range of 45 - 50 °C and at pH 4.5

- *Acremonium* sp. EA0810 endoglucanase is a promising enzyme to future industrial applications due to its high activity en neutral pH and high thermostability

- *Acremonium* sp. EA0810 xylanase and endoglucanase appeared in the zymogram analysis as one form of 27.8 and 61.6 kDa, respectively

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