MAÍRA NICOLAU DE ALMEIDA

COMPLEXO CELULOLÍTICO E HEMICELULOLÍTICO DO FUNGO ENDOFÍTICO *FUSARIUM VERTICILLIOIDES* E SUA APLICAÇÃO PARA SACARIFICAÇÃO DO BAGAÇO DE CANA

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

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APROVADA: 22 de fevereiro de 2013

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BIOGRAFIA

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RESUMO

ALMEIDA, Maíra Nicolau de, D. Sc., Universidade Federal de Viçosa, fevereiro de 2013. **Complexo celulolítico e hemicelulolítico do fungo endofítico *Fusarium verticillioides* e sua aplicação para sacarificação do bagaço de cana.** Orientador: Sebastião Tavares de Rezende. Coorientadores: Valéria Monteze Guimarães e Olinto Liparini Pereira.

Neste trabalho o fungo endofítico *Fusarium verticillioides* foi avaliado quanto ao seu potencial para produção de enzimas celulases e hemicelulases visando sua aplicação no processo de sacarificação da biomassa para produção de etanol. O meio de cultivo utilizado para produção de celulases e xilanase por *F. verticillioides* foi otimizado. Níveis ótimos de fonte de carbono (palha de milho), fonte de nitrogênio (nitrato de sódio) e tempo de cultivo foram determinados. As atividades de endoglicanase e xilanase aumentaram de 2,8 U/mL para 8,0 U/mL e de 3,4 U/mL para 114,0 U/mL, respectivamente. Temperatura e pH ótimos foram determinados para endoglicanse (5,6; 80 °C), celobiase (5,6; 60 °C), FPase (6,0; 55 °C) e xilanase (7,0; 50 °C). O extrato enzimático otimizado foi usado na sacarificação e fermentação simultâneas de bagaço de cana. Neste processo 9,7 g/L de etanol foram obtidos com um rendimento de etanol/ biomassa (g/g) de 0,19. Um segundo meio de cultivo contendo diferentes fontes de nitrogênio (ureia, extrato de levedura, peptona e sulfato de amônio) e forrageira como fonte de carbono foi estabelecido por meio de delineamento de Plackett Burman para produção de celulases. Dois meios contrastantes, M1 e M2, foram definidos para maior produção de endoglicanase e celobiase, respectivamente. Misturas destes dois extratos foram testadas para a sacarificação do bagaço de cana e o extrato M1 apresentou o maior rendimento para conversão de glicanas 26,6 %, e de xilanases 40,4 %. Dosagens diferentes de proteína (10 mg/g, 20 mg/g e 40 mg/g de biomassa seca) do extrato M1 foram avaliadas em relação à sacarificação de bagaço de cana. Rendimentos de 43,4 % e 73,1 % foram observados para conversão de glicanas e xilanas, respectivamente quando 40 mg/g foi utilizado. Adsorção de proteína foi observada durante a sacarificação o que pode explicar a observada diminuição da produtividade durante o processo de sacarificação. O fungo *F. verticillioides* e o fungo *Acremonium zeae* foram avaliados em relação as suas capacidades de fermentação de açúcares simples e de biomassa lignocelulósica. *Fusarium verticillioides* produziu etanol a partir de glicose,

xilose e de uma mistura dos destes dois açúcares com rendimentos de 0,47 g/g, 0,46 g/g e 0,50 g/g de etanol por açúcar utilizado. O fungo *Acremonium zeae* produziu etanol a partir de glicose, xilose e da mistura com rendimento de 0,37 g/g, 0,39 g/g e 0,48 g/g de açúcar utilizado. Os dois fungos foram capazes de co-fermentar glicose e xilose. *Fusarium verticillioides* e *A. zeae* produziram altas atividades de endoglicanase e de xilanase utilizando bagaço de cana como fonte de carbono. A produção de etanol a partir de 40 g/L de bagaço de cana foi de 4,6 g/L e 3,9 g/L para *F. verticillioides* e *A. zeae*, respectivamente. Grande interesse foi gerado pelas características bioquímicas da atividade de endoglicanase, como atividade em temperaturas elevadas e estabilidade em diversos pHs. Assim, foi desenvolvido um estudo de purificação e caracterização da atividade de endoglicanase do fungo *F. verticillioides*. Um complexo multienzimático, $E1_C$, e uma endoglicanase livre, E2, foram purificados. O complexo $E1_C$ contém duas endoglicanases (GH3 e GH10), uma celobiohidrolase (GH7) e uma xilanase (GH10). A temperatura de máxima atividade foi 80 °C para $E1_C$ e para E2. A enzima livre e o complexo foram termoestáveis a 50 °C e 60 °C. A energia de ativação para $E1_C$ e E2 foram 21,3 KJ/mol e 27,5 KJ/mol, respectivamente. Atividade máxima de $E1_C$ foi em pH 4,5 e de E2 foi em pH 5,5; $E1_C$ apresentou alta estabilidade após 24 h de pré-incubação em pHs variando de 2,6 a 8,0. O valor de K_M para $E1_C$ foi 10,25 g/L enquanto para E2 foi 6,58 g/L usando carboximetilcelulose como substrato. Tanto $E1_C$ quanto E2 foram significativamente ativadas por Mn^{2+}, $CoCl_2$, furfural, hidroximetilfurfural and ditiotreitol enquanto foram inibidas por SDS, $CuSO_4$, $FeCl_3$, $AgNO_4$, $ZnSO_4$ e $HgCl_2$. O complexo $E1_C$ e a enzima livre E2 apresentaram atividade mais alta utilizando o substrato glicana de cevada (Barley-β-glucan) do que utilizando o substrato padrão carboxymetilcelulose (CMC), indicando atividade de endo-β-1,3-1,4-glicanase. As enzimas foram capazes de hidrolisar celopentaose, celotetraose e celotriose, entretanto com modos de ação diferentes. Em todos os ensaios que avaliavam estabilidade e eficiência, $E1_C$ apresentou melhores resultados do que E2, o que sugere vantagens geradas pela interação física de proteínas presente nos complexos.

ABSTRACT

ALMEIDA, Maíra Nicolau de, D. Sc., Universidade Federal de Viçosa, February, 2013. **Cellulolytic and hemicellulolytic complex from the endophytic fungus *Fusarium verticillioides* and its application on sugarcane bagasse saccharification.** Adviser: Sebastião Tavares de Rezende. Co-Advisers: Valéria Monteze Guimarães and Olinto Liparini Pereira.

In this work, the endophytic fungus *Fusarium verticillioides* was evaluated in relation to its potential to produce cellulases and hemicellulases aiming to its application on biomass saccharification for ethanol production. The culture medium used for cellulase and xylanase production was optimized. Optimum levels of carbon source concentration (corn straw), nitrogen source concentration (sodium nitrate) and time of cultivation were obtained. Endoglucanase and xylanase activities increased from 2.8 U/mL to 8.0 U/mL and from 3.4 U/mL to 114.0 U/mL, respectively. The optimal pH and temperature were determined for endoglucanase (5.6, 80 °C), cellobiase (5.6, 60 °C), FPase (6.0, 55 °C) and xylanase (7.0, 50 °C). The optimized crude extract was applied in saccharification and fermentation of sugarcane bagasse from which 9.7 g/L of ethanol was produced at an ethanol/biomass (g/g) yield of 0.19. A second cultivation medium containing different nitrogen sources (urea, yeast extract, peptone and ammonium sulfate) and forage as carbon source was established throught Plackett Burman design for cellulases production. Two contrasting media, M1 and M2 were defined for higher endoglucanase and cellobiase production, respectively. Mixtures of these two extracts were tested on a sugarcane bagasse saccharification and extract M1 presented the best performance, yielding 26.6 % of glucan conversion and 40.4 % of xylan conversion. Different dosages of protein (10 mg/g, 20 mg/g and 40 mg/g of dry biomass) were tested intending to improve the hydrolysis performance of M1 extract. Saccharification rates of 43.4 % and 73.1 % were observed for glucan and xylan fractions respectively when 40 mg/g was used. A high protein adsorption on substrate was observed during saccharification assays employing M1 extract and that can explain de lower sugar productivity in the saccharification process along the time. The fungus *F. verticillioides* and the fungus *Acremonium zeae* were evaluated in relation to its abilities to ferment simple sugars and lignocellulosic biomass. The fungus *Fusarium verticillioides* produced ethanol from glucose, xylose and a mixture of these two sugars in

limited oxygen conditions with yields of 0.47 g/g, 0.46 g/g and 0.50 g/g of ethanol per sugar utilized. The fungus *Acremonium zeae* produced ethanol from glucose, xylose and mixture of these two sugars with yields of 0.37 g/g, 0.39 g/g and 0.48 g/g of ethanol per sugar utilized. Both fungi were able to co-ferment glucose and xylose. *Fusarium verticillioides* and *A. zeae* produced high endoglucanase and xylanase activities using sugarcane bagasse as substrate. Ethanol production from 40 g/L of pre-treated sugarcane bagasse was 4.6 g/L and 3.9 g/L for *Fusarium verticillioides* and *A. zeae*, respectively. Both fungi showed features suitable for consolidated bioprocessing. Great interest was generated by the biochemical characteristics of the endoglucanase activity, as activity in high temperatures and stability in various pHs. Then it was developed a purification and characterization study of endoglucanase activity from the fungus *F. verticillioides*. A multienzyme complex, $E1_C$, and a free endoglucanase, E2 were purified. The $E1_C$ contained two endoglucanases (GH6 and GH10), one cellobiohydrolase (GH7) and one xylanase (GH10). The temperature of maximal activity was 80 °C for both $E1_C$ and E2. The free enzyme and the complex were very thermostable at 50 °C and 60 °C. The activation energies for $E1_C$ and E2 were 21.3 KJ/mol and 27.5 KJ/mol, respectively. Maximum activity of $E1_C$ was encountered at pH 4.5 and of E2 was at pH 5.5; $E1_C$ presented high stability after 24 h of pre-incubation at pH ranging from 2.6 to 8.0. The K_M value for $E1_C$ was 10.25 g/L while for E2 was 6.58 g/L using carboxymethylcellulose as substrate. Both $E1_C$ and E2 were significantly activated by Mn^{2+}, $CoCl_2$, furfural, hydroxymethylfurfural and dithiothreitol while they were inhibited by SDS, $CuSO_4$, $FeCl_3$, $AgNO_4$, $ZnSO_4$ and $HgCl_2$. Both $E1_C$ and E2 presented higher activity towards barley-β-glucan than carboxymethylcellulose (CMC), indicating endo-β-1,3-1,4-glucanase activity. The enzymes were able to hydrolyze cellopentaose, cellotetraose and cellotriose, however, in different mode of action. In all the assays evaluating stability and efficiency $E1_C$ showed better performance than E2, suggesting advantages generated by the physical interaction between proteins.

1- Introdução

O etanol obtido a partir de biomassas lignocelulósicas, etanol de segunda geração, vem sendo considerado uma alternativa promissora para completar a plataforma energética já estabelecida para a produção do etanol de cana de açúcar. A acessibilidade local e baixo custo da matéria prima estão entre as suas maiores vantagens. Além disso, o desenvolvimento dessa tecnologia contribui para a diminuição da competição por terras férteis para produção de energia ou para alimentação, uma vez que permite maior rendimento de etanol por hectare de terra plantada.

Porém, apesar de oferecer muitas vantagens, o etanol lignocelulósico ainda não é uma realidade porque a tecnologia para sua produção não está totalmente desenvolvida e muitos gargalos tecnológicos precisam ser superados. O processo de produção de etanol a partir de biomassa pode ser dividido em três etapas críticas: pré-tratamento, sacarificação e fermentação.

O pré-tratamento consiste em processos físicos e químicos aplicados à biomassa com o objetivo de diminuir o teor de lignina, diminuir o grau de polimerização e cristalinidade da celulose e aumentar a porosidade e a superfície de contato da biomassa. Essas alterações facilitam a atuação das enzimas hidrolíticas no passo seguinte que é a sacarificação enzimática. O pré-tratamento é uma etapa onerosa e que favorece a produção de uma série de compostos tóxicos que inibem os micro-organismos fermentadores.

A sacarificação enzimática consiste na utilização de celulases e hemicelulases para depolimerizar a biomassa. O obstáculo inerente a esta etapa é o alto custo da produção das enzimas e a complexidade do processo de hidrólise enzimática da biomassa. Os complexos enzimáticos comerciais atualmente utilizados no processo são produzidos por fungos dos gêneros *Trichoderma* e *Aspergillus*. Há muitos anos, grandes investimentos em pesquisa vêm sendo aplicados no melhoramento de fungos pertencentes a estes gêneros para a produção de celulases. Entretanto, apesar dos maciços investimentos, a produção dos extratos enzimáticos a partir destes fungos apresenta alto custo e os extratos necessitam ser utilizados em grandes quantidades para uma degradação eficiente da biomassa o que onera ainda mais o processo.

Na fermentação os açúcares obtidos na sacarificação são convertidos a etanol. Os gargalos da fermentação são a obtenção de micro-organismos que fermentem em alto rendimento hexoses e pentoses e apresentem alguma tolerância a inibidores presentes no meio reacional. A sacarificação e fermentação simultâneas é um processo que exige, além dessas características, micro-organismos termotolerantes afim de que as condições ótimas de sacarificação e fermentação estejam mais próximas do ideal de cada uma delas. O bioprocesso consolidado também tem sido apontado como uma alternativa para a produção de etanol lignocelulósico. Neste processo, as características desejadas para os micro-organismos são ainda mais abrangentes, uma vez que a produção de enzimas, sacarificação e fermentação são realizadas em uma única etapa pelo mesmo micro-organismo.

Fungos endofíticos se desenvolvem no interior de sementes, raízes, caules e folhas e apresentam grande potencial para a produção de celulases e hemicelulases eficientes, uma vez que para sua sobrevivência dentro de materiais vegetais vivos é necessário a atuação destas enzimas. Neste trabalho, focalizamos o estudo na otimização da produção de extratos enzimáticos pelo fungo endofítico *Fusarium verticillioides*. Extratos enzimáticos deste fungo foram utilizados para sacarificação de bagaço de cana seguida de produção de etanol por levedura. Além disso, também foi estudado o potencial dos fungos endofíticos *F. verticillioides* e *Acremonium zeae* para produção de etanol pelo bioprocesso consolidado a partir de bagaço de cana.

2. Objetivos

- Otimizar as condições de cultivo do fungo *Fusarium verticillioides* para produção de celulases e hemicelulases.
- Avaliar condições ideais para a sacarificação do bagaço de cana utilizando os extratos otimizados do fungo.
- Avaliar o potencial de fermentação do fungo utilizando açúcares simples e biomassa lignocelulósica.
- Purificar e caracterizar bioquímica e cineticamente endoglucanases do fungo *F. verticillioides* e estudar o modo de ação dessas enzimas em diferentes substratos.

3- Revisão bibliográfica

3.1- Etanol

Etanol é um combustível líquido que pode ser obtido a partir de açúcares e grãos. Este combustível se destacou no Brasil desde a década de 70 como uma fonte de energia renovável, alternativa aos combustíveis fósseis, principalmente a gasolina [1]. O etanol apresenta um maior valor de octanagem (medida da qualidade do combustível), limites mais amplos de inflamabilidade, maior velocidade de inflamação e maior temperatura de vaporização do que a gasolina. Essas propriedades conferem ao etanol maior taxa de compressão e menor tempo de combustão, o que leva a uma maior eficiência teórica em relação à gasolina em um motor de combustão interna. Desvantagens do etanol em relação à gasolina incluem menor densidade de energia, maior corrosividade, baixa luminosidade de chama, menor pressão de vapor (dificultando a ligação em clima frio) e miscibilidade com água [2].

A produção mundial de etanol em 2012 foi de 85,2 bilhões de litros [3]. A produção de etanol no Brasil cresceu nos últimos 13 anos, entretanto tem se estabilizado nos últimos 4 anos (Tabela 1). Na safra 2012/2013 foram produzidos 22,84 bilhões de litros de etanol [4].

Tabela 1: Produção brasileira de etanol

Ano-Safra	Etanol total (bilhões de litros)
00/01	10,52
01/02	11,47
02/03	12,48
03/04	14,64
04/05	15,21
05/06	15,81
06/07	17,94
07/08	22,44
08/09	27,68
09/10	25,74
10/11	27,60
11/12	22,74
12/13	22,84

Adaptado de [4]

O Brasil tem uma localização geográfica privilegiada e seu clima permite o cultivo da cana-de-açúcar com alto rendimento e com pouca irrigação. Além disso, o Brasil é o único grande produtor de álcool que pode aumentar

significativamente sua produção para suprir a demanda interna e ainda ter excesso para exportação. Com a contínua introdução de novas variedades de cana no mercado e com o desenvolvimento da tecnologia de produção de etanol a partir do bagaço de cana o aumento da produção de etanol é viável [1, 5].

3.2- A biomassa lignocelulósica

A única fonte renovável de carbono grande o suficiente para substituir parcialmente os combustíveis fósseis é a biomassa. Materiais lignocelulósicos são particularmente atraentes neste contexto devido ao seu relativo baixo custo e disponibilidade local [6]. Em geral, as biomassas lignocelulósicas podem ser divididas em seis grupos: resíduos agroindustriais (bagaço de cana, resíduos de milho, palha de arroz, palha de trigo, sorgo entre outros), madeiras; resíduos de celulose (papéis de jornal, papéis de reciclagem), biomassas herbáceas (alfafa, switchgrass, entre outras plantas forrageiras) e resíduos sólidos municipais [2, 7].

Estes resíduos são ricos em fibras que quando processadas liberam açúcares fermentáveis para produção de etanol, butanol, biogás e outros produtos de alto valor agregado, os *building blocks*, moléculas com grupos funcionais múltiplos que possuem o potencial para serem transformadas em novos grupos de moléculas úteis. Doze *building blocks* foram identificados: 1,4-diácidos (succínico, fumárico e málico), ácido 2,5-furano carboxílico, ácido 3-hidróxido propiônico, ácido aspártico, ácido glucárico, ácido glutâmico, ácido itacônico, ácido levilínico, 3-hidróxidobutillactona, glicerol, sorbitol, e xilitol/arabinitol [8, 9].

O bagaço de cana, o principal resíduo lignocelulósico no Brasil é um material fibroso obtido após a extração do suco da cana no processo de produção de açúcar e etanol. Em média, é constituído por lignina (19 – 24 %), celulose (32 – 44 %), hemicelulose (27 – 32 %) e cinzas (4,5 – 9 %) [1].

Usinas de açúcar geram aproximadamente 270-280 Kg de bagaço por tonelada de cana-de-açúcar e atualmente 540 milhões de toneladas de bagaço seco são processados anualmente em todo mundo [1, 10]. A produção anual de bagaço no Brasil é estimada em 186 milhões de toneladas. Cerca de 92 % deste resíduo é utilizado para produção de energia a partir de sua queima. Se os 8 % restantes fossem convertidos em etanol de segunda geração, poderia-

se esperar um adicional de 2200 L por hectare de terra plantada, aumentando o rendimento de etanol por hectare brasileiro de 6000 L para 8200 L [1].

3.2.1- Natureza química da biomassa e sua hidrólise

A lignocelulose é composta por microfibrilas de celulose inseridas em uma matriz de hemicelulose, pectina, lignina e uma pequena quantidade de proteínas estruturais.

A celulose é um homopolissacarídeo composto por resíduos de β-D-glicopiranos ligados entre si por ligações (1 - 4) glicosídicas. Os modelos atuais da organização microfibrilar sugerem que ela tem uma subestrutura constituída de domínios altamente cristalinos unidos por ligações amorfas. Para a degradação completa da celulose são necessárias as atuações sinergísticas de quatro tipos de celulases (Figura 1).

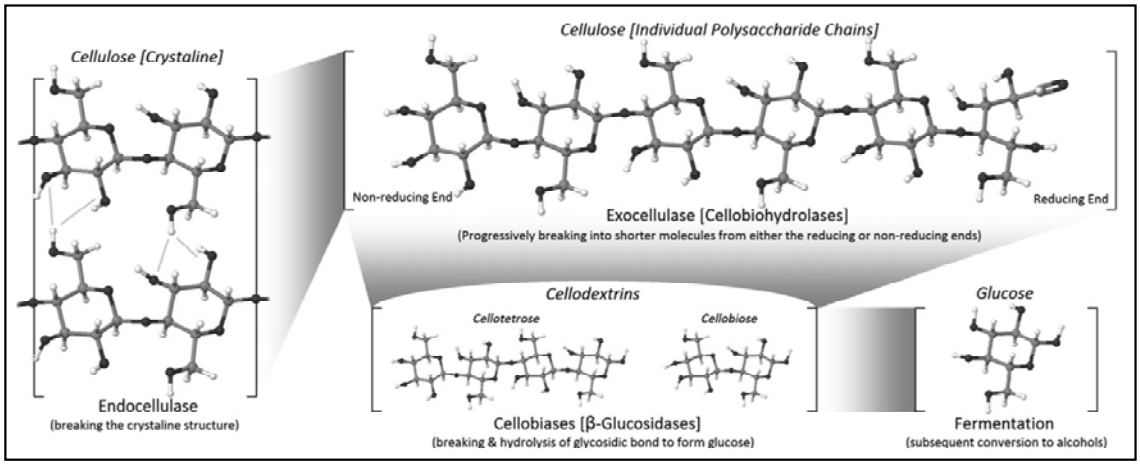

Figura 1: Atuação sinergística das quatro celulases envolvidas na hidrólise da celulose [11].

As endoglicanases (1,4- β -D- glicana-4-glicanohidrolase; EC 3.2.1.4) hidrolisam ligações β- (1 → 4) internas da celulose. As celobiohidrolases (1,4-β-D-glicana celobiohidrolase; EC 3.2.1.91) e as exoglicohidrolases (1,4-β-D-glicana glicobiohidrolase, EC 3.2.1.74) são exocelulases. Celobiohidrolases (CBH) degradam celulose por remoção consecutiva de celobiose dos terminais redutores (CBH I) e não redutores (CBH II) do polissacarídeo. Exoglicohidrolases hidrolisam consecutivamente a remoção de unidades de glicose de terminais não redutores de celodextrinas. E finalmente as β-glicosidases, ou celobiases, (β-D-glicosideoglicohidrolase; EC 3.2.1.21) que clivam celobiose em glicose e removem glicose de terminais não redutores de pequenas celodextrinas. 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 da biomassa [12, 13].

As hemiceluloses, também chamadas de glicanas de ligação cruzada, são constituídas principalmente pelos açúcares D-glicose, D-manose, D-galactose, D-xilose, L-arabinose e D-ácido glicurônico. No geral, as hemiceluloses apresentam um baixo grau de polimerização (média de 100 - 200) e a qualidade e quantidade de açúcares presentes em sua composição depende do tipo de parede celular. 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 [14, 15].

Para a sua hidrólise é necessário um conjunto de enzimas que depende de quais açúcares estão presentes na biomassa. Para degradação de xilanas em geral são necessárias basicamente sete enzimas diferentes. Endo-1,4-β-D-xilanases (EC 3.2.1.8) são enzimas que clivam aleatoriamente o esqueleto de xilana 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 [16, 17] (Figura 2).

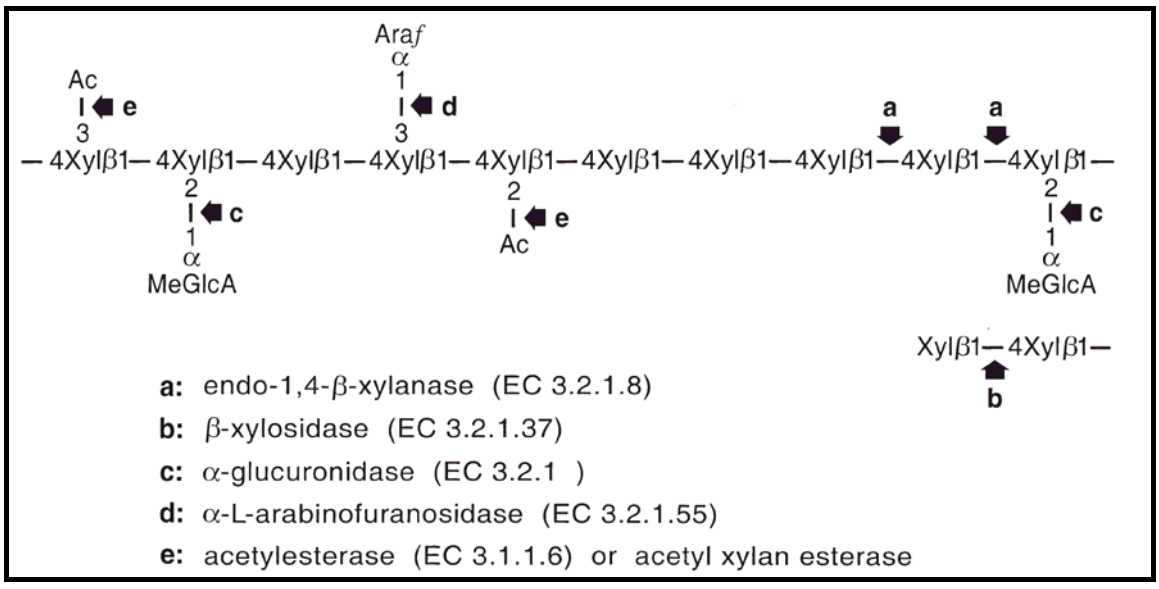

Figura 2: Pontos de atuação de diversas enzimas para hidrólise da xilana [15]

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. 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. 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 [15, 18].

Lignina é um composto que está covalentemente ligado à celulose e outros polissacarídeos da parede celular. Essa estrutura empacota as microfibrilas de polissacarídeos protegendo-os contra a atividade hidrolítica de enzimas e outros fatores externos e estabiliza o complexo lignocelulose [19]. A lignina é composta basicamente de unidade de fenilpropanóides ramificados dispostas aleatoriamente formando uma macromolécula tridimensional e amorfa [20]. 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 3). Esses alcoóis formam um polímero por ação enzimática.

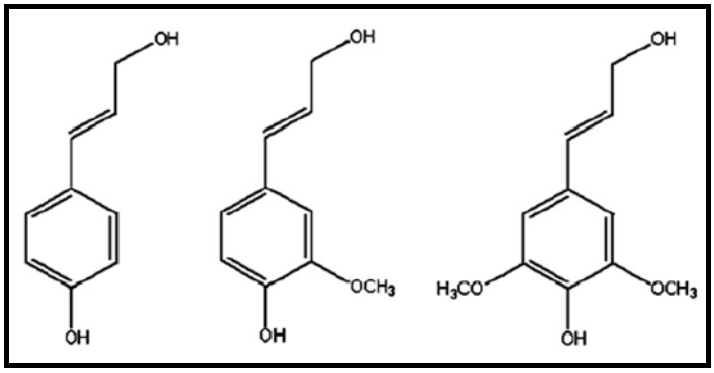

Figura 3: estruturas dos compostos fenólicos precursores de lignina: cumaril, coniferil e sinapil [21]

A estrutura precisa da lignina não é conhecida devido à dificuldade para sua extração das plantas, mas algumas estruturas hipotéticas são propostas (Figura 4).

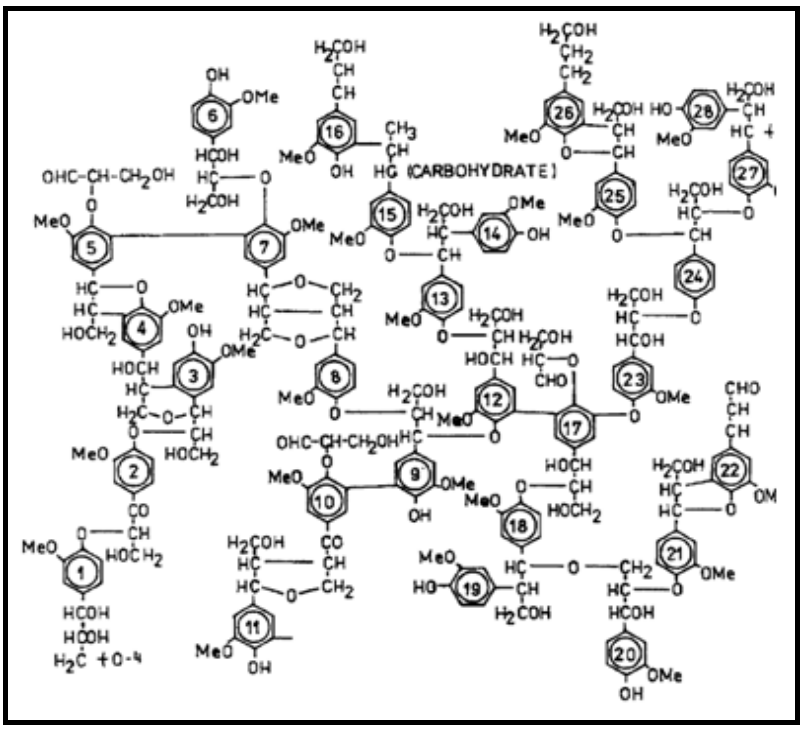

Figura 4: Estrutura hipotética proposta para a lignina [19]

As funções da lignina estão relacionadas a questões estruturais, impermeabilização do tecido vegetal, resistência ao ataque de insetos e micro-organismos e proteção contra o estresse oxidativo. A lignina é extremamente recalcitrante e sua presença no tecido vegetal é um dos maiores entraves para o processo de solubilização da biomassa.

A conversão enzimática da lignina, diferentemente da hidrólise de celulose e hemicelulose, é realizada por oxidases. As oxidases são as lacases, manganês peroxidase e lignina peroxidase. Essas enzimas são produzidas principalmente por fungos de podridão branca [21, 22].

3.3- Etanol lignocelulósico: o processo e suas perspectivas

A produção de etanol a partir de biomassa lignocelulósica é baseada na solubilização de seus componentes em açúcares fermentáveis. O processo de produção de etanol a partir de biomassa pode ser dividido basicamente em três etapas: pré-tratamento, sacarificação e fermentação e destilação. O etanol lignocelulósico vem complementar a plataforma de produção de etanol já estabelecida e pode contribuir para o maior aproveitamento das terras cultiváveis diminuindo a competição das mesmas para finalidade alimentícia ou energética.

Apesar das expectativas em relação ao etanol de segunda geração, alguns empecilhos técnicos e econômicos ainda devem ser contornados. Os gargalos chaves que impedem a viabilidade econômica deste processo são: seleção e otimização do pré-tratamento mais eficiente e de menor custo; diminuição dos custos da hidrólise enzimática; e maximização da conversão de açúcares a etanol por cepas de leveduras mais adaptadas. Além dos gargalos do próprio processo, também se faz necessária uma integração com outras tecnologias para minimizar o consumo de água e energia e aproveitar co-produtos como a lignina.

3.3.1- Pré-tratamentos

O pré-tratamento da biomassa é um passo fundamental que tem o objetivo de desestruturar a parede celular da biomassa, reduzindo o teor de lignina, aumentando a porosidade do material e rompendo as cadeias de celulose (redução da cristalinidade e grau de polimerização). Assim, a superfície de contato é aumentada facilitanto a acessibilidade das enzimas e aumentando eficiência do processo de sacarificação enzimática [2, 23].

A moagem é um pré-tratamento mecânico que tem por objetivo reduzir o tamanho das partículas e a cristalinidade da celulose. A moagem promove um grande aumento na superfície específica da biomassa com uma concomitante redução no grau de polimerização de seus constituintes. Entretanto, este processo isolado é insuficiente para se obter taxas de sacarificação economicamente viáveis, logo é um pré-tratamento que precede outros [24, 25].

Pré-tratamentos térmicos como explosão a vapor e água líquida quente afetam principalmente a fração hemicelulósica e lignínica da biomassa. Temperaturas superiores a 150 - 180 °C promovem inicialmente a solubilização da hemicelulose e a fragmentação da lignina. Durante os tratamentos térmicos, as ramificações laterais presentes na hemicelulose são inicialmente clivadas levando a geração de grupos ácidos. Estes grupos ácidos em um segundo momento contribuem para a solubilização dos demais componentes da hemicelulose catalisando a hidrólise de seus diversos polímeros componentes, caracterizando assim um processo autolítico, onde os próprios constituintes da hemicelulose contribuem para a sua degradação. Temperaturas elevadas conduzem à degradação da lignina promovendo a geração de compostos

fenólicos solúveis. Estes compostos são reativos e se não forem eliminados rapidamente da solução podem reagir ou re-precipitar sobre as fibras afetando negativamente o efeito do pré-tratamento [24].

O pré-tratamento ácido é uma das alternativas mais utilizadas para se promover modificações na biomassa e podem ser executados em condições de temperatura ambiente ou temperatura moderada. Os tratamentos ácidos podem ser realizados em altas ou baixas concentrações de diferentes ácidos como HCl, H_2SO_4, ácido nítrico e ácido fosfórico. O principal efeito dos tratamentos ácidos é promover a degradação de hemicelulose. A celulose também pode ser clivada em alguns pontos de sua estrutura durante o tratamento ácido o que provoca redução de sua cristalinidade e redução no grau de polimerização. Geralmente, os tratamentos ácidos são pouco efetivos para promover a degradação ou alteração da lignina. O grande inconveniente dos tratamentos ácidos é a geração de derivados de furano e de ácidos alifáticos de cadeias curtas, os quais inibem a fermentação [24, 25].

Tratamentos alcalinos promovem um aumento na digestibilidade da biomassa e os resíduos vegetais de natureza herbácea geralmente são mais suscetíveis a este tipo de tratamento do que os resíduos derivados de materiais lenhosos. Os efeitos do tratamento alcalino sobre a biomassa incluem inicialmente a solvatação e a saponificação dos seus componentes por meio da clivagem de ligações ésteres existente entre as cadeias de polímeros hemicelulósicos (principalmente xilanas) e a lignina. A eliminação das ligações cruzadas entre diferentes componentes da hemicelulose e entre a hemicelulose e a lignina favorece o aumento da porosidade na biomassa. Além disto, nota-se que os tratamentos alcalinos promovem um “inchaço” das cadeias celulósicas e isto provoca a redução da cristalinidade e aumenta a porosidade entre as cadeias do polímero. A lignina é moderadamente alterada durante os tratamentos alcalinos observando-se a solubilização e a oxidação de seus componentes e isto também contribui para um aumento no rendimento do processo de sacarificação subseqüente.

Tratamentos alcalinos são realizados utilizando soluções diluídas de NaOH e KOH sob condições de temperatura ambientes ou em autoclave (121 °C). As condições alcalinas do processo desfavorecem a formação de derivados de furanos caracterizando-se assim uma vantagem em relação aos tratamentos ácidos e térmicos. Por outro lado, a solubilização mais

pronunciada da lignina favorece a geração de compostos fenólicos tóxicos e este, juntamente com o custo do NaOH podem ser considerados aspectos negativos deste tipo de pré-tratamento [24, 25].

Tratamentos oxidativos têm como principal objetivo remover a lignina da biomassa e aumentado a porosidade do material. Os processos oxidativos podem ser realizados utilizando-se fortes agentes oxidantes como H_2O_2 e ácido paracético e as principais reações químicas catalisadas por estes reagentes são: substituições eletrofílicas, substituições de cadeias laterais, clivagem de ligações aril-alquil éter e clivagem oxidativa de núcleos aromáticos. Tratamentos oxidativos não favorecem a formação de derivados de furano, entretanto, pode gerar compostos fenólicos e grupos alifáticos de cadeias curtas [24].

A explosão das fibras pela amônia também conhecida como AFEX (Ammonia Fiber Explosion) é um tipo de pré-tratamento físico-químico no qual a biomassa é exposta à amônia liquida em condições de alta temperatura e pressão por alguns minutos, sendo então a pressão rapidamente reduzida. A rápida descompressão favorece uma rápida expansão da amônia impregnada na biomassa promovendo a explosão do material lignocelulósico. O tratamento AFEX tem pouco efeito sobre a solubilização da hemicelulose e da lignina. O principal efeito benéfico deste tipo de tratamento consiste na redução da cristalinidade e do grau de polimerização da celulose. O tratamento AFEX praticamente não altera a composição química da biomassa, diferentemente dos tratamentos térmicos, oxidativos, ácidos e alcalinos [25].

Diversos outros pré-tratamentos vêm sendo amplamente estudados. Entretanto, até o momento nenhum pré-tratamento reúne todas as condições necessárias simultaneamente, cada tipo de pré-tratamento possui vantagens e desvantagens e desta forma a escolha do pré-tratamento é uma equação extremamente complexa que depende de vários fatores, sendo que o custo, as características da biomassa e as condições operacionais utilizadas são aquelas que exercem maior influência no momento de se escolher o pré-tratamento mais adequado.

3.3.2- Sacarificação enzimática

A sacarificação ou hidrólise enzimática é considerada um dos mais importantes gargalos do processo, pois as enzimas são produtos de alto custo

que oneram consideravelmente o processo. A eficiência das enzimas na sacarificação da biomassa é um processo complexo que depende de vários fatores como estabilidade térmica e em pH, inibição das enzimas pelos seus produtos, sinergismo entre as enzimas, ligação produtiva e improdutiva à celulose e dosagem e composição dos extratos enzimáticos [11, 26, 27].

A escolha de coquetéis enzimáticos eficientes para sacarificação da biomassa lignocelulósicas tem sido um grande desafio para as pesquisas recentes sobre o etanol de segunda geração. A eficiência hidrolítica de um coquetel é dependente de propriedades individuais das enzimas e também da interação entre elas. Um coquetel ideal deve apresentar alta atividade hidrolítica sobre a biomassa lignocelulósica, atuar em valores de pHs relativamente próximos ao pré-tratamento escolhido, apresentar pouca inibição pelo produto e ter um bom custo benefício [28].

Apesar de a alta atividade hidrolítica ser uma característica muito importante do coquetel eficiente, também é igualmente importante a proporção das enzimas presentes neste extrato. A sacarificação enzimática da biomassa depende da atuação sinergística das celulases e hemicelulases.

A combinação de diferentes concentrações destas enzimas em um coquetel é um grande desafio. Coquetéis com alta atividade sobre papel de filtro (FPase) são normalmente mais desejáveis, já que a atividade de FPase é dependente das atividades de endoglicanases, exoglicohidrolases, celobiohidrolases e celobiases. Porém, apesar da atividade de FPase ser um indicativo forte do potencial de sacarificação de um coquetel não existe uma correlação direta entre estes fatores [29].

Diferentes estratégias têm sido desenvolvidas para a obtenção de um coquetel eficiente. Uma estratégia consiste na montagem de coquetéis por misturas de enzimas como β-glicosidases, pectinases e xilanases às preparações enzimáticas naturais que contém principalmente exocelulases e endoglicanases. Também tem sido estudada a engenharia genética dos micro-organismos produtores de celulases para induzir à produção de um coquetel equilibrado [30, 31].

Os coquetéis comerciais enzimáticos são principalmente produzidos a partir de espécies de *Trichoderma*. Entretanto, estes fungos produzem extratos com altas atividades das exocelulases e das endoglicanases e com baixa atividade de celobiase. Estudos com suplementação de celobiase nestes

extratos têm apresentados bons resultados, apesar de que a alta atividade de celobiase não garante um bom rendimento de sacarificação caso as outras celulases não estejam presentes em quantidades adequadas [32].

Em uma comparação entre extratos de *Penicillium echinulatum* e de *Trichoderma reesei* para hidrólise de polpa Kraft, foi feita suplementação do coquetel de Trichoderma para igualar as proporções de β-glicosidase/FPase. O extrato de *Trichoderma* apresentou melhor resultado de sacarificação, mesmo com proporções semelhantes de enzimas. Muitas atividades enzimáticas foram determinadas (endoglicanases, exocelulases, celobiases, β-glicosidases, xilanases e FPases) e não foi encontrada nenhuma grande diferença entre os dois extratos que explique o maior rendimento do extrato de *Trichoderma* após a suplementação com β-glicosidase [33]. Este resultado é mais um indicativo de quão complexa é a formulação de um coquetel ideal.

Até o momento a comunidade científica ainda não chegou a uma conclusão sobre qual a proporção ideal das enzimas para hidrólise da biomassa lignocelulósica. Além do complexo sinergismo entre as enzimas, essa indefinição também se deve à adequação do coquetel a uma determinada biomassa e ao tipo de pré-tratamento ao qual ela foi submetida. Como as biomassas têm distintas composições, a proporção de enzimas também deve ser distinta para cada substrato.

Na busca por um coquetel ideal, também tem se falado da importância da presença de hemicelulases em coquetéis. A presença de hemicelulose residual do pré-tratamento mesmo que em baixas concentrações podem diminuir a eficiência da hidrólise por celulases. A utilização de hemicelulases, principalmente as xilanases podem auxiliar hidrolisando a xilana que não foi solubilizada no pré-tratamento e possibilitando assim, maior acesso das celulases à celulose.

Quais hemicelulases são importantes para este processo depende da composição da biomassa. Como a hemicelulose tem uma estrutura muito mais diversa do que a celulose, a escolha das enzimas para sua degradação é ainda mais complexa. Por exemplo, para a sacarificação de farelo de trigo que apresenta uma hemicelulose rica em xilana com cadeias laterais de arabinose a suplementação de xilanase e de α-arabinofuranosidases em extratos comerciais de celulases aumentou o rendimento da sacarificação [34].

Além das enzimas, a atuação de algumas proteínas sem atividade hidrolítica vem sendo estudada. As expansinas são proteínas que podem auxiliar na hidrólise da celulose enfraquecendo as ligações da mesma. Essas proteínas não têm atividade para degradação de celulose, elas atuam rompendo interações de hidrogênio entre as fibras da celulose ou entre a celulose e a hemicelulose. Isso leva a um enfraquecimento da estrutura, facilitando assim, a atuação das enzimas hidrolítica [35, 36].

Atualmente as enzimas comerciais utilizadas em estudos de produção de etanol de segunda geração são produzidas por fungos dos gêneros *Trichoderma* e *Aspergillus*. A busca por complexos enzimáticos produzidos por fungos diferentes daqueles destes gêneros já extensivamente estudados é uma alternativa para a superação dos entraves relacionados à sacarificação enzimática [37]. O desenvolvimento de novos coquetéis enzimáticos, mais eficientes e específicos para o bagaço de cana pode ser um grande avanço para a viabilidade do etanol de segunda geração a partir desta biomassa.

3.3.3- Sacarificação e fermentação simultâneas, sacarificação e fermentação separadas e bioprocesso consolidado

Quando a hidrólise enzimática da biomassa pré-tratada e a fermentação por leveduras ocorrem simultaneamente em um mesmo meio reacional o processo é denominado sacarificação e fermentação simultâneas (SSF). Entretanto, quando a sacarificação e a fermentações são etapas separadas e sequenciais, o processo é denominado sacarificação e fermentação separadas (SHF). Os dois processos apresentam pontos positivos e negativos que vem sendo amplamente discutidos na literatura.

A SSF tem sido considerada a mais promissora forma de produção de etanol devido principalmente à diminuição dos custos atrelada ao processo. Na SSF, a biomassa, após ser pré-tratada, é hidrolisada enzimaticamente e fermentada produzindo ao mesmo tempo, açúcares para o crescimento da levedura e para a fermentação a etanol. A temperatura de reação deve ser mantida naquela ideal para a viabilidade do micro-organismo fermentador que normalmente apresenta crescimento e fermentação ideal entre 32 – 40 °C, dependendo da cepa utilizada. Esta temperatura é inferior àquela considerada ideal para a hidrólise pela maioria das enzimas. Celulases e hemicelulases apresentam em média temperatura de máxima atividade igual ou superior a 50

°C. Logo, em SSF as enzimas atuam em condições subótimas, o que diminui o seu rendimento [38].

A dificuldade de conciliação entre a temperatura de atividade das enzimas e a temperatura de fermentação é um grande gargalo do processo. Para tentar contornar-lo muito tem sido pesquisado sobre a prospecção de micro-organismos fermentadores termotolerantes [39]. Existe, porém, um limite para o aumento de temperatura da fermentação, devido à evaporação do etanol. Mesmo que se encontrem micro-organismos fermentadores termotolerantes dificilmente o processo poderá ser conduzido à mesma temperatura de maior atividade das enzimas.

Uma solução plausível para este problema também pode partir de enzimologistas. Pensamento muito recorrente é o de que enzimas altamente termoestáveis são mais apreciáveis para processos industriais. Este raciocínio tem se comprovado correto para a maioria dos processos industriais, pois diminui contaminações, possibilita maiores rendimentos e pode evitar a diminuição da temperatura por um processo que esteja em andamento anteriormente à hidrólise enzimática. Entretanto, para a aplicação das enzimas especificamente no processo SSF, a prospecção por enzimas que apresentem altas atividades em temperaturas mais amenas pode começar a ser uma tendência. Este caminho ainda é árduo, pois a própria natureza das enzimas exige certa quantidade de calor para promover as necessárias alterações das estruturas para hidrólise dos substratos.

Apesar da limitação em relação à temperatura, ótimos resultados têm sido relatados utilizando SSF. Artigos que relatam média de conversão de 0,4 g de etanol produzidos a partir de 1 g de biomassa são comuns na literatura. Este rendimento é próximo ao teórico para fermentação de glicose 0,51 g/g [40]. Também se deve considerar que a realização da sacarificação e fermentação na mesma temperatura pode gerar economia de energia no processo, pois não é necessário que se faça o resfriamento do reator após a hidrólise para proceder à fermentação.

Dentre as vantagens que SSF apresenta em relação à SHF, uma das mais destacadas é a diminuição da inibição enzimática pelo produto. Como já foi abordado, a celobiose e a glicose são grandes inibidores das enzimas endo e exocelulases. Durante a SSF, à medida que estes açúcares vão sendo liberados eles são prontamente utilizados na fermentação. Desta maneira, as

enzimas não são inibidas e o rendimento de etanol pode ser aumentado. Os efeitos benéficos da SSF em relação à inibição de enzimas têm sido relatados por vários autores [2, 32, 33].

Outra vantagem seria o efeito protetor do etanol ao hidrolisado. Como o hidrolisado é uma fonte rica em açúcares, podem ocorrer contaminações com micro-organismos que consomem os açúcares improdutivamente, sem a produção de etanol [33, 41].

Apesar de SSF ser atualmente o processo considerado mais vantajoso por grande parte dos pesquisadores, SHF também apresenta vantagens interessantes ao processo. A principal delas, já citada é a possibilidade de proceder à hidrólise e fermentação em suas condições ótimas. Como os dois processos são realizados em momentos distintos, é possível fazer a separação do hidrolisado líquido rico em açúcares da lignina residual que não é hidrolisada. Esta lignina pode ser utilizada em um processo acoplado de produção de energia como a combustão. Além disso, na SHF as células das leveduras podem ser recicladas para outras bateladas de fermentação ou podem ser utilizadas como suplemento de alimentação animal. Esta prática é muito comum na indústria alcooleira do Brasil [1].

O bioprocesso consolidado (CBP) é a combinação da produção de enzimas, sacarificação da biomassa e fermentação dos açúcares liberados em apenas uma etapa [42]. Preferencialmente, todas estas etapas são realizadas pelo mesmo micro-organismo, entretanto, estudos de co-fermentação do fungo *Acremonium cellulolyticus* e da levedura *Saccharomyces cerevisiae* apresentou resultados promissores com rendimento de 0,19 g/g para sacarificação e fermentação de Avicel [43]. O CBP tem sido considerado uma alternativa para diminuir os custos do etanol uma vez que o investimento necessário para produção de enzimas e preparo dos extratos enzimáticos comerciais podem ser evitados. Além da produção de etanol, também podem ser produzidos outros produtos importantes como ácido lático, ácido glutâmico, ácido succínico e isobutanol, dependendo de qual micro-organismo é utilizado [44].

Um micro-organismo ideal para ser utilizado no bioprocesso consolidado precisa ser capaz de solubilizar a biomassa e também de produzir etanol com alto rendimento em condições industriais. Muitos estudos propõem modificações genéticas para expressão de celulases por micro-organismos naturalmente fermentadores, principalmente em leveduras [42, 45, 46]. Alguns

micro-organismos como *Fusarium oxysporum* [47], *Trametes hirsuta* [48] e *Phlebia* sp. [49] são naturalmente capazes de produzir etanol diretamente da biomassa lignocelulósica e apresentam grande potencial para serem utilizados no CBP.

Apesar de apresentar muitas vantagens, alguns desafios ainda precisam ser superados para a viabilidade do CBP. Assim como a SSF, o CBP é conduzido em condições subótimas para a hidrólise enzimática (acima de 50 °C) e para a fermentação (28 – 37 °C). Dessa forma, o rendimento da sacarificação é muitas vezes baixo o que diminui o rendimento total do processo. Além disso, a conciliação de alta atividade enzimática, alta capacidade de fermentação e tolerância a etanol e a inibidores é difícil de ser conseguida [39].

A natureza dos micro-organimos, bactérias, leveduras ou fungos, também tem sido amplamente discutida. Quando leveduras e bactérias engenheiradas para expressão de celulases são utilizadas no CBP, o gargalo está na baixa atividade de enzimas que esses micro-organimos podem expressar. Além disso, em muitos casos, além de inserir os genes para expressão das enzimas, também são necessárias outras alterações genéticas para possibilitar a fermentação de pentoses. As vantagens da utilização de leveduras e bactérias são a maior disponibilidade de espécies termotolerantes, a maior rapidez para fermentação e no caso de leveduras, maior tolerância ao etanol. Fungos, por outro lado, secretam altas atividades de enzimas e muitos são capazes de fermentar xilose naturalmente. As desvantagens da utilização de fungos no CBP é a menor tolerância ao etanol, o longo tempo de fermentação e a formação concomitante de outros produtos como ácido acético, ácido lático e glicerol [42, 50-52].

3.3.4- Micro-organismos fermentadores

Devido à complexidade dos processos de fermentações da biomassa lignocelulósica, a escolha do micro-organismo também vai ser diferente daqueles utilizados na fermentação tradicional. A busca pelo micro-organismo ideal vem sendo realizada por grupos de pesquisa que seguem por diferentes caminhos: micro-organismos tolerantes a inibidores, micro-organismos fermentadores de pentose e micro-organismos termotolerantes.

Biomassas lignocelulósicas podem conter 5 – 27 % de pentoses como xilose e arabinose que não são fermentados pelo micro-organismo mais utilizado nas fermentações industriais, a *Saccharomyces cerevisiae*. Devido às altas concentrações destes açúcares nas biomassas a sua utilização para a produção de etanol poderia trazer grande benefício ao processo.

Micro-organismos que fermentam xilose são encontrados entre fungos filamentosos, bactérias e leveduras. Bactérias anaeróbicas fermentam pentoses a etanol, mas são inibidas em baixas concentrações de etanol de açúcares. Cepas de leveduras naturalmente fermentadoras de pentoses a etanol são relativamente comuns. Um exemplo é a *Sheffersomyces stipitis* (*Pichia stipitis*) que fermenta xilose em etanol com um rendimento e produtividade razoáveis para um processo industrial, entretanto, essas leveduras são inibidas por produtos gerados nos pré-tratamentos. Já alguns fungos filamentosos são tolerantes a inibidores, porém apresentam uma fermentação muito lenta [23].

Existem basicamente duas vias metabólicas para fermentação de xilose e arabinose, as duas principais pentoses das biomassas: via da xilose isomerase, comum em bactérias e via da xilose redutase e xilitol desidrogenase, comum em fungos e leveduras (Figura 5). Uma estratégia para obtenção de micro-organismos fermentadores de pentoses são manipulações genéticas para expressão heteróloga dessas enzimas que metabolizam xilose em micro-organismos fermentadores de glicose. As duas vias já foram introduzidas em *S. cerevisiae* e alguns estudos indicam que a via proveniente de fungos apresenta maiores rendimentos de fermentação do que aquela proveniente de bactérias [53].

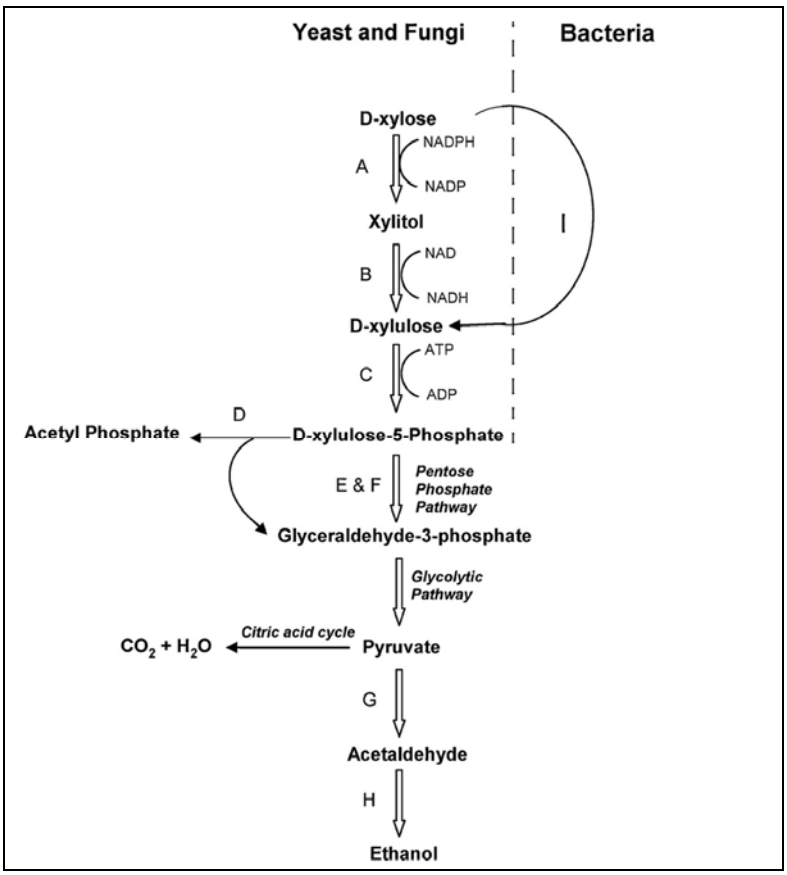

Figura 5: Esquema do metabolismo de xylose. (A) Xilose redutase; (B) xilitol desidrogenase; (C) xilulocinase; (D) fosfocetolase; (E) transaldolase; (F) transcetolase; (G) piruvato decarboxilase; (H) alcool desidrogenase e (I) xilose isomerase [54].

A busca por micro-organismos com tolerância a inibidores também é um importante passo para a viabilização do processo de produção de etanol lignocelulósico. Certas leveduras inclusive algumas linhagens de *S. cerevisiae* já são naturalmente tolerantes a inibidores derivados de furaldeídos convertendo-os em seus alcoóis menos tóxicos (Figura 6).

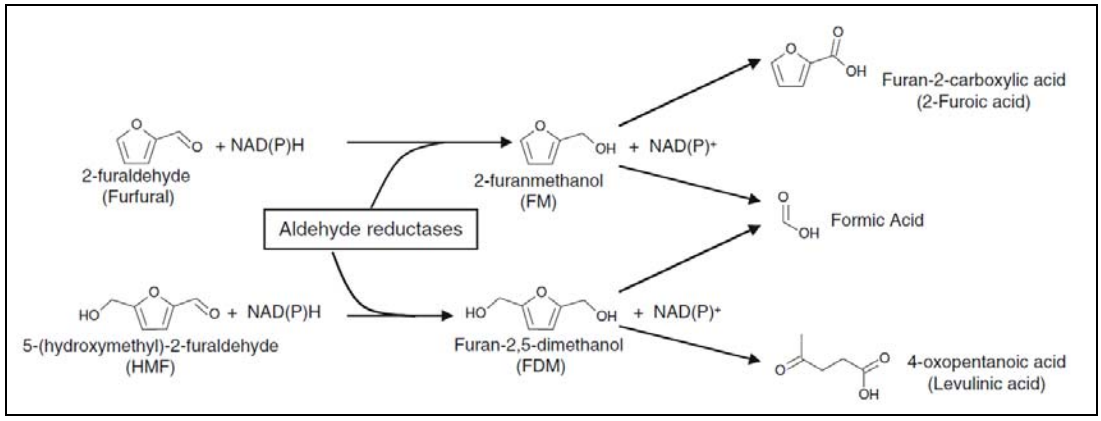

Figura 6: Via de conversão de 2- furaldeído (furfural) e 5-(hidroximetil)-2-furaldeído (HMF-hidroximetilfurfural) em 2-furanometanol (FM) e em furano-2,5-dimetanol (FDM) catalisada por múltiplas redutases e possível quebra destes alcoóis em ácidos orgânicos[55].

A obtenção de micro-organismos fermentadores de pentose com possível tolerância a inibidores também tem sido estudada. A bactéria *Escherichia coli* MM160 foi inicialmente modificada para fermentar pentoses e depois para aumentar a sua tolerância a inibidores como acetato, ácido fórmico, furfural e hidroximetilfurfural. Esta bactéria foi utilizada para fermentar bagaço de cana pré-tratado com ácido fosfórico em um processo de sacarificação e fermentação simultânea precedida de 6 h de pré-hidrólise. A fermentação foi realizada com a fibra hidrolisada e com o hidrolisado líquido, eliminado etapas de separação e lavagem da biomassa. A bactéria recombinante apresentou maior tolerância à furfural, hidroximetilfurfural e acetato, entretanto não apresentou tolerância ao ácido fórmico. Foi produzido por esta levedura etanol na concentração de 30 g/L com uma eficiência de conversão de 0,21 g/g [56].

Fungos filamentosos têm sido amplamente estudados em relação à fermentação de hexoses e de pentoses. Alto rendimento de etanol em relação à fonte de carbono é relatado, entretanto, o tempo de fermentação para atingir o rendimento máximo é muito longo e a tolerância ao etanol é menor do que a observada para leveduras [52, 57-60].

3.4- Produção de enzimas

Técnicas de produção de celulases e hemicelulases a partir de fungos e bactérias vêm sendo aprimoradas ao longo de muitos anos. Essas enzimas já são utilizadas industrialmente principalmente na indústria alimentícia, de rações animais, têxtil e de detergentes [16, 61]. Entretanto, a produção dessas enzimas para a sacarificação da biomassa obedece a princípios diferentes, devido à complexa natureza da biomassa.

A comercialização de enzimas para a produção de etanol conta com empresas importantes como Genencor e Novozymes. Estas empresas utilizam celulases produzidas principalmente por fungos filamentosos dos gêneros *Aspergillus*, *Trichoderma* e *Penicillium*, nativos ou modificados geneticamente. Em alguns trabalhos mais recentes têm sido utilizadas celulases produzidas por *Acremonium cellulolyticos,* comercializada pela empresa japonesa Meiji Seika Kaisha. Essas enzimas têm mostrado resultados melhores para produção de etanol do que aquelas enzimas comerciais de *Aspergillus*, *Trichoderma* e *Penicillium*. Este resultado é creditado ao alto teor de celobiase produzido naturalmente pelo fungo *Acremonium* [28, 62-65].

3.4.1- Cultivo de micro-organismos em fermentação no estado sólido

Em estudos de produção de celulases têm sido testadas as estratégias de cultivo do micro-organismo em meio líquido e em fermentação no estado sólido.

A fermentação em estado sólido (FES) é definida como a fermentação na ausência ou quase ausência de água livre, essa condição mimetiza as condições naturais de crescimento do fungo e por isso pode proporcionar bons resultados. Neste método o fungo é crescido diretamente sobre a biomassa umedecida. Para a extração das enzimas é necessário apenas a adição de tampão sobre a biomassa e agitação por um curto período (0,5 - 1 h). Devido a este método de extração, preparados enzimáticos bem concentrados são obtidos, o que evita uma possível etapa de concentração, gerando redução nos custos do processo. Além disso, uma menor quantidade de efluentes é gerado em relação ao cultivo em meio líquido [28]. Muitos autores apontam FES como o método mais promissor para produção de enzimas lignocelulósicas devido ao seu baixo custo. Estudos indicam que a produção de celulases por FES pode ter um custo de 10 - 100 vezes menor do que aquela por cultura submersa [28, 66, 67]. Além disso, para a utilização de celulases na produção de etanol, é mais importante um extrato enzimático concentrado do que com qualquer grau de pureza. Fato que endossa ainda mais o argumento das maiores vantagens do FES sobre cultura submersa [67].

Mesmo em meio a tantas vantagens citadas na literatura a produção de celulases em FES vem recebendo menos atenção do que o esperado. Isso se deve a alguns fatores que dificultam o processo, a começar pela necessidade de uma escolha criteriosa do micro-organismo a ser cultivado. Fungos, bactérias e leveduras têm sido cultivados em FES, entretanto, existe uma dificuldade na adaptação do micro-organismo às condições do FES que são mais árduas do que aquelas da cultura submersa. Um micro-organismo pode ter excelente crescimento e produção de enzimas em cultura submersa e o mesmo não se repetir no FES. Isso se deve às condições mais difíceis como acessibilidade ao substrato, oxigenação do meio de cultura e transferência de solutos. Devido a estes mesmos fatores o escalonamento do processo é apondado como o maior entrave para a comercialização de extratos produzidos em FES [66-68].

A otimização do processo de FES e a escolha de micro-organismos adaptáveis ao método e com potencial para produção de celulases é uma alternativa que vem sendo buscada por muitos pesquisadores para possibilitar a superação deste importante gargalo na produção de etanol de segunda geração.

3.4.2- Cultivo de micro-organismos em cultura submersa

Cultura submersa é definida como a fermentação na presença de excesso de água, também é conhecida como cultivo em meio líquido. Praticamente toda produção de enzimas comerciais utiliza esta técnica. A grande vantagem da cultura submersa em relação à FES é a facilidade de controle de parâmetros como pH do meio de cultura, disponibilidade de nutrientes, suplementação de indutores e temperatura da fermentação. Esta vantagem é crucial para a produção de enzimas em escala industrial, pois para isso é necessário um controle sobre o crescimento e produção de enzimas pelos micro-organismos.

A produção de celulases é dependente de mecanismos de indução e repressão e o processo de produção e a formulação do meio de cultivo devem ser planejados de forma a superar esses fatores. Lactose tem sido citada como um indutor de celulases de baixo custo e glicose como um repressor da produção de celulases. Normalmente a produção das enzimas é realizada em bateladas, entretanto, com o objetivo de maior controle de fatores como esses indutores e repressores, a produção por batelada alimentada tem sido estudada [28].

Inúmeras fontes de carbono já foram avaliadas para produção de celulases. Muito já se estudou sobre a indução de celulases por celuloses pura como Avicel e Solka-Floc. Com o objetivo de desonerar o processo de produção de enzimas, fontes de carbono que são resíduos agro-industriais como bagaço de cana, palha de milho, palha de arroz, farelo de trigo, têm sido avaliadas. A utilização destes materiais tem proporcionado ótimos resultados em termos de produção de enzimas [62, 69, 70].

Apesar de a produção de enzimas ainda ser um gargalo importante para a viabilidade econômica do etanol de segunda geração, os custos de produção de celulases em cultura submersa já diminuiu muito nos últimos anos. Isso se deve a incentivos financeiros do governo dos EUA oferecidos às duas maiores

empresas produtoras de enzimas: Genencor e Novozymes. Essas empresas receberam investimentos do governo para desenvolver pesquisas que visem à diminuição dos custos de produção das celulases.

3.5- Fungos endofíticos

Fungos endofíticos são micro-organismos que se desenvolvem no interior das plantas, podendo ser encontrados em caules, raízes, sementes e folhas. Micro-organismos endofíticos normalmente não causam danos às plantas e estudos apontam que eles podem proporcionar um efeito protetor às mesmas [71]. Estes micro-organismos são promissoras fontes de enzimas hidrolíticas já que essas enzimas estão diretamente envolvidas na penetração e desenvolvimento do fungo nas plantas. A presença do conjunto completo de celulases e hemicelulases propicia ao fungo endofítico vantagens competitivas em relação a outros micro-organismos para um maior sucesso na colonização das plantas [72-74].

Fungos endofíticos dos gêneros *Acremonium* e *Fusarium* vêm sendo avaliados quanto à produção de enzimas celulases e hemicelulases. Trabalhos já indicaram um grande potencial de produção de enzimas produzidas por estes micro-organismos que podem ser utilizadas na sacarificação da biomassa lignocelulósica [68, 75].

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4- Artigo 1: Optimization of endoglucanase and xylanase activities produced from *Fusarium verticillioides* and their application in the production of ethanol from sugarcane bagasse

4.1- Abstract

Enzymatic hydrolysis is an important but expensive step in the production of ethanol from biomass. Thus, the production of efficient enzymatic cocktails is of great interest for this biotechnological application. The production of endoglucanase and xylanase activites from *F. verticillioides* were optimized in a factorial design (2^5) followed by a CCDR design. Endoglucanase and xylanase activities increased from 2.8 to 8.0 U/mL and from 13.4 U/mL to 114 U/mL, respectively. The optimal pH and temperature were determined for endoglucanase (5.6, 80 °C), cellobiase (5.6, 60 °C), FPase (6.0, 55 °C) and xylanase (7.0, 50 °C). The optimized crude extract was applied in saccharification and fermentation of sugarcane bagasse from which 9.7 g/L of ethanol was produced at an ethanol/biomass yield of 0.19.

Key words: *Fusarium*; endoglucanase; xylanase; ethanol.

4.2- Introduction

Global interest in the production and consumption of biofuels (especially ethanol and biodiesel) has been growing since the turn of the century. This interest has in part been caused by environmental concerns and mainly due to the need to mitigate greenhouse gas emissions [1].

Among potential alternative bioenergy resources, lignocellulose has been identified as an important source of biofuels and other value-added products. Lignocelluloses from agricultural, industrial and forest sources account for the majority of total biomass present in the world and comprise a vast renewable resource. Lignocellulosic raw materials, which do not compete with food production, can provide environmental, economic, and strategic benefits for the production of fuels [2].

Lignocellulosic biomasses are mainly composed of cellulose, hemicelluloses 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)[3]. Hemicellulose is a heterogeneous and branched polymer of pentoses, hexoses and uronic acids. Complete enzymatic hydrolysis of xylan , the major polymer founded in hemicelluloses, 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) [4, 5].

Production of ethanol from lignocelluloses includes four main steps: pretreatment, enzymatic hydrolysis, fermentation and distillation. The high costs of enzyme production and the excessive enzyme loadings necessary to hydrolyze pretreated biomass are often considered the major bottlenecks for the commercial production of lignocellulosic ethanol [6].

Filamentous fungi are the major source of commercial cellulases. Cellulolytic fungi belonging to the genera *Trichoderma* have long been considered the most productive and powerful to hydrolyze crystalline cellulose, and the majority of commercial preparations are based on mutant strains of *T. ressei*. However, recent publications have increasingly demonstrated that there are alternatives to *T. reesei* enzymes for the production of second generation ethanol [7]. Enzymatic extract from *Chrysoporthe cubensis* [8], *Acremonium* [9], *Penicillium* [10], *Aspergillus* [11], *Fusarium* [12] and others are being studied.

Endophytic microorganisms are a promising source of enzymes since hydrolytic enzymes appear to be involved in the penetration and development of fungi in plants [13, 14]. *Fusarium verticillioides* alson known as *Gibberella moniliformis* is primarily a pathogen of maize. This fungus was isolated from commercial corn seeds and an initial screening of cellulases and hemicellulases activities has been studied previously [15]. This fungus is an important endophytic pathogen of small grain cereals, including corn and wheat, grasses and roots [16]. *Fusarium oxysporum* is the most studied species among the *Fusarium* genera in relation to its application in lignocellulose hydrolysis [17-20].

The objective of the present study was optimize endoglucanase and xylanase productions by the maize endophyte *Fusarium verticillioides* and study the application of the optimized extract for saccharification and ethanol production from sugarcane bagasse.

4.3- Materials and methods

4.3.1- Materials

Carboxymethylcellulose (CMC), Avicel, birchwood xylan, cellobiose and the substrates ρ-nitrophenyl-α-L-arabinofuranoside (ρNPAra) and ρ-nitrophenyl-β-D-xylopyranoside (ρNPXyl) were obtained from Sigma Chemical Co. (St. Louis, MO). The sugarcane bagasse, corn cobs and corn straw were acquired from a local farm; and wheat bran was acquired from the local market. All other chemicals used were of analytical grade.

4.3.2-Enzyme assay

FPase activity was determined using Whatman no. 1 filter paper (1 x 6 cm, 50 mg) in a total volume reaction of 1.5 mL for 60 minutes. Endoglucanase activity was determined using CMC 1 % in a final volume of 0.5 mL for 30 minutes. Xylanase activity was determined using birchwood xylan 1 % in a final volume of 0.5 mL for 15 minutes. All these assays were carried out in 0.05 M sodium acetate buffer pH 5.0 at 50 °C. The release of reducing sugars for these assays was determined using the 3,5-dinitrosalicylic acid (DNS) method [21] and calculated according to the standard curve (0.11 – 1.11 μmol of glucose). The assays were based on those described by Ghose [22].

Cellobiase activity was determined using cellobiose as substrate in a final concentration of 6.4 mM and 0.05 M sodium acetate buffer pH 5.0 in final volume of 0.5 mL for 30 minutes at 50 °C. The reaction was terminated by boiling the samples for 5 min. The glucose liberated was quantified using a commercial analytical kit based on glucose oxidase and peroxidase (Quibasa Basic Chemical, Belo Horizonte, MG, Brazil).

The activity of α-L-arabinofuranosidase and β-xylosidase were measured using 0.5 mM ρNPAra and ρNPXyl, respectively. These enzymes assays were carried out at 50 °C for 15 minutes in 0.05 M sodium acetate buffer, pH 5.0 in a final volume of 0.5 mL. Absorbance of the mixtures was then measured at 410 nm. The amount of ρNP released was calculated according to the standard curve (0.0017 - 0.0625 μmol of ρNP).

For all enzymatic activities one enzyme unit was defined as the amount of enzyme that released 1 μmol of product per minute under the assay conditions.

4.3.3- Microorganism and inoculum preparation

The fungus *F.verticillioides* was isolated from commercial corn seeds, belongs to mycological collection of Laboratory of Biochemical Technology at Federal University of Viçosa, MG, Brazil, and was routinely propagated in potato dextrose agar slants at 28 °C. The sporulated slants obtained after 7 days were used for preparing the spore inoculums with sterile water. For inoculation, 2 mL of water containing 1.5 x 10^6 spores was used in 50 mL culture medium.

4.3.4- Microorganism identification

The identification of the species of this isolate was conducted using the genomic DNA. The DNA extraction was performed using genomic DNA purification kit from Promega (Wizard Genomic DNA Purification Kit. For PCR reaction, the DreamTaq™ Master Mix (MBI Fermentas, Vilnius, Lithuania) was used, following the manufacturer's protocol.

Two loci were amplified including fragments of the ITS gene region using primers ITS1 (5´TCCGTAGGTGAACCTTGCGG 3´) and ITS4 (5´ TCCTCCGCTTATTGATATGC 3´) and elongation factor (EF1α) using primers EF2 (GGARGTACCAGTSATCATGTT) and EF1 (ATGGGTAAGGAGGACAAGAC). Amplification was performed with an initial denaturing step at 96 ° C for 5 min followed by 35 cycles of denaturation at 96 ° C for 30 s, annealing at 52 ° C for 30 sec, extension initial 72 ° C for 1 min and 4 min final extension at 72 ° C. The PCR product was visualized on agarose gel, at 2% to determine fragment size and purity. PCR products were purified with an ExoSAP-IT ® kit, according to the manufacturer protocol.

The sequencing was performed at the Laboratory of Genomics of the Institute of Biotechnology Applied to Agriculture (BIOAGRO) at the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. The quality of sequences were checked in a Sequence Scanner Software v1.0 (Applied Biosystems) and edited using the software package Seqman from DNAStar Inc. Consensus regions of the sequences edited were compared in the GenBank database using the Mega BLAST program.

The identification of this isolate was performed using *Fusarium* MLST database (http://www.cbs.knaw.nl/Fusarium/).

4.3.5- Selection of growth carbon source

Carboxymethylcellulose, Avicel, sugarcane bagasse, corn straw, corn cobs or wheat bran were initially tested as carbon sources for cellulase and xylanase production by *F. verticillioides*.

Two base media were evaluated for all carbon sources. Medium 1 was based on Mandels medium [23] and contained (in g/L): urea, 0.3; $(NH_4)_2SO_4$, 1.4; KH_2PO_4 2.0; $CaCl_2$, 0.3; $MgSO_4$, 0.3; yeast extract, 0.25; and peptone, 0.75. Medium 2 contained (in g/L): $NaNO_3$, 6; KH_2PO_4, 1.5; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01; and $ZnSO_4$, 0.01; both mediums 1 and 2 contained the carbon source at 10.0 g/L [15]. The microorganism was grown at 28 °C with agitation (180 rpm) for 5 days in a rotary shaker (Tecnal, TE-421, Piracicaba, São Paulo, Brazil). Samples were centrifuged for 15 min at 10000 x *g* and the supernatants were used as the crude enzyme extract. All experiments were performed in triplicates.

4.3.6- Optimization of enzyme production

Two factorial designs were carried out. In the first, a 2^5 factorial design without repetition was performed in order to study the main effects and the effects of double interaction of the factors sodium nitrate concentration (N), corn straw concentration (C), cultivation time (T), initial pH (pH) and lactose concentration (L) for the production of endoglucanase, FPase, cellobiase and xylanase activities (dependent variables). In this experimental design, 32 experimental assays were carried out in a randomized design. The levels of the factors were: sodium nitrate concentration: 2 and 10 g/L, corn straw concentration: 1 and 4 %, cultivation time: 72 and168 hours, initial pH: 4 and 6 and lactose concentration: 0 and 1 % (Table 1).

Table 1: Real Values and coded values (in parentheses) for the factorial design and the responses

Run	C	N	pH	L	T	Endo.	FP.	Cello.	Xyl.
1	1 (-1)	2 (-1)	4 (-1)	0 (-1)	72 (-1)	3.59	0.24	0.11	57.96
2	4 (+1)	2 (-1)	4 (-1)	0 (-1)	72 (-1)	0.98	0.24	0.05	37.50
3	1 (-1)	10 (+1)	4 (-1)	0 (-1)	72 (-1)	3.92	0.27	0.19	55.86
4	4 (+1)	10 (+1)	4 (-1)	0 (-1)	72 (-1)	8.38	0.50	0.12	97.66
5	1 (-1)	2 (-1)	6 (+1)	0 (-1)	72 (-1)	3.68	0.22	0.12	63.63
6	4 (+1)	2 (-1)	6 (+1)	0 (-1)	72 (-1)	0.86	0.05	0.05	41.01
7	1 (-1)	10 (+1)	6 (+1)	0 (-1)	72 (-1)	3.74	0.28	0.20	63.00
8	4 (+1)	10 (+1)	6 (+1)	0 (-1)	72 (-1)	6.64	0.34	0.11	96.14
9	1 (-1)	2 (-1)	4 (-1)	1 (+1)	72 (-1)	0.17	0.06	0.04	30.57
10	4 (+1)	2 (-1)	4 (-1)	1 (+1)	72 (-1)	0.10	0.08	0.03	20.92
11	1 (-1)	10 (+1)	4 (-1)	1 (+1)	72 (-1)	2.58	0.24	0.07	60.08
12	4 (+1)	10 (+1)	4 (-1)	1 (+1)	72 (-1)	0.87	0.06	0.05	56.07
13	1 (-1)	2 (-1)	6 (+1)	1 (+1)	72 (-1)	0.16	0.06	0.05	24.55
14	4 (+1)	2 (-1)	6 (+1)	1 (+1)	72 (-1)	0.08	0.06	0.02	19.57
15	1 (-1)	10 (+1)	6 (+1)	1 (+1)	72 (-1)	2.70	0.24	0.10	54.80
16	4 (+1)	10 (+1)	6 (+1)	1 (+1)	72 (-1)	0.82	0.13	0.04	59.72
17	1 (-1)	2 (-1)	4 (-1)	0 (-1)	168 (+1)	2.52	0.19	0.25	30.94
18	4 (+1)	2 (-1)	4 (-1)	0 (-1)	168 (+1)	1.05	0.15	0.09	23.37
19	1 (-1)	10 (+1)	4 (-1)	0 (-1)	168 (+1)	2.52	0.21	0.35	39.90
20	4 (+1)	10 (+1)	4 (-1)	0 (-1)	168 (+1)	7.46	0.25	0.20	66.21
21	1 (-1)	2 (-1)	6 (+1)	0 (-1)	168 (+1)	2.69	0.15	0.24	30.85
22	4 (+1)	2 (-1)	6 (+1)	0 (-1)	168 (+1)	1.14	0.16	0.11	27.03
23	1 (-1)	10 (+1)	6 (+1)	0 (-1)	168 (+1)	2.61	0.11	0.33	43.80
24	4 (+1)	10 (+1)	6 (+1)	0 (-1)	168 (+1)	6.99	0.25	0.30	74.15
25	1 (-1)	2 (-1)	4 (-1)	1 (+1)	168 (+1)	0.83	0.13	0.12	22.60
26	4 (+1)	2 (-1)	4 (-1)	1 (+1)	168 (+1)	0.21	0.14	0.12	17.18
27	1 (-1)	10 (+1)	4 (-1)	1 (+1)	168 (+1)	2.28	0.14	0.35	44.04
28	4 (+1)	10 (+1)	4 (-1)	1 (+1)	168 (+1)	7.77	0.45	0.30	88.50
29	1 (-1)	2 (-1)	6 (+1)	1 (+1)	168 (+1)	1.01	0.11	0.18	20.69
30	4 (+1)	2 (-1)	6 (+1)	1 (+1)	168 (+1)	0.20	0.12	0.13	17.40
31	1 (-1)	10 (+1)	6 (+1)	1 (+1)	168 (+1)	2.51	0.19	0.36	44.95
32	4 (+1)	10 (+1)	6 (+1)	1 (+1)	168 (+1)	7.44	0.49	0.29	69.36

C: Corn straw concentration (%); N: sodium nitrate concentration (g/L); L: lactose concentration (%); T: cultivation time (hours); All the activities are expressed in U/mL.

The analysis of variance (ANOVA) of the *F* test was preliminary performed to evaluate if there was any effect of the factors different from zero, followed by utilizing the *t* test to determine which coefficients were different from zero at 5 % probability. In this analysis the third, fourth and fifth order interaction effects were used as the residual effect. The effects were calculated according to the equations:

$$E_m = m_+ - m_-$$

and

$$E_i = \frac{(m_{++} + m_{--}) - (m_{+-} + m_{-+})}{2}$$

where E_m is the main effect, E_i is the interaction effect, m_+ is the mean of the dependent variable values for the highest level of one factor, m_- is the mean of the dependent variable values for the lowest level of one factor, m_{++} is the mean of the dependent variable values of the highest level of two factors, m_{--} is the mean of the dependent variable values of the lowest level of two factors, m_{+-} is the mean of the dependent variable values of the highest level of factor 1 and lowest level of factor 2, and m_{-+} is the mean of the dependent variable values of the highest level of factor 2 and lowest level of factor 1.

The preliminary factorial design allowed for selection of the statistically significant variables with respect to enzyme production according to the *t* test applied to the regression coefficients with 95 % confidence level.

The variables which presented a significant effect in the 2^5 factorial design were selected to be studied in a central composite rotatable design (CCRD). Levels were modified in an attempt to increase enzyme activities. This design was utilized to obtain a second-order model for the prediction of enzyme production (dependent variable) as a function of the variables studied (independent variables). Fifteen experimental assays were carried out, including 8 factorial points, 6 axial points and 4 central points in a completely randomized design. The central points were defined by the following combination: sodium nitrate concentration: 5-15 g/L, corn straw concentration: 2-6 % and time of cultivation: 24-168 hours; and these were evaluated by the *t* test.

Data obtained from CCRD (Table 2) was used to fit an empirical quadratic polynomial model related to the response by a multiple regression procedure, represented by the following equation.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \varepsilon_{ij}$$

where Y is the predicted response, *i* and *j* assume a value from 1 to the total number of variables (*n*), β_0 is the intercept term, β_i is the linear coefficient, β_{ij} is the interaction coefficient, β_{ii} is the coefficient of the quadratic term and x_i and x_j are the levels of the independent variables. ε_{ij} is the random error.

Table 2: Real values and coded values (in parentheses) for the response surface design and the responses

				Endo.		Cello.		FPase		Xyl.	
Run	N	C	T	Obs.	Pred.	Obs.	Pred.	Obs.	Pred.	Obs.	Pred.
1	7.0 (-1)	2.8 (-1)	53 (-1)	5.19	5.09	0.12	0.09	0.37	0.36	69.9	56.3
2	12.9 (+1)	2.8 (-1)	53 (-1)	5.22	5.09	0.15	0.18	0.34	0.34	61.9	59.2
3	7.0 (-1)	5.2 (+1)	53 (-1)	1.14	1.27	0.06	0.08	0.15	0.15	39.2	39.8
4	12.9 (+1)	5.2 (+1)	53 (-1)	1.15	1.27	0.09	0.08	0.14	0.13	48.1	38.0
5	7.0 (-1)	2.8 (-1)	138 (+1)	5.58	5.69	0.53	0.54	0.39	0.38	52.3	60.8
6	12.9 (+1)	2.8 (-1)	138 (+1)	5.50	5.69	0.71	0.69	0.41	0.38	68.9	66.8
7	7.0 (-1)	5.2 (+1)	138 (+1)	8.11	8.22	0.51	0.47	0.59	0.57	82.5	83.7
8	12.9 (+1)	5.2 (+1)	138 (+1)	7.56	8.22	0.50	0.52	0.60	0.58	73.1	85.1
9	5 (-1.68)	4 (0)	96 (0)	7.91	7.36	0.18	0.20	0.56	0.57	72.2	73.4
10	15 (+1.68)	4 (0)	96 (0)	5.81	7.36	0.34	0.32	0.54	0.56	75.9	76.9
11	10 (0)	2 (-1.68)	96 (0)	5.22	5.31	0.42	0.42	0.31	0.32	59.0	64.2
12	10 (0)	6 (+1.68)	96 (0)	4.70	4.23	0.27	0.27	0.30	0.31	68.6	65.6
13	10 (0)	4 (0)	24 (-1.68)	0.16	0.29	0.00	0.00	0.03	0.03	16.3	30.8
14	10 (0)	4 (0)	168 (+1.68)	7.15	6.64	0.74	0.757	0.40	0.43	86.5	74.2
15	10 (0)	4 (0)	96 (0)	7.41	7.36	0.20	0.24	0.49	0.50	103.1	112.9
16	10 (0)	4 (0)	96 (0)	7.02	7.36	0.26	0.24	0.44	0.50	114.2	112.9
17	10 (0)	4 (0)	96 (0)	8.28	7.36	0.23	0.24	0.53	0.50	118.3	112.9
18	10 (0)	4 (0)	96 (0)	8.05	7.36	0.28	0.24	0.55	0.50	116.5	112.9

All data was treated with the aid of Minitab 16. The quality of fit of the second-order model equation was expressed by the coefficient of determination (R2).

4.3.7- Biochemical characterization of the enzymes

The effect of temperature on enzyme activities was determined in 0.05 M sodium acetate buffer, pH 5.0 and a temperature range of 10 - 90 °C.

The effect of pH on endoglucanase, FPase, cellobiase and xylanase activities were investigated at different pH values (from 3.6 to 8.0) using McIlvaine buffer (citric acid/sodium phosphate) [24] at the optimum temperature for each enzyme activity. Results of the analyses are presented as mean ±SD for three measurements.

4.3.8- Biomass pretreatment

Sugarcane bagasse was dried in an oven at 70 °C until the weight was constant. The dry sugarcane bagasse was milled in rotary mill (model MA-580, Marconi Co. Piracicaba – SP) and passed through a 1.0 mm screen. Sodium hydroxide at a concentration of 1 % was used to pretreat 25 g of milled sugarcane bagasse samples at a solid loading of 10 % (w/v). Treatments were performed in duplicate in an autoclave at 121 °C for 60 min. The pretreated

materials were separated into solid and liquid fractions using a Buchner funnel with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at -20 °C.

The chemical composition of the untreated and alkaline pretreated sugarcane bagasse was determined as described in previous study using a modified Klason lignin method derived from the TAPPI Standard Method T222 (OM-98) and TAPPI Useful Methods (UM-250) [25].

4.3.9- Yeast cultivation

Kluyveromyces marxianus ATCC8554 was used to ferment glucose generated during enzymatic hydrolysis. The yeast previously grown in petri dishes was inoculated in 50 mL of an YPD (yeast peptone dextrose) medium and placed in a shaker at 28 °C and 180 rpm for 12 h. After this time the yeast suspension was centrifuged for 10 min at 10,000 *g*, and the supernatant was discarded. The yeast cells were resuspended in approximately 4 mL of YP medium and transferred to the appropriate SSF flask.

4.3.10- Simultaneous saccharification and fermentation (SSF)

The SSF experiments were carried out in 125-mL glass Erlenmeyer-flasks with a 50 mL working volume. Phosphate buffer 0.1 M, pH 6.0 and enzyme solution were added to the bagasse to maintain a solids loading of 5 % dry matter (w/v). The reaction was performed in a bench agitator at 40 °C for 120 h. Fifteen samples of 0.5 mL were removed at determined times using sterilized equipment to avoid contamination. The SSF results were analyzed by HPLC on a Shimadzu series 10A chromatograph. An analytical column (Aminex HPX-87H, 300 x 7.8 mm) was fitted to the HPLC, eluted with 5 mM sulfuric acid at 60 ºC and flow rate of 0.6 mL/min. Nine hours after addition of the enzyme solution, the produced *K. marxianus* cell suspension was added to provide a final D.O of 2.0. Gaseous nitrogen was periodically bubbled in the SSF solution to minimize oxygen levels. The experiment was performed with two repetitions.

The ethanol yield (Y_{Et}) was calculated as follow:

$$Y_{Et} = \frac{E_f - E_i}{0.567 \times G} \times 100$$

where Y_{Et} is the ethanol yield (%), E_f is the final ethanol mass (g), E_i is the initial ethanol mass (g), G is the is the glucan mass present in the pretreated biomass (g) and 0.567 is the stoichiometric yield of ethanol from cellulose.

The ethanol yield per gram of dry biomass was calculated as follow:

$$Y = \frac{E_f - E_i}{B}$$

where Y is the ethanol yield (g/g), E_f is the final ethanol mass (g), E_i is the initial ethanol mass (g) and B is the total biomass (g).

4.4- Results and discussion

4.4.1- Microorganism identification

The endophytic fungus was isolated from commercial corn seeds and based on morphological characteristics was classified as *Acremonium* sp. EA0810 (deposited in the mycological collection of the Laboratory of Biochemical Technology at the Federal University of Viçosa, MG, Brazil).

After sequence analysis of the ITS (rDNA) region and elongation factor region, the fungal taxonomical classification was revisited. Based on these molecular studies the fungus was classified as *Fusarium verticillioides*, which had 99.5 % identity with the isolates CBS 734.97 and CBS 119825, two *Fusarium verticillioides*/*Gibberella moniliformis* strains deposited at the Centraalbureau voor Schimmelcultures (CBS).

4.4.2- Carbon source selection

Several carbon sources and two different culture mediums were analyzed to induce the production of endoglucanase, cellobiase, FPase and xylanase activities on *F. verticillioides* (Figure 1A, 1B, 1C and 1D).

The natural carbon sources, including corn straw, corn cobs and sugarcane bagasse induced higher FPase activity than the synthetic substrates Avicel and CMC (Figure 1 C). Corn straw and corn cobs were the best carbon sources for inducing endoglucanase and xylanase activities, while cellobiase activity was highest when corn straw was used as the carbon source (Figure 1 A, 1B and 1D).

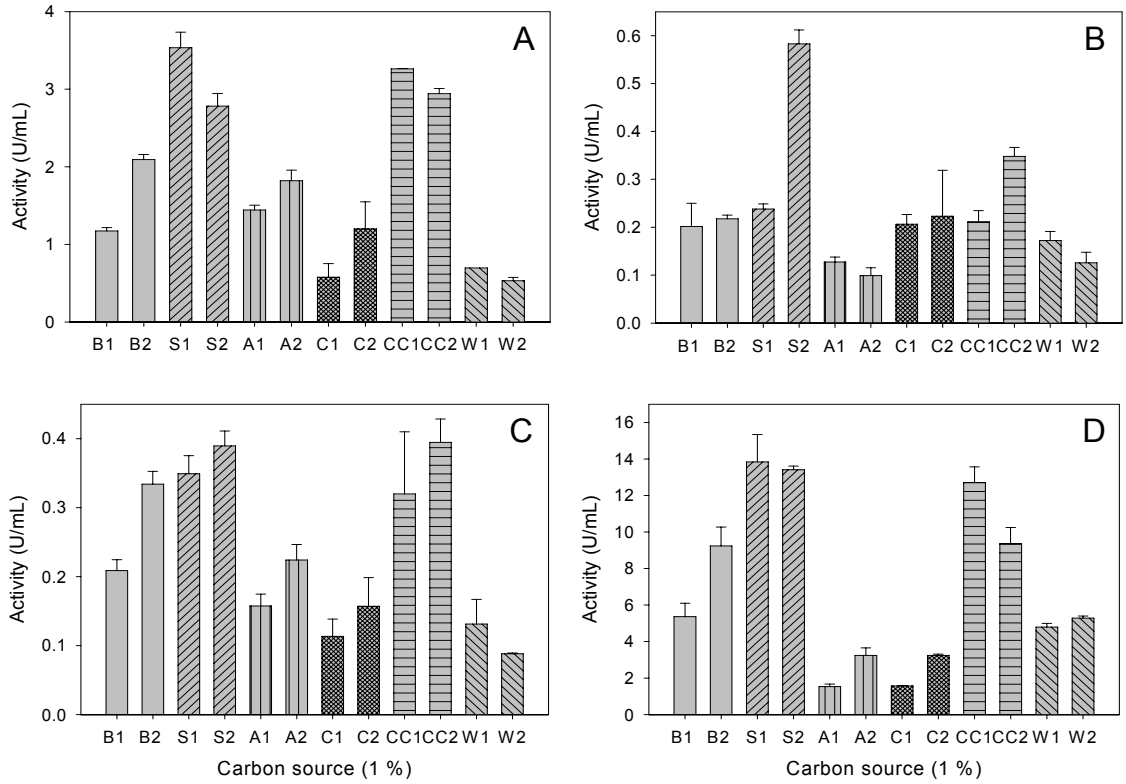

Figure 1: Effect of the carbon source on endoglucanase (A), cellobiase (B), FPase (C) and xylanase (D) activities. 1: Medium 1; 2: Medium 2; B: sugarcane bagasse; S: corn straw; A: Avicel; C: carboxymethylcellulose; CC: corn cobs; W: wheat straw.

Elevated induction of cellulases by the natural carbon sources can be explained by the endophytic nature of the fungi. Evolution of endophytic in lignocellulosic rich environments can result in production of enzymes capable of degrading this type of natural biomass. The fact that *F. verticillioides* EA 0810 was isolated from corn seeds may explain its preference for this carbon source.

Contrary to the results obtained in the present study, it is generally reported that synthetic carbon sources such as pure cellulose and CMC induce high cellulase activities [26, 27]. Replacing synthetic with natural carbon sources to produce cellulases has been proposed to reduce production costs since the natural cellulose is significantly cheaper [28, 29].

For corn cobs and corn straw, the best carbon sources for inducing cellulase and xylanase activities, medium 1 and medium 2 presents few differences with the except of cellobiase activity which was much higher in medium 2 (Figure 1A-D). Cellobiase is a crucial enzyme in the saccharification process, since this enzyme catalyzes the hydrolysis of cellobiose, which is a potent inhibitor of cellulolytic activities. Therefore, the enzymatic cocktail produced by the fungal culture containing corn straw as the carbon source in

medium 2 was used for enzyme production optimization and biomass saccharification experiments.

4.4.3- Optimization of enzymes production

4.4.3.1-Factorial design

The experimental conditions and the results for cellulase and xylanase activities in the first factorial design are shown in Table 1. Sodium nitrate concentration had a positive effect in all enzymes activities, indicating that increasing the sodium nitrate concentration promoted an increase in activities (Table 3). Therefore, the range for this variable was redefined from 2 – 10 g/L used in the first factorial design to 5 – 15 g/L in the CCRD.

Table 3: The main and double interaction effects observed at factorial design for corn straw concentration (C), sodium nitrate concentration (N), initial pH (pH), lactose concentration (L) and cultivation time (T) on the enzymatic activities. Values represent coded units.

	Effect			
Factor	Endo.	Cello.	FPase	Xyl.
C	0.842	-0.065*	0.038	7,72*
N	3.122*	0.101*	0.125*	33,03*
pH	-0.122	0.012	-0.024	0,081
L	-1.81*	-0.034	-0.056	-12,38*
T	0.622	0.147*	0.012	-11,13*
CxN	2.096*	-0.002	0.059	17,45*
CxpH	-0.209	0.002	-0.009	-0,46
CxL	-0.186	0.030	0.004	-1,92
CxT	1.069	-0.014	0.058	5,45
NxpH	-0.169	-0.001	0.013	-0,38
NxL	-0.096	0.006	0.023	4,97
NxT	0.619	0.054	-0.008	2,08
pHxL	0.136	-0.0004	0.036	-3,69
pHxT	0.116	0.008	0.014	-0,64
LxT	1.224	0.035	0.095*	10,93*

* Significant effect at 5 % probability.

Interaction between corn straw and sodium nitrate has a positive effect on endoglucanase and xylanase activities. Corn straw concentration showed a positive effect on xylanase, endoglucanase and FPase activities although the effect in the last two activities was not significant. Contrarily, corn straw concentration had a negative effect on cellobiase activity (Table 3). Because the

sodium nitrate range was redefined and the interaction between corn straw and sodium nitrate concentrations was positive for endoglucanase and xylanase activities, it was decided to redefine the corn straw level as well. The concentration was changed from 1 – 4 % to 2 – 6 % in the CCRD.

Lactose concentration presented a negative effect on endoglucanase and xylanase activities (Table 3). Lactose is known to be a cellulase inducer in *Acremonium* and *Trichoderma* species [30, 31], but in the case of *F. verticillioides* lactose decreased cellulase activities (Table 1); thus, lactose was excluded in the CCRD. Lactose and cultivation time presented positive interactions on FPase and xylanase activity, and in the CCDR the shortest cultivation time was decreased to 24 hours. None of the variables were significantly affected by the pH so it was maintained at pH 6.0, which is the natural pH of the medium.

4.4.3.2-Central composite rotatable design

The CCRD with the variables of sodium nitrate concentration, corn straw concentration and cultivation time, as well as the results for enzyme activities are presented in Table 2.

Using a regression analysis, second–order models (Eq. 2 – 5, Table 4) were obtained for endoglucanase, cellobiase, FPAse and xylanase activities as a function of the statistically significant parameters ($p<0.05$) for each enzyme. Because the *t*-values for the regressions were highly significant ($P<0.05$) and the percentages of variation explained by the models were suitable ($R^2 \geq 91.6\%$), the models could be considered as predictive and were therefore used to generate the response surface plots (Figure 2) for endoglucanase, FPase, cellobiase and xylanase activities. Addictionally, fit of the statistical models to experimental data was confirmed by the predicted values (Table 2).

Table 4: Second-order equations obtained for enzyme activities.

Activity (U/mL)	Regression	R^2(%)	
Endoglucanase	$Y=-7.36+1.89T-0.92C^2-1.38T^2+1.59CT$	95.1	Eq.2
Cellobiase	$Y=0.24+0.03N-0.04C+0.22T+0.04C^2+0.05T^2$	98.8	Eq.3
FPase	$Y=0.50+0.12T-0.02N^2-0.064C^2-0.09T^2+0.10CT$	97.9	Eq.4
Xylanase	$Y= 112.9+12.9T-13.3N^2-16.9C^2-21.4T^2+9.9CT$	91.6	Eq.5

N: sodium nitrate concentration; C: corn straw concentration; T: cultivation time. Coefficients are expressed in coded values. Statistically significant parameters were used according to t-test ($p<0.05$)

The highest endoglucanase activity was 8.28 U/mL in run 17, one of the central points. The central points (runs 15 – 18) presented little variation, indicating good reproducibility of the experimental data. Interaction between corn straw concentration and cultivation time was the most important factor for endoglucanase activity, where the interaction between them was positive (Figure 2). It can be observed that changes in corn straw concentration and cultivation time levels must be equal. Therefore, increasing corn straw concentration must be followed by an increase in cultivation time; while a decrease in corn straw concentration must be followed by a decrease in cultivation time. The optimum corn straw concentration for endoglucanase activity was 5 % and optimum cultivation time was 146 hours.

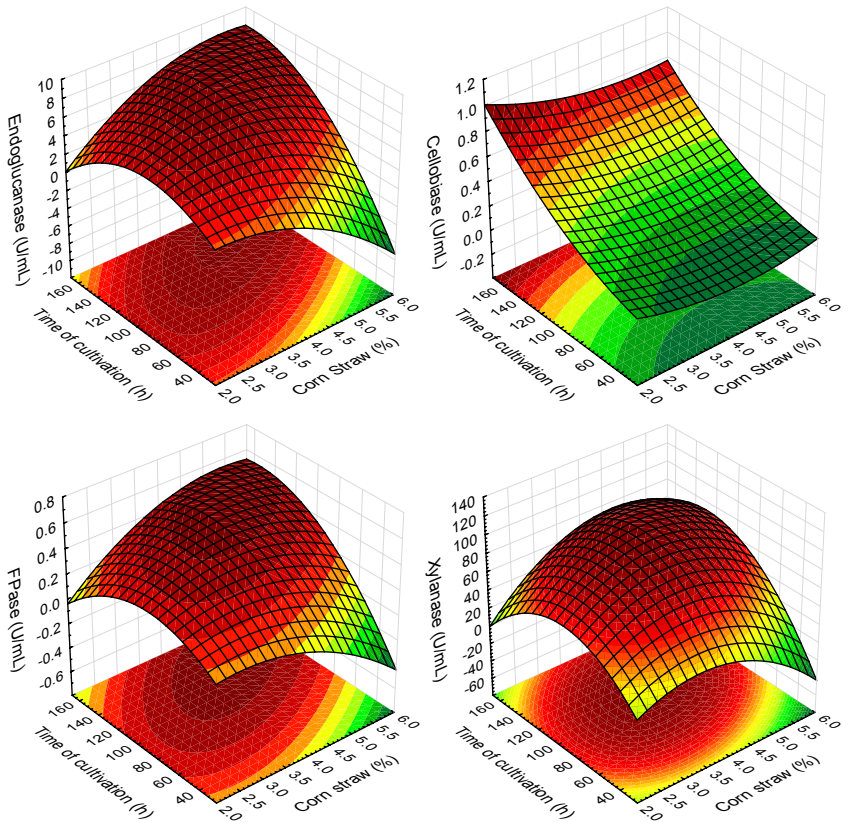

Figure 2: Response surface plots for the enzyme activity production by *Fusarium verticillioides* in relation to the factors of corn straw concentration and cultivation time.

In the factorial design it was observed that high levels of sodium nitrate and corn straw resulted in higher endoglucanase activity along the tested

intervals and in this experiment it was observed that sodium nitrate was responsible for the greatest increase in endoglucanase activity. However, as the sodium nitrate concentration increased, its effect was less intense and was considered non-significant. This result indicated that *F. verticillioides* has a limited endoglucanase production in relation to nitrogen source concentration. Thus, sodium nitrate could be maintained at the lowest level, 5 g/L, since it was not significant.

Corn straw concentration had a positive effect on endoglucanase activity in the factorial design, and it appears to be due to the positive interaction with sodium nitrate concentration. Because the effect of sodium nitrate is lost with the increase in concentration corn straw also presented a small effect, although it did present a quadratic effect which permitted definition of an ideal concentration. However, accordingly to Figure 2 it can be noted that changes in the ideal concentration resulted in minimal changes in enzyme activity.

The effect of corn straw concentration and cultivation time on FPase activity was similar to that observed for endoglucanase activity (Figure. 2). The optimum corn straw concentration for FPase activity was 4.9 % and optimum cultivation time was 140 hours. Different from its effect on endoglucanase activity, sodium nitrate presented a positive effect on FPase activity. This positive effect was similar to that observed for cellobiase activity. Since FPase activity is dependent on endoglucanase, cellobiase and other enzymes, the effect of sodium nitrate concentration may be explained by the improved cellobiase activity.

Corn straw concentration and cultivation time also presented an elevated effect on xylanase activity (Figure 2). The optimum corn straw concentration for xylanase activity was 4.1 % and optimum cultivation time was 110 hours. Process optimization resulted in a great increase in xylanase activity. In total the xylanase activity improved 8.7 times, increasing from 13 U/mL using corn straw in medium 2 to about 113 U/mL in the optimized medium. Sodium nitrate presented no significant effect on xylanase activity.

All three factors analyzed showed significant effects on cellobiase activity. Corn straw concentration had a similar negative effect to the tendency observed in the factorial design. Sodium nitrate concentration and cultivation time presented positive effects on cellobiase activity. Cellobiase production appears to be greatest induced by corn straw concentrations lower than 2 %, sodium

nitrate concentration exceeding 15 g/L and cultivation time greater than 168 hours. With respect to cellobiase activity the optimized values of the parameters evaluated could not be achieved because inadequate levels were used in this study.

The conditions that result in increased cellobiase activity were significantly different than those observed for the highest endoglucanase and xylanase activities. Effect of the nitrogen source concentration was different for endoglucanase and cellobiase activities as was also observed for *Penicillium funiculosum*, however in that study higher levels of the nitrogen source induced higher endoglucanase activity and lower levels could induce higher β-glucosidase activity (which can be direct related to cellobiase activity) [32]. The effect of nitrogen on cellobiase and endoglucanase activities obtained for *F. verticillioides* was similar to that observed for *Fusarium oxysporum* in a step to step optimization process [33].

For use in the following experiments, the fungus was cultivated in the base medium with sodium nitrate 5 g/L and corn straw 5 % for 140 hours. This condition was within the optimum interval for endoglucanase, FPase and xylanase activities. Endoglucanase and xylanase activities were those most improved in the optimization process, increasing from 2.8 to 8.0 U/mL and from 13.4 U/mL to 114 U/mL, respectively when comparing with the initial conditions used in this work. It was not possible to cultivate the fungus in the optimized conditions for the four enzyme activities, since for cellobiase the responses were opposite for the factors analyzed.

4.4.4- Biochemical characterization

The effects of temperature and pH on endoglucanase, FPase, cellobiase and xylanase activities were analyzed. The effects of temperature were analyzed in a reaction medium of pH 5.0, and after that the effects of pH were analyzed for each enzyme at its optimal temperature.

Endoglucanase activity was maximized at 80 °C (Figure 3). It was previously verified that endoglucanase from *F. verticillioides* has a high optimum temperature, however it was observed maximal activity at 70 °C [15]. This difference may be due to the production of different forms of the enzymes since the culture medium was different. The optimum pH for endoglucanase activity at 80 °C was 5.6 (Figure 3). A neutral optimum pH was also verified for

endoglucanase from *Mucor circinelloides* [34]; and a similar optimum temperature (75 °C) was observed for endoglucanase of *Fusarium oxysporum* [35].

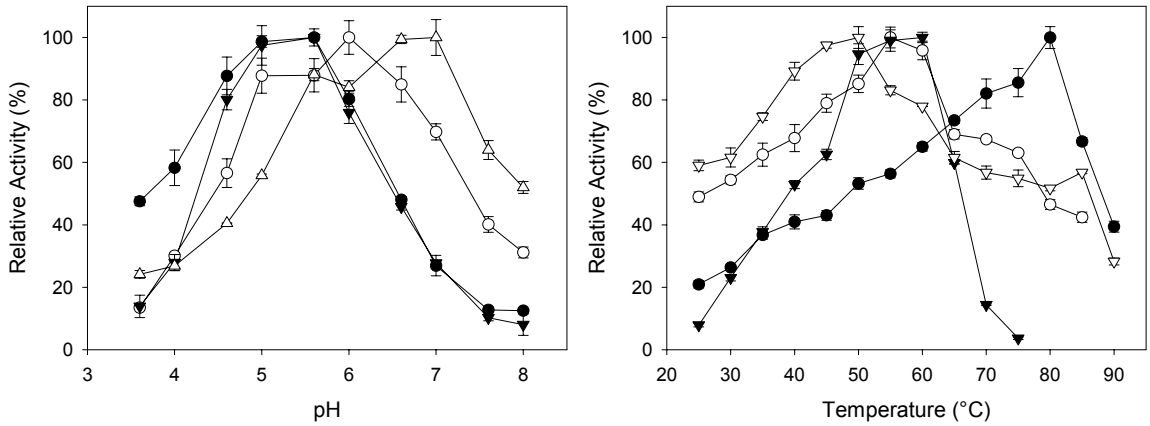

Figure 3: Effect of pH and temperature on endoglucanase (●), cellobiase (▼), FPase (○) and xylanase (△) activities.

FPase activity was highest at 55 °C and pH 6.0, while xylanase presented maximum activity at 50 °C and pH 7.0 (Figure 3). These data according to that obtained in [15]. Maximum cellobiase activity was observed between 50 – 60 °C, and the optimum temperature was 60 °C, while the optimum pH was 5.6 (Figure 3). The temperature activity profiles of FPase, cellobiase and xylanase from *F. verticillioides* showed features similar to those reported for other cellulolytic fungal species [26, 35-37].

4.4.5- Biomass pretreatment

Sugarcane bagasse was submitted to alkaline pretreatment before being hydrolyzed by the enzymatic crude extract. The pretreatment process is essential to remove lignin, reduce cellulose crystallinity and increase the porosity of lignocellulosic material, thus facilitating access of cellulases to the substrate [38]. Alkaline pretreatment was selected based on the results obtained in a previous study which compared the saccharification efficiency of biomass pretreated with diluted sulfuric acid or sodium hydroxide [25]. Alkaline pretreatment has been shown to be advantageous, mainly due to increased lignin removal that plays a crucial role in enhancing enzymatic digestibility [25, 39].

Chemical composition of the raw sugarcane bagasse sample used in the present study was as follows: cellulose 45.4%, hemicellulose 26.8% (sum of

xylans, galactans, mannans, and arabinans), and lignin 22.0%. Proteins, extractives, and ash were not measured which likely consist of the remaining 5.8%. After pretreatment the chemical composition was: cellulose 66 %, hemicellulose 25.8 % and lignin 5.9 %.

4.4.6- Simultaneous saccharification and fermentation

In order to analyze the potential of the optimized *F. verticillioides* crude extract to hydrolyze biomass as part of the ethanol production process, SSF experiments were performed. Based on the information obtained during biochemical characterization the pH of 6.0 was used to perform the SSF, because at this pH elevated enzyme activity is acquired for the four enzymes analyzed.

Considering that enzymatic hydrolysis is a very expensive step in the ethanol production process, SSF was performed using the crude extract without any preparation. The enzymatic extract was mixed with sodium acetate buffer at a ratio of 1:1 and added to the pretreated sugarcane bagasse. Enzyme load was: 8.0 U FPase, 108.6 U endoglucanase, 5.3 U cellobiase, 1.35 U α-arabinofuranosidase, 1.0 U β-xylosidase and 1501.9 U xylanase per gram of dry biomass. By conducting SSF with a low enzyme loading it was also possible to observe the real efficiency of the crude extract, because limitations of the extract are not hidden by excessive enzyme loading.

The thermotolerant yeast *Kluyveromyces marxianus* ATCC8554 was used in the SSF experiments. This yeast is able to ferment glucose to ethanol at 40 °C, thus permitting that enzymatic hydrolysis of biomass occurs at a temperature high enough to obtain adequate saccharification levels. A pre-saccharification was performed for 9 hours after which the yeast was added to the reaction mixture. This pre-saccharification period permits for accumulation of a substantial initial glucose concentration that allows the yeast to ferment glucose to ethanol and not use it as carbon source for respiration. The importance of pre-saccharification for high ethanol yields has been previously demonstrated [40].

After three hours of yeast inoculation glucose levels begin to fall followed shortly by increase in ethanol concentrations (Figure 4). After sixteen hours the glucose concentration was no longer detectable while the ethanol concentration continued to increase, indicating that the glucose liberated was immediately

fermented by the yeast to ethanol. The ethanol concentration continued increasing until 108 hours and the final concentration was 9.7 g/L. Considering the glucan content on pretreated sugarcane bagasse, this value means 52 % of the maximal theoretical yield. Similar yields were obtained when conducting SSF with sugarcane bagasse and agave bagasse [41]. When pretreated switchgrass was hydrolyzed using similar solids and enzyme loading conditions to those used in the present study, but instead using a commercial enzyme, a lower ethanol concentration was achieved (5.2 g/L). As expected, better results were obtained when increasing both enzyme and solids loadings [42]. The final ethanol concentration is closely related to the enzyme loading and the initial biomass concentration. Higher ethanol yields are obtained in SSF using biomass loading of 10 % or greater [42, 43].

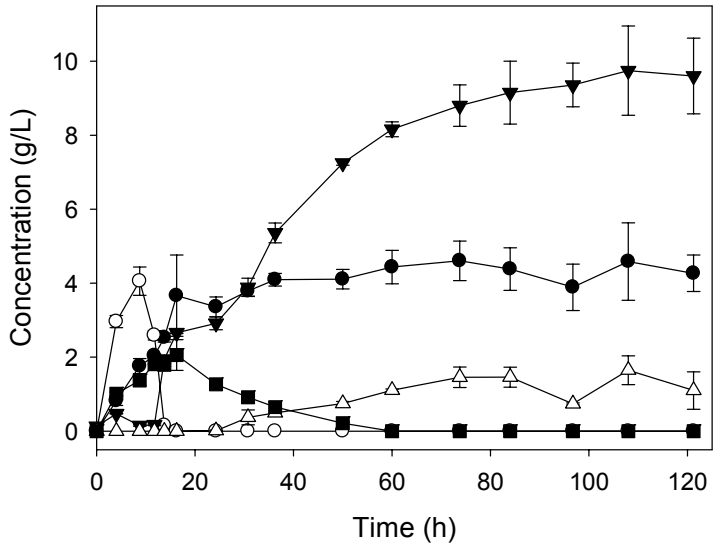

Figure 4: Simultaneous saccharification and fermentation of sugarcane bagasse using the optimized *F. verticillioides* extract. Glucose (○), xylose (●), cellobiose (■), ethanol (▼) and xylitol (△).

The ethanol yield per gram of dry biomass was 0.19 g/g, similar to 0.21 g/g obtained from hydrolyzed sugarcane bagasse fermented by *Escherichia coli* [43] and 0.17 g/g obtained from hydrolyzed sugarcane bagasse fermented by *Saccharomyces cerevisiae* [44].

Cellobiose was not observed after 60 h of fermentation, indicating that the cellobiase level was sufficient for the reaction and there was no inhibition by this sugar. Because cellotriose and cellotetraose were not detected in the analysis, it was assumed that the limiting step of the hydrolysis was one or more steps before the production of these sugars. Endoglucanase is the first cellulase to

act at the cellulose chain, and after its action the substrates for the other cellulases are released, thus it seems that more endoglucanase enzymes are required to increase the sugar concentration. Another possibility is the low exoglucanase activity, however this activity was not evaluated.

A tendency to decrease ethanol concentration was observed after reaching the maximum at 100 h, which may suggest ethanol respiration by the yeast after consumption of all available glucose in the medium. This was also observed during SSF of *Jatropha curcas* shells when using *Saccharomyces cerevisiae* as the fermenting microorganism [45].

The xylose concentration increased constantly until sixteen hours after which its concentration began to stabilize. After thirty hours the xylitol concentration started to increase. It is assumed that xylose liberated by the action of xylanases was converted to xylitol or used as a carbon source for yeast growth. Although it appears that xylose was not fermented into ethanol, the presence of xylanase enzymes does increase cellulose accessibility and subsequently improves enzymatic saccharification. A negative correlation between xylan content in biomass and glucan conversion was demonstrated for sugarcane bagasse and switchgrass saccharification [46].

4.5- Conclusion

In this study, the corn straw concentration, sodium nitrate concentration and cultivation time of *F. verticillioides* to produce endoglucanase and xylanase activities were successfully optimized, increasing the activities 2.9 and 9 times, respectively. The enzymes were characterized in relation to temperature and pH. The optimized crude extract produced 9.7 g/L from alkali pre-treated sugarcane bagasse showing to be effective to produce ethanol from lignocellulosic biomass.

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5- Artigo 2: Cellulases production from *Fusarium verticillioides* and its application on the saccharification process of sugarcane bagasse

5.1- Abstract

Forage was used as carbon source to cellulase and hemicellulases production by *F. verticillioides*. A Plackett Burman design was employed to evaluate the effects of different factors on enzymatic production and two contrasting media composition were found, M1 and M2. In M1 the endoglucanase and celobiase production were 6.5 and 0.39 U/mL, respectively. By another hand, the production of endoglucanase and celobiase in M2 conditions were 2.6 and 6.8 U/mL, respectively. Mixtures of these two extracts were tested on a sugarcane bagasse saccharification and M1 presented the best performance, yielding 26.6 % of glucan conversion and 40.4 % of xylan conversion. Different dosages of protein (10, 20 and 40 mg/g of dry biomass) were tested intending to improve the hydrolysis performance of M1 extract. Saccharification rates of 43.4 and 73.1 % were observed for glucan and xylan fractions respectively when 40 mg/g was used. A high protein adsorption on substrate was observed during saccharification assays employing M1 extract and that can explain de lower sugar productivity in the saccharification process along the time.

Key words: endoglucanase, cellobiase, *Fusarium verticillioides*, sugarcane bagasse

5.2- Introduction

Lignocellulosic biomass, composed mainly of cellulose, hemicellulose and lignin, are widely studied as sources for second generation ethanol [1, 2]. The use of these materials for production of other value added product, mainly in a biorefinery concept is also an alternative. Ideally, a biorefinery should integrate biomass conversion processes to produce a range of fuels, power, materials, and chemicals from biomass [3].

Lignocellulose conversion on accessible products is a bottleneck. Two main approaches have been developed in parallel for conversion of lignocellulosic materials to commodity chemicals- ‘‘acid based’’ and ‘‘enzyme

based''. The highly complex enzyme based approach is advantageous over chemical treatments because of moderate operating conditions, higher conversion efficiencies, absence of substrate loss due to chemical modifications and no generation of toxic chemicals [4]. However, enzymatic depolymerization of polysaccharides is less adequate to industrial scale implementation due to the enzyme loading requirements and associated costs [5].

For complete cellulose degradation the synergistic action of the four cellulases is necessary. Endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.176), exoglucohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21). The hemicellulose fraction hydrolysis requires a more complex group of enzymes, the hemicellulases. Complete enzymatic hydrolysis of xylan, the major polymer founded in hemicelluloses, 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) [6, 7].

The parameters to optimize enzymatic saccharification of biomass are subject of diverse studies, however it is not possible to standardize the process due its high complexity [8-12]. The biomass nature concerning to sugar and lignin composition are key factors to optimize the saccharification. Several pretreatments techniques are being studied intending to improve the lignin removal and reducing crystallinity and polymerization degree of the cellulose. Nevertheless, these pretreatment technique shall guarantee the sugar preservation [13]. Solid loading, moisture content and enzyme dosage also are critical bottlenecks concerning to the economical feasibility of the biomass saccharification and many works have been developed to evaluate the influence of these factors on efficiency of saccharification processes [7, 9, 10, 12, 14, 15].

The corn endophytic *Fusarium verticillioides* (*Gibberella moniliformis*) was isolated from commercial corn seeds and an initial screening of cellulases and hemicellulases activities has been studied previously [16]. In the present study, different conditions of cultivation to fungi *F. verticillioides* were tested using a Plackett-Burman design. Two contrasting enzymatic extract were obtained and both were employed singly or in a mixture in biomass saccharification assays.

5.3- Materials and methods

5.3.1- Materials

The carboxymethylcellulose (CMC), Avicel, birchwood xylan, cellobiose and the substrates ρ-nitrophenyl-α-L-arabinofuranoside (ρNPAra) and ρ-nitrophenyl-β-D-xylopyranoside (ρNPXyl) were obtained from Sigma Chemical Co. (St. Louis, MO). The forage *Andropogum guayanus* was acquired in a local farm. All other chemicals used were of analytical grade.

5.3.2- Enzyme assay

FPase activity was determined using Whatman no. 1 filter paper (1 x 6 cm, 50 mg) in a total volume reaction of 1.5 mL for 60 minutes at 50 °C. Endoglucanase activity was determined using carboxymethilcellulose 1 % in a final volume of 0.5 mL for 30 minutes at 50°C. Xylanase activity was determined using birchwood xylan 1 % in a final volume of 0.5 mL for 15 minutes at 50°C. All these assays were carried out in 0.05 M sodium acetate buffer pH 5.0. For all these assays, the reducing sugar released was determined using the 3,5-dinitrosalicylic acid (DNS) reagent [17]. The amount of product released was calculated according to the standard curve (0.11 – 1.11 µmol of glucose). The assays for endoglucanase and FPase were based on those described by Ghose [18].

Cellobiase activity was determined using cellobiose as substrate in a final concentration of 6.4 mM and 0.05 M sodium acetate buffer pH 5.0 in final volume of 0.5 mL for 30 minutes at 50°C. The reaction was terminated by boiling the samples for 5 min. The glucose liberated was quantified using a commercial analytical kit based on glucose oxidase and peroxidase (Quibasa Basic Chemical, Belo Horizonte, MG, Brazil).

The activity of α-L-arabinofuranosidase and β-xylosidase were measured using 0.5 mM ρNPAra and ρNPXyl, respectively. These enzymes assays were carried out at 50 °C for 15 minutes in 0.05 M sodium acetate buffer, pH 5.0 in a final volume of 0.5 mL. Absorbance of the mixtures was then measured at 410 nm. The amount of ρNP released was calculated according to the standard curve (0.0017 - 0.0625 µmol of ρNP).

For all enzymatic activities one enzyme unit was defined as the amount of enzyme that released 1 µmol of product per minute under the assay conditions.

Protein concentration in the enzymatic extracts was determined by the Coomassie Blue binding method using bovine serum albumin as the standard [19].

5.3.3- Microorganism and inoculum preparation

The fungus *F.verticillioides* was isolated from corn seeds, belongs to mycological collection of Laboratory of Biochemical Technology at Federal University of Viçosa, MG, Brazil, and was routinely propagated in potato dextrose agar slants at 28 °C. The sporulated slants obtained after 7 days were used for preparing the spore inoculum with sterile water. For inoculation, 2 mL of water containing 1.5 x 10^6 spores was used in 50 mL culture medium.

5.3.4- Enzymes production

Plackett Burman design was carried out aiming to identify factors that could affect the cellulases production by *F. verticillioides*. A 2^9 + 1 factorial design with three repetition at central point was performed in order to study the main effects of the following factors: urea, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2$, $MgSO_4$, yeast extract, peptone, forage concentration and forage size (independent variables). The cellulases production was monitored by measuring of endoglucanase and cellobiase activity (dependent variables). The level of the studied factors were based on Mandel's medium [20]. In this experimental design, 21 runs were carried out under completely randomized design. The levels of the factors were: urea (0.1, 0.3 and 0.5 g/L), $(NH_4)_2SO_4$ (0.7, 1.4 and 2.1 g/L), KH_2PO_4 (1, 2 and 3 g/L), $CaCl_2$ (0.1, 0.3 and 0.5 g/L), $MgSO_4$ (0.1, 0.3 and 0.5 g/L), yeast extract (0.1, 0.25 and 0.4 g/L), peptone (0.3, 0.75 and 1.2), concentration of forage (10, 25 and 40 g/L) and particle size of the forage (mesh 30, 20 and 10 of the sieve) (Table 1). The time of cultivation was also evaluated (2, 4 and 7 days) however this factor was not included in Plackett Burnman design and a complete set of runs was carried out for each time of cultivation evaluated. All cultivations were carried out at 28 °C under agitation (180 rpm) in a rotary shaker (Tecnal, TE-421, Piracicaba, São Paulo, Brazil).

Table 1: Uncoded values of independent variables for Plackett Burman design

Run	Urea	$(NH_4)_2SO_4$	KH_2PO_4	$CaCl_2$	$MgSO_4$	YE	P	F	M
1	0.5	0.7	3	0.5	0.1	0.1	0.3	10	30
2	0.5	2.1	1	0.5	0.5	0.1	0.3	10	10
3	0.1	2.1	3	0.1	0.5	0.4	0.3	10	10
4	0.1	0.7	3	0.5	0.1	0.4	1.2	10	10
5	0.5	0.7	1	0.5	0.5	0.1	1.2	40	10
6	0.5	2.1	1	0.1	0.5	0.4	0.3	40	30
7	0.5	2.1	3	0.1	0.1	0.4	1.2	10	30
8	0.5	2.1	3	0.5	0.1	0.1	1.2	40	10
9	0.1	2.1	3	0.5	0.5	0.1	0.3	40	30
10	0.5	0.7	3	0.5	0.5	0.4	0.3	10	30
11	0.1	2.1	1	0.5	0.5	0.4	1.2	10	10
12	0.5	0.7	3	0.1	0.5	0.4	1.2	40	10
13	0.1	2.1	1	0.5	0.1	0.4	1.2	40	30
14	0.1	0.7	3	0.1	0.5	0.1	1.2	40	30
15	0.1	0.7	1	0.5	0.1	0.4	0.3	40	30
16	0.1	0.7	1	0.1	0.5	0.1	1.2	10	30
17	0.5	0.7	1	0.1	0.1	0.4	0.3	40	10
18	0.5	2.1	1	0.1	0.1	0.1	1.2	10	30
20	0.1	0.7	1	0.1	0.1	0.1	0.3	10	10
21	0.3	1.4	2	0.3	0.3	0.25	0.75	25	20
22	0.3	1.4	2	0.3	0.3	0.25	0.75	25	20
23	0.3	1.4	2	0.3	0.3	0.25	0.75	25	20

YE: yeast extract; P: peptone; F: concentration of forage; M: mesh of the sieve.

The analysis of variance (ANOVA) of the *F* test was preliminary performed to evaluate if there was any effect different from zero and after that the data were evaluated by the *t* test to determine which coefficients were different from zero at 10 % of probability.

5.3.5- Biomass pretreatment

Sugarcane bagasse pretreatment was based on reported by Falkoski *et al* [21]. Sugarcane bagasse was dried in an oven at 70 °C until the weight was constant. The dry sugarcane bagasse was milled in rotary mill (model MA-580, Marconi Co. Piracicaba – SP) and passed through a 1.0 mm screen. Sodium hydroxide at a concentration of 1 % was used to pretreat 25 g of milled sugarcane bagasse samples at a solid loading of 10 % (w/v). Treatments were performed in duplicate in an autoclave at 121 °C for 60 min. The pretreated materials were separated into solid and liquid fractions using a Buchner funnel

with filter paper. The solid fraction was washed thoroughly with distilled water, dryed at 40 °C, sealed in a hermetic vessel and stored at -20 °C.

The chemical composition of the untreated and alkaline pretreated sugarcane bagasse was determined as described in previous study using a modified Klason lignin method derived from the TAPPI Standard Method T222 (OM-98) and TAPPI Useful Methods (UM-250) [21].

5.3.6- Saccharification experiments

Enzymatic saccharification of alkali pretreated sugarcane bagasse was performed in 15 mL sample tubes containing 10 mL of total volume. The assay consisted of pretreated sugarcane bagasse at a solid loading of 6 % (dry matter, w/v), appropriately diluted enzymes in 50 mM sodium acetate buffer, pH 5.0, sodium azide (10 mM), tetracycline (40 µg/mL) and tween 20 (0,1 %). The crude enzymatic extracts produced by *F. verticillioides* in the selected culture media were concentrated 10-fold using an Amicon Ultrafiltration system (Millipore Co. – Billerica, MA, USA) using a YM-10 (Cut-off Mr 10,000 Da) membrane filter.

The enzyme loading changed for each experiment and was always determined as a function of protein concentration. All saccharification reactions were carried out in an orbital shaker at 250 rpm and 50 °C for up to 120 h. Samples (100 µL) were taken from the reaction mixture at different time intervals and immediately centrifuged for 10 min at 10000 x g, incubated in boiling bath to denature the enzymes and stored at -20 °C. The samples were analyzed in HPLC on a Shimadzu series 10A chromatograph. It was used a column Aminex HPX87P (300 x 7.8 mm, 9 µm particle size). The mobile phase was water in a flow of 0.6 mL/min, at 80 °C.

The glucose conversion was calculated using the following equations [7]:

$$\text{Conversion (\%)} = \frac{\text{glucose (g/L)} \times 0.90 \times 100}{\text{initial glucan (g/L)}}$$

The xylose conversion was calculated using the following equation:

$$\text{Conversion (\%)} = \frac{\text{xylose (g/L)} \times 0.88 \times 100}{\text{initial xylan (g/L)}}$$

5.3.7- Thermostability

For determination of thermostability, the enzyme fractions were pre-incubated with sodium acetate buffer, 100 mM, pH 5.0 at 50, 60, 70 and 80 °C. Samples were collected at several periods of time and kept on ice for at least 1 hour. Residual activity was determined using the standard assay.

5.4- Results and discussion

5.4.1- Enzyme production

5.4.1.1 – Plackett Burman design

A Plackett and Burman design was employed to determine the individual effect of nine variables over the production of endoglucanase and cellobiase activity by *F. verticillioides* under submerged fermentation. The variables and the experimental conditions used are showed in Table 1. The samples were taken after 2, 4 and 7 days of cultivation and the activities were measured (Table 2).

Observing the average of enzymatic production after different cultivation times it was clear that the highest activities were obtained after 7 days of cultivation to both endoglucanase and cellobiase activity. Thus, the set of dates obtained after this time was utilized to perform the statistical analysis.

Table 2: Results of the Plackett Burman design for production of endoglucanase and cellobiase activities at 2, 4 and 7 days of cultivation

Run	E 2	E 4	E 7	C 2	C4	C 7
1	2.54	2.91	3.76	0.05	0.19	0.28
2	2.38	1.96	3.80	0.06	0.10	0.25
3	2.54	2.85	3.23	0.06	0.17	0.30
4	3.11	3.24	3.94	0.04	0.22	0.31
5	2.37	4.86	6.58	0.16	0.52	1.14
6	2.59	4.85	7.80	0.09	0.47	1.12
7	3.31	3.44	4.28	0.05	0.21	0.37
8	2.34	4.30	6.32	0.13	0.72	1.49
9	2.45	3.51	4.18	0.23	0.77	1.46
10	2.47	3.16	4.16	0.06	0.21	0.33
11	2.95	3.19	4.10	0.07	0.17	0.29
12	2.69	4.11	6.10	0.11	0.61	1.33
13	2.29	4.48	6.44	0.05	0.34	1.21
14	2.02	3.91	5.25	0.09	0.54	1.50
15	2.04	3.24	4.04	0.09	0.47	1.23
16	2.96	3.38	4.27	0.05	0.19	0.49
17	2.04	4.79	6.09	0.11	0.52	1.12
18	3.52	3.26	3.86	0.05	0.17	0.33
20	1.66	2.96	4.59	0.07	0.28	0.43
21	2.96	4.68	6.99	0.12	0.44	1.00
22	3.11	4.90	7.02	0.14	0.45	1.00
23	3.00	4.70	7.99	0.11	0.44	1.06
Average Activity	2.61± 0.47	3.76 ±0.84	5.22±1.47	0.09 ±0.05	0.37 ±0.19	0.82 ±0.47

E: endoglucanase; C: cellobiase.

The t-test was used to identify the effect of every factor on endoglucanase and cellobiase production. At Table 3 are showed the coefficients (effect), the t-values and the p-values associated to each factor evaluated in the Plackett Burman design. The larger the magnitude of ‘t’ in t-test and smaller the P-value, the more significant is the corresponding coefficient. At significance level of 10 %, forage concentration was the unique variable that had positive and significant effect on the endoglucanase production by *F. verticillioides*. On the other hand, the production of cellobiase activity was affected significantly by KH_2PO_4 and forage concentration in a positive way and by urea and yeast extract concentration in a negative way.

Table 3: The main effects observed at Plackett Burman design for independent variables on the enzymatic activities. Values represent coded units.

	Endoglucanase			Cellobiase		
Factor	Effect	t-value	p-value	Effect	t-value	p-value
Urea	0.8082	1.35	0.201	-0.1469*	-2.22	0.044
$(NH_4)_2SO_4$	-0.0131	-0.02	0.983	0.0663	1.00	0.334
KH_2PO_4	-0.5716	-0.95	0.358	0.1786*	2.70	0.018
$CaCl_2$	-0.2802	-0.47	0.648	-0.1003	-1.52	0.153
$MgSO_4$	0.1524	0.25	0.803	-0.0601	-0.91	0.379
Yeast extract	0.2900	0.48	0.637	-0.1741*	-2.64	0.021
Peptone	0.4853	0.81	0.433	-0.0073	-0.11	0.914
Forage concentration	1.7468*	2.91	0.012	1.0228*	15.49	0.000
Forage particle size	-0.1347	-0.22	0.826	-0.0343	-0.52	0.612

* Significant effect at 10 % probability.

Forage concentration had a strong positive effect over the production of both endoglucanase and cellobiase activity. Thus, an additional experiment was carried out intending to determine the ideal forage concentration to produce endoglucanase and cellobiase activity by *F. verticillioides*.

Although the factors urea, KH_2PO_4 and yeast extract were significant to production of cellobiase they were not studied in the next experiment because the effects related with these factors (-0.146, 2.70 and -2.64 respectively) were much lower in comparison with effect of the forage concentration (15.49).

Nevertheless, it was noteworthy that some factors tested in the Plackett Burman design presented contrasting effects over production of endoglucanase and cellobiase activity by *F. verticillioides*. Although not significantly, the factors urea, $MgSO_4$, yeast extract and peptone had a positive effect over the production of endoglucanase activity. However the same factors presented a negative effect to cellobiase production. Besides that, it was also observed that KH_2PO_4 affected positively and significantly the production of cellobiase, but the effect of this factor on production of endoglucanase was negative. Thus, two different medium, M1 and M2, were used in the experiment to optimize the forage concentration to production of endoglucanase e cellobiase activity by *F. verticillioides*.

Considering the levels employed in the execution of Plackett Burman design the composition of medium M1 was adjusted to favor the production of endoglucanase. Thus, the higher levels were utilized to those factors that presented a positive effect on endoglucanase production. In another hand,

lower levels were used to factors that presented negative effect. The following composition was obtained to medium M1 (g/L): 0.5 urea; 0.7 $(NH_4)_2SO_4$; 1.0 KH_2PO_4; 0.1 $CaCl_2$; 0.5 $MgSO_4$; 0.4 yeast extract; 1.2 peptone; particle size: 10 mesh.

Following the same procedure the medium M2 was composed to favor the production of cellobiase activity and the composition was (g/L): 0.1 urea; 2.1 $(NH_4)_2SO_4$; 3.0 KH_2PO_4; 0.1 $CaCl_2$; 0.1 $MgSO_4$; 0.1 yeast extract; 0.3 peptone; particle size: 10 mesh.

5.4.1.2 – Study of forage concentration and cultivation time

The endoglucanase production by *F. verticillioides* in different forage concentrations was evaluated during 16 days, employing media M1 and M2 (Figure 1).

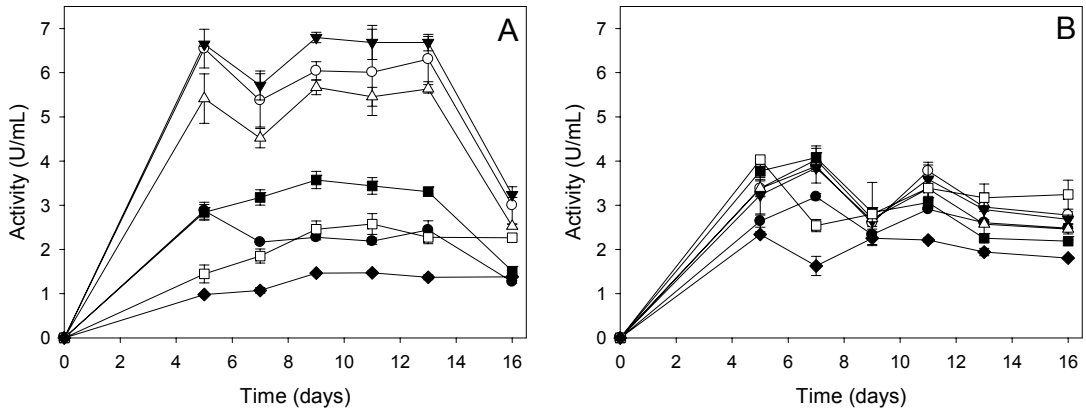

Figure 1: Endoglucanase production on different forage concentration: (●) 1 %, (○) 2.5 %, (▼) 4 %, (△) 5.5 %, (■) 7 %, (□) 8.5 %, (◆) 10 %, in M1 (A) and M2 (B) media.

The endoglucanase activity was produced in higher amounts in M1 than in M2, confirming that the determination of medium composition based in Plackett Burman analysis was adequate. For endoglucanase production, the best forage concentration was between 2.5 to 5.5 %. Concentrations below or above these values induced lower endoglucanase production. The chosen condition for endoglucanase production was M1, 2.5 % forage and 5 days of cultivation time. In this condition, 6.5 U/mL of endoglucanase and 0.39 U/mL of cellobiase activity were produced. Then, endoglucanase rich enzymatic extract obtained in the conditions cited above was referred to M1 extract.

For cellobiase production the best medium was M2 confirming once more what it was firstly suggested (Figure 2).

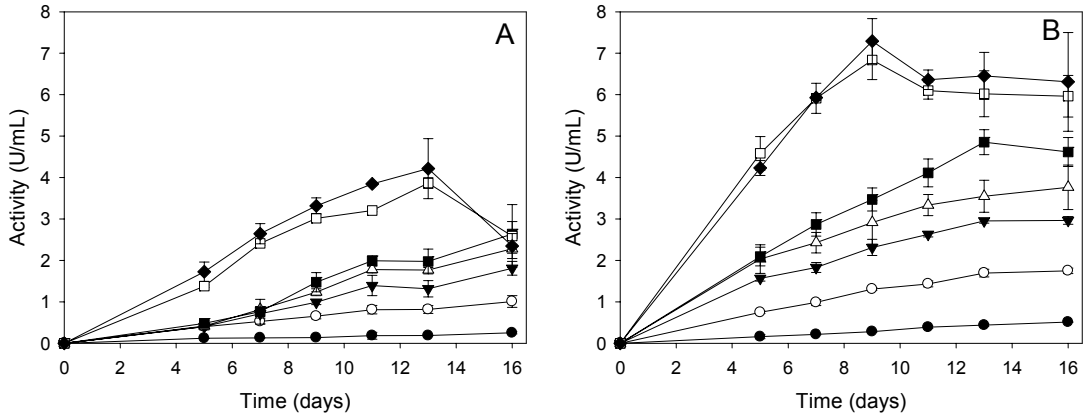

Figure 2: Cellobiase production on different carbon source concentration 1 % (●), 2.5 % (○), 4 % (▼), 5.5 % (△), 7 % (■), 8.5 % (□), 10 % (◆), in M1 (A) and M2 (B) media.

Increasing forage concentrations induced higher cellobiase activity. The higher cellobiase activity was obtained when 8.5 or 10 % forage was used. The best condition to cellobiase production was M2, 8.5 % forage and 9 days of cultivation time. In this condition it was produced 6.8 U/mL of cellobiase and 2.6 U/mL of endoglucanase. Then, cellobiase rich enzymatic extract obtained in the conditions cited above was referred to M2 extract. The differences about endoglucanase and cellobiase between M1 and M2 extract confirm that the medium composition interfere decisively in enzymatic profile secreted by fungus *F. verticillioides*.

5.4.2- Sugarcane bagasse saccharification

5.4.2.1- Mixtures experiments

Endoglucanases produces nicks in the cellulose polymer exposing reducing and non-reducing ends, cellobiohydrolases acts upon these reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose units, and cellobiase cleaves the cellobiose to liberate glucose, thereby completing the hydrolysis [22]. Cellobiose is known as an exocellulases and endoglucanase inhibitor, decreasing the cellulose hydrolysis yield. Cellobiase has been added to commercial cocktails to increase its efficiencies [8, 23].

In this study it was obtained distinct enzymatic extract produced by *F. verticillioides* employing different medium composition. The extract M1 was characterized by higher endoglucanase activity while the extract M2 showed a superior cellobiase activity. As endoglucanase activity is crucial in the beginning

of saccharification processes and cellobiase activity is fundamental in the final part of the process, it could be suggested that, in a biomass saccharification process, using mixtures of these two extracts would be more efficient than using each extract separately.

To test this assumption, the extracts M1 and M2 were applied separated or in different mixtures in saccharification of sugarcane bagasse. The mixtures were defined based on protein content in the extracts and it was used 10 mg of protein per gram of dry biomass. M1 and M2 were mixed in the following proportions: 100 % M1, 75 % M1 + 25 % M2, 50 % M1 + 50 % M2, 25 % M1 + 75 % M2 and 100 % M2.

Cellulases and some important hemicellulases activities involved in biomass depolymerization process were measured in M1 and M2 extracts and in the mixture cocktails produced. The results obtained are showed at Table 4.

Table 4: Final activities on each cocktail mixture used for saccharification assays

	Enzyme Activities (U/g)					
Mixture	Endo	Cello	FPase	Xyl	β-Xyl	α-Arab
100 % M1	127.5	11.0	9.7	849.0	1.4	1.9
75 % M1 + 25 %M2	102.7	28.5	10.8	756.1	3.1	3.8
50 % M1 + 50 % M2	93.0	31.4	9.3	705.8	4.6	5.5
25 % M1 + 75 % M2	77.2	133.2	9.1	693.7	6.8	7.2
100 % M2	69.9	152.4	7.6	600.2	8.2	8.7

Endo: endoglucanase, Cello: cellobiase, Xyl: xylanase, β-Xyl: β-xylosidase, α-Arab: α-Arabinofuranosidase

The FPase activity, which depends on the mixture of the four cellulases were almost equal on mixtures and it was slightly lower in 100 % M2 extract. In general, it could be noted that enzymes for polymeric substrates (endoglucanase and xylanase) followed the same tendency showing higher activity on M1 extract. On the other hand, enzymes for oligomeric substrates (cellobiase, β-xylosidase and α-arabinofuranosidase) presented higher activity on M2.

Sugarcane bagasse was used for saccharification experiments because it is produced in large quantities in Brazil. The annual Brazilian production of sugarcane bagasse is currently estimated at 186 million tons per year and this amount of biomass represents a potential to produce around 11.160 billion gallons of lignocellulosic ethanol per year [24]. Sugarcane bagasse was submitted to alkaline pretreatment before being hydrolyzed. Chemical

composition of the raw sugarcane bagasse sample used in the present study was as follows: glucan 45.4%, hemicellulose 26.8% (sum of xylans, galactans, mannans, and arabinans), and lignin 22.0%. Proteins, extractives, and ash were not measured which likely consist of the remaining 5.8%. After alkaline pretreatment the chemical composition of the pretreated biomass was: cellulose 66.1 %, xylan 23.6 %, galactan 0.3 % and arabinan 1.9 %.

The releasing of sugars on sugarcane bagasse saccharification were highly dependent on the mixture used (Figure 3). Higher glucose concentration was obtained increasing M1 proportion. In assays carried out with 100% M1 extract was obtained 11.8 g/L of glucose (26.6% of glucan conversion) and in assays with 100 % M2 extract only 6.8 g/L were obtained (14.5% of conversion). Intermediate values to glucose production were observed when assays were carried out with mixtures of M1 and M2 extracts.

The percentages of conversion of xylan into xylose were 40.4, 45.9, 42.4, 46.3 and 41.5 % for assays carried out with 100 % M1 extract until 100 % M2 extract, respectively. The xylan conversion is more complex than the cellulose, since it is dependent on many different enzymes. In this case the mixtures presented a rate of xylan conversion slightly superior than pure extracts. This result suggests that the mixtures have a better balance between xylanase, which hydrolyze the main polymer and the accessories enzymes β-xylosidase and α-arabinofuranosidase. β-Xylosidase hydrolyze the xylooligosacharides released by the xylanase action and also any xylose residue in the xylan ramification. α-Arabinofuranosidase together with other enzymes like α-galactosidases, β-galactosidases and acetyl esterases, hydrolyze the carbohydrate residues presents on the ramification of xylan, contributing to the complete hydrolysis of the polymer and also facilitating the access of the xylanase on this own substrate [7]. The synergy between these enzymes may be maximized on the mixtures.

Cellobiose accumulated in the beginning of the process in the assays with 100 % M1 extract, but any amounts of M2 added helped to avoid this accumulation on the others mixtures. Despite this initial difference, after 24 h of saccharification, cellobiose was already consumed and in the end of the process, cellobiose concentration was similar to all mixtures tested (between 0.29 to 0.33 g/L). The ratio endoglucanase:cellobiase, 12:1, present on M1 was sufficient to avoid cellobiose inhibition. Among the enzyme activities analyzed,

endoglucanase was the main difference between the mixtures and was probably responsible for the better performance of 100 % M1 extract.

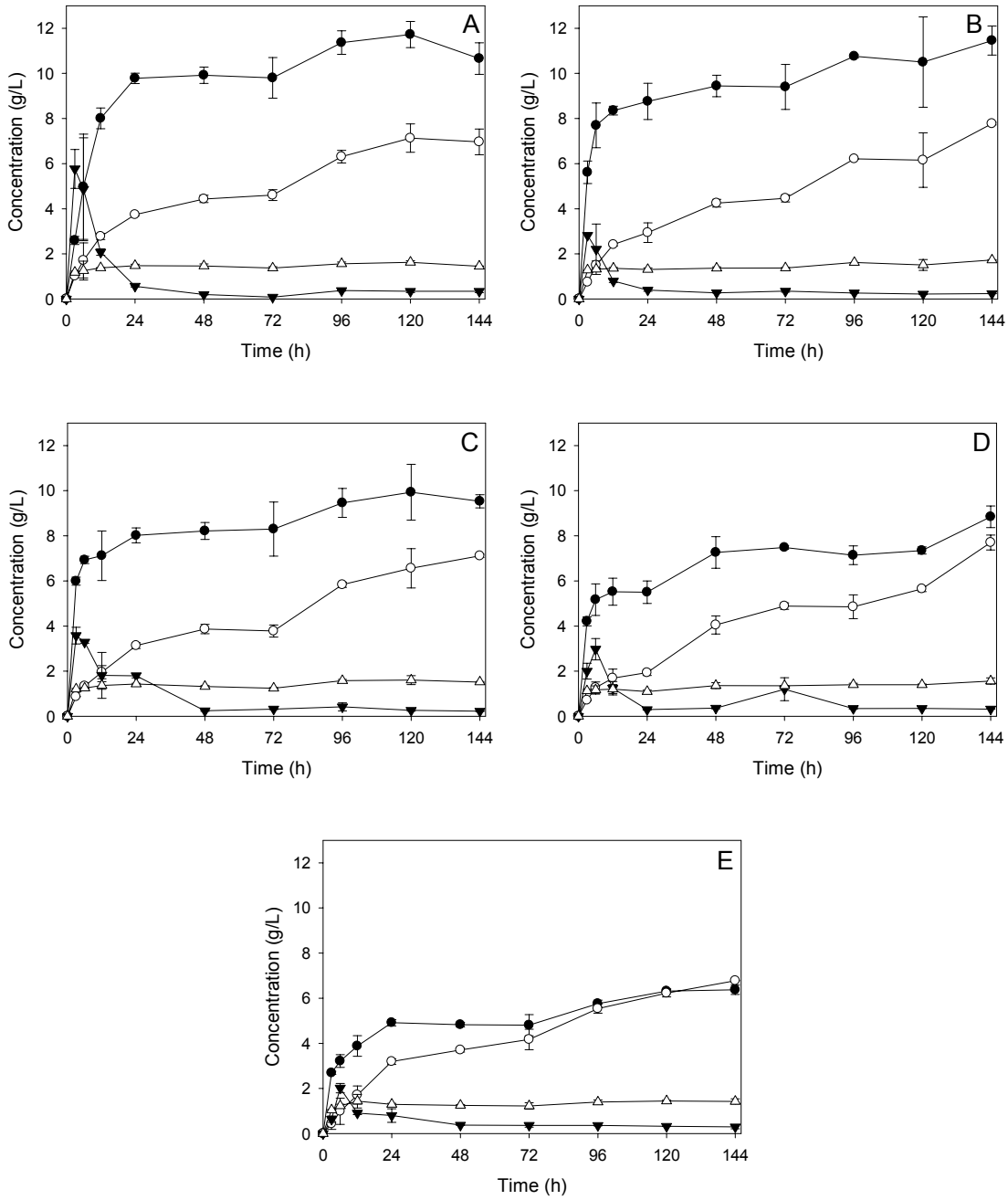

Figure 3: Saccharification of sugarcane bagasse using different enzymatic mixtures between M1 and M2 extract produced by fungus *F. verticillioides*. 100 % M1 (A), 75 % M1 – 25 % M2 (B), 50 % M1 – 50 % M2 (C), 25 % M1 – 75 % M2 (D) and 100 % M2 (E). Glucose (●), xylose (○), Cellobiose (▼), Arabinose (△).

Similar results were observed for sugarcane bagasse saccharification using enzymatic extract from *Trichoderma* and *Aspergillus*. It was reported that a rich cellobiase (β-glucosidase) extract do not presents best results for saccharification if it do not contain an equilibrated cellulase composition [25].

Another fact that can be analyzed is the great increase in glucose and xylose concentration until 24 h, followed by slight increase until 144 h. The glucose and xylose productivities in the saccharification assays decreases quickly along the time (Figure 4).

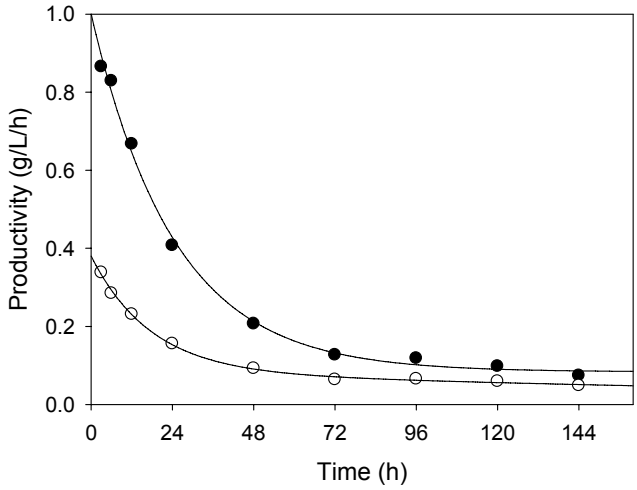

Figure 4: Productivity of glucose (●) and xylose (○) along the saccharification time for 100 % M1 extract.

5.4.2.2- Analyses on the non-productive factors

Non-productive protein adsorption on the substrate, enzyme deactivation and other rate limitations, such as decrease in substrate reactivity and inaccessibility caused by lignin are usually pointed as reasons to the reduction on hydrolysis during saccharification process [26]. Trying to elucidate this loss of productivity it was performed a new saccharification using M1 extract. The assay was the same as the early experiment, however, in a final volume of 50 mL. The soluble protein concentration, enzymatic activities and products presents on supernatant of saccharification were analyzed (Figure 5).

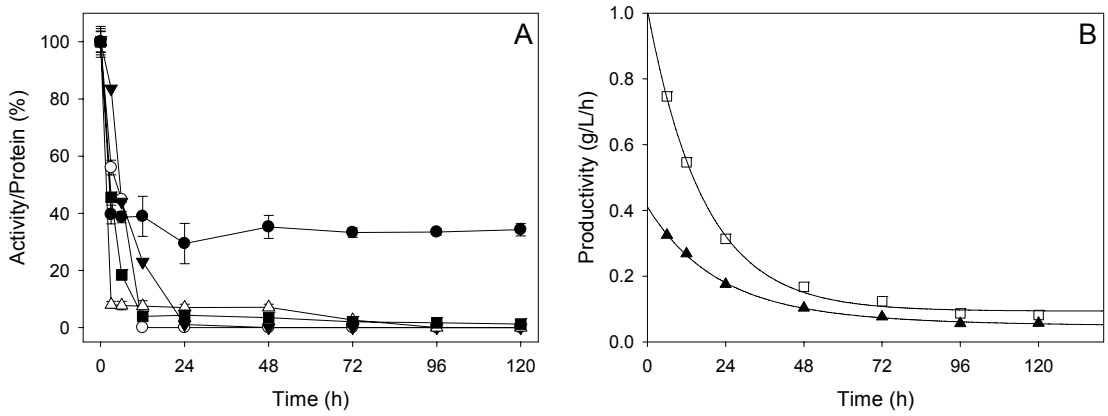

Figure 5: Determination of soluble protein and enzymatic activities along saccharification of sugarcane bagasse employing M1 extract produced by *F. verticillioides* (A): protein concentration (●), endoglucanase (○), FPase (▼), cellobiase (△) and xylanase activities (■). The values equivalents to 100% were: protein 0.6 mg/mL, endoglucanase 4.2 U/mL, FPase 0.45 U/mL, cellobiase 0.16 U/mL and xylanase 51.9 U/mL Productivity (B): glucose (□), xylose (▲).

In the first three hours of saccharification it was already significant the decrease of the proteins on the supernatant. The total protein decreased to 40 % of the initial concentration and maintained this level until 120 h. This fact evidence the adsorption to substrate phenomenon, which usually occurs with cellulases [27]. After three hours, the endoglucanase, FPase, xylanase and cellobiase activities were 55 %, 83 %, 45 % and 8 %, of the initial activity respectively. It was not detected endoglucanase, FPase and cellobiase activities after 12, 24 and 96 h, respectively. The xylanase activity maintained about 2 % until 120 h of saccharification. The glucose and xylose productivity followed the same performance observed in the previous experiment. Slight increases in glucose and xylose were observed after 24 h of saccharification and the remarkable fall in saccharification yields can be correlated with the low enzymatic activities detected in reaction medium after the first hours of saccharification.

After 120 h of saccharification the final slurry was centrifuged and the supernatant was dialyzed and utilized to determine the residual cellulases and xylanases activities. The objective of this step was to evaluate the enzymatic activities remaining on the supernatant in the absence of sugars, which is known to inhibit cellulases and hemicellulases activities. However, no increase on the activities was observed, excluding the possibility of enzyme product inhibition.

Thermal stability of the main cellulases and xylanases activities produced by fungus *F. verticillioides* was carried out at the same conditions employed in saccharification experiments (pH 5.0, 50 °C) (Figure 6).

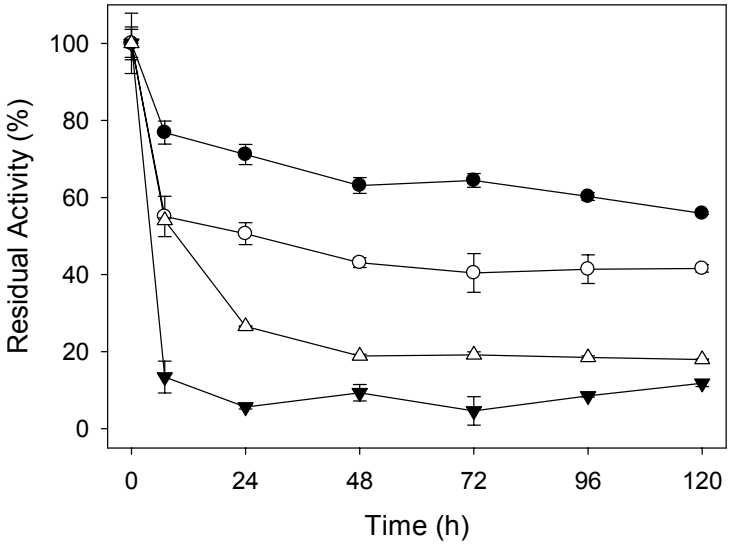

Figure 6: Stability of endoglucanase activity (●), FPase activity (○), cellobiase activity (▼) and xylanase activity (△) produced by fungus *F. verticillioides*. The activity without pre-incubation was considered 100%.

Endoglucanase and FPase activities were very stable retaining 56 and 42 %, respectively, of the initial activity after 120 h of pre-incubation. This high stability excludes thermal or pH inactivation of the enzymes along saccharification process.

In general, cellulases are bimodular proteins with a large catalytic and a small carbohydrate binding domain (CBD), which bind to cellulose and increase the efficiency of the process. However, some non-productive binding to cellulose and lignin can also occur and is not possible to differentiates between these two kinds of binding [26, 27]. The adsorption effect seems to be the reason of the loss of endoglucanase and FPase activities on the supernatant. The activity of tightly adsorbed enzymes is known to decrease gradually in the course of hydrolysis of insoluble cellulose as a result of the enzyme limited mobility along the substrate surface [11]. This fact, together with some non-productivity adsorption of cellulases on the substrate could have contributed to the loss of productivity observed. Surfactants, like tween 20, tween 80, Triton and others, are reported as alternative to decrease enzyme adsorption and to increase enzyme stability [28]. In this study it was used 0.1 % of Tween 20 in the saccharification assay but it was not enough to avoid the observed

adsorption and tests using higher concentration of Tween 20 or others surfactants are required.

The xylanolytic complex from *F. verticillioides* retained 18 % of the initial activity since 48 h until 120 h of pre-incubation. Xylanase are also reported to contain a CBM and to adsorb on substrate [29]. The enzyme inactivation may have contributed, together to enzyme adsorption to the slow xylose releasing after 24 h.

Cellobiase showed to be the less stable enzymatic activity in the conditions tested. In the first 7 hours, the residual activity decreased to 13 % of the initial activity and this level was maintained relatively constant until 120 h. It is not common the presence of CBM on cellobiases and as was observed for saccharification using enzymes from *Trichoderma*, the adsorption of cellobiase usually do not occur [26]. It can be deduced that the low cellobiase activity on the saccharification supernatant is due to the low stability of this enzyme on the conditions used. However, the remaining cellobiase presents on the saccharification was still able to hydrolyze its substrate since no cellobiose accumulation was observed.

5.4.2.3- Concentration and feed of enzyme on saccharification

Different doses were used for sugarcane bagasse saccharification aiming to increase the efficiency of the process. It was also analyzed the effect of fractioned enzymatic feed on the final saccharification yields. In this experiment three enzymatic doses were tested: 10, 20 and 40 mg of protein per gram of dry biomass. Each dose was evaluated at two different manners. At first way the enzymatic dose was added completely at the beginning of saccharification reaction. At second way the adding of enzyme was divided in two parts where 50 % was applied in medium reaction at the beginning and 50 % added after 24 h of saccharification (Figure 7).

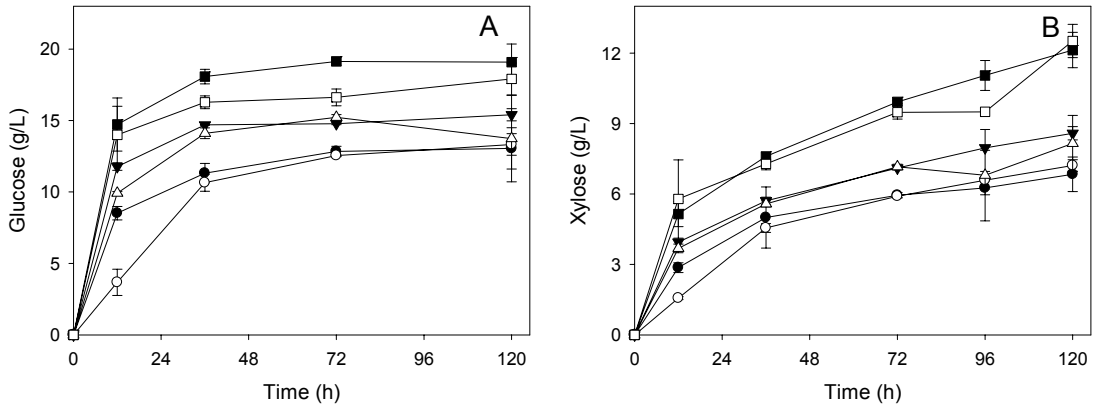

Figure 7: Saccharification using different doses and feed of enzymatic cocktail. Glucose concentration (A) and xylose concentration (B): 10 mg/g (●), 5 and 5 mg/g (○), 20 mg/g (▼), 10 and 10 mg/g (△), 40 mg/g (■), 20 and 20 mg/g (□).

The higher glucose and xylose releasing were observed using 40 mg/g in unique dose. The fractioned enzyme feed produces slightly lower yield on product formation than when the enzymatic dose was entirely applied at the start reaction. Concerning the treatments carried out with no fractioned enzyme loading the glucan conversions were 29.6, 35.0 and 43.4 % for treatments with 10, 20 and 40 mg of protein per gram of biomass, respectively. The xylan conversions were 41.2, 51.7 and 73.1 % for 10, 20 and 40 mg/g, respectively. Some studies reported the stabilization of product formation using 10 FPU/g [25] and 15 mg/g (49.5 FPU/g) [30] for sugarcane bagasse hydrolysis. In the present study, this stabilization was not verified (Figure 8).

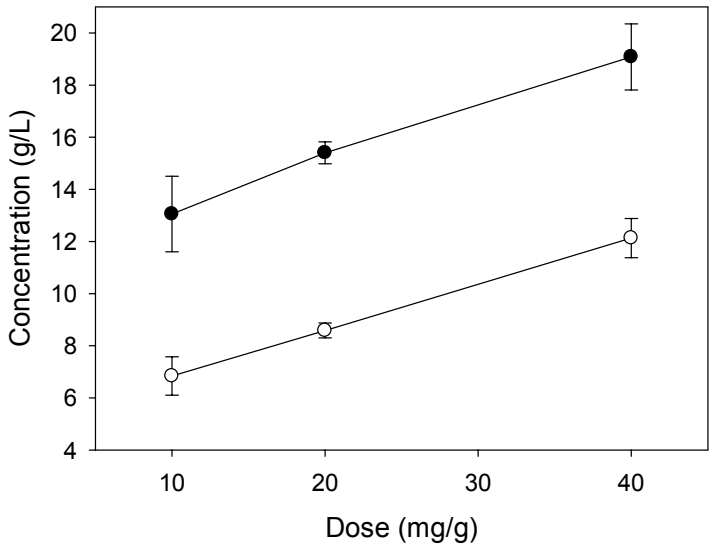

Figure 8: Cocktail dosage effect after 120 h of saccharification. Glucose 10 mg/g (●) and xylose (○).

The minimal enzyme dosage as well as the total biomass conversion is highly dependent not only on cocktail efficiency, but also on others factors. The biomass composition is one of the most important factors to hydrolysis efficiency. High lignin content, cellulose crystallinity and polymerization degree are crucial features that decrease the hydrolysis. Many studies about pretreatments aimed to overcome this bottlenecks [13]. The initial solid loading also interferes on the dosage needed and efficiency of the process, since low solid loading yields better enzymatic hydrolysis. On the other hand, only using high solid loading is possible achieve ideal product concentrations [9]. The pH and temperature are others parameters which need to be optimized for each enzymatic cocktail towards minimizing enzyme dosage [7].

5.5- Conclusion

Fusarium verticillioides produced two different enzymatic cocktails, rich on endoglucanase or rich on cellobiase. Sinergistic studies on the two preparation revealed the best endoglucanase:cellobiase ratio of 12:1. Different protein dosages were tested and the best sugarcane bagasse conversion was achieved using 40 mg of protein/g of biomass. In this assay, conversions of 43.4 % for glucan and 73.1 % for xylan were obtained.

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6- Artigo 3: Direct ethanol production from glucose, xylose and sugarcane bagasse by the corn endophytic fungi *Fusarium verticillioides* and *Acremonium zeae*

6.1- Abstract

The fungus *Fusarium verticillioides* produced ethanol from glucose, xylose and a mixture of these two sugars in limited oxygen conditions with yields of 0.47, 0.46 and 0.50 g/g of ethanol per sugar utilized. The fungus *Acremonium zeae* produced ethanol from glucose, xylose and mixture of these two sugars with yields of 0.37, 0.39 and 0.48 g/g of ethanol per sugar utilized. Both fungi were able to co-ferment glucose and xylose. *Fusarium verticillioides* and *A. zeae* produced high endoglucanase and xylanase activities using sugarcane bagasse as substrate. Ethanol production from 40 g/L of pre-treated sugarcane bagasse was 4.6 and 3.9 g/L for *Fusarium verticillioides* and *A. zeae*, respectively. Both fungi studied were capable of co-fermenting glucose and xylose at high yields. Moreover, they were able to produce ethanol directly from lignocellulosic biomass, indicating to be suitable microorganisms for consolidated bioprocessing.

Keywords: consolidated bioprocessing, ethanol, sugarcane bagasse, *Fusarium*, *Acremonium*

6.2- Introduction

Fuel ethanol production from renewable resources such as plant biomass is one of the most promising alternatives to conventional petroleum-based transportation fuels. Lignocellulose, a polymer constituting of cellulose, hemicelluloses and lignin, is considered an attractive feedstock for the production of fuel ethanol, because of its availability in large quantities at low cost and for reducing competition with food [1, 2].

Lignocellulosic ethanol is not yet produced at a competitive level due to the high cost of the process. Among the main routes to advanced lignocellulosic ethanol, consolidated bioprocessing which integrates enzyme production, saccharification and fermentation into one step, holds tremendous potential to reduce ethanol production costs [2, 3]. An ideal microorganism for a

consolidated bioprocess must be able to both solubilize biomass and produce ethanol at a high yield under industrial conditions. Many studies have proposed engineering a cellulase producer to make it ethanologenic or engineering an ethanologenic microorganism to be cellulolytic [3-7]. Some microbial species such as *Fusarium oxysporum* [8], *Trametes hirsuta* [9] and *Phlebia* sp. [10] have been reported to naturally produce ethanol from cellulose.

Cellulose and hemicelluloses typically comprise up to two thirds of the lignocellulosic material and are the substrates for second generation ethanol production. Cellulose hydrolysis releases glucose, while hemicellulose hydrolysis mainly releases xylose and small amounts of arabinose and galactose [11]. The fermentation of both glucose and xylose is very important to the feasibility of the second generation ethanol production and some of the filamentous fungi presenting this interesting feature are being studied [12-14].

Furthermore, in order to be applied in consolidated bioprocessing, the microorganism must produce and secrete a variety of cellulases and hemicellulases. *Fusarium verticillioides* was studied in relation to its production of cellulases and hemicellulases [15]. *Acremonium zeae* was also evaluated in relation to enzyme production and presented significant enzyme production levels [15, 16]. However, the fermentation ability of this fungus has not yet been reported.

The present study evaluated the performance of *F. verticillioides* and *A. zeae* for fermentation of glucose and xylose, both separately or in a mixture in aerobic or oxygen limited conditions. It was also analyzed enzyme production by this fungi using pre-treated sugarcane bagasse as a substrate, as well as its hydrolysis and fermentation in a consolidated bioprocessing for ethanol production.

6.3- Materials and methods

6.3.1- Microorganism

The endophytic fungi *Fusarium verticillioides* and *Acremonium zeae* were isolated from commercial corn seeds and belong to the mycological collection of the Laboratory of Biochemical Technology at Federal University of Viçosa, MG, Brazil, and was routinely propagated on potato dextrose agar slants at 28 °C for 7 days and maintained at 10 °C for 2-10 days.

6.3.2- Microorganism cultivation

The basal medium for fungi growth was composed of (in g/L) $NaNO_3$, 6; KH_2PO_4, 1.5; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01; and $ZnSO_4$, 0.01. Sporulated slants were used for preparing the spore inoculums with sterile water. For inoculation 2 mL of water containing $1.5 \pm 0.2 \times 10^6$ spores was used in 50 mL of the basal medium with 10 g/L of glucose and 10 g/L of xylose as carbon sources. Fungi were grown at 28 °C under agitation (180 rpm) for 48 hours and this fungal culture was defined as the pre-inoculum.

Five milliliters of the pre-inoculums were aseptically transferred to 125-mL glass Erlenmeyer-flasks with a 50-mL working volume containing the basal medium with different carbon sources. The carbon sources were glucose 20 g/L, xylose 20 g/L or a mixture of glucose and xylose at 10 g/L each. Aerobic growth was performed using cotton-plugged Erlenmeyer flasks. Oxygen limited growth was performed using rubber-plugged Erlenmeyer flasks which were perforated with two needles. One, coupled with a hose, for introduction of gaseous nitrogen and the other as a gas vent. After the addition of pre-inoculum, gaseous nitrogen was bubbled for 10 minutes in each flask for the oxygen limited condition.

6.3.3- Fungal biomass estimation

The dry mass of the fungi grow in each Erlenmeyer flask was determined by filtering the culture media using Whatman n°1 filter paper with a vacuum pump. The filter paper was then dried in an oven at 100 °C for 48 hours and weighed using an analytical balance.

6.3.4- Fermentation product analysis

The products after fungal cultivation were analyzed via HPLC on a Shimadzu series 10A chromatograph. For this purpose, an analytical column (Aminex HPX-87H, 300 x 7.8 mm) was used, eluted with 5 mM sulfuric acid at 60 ºC and a flow rate of 0.6 mL/min.

6.3.5- Biomass pretreatment

Sugarcane bagasse was dried in an oven at 70 °C until the weight was constant. The dry sugarcane bagasse was milled in rotary mill (model MA-580,

Marconi Co. Piracicaba – SP) and passed through a 1.0 mm screen. Sodium hydroxide at a concentration of 1 % was used to pretreat milled sugarcane bagasse samples at a solid loading of 10 % (w/v). Treatments were performed in duplicate in an autoclave at 121 °C for 60 min. The pre-treated materials were separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed twice with distilled water, dryed at 40 °C and stored at -20 °C. The chemical composition of sugarcane bagasse was determined as described previously [17].

6.3.6- Enzyme production

Five milliliters of the pre-inoculums were aseptically transferred to 125-mL glass Erlenmeyer-flasks with a 50-mL working volume containing the basal medium and 20 g/L of pre-treated sugarcane bagasse as carbon source. The fungi were grown aerobically at 28 °C under agitation (180 rpm) for 9 days, and aliquots of 1 mL were taken aseptically every day to verify enzyme production.

6.3.7- Enzyme assay

Endoglucanase activity was determined using 250 µL of carboxymethilcellulose 2 % and 250 µL of the enzyme appropriately diluted in sodium acetate buffer pH 5.0 at 50 °C for 30 minutes. Xylanase activity was determined using 250 µL of birchwood xylan 2 % and 250 µL of the enzyme appropriately diluted in sodium acetate buffer pH 5.0 for 15 minutes. The release of reducing sugars for these assays was determined using the 3,5-dinitrosalicylic acid (DNS) reagent [18] and was calculated according to the standard curve (0.11 – 1.11 µmol of glucose for endoglucanase and 0.11 – 1.11 µmol of xylose for xylanase) [19].

6.3.8- Biomass fermentation

The fungus was cultivated under aerobic conditions for enzyme production as described above for 96 h at 28 °C, which is referred to as the growth phase. Thereafter, the production phase cultivation was carried out. The cotton stopper was replaced by a rubber stopper with two perforations for introduction of nitrogen and venting of gases. Sterilized pre-treated sugarcane bagasse was added to a final concentration of 40 g/L, considering the amount of biomass

previously added for enzyme production and gaseous nitrogen was bubbled into the flasks to provide the oxygen limited condition. The flasks were incubated at 28 °C and 180 rpm.

6.4- Results and discussion

6.4.1- Growth under aerobic conditions

The concentrations of glucose, xylose, ethanol and dry cell mass during the time course of *F. verticillioides* and *A. zeae* cultivation using glucose as carbon source are shown on Figure 1.

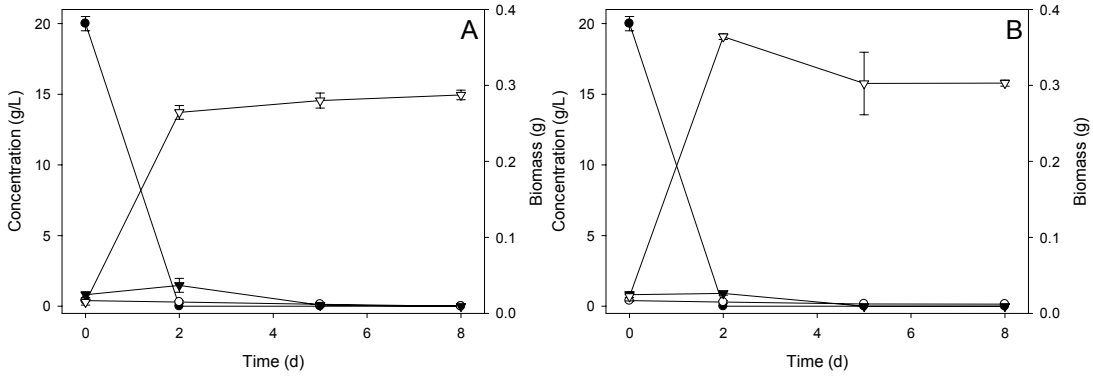

Figure 1: Aerobic growth of *F. verticillioides* (A) and *A. zeae* (B) using glucose as carbon source. Glucose (●); Xylose (○); Ethanol (▼); Biomass (▽).

After 2 days of cultivation, glucose was completely consumed by both fungi; and small concentrations of ethanol, 1.4 g/L for *F. verticillioides* and 0.9 g/L for *A. zeae* were detected. However, this ethanol was consumed upon exhaustion of glucose. The biomass yield on sugars was 0.29 g/g (g biomass/g of sugar consumed) for *F. verticillioides* and 0.36 for *A. zeae*. Biomass yields of 0.5 g/g are normally found for aerobic growth of filamentous fungi, however, similar yields was observed for *Fusarium oxysporum*. These low yields are normally associated to extensive by-product formation [14]. To calculate biomass yield the higher biomass obtained was considered.

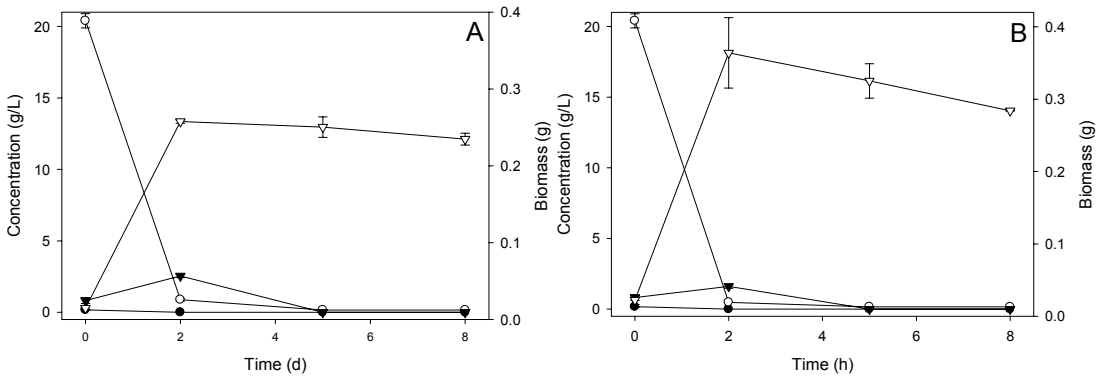

Figure 2: Aerobically growth of *F. verticillioides* (A) and *A. zeae* (B) using xylose as carbon source. Glucose (●); Xylose (○); Ethanol (▼); Biomass (▽).

When xylose was used as carbon source the consumption profiles were similar to those obtained with glucose. However, after 2 days of cultivation xylose was still detected, 0.88 g/L for *F. verticillioides* and 0.48 g/L for *A. zeae*, indicating that these fungi consume xylose at a slightly slower rate. Small amounts of ethanol were also detected after 2 days of cultivation, 2.5 g/L for *F. verticillioides* and 1.6 g/L for *A. zeae*, after which this ethanol was consumed. The biomass yields were 0.26 and 0.36 g/g for *F. verticillioides* and *A. zeae*, respectively.

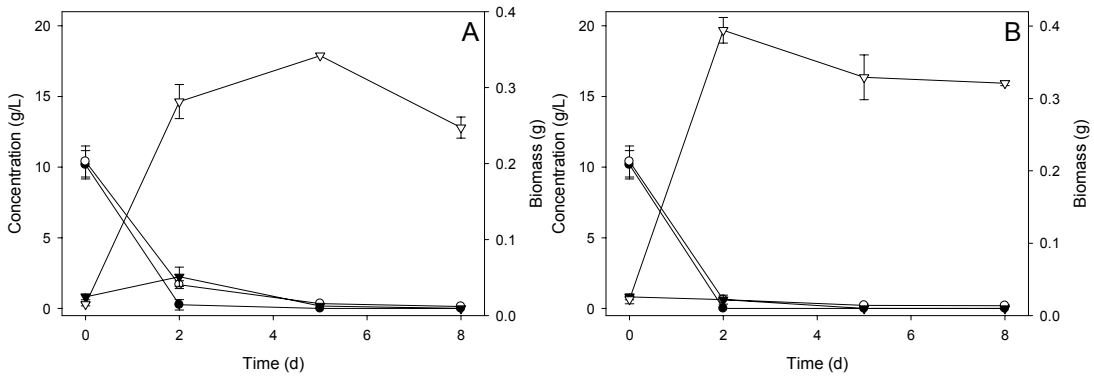

Figure 3: Aerobically growth of *F. verticillioides* (A) and *A. zeae* (B) using glucose and xylose as carbon sources. Glucose (●); Xylose (○); Ethanol (▼); Biomass (▽).

In the presence of glucose and xylose, both sugars were consumed until exhausted by the fungi. *Fusarium verticillioides* consumed xylose slightly slower than glucose. In a similar experiment *Fusarium oxysporum* also hydrolyzed glucose first which was totally consumed after 24 h of fermentation, and after 48 h of fermentation about 4 g/L of xylose was still detected [14]. The biomasses yields were 0.34 and 0.39 g/g for *F. verticillioides* and *A. zeae*, respectively, higher than those obtained using glucose or xylose as the only carbon source. Biomass concentrations of both fungi at all cultivation conditions slightly

decreased as the carbon sources were exhausted. This effect is possibly due to autolysis of the cells under starvation conditions.

6.4.2- Growth in the oxygen limited condition

Under oxygen limited conditions the glucose consumption was notably lower than under aerobic conditions. Glucose was completely consumed after 8 days of cultivation of the two fungi studied. The maximum ethanol productions, 9.44 g/L for *F. verticillioides* and 7.47 for *A. zeae*, were observed at day 8 (Figure 4). *A. zeae* presented a lower ethanol concentration and a higher biomass concentration than *F. verticillioides*, indicating that this fungus used part of the glucose for biomass instead of ethanol production.

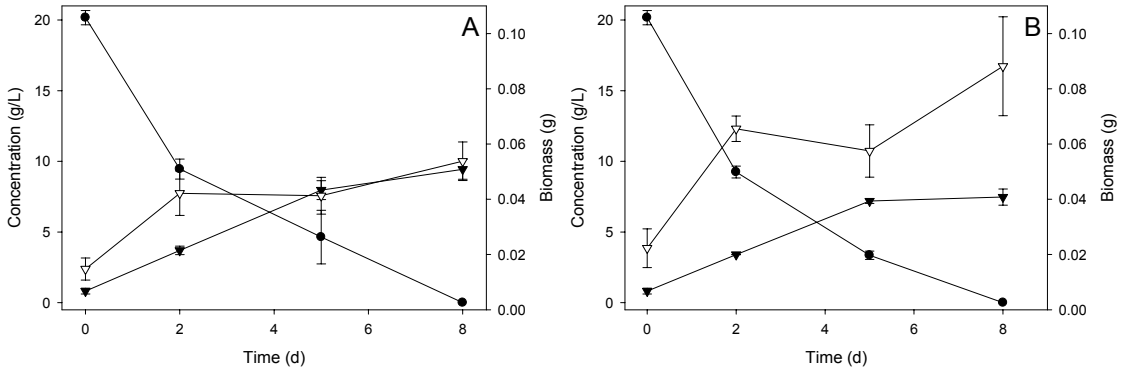

Figure 4: Oxygen limited growth of *F. verticillioides* (A) and *A. zeae* (B) using glucose as a carbon source. Glucose (●); Ethanol (▼); Biomass (▽).

The ethanol yields (ethanol/ glucose) were 0.47 g/g for *F. verticillioides* and 0.37 g/g for *A. zeae*. These values are, respectively, 83 % and 66 % of the maximal theoretical yield. A yield of 80 % was observed for anaerobic glucose fermentation by *Fusarium oxysporum*. However, under oxygen limited condition this yield was 1.5 fold lower [20].

The ethanol yields obtained by *F. verticillioides* and *A. zeae* were compared to those obtained by other natural or genetically modified fermenting microorganisms (Table 1). The yield of ethanol per gram of glucose obtained by *F. verticillioides* was similar to or even higher than yields obtained for yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, commonly used in fermentative process, however, *F. verticillioides* required more time to reach this yield.

Table 1: Glucose fermentation performance for diverse microorganisms

Microorganism	S (g/L)	FC	Y_{ME} (g/g)	Y_E (g/g)	B (g/g)	Reference
Fungi						
F. verticillioides	20	OL	0.47	0.47	0.05	This study
F. verticillioides	20	A	0.07	0.07	0.28	This study
A. zeae	20	OL	0.37	0.37	0.09	This study
A. zeae	20	A	0.05	0.05	0.36	This study
Neolentinus lepideus	20	OL	0.38	0.38	-	[13]
Trametes hirsute	20	AN	0.49	0.49	-	[9]
Fomitopsis palustris	20	AN	-	0.46	-	[21]
Phlebia sp.	20	OL	-	0.44	-	[10]
Fusarium oxysporum	40	AN	0.44*	0.44*	-	[20]
Fusarium oxysporum	40	OL	0.29*	0.29*	-	[20]
Mucor indicus	15	AN	0.46	0.46	0.14	[22]
Mucor indicus	15	A	0.41	-	0.30	[22]
Rhizopus oryzae	50	A	0.43	-	0.13	[12]
Mucor hiemalis	50	A	0.39	-	0.09	[12]
Rhizopus microsporrus	50	A	0.39	-	0.15	[23]
Rhizomucor	50	OL	0.47	0.46	-	[23]
Rhizopus sp.	50	A	0.26	-	0.15	[23]
Yeasts						
Pichia stipitis	15	AN	0.46	-	0.04	[22]
*Saccharomyces cerevisiae***	50	AN	0.38	-	-	[24]
*Saccharomyces cerevisiae***	49	A	0.49	0.49	-	[25]
Kluyveromyces marxianus	20	AN	0.35	-	-	[26]
Spathaspora Passalidarum	100	OL	0.31	0.31	0.22	[27]

S: Substrate concentration; FC: fermentation condition; Y_{ME}: yield of metabolized ethanol (consumed ethanol); Y_E: ethanol yield considering sugar available; B: biomass yield per gram of substrate available; A: aerobic; AN: anaerobic; OL: oxygen limited; *Adapted results; **Genetically modified microorganism to ferment glucose and xylose

Hexoses sugars are generally considered to be readily fermented to ethanol, whereas pentoses sugars are not fermented by many alcohol-fermenting fungi, including baker's yeast, filamentous fungi and basidiomycete fungi [9]. Because xylose is the main pentose produced from hydrolysis of plant hemicelluloses, organisms capable of fermenting xylose to ethanol at good yields are required for biomass conversion to ethanol. Most yeasts commonly used for fermentation do not naturally ferment xylose, therefore many strains have been genetically modified or adapted to ferment xylose to ethanol [28, 29].

When xylose was used as a carbon source both *F. verticillioides* and *A. zeae* were able to produce ethanol (Figure 5). *Fusarium verticillioides* produced

5.68 g/L, a yield of 0.28 g/g (0.46 g/g of consumed xylose), and *A. zeae* produced 4.09 g/L, a yield of 0.20 g/g (0.39 g/g of consumed xylose).

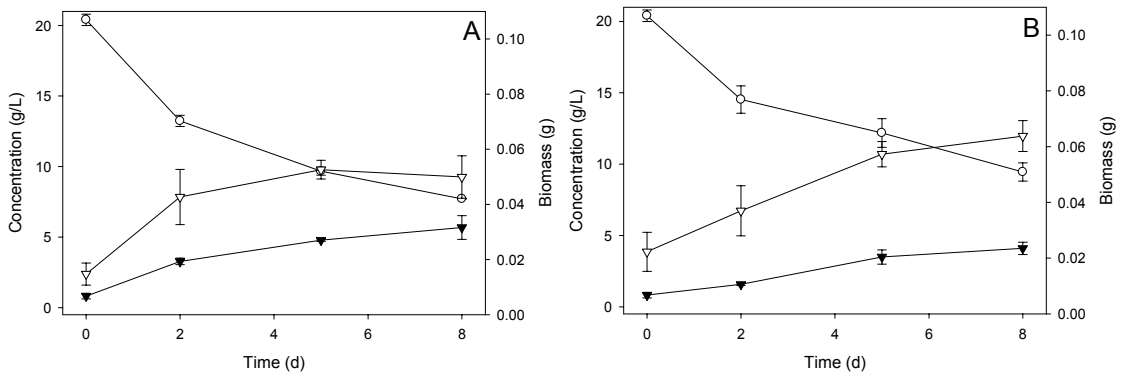

Figure 5: Oxygen limited growth of *F. verticillioides* (A) and *A. zeae* (B) using xylose as a carbon source. Xylose (○); Ethanol (▼); Biomass (▽).

Different from that observed for glucose fermentation, xylose was not completely consumed. *Fusarium verticillioides* consumed 12.3 g/L of xylose while *A. zeae* presented slightly lower xylose uptake, consuming 10.6 g/L. Xylose uptake has been indicated as a bottleneck of xylose fermentation. However, in recombinant yeasts displaying the fungal or bacterial xylose metabolism system, it seems that xylose transport is not the limiting factor but instead another unknown factor [30].

The ethanol yields obtained by *F. verticillioides* and *A. zeae* are compared with other fungi and yeasts in Table 2.

Table 2: Xylose fermentation performance for diverse microorganism

Microorganism	S (g/L)	FC	Y_{ME} (g/g)	Y_E (g/g)	B (g/g)	Reference
Fungi						
F. verticillioides	20	OL	0.46	0.28	0.053	This study
F. verticillioides	20	A	0.13	0.13	0.257	This study
A. zeae	20	OL	0.39	0.20	0.064	This study
A. zeae	20	A	0.08	0.08	0.364	This study
Neolentinus lepideus	20	OL	0.34	0.34	-	[13]
Trametes hirsute	20	AN	0.44	0.19*	-	[9]
Phlebia sp.	20	OL	-	0.33	-	[10]
Fusarium oxysporum	40	AN	0.25	0.22	-	[31]
Mucor indicus	50	A	-	0.19	0.23	[22]
Rhizopus oryzae	50	A	0.28	-	0.17	[12]
Mucor hiemalis	50	A	0.18	-	0.11	[12]
Rhizomucor	50	A	0.22	0.13	0.15	[23]
Yeasts						
*Saccharomyces cerevisiae***	50	AN	0.40	0.32*	-	[24]
Pichia stipitis	15	A	-	0.36	0.16	[22]
Spathaspora Passalidarum	100	OL	0.41	0.41	0.12	[27]
*Saccharomyces cerevisiae***	50	OL	0.38	-	-	[32]
*Saccharomyces cerevisiae***	40	AN	0.21	-	-	[33]

S: Substrate concentration; FC: fermentation condition; Y_{ME}: yield of metabolized ethanol (consumed ethanol); Y_E: ethanol yield considering sugar available; B: biomass yield per gram of substrate available; A: aerobic; AN: anaerobic; OL: oxygen limited; *Adapted results; **Genetically modified microorganism to ferment glucose and xylose

In the *F. verticillioides* culture the by-products detected from xylose fermentation were acetic acid (0.36 g/L) and xylitol (0.23 g/L). In the *A. zeae* culture 0.17 g/L of acetic acid and 0.22 g/L of xylitol were produced. The formation of these by-products, which were not produced in detectable amounts during glucose fermentation, contributed to the lower ethanol yield. The formation of acetic acid and xylitol as by-product was previously reported for many microorganisms. However, the concentrations of these by-products from fermentation by *F. verticillioides* and *A. zeae* were very low comparing to others studies [8, 10, 20, 23, 28, 31].

When the mixture of glucose and xylose was used as the carbon source, *F. verticillioides* produced 5.6 g/L ethanol and *A. zeae* produced 5.9 g/L ethanol. Considering both the total sugar available and the consumed sugar, yields were 0.28 and 0.5 g/L for *F. verticillioides* and 0.29 and 0.48 g/L for *A. zeae*.

Fusarium verticillioides started to consume xylose after glucose exhaustion. At day 8, 12 % of the xylose was consumed. Contrarily, *Acremonium zeae* was able to co-metabolize glucose and xylose, however,

glucose was preferentially consumed. After 8 days of cultivation, 22 % of the xylose was consumed (Figure 6). Xylose consumption after glucose depletion has been reported for many yeasts and fungi [14, 25, 32].

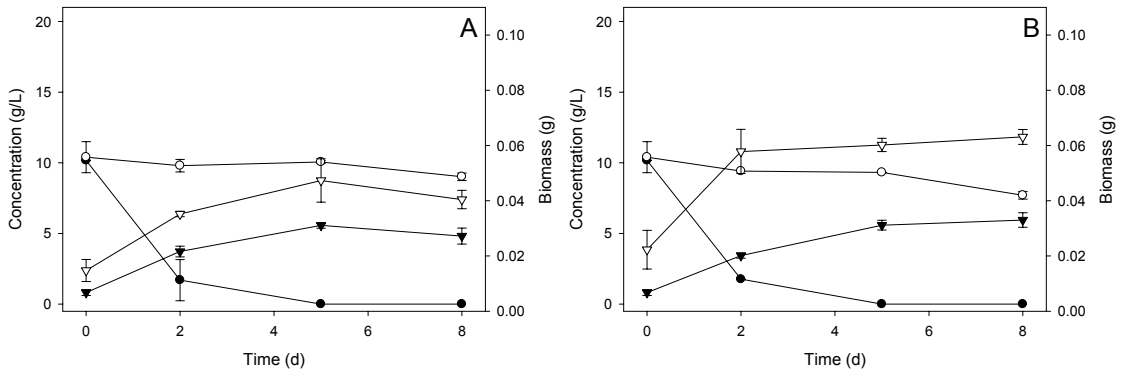

Figure 6: Oxygen limited growth of *F. verticillioides* (A) and *A. zeae* (B) using a mixture of glucose and xylose as the carbon source. Glucose (●); Xylose (○); Ethanol (▼); Biomass (▽).

The inhibition of xylose utilization by glucose in *Saccharomyces cerevisiae* has been associated with competition of glucose and xylose for the same transporters, which have higher affinity to glucose. However, increase in xylose uptake in the presence of low concentrations of glucose has also been related [26, 34]. The inhibition of putative xylose transporter gene expression by glucose was reported in a transcriptome analysis of *Aspergillus niger* [35]. Another possible explanation is the evidence verified in metabolite analysis of *Fusarium oxysporum* fermentation which shows that in the presence of glucose and xylose, in oxygen limited conditions, the pentose phosphate pathway was blocked which induced the formation of acetate and reduced xylose consumption [14]. These are some possible explanation, but glucose/xylose fermentation by fungi is not well known.

Despite the low consumption of xylose, both fungi were able to co-ferment glucose and xylose to ethanol. Considering the theoretical ethanol yield, which is 0.51 g/g, it is noted that the xylose consumed was used for ethanol production by the two fungi, since the ethanol concentrations observed could not be achieved if using only 10 g/L of glucose as presented in the culture medium.

The ethanol yields obtained by *F. verticillioides* and *A. zeae* were compared with the yields reported for other microorganisms (Table 3). It can be noted that both fungi presented high ethanol yields compared with other fungi and also with yeasts. The ability to co-ferment glucose and xylose is

indispensable for the economic feasibility of second generation ethanol, since these are the two main sugars presented in most lignocellulosic biomass. Therefore, both fungi studied present potential for application in this process.

Table 3: Fermentation performance for diverse microorganism using a mixture of glucose and xylose

Microorganism	S (g/L)	FC	Y_{ME} (g/g)	Y_E (g/g)	B (g/g)	Reference
Fungi						
F. verticillioides	20	OL	0.50	0.28	0.05	This study
F. verticillioides	20	A	0.11	0.11	0.34	This study
A. zeae	20	OL	0.48	0.29	0.06	This study
A. zeae	20	A	0.03	0.03	0.39	This study
Fusarium oxysporum	40	OL	-	0.22*	-	[14]
Fusarium oxysporum	40	AN	-	0.16*	-	[14]
Rhizomucor	15	OL	0.38	0.31	-	[23]
Yeasts						
*Zymomonas mobilis***	50	A	0.45	0.44	-	[36]
Spathaspora Passalidarum	100	OL	0.42	-	-	[27]
*Saccharomyces cerevisiae***	100	AN	0.40	-	-	[37]
*Saccharomyces cerevisiae***	100	A	0.44	0.39*	-	[24]
*Saccharomyces cerevisiae***	40	AN	0.38	-	-	[33]

S: Substrate concentration; FC: fermentation condition; Y_{ME}: yield of metabolized ethanol (consumed ethanol); Y_E: ethanol yield considering sugar available; B: biomass yield per gram of substrate available; A: aerobic; AN: anaerobic; OL: oxygen limited; *Adapted results; **Genetically modified microorganism to ferment glucose and xylose

When compared to *A. zeae*, *F. verticillioides* presented higher efficiency for glucose or xylose fermentation when utilized as individual carbon sources. However, *A. zeae* presented an equivalent fermentation performance when the mixture of both sugars was used, but the consumed sugars were not totally converted to ethanol. The *A. zeae* culture produced 0.20 g/L of xylitol as a by-product while *Fusarium verticillioides* produced 0.09 g/L of xylitol. Formation of by-products, together with the higher biomass production by *A. zeae*, explains the higher ethanol yield per consumed sugar observed for *F. verticillioides*.

6.4.3- Enzyme production

Fusarium verticillioides and *A. zeae* were cultivated aerobically on basal media using pre-treated sugarcane bagasse as the carbon source. This was referred to as the growth phase and results in enzyme production by the fungi. In the *F. verticillioides* culture the maximal endoglucanase activity (3.4 U/mL)

was observed after 96 hours of cultivation, remaining stable for up to 216 hours. The highest xylanase activity, 48 U/mL, was observed after 48 hours of cultivation and dropped to 32 U/mL after 96 hours of cultivation.

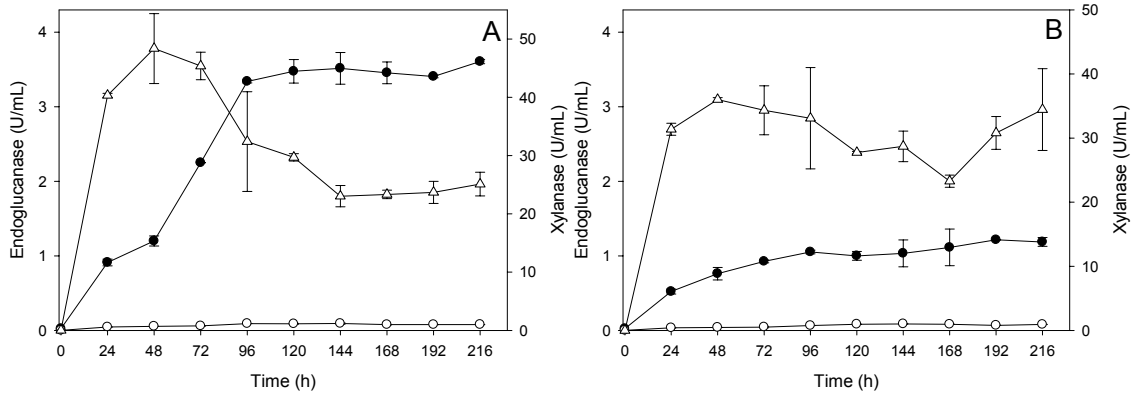

Figure 6: Enzyme production by the fungus *Fusarium verticillioides* (A) and *Acremonium zeae* (B) using pre-treated sugarcane bagasse as the carbon source. Endoglucanase (●), xylanase (△).

The fungus *Acremonium zeae* was able to produce significant endoglucanase and xylanase activities, however, lower than those obtained by *F. verticillioides*. Endoglucanase activity reached 1.0 U/mL after 96 hours and increased slightly until 216 hours, reaching 1.2 U/mL. The maximal xylanase activity, 36 U/mL, was observed after 48 hours of cultivation and decreased slightly until 216 hours.

The optimal growth phase was defined as 96 hours for *F. verticillioides* and *A. zeae* since at this time high endoglucanase and xylanase activities were observed for both fungi.

6.4.4- Fermentation of pre-treated sugarcane bagasse

After the growth phase the flasks were maintained in limited oxygen conditions, referred to as the production phase, when sugarcane bagasse hydrolysis and sugar fermentation were performed simultaneously. Chemical composition of the raw sugarcane bagasse sample used in the present study was as follows: glucan 45.4 %, xylan 23.7 %, galactans 0.2 %, arabinans 2.0 %, and lignin 22.0 %. After pretreatment the chemical composition was: glucan 51.8 %, xylan 25.9 %, arabinan 2.3 % and lignin 7.7 %.

Fusarium verticillioides fermentation produced a maximum ethanol concentration of 4.6 g/L, while *A. zeae* produced 3.9 g/L after 144 hours (Figure 7). The sugars released from sugarcane bagasse were probably assimilated

immediately; therefore they were not significantly detected in the culture after 2 days.

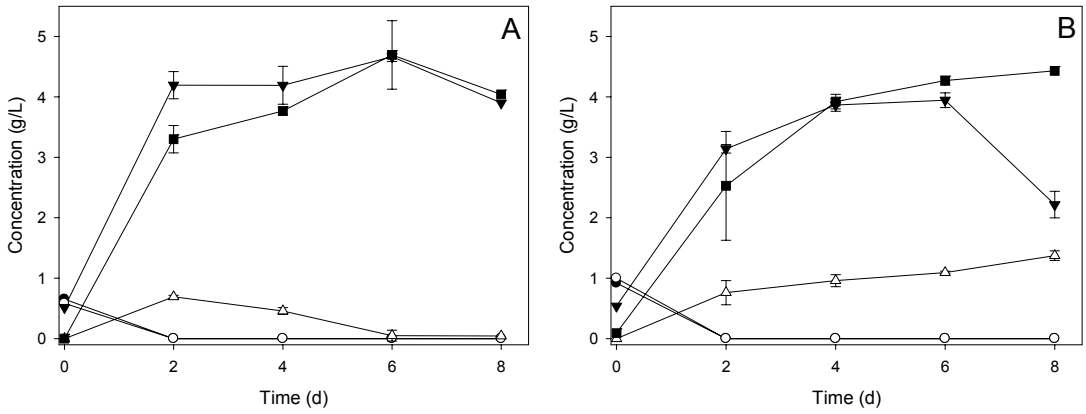

Figure 7: Pre-treated sugarcane bagasse fermentation by *Fusarium verticillioides* (A) and *Acremonium zeae* (B). Glucose (●), xylose (○), ethanol (▼), xylitol (△) and acid acetic (■).

Considering the glucan and xylan fraction of the sugarcane bagasse, the yields of ethanol per gram of biomass were 0.15 g/g and 0.13 g/g for *F. verticillioides* and *A. zeae*, respectively. The fungus *F. verticillioides* consumed 41.5 % of glucan and 59.2 % of xylan presented on the pretreated sugarcane bagasse. The fungus *A. zeae* consumed 32.2 % of glucan and 55.1 % of xylan presented on the pretreated sugarcane bagasse. The ethanol yield considering the biomass (glucan and xylan) consumed were 0.31 g/g for both fungi. The ethanol yield from biomass fermentation is related to the biomass composition. Some ethanol yields from direct biomass hydrolysis and fermentation are compared in Table 4.

Table 4: Fermentation performance of diverse microorganisms using lignocellulosic biomasses

Microorganism	Biomass	S (g/L)	Main sugars	Y_E (g/g)	Reference
F. verticillioides	PSB	40	Glucose, xylose	0.15	This study
A. zeae	PSB	40	Glucose, xylose	0.13	This study
Fusarium oxysporum	Corn cobs	40	Glucose, xylose	0.05	[38]
Fusarium oxysporum	Wheat bran	40	Glucose, xylose, arabinose	0.14	[38]
*Fusarium oxysporum***	Corn cobs	40	Glucose, xylose	0.07	[38]
*Fusarium oxysporum***	Wheat bran	40	Glucose, xylose, arabinose	0.22	[38]
Trametes hirsute	Wheat bran	20	Glucose, xylose, arabinose	0.21	[9]
Trametes hirsute	Rice straw	20	Glucose, xylose, galactose, mannose	0.15	[9]
*C. cellulolyticum***	Switchgrass	10	Glucose; xylose	0.13	[39]
Fusarium oxysporum	Cellulose	20	Glucose	0.35	[8]
Fusarium oxysporum	Cellulose	20	Glucose	0.17	[8]
Phlebia sp.	Kraft pulp	20	Glucose	0.42	[10]
Phlebia sp.	Newspaper	20	Glucose	0.20	[10]
Clostridium phytofermentans	Filter paper	10	Glucose	0.35*	[40]
*Kluyveromyces marxianus***	β-glucan	10	Glucose	0.42	[7]
*Saccharomyces cerevisiae***	CMC	10	Glucose	0.34	[5]
*Saccharomyces cerevisiae***	β-glucan	20	Glucose	0.46	[6]
*C. cellulolyticum***	Avicel	10	Glucose	0.27	[39]

S: Substrate concentration; Y_E: ethanol yield considering sugar available; PSB: pre-treated sugarcane bagasse; CMC: Carboxymethylcellulose; *Adapted results; **Genetically modified microorganism

It can be noted that the process which used substrates releasing only glucose presented higher ethanol yields than those which used complex substrates releasing xylose, galactose and mannose. This result can be attributed to the difficulty of substrate hydrolysis due to its complexity, which requires a wide range of enzymes and also to the limited fermentation capacity of the microorganisms for these sugars. With regards to the biomass used in this study, its complexity and also the alkaline pre-treatment may have generated some phenol compounds which are normally associated with inhibition of microorganism growth [41].

Another fact that must be considered in all processes that involve simultaneous saccharification and fermentation is the difference in optimal temperature of each process. Enzymes typically present higher activity at temperatures exceeding 40 °C and this was previously reported for *F. verticillioides* and *A. zeae* enzymes [15]. The fermentation process, on the other hand, is normally performed between 28 – 35 °C for mesophilic microorganisms. Since no glucose or xylose was detected, it appears that the bottleneck of the process was biomass hydrolysis. Further studies are required to determine the best temperature and other conditions in order to optimize hydrolysis and fermentation by *F. verticillioides* and *A. zeae*.

6.5- Conclusion

This is the first report on the fermentation performance of the fungi *F. verticillioides* and *A. zeae*. Both fungi presented high ethanol yields for glucose and xylose fermentation, and were able to co-ferment these two sugars. Furthermore, the fungi were able to produce ethanol directly from lignocellulosic biomass, indicating that they are promising microorganisms for application in consolidated bioprocessing.

6.6-References

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7- Artigo 4: Purification, properties and mode of action of a multienzyme complex and a free endoglucanase from *Fusarium verticillioides*

7.1- Abstract

A novel multienzyme complex, $E1_C$, and a free endoglucanase, E2 were purified from culture supernatants of the endophytic fungi *Fusarium verticillioides*. The $E1_C$ contained two endoglucanases (GH6 and GH10), one cellobiohydrolase (GH7) and one xylanase (GH10). The temperature of maximal activity was 80 °C for both $E1_C$ and E2. The free enzyme and the complex were very thermostable at 50 and 60 °C. $E1_C$ presented a half-life of 277 h at 50 °C and 192.8 h at 60 °C, and E2 presented half-life of 16 h at 50 °C and 11.6 at 60°C. The activation energies for $E1_C$ and E2 were 21.3 and 27.5 KJ/mol, respectively. Maximum activity of $E1_C$ was encountered at pH 4.5 and of E2 was at pH 5.5; $E1_C$ presented high stability after 24 h of pre-incubation at pH ranging from 2.6 to 8.0. The K_M value for $E1_C$ was 10.25 g/L while for E2 was 6.58 g/L. Both $E1_C$ and E2 were significantly activated by Mn^{2+}, $CoCl_2$, furfural, hydroxymethylfurfural and dithiothreitol while they were inhibited by SDS, $CuSO_4$, $FeCl_3$, $AgNO_4$, $ZnSO_4$ and $HgCl_2$. Both $E1_C$ and E2 presented higher activity towards barley-β-glucan than carboxymethylcellulose (CMC), indicating endo-β-1,3-1,4-glucanase activity. The enzymes were able to hydrolyze cellopentaose, cellotetraose and cellotriose, however, in different mode of action. The enzyme complex $E1_C$ showed preference for large chain substrates while no preference was observed for E2. Enzyme E2 presented an exo-type hydrolysis and $E1_C$ an endo-type hydrolysis when cellotetraose was the substrate. In all the assays evaluating stability and efficiency $E1_C$ showed better performance than E2, suggesting advantages generated by the physical interaction between proteins.

Key words: multienzyme complex, endoglucanase, cellobiohydrolase, xylanase, purification, *Fusarium verticillioides*.

7.2- Introduction

Cellulases are the enzymes involved in the hydrolysis of cellulose, the major polysaccharide of the plant cell wall. For complete cellulose degradation the synergistic action of the four cellulases is necessary: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.176), exoglucohydrolases (EC 3.2.1.74) and β-glucosidases (EC 3.2.1.21). Endoglucanases mainly catalyze the hydrolysis of internal β-1,4-glycoside bonds of cellulosic chains producing free chain ends for cellobiohydrolases or exoglucohydrolases, which release cellobiose and glucose, respectively. β-Glucosidases, the latest enzyme to act, hydrolyze cellobiose into glucose [1].

Cellulases have attracted considerable research interest because of their potential application in the biofuel industry. Enzymatic hydrolysis of lignocellulosic biomass to produce second generation ethanol is an important step and remains a bottleneck in the process due to the high cost and low efficiency of enzymes [2, 3]. For complete lignocelluloses hydrolysis the synergistic action of cellulases and hemicellulases is required.

It is known that cellulases from *Clostridium* sp. and some other bacteria exist as a multienzyme complex with cellulolytic and hemicellulolytic activities, referred to as cellulosomes. Fungi typically depolymerize lignocellulose biomass due to synergism between free enzymes. However, fungal multienzyme complexes have been reported for some fungi including *Trichoderma harzianum* [4], *Chaetomium* sp [5] and *Penicillium purpurogenum* [6].

Based on their sequence and three-dimensional (3D) structure, endoglucanases are grouped, along with other enzymes, into 11 glycoside hydrolase (GH) families, including GH 5, 6, 7, 8, 9, 10, 12, 44, 45, 48, 51, 74 and 124 (http://www.cazy.org). In general, cellulases are bimodular proteins with a large catalytic and a small carbohydrate binding domain (CBD) linked by a short highly glycosylated protein sequence rich in serine and proline. Progress on the mode of action, active site architecture and 3D structure of bacterial and fungal cellulases has permitted that their role in cellulose hydrolysis be predicted and their application in a wide range of processes [7].

Fusarium verticillioides (*Gibberella moniliformis*) is primarily a pathogen of maize and was isolated from commercial corn seeds. This fungus is an important endophytic pathogen of small grain cereals, including corn and wheat,

grasses and roots [8]. In the present study we purified and studied the properties and mode of action of one free endoglucanase and one multienzyme complex from *F. verticillioides*. This is the first report on endoglucanase from *F. verticillioides* and also the first study reporting a multienzyme complex in a *Fusarium* specie. Studies on the cellulase system of *F. verticillioides* may provide useful information to improve its efficiency for cellulose hydrolysis in biotechnological applications.

7.3- Materials and methods

7.3.1- Microorganism, inoculum preparation and enzyme production

The fungus *Fusarium verticillioides* was isolated from corn seeds and belongs to mycological collection of the Laboratory of Biochemical Technology at the Federal University of Viçosa, MG, Brazil, and was routinely propagated on potato dextrose agar slants at 28 °C for 5 days, after which it was maintained at 8-10 °C.

The slants were used for preparing the spore inoculums with sterile water. In every 50 mL of culture medium, 2 mL of water containing 6.7 x 10^5 spores/mL was inoculated. The medium contained (in g/L): $NaNO_3$, 6; KH_2PO_4, 1.5; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; and corn straw, 10.0 [9]. The microorganism was grown at 28 °C with agitation (180 rpm) for 5 days. Samples were centrifuged for 15 min at 10000 x *g* and the supernatants were used as the crude enzyme.

7.3.2- Enzyme assay

The endoglucanase standard assay consisted of a reaction system containing 150 µL of 100 mM sodium acetate buffer pH 5.0, 100 µL of an enzyme solution and 250 µL of sodium carboxymethyl cellulose (CMC) 2 % (w/v). CMC used in the standard reaction had an average molecular mass of 90000 g/mol and 0.7 carboxymethyl groups per anhydroglucose unit. The reaction was carried out for 30 minutes at 50 ºC or the temperature indicated in each assay. Release of reducing sugars was determined using the 3,5-dinitrosalicylic acid reagent [10] and was calculated according to the standard curve (0.11 – 1.11 µmol of glucose). One enzyme unit was defined as the

amount of enzyme necessary to produce 1 μmol of glucose equivalent per minute.

7.3.3- Protein determination

Protein concentration in the enzymatic extracts was determined by the Coomassie Blue binding method using bovine serum albumin as the standard [11].

7.3.4- Endoglucanase purification

The enzymatic extract was concentrated by Amicon ultrafiltration with a 10 kDa molecular cut-off membrane. This concentrated sample was subjected to gel filtration chromatography in a Sephacryl S-300 column (450 mL) equilibrated with 10 mM sodium acetate buffer, pH 5.0. The proteins were eluted at a flow rate of 30 $mL.h^{-1}$ at 10 °C. Two peaks containing endoglucanase activity were observed and denominated $E1_C$ and E2.

$E1_C$ was pooled and passed through a HiTrap Phenyl-Sepharose column (5 mL) equilibrated with 1 M ammonium sulfate in 20 mM sodium acetate buffer, pH 5.0. Proteins were eluted at a flow rate of 240 mL/h^{-1} for 30 minutes with a linear ammonium sulfate gradient (1–0 M) in 20 mM sodium acetate buffer, pH 5.0. Protein fraction with endoglucanase activity was pooled, dialyzed and submitted to a HiTrap CM-Sepharose column (5 mL) equilibrated with 10 mM sodium acetate buffer, pH 5.0. Proteins were eluted at a flow rate of 240 mL/h^{-1} for 30 minutes with a linear NaCl gradient (0–1 M) in 10 mM sodium acetate buffer, pH 5.0.

E2 was pooled and passed through a HiTrap Q-Sepharose column (5 mL) equilibrated with 10 mM sodium acetate buffer, pH 5.0. Proteins were eluted at a flow rate of 120 mL/h^{-1} for 30 minutes with a linear NaCl gradient (0–0.2 M) in 10 mM sodium acetate buffer, pH 5.0. All chromatographic materials were acquired from GE Healthcare. Other than gel filtration, all chromatographic separations were performed at room temperature (about 23 °C).

7.3.5- Molecular mass determination and zymogram analysis

Enzyme molecular mass was estimated by SDS-PAGE using a 12.5 % polyacrylamide gel [12] and the molecular mass standards from Sigma and Fermentas. The obtained gel was then silver-stained [13].

For zymogram analyze enzyme samples were applied to a 12 % SDS-PAGE gel containing 0.2 % (w/v) CMC polymerized within the gel matrix. After electrophoresis the gel was divided into two parts. One part, containing the sample and molecular marker (Fermentas, USA), was silver stained. The other part of the gel containing only the enzymatic sample was washed twice for 20 min in 20 % isopropanol dissolved in 100 mM sodium acetate buffer, pH 5.0, followed by two washes (20 min each) in the same buffer. The gel was incubated at 50 °C in sodium acetate buffer for 60 min, stained with congo red 0.1 % (w/v) for 15 min, and destained by 2 washes (15 min each) with 1 M NaCl. Endoglucanase activity was visible as a clear band, and in order to increase the contrast with the background, the gel was washed with acetic acid 5 % (w/v).

7.3.6- Protein digestion and identification by mass spectrometry (MS)

Protein Digestion: Protein spots were removed manually from the gels, reduced by DTT and alkylated by iodoacetamide followed by digestion with trypsin in a buffer 50 mM ammonium bicarbonate, pH 7.8, containing 20.0 ng/µL of sequencing grade trypsin (Promega). Digestion was performed overnight at 37 °C and the supernatant was recovered to a new 0.5-mL plastic microcentrifuge tube. Peptides were extracted from the spots with 30 µL of 50 mM ammonium bicarbonate, followed by incubation for 10 min with occasional vortex mixing. After that, the supernatant was collected and transferred to a 0.5 mL plastic microcentrifuge tube. This extraction was performed two more times by adding 30 µL of the extraction buffer (50 % (v/v) acetonitrile and 1% (v/v) TFA) to the tube containing the gel pieces, and incubating the sample for 10 min with occasional vortex mixing. The supernatant was collected and combine in the 0.5-mL plastic micro-centrifuge tube. The volume of the extract was completely dried by evaporation in a speed-vac. Tryptic peptides were solubilized in 30 µL of MS grade water (Sigma-Aldrich) containing formic acid 0.1% (v/v).

Protein identification by MS: Ten microliter volumes of trypic digest were used for LC-MS analysis using a HPLC system (Shimadzu, Prominence UFLC), containing a C18 5-Å 300 µm × 100-mm capillary column operating with a flow rate of 3.0 µl/min. The eluted peptides were injected in a micrOTOF-Q (Bruker) mass spectrometry on-line using a microESI ionization needle. Mobile phase buffers used for the gradient program were water with 0.1 % (v/v) formic acid (A) and acetonitrile with 0.1 % (v/v) formic acid (B). The gradient program consisted of 5% B for 5 min, linear ramping to 50 % B over 35 min, linear ramping to 95 % B over 10 min, holding at 95 % B for 10 min, ramping back to 5 % B over 5 min, followed by holding at 5 % B for 5 min. The mass spectrometer scanned between 300 and 1500 m/z in positive mode, and data was acquired for 70 min in each LC-MS/MS run. Data acquisition by both LC-MS instruments was managed by Hystarpackage (Bruker) and the spectrums were processed with the Data Analysis package (Bruker) using the default settings for proteomics. The mass spectrometer operated in auto-MSn mode which collected MS2 spectra for the more intense ions in each full scan spectrum, excluding single charge ions. The scan time was set to 0.5 s for the survey scan, and the MS2 spectra were recorded for five spectrums to allow for collection of the maximum number of MS2 spectra during the analysis. Peak lists were generated as a mascot generic format (mgf) by Data Analysis (Bruker) and used for protein identification by the MASCOT algorithm (Matrix Science). Batch Mascot searches were performed locally against the NCBI Fungi protein database using the MASCOT DAEMON CLIENT, with a precursor tolerance of 0.1 Da for the product ions, allowing for methionine oxidation and deamidated (NQ) as a variable modification, carbamidomethylation as a fixed modification, one missed cleavage, charge states 2, 3, and 4, and trypsin as the enzyme. The peptide and protein identification were statistically evaluated and validated by the Peptide Prophet and Protein Propht algorithms using the Scaffold package (Proteome Software) for 90 % probability.

7.3.7- Effect of pH and temperature

The influence of pH and temperature on endoglucanase activities were studied using the standard assay described above, but varying the pH using McIlvaine buffer solutions (pH 3.0-7.5) [14] and the glycine-HCl buffer solution while varying the incubation temperatures (30 - 95 °C). For determination of

thermal stability, the enzyme fractions were preincubated with sodium acetate buffer, pH 5.0, at 50, 60, 70 and 80 °C. Samples were collected at several periods of time and kept on ice for at least 1 hour. Residual activity was determined using the standard assay at the optimum temperature.

7.3.8- Activation energy and temperature coefficient

The activation energies (Ea) for the reaction catalyzed by the endoglucanases were calculated using CMC as the substrate at temperatures varying from 30 to 70 ºC at pH 5.0. Values were calculated by plotting the data as Arrhenius: log of velocity on the ordinate versus 1/T in Kelvin on the abscissa. The Arrhenius equation used was: slope = - Ea/2.3R, where R is the universal gas constant (8.314 $J.K^{-1}.mol^{-1}$).

The temperature coefficient (Q_{10}) was calculated according to the equation: $lnQ_{10}= (E_a \times 10)/RT^2$, where Ea is the activation energy of the enzymes (J/mol), R is the universal gas constant and T is the absolute temperature (K).

7.3.9- Kinetic Studies

Kinetics experiments were performed at 80 °C and pH 5.0. The Michaelis-Menten constant (K_M) and maximum reaction rates (V_{max}) were calculated according to the Michaelis-Menten plot with sucrose as the substrate, using the computer software Curve Expert version 1.3 for Windows. The CMC concentration varied from 0.16 to 6.66 % (w/v).

7.3.10- Effect of ions and reducing agents

The effects of ions and reducing agents on endoglucanase activities were assayed by performing the standard assay and including the compound under analysis at final concentration of 1 or 6 mM in 100 mM sodium acetate buffer, pH 5.0.

7.3.11- Substrate specificity

Enzymatic assays were performed with various synthetic and natural substrates. The reaction consisted of 150 µL of 100 mM sodium acetate buffer pH 5.0, 100 µL of an enzyme solution and 250 µL of substrates.

Hydrolysis of ρ-nitrophenyl-β-D-xylopyranoside, ρ-nitrophenyl-β-D-glucopyranoside and ρ-nitrophenyl-β-D-cellobioside were determined using a final substrate concentration of 1 mM, for 30 min. The reaction was terminated using sodium carbonate 0.5 M and the amount of p-nitrophenol released was determined at 410 nm.

The hydrolysis of CMC (average molecular mass of 90000 g/mol and 0.7 carboxymethyl groups per anhydroglucose unit), CMC 2 (average molecular mass of 250000 g/mol and 0.9 carboxymethyl groups per anhydroglucose unit), birchwood xylan and barley- β- glucan were determined using a final substrate concentration of 1 % (w/v). Hydrolysis of locust bean gum was assessed using a final concentration of 0.5 % (w/v). The hydrolysis of filter paper was determined using 1 x 6 cm (50 mg) filter paper Whatman n°1. All reducing sugars released by the above reactions were determined using the DNS reagent [10].

Starch hydrolysis was evaluated using the final concentration of 0.5 % (w/v) and the starch concentration was determined by the iodine reagent (0.3 % iodine and 3 % potassium iodide) based on the Fuwa method [15].

Hydrolysis of cellobiose was determined using a final concentration of 6.0 mM. The reaction was terminated by boiling the samples for 5 min and the glucose liberated was quantified using a commercial analytical kit based on glucose oxidase and peroxidase (Quibasa Basic Chemical, Belo Horizonte, MG, Brazil).

For Avicel hydrolysis, 0.1 g of Avicel was used at a final volume of 2 mL (enzyme solution and buffer). The reaction was conducted in a rotary shaker (180 rpm, 60 °C) for 4 hours and the reducing sugar released was determined using the DNS reagent [10].

With the exception of Avicel hydrolysis, all others reactions were performed at 80 °C for 30 minutes. For each a control reaction was conducted with CMC at the same condition and enzyme concentration. This control was considered 100 % of activity.

7.3.12- Cello-oligosaccharide hydrolysis

Hydrolysis of cellopentaose, cellotetraose and cellotriose were evaluated. The reaction mixture consisted of 50 µL containing 10 mM sodium acetate buffer pH 5.0, enzyme solution in appropriated dilution and the substrate with a

final concentration of 4 μM. Enzymes were standardized in a final concentration of 0.03 U/mL in the reaction mixture. The reaction was conducted at 60 °C for 1 hour. After this time, the reaction mixtures were frozen and then analyzed. The cello-oligosaccharides were analyzed in an HPLC, Shimadzu series 10A chromatograph equipped with a Supelcosil LC-NH_2 column (25 cm x 4.6 mm, 5 μm particle size). The mobile phase was acetonitrile: water (80:20) at a flow of 1mL/min, at 35 °C.

7.3.13- CMC hydrolysis

The reaction mixture consisted of 2 mL containing 10 mM sodium acetate buffer, pH 5.0, enzyme in appropriated dilution and the substrate with a final concentration of 1 %. Enzymes were standardized in a final concentration of 0.03 U/mL in the reaction mixture. The reaction was conducted at 60 °C for 67 hours and 6 aliquots of 100 μL were taken along this time, which were boiled for 10 minutes and frozen until analyses. The products were analyzed in an HPLC, Shimadzu series 10A chromatograph equipped with a Supelcosil LC-NH_2 column (25 cm x 4.6 mm, 5 μm particle size). The mobile phase was acetonitrile: water (80:20) at a flow of 1mL/min, at 35 °C.

7.4- Results and discussion

7.4.1- Endoglucanase purification

Size exclusion chromatography of *Fusarium verticillioides* cell-free culture supernatants resulted in two main forms of proteins with endoglucanase activities (Figure 1).

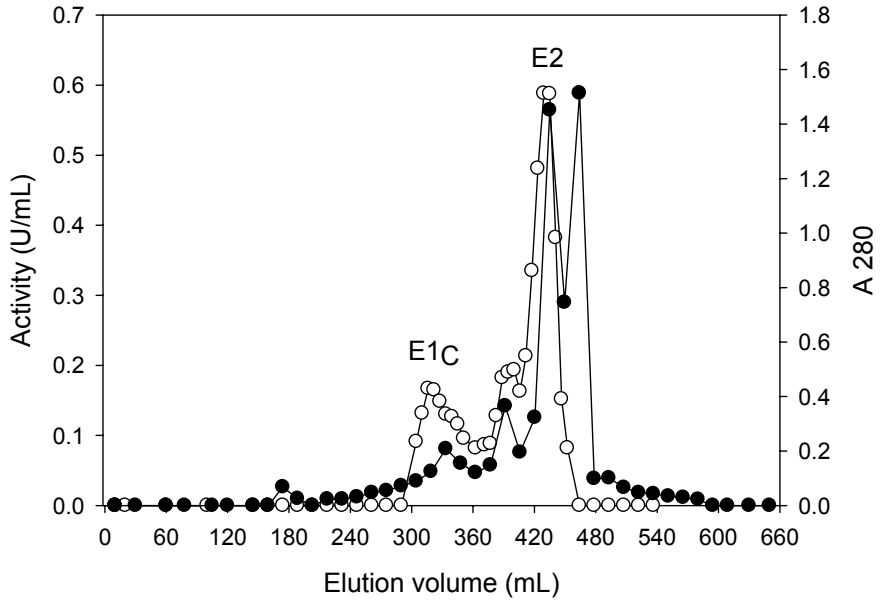

Figure 1: Size exclusion chromatography of the crude extract from *Fusarium verticillioides*. (○) endoglucanase activity; (●) protein (A 280).

The first form eluted was designated $E1_C$ and was submitted to Phenyl-Sepharose (Figure 2). The fractions containing endoglucanase activity were pooled, dialyzed and then submitted to ion-exchange chromatography, CM-Sepharose (Figure 3).

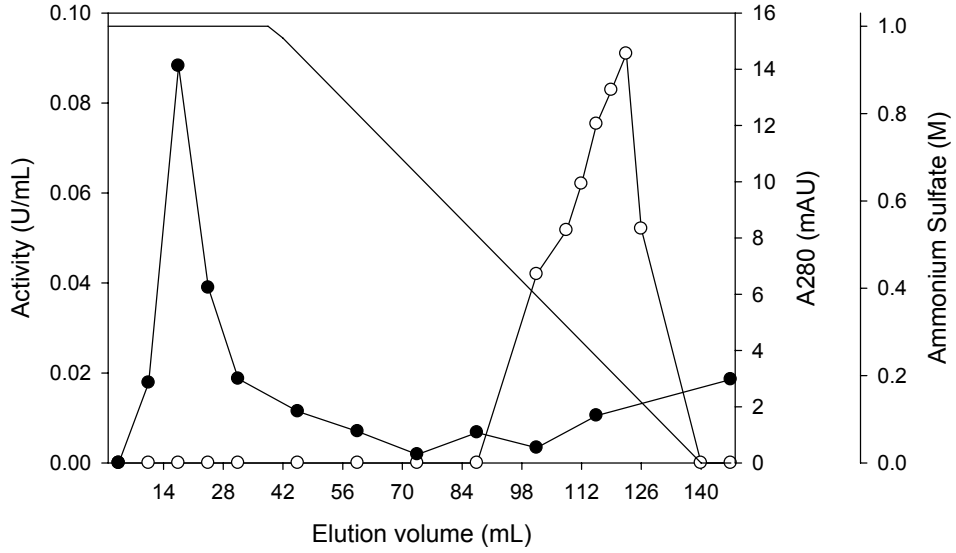

Figure 2: Hydrophobic Interaction chromatography of $E1_C$ from *Fusarium verticillioides* (○) endoglucanase activity; (●) protein (A 280); (-) Ammonium sulfate.

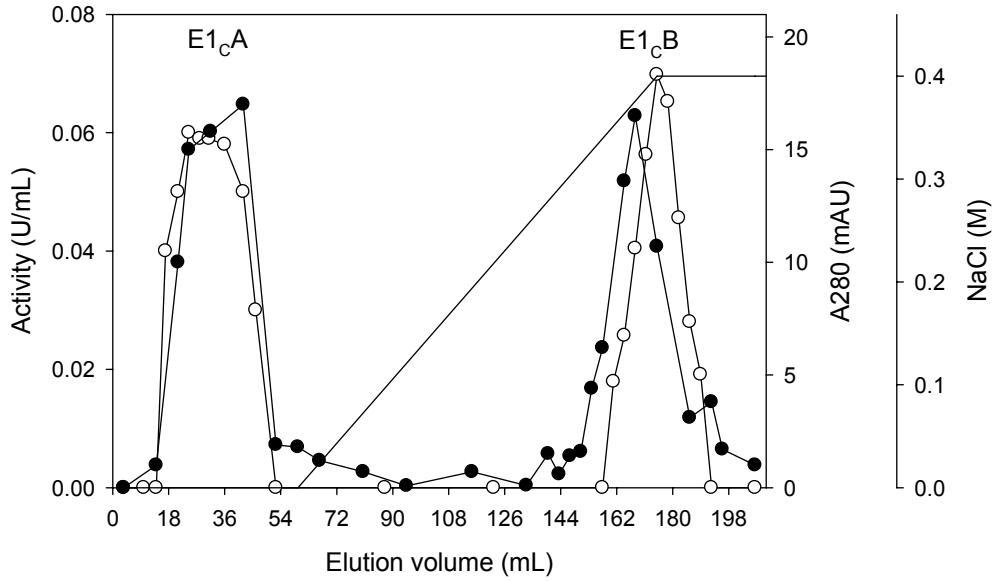

Figure 3: Ion exchange chromatography of $E1_C$ from *Fusarium verticillioides*. (○) endoglucanase activity; (●) protein (A 280); (-) NaCl

After CM-Sepharose chromatography two endoglucanase peaks were observed, one ($E1_CA$) eluted with the buffer wash and the second ($E1_CB$) eluted in the saline gradient. Purification results are summarized in Table 1.

Table 1: Summary of the results obtained during the purification process of $E1_C$ and E2 from *Fusarium verticillioides*

Purification step	**Activity (U)**	**Protein (mg)**	**Specific Activity (U/mg)**	**Purification Factor**	**Yield (%)**
Concentrated crude extract	220.00	26.10	8.43	1.00	100.0
Endoglucanase $E1_C$					
Sephacryl S-300	12.19	1.49	8.17	0.97	5.54
Phenyl-Sepharose	3.19	1.43	2.22	0.26	1.45
CM-Sepharose $E1_CA$	1.35	1.00	1.35	0.16	0.61
CM-Sepharose $E1_CB$	1.44	1.10	1.31	0.16	0.66
Endoglucanase E2					
Sephacryl S-300	26.59	1.88	14.17	1.68	12.09
Q-Sepharose	14.56	0.39	37.04	4.39	6.62

The specific activity of $E1_C$ after Phenyl Sepharose and CM-Sepharose was lower than the initial specific activity, which is not common in the purification process. This fact was probably due to the great loss of enzyme activity during the purification process. Another possibility is the lack of synergism between the enzymes once they were separated. However, the

efficiency of the purification process can be confirmed by the SDS-PAGE analysis in Figure 4 and Figure 5.

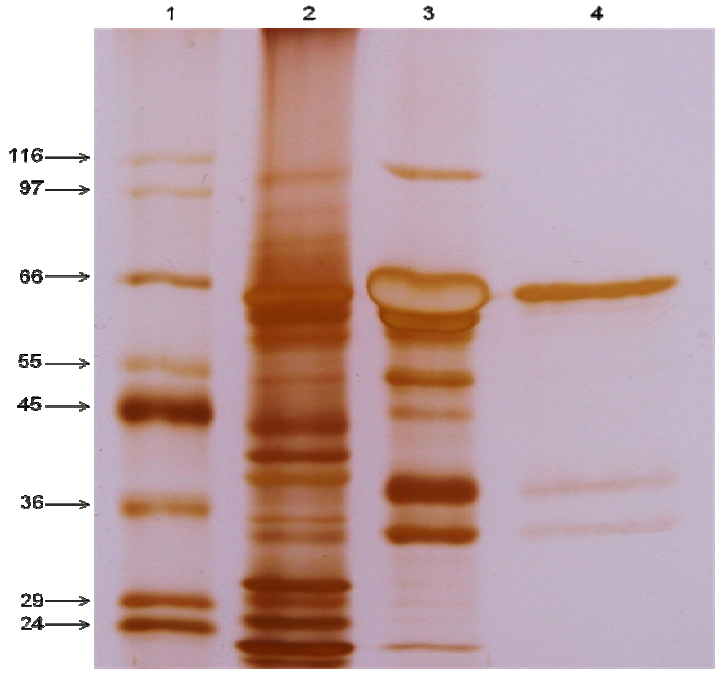

Figure 4: SDS-PAGE of purified endoglucanase $E1_C$ from *Fusarium verticillioides*. 1: molecular marker (Sigma); 2: crude extract; 3: $E1_C$ after Sephacryl S-300; 4: E1C after Phenyl-Sepharose.

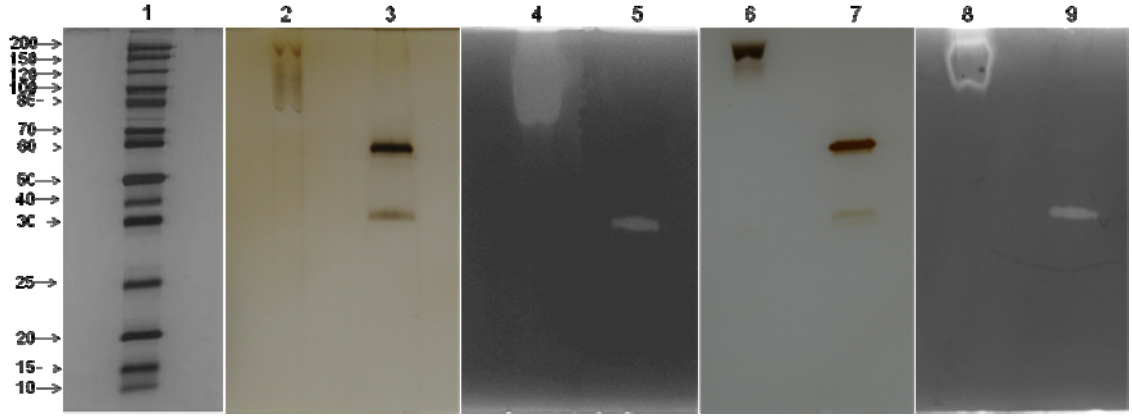

Figure 5: SDS-PAGE of endoglucanase $E1_C$ from *Fusarium verticillioides* after CM-Sepharose chromatography. 1: molecular marker (Fermentas); 2: non-boiled $E1_CA$ silver-stained; 3: boiled $E1_CA$ silver-stained; 4: non-boiled $E1_CA$ zymogram; 5: boiled $E1_CA$ zymogram; 6: non-boiled $E1_CB$ silver-stained; 7: boiled $E1_CB$ silver-stained; 8: non-boiled $E1_CB$ zymogram; 9: boiled $E1_CB$ zymogram.

After Phenyl-Sepharose chromatography three proteins remain in the sample, observed at 70.7, 38.8 and 34.0 kDa. After CM-Sepharose, it is observed that fraction $E1_CA$ contains only two bands with 70.7, 38.8 kDa. The $E1_CB$ fraction, however, contains the same three bands detected in the Phenyl-Sepharose fraction. When $E1_CA$ and $E1_CB$ were submitted to heat treatment the bands were separated in the gel and only the band of 38.8 kDa presented

endoglucanase activity. However, when they were not boiled, the proteins are grouped in a big protein band, located in the beginning of the gel, which also presented endoglucanase activity. It can therefore be concluded that these three proteins exist in nature form as a multienzyme complex. The protein with 38 kDa is the main endoglucanase present in the complex and it presents activity on the zymogram independently of the presence of other proteins.

Among the three proteins which form the complex, the 34.0 kDa protein can be separated after pH changes, as was observed in CM-Sepharose chromatography. However, because this protein was not separated from the 70.7 kDA protein in gel filtration chromatography, it can be deduced that in these conditions they were physically associated. The 70.7 and 38.8 kDa proteins, however, can be separated after heat treatment only.

The other form, designated E2, was submitted to an Q-Sepharose column (Figure 6). It was purified to a specific activity of 37.0 U/mg with a yield of 6.6 % (Table 1). The SDS-PAGE analysis indicated that this form had a mass of 54.0 kDa; and the zymogram analysis confirmed the endoglucanase activity of this protein (Figure 7).

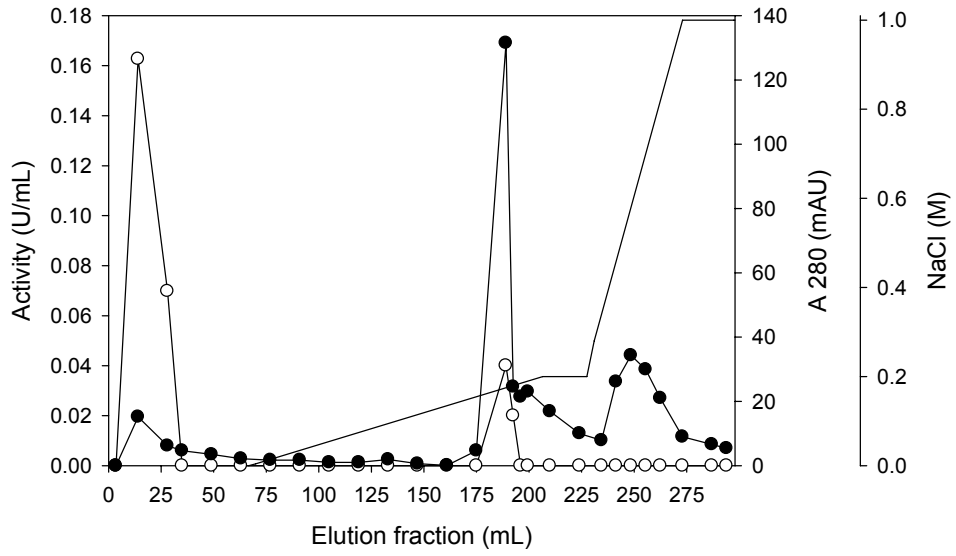

Figure 6: Ion exchange chromatography of E2 (○) endoglucanase activity; (●) protein (A 280); (-) NaCl

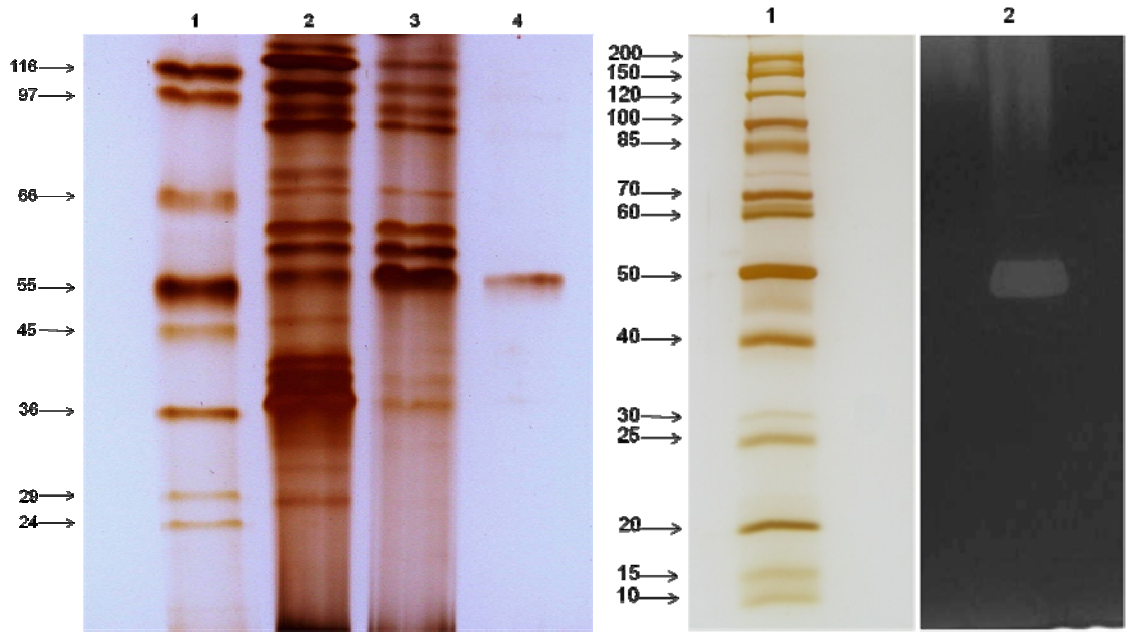

Figure 7: SDS-PAGE of purified endoglucanase E2 from *Fusarium verticillioides* (left). 1: molecular marker standards (Sigma); 2: crude extract; 3: E2 after Sephacryl S-300; 4: E2 after Q-Sepharose. Zymogram analysis of purified E2 (right). 1: molecular marker standards (Fermentas); 2: Purified E2.

7.4.2- Mass spectrometry analysis

Bands were cut from the SDS-PAGE and analyzed via mass spectrometer. The $E1_C$ proteins were named as: $E1_C$.1, protein with 70.7 kDa; $E1_C$.2, protein with 38.8 kDa; $E1_C$.3, protein with 34 kDa. The peptides identified are in Table 2.

After a BLAST search of the GenBank database, it was verified that the bands presented in the sample $E1_C$ contained at least two endoglucanases, one cellobiohydrolase and one xylanase. Contrarily, in the E2 band only an endoglucanase from GH5 family was detected. In the $E1_C$.1 band a high concentration of cellobiohydrolase from family GH7 was detected along with a minor concentration of endoglucanase from the family GH6. In the $E1_C$.2 band endoglucanase was detected. Although no conserved peptide was detected, it can be deduced that the endoglucanase in $E1_C$.2 belongs to the family GH10, since it is similar to two endoglucanases from this family. In the $E1_C$.3 band a xylanase from the family GH10 was detected. The sequences of this protein were quite similar to those observed on $E1_C$.2, however, this protein showed no endoglucanase activity in the zymogram (data not shown). The GH5, GH7 and GH10 families grouped several enzymes that present retaining mechanisms for

substrate hydrolysis and the catalytic residues are two glutamates. Differently, the GH6 family grouped enzymes which presented inverting mechanisms and the catalytic residues are two aspartates (http://www.cazy.org/GH6.html).

Table 2: Peptides identified by spectrometry analysis for proteins $E1_C$ and E2

Band	Name	Coverage (%)	Sequences	Family
$E1_C$.1	1,4-beta-cellobiohydrolase from *Fusarium oxysporum* (P46238)	26	WTHQTSGSTNCYTGNK* YGTGYCDAQCPR* YGGTCDADGCDFNAYR* KMTVVTQFHK MTVVTQFHK LSEITR LYVQNGK VIANSESK IAGNPGSSLTSDFCSK GSCATTSGKPSDLER VSFSNIK FGPIGSTYK SPFTCK KINDFYSQCQ	GH7
$E1_C$.1	Endoglucanase from *F. oxysporum* (P46236)	8	LSTKPDYTESNPNYDEQR VILVIEPDSLANLVTNLNVDK*	GH6
$E1_C$.2	Endoglucanase from *Magnaporthe oryzae* - GH10 (XP_003709018)	7	VLGEDFVGIAFR LYINDYNLDIANYAK	-
$E1_C$.3	Xylanase from *F. oxysporum* (AAC06239)	13	NSFTFSNADK VLGEDFVGIAFR ILQWDVVNEIFAEDGNLR* AQFGQVTCENSMK* LYINDYNLDIANYAK	GH10
E2	Hypothetical protein from *F. oxysporum* (EGU78866)	21	TCVSGYK AATEWLR ENSDVWK NMLDYLK GANVILDPHNYGR IITSTSDFQTWWK IVYEMHQYLDSDSSGTSPNCVSTTIGVER*	GH5
	Endoglucanase from *Aspergillus niger* (XP_001397982.2)	Maximal identity on Blast search (83 %)		

* Conserved sequences of the indicated families. $E1_C$.1: protein with 70.7 kDa. $E1_C$.2: protein with 38.8 kDa. $E1_C$.3: protein with 34 kDa. The accession numbers of the sequences are in parenthesis after the names of the parents.

Multienzyme complexes containing cellulases and hemicellulases have been reported for some microorganisms. *Penicillium purpurogenum* produced a

complex containing mainly hemicellulosic activities including α-glucosidases, α-arabinofuranosidase, xylanase, acetylxylanesterase and dehydrogenase [6]. *Trichoderma harzianum* produced three complexes containing cellulases and hemicellulases, where one of the complexes presented two cellobiohydrolases, one α-arabinofuranosidase and one xylanase [4]. The bacteria *Paenibacillus curdlanolyticus* produced a complex containing fourteen proteins, ten of which presented endoglucanase activities and seven presenting xylanase activity [16]. Diversity of the multienzyme complex composition may result from adaptation of the fungi to the environment, as was observed for *Penicillium purpurogenum* [6]. The activities presents in each multi-enzyme complexes can vary, but enzymes usually act synergistically and in some cases there are non-catalytic proteins which may function to stabilize the complexes [6, 17]. Physical proximity of the enzymes can provide substrate channeling, which is a process of direct transfer of the product of an enzyme to another proximate enzyme as its substrate without equilibration with the bulk phase [18]. This substrate proximity can act as an advantage by increasing the efficiency of hydrolysis. Therefore, the multienzyme complex may be a mechanism used by microorganisms to improve catalysis and enzyme stability, as well as better adaptation to different environments.

Despite evidence on the existence of a multienzyme complex in the *F. verticillioides* culture, it was not verified how the proteins are associated. Association could be mediated by direct protein–protein interactions as well as by glycosylations [6]. In the most studied multienzyme complexes, the cellulosomes, proteins are associated via scaffolding protein mediation. In this system the structural proteins dockerins and cohesins mediate the association of cellulases and hemicellulases for efficient cellulose hydrolysis. The most studied cellulosome is that from the anaerobic bacteria *Clostridium Thermocellum* and some anaerobic fungi also presents this complex structure [19, 20]. In the case of the multienzyme complex from *F. verticillioides*, no structural proteins were detected in the complex, suggesting a different nature of this multienzyme complex.

For biochemical and kinetic characterization, the $E1_CB$ was used. This sample, obtained after saline elution by CM-Sepharose chromatography, is the complete and purified multienzyme complex.

7.4.3- Effect of temperature

Enzymes were assayed from 35 to 95 °C in buffer at pH 5.0. The optimal temperatures for both $E1_C$ and E2 were 80 °C (Figure 8).

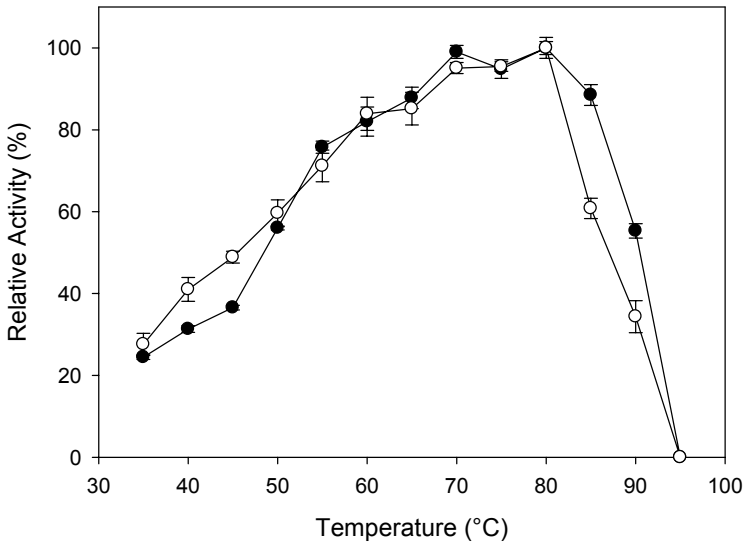

Figure 8: Effect of temperature on $E1_C$ (●) and E2 (○) activities at pH 5.0.

In general, endoglucanases from fungi present optimal temperatures between 50 and 70 °C [21-25]. Thermophilic endoglucanases presenting 80 °C as the optimum temperature have been mostly reported for thermophilic microorganism such as *Fervidobacterium nodosum* [26], *Thermoascus aurantiascus* [27] and *Acidothermus cellulolyticus* [28], but was also found in the mesophilic fungus *Daldinia eschscholzii* [7].

The two endoglucanases showed high thermostability mainly at 50 and 60 °C (Figure 9). Equations were constructed for enzyme activity loss and the half lives were calculated according to each equation. The half life is the time at which the enzyme losses 50 % of its initial activity. $E1_C$ presented higher thermostability than E2 at 50 and 60 °C, obtaining half-lives of 277.8 and 192.8 h, respectively, while E2 presented 16.37 and 11.61 h for the same temperatures (Table 2). The high stability of multienzyme complex $E1_C$ can be attributed to the presence of the other proteins which may promote a stabilizing effect. Similar results were obtained when comparing the cellulosome from *Clostridium thermocellum* and free cellulases from *Trichoderma* sp. [29].

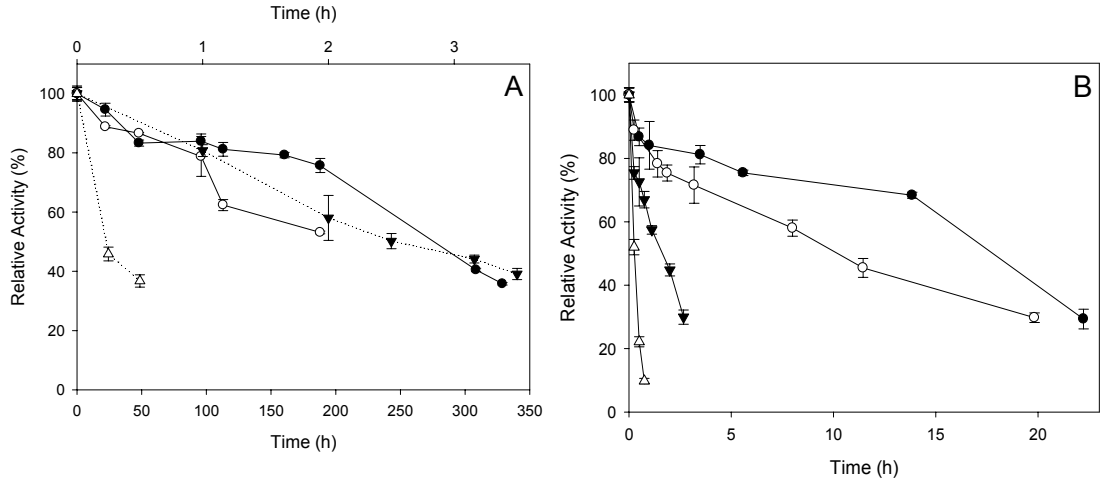

Figure 9: Thermostability of $E1_C$ (A) and E2 (B) at 50 (●), 60 (○), 70 (▼) and 80 °C (△) at pH 5.0 in sodium acetate buffer. In E1C, dotted lines correspond to the top x axis and solid lines corresponding to bottom x axis. The activity without pre-incubation was considered 100%.

Table 2: Half-life of $E1_C$ and E2 at different temperatures and pH 5.0

	$T_{1/2}$ (h)	
Temperature (°C)	**$E1_C$**	**E2**
50	277.8	16.37
60	192.8	11.61
70	2.71	1.66
80	0.34	0.34

Although $E1_C$ and E2 showed the highest activities at 80 °C, they did not present high stability at this temperature, where both presented half-lives of 0.34 h. *Fusarium verticillioides* is a mesophilic fungus and does not grow at temperatures above 35 °C, however, the two main endoglucanases produced by this fungus were thermostable at high temperatures.

Endoglucanases from a *Fusarium oxysporum* presented optimal temperatures of 50 and 55 ° but do not present high thermostability [30, 31]. In another study the production of a thermostable endoglucanase from *F. oxysporum* was reported, which presented an optimal temperature of 75 °C. However that enzyme was not as thermostable as $E1_C$ and E2, presenting half-lives of 25.8, 21.2 and 15.1 minutes for 50, 60 and 70 °C, respectively [32].

The activation energy (E_a) for hydrolysis of CMC was 21.38 kJ/mol for $E1_C$ and 27.48 kJ/mol for E2 (Figure 10).

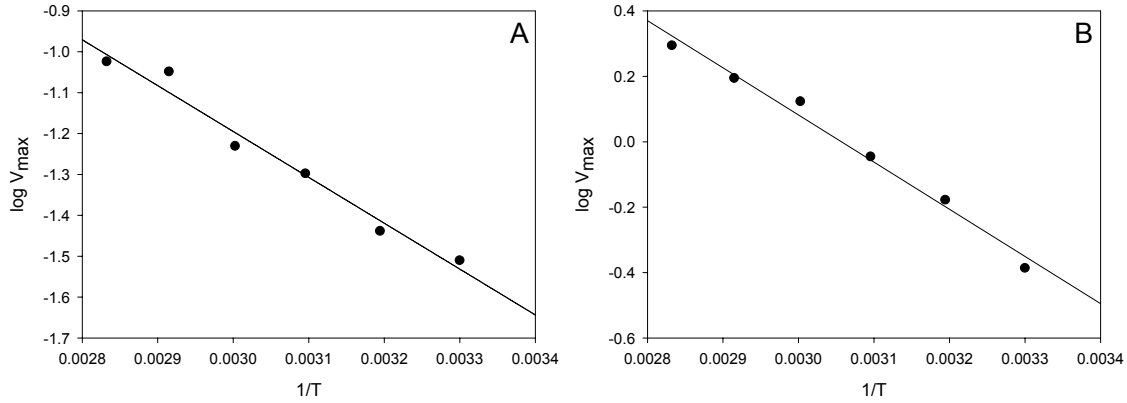

Figure 10: Plots for $E1_C$ (A) and E2 (B) endoglucanases from Fusarium verticillioides used for activation energy calculation using the Arrhenius equation (slope = - Ea/2.3R)

The lower E_a for $E1_C$ showed that this enzyme must overcome a lower energy for hydrolyzing CMC, which can be facilitated by the presence of cellobiohydrolase on the multienzyme complex $E1_C$. This also implies that E2 catalyzed the hydrolysis of CMC less efficiently than $E1_C$. The two endoglucanases from *F. verticillioides* presented lower E_a than the endoglucanases produced by *Aspergillus fumigatus*, which presented 51 and 32.7 kJ/mol, suggesting that $E1_C$ and E2 catalyzed CMC hydrolysis more efficiently than those from *A. fumigatus* [25].

The E_a gives rise to another activity parameter, the temperature coefficient (Q_T), which is the factor by which the rate of a reaction changes for every 10 °C increase in temperature. The Q_T values at 50 and 60 °C were 1.27 and 1.36 for $E1_C$ and E2, respectively. The Q_T for enzymes generally range between 1 and 2. A higher Q_T for E2 indicated that it was more temperature sensitive than $E1_C$. This fact also supported the implication that the E2 had a lower thermostability than $E1_C$.

7.4.4- Effect of pH

Enzymes were assayed at 80 °C in a discontinuous buffer system (Figure 11). $E1_C$ maintained at least 98 % of the maximal activity between 4.5 and 5.5 and the optimal pH for $E1_C$ was 4.5. In the glycine HCl buffer the enzyme showed higher activity than at the same pH in McIlvaine buffer, probably due an unspecific stabilization effect of glycine.

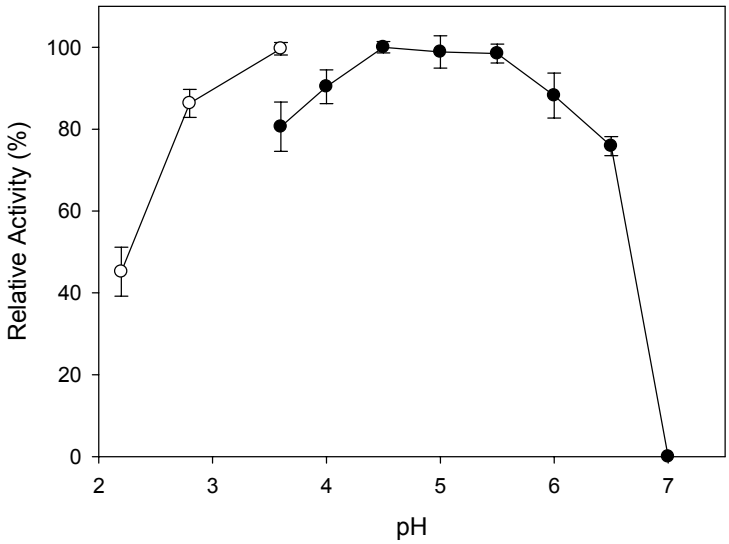

Figure 11: Effect of pH on $E1_C$ activity at 80 °C in a discontinuous buffer system. (○) Glycine HCl buffer; (●) McIlvaine buffer.

When $E1_C$ was pre-incubated for 12 hours at 24 °C with pH varying between 2.2 and 8.5, the enzyme showed high stability, maintaining at least 80 % of the maximal activity at all pH levels (Figure 12). Between 5.0 and 7.5 $E1_C$ maintained at least 95 % of the maximal activity. After 24 hours of pre-incubation $E1_C$ maintained stability between 3.6 and 7.5, however at the more extremes pH levels such as 2.2, 3.0 and 8.0, $E1_C$ presented activities between 53 and 63 % of the maximum.

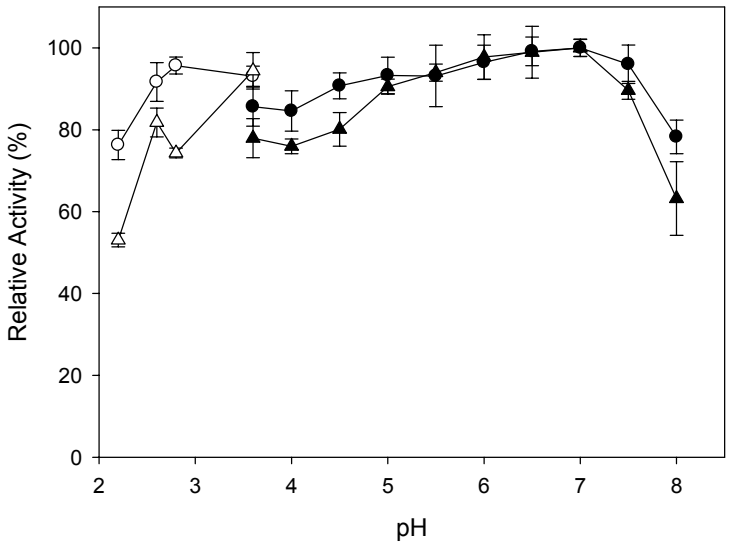

Figure 12: Stability of $E1_C$ for different pH levels at 24 °C. (○) Glycine HCl buffer, 12 h of pre-incubation; (●) McIlvaine buffer 12 h of preincubation; (△) Glycine HCl buffer, 24 h of pre-incubation; (▲) McIlvaine buffer 24 h of preincubation. The highest activity obtained after each pre-incubation time was considered as 100%.

E2 maintained at least 92 % of the maximal activity between 4.0 and 6.0 and the optimal pH for E2 was 5.5, slightly higher than the optimal pH observed for $E1_C$ (Figure 13).

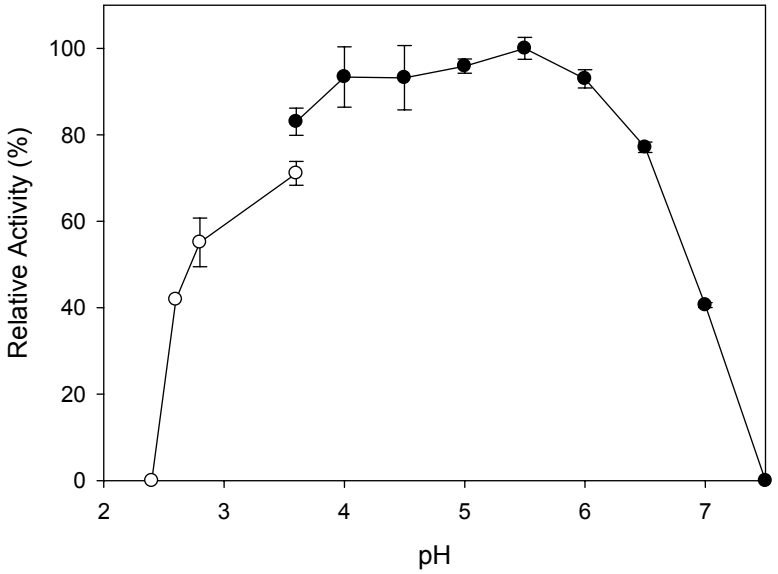

Figure 13: Effect of pH on E2 activity at 80 °C in a discontinuous buffer system. (○) Glycine HCl buffer; (●) McIlvaine buffer.

Although thermostability was observed, E2 showed to be less stable than $E1_C$ in relation to pH (Figure 14). After 12 hours of pre-incubation at different pH levels, E2 maintained at least 80 % of the maximal activity in pH between 2.2 and 6.5. After 24 hours of pre-incubation, the enzyme showed a loss in activity at pH levels of 2.2 to 4.0 and 7.0 to 8.0. $E1_C$ showed to be more stable in a broad pH range, different from E2 which lost activity in more extreme pH.

The endoglucanases $E1_C$ and E2 showed optimum pH levels similar to other fungal endoglucanases, however they presented high activities at low pH (2.5 – 4.0). This pH profile was similar to that observed for the endoglucanases from *Mucor circinelloides* [24] and from *Bacillus subtilis* [33]. In general, endoglucanases do not present activity at low pH as observed for $E1_C$ and E2 [22, 26, 34-36]. Endoglucanases, which present these feature are usually termed as acidophilic enzymes [28, 33]. Some endoglucanases, which presented optimal activities below pH 3.0, are also termed this way, however they do not present activity at higher pH as was observed for $E1_C$ and E2, indicating that the two enzymes from *F. verticillioides* are useful in a wide pH range [37, 38].

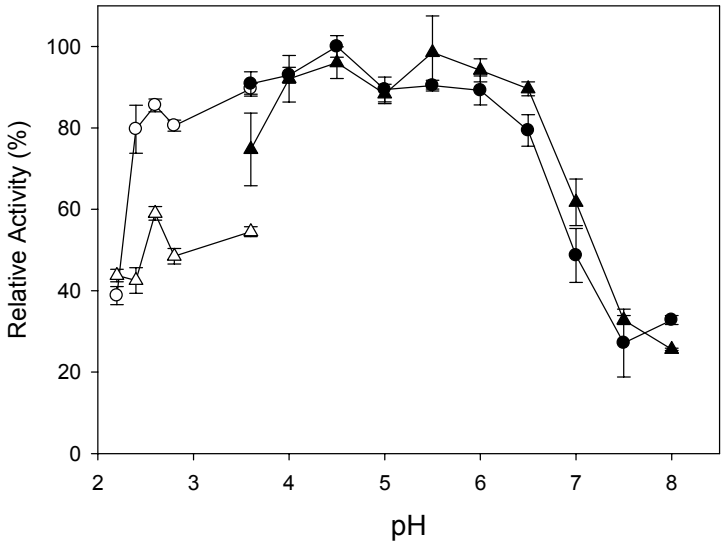

Figure 14: Stability of E2 for different pH levels at 24 °C. (○) Glycine HCl buffer, 12 h of pre-incubation; (●) McIlvaine buffer 12 h of preincubation; (△) Glycine HCl buffer, 24 h of pre-incubation; (▲) McIlvaine buffer 24 h of preincubation. The highest activity obtained after each pre-incubation time was considered as 100%.

7.4.5- Effect of ions and other reagents

The multienzyme complex $E1_C$ and the enzyme E2 were assayed in the presence of certain ions and other reagents at 1 and 6 mM (Table 3 and Table 4), at 80 °C and pH 5.0. The enzymes $E1_C$ and E2 showed very similar behavior in the presence of the ions.

The activities of the two enzymes were significantly increased by the ions Co^{2+} and Mn^{2+}. Higher activity in the presence of these two ions was observed in other studies for endoglucanases from *Anabaena laxa* [39] and from *Daldinia eschscholzii* [7]. Endoglucanses from the family 61 were also activated in the presence of some divalent ions, including Mn^{2+} and Co^{2+}. The structure of the endoglucanase GH61 was studied and it was suggested that there is a functional metal ion-binding site, prompting a series of experiments which show that divalent metal ions are essential for the ability of GH61 to stimulate biomass hydrolysis [40]. The opposite, a inhibitory effect promoted by these two ions, was observed for endoglucanases from *Penicillium purpurogenum* [22] and *Bacillus* sp. [36].

Table 3: Effect of metal ions on $E1_C$ and E2 activities

	Relative Activity (%)			
	$E1_C$		E2	
	1 mM	**6 mM**	**1 mM**	**6 mM**
Control	100.0±1.2	100.0±2.5	100.0±3.3	100.0±3.8
$CoCl_2$	133.9±3.4	113.0±4.1	132.0±2.5	104.7±6.9
$CaCl_2$	103.2±1.3	90.2±3.6	96.3±2.9	89.1±3.7
$CaCO_3$	93.4±2.1	66.7±3.9	89.7±3.7	38.7±6.1
KCl	95.4±1.5	83.5±2.1	94.9±2.0	93.3±2.9
NaCl	85.1±1.4	81.0±0.3	94.6±0	97.7±3.5
KI	85.0±1.4	89.6±3.3	99.1±1.4	99.7±2.9
$HgCl_2$	0.0±0	0.0±0	0.0±0	0±0
$ZnCl_2$	82.5±4.0	0.0±0	78.3±3.5	0.0±0
$ZnSO_4$	75.7±1.6	0.0±0	79.4±4.2	0.0±0
$MgSO_4$	97.4±0.7	80.2±4.2	100.1±1.7	93.4±3.9
$AgNO_3$	77.8±3.5	0.0±0	78.0±3.9	31.2±1.3
$MnSO_4$	159.1±3.3	138.2±4.1	142.5±2.4	138.4±2.6
$MnCl_2$	157.8±3.0	142.2±0.2	145.6±3.7	123.0±3.3
$AlCl_3$	86.7±2.3	60.1±2.6	90.5±4.7	93.4±4.1
Na_2SO_4	86.8±2.5	80.1±4.2	90.7±3.8	86.7±2.7
NaF	100.1±5.8	95.9±3.7	103.5±8.6	115.1±7.0
$FeCl_3$	93.9±4.5	52.2±0.8	110.5±3.7	53.9±1.7
$CuSO_4$	76.4±3.3	0.0±0	48.1±0.8	0±0
$MgCl_2$	99.4±1.0	81.3±3.3	100.1±3.5	98.4±4.5
$NaNO_3$	97.4±1.5	74.2±4.2	95.3±4.5	89.7±2.9
$(NH_4)_2SO_4$	92.2±2.1	95.5±3.3	95.8±3.8	109.9±1.7

100 % was considered for the enzyme activity with no ions

At different concentrations the ions Cu^{4+}, Hg^{2+}, Zn^{2+}, Zn^{4+} and Ag^{3+} caused total loss of $E1_C$ and E2 activities and Fe^{3+} causes great activity loss at 6 mM. The inhibitory effect of the metal cations Cu^{4+}, Hg^{2+}, Zn^{2+}, Zn^{4+} , Fe^{3+} and Ag^{3+} is a common feature in glycosyl hydrolases and it is usually associated with the presence of at least one sulfhydryl and/or carboxyl group, amino and imidazolium groups of histidine in the active site. Oxidation of this group by the metal cations destabilizes the conformation folding of the enzyme or leads to formation of chemical interaction at irregular positions within the protein [7, 22, 41].

The ion Ca^{2+} tended to cause activity losses of $E1_C$ and E2 mainly at the concentration of 6 mM. Ca^{2+} does not usually cause activity loss in endoglucanases [7, 22, 24, 39]. However, this type of inhibition could also be found for endoglucanase from *Phialophora* sp. [42] and for 1,3-1,4-β-D-glucanase, a specific endoglucanase from family 16. The inhibitive effect of

Ca^{2+} on 1,3-1,4-β-D-glucanase was studied and non-competitive inhibition was observed. Ca^{2+} binds to a specific site in the vicinity of the active site of the enzyme and causes activity loss [43]. The other ions tested presented little or none influence on $E1_C$ and E2 activities.

Table 4: Effect of various reagents on $E1_C$ and E2 activities

	Relative Activity (%)			
	$E1_C$		E2	
	1 mM	6 mM	1 mM	6 mM
Control	100.0±1.2	100.0±2.5	100.0±3.3	100.0±3.8
EDTA	75.0±2.0	73.8±1.4	87.8±1.5	87.3±2.8
SDS	62.3±3.3	0.0±0	0.0±0	0.0±0
TritonX-100	98.7±2.4	99.1±2.1	113.8±3.7	132.0±1.9
Tween 20	94.8±2.6	93.4±2.0	108.5±2.1	105.5±2.0
Glycerol	96.3±3.3	96.8±3.4	103.8±1.4	114.6±0.7
Urea	95.2±3.8	100.1±3.3	108.9±2.0	112.3±3.6
Acetic acid	97.1±2.4	94.2±0.8	105.6±8.7	107.0±6.1
Ethanol	96.6±3.1	98.4±3.3	93.9±1.2	98.5±2.7
Furfural	103.6±1.7	125.3±0.9	102.9±9.2	102.3±6.5
HMF	111.7±4.2	133.2±3.0	110.4±6.5	105.3±5.0
Azide	101.1±2.9	95.7±1.4	94.6±5.4	98.9±3.1
BME	103.3±4.5	98.4±3.4	97.0±2.2	89.0±7.4
DTT	117.1±1.1	107.0±0.4	107.9±11.1	89.4±9.8
DMSO	106.8±2.5	101.0±2.1	104.7±2.5	96.4±1.7

100 % was considered the enzyme activity without addition of the ion. EDTA: Ethylenediamine tetraacetic acid; SDS: Sodium dodecyl sulfate; HMF: Hydroxymethylfurfural; BME: β-Mercaptoethanol; DTT: Dithiothreitol; DMSO: Dimethyl sulfoxide

Activities of $E1_C$ and E2 were lower when incubating with the chelating agent EDTA. It may be suggest that these are two methaloenzymes. The inhibition effect of EDTA was also observed for endoglucanases from *Bacillus* sp. [36], *Fervidobacvterium nodosum* [26], and *Penicillium purpurogenum* [22].

The SDS, an anionic surfactant and denaturing detergent promoted completely activity loss at 6 mM, however $E1_C$ showed some resistance at 1 mM of SDS, retaining 63 % of the activity. The other surfactant agents tested, Triton X-100, Tween 20 and glycerol which are non-ionic surfactants, generally promoted an increase in E2 activity and no effect on $E1_C$ activity. The stabilization effect of non-ionic surfactants is a widely recognized phenomenon and they are commonly used in protein formulations to inhibit protein aggregation [44].

Urea is one of the most commonly used denaturants of proteins. The effects of this osmolyte on protein stability have been examined extensively and it normally acts directly on the protein backbone. Urea presents a positive interaction with the protein backbone while displacing water from the first hydration shell. However, in the concentrations tested in the present study, urea had no effect on $E1_C$ activity and promoted an increase in E2 activity. The positive effect of low urea concentrations on endoglucanase activity was also observed for the enzyme from *Aspergillus aculeatus* [45].

Furfural and Hydroxymethylfurfural (HMF) are residual products from acid pretreatment of lignocellulosic biomass. Considering the application of endoglucanases in biomass hydrolysis, activity of the enzyme in the presence of these compounds is of great importance. An increase in $E1_C$ activity was observed as the concentration of furfural and HMF increased. The E2 activity, however, showed significant increase in the presence of these two compounds but maintain its maximal activity. Based on this results it is affirmed that $E1_C$ and E2 can be successfully used for cellulose hydrolysis in the presence of furfural and HMF, however studies using higher concentrations are required. Xu and collaborators [29] studied the effect of furfural on cellulase activity of the cellulosome from *C. thermocellum* and on commercial cellulase from *Trichoderma reesei*. They observed that cellulase from the cellulosome was resistant to furfural at 5 mM and the cellulase from *T. reesei* was resistant to this compound at 100 mM. However, no increase in enzyme activity was observed.

The reducing agents DTT and BME did not have significant effects on E2 activity, which suggests that any disulfide bond is important for the activity of this enzyme. On the other hand, these two reducing agents had different effects on $E1_C$ activity. BME caused no significant changes to $E1_C$, however DTT increased the activity of $E1_C$. This increase suggests the presence of a potentially reactive thiol group. These results are according to enzyme inhibition caused by the metal cations. Higher activity in the presence of a DTT was also observed in endoglucanase from *Bacillus subtilis* [33].

7.4.6- Kinetic studies

Using CMC as substrate, the Michaelis constant (K_M) of the endoglucanase activities were determined (Figure 15). $E1_C$ presented a K_M of

10.25 mg/mL while E2 presented a K_M of 6.58 mg/mL. The K_M value represents the relation of the dissociation constant per the enzyme-substrate association constant. Because the K_M of E2 was lower than that observed for $E1_C$, in general it indicates that E2 presented more relative affinity for CMC than $E1_C$. This data corroborates with that included in the activation energy discussion. Because E2 presented more relative affinity to CMC, it may demand more energy to disrupt the chemical interactions important for enzyme-substrate association; therefore it presented higher activation energy.

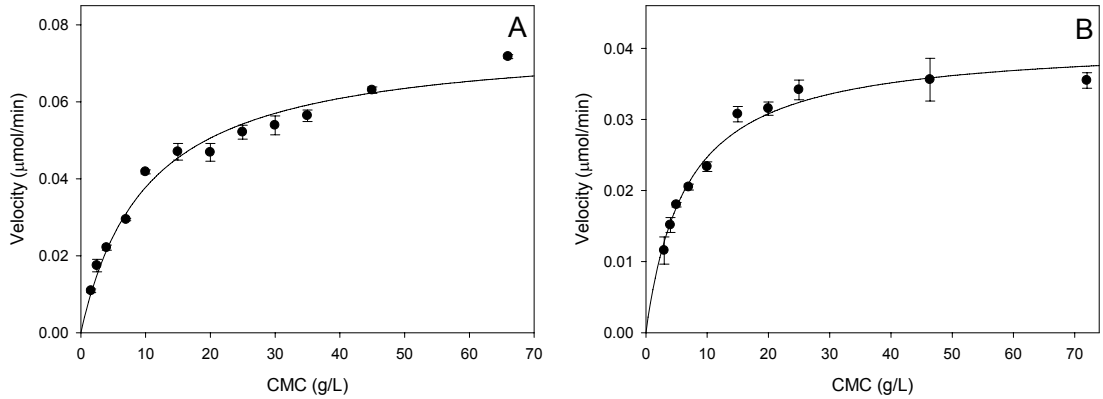

Figure 15: Michaelis-Menten plot for carboxymethylcellulose hydrolysis by $E1_C$ (A) and E2 (B) at 80 °C pH 5.0

The K_M values observed for $E1_C$ and for E2 are higher than most reported in literature. Endoglucanases from *Penicillium purpurogenum*, *Daldinia eschscholzii* and *Trichoderma reesei* presented K_M of 1.15, 1.74 and 0.84 mg/mL, respectively [7, 22, 46]. However, endoglucanases from the fungus of the same genera, *Fusarium oxysporum*, presented K_M values of 23.2 and 13.5 mg/mL, higher than those observed for $E1_C$ and E2 [30, 31].

7.4.7- Substrate specificity

The substrate specificities of $E1_C$ and E2 towards various soluble and insoluble substrates were tested (Table 5). Based on substrate specificity and mode of action, cellulases are distinguished either as endoglucanases, cellobiohydrolases, or β-glucosidases.

Table 5: Substrate specificity of $E1_C$ and E2 for various substrates

	Relative Activity (%)	
Substrate	$E1_C$	E2
CMC	100.0±1.3	100.0±3.5
CMC 2	89.6±3.9	74.8±4.0
Barley-β-glucan	333.5±15.0	247.0±10.1
Locust bean gum	38.4±0.6	16.4±1.0
Birchwood xylan	281.6±7.3	2.7±0.1
Filter paper	n.d	1.9±0.2
Avicel	n.d	n.d
Starch	n.d	n.d
pNP-β-D-Xylopyranoside	n.d	n.d
pNP-β-D-Glycopyranoside	n.d	n.d
pNP-β-D- Cellobioside	n.d	n.d
Cellobiose	n.d	0.28±0.04

n.d. not detectable

Two types of CMC were tested. The first, CMC, which presented lower molecular weight and lower degree of substitution, was better hydrolyzed by $E1_C$ and E2 than CMC 2. This result can be attributed to the higher viscosity of CMC 2 which can lower the diffusion rate and thus complicate hydrolysis, or to more difficult access of the enzyme to the substrate, since it had a higher degree of substitution. Endoglucanases from *Melanocarpus* sp. presented the same behavior, preferring the low viscosity CMC [35].

Both $E1_C$ and E2 showed higher activity toward barley-β-glucan than toward CMC, presenting 333.5 and 247.0 % greater activity, respectively. Barley-β-glucan is a glucose polymer linked with β-1,3-glycosidic bonds. These results indicate that the endoglucanase E2 and the endoglucanases presents in $E1_C$ are β-1,3-1,4-glucanases. Similar results were observed for endoglucanase from *Phialophora* sp. [42], *Fervidobacterium nodosum* [26] and *Aspergillus niger*, which were also denominated this way [47].

Activity towards hemicellulose substrates appears to have an apparent dependence on the GH family. Endoglucanases from the families GH5 and GH6 present activity towards galactomannan and xylan [48]. Similar results were observed for $E1_C$ (GH6 and GH10) and E2 (GH5) which hydrolyzed Locust bean gum (galactomannan) and birchwood xylan. Activity towards galactomannan can be attributed to a similar structural homology between endoglucanases and mannanases from the same family and to a relatively flexible active site structure of the enzymes [48]. In the case of $E1_C$, the high xylanase activity is due to the presence of the enzyme xylanase presents on the

multienzyme complex. When the sample $E1_CA$, which had no xylanase enzymes in it, was assayed towards xylan, the activity was 16 % of that towards CMC. In this case the activity can be attributed to endoglucanases of the multienzyme complex. This broad specificity is an advantageous feature for endoglucanases from *F. verticillioides*, since lignocellulosic biomass, its natural substrate, is highly heterogeneous. From this point of view $E1_C$ presents an advantage compared to E2 which acts alone and probably less efficiently than $E1_C$.

The insoluble substrates Avicel and filter paper, crystalline celluloses, were not hydrolyzed by $E1_C$. On the other hand, E2 was able to poorly hydrolyze filter paper. This result may be due to the facility of a single enzyme to access the crystalline and amorphous cellulose which may be more difficult for a multienzyme complex. Activity against this substrate is reported for endoglucanases from family 5 and 6, however in low quantities [48]. The endoglucanases from *Fusarium oxysporum* were also incapable of hydrolyzing insoluble substrates [30, 31].

Soluble starch was tested to verify the ability of the enzymes to hydrolyses α-1,4 linkages. Neither $E1_C$ or E2 presented this activity, in accordance with results reported in literature for endoglucanases from other microorganisms, indicating specificity for β-linkages [7, 26, 35].

No activity was detected towards the synthetic substrates ρNP-β-D-xylopyranoside, ρNP-β-D-glycopyranoside and ρNP-β-D-cellobioside. Cellobiose was also tested and $E1_C$ did not present activity, according to features observed for other GH6 endoglucanases whose minimum substrate is cellotriose [49]. On the other hand, E2 presented traces of activity towards cellobiose. This indicated the general lack of ability of $E1_C$ and E2 to hydrolyze small carbohydrates. This result is in accordance with literature [30, 31, 36].

7.4.8- Mode of action

7.4.8.1- Hydrolysis of cellooligossacarides

Hydrolysis of cellopentaose (G_5), cellotetraose (G_4) and cellotriose (G_3) by $E1_C$ and E2 was evaluated. The same units of enzyme activity (µmol/min/mL) were used for the two samples. Enzymes making up $E1_C$ had the ability to hydrolyze all cello-oligosaccharides (Figure 16).

When using $E1_C$, the main G_5 hydrolysis products were G_3 (62 %) and G_2 (30 %) and the main hydrolysis product of G_4 was G_2 (77 %) (Figure 12). These results indicated that $E1_C$ preferentially cleaved the internal glycosidic bond in G_5 and G_4. However, the enzyme was also capable of act on the external linkages as can be observed by the release of different products at lower concentrations.

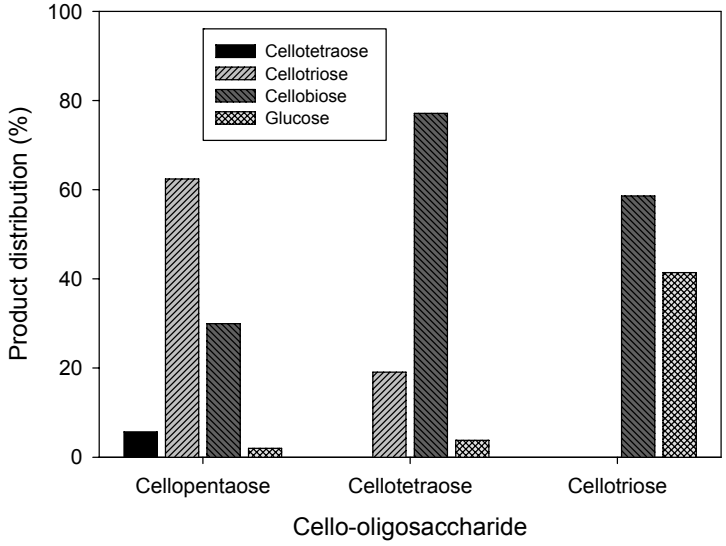

Figure 16: Hydrolysis of cello-oligosaccharides by the endoglucanase $E1_C$ after 1h of reaction

The large concentration difference between G_3 and G_2 for G_5 hydrolysis, between G_3 and G_1 for G_4 hydrolysis and between G_2 and G_1 for G_3 hydrolysis is an indicator of transglycosilation activity. These features are similar to those observed for endoglucanases from *Thermoascus aurantiacus* [27] and from a ruminal bacterium [50]. Similar results were also observed for endoglucanase from *Fusarium oxysporum*, however, enzymes of this organism preferentially release G_2 from substrates, even in G_5 hydrolysis [30, 31]. After 1 h of reaction the yields of G_5, G_4 and G_3 hydrolysis were 34.5, 21.5 and 3.6, indicating that as the chain length of substrate increased the enzyme efficiency also increased, which was indicated as a common feature for endoglucanases of the family GH6 [49]. Endoglucanases from *Anabaena laxa* also presented less efficiency for hydrolysis of short chain cello-oligosaccharides [39].

The hydrolysis of cello-oligosaccharides by E2 was also evaluated (Figure 17). As was observed for $E1_C$, G_3 and G_2 were the main products from hydrolysis of G_5. The presence of G_4 was also observed, however no G_1 was

detectable. It is possible that E2 presented a high transglycosilation activity and perhaps the glucose formed was rapidly transferred to any other sugar.

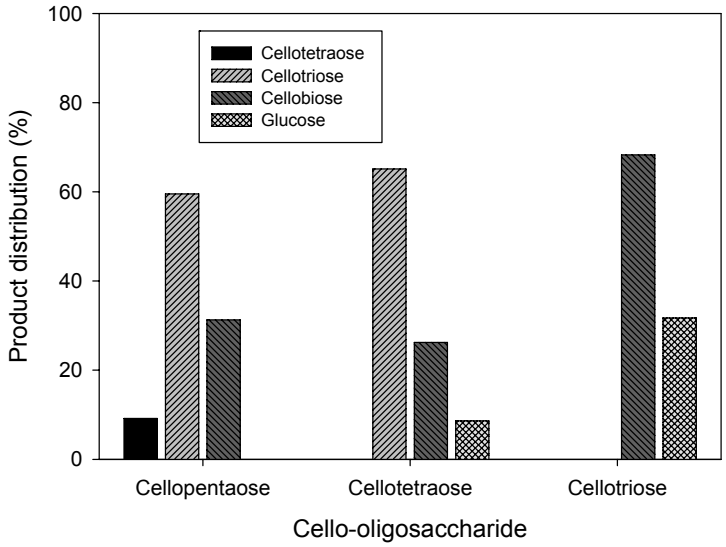

Figure 17: Hydrolysis of cello-oligosaccharides by the endoglucanase E2 after 1h of reaction

Hydrolysis of G_4 presented different results when comparing the enzymes $E1_C$ and E2. The enzyme E2 seems to preferentially cleave the end glycosydic bond to release G_3 as the main product, an exo-type hydrolysis, while $E1_C$ presented mainly endo-type hydrolysis. This result was also observed for G_5 and for G_4 hydrolysis. As was observed for $E1_C$, hydrolysis products were not released in an equimolar fashion, indicating a potential transglycosylating activity. After 1 h of reaction the yields for G_5, G_4 and G_3 hydrolysis were 2.2, 2.5 and 2.44. Different from $E1_C$, E2 did not shown high differences in efficiency as the chain length of the substrate varied from 5 or 3 monomers. Moreover, the total hydrolysis efficiency of $E1_C$ was much higher than that obtained by E2 hydrolysis. The better performance of $E1_C$ can be attributed to its multienzyme complex nature, which relies on the synergy between one cellobiohydrolase and two endoglucanases. These enzymes probably worked together to obtain improved hydrolysis performance and substrate channeling may also contributes to its better performance.

The enzyme E2 presented features common to others endoglucanases from the family GH5. Endoglucanases from this family preferentially release cellotriose from cello-oligosaccharides and the efficiency of the enzyme is unaffected by the substrate chain length [49].

7.4.8.2- Hydrolysis of CMC

Hydrolysis of CMC by $E1_C$ produced a mixture of G_5, G_4, G_3, G_2 and G_1 (Figure 18).

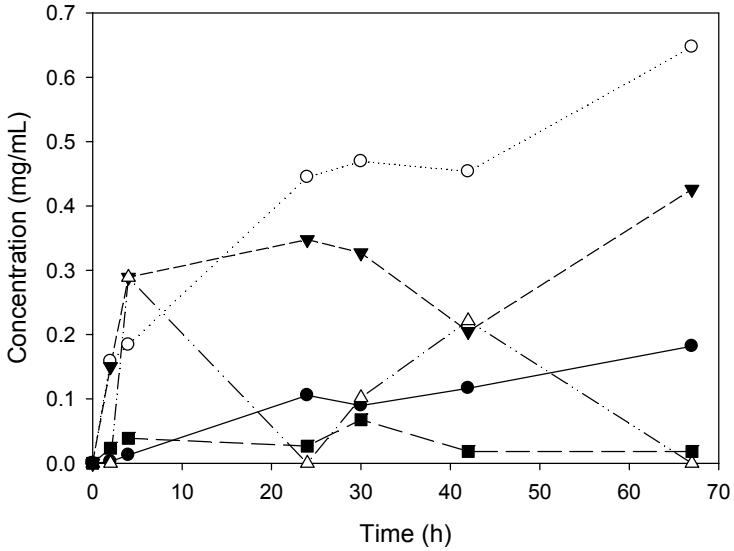

Figure 18: Carboxymethyl cellulose hydrolysis by $E1_C$ at 60 °C. Glucose (●), Cellobiose (○), Cellotriose (▼), cellotetraose (△), cellopentaose (■).

In the initial hours, G_4, G_3 and G_2 were the main products of CMC hydrolysis. This suggests that $E1_C$ preferentially acts on internal glycosidic bonds of a polymer to release these small products. Cellopentaose was detected all reaction times, however in lower concentrations than G_4, G_3 and G_2. It is possible that as soon as G_5 was released it was readily hydrolyzed, since $E1_C$ presented high efficiency for hydrolyzing this substrate. Because G_4 and G_3 are both substrate and products, they alter between increasing and decreasing stages as they are being releasing or hydrolyzed. On the other hand, cellobiose presented continuously increasing concentrations. This effect is in accordance with the result observed for substrate specificity, where it was indicated that cellobiose is not a substrate for $E1_C$. Cellobiose accumulation was also observed in CMC hydrolysis when using endoglucanase from *Daldinea eschscholzii* [7] and from *Gloeophyllum trabeum* [51]. The final hydrolysis yield, considering the production of G_5, G_4, G_3, G_2 and G_1 was 12.7 % after 67 h of hydrolysis.

Hydrolysis of CMC by E2 produced a mixture of G_5, G_3, G_2 and G_1 (Figure 19). The cello-oligosaccharides G_4, G_3 and G_2 were the main products in the initial hours of hydrolysis, indicating preferentially of hydrolysis similar to that

observed for $E1_C$. However, after 16 hours of hydrolysis, the main products were G_5, G_3 and G_2. The G_4 was completely hydrolyzed and after 30 hours it was no longer detected. As was observed in the hydrolysis of cello-oligosaccharides, E2 did not present any preference of hydrolysis according to chain length of the substrate, where long chain polymers such as G_5 accumulated just as the short chains like G_2, differently from that observed for $E1_C$. The final hydrolysis yield, considering the production of G_5, G_4, G_3, G_2 and G_1 was 19.6 % after 67 of hydrolysis. The better performance of E2 to hydrolyze the polymer CMC comparing with $E1_C$, may be attributed to the multienzyme complex nature of $E1_C$ which can difficult the access to the substrate.

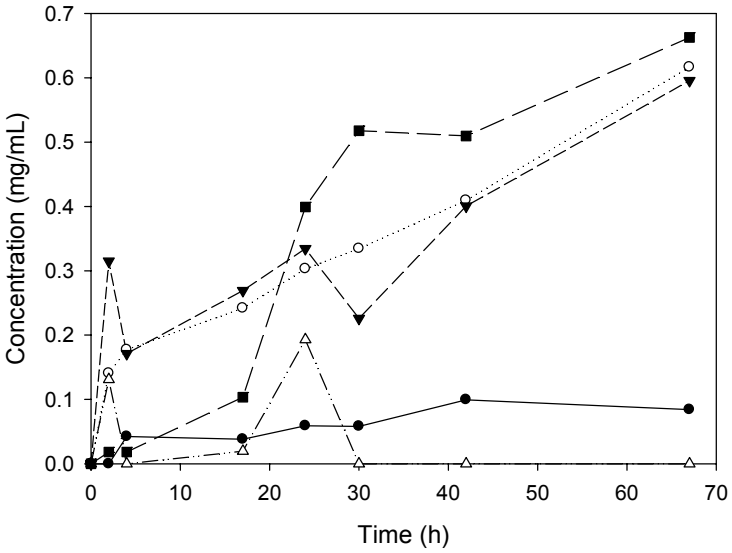

Figure 19: Carboxymethyl cellulose hydrolysis by E2 at 60 °C. Glucose (●), Cellobiose (○), Cellotriose (▼), cellotetraose (△), cellopentaose (■).

7.5- Conclusion

One multienzyme complex, $E1_C$, and one free endoglucanase, E2, from *F. verticillioides* were purified and presented interesting features in relation to biotechnological application. The multienzyme complex generally showed high stability and hydrolysis efficiency when compared to the free enzyme. Both $E1_C$ and E2 presented activities over a wide pH and temperature range. This is an advantage because different applications require different reaction conditions. The high thermostability is one of the remarkable features of the multienzyme complex $E1_C$ and it was also observe less extensively for E2. Thermostability is an important feature for industrial application, since it can mean less frequent enzyme replacement and ability to conform to changes in reactor temperatures.

Additionally, $E1_C$ was activated by furfural and hydroxymethylfurfural and E2 was not affected by them. These two compounds may potentially be present for conversion of lignocellulosic biomass to ethanol, an important application for endoglucanases. All of theses features indicate that the cellulase system from *F. verticillioides* can be used successfully in biotechnological applications.

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8- Conclusões gerais

- O fungo endofítico *Fusarium verticillioides* apresenta um relevante potencial para produção de hidrolases visando aplicação em processos de sacarificação de biomassa lignocelulósica e produção de etanol.
- O perfil de secreção de celulases e xilanase do fungo *F. verticillioides* pode ser alterado por meio de manipulação das condições de cultivo.
- As condições para a produção de diferentes atividades enzimáticas pelo fungo *F. verticillioides* foram otimizadas e as máximas atividades obtidas para as atividades de endoglicanase, FPase, xilanase e celobiase foram 8,0 U/mL, 0,6 U/mL, 114,0 U/mL e 6,8 U/mL, respectivamente.
- O fungo *F. verticillioides* apresentou grande potencial para ser utilizado na produção de etanol no Bioprocesso Consolidado, uma vez que foi capaz de produzir etanol diretamente de bagaço de cana.
- Duas endoglicanases produzidas pelo fungo *F. verticillioides*, $E1_C$ e E2, foram purificadas e caracterizadas bioquímica e cinéticamente.
- A enzima $E1_C$ é secretada na forma de um complexo multi-enzimático contendo duas endoglicanases, uma celobiohidrolase e uma xilanase.
- $E1_C$ e E2 apresentaram alta termoestabilidade a 50 e 60 °C o que é uma vantagem para uso destas enzimas em processos biotecnológicos que necessitem temperaturas elevadas.