DANIEL LUCIANO FALKOSKI

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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: 25 de fevereiro de 2011

Prof. Acelino Couto Alfenas
(Coorientador)

Prof. Jorge Luiz Colodette
(Coorientador)

Profª. Elza Fernandes Araújo

Prof. Cirano José Ulhoa

Prof. Sebastião Tavares de Rezende
(Orientador)

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BIOGRAFIA

DANIEL LUCIANO FALKOSKI, filho de Isidoro Antônio Falkoski e Terezinha Maria Falkoski, nasceu na cidade de Santo Antônio da Patrulha, Rio Grande do Sul, em 24 de outubro de 1980.

Em agosto de 1999, formou-se Técnico em Agropecuária pela Escola Estadual Ildefonso Simões Lopes, Osório, Rio Grande do Sul, Brasil.

Em janeiro de 2005, graduou-se em Engenharia Agronômica pela Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brasil.

Em fevereiro de 2007, tornou-se mestre em Bioquímica Agrícola pela Universidade Federal de Viçosa. Em março do mesmo ano iniciou o curso de doutorado no Programa de Pós-Graduação em Bioquímica Agrícola, nessa mesma Universidade, submetendo-se a defesa de tese em 25 de fevereiro de 2011.

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RESUMO

FALKOSKI, Daniel Luciano, D. Sc., Universidade Federal de Viçosa, fevereiro de 2011. **Enzimas lignocelulolíticas de fungos de podridão branca e fitopatógenos: produção, caracterização e aplicação em processos de sacarificação da biomassa.** Orientador: Sebastião Tavares de Rezende. Coorientadores: Valéria Monteze Guimarães, Acelino Couto Alfenas e Jorge Luiz Colodette.

Neste trabalho, seis diferentes cepas fúngicas isoladas de plantações de eucalipto foram avaliadas quanto aos seus potenciais para produção de enzimas lignocelulolíticas visando suas aplicações em processos biotecnológicos, mais precisamente, processos de sacarificação da biomassa. Os fungos *Pycnoporus sanguineus, Trametes* sp. J2, *Trametes* sp J5, isolado J-129 (um basidiomiceto não identificado), *Chrysoporthe cubensis* e *Cylindrocladium pteridis* foram cultivados em meio líquido (ML) e meio semi-sólido (MSS) contendo farelo de trigo, sabugo de milho, polpa Kraft e celulose microcristalina (Avicel) como fonte de carbono. Após 4, 8 e 12 dias de fermentação os extratos enzimáticos produzidos foram coletados e analisados para determinação das atividades de xilanase, endoglicanases, β-glicosidases, lacases e celulase total (FPase). *Pycnoporus sanguineus* apresentou as maiores atividades para FPase (polpa Kraft-MSS) e lacase (farelo de trigo-ML) sugerindo que este microrganismo pode ser utilizado como um eficiente produtor de ambos os grupos de enzimas: cellulases e ligninases. *Chrysoporthe cubensis* propiciou as maiores atividades para endoglicanase e β-glicosidase (farelo de trigo-SSF). Sendo assim, foi demonstrado pela primeira vez que *C. cubensis* possui um grande potencial para produção de enzimas (principalmente celulases) para aplicação em processos de sacarificação da biomassa. As maiores atividades xilanolíticas foram encontradas em extratos produzidos pelo fitopatógeno *C. pteridis*, o qual foi apto a secretar quantidades incomuns de xylanase (aproximadamente 150000 U L^{-1}) quando cultivado em meio semi-sólido usando farelo de trigo como substrato. *Pycnoporus sanguineus* e *C. cubensis* secretaram as maiores atividades celulolíticas e por isto os extratos enzimáticos produzidos por estes microrganismos foram caracterizados e subseqüentemente aplicados em testes de sacarificação da biomassa. Além de celulases e xilanases, *P. sanguineus* e *C. cubensis* também mostraram uma marcante capacidade para secretar atividades de α-

arabinofuranosidase, α-galactosidase, β-mananase e poligalacturonase. Além disto, baixas atividades para β-xilosidase e β-manosidase também foram detectados em ambos os extratos enzimáticos. As atividades celulolíticas (endoglicanase, FPase e β-glicosidase) e xilanolíticas produzidas por *P. sanguineus* e *C. cubensis* foram caracterizadas em relação a pH e temperatura ótimos e foi observado que todas as atividades enzimáticas analisadas foram maximamente ativas quando incubadas em uma faixa de pH entre 3,5 e 4,5. Os valores de temperaturas ótimas variaram entre 50 e 60 °C. Além disto, todas as atividades enzimáticas foram altamente estáveis nas temperaturas de 40 e 50 °C tendo mantido mais de 70 % de suas atividades residuais após 48 h de incubação. Os extratos celulolíticos de *P. sanguineus* e *C. cubensis* foram aplicados em experimentos de sacarificação utilizando bagaço de cana, previamente submetido à pré-tratamento ácido ou básico, como substrato e os resultados de sacarificação foram comparados com aqueles obtidos utilizando-se uma preparação comercial de celulases. O uso de pré-tratamento ácido foi pouco eficiente na remoção da lignina junto ao bagaço de cana e como conseqüência disso observou-se um baixo rendimento de sacarificação quando este substrato foi utilizado, independentemente do extrato enzimático aplicado. Por outro lado, quando bagaço pré-tratado com base foi utilizado como substrato, elevadas taxas de hidrólise foram observadas. Considerando-se a produção de equivalentes açúcares redutores, foram observados rendimentos de sacarificação de 89,0, 60,4 e 64,0 % após 72 h de reação nos ensaios conduzidos com extrato de *C. cubensis*, extrato de *P. sanguineus* e com a preparação de celulases comercial, respectivamente. A produção de glicose também foi avaliada e, similarmente, se observou uma maior liberação deste monossacarídeo nos ensaios realizados com extrato do fungo *C. cubensis* (52,7 %). Para os ensaios de sacarificação utilizando extrato de *P. sanguineus* e celulase comercial a produção de glicose observada correspondeu a 22,6 e 36,6 % do rendimento teórico possível, respectivamente. Os resultados obtidos sugerem que as seis cepas fúngicas avaliadas neste trabalho têm grande potencial como produtoras de enzimas para aplicações em processos biotecnológicos. Os extratos celulolíticos de *P. sanguineus* e *C. cubensis* demonstraram um grande potencial para serem utilizados em processos de sacarificação da biomassa uma vez que o desempenho de hidrólise observado

para estes extratos foi similar ou superior àquele observado em ensaios utilizando-se uma preparação de celulases comercial.

ABSTRACT

FALKOSKI, Daniel Luciano, D. Sc., Universidade Federal de Viçosa, February, 2011. **Ligninolytic enzymes from White-rot and phytopathogenic fungi: production, characterization and application in biomass saccharification process.** Adviser: Sebastião Tavares de Rezende. Co-advisers: Valéria Monteze Guimarães, Acelino Couto Alfenas and Jorge Luiz Colodette.

In this work, six different fungi strain isolated from eucalyptus plantations were evaluated in relation to their ability to produce ligninolytic enzymes to biotechnological application, specifically, biomass saccharification for bioethanol production. The fungi *Pycnoporus sanguineus, Trametes* sp. J2, *Trametes* sp J5, isolated J-129 (a Basidiomycete unidentified), *Chrysoporthe cubensis* e *Cylindrocladium pteridis* were cultivated in submerged fermentation (SmF) or in solid state fermentation (SSF) using wheat bran, corn cobs, Kraft pulp or microcrystalline cellulose (Avicel) as carbon source. After 4, 8 and 12 days of fermentation, the enzymatic extract produced were analyzed in relation to xylanase, endoglucanase, β-glucosidase, laccase and total cellulase (FPase) activities. The white-rot Basidiomycete *P. sanguineous* presented the highest FPase (Kraft pulp-SSF) and laccase (wheat bran-SmF) activities, suggesting that this microorganism could be used as an efficient producer for the two groups: cellulases and ligninases. *Chrysoporthe cubensis* presented the highest endoglucanase and β-glucosidase (wheat bran-SSF) activities. Therefore, for the first time, it was demonstrated that *C. cubensis* has a great potential to enzyme production (mainly cellulases) that could be applied in biomass saccharification processes. The highest xylanolytic activities were detected in enzymatic extracts produced by the plant pathogenic *C. pteridis*, which was able to secrete uncommon amounts of xylanase activity (approximately 150000U L^{-1}) when cultivated in SSF using wheat bran. *P. sanguineus* and *C. cubensis* were able to secrete the highest amounts of cellulolytic activities and therefore the enzymatic extracts produced by these microorganisms were characterized and subsequently applied in biomass saccharification experiments. Besides cellulases and xylanases, *P. sanguineus* and *C. cubensis* were also able to produce α-arabinofuranosidase, α-galactosidase, β-mananase and polygalacturonase. It was also detected low activities of β-xylosidase and β-mannosidase in both enzymatic extracts. The cellulolytic activities

(endoglucanase, FPase and β-glucosidase) and xylanolytic activities produced by *P. sanguineus* and *C. cubensis* were characterized in relation to pH and temperature and it was observed that all activities were maximized in a pH range of 3.5-4.5. The optimum temperatures for those enzymes varied between 50 and 60 °C. Besides, all enzymatic activities were highly stable at 40 and 50 °C, maintaining more than 70 % of its residual activities after 48 h of pre incubation at the aforementioned temperatures. The enzymatic extracts from *P. sanguineus* and *C. cubensis* were employed in saccharification experiments using acid-treated or alkali-treated sugarcane bagasse as substrate. The saccharification results were compared to those obtained employing a commercial cellulase. The acid pretreatment presented low efficiency in lignin removal from sugarcane bagasse and as a consequence, it was observed a low yield of saccharification when acid-treated bagasse was hydrolyzed, independently from the cellulase extract utilized. On the other hand, when alkali-treated sugarcane bagasse was used as substrate for saccharification, high hydrolysis rates were observed. Considering equivalent reducing sugars production, it was observed saccharification yields of 89.0, 64.4 and 64.0 % after 72 h of hydrolysis reaction using extract from *C. cubensis*, extract from *P. sanguineous* and a commercial cellulase, respectively. The glucose production was also evaluated and, similarly it was observed a higher yield in the saccharification using extract from *C. cubensis* (52.7 %) than in those using extract from *P. sanguineous* (22.6 %) or commercial cellulase (36.6 %). The results obtained suggest that the six fungi strains evaluated in this work present a great potential to produce enzymes for application in biotechnological processes. The cellulolytic extracts from *P. sanguineous* and *C. cubensis* demonstrated a great potential to be employed in biomass saccharification processes, since their observed hydrolysis performances were similar or superior to that observed performance for commercial cellulase.

1. Introdução e justificativa

A produção de bioetanol a partir de biomassa lignocelulósica desponta atualmente como uma das alternativas mais promissoras para a substituição dos combustíveis fósseis. Dentre as principais vantagens relacionadas ao uso do bioetanol derivado de biomassa destaca-se o fato de que se trata de um combustível renovável e que sua utilização contribui para a redução da emissão de gases causadores do efeito estufa. Considerando também que atualmente a maioria do bioetanol produzido no mundo deriva da cana-de-açúcar e do milho, o uso do bioetanol lignocelulósico representaria uma diminuição da utilização de grandes áreas para o cultivo destas culturas, o que reduziria as oscilações nos preços dos alimentos e os riscos de avanços das fronteiras agrícolas para regiões de preservação. As principais fontes de biomassas com grande potencial para serem utilizadas na fabricação de bioetanol são os resíduos agrícolas, os resíduos florestais e resíduos de lixo urbano.

Entretanto, apesar das inúmeras vantagens sugeridas, o uso do bioetanol obtido a partir de biomassa lignocelulósica ainda é uma realidade distante, principalmente devido a uma série de gargalos econômicos e tecnológicos. O processo de produção de etanol a partir de biomassa pode ser dividido em três etapas críticas:

- Pré-tratamento - nesta etapa a biomassa é tratada física ou quimicamente com o objetivo de eliminar, ou reduzir, a lignina e promover a desorganização da estrutura cristalina da celulose, facilitando o ataque das enzimas hidrolíticas no passo seguinte que é a sacarificação. É uma etapa cara, exigente em energia e reagentes químicos, e que ainda favorece a produção de uma série de compostos tóxicos como furfural, hidroximetilfurfural e outros ácidos orgânicos que inibem os micro-organismos fermentadores.
- Sacarificação - a sacarificação consiste na utilização de enzimas para a hidrólise da celulose presente na biomassa em glicose, um açúcar prontamente fermentável. As principais enzimas utilizadas nesse processo são as celulases e estas enzimas são obtidas principalmente de fungos dos gêneros *Aspergillus* e *Trichoderma*. A eficiência desta etapa é bastante alta, entretanto, os elevados custos para a produção destas enzimas, somados a grande complexidade da

biomassa vegetal requer que coquetéis enzimáticos mais eficientes sejam desenvolvidos e que o custo da produção destas enzimas seja reduzido.

- Fermentação – nesta etapa o xarope rico em glicose obtido na sacarificação é fermentado, e, após a destilação deste material, podemos recupera o álcool combustível. Esta etapa é primeiramente limitada pela presença de inibidores da fermentação que são formados principalmente durante o pré-tratamento. Outro fato que limita a eficiência desta etapa é o alto teor de pentoses, principalmente xilose, contido no hidrolisado. O principal micro-organismo utilizado para a produção de etanol, a levedura *Saccharomyces cerevisiae*, é inapta para a fermentação de pentoses e isto certamente acarreta em uma redução da produção de etanol a partir de biomassa lignocelulósica. Pesquisas envolvendo engenharia metabólica e busca por novos micro-organismos fermentadores de pentoses vem sendo realizadas com o objetivo de maximizar a utilização do hidrolisado para a produção de etanol, entretanto os resultados ainda são economicamente insatisfatórios.

A crescente demanda por energia somada ao esgotamento das fontes de combustível fósseis e a necessidade da redução da emissão de gases causadores de efeito estufa requerem pesquisas que possam contribuir para a redução destes gargalos tecnológicos, de forma que a produção de etanol a partir de biomassa lignocelulósica seja viabilizada técnica e economicamente.

Considerando que a tecnologia de produção e aplicação enzimática é um dos maiores limitantes para a produção de bioetanol lignocelulósico, focalizamos neste trabalho, a avaliação do potencial de fungos basidiomicetos causadores de podridão branca e fungos fitopatogênicos em produzir enzimas lignocelulolíticas. Estudos com estes fungos são particularmente interessantes devido à alta capacidade que esses micro-organismos têm para degradar a celulose em seus ambientes naturais. Não obstante, foi de nosso interesse também avaliar o real potencial de utilização dos extratos enzimáticos produzidos por esses fungos em processos de sacarificação de biomassas de grande interesse econômico, como o bagaço de cana.

2. Objetivos

- Avaliar o potencial das cepas fúngicas *Pycnoporus sanguineus*, *Trametes* sp. J2, *Trametes* sp. J5, isolado J-129 (não identificado), *Chrysoporthe cubensis* e *Cylindrocladium pteridis* para a produção de xilanases, endoglicanases, β-glicosidases, lacases e atividade de celulase total (FPase) sob diferentes condições de cultivo utilizando diferentes fontes de carbono.

- Selecionar os micro-organismos e as condições de cultivo que proporcionem a obtenção de extratos com as maiores atividades enzimáticas.

- Caracterizar as atividades enzimáticas presentes nos extratos mais promissores para sacarificação da biomassa, determinando temperatura e pH ótimos e termoestabilidade.

- Avaliar o efeito de diferentes processos de pré-tratamento químico sobre a composição da biomassa (bagaço de cana) e determinar os efeitos de cada pré-tratamento sobre a subseqüente hidrólise enzimática.

- Testar o potencial dos extratos enzimáticos mais promissores em um experimento de sacarificação utilizando bagaço de cana pré-tratado como substrato e contrastar os resultados alcançados com esses extratos com àqueles obtidos utilizando-se uma preparação de celulases comercial.

3. Revisão bibliográfica

3.1. Panorama da matriz energética mundial

A demanda por energia é crescente devido ao rápido crescimento da população e ao desenvolvimento dos processos industriais. Entretanto, o desenvolvimento de novas fontes de energia não vem acompanhando esta demanda de consumo. Nem mesmo os países desenvolvidos estão habilitados a compensar esta crescente demanda por energia, mesmo que estes estejam constantemente investindo na diversificação de sua matriz energética. Atualmente, a energia utilizada para suprir as necessidades da maior parte dos setores de produção e de transporte advém de fontes convencionais como petróleo, carvão mineral e gás natural. Dentro deste cenário a depleção dos combustíveis fósseis e os graves problemas ambientais advindos da combustão indiscriminadas destes combustíveis surgem como os dois principais problemas relacionados à atual matriz energética no mundo *[1, 2]*.

Em estudo realizado pela Companhia Britânica de Petróleo (BP) em 2008 foi estimado que as reservas mundiais de petróleo, gás natural e carvão são de 168,3 bilhões de toneladas, 177,4 trilhões de m^3 e 847,5 bilhões de toneladas, respectivamente *[3]*. Considerando a atual tendência de consumo destes combustíveis estima-se que, em aproximadamente 40 anos as reservas de petróleo no mundo estarão praticamente exauridas. Observou-se no ano de 2007 uma produção de 3,90 bilhões de toneladas de petróleo no mundo. Este valor foi 0,2% menor do que registrado no ano anterior. Apesar de efêmera, esta redução/estagnação pode estar confirmando os estudos realizados por Campbell & Laherrere *[4]* que utilizando diferentes modelos de estimativas concluíram que a produção mundial declinaria a partir do ano de 2010, partindo de uma produção estimada de 25 bilhões de barris/ano para aproximadamente 5 bilhões de barris/ano no ano de 2050.

Considerando ainda os dados da BP, estima-se que as reservas de gás natural e carvão seriam suficientes para abastecer as atuais necessidades mundiais durante os próximos 60 e 133 anos respectivamente. Estas previsões são menos dramáticas, porém não menos preocupantes que aquelas relacionadas ao consumo de petróleo.

O fato da economia dos países desenvolvidos e em desenvolvimento ser altamente dependente de petróleo e de outros combustíveis fósseis sugere que

a inadequada disponibilidade destes recursos poderia gerar graves desequilíbrios econômicos, sociais e políticos em todo mundo. Desta forma, existe um grande interesse na exploração de fontes alternativas de energia que possam vir a substituir os combustíveis fósseis *[5]*.

Se do ponto de vista econômico e tecnológico o futuro da matriz energética mundial, sedimentada em combustíveis fósseis, encontra-se ameaçada, observa-se que do ponto de vista ambiental as questões são ainda mais ameaçadoras. A extração de energia dos combustíveis convencionais é um processo extremamente poluente *[1]*. A queima de combustíveis fósseis é a principal causa da liberação de gases como CO, CO_2, SO_2 e NO_x na atmosfera *[6]*.

A emissão de SO_2 produzido na queima de combustíveis fósseis é a maior causa de chuvas ácidas. Globalmente, o aumento nas taxas de emissão dos chamados gases de efeito estufa, como o CO_2 e NO_x representam uma ameaça ao clima mundial *[7]*. Estima-se que nos últimos 40 anos cerca de 15.000 milhões de toneladas de CO_2 foram liberadas na atmosfera devido à queima de combustíveis fósseis *[8]*. O acúmulo destes gases na atmosfera é apontado como uma das principais causas para as alterações climáticas que são observadas atualmente *[9]*. O atual cenário e a manutenção destas taxas de emissão apontam para a ocorrência de algumas calamidades naturais tais como: inundações, secas prolongadas, elevação da temperatura média em todo o globo, elevação do nível dos oceanos, alteração dos regimes pluviométricos, alterações das fronteiras agrícolas *[1, 10]* e em última instância todas estas possibilidades podem até mesmo ameaçar a vida no planeta *[11]*.

3.2. Biomassa e biocombustíveis

Atualmente, existe a necessidade do desenvolvimento de fontes alternativas de energia que sejam pouco onerosas, renováveis e não poluentes. Por isto uma grande atenção vem sendo dispensada para fontes de energia como a energia solar, eólica, térmica, hidrelétrica e proveniente de biomassa *[1]*.

Nos últimos anos muitos esforços têm sido dirigidos para o desenvolvimento de tecnologias que empregam biomassa como matéria-prima para a obtenção de energia *[5, 10, 12-15]*.

Biomassa compreende toda a matéria viva, ou proveniente de um ser vivo, existente na Terra. Do ponto de vista energético, a biomassa derivada de tecidos vegetais é aquela com maior potencial para ser aplicada em processos de produção energia ou combustíveis. As fontes de biomassa vegetal são materiais orgânicos no qual a energia solar está armazenada em ligações químicas. Ela é geralmente constituída de carbono, oxigênio, hidrogênio e nitrogênio. Enxofre também pode ser encontrado, porém em menores quantidades *[16]*. Dentre as principais vantagens que colocam o uso de biomassa vegetal como uma das mais promissoras alternativas para a substituição dos combustíveis fósseis pode-se citar:

- As fontes de biomassa são renováveis e potencialmente sustentáveis;
- Os danos ambientais advindos do uso de biomassa como fonte de energia são menores do que aqueles provocados pelo uso de combustíveis fósseis;
- Entre todas as fontes alternativas de energia a biomassa vegetal é a única que armazena a energia solar de forma efetiva *[1]*;
- Biomassa vegetal é a única fonte de energia renovável que pode ser convertida em combustíveis sólidos, líquidos e gasosos, por meio de diferentes processos de conversão *[17]*;
- Quando a biomassa vegetal é utilizada como fonte de energia pela combustão direta (como lenha, por exemplo) uma menor quantidade de resíduos é produzida, comparativamente ao uso do carvão mineral. Posteriormente estes resíduos podem ser empregados em processos para a produção de fertilizantes *[18]*;
- Excetuando-se condições de sazonalidade, praticamente todas as regiões do mundo produzem algum tipo de biomassa vegetal. O desenvolvimento de tecnologias apropriadas para a transformação de cada tipo de biomassa em combustíveis líquidos e gasosos pode contribuir para uma maior independência dos países em desenvolvimento com relação aos combustíveis fósseis *[18]*;
- A utilização da biomassa como matéria-prima para a produção de energia pode contribuir para a redução dos níveis de CO_2 na atmosfera uma

vez que a fotossíntese, processo biológico diretamente ligado à síntese de biomassa, é um forte dreno do CO_2 atmosférico *[1]*.

Biocombustível, seja ele líquido ou gasoso, é todo combustível que é derivado de algum tipo de biomassa. Biocombustíveis podem ser produzidos a partir de açúcares, óleos vegetais, cereais, lixo orgânico e resíduos agrícolas (palha de milho, bagaço de cana, casca de arroz, etc.). O emprego dos biocombustíveis como substituintes dos combustíveis fósseis está diretamente relacionado à eficiência da produção dos mesmos. Por isso, inúmeras pesquisas envolvendo o desenvolvimento e o aprimoramento de tecnologias de produção desses combustíveis vem sendo realizadas em todos os países do mundo *[10, 19]*.

Biocombustíveis são convencionalmente produzidos usando-se processos de catálises químicas e biológicas. Por outro lado, recentes avanços têm promovido a ampliação do uso de inúmeras enzimas e micro-organismos para o desenvolvimento de produtos e processos. Existem três tecnologias destacadas para a produção de biocombustíveis, cada uma derivada de diferentes tipos de biomassas e que resultam na produção de três diferentes biocombustíveis: biogás, biodiesel e bioetanol *[10]*.

Bioetanol: o bioetanol ou álcool etílico (C_2H_5OH) é uma molécula orgânica obtida pela fermentação de açúcares simples (hexoses e pentoses) por micro-organismos como *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia pastoris* e *Thermoanaerobacterim saccharolyticum [20]*.

Bioetanol é um combustível transparente de alta octanagem e que possui propriedades desejáveis para a utilização em motores de combustão interna *[1, 14]*. O bioetanol pode ser empregado de várias maneiras como um combustível de transporte, podendo ser usado de forma direta ou misturado à gasolina. A mistura álcool/gasolina pode ser diretamente utilizada em motores de combustão interna desenvolvidos para gasolina sem prejuízos significativos para seu desempenho. Em países como o EUA o bioetanol é comumente misturado à gasolina em uma proporção de 10:90 sendo denominado E10 ou “gasohol”. No Brasil, o etanol é utilizado puro e também misturado à gasolina em uma proporção de 24:76 *[21]*.

O bioetanol é um combustível que contém 35% de oxigênio e por este fato sua combustão gera baixa emissão de gases derivados de oxido nitroso (NO_x). O alto teor de oxigênio do bioetanol também melhora a combustão da gasolina quando esta é misturada a este biocombustível, reduzindo a emissão de gases derivados da combustão incompleta nos motores, que geralmente liberam CO e hidrocarbonetos *[19]*. Devido a estas propriedades, o uso do bioetanol misturado à gasolina (ou puro) pode reduzir significativamente o uso do petróleo e a emissão de gases poluentes *[22]*.

Biodiesel: o biodiesel é um liquido quase incolor produzido a partir da transesterificação de óleos vegetais e gorduras animais e que possui propriedades muito semelhantes às do óleo diesel derivado do petróleo. Considerando-se o índice de cetanos, um parâmetro utilizado para avaliar a qualidade de óleos combustíveis, observa-se que 1 L de óleo diesel pode gerar 35,6 MJ de energia ao passo de que 1 L de biodiesel pode gera 33,1 MJ. Tamanha semelhança indica o biodiesel como uma das mais viáveis alternativas para a substituição de combustíveis oleosos derivados do petróleo seja ele de forma direta ou como mistura.

A atual produção mundial de biodiesel é de cerca de 5 bilhões de galões e o cenário aponta para um grande aumento na produção deste biocombustível, uma vez que muitos países estão implementando a utilização de uma mistura 10:90 entre biodiesel e diesel convencional *[10, 23]*. Atualmente a produção de biodiesel se dá principalmente por meio de processos químicos baseados na metanólise e empregando catálise básica ou ácida. Entretanto, veem sendo desenvolvidos em escalas laboratoriais métodos que utilizam lipases como catalisadoras do processo de transesterificação de óleos vegetais *[24]*.

O emprego de enzimas na produção de biodiesel apesar de oneroso é altamente desejável. A transesterificação por metanólise ácida ou básica, apesar de eficiente, é extremamente poluente, gera resíduos tóxicos que não podem ser reaproveitados, exige um grande volume de água que precisa ser posteriormente tratada e exige que processos de purificação sejam empregados na recuperação do glicerol, um subproduto da reação de grande valor comercial *[25]*. Uma das alternativas mais atrativas para a redução de custos atrelados à utilização de enzimas seria o desenvolvimento de processos

de imobilização de lipases ou de células contendo lípases. Processos de imobilização de enzimas geralmente melhoram as propriedades catalíticas das enzimas e permitem a sua reutilização promovendo quedas significativas no custo final do processo *[10, 26]*.

Biogás: considerando-se a atual conjectura da matriz energética mundial, a utilização do gás metano também desponta como uma alternativa promissora para a substituição dos combustíveis fósseis *[10, 27, 28]*. O biogás, ou metano derivado de biomassa, é obtido a partir da ação de bactérias metanogênicas sobre a matéria orgânica na ausência de oxigênio.

O processo pode ser subdividido em três etapas. Em um primeiro momento a biomassa sofre um processo de hidrólise. Isto pode ser alcançado naturalmente, como por exemplo, pela ação de bactérias do gênero *Clostridia* e *Streptococci* ou por meio de hidrólise química. Em um segundo momento, por ação de bactérias anaeróbicas, o hidrolisado passa por um processo de fermentação acidogênica, onde os açúcares e demais substratos são metabolicamente transformados em CO_2, acido acético, H_2 e outros ácidos orgânicos. No ultimo estágio, bactérias metanogênicas como *Methanosarcina* spp e *Methanothrix* spp convertem ácidos, CO_2 e H_2 em metano que pode ser recolhido e utilizado como combustível *[10]*.

Considerando a rusticidade e a ampla gama de substratos que podem ser metabolizados por estes micro-organismos anaeróbicos a produção de biogás traz algumas vantagens sobre as demais metodologias de produção de biocombustíveis. Materiais das mais diferentes origens podem ser diretamente utilizados como substrato tais como lixo urbano, dejetos animais, esgoto urbano, lixo industrial, resíduos agrícolas, etc. Esses materiais requerem pré-tratamentos mínimos, diferente do que ocorre no caso da produção de biodiesel e bioetanol, e ao final do processo o resíduo ainda pode ser utilizado como fertilizante *[27]*.

Kaparaju, et al. *[28]* defendem que a produção de biogás seja atrelada a produção de bioetanol e biodiesel constituindo-se o conceito de biorefinaria. A produção de biodiesel e de bioetanol pode gerar resíduos ainda ricos em substratos (açúcares e ácidos graxos) além de algumas substâncias tóxicas ao meio ambiente. Esses resíduos são facilmente fermentados por bactérias anaeróbicas e a sua utilização para geração de biogás poderia dobrar a

produção de energia considerando-se a mesma quantidade de biomassa e ainda reduziria a contaminação ao meio ambiente.

3.3. Bioetanol

Atualmente, Brasil e EUA respondem como os maiores produtores de bioetanol no mundo. A produção de bioetanol nos EUA em 2010 foi de aproximadamente 50,0 bilhões de litros e foi praticamente (superior a 98%) toda derivada do amido de milho caracterizando-o assim como o maior produtor mundial deste combustível *[29]*.

No Brasil, diferentemente do EUA, utiliza-se a cana-de-açúcar como principal matéria prima para a produção de bioetanol. O Brasil é atualmente o segundo maior produtor mundial de bioetanol e o país produziu em 2010 cerca de 27,7 bilhões de litros deste biocombustível *[30]*.

Os processos tecnológicos para a obtenção de álcool a partir de milho e de cana-de-açúcar já estão plenamente estabelecidos e são economicamente viáveis, entretanto, é preciso destacar que a obtenção de etanol derivado da cana-de-açúcar é economicamente favorável uma vez que a produção de 1 m^3 de álcool a partir de milho nos EUA tem um custo final que varia entre 250 e 420 US$, ao passo que a produção de 1 m^3 de álcool a partir de cana-de-açúcar no Brasil custa cerca de 160,00 US$ *[31]*. Fatores como menor custo de produção, alta produtividade da cultura, altos rendimentos na produção de bioetanol por área cultivada, grande disponibilidade de terras cultiváveis e aplicação de políticas públicas que garantem alta demanda por álcool são apontados como os principais motivos para o baixo custo da produção de etanol derivado de cana-de-açúcar no Brasil *[14, 31, 32]*. Outro fator crucial para a elevação do custo do etanol derivado do milho é a necessidade da etapa de hidrólise do amido em glicose para que esta seja fermentada a etanol *[33]*. Este conjunto de fatores contribui para que o programa de produção e utilização de bioetanol no Brasil seja o mais eficiente do mundo *[32]*.

Contudo, considerando o atual potencial de produção de álcool a partir destas duas tecnologias e a crescente necessidade de ampliação da produção de biocombustíveis projeta-se uma série de dificuldades, principalmente em termos de sustentabilidade, que limitam a ampliação da utilização do etanol derivado do milho ou da cana-de-açúcar *[34]*.

A síntese de bioetanol a partir de culturas agrícolas que são empregadas também na produção de alimentos, como a cana-de-açúcar e o milho, pode provocar uma competição pelo uso da terra para a produção destas culturas *[35]*. Esta competição afeta diretamente as commodities dos produtos agrícolas provocando alta nos preços dos alimentos, o que pode gerar graves desequilíbrios sociais como já observado no ano de 2008 *[36]*. Além disto, a grande necessidade de áreas cultiváveis, que o cultivo destas culturas demanda, poderá levar a grandes alterações no uso da terra e da água, podendo provocar aumento do desmatamento e redução na disponibilidade de água em algumas regiões *[36]*.

Baseando-se nestas circunstâncias a utilização de biomassa lignocelulósica desponta como uma das alternativas mais promissoras para a produção do bioetanol *[13]*. Considerando-se a atual disponibilidade mundial de resíduos agrícolas lignocelulósico e resíduos de lixo urbano, principalmente papel, estima-se um potencial para uma produção de 491 bilhões de litros deste biocombustível ao ano, valor cerca de 6 vezes superior à produção de bioetanol derivada de milho e de cana-de-açúcar atualmente *[14, 37]*.

Dentro destas perspectivas e considerando o seu atual "status" na produção mundial de bioetanol derivado de cana, o Brasil possui o potencial para se tornar um dos maiores fornecedores mundiais de energia no futuro. Em uma análise sobre o potencial do bioetanol derivado de cana feita por Cerqueira Leite et al. *[38]* foi estimado que o Brasil poderá, dentro de um espaço de 15 anos, produzir etanol suficiente para substituir cerca de 5% de toda gasolina utilizada no mundo. Para alcançar este patamar a produção brasileira precisará passar dos atuais 20-25 bilhões de litros/ano para aproximadamente 105 bilhões de litros/ano em 2025. Para que esta evolução seja sustentável os autores descrevem ainda que este avanço não poderá ser dependente somente da expansão da área cultivada, mas sim, dependente do desenvolvimento de novas variedades de cana-de-açúcar mais produtivas e principalmente, dependente do desenvolvimento de tecnologias para produção de bioetanol lignocelulósico. Hussuani et al. *[39]* estimaram que se a palhada da cana-de-açúcar e 50% do bagaço produzido forem convertidos, em seu máximo potencial, a etanol, poderíamos obter um produção extra de bioetanol na ordem de 3700 a 4000 L/ha/ano. Em outras palavras, seria possível

praticamente dobrar a produção de bioetanol sem a necessidade de plantio de cana-de-açúcar em novas áreas.

Entretanto, apesar de todo este potencial o bioetanol lignocelulósico ainda não é uma realidade economicamente viável *[36]*. A obtenção do etanol lignocelulósico ou etanol de segunda geração, como também é conhecido, é um processo complexo que será descrito nos próximos itens, considerando-se os gargalos tecnológicos que impedem que o processo já esteja estabelecido.

3.4. Natureza química da biomassa

Para que uma biomassa seja efetivamente empregada na produção de bioetanol, ela deve ser degradada em suas unidades fundamentais constituintes, ou seja, os açúcares. Os maiores desafios para a obtenção de etanol de segunda geração são exatamente a complexidade, a variedade e o caráter recalcitrante destes materiais. Todos estes atributos tornam a degradação da biomassa um processo extremamente complexo.

A biomassa é composta por celulose, hemicelulose, lignina, extrativos, cinzas e outros componentes *[14]*. Na tabela 1 podemos observar a composição média de algumas biomassas com grande potencial para serem empregadas em processos de produção de bioetanol.

Tabela 1: Composição média de algumas biomassas com potencial para serem utilizadas na produção de bioetanol.

Biomassa	Composição da Biomassa (%, base seca)				
	Celulose	Hemicelulose	Lignina	Cinzas	Ref.
Bagaço de cana	32-44	27-32	19-24	4,5-9	*[40]*
Capim elefante	22	24	24	6	*[40]*
Palha de trigo	29-35	26-32	16-21	N.D.	*[40]*
Bambu	26-43	15-26	21-31	1.7-5	*[40]*
Sabugo de milho	45	35	15	1.36	*[41]*
Madeira (“Hardwood”)	40-55	24-40	18-25	N. D.	*[41]*
Madeira (“Softwood”)	45-50	25-35	25-35	N. D.	*[41]*
Palha de arroz	32.1	24	18	N. D.	*[41]*
Papel de Jornal	40-55	25-40	18-30	5-8	*[41]*
Capins (média)	25-40	25-50	10-30	1.5	*[41]*

N. D. = não disponível

O principal composto presente na biomassa é a celulose, um homopolissacarídeo composto por resíduos de β-D-glicopiranos ligados entre si por ligações (1-4) glicosídicas. A principal função da celulose no tecido vegetal é conferir resistência e rigidez. As moléculas de celulose são longas, lineares e podem interagir entre si formando estruturas cristalinas (bem organizadas) que conferem ao tecido vegetal grande resistência. Em determinadas extensões das microfibrilas as moléculas de celulose não estão tão bem organizadas formando estruturas amorfas e menos rígidas *[42]*.

A hemicelulose é o segundo maior constituinte dos tecidos vegetais. Trata-se de uma mistura de polímeros complexos de vários monossacarídeos e derivados dos quais podemos citar glicose, manose, xilose, arabinose, ácido 4-o-metil glicurônico e ácido 4-o-metil galacturônico *[43]*. A composição da hemicelulose é extremamente complexa e variável podendo ser afetada pela espécie vegetal, pelo clima e pelo estágio de desenvolvimento da planta *[14, 44]*. Xilose, um açúcar de cinco carbonos, é o componente mais abundante na estrutura hemicelulósica, principalmente em se tratando de tecidos lenhosos. Arabinose, também uma pentose, representa cerca de 2-4% do total de açúcares presentes na hemicelulose de tecidos lenhosos, entretanto, esta proporção sobe para 10-20% em tecidos de plantas herbáceas podendo chegar até a 40% em fibras de milho *[45]*.

A hemicelulose tem uma massa molecular bem inferior ao da celulose e diferentemente da mesma, possui ramificações laterais que podem ser constituídas por múltiplos açúcares (xilose, arabinose, glicose, galactose, ácidos urônicos, entre outros) que são facilmente hidrolisáveis *[46]*. A hemicelulose age como uma conexão entre a celulose e a lignina conferindo ao tecido vegetal a rigidez característica *[42]*.

A lignina é o terceiro componente mais importante na constituição dos tecidos vegetais. Consiste de um heteropolímero amorfo constituído de três diferentes tipos de unidades derivadas de fenil propano (p-coumaril álcool, coniferil álcool e sinapil álcool) que podem ser arranjadas de infinitas maneiras. As funções da lignina estão relacionadas à questões estruturais, impermeabilização do tecido vegetal, resistência ao ataque de insetos e micro-organismos e proteção contra o estresse oxidativo *[46]*. A lignina é extremamente recalcitrante e sua presença no tecido vegetal é um dos maiores entraves para o processo de solubilização da biomassa *[44]*.

3.5. Bioetanol Lignocelulósico

A produção de etanol a partir de biomassa lignocelulósica consiste de três etapas principais: pré-tratamento, hidrólise ou sacarificação e fermentação *[14]*.

Atualmente, a maioria dos projetos pilotos que propõe a produção de etanol lignocelulósico promovem a degradação da biomassa por meio de hidrólise ácida. Entretanto, este processo não se mostra suficientemente eficiente porque esta reação é extremamente inespecífica e promove, além da liberação de monossacarídeos, a formação de compostos tóxicos que inibem o processo subseqüente de fermentação. A hidrólise ácida também reduz o rendimento do processo como um todo porque muito dos monossacarídeos são degradados ou transformados em reações secundárias, tornando-se impróprios para fermentação. Além disso, o bioetanol produzido a partir de mostos obtidos de hidrólises ácidas é rico em componentes corrosivos que podem danificar os motores e outros equipamentos *[10, 44]*.

Por estes motivos os atuais projetos de desenvolvimento de etanol lignocelulósico priorizam a utilização de enzimas produzidas por micro-organismos para a realização do processo de sacarificação. Entretanto, fatores como alta cristalinidade da celulose, porosidade reduzida da biomassa, tamanho das partículas e a presença da lignina limitam a atividade enzimática e consequentemente a eficiência do processo *[47-49]*. Por estas razões pré-tratamentos da biomassa são utilizados para que o processo de sacarificação enzimática seja eficiente.

3.5.1. Pré-tratamentos

O pré-tratamento tem como função eliminar/reduzir a hemicelulose e a lignina, desorganizar a estrutura cristalina da celulose e promover a abertura de poros na estrutura da biomassa de forma que as enzimas, principalmente as celulases, possam atuar de forma mais efetiva e promover a rápida hidrólise do material *[5]*. Diferentes tipos de pré-tratamentos são utilizados com este propósito promovendo diferentes tipos de alterações na biomassa.

Tratamentos como a pirólise, pré-tratamento a vapor, explosão a vapor e pré-tratamento à base de água quente atuam principalmente promovendo a remoção da lignina e da hemicelulose uma vez que estes componentes são

instáveis quando expostos a temperaturas superiores a 180 °C *[50, 51]*. Os tratamentos térmicos geralmente são bastante eficientes e promovem a remoção de boa parte da lignina e da hemicelulose. Entretanto, os mesmos apresentam a desvantagem de necessitarem de grandes quantidades de energia para serem realizados. Além disso, esses tipos de pré-tratamentos conduzem a formação de vários compostos tóxicos como o furfural, hidroximetilfurfural (derivados da hidrólise da hemicelulose), vanilina e vanilina álcool (derivados da degradação da lignina). Esses compostos são extremamente nocivos para micro-organismos fermentadores e podem inibir os processos subseqüentes *[44]*.

Tratamentos químicos à base de ácidos diluídos e álcalis também podem ser empregados para o pré-tratamento da biomassa utilizando condições amenas de temperatura. Tratamentos ácidos atuam principalmente removendo a hemicelulose enquanto que os tratamentos alcalinos afetam principalmente a lignina. Observa-se também que estes tipos de tratamentos alteram as propriedades da lignina promovendo a liberação de compostos tóxicos, o que é indesejável *[46, 52-54]*.

Outros tipos de tratamentos físico-químicos como a explosão com CO_2 e a explosão das fibras com amônia também são bastante estudados *[5]* tendo se obtido altos rendimentos de hidrólise de biomassas tratadas por estes métodos *[55, 56]*. Esses pré-tratamentos diferenciam-se dos outros citados anteriormente por promoverem uma menor formação de compostos tóxicos. Além disso, o aumento no rendimento na sacarificação nestes casos se dá principalmente pelo aumento da porosidade da biomassa e não tanto pela solubilização da hemicelulose e da lignina *[57]*.

De fato, pesquisas ainda necessitam ser realizadas para que se aperfeiçoem os diferentes tipos de pré-tratamentos e se reduza os seus custos, que ainda são altos. O tipo de biomassa também é um fator a ser considerado na escolha do melhor tratamento, podendo-se inclusive combinar diferentes pré-tratamentos para que se possa obter a máxima hidrólise da biomassa com a menor formação de agentes tóxicos e inibidores *[44]*.

3.5.2. Sacarificação enzimática

Após o pré-tratamento a hidrólise da biomassa é conduzida utilizando-se enzimas, principalmente celulases, as quais são altamente especificas *[58]*. O

produto resultante desta hidrólise é um xarope rico em açúcares redutores, principalmente glicose, que podem ser facilmente convertidos a etanol pelo processo de fermentação *[5]*. As celulases utilizadas em processos biotecnológicos, como a sacarificação enzimática, são produzidas principalmente por micro-organismos, mas esta questão será debatida no próximo tópico.

As celulases empregadas na sacarificação enzimática são geralmente misturas de várias enzimas. Ao menos três maiores grupos estão envolvidos no processo *[59]*:

- Endoglicanases (E.C. 3.2.1.4.): são enzimas que hidrolisam a celulose atacando aleatoriamente a estrutura amorfa (e até mesmo a estrutura cristalina) do polímero gerando extremidades redutoras e não redutoras;
- Exoglicanases (E.C. 3.2.1.91): são enzimas que hidrolisam as fibras de celulose a partir de sua extremidade, podendo ela ser redutora ou não; a ação dessas enzimas produz principalmente celobiose, um dissacarídeo de glicose, e celodextrinas, que são oligossacarídeos que contem de 3 a 6 resíduos de glicoses em sua estrutura;
- β-glicosidases (E.C.3.2.1.21): são enzimas que hidrolisam principalmente celobiose e celodextrinas produzindo glicose.

Como as β-glicosidases hidrolisam apenas celobiose e pequenas celodextrinas sua atividade é dependente da ação das enzimas exoglicanases, e estas, por sua vez, dependem da ação das endoglicanases. Todas estas interdependências caracterizam que o processo de hidrólise da celulose é um evento onde um grande grupo de enzimas atua de maneira sinérgica *[60]*.

Além das celulases, existe também um grande grupo auxiliar de enzimas que podem atuar sobre a hemicelulose residual e contribuir para uma hidrólise mais eficiente da biomassa. Dentre estas enzimas, conhecidas como hemicelulases, pode-se destacar as glucoronidases, acetilesterases, xilanases, β-xilosidases, β-manosidases, galactomananases, α-arabinofuranosidases, α-galactosidases e glucomananases *[61]*.

Comparando ao emprego da hidrólise ácida, o uso de coquetéis de celulases para a sacarificação da biomassa é vantajoso porque a reação é específica e não gera compostos tóxicos à fermentação. A hidrólise é

conduzida em condições mais amenas de pH e temperatura (na faixa de 45-50°C e pH entre 4-5) e por isto uma menor quantidade de energia é requisitada no processo. Durante a hidrólise enzimática também se observa que os equipamentos utilizados no processo (tanques, tubulações) sofrem menos danos quando comparados ao uso de processos químicos que usam ácidos concentrados. O etanol produzido de um mosto obtido por hidrólise enzimática é de melhor qualidade, contendo menos agentes corrosivos. Também se destaca o menor custo com relação ao tratamento de água e de resíduos quando se aplica a sacarificação enzimática em detrimento da sacarificação ácida *[10, 13]*.

Entretanto, apesar de todas estas vantagens e de se encontrarem na literatura taxas de conversão enzimática de celulose à glicose acima de 90% *[62-64]*, esta não é ainda uma tecnologia estabelecida. Um dos maiores limitantes da sacarificação enzimática é o alto custo atrelado à produção de enzimas *[65]*. Nos últimos anos, companhias como a Novozyme Inc. e a Genecor International vêm recebendo incentivos na ordem de milhões de dólares para desenvolver pesquisas relacionadas à redução de custos na produção de enzimas. Entretanto, os resultados ainda estão aquém daqueles necessários para tornar o processo competitivo *[13]*. Soma-se ao alto custo de produção, o fato das enzimas serem altamente instáveis e não suportarem longos períodos de armazenamento. A alta complexidade e a variedade das biomassas passíveis de serem utilizadas na produção de etanol também pode ser um problema, pois um coquetel enzimático eficiente para a hidrólise de bagaço de cana pode, por exemplo, não ser tão eficiente para a hidrólise de outros tipos de resíduos *[10]*.

A sacarificação enzimática também é limitada devido ao fato de endoglicanases e exoglicanases serem inibidas pelos produtos decorrentes de suas ações catalíticas: celobiose e glicose *[5, 65]*. Isso, entretanto, pode ser amenizado suplementado-se o meio de reação com altas concentrações de enzimas β-glicosidases *[61]* ou então promovendo as etapas de sacarificação e fermentação simultaneamente em um processo conhecido como SSF (Sacarificação e fermentação simultânes) *[66]*.

Entretanto, o estabelecimento de tecnologias para sacarificação enzimática não depende somente de pesquisas do ponto de vista aplicado. Inúmeras lacunas sobre os mecanismos senergísticos de ação das celulases

precisam ser preenchidas para que estas enzimas possam ser bioquimicamente melhoradas e utilizadas de forma cataliticamente mais eficiente em processos biotecnológicos. Dentre as pesquisas básicas atualmente desenvolvidas com celulases destacam-se aquelas que estudam os mecanismos de ligação das celulases ao seu substrato, o processo de montagem do complexo multiprotéico denominado celulossomo e a importância e a função dos módulos de ligação a carboidratos (MLC) para o processo de hidrólise *[67, 68]*.

Por todos estes fatores, o desenvolvimento de estudos para produção, expressão, aplicação e compreensão do modo de ação de celulases de diferentes micro-organismos são imprescindíveis para a implantação do processo de produção de bioetanol lignocelulósico *[67]*.

3.5.3. Fermentação

Diferentemente do que ocorre na produção de etanol a partir de caldo de cana ou de amido de milho, o xarope rico em açúcares obtido após o pré-tratamento e a sacarificação enzimática da matéria lignocelulósica é uma mistura complexa composta por açúcares, derivados de furano, ácidos orgânicos fracos e compostos fenólicos *[69]*. Devido à hemicelulose, os hidrolisados de biomassa são extremamente ricos em pentoses como arabinose e principalmente xilose. Estes açúcares não podem ser metabolizados a etanol porque as cepas industriais da levedura *Saccharomyces cerevisiae*, principal micro-organismo utilizado na produção de etanol, não são capazes de fermentar estes monossacarídeos. Isto certamente acarreta em uma grande queda ao rendimento total do processo *[13]*.

Existem na natureza inúmeros micro-organismos capazes de fermentar pentose produzindo etanol *[70]*, mas a utilização dos mesmos é limitada por fatores como baixa tolerância ao etanol e a realização de fermentações mistas. Algumas espécies de bactérias anaeróbicas como *Thermoanaebacterium saccharolyticum* e *Clostridium thermocellum* realizam a fermentação alcoólica de pentoses, entretanto, estes organismos são sensíveis a altas concentrações de etanol. Além disso, estas bactérias dispõem de outras rotas fermentativas que geram acetato e ácido lático e isto faz como que o rendimento final na produção de etanol seja comprometido *[20, 71]*.

Fungos filamentosos como *Rhizopus oryzae*, *Mucor corticolous* e *Rhizomucor pusillus* são capazes de produzir consideráveis quantidades de etanol (10-15 $g.L^{-1}$) fermentando xilose e xaropes obtidos de hidrólise ácida de resíduos florestais, demonstrando que estes micro-organismos têm alta tolerância aos compostos tóxicos gerados no pré-tratamento e na hidrólise *[72]*. Entretanto a produtividade etanólica e a velocidade do processo fermentativo destes fungos ainda não permitem suas aplicações na indústria de forma competitiva *[13]*.

Algumas leveduras como a *Pichia stipidis* podem naturalmente fermentar xilose e arabinose a etanol com bom rendimento e produtividade Entretanto, este micro-organismo é extremamente sensível aos compostos tóxicos gerados no pré-tratamento e na hidrólise, o que limita seu emprego na produção de etanol lignocelulósico *[13]*. Uma das alternativas mais promissoras para a resolução deste problema passa pela engenharia genética de *S. cerevisiae,* introduzindo em seu genoma genes que codificam para a síntese de enzimas necessárias à fermentação de xilose e outras pentoses *[73]*. Zhao e Xia *[66]* utilizaram a levedura *S. cerevisiae* ZU-10 para a fermentação de hidrolisados de resíduos de milho, ricos em xilose e glicose. A cepa ZU-10 tem inserido em seu genoma genes de *P. stipidis* que codificam enzimas envolvidas na metabolização da xilose. Tanto glicose como xilose foram metabolizadas rapidamente alcançando-se produtividade de até 30 $g.L^{-1}$ de etanol.

A questão da metabolização das pentoses é uma limitação importante no contexto da produção de etanol lignocelulósico, entretanto, a presença de inibidores como furfural, hidroximetilfurfural e fenóis, que são gerados durante o processo de pré-tratamento, é o principal limitante do processo fermentativo, pois a presença destes compostos, quando em altas concentrações conduz à ausência completa da fermentação. Avaliações de alguns processos de detoxificação veem sendo executadas, entretanto estes processos são extremamente onerosos e favorecem a perda de açucares fermentáveis *[74, 75]*. Como a maioria dos inibidores da fermentação é gerada no pré-tratamento a alternativa mais aplicada atualmente é a remoção da fração líquida gerada no pré-tratamento substituindo-a por tampão *[13]*. Estas frações, porém, são ricas em açúcares, principalmente pentoses, e seu descarte representaria uma redução no potencial final de produção de combustíveis além de gerar possíveis problemas ambientais. Kaparaju et al, *[28]* sugere que estas frações

ricas em pentose e substâncias tóxicas sejam empregadas na produção de gás metano ou H, uma vez que micro-organismos responsáveis por este processo são menos sensíveis aos agentes tóxicos e podem utilizar toda sorte de componentes orgânicos para esta proposta metabólica.

3.6. Produção de celulases

Devido à grande quantidade de celulases requerida para a hidrólise de biomassa, o custo da produção das enzimas é um dos grandes obstáculos para o estabelecimento da tecnologia do etanol lignocelulósico. De acordo com dados providenciados pela empresa Novozyme, uma quantidade de enzimas cerca de 40 a 100 vezes superior é necessária para se produzir uma equivalente quantidade de etanol a partir de biomassa, comparando-se com a produção de bioetanol a partir de amido de milho *[76]*. Isso ocorre porque as celulases precisam superar uma série de obstáculos para promover sua catálise. As celulases precisam atuar sobre um substrato insolúvel e promover a ruptura de uma estrutura altamente compacta e organizada que oferece poucos pontos para o ataque enzimático. Como conseqüência disto, um ataque combinado de múltiplas enzimas é critico para a hidrólise da celulose *[77]*.

A obtenção de celulases para aplicação na indústria, atualmente é apoiada na utilização de micro-organismo. Bactérias e fungos cultivados em meios líquidos ou sólidos podem produzir grandes quantidades celulases para a hidrólise de materiais lignocelulósico *[5]*. Bactérias pertencentes aos gêneros *Clostridium*, *Cellulomonas*, *Bacillus, Thermomonospora, Ruminococcus, Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora* e *Streptomices* podem produzir celulases e são extensivamente estudadas para a produção destas enzimas *[5, 78]*. Esses micro-organismos, na grande maioria, anaeróbicos, produzem celulases com alta atividade especifica, no entanto, os níveis totais de enzimas encontrados em seus meios de cultivo são pobres. Devido ao fato destas bactérias terem um crescimento muito lento e exigirem ambientes livres de oxigênio para o seu cultivo a maior parte da produção industrial de celulases é focada na utilização de fungos *[61]*.

Fungos do gênero *Penicillium [79]*, *Trichoderma [80]* e *Aspergillus [81]* veem sendo amplamente estudados devido a sua alta capacidade na produção de enzimas hidrolíticas sendo que estes micro-organismos constituem a base

da indústria produtora de enzimas celulolíticas *[82]*. O cultivo de fungos para produção de enzimas é altamente vantajoso por diversos fatores dentre os quais pode-se destacar: alta produção de enzimas, secreção eficiente de enzimas (que facilita a recuperação destas enzimas para posterior uso) e capacidade de crescer e produzir enzimas em uma variada gama de substratos orgânicos, como resíduos agrícolas *[80-84]*. Outra vantagem interessante atribuída ao uso de fungos é sua capacidade de fermentar diferentes substratos sendo cultivados em meio sólido produzindo grandes quantidades de enzimas *[81, 85, 86]*. Fermentações em meio sólido podem ser vantajosas por promoverem: a obtenção de extratos altamente concentrados, alta estabilidade dos produtos, baixa repressão catabólica, cultivo de micro-organismos em substratos insolúveis e menor demanda por esterilidade devido à baixa atividade de água neste tipo de sistema de cultivo *[87]*.

Fungos pertencentes ao gênero *Trichoderma* são os mais utilizados para a produção de celulases para aplicação industrial devido aos altos níveis de expressão destas enzimas por estes micro-organismos *[82]*. O complexo celulolítico do fungo *T. reesei* é composto por no mínimo duas exoglicanases, cinco endoglicanases e duas β-glicosidases *[1]*. Entretanto, o uso de extratos enzimáticos deste fungo é ainda limitado pela baixa termoestabilidade de suas enzimas e também devido à baixa produção de enzimas β-glicosidases, o que resulta em um acúmulo de celobiose durante a hidrólise e conseqüente inibição do processo *[82]*. Uma das alternativas apresentadas para amenizar este problema é a combinação de extratos enzimáticos de *T. reesei* com extratos enzimáticos ricos em β-glicosidases produzidos por outros micro-organismos. Zhao e Xia *[66]* obtiveram o dobro de produção de etanol quando sacarificaram palha de milho com extrato enzimático de *T. reesei* suplementado com extrato enzimático de *A. niger* rico em β-glicosidases.

Em todo o mundo vêm sendo realizadas pesquisas que tem como o intuito aumentar o a produção de celulases e reduzir o custo de produção das enzimas. Estas pesquisas focalizam principalmente no estudo de novos organismos produtores de celulases bem como na avaliação de sistemas de cultivo e utilização de fontes de carbono que induzam eficientemente a produção das enzimas e que sejam de baixo custo *[84, 86, 88-91]*.

Deve-se salientar também que o uso de ferramentas de biologia molecular constitui-se em uma importante estratégia para que se obtenham organismos super produtores de celulases. O sequenciamento do genoma de micro-organismos celulolíticos como *T. reesei* e *P. chrysosporium* proporcionaram informações importantes sobre como estes micro-organismos degradam a celulose e permitiram também a identificação de novas seqüências que codificam celulases *[67, 92]*. Utilizando-se técnicas apropriadas de engenharia genética, múltiplas cópias desses genes podem ser re-introduzidas nos genomas destes fungos obtendo-se assim micro-organismos com alta capacidade para superexpressar essas enzimas *[67, 93]*. Além disso, técnicas de engenharia de proteínas como a evolução dirigida e o desenho racional de proteínas podem ser aplicadas para o desenvolvimento de celulases mais termoestáveis e cataliticamente mais eficientes para serem utilizadas em processos de hidrólise da celulose cristalina *[94, 95]*.

Contudo, a produção comercial de celulases por fungo é um processo que ainda carece de estudos e seu custo é extremamente elevado. Por isso a exploração de novos micro-organismos e distintas condições de crescimento são necessárias para que se obtenham extratos realmente efetivos para a degradação de celulose a custos competitivos.

3.7. Basidiomicetos causadores de podridão branca

Dentre os inúmeros micro-organismos que vem sendo estudados em todo mundo como produtores de enzimas hidrolíticas os fungos da classe Basidiomycete merecem uma especial atenção, principalmente aquelas espécies classificadas como causadoras da podridão branca em madeira.

Os fungos causadores de podridão branca são os únicos organismos capazes de degradar todos os componentes da biomassa lignocelulósica, inclusive a lignina *[96]*. Esta capacidade em degradar lignina se deve ao sistema enzimático extracelular inespecífico composto pelas enzimas lacase (E.C. 1.10.3.2), manganês peroxidase (E.C. 1.11.1.13) e lignina peroxidase (E.C. 1.11.1.14) as quais atuam juntamente com H_2O_2 oxidando a lignina e provocando sua degradação. Fungos de podridão branca produzem uma ou mais dessas enzimas responsáveis pela degradação da lignina *[97, 98]*.

Essas propriedades garantem às enzimas lacases, manganês peroxidases e lignina peroxidases fundamental importância para uma eficiente

ciclagem dos resíduos vegetais na natureza. Além disso, estas enzimas apresentam um enorme potencial para aplicação em processos biotecnológicos.

Na indústria de papel e celulose uma etapa onerosa e passível de formação de resíduos tóxicos é a etapa de branqueamento realizada a partir do processo Kraft *[99]*. Estudos evidenciam que a utilização de lacases e xilanases podem reduzir a energia e a quantidade de reagentes utilizados no processo de branqueamento da polpa gerando ainda uma menor quantidade de resíduos tóxicos *[100, 101]*.

Diversos segmentos da indústria fazem uso de corantes sintéticos em algumas etapas de fabricação de seus produtos, em especial a indústria têxtil. Durante o processo de coloração de tecidos, cerca de 15% dos corantes empregados são liberados ao meio ambiente juntamente com a água utilizada no processo. Esses efluentes são de difícil tratamento e os compostos neles contidos são tóxicos, mutagênicos e carcinogênicos *[102]*. Uma das formas mais promissoras de tratamento desses resíduos é a utilização de extratos enzimáticos produzidos por basidiomicetos ou a utilização direta dos próprios fungos. Isto é possível devido à capacidade das enzimas lacases, manganês peroxidases e lignina peroxidases em degradar estas moléculas corantes que são geralmente compostos polifenólicos *[102]*.

O complexo de enzimas oxidativas produzido pelo basidiomiceto *Phanerochaete chrysosporium* degrada corantes como Índigo Carmine, Neutral Reed^{HC}, Amarelo de Clorazol, Cristal Violeta e Remazol Brilhant Blue R ^{PAQ} *[103]*. *Pycnoporus sanguineos*, *Coriolus versicolor* e *Pleorotus ostreatus* são também fungos basidiomicetos que podem degradar, por meio de seus complexos enzimáticos, dezenas de corantes usados na indústria têxtil *[103-105]*. Além dessas aplicações, estas enzimas oxidativas também podem ser usadas na indústria alimentícia, de bebidas, de cosméticos, na agricultura, em processo de biorremediação e em métodos de análises bioquímicas *[86]*.

Até o final da ultima década, a maioria das pesquisas realizada com fungos de podridão branca focalizaram principalmente no estudo de suas enzimas oxidativas. Entretanto, a necessidade de novas fontes de enzimas celulases e hemicelulases fizeram com que muitos pesquisadores voltassem suas atenções para basidiomicetos e seu arsenal de enzimas hemicelulolíticas *[86, 106-108]*.

Em trabalhos como os realizados por Elisashivili et al. *[86]* foram feitas avaliações da produtividade de celulases e hemicelulases de basidiomicetos como *Trametes versicolor*, *Cerrena maxima*, *Coriolopsis polyzona* e *Pleorotus ostreatus* em cultivos com diferentes fontes de carbono. Os autores obtiveram valores de atividade para celulases e hemicelulases comparáveis àqueles obtidos por outros autores que utilizaram em seus trabalhos os fungos *Trichoderma reesei [80]*, *A. niger [81]* e *Penicillium echinulatum [83]* evidenciando assim o potencial dos fungos basidiomicetos na produção de enzimas que podem ser utilizadas para sacarificação de biomassa visando à produção de bioetanol.

Curiosamente, apesar do desenvolvimento de inúmeros estudos recentes envolvendo a produção e a caracterização de hemicelulases de basidiomicetos, existem escassos trabalhos de avaliação da aplicação de extratos enzimáticos produzidos por esta classe de fungos em processos de sacarificação de biomassas para produção de etanol de segunda geração. Por este motivo, faz-se necessário não somente a realização de pesquisas envolvendo a produção de cellulases e hemicelulases por fungos causadores de podridão branca, mas principalmente na avaliação da aplicação de seus arsenais enzimáticos em processos biotecnológicos de degradação de biomassa para produção de biocombustíveis.

3.8. Fungos fitopatôgenicos

A busca por novos micro-organismos que apresentem destacada capacidade para a produção de celulases e hemicelulases vem ocorrendo de maneira intensa em todo mundo e os mais diversos habitats vêm sendo explorados na busca por micro-organismos de grande potencial para a produção de enzimas de interesse biotecnológico. Curiosamente, em meio a esta constante procura, observa-se que uma importante classe de micro-organismos vem sendo posta em segundo plano pela grande maioria dos pesquisadores. Trata-se dos fungos fitopatôgenicos.

Os fungos fitopatogênicos compreendem um número incontável de espécies pertencentes às mais diferentes classes, ordens, famílias e gêneros, mas que possuem, em comum, a habilidade de degradar e romper os tecidos vegetais durante a invasão e colonização de seus hospedeiros. Fungos fitopatogênicos secretam diversas enzimas (celulases e hemicelulases) para

degradar os componentes da parede celular de plantas e então posteriormente utilizam os monossacarídeos e aminoácidos derivados desta ação hidrolítica como fonte de energia e de nutrientes para o seu desenvolvimento *[109]*.

Espécies fitopatogênicas como *Fusarium graminearum [110], Bipolaris sorokiniana [111]* e *Sclerotinia sclerotiorum [112]* foram avaliadas com relação às suas habilidades para secreção de hemicelulases, pectinases e celulases e foi observado que existe uma estreita correlação entre a capacidade destes organismos em secretarem enzimas hidrolíticas e a sua virulência.

No entanto, apesar de inúmeros trabalhos divulgarem a habilidade de fungos fitopatogênicos em produzir hidrolases de interesse biotecnológico pouco se sabe sobre as possibilidades destes organismos serem utilizados para a produção dessas enzimas em larga escala, pois são raros os trabalhos que focam na otimização da produção de celulases e hemicelulases por fitopatógenos.

Existem ainda menos investigações envolvendo a aplicação das enzimas produzidas por estes micro-organismos em processos biotecnológicos, tal como a sacarificação da biomassa. Desta forma, acredita-se que existe uma série de oportunidades inexploradas uma vez que aparentemente os fungos fitopatogênicos tem um grande potencial para serem bons produtores de celulases e hemicellulases.

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4. Artigo I – Production and characterization of enzymes produced by wood white-rot and plant pathogenic fungi.

4.1. Abstract

Microbial enzymes, mainly hydrolases and oxidases, play a key role in developing of new technologies for processing and utilization of vegetal biomass. In the recent years several researches have been carried out aiming the prospection of new microorganisms and new enzymes which have potential to be applied in industrial bioprocesses. In this work, it was evaluated the effect of carbon source, time of fermentation and cultivation condition (solid state fermentation and submerged fermentation) on production of endoglucanases, β-glucosidases, xylanases and laccases to four Basidiomycetes strains fungi (*Pycnoporus sanguineos*, *Trametes sp.* J-2, *Trametes sp*. J-5 and a unidentified isolated J-129) and two plant pathogenic strains fungi (*Chrysoporthe cubensis* and *Cylindrocladium pteridis*) isolated from eucalyptus plantation. The highest FPase activity (136 U L^{-1}) was reached by *P. sanguineus* cultivated under solid state fermentation using Kraft pulp as a substrate. *P. sanguineus* was also the best laccase producer and a volumetric activity of 11032 U L^{-1} was observed at submerged fermentation with wheat bran. *Chrysoporthe cubensis* secreted the highest endoglucanase (20335 U L^{-1}) and β-glucosidase (1487 U L^{-1}) activities when cultivated on solid state fermentation using wheat bran as support. Meanwhile*, Cylindrocladium pteridis* was the best xylanase producer and a yield of 142519 U L^{-1} was observed in solid state fermentation employing wheat bran as carbon source. The optimum pH of cellulases and xylanases produced by strains here studied were in a range of 3.5-6.0, however, the optimum pH to laccases has been found in a range of 2.5-3.5. The optimum temperatures to all enzymatic complexes studied were observed in a range of 50-70°C. These results suggest that the fungal strains studied in this work have a great potential to produce enzymes with adequate features for application in biotechnological processes.

4.2. Introduction

The use of enzymes in transformation industry is rising day by day and nowadays innumerable industrial processes are carried out employing these

biological catalysts. The enzymatic catalysis is more advantageous than chemical routes because it provides higher yields of products, diminution at demand for raw materials and reducing emissions and waste resulting in cost savings and reduced generation of pollutants *[1]*. Lignocellulolytic enzymes such as xylanases, cellulases and laccases are of fundamental importance for the efficient bioconversion of plant residues and they are prospective for the various biotechnological applications *[2-4]*.

Xylanases (E.C. 3.2.1.8) are enzymes that cleave the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate and releasing xylooligosaccharides. Xylanases are utilized in the food, animal feed, fuel and textile industry; however, the most prominent use of xylanases is in the paper and pulp industry where it is employed as a tool in biobleaching processes *[2]*.

Cellulases comprise a system of three groups of hydrolytic enzymes that act synergistically promoting the degradation of cellulose. Endoglucanases (E.C. 3.2.1.4) randomly attack cellulose chains generating reducing and non-reducing ends. Cellobiohydrolases (E.C. 3.2.1.91) act over the reducing and non-reducing ends, releasing cellobiose units which by action of enzyme β-glucosidase (E.C. 3.2.1.21) are converted to glucose *[3]*. Cellulases are usually applied in industrial processes such as: paper recycling, juice extraction, biostoning, animal feed additives and others *[5]*. However the most relevant application of cellulase is in biorefinery processes. In this context, cellulases are employed to degrade lignocellulosic biomass to simple sugars, which are versatile starting materials for further conversion by fermentation, biocatalytic, and chemo catalytic processes to value-added products, including biofuels, biopolymers and chemicals *[6]*

Laccases are oxidoreductases which oxidize a variety of aromatic compounds using oxygen as the electron acceptor and producing water as by-product. The presence of redox mediators is generally required for a number of biotechnological applications, providing the oxidation of complex substrates not oxidized by the enzyme alone [4]. Laccases are versatile enzymes that when act over lignin (or other aromatic compounds) may display ligninolytic and/or polymerizing (cross-linking) abilities, depending on the conditions under which the reaction is conducted *[7]*. Because of such skills laccases can be applied in several biotechnological processes such as: biobleaching of Kraft pulp *[8]*,

bioremediation of phenolic compounds *[9]* and fiber modification to obtaining functionalized papers *[10, 11]*.

Large amounts of enzymes are required for application in biotechnological processes and the cost for production and acquisition of these biocatalysts are the largest limitations for the expansion and consolidation of enzymatic industrial processes yet. Microorganisms, especially fungi, are the main sources of enzymes for the industry. Fungi belonging to the genera *Trichoderma*, *Aspergillus* and *Penicillium* are among the greatest producers of cellulases and hemicellulases and species such as *Trichoderma reesei*, *Trichoderma longibrachiatum* and *Aspergillus niger* are the most commonly used for the production of these enzymes in large scale [2, 5]. Industrial laccase production is generally obtained from cultures of white-rot Basidiomycete fungi, the unique microorganisms that are able to degrade enzymatically all biomass components (including lignin) and the main species used for this propose belong to the *Trametes* genus *[4, 12]*.

Although various groups of known lignocellulolytic microorganisms have its enzymatic systems well characterized and some of them are already used in industrial scale, worldwide researches have demonstrated that the activities of new isolates have been comparable or superior to the traditional strains. Nature represents an interminable source of lignocellulolytics microorganisms and especially tropical countries as Brazil, which presents a very diversified microbial flora, certainly shelters species of unknown microorganisms of optimum industrial interest *[13]*. In this context, the exploration of new fungal strains is very important to provide microorganism with properties that match with the requirements existing in the industrial marked.

This paper describes the lignocellulolytic enzymes activities produced by four wood-rot Basidiomycetes and two plant pathogenic fungi isolated from eucalyptus plantation. All fungal strains were cultivated under submerged and solid-state fermentation employing different carbon sources and the results to enzymes production were discussed regarding the fungal strain, carbon source and system of cultivation. Furthermore, all enzymatic activities obtained were characterized in relation of pH and temperature optimal to evaluate the feasibility of using these enzymes in industrial processes

4.3. Material and Methods

4.3.1. Materials

Substrates including ρ-nitrophenyl-β-D-glucopyranoside (pNPGlc), carboxymethilcellulose (CMC), xylan from birchwood, 2,2'-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS), Avicel PH101 (microcrystalline cellulose) and also the chemical reagents monopotassium phosphate, ammonia nitrate, magnesium sulfate, calcium chloride, cuprum sulfate, sodium acetate, sodium carbonate, dinitrosalicylic acid (DNS) and potato dextrose agar (PDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Yeast extract was obtained from Himedia Laboratories Co. (Mumbai, Maharashtra, India). Potassium sodium tartrate was acquired from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). Kraft pulp was kindly supplied by the Pulp and Paper Laboratory of the Federal University of Viçosa, Viçosa, MG, Brazil. Milled corn cob and wheat bran were obtained locally. All others reagents used in this study were of analytical grade.

4.3.2. Microorganisms and inoculum preparation

Four white-rot basidiomycete and two plant pathogenic fungi collected from eucalypts plantation in several regions of Brazil were studied in this work. The identification and other pertinent informations about each fungal strain can be observed in Table 1. All fungal strains belong to the mycological collection of the Forest Pathology Laboratory at the Federal University of Viçosa, MG, Brazil.

Table 1: White-rot Basidiomycetes and plant pathogen fungi evaluate during this study. The isolation procedures to each strain were described at respective references

Strain	Classification	Local of isolation	Ref.
Pycnoporus sanguineus PF-2	White-rot basidiomycete	Viçosa, MG, Brazil	*[14]*
Trametes sp. J2	White-rot basidiomycete	Monte Dourado, PA, Brazil	*[14]*
Trametes sp. J5	White-rot basidiomycete	Monte Dourado, PA, Brazil	*[14]*
Isolated J-129	White-rot basidiomycete	Monte Dourado, PA, Brazil	*[14]*
Cylindrocladium pteridis PF-1	Plant pathogen	Lençóis Paulistas, SP, Brazil	*[15]*
Chrysoporthe cubensis LPF-01	Plant pathogen	Belo Oriente, MG, Brazil	*[16]*

The fungi were maintained on PDA plates at 28°C and subcultured periodically. The inoculum was prepared by growing the different strains under submerged fermentation in 250 mL erlenmeyer flasks containing 100 mL of

medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2. Each flask was inoculated with 10 agar plugs (cut out of a 5 day-old colony of each strain grown on the PDA plates) and incubated in a rotary shaker for 5 days at 150 rpm and 28 °C. The obtained culture was aseptically homogenized with a Polytron® device and immediately used to inoculate both, submerged culture media and solid state culture media. This routine for inoculum preparation was employed in all experiment developed in this work.

4.3.3. Culture condition

The potential for lignocellulolytic enzyme production by wood-root Basidiomycetes and plant pathogenic fungi cultured under submerged and solid state fermentation employing different carbon sources was investigated. The conditions used at each fermentation system are described as follow.

4.3.3.1. Solid state fermentation (SSF)

SSF was carried out using Kraft pulp, wheat bran and milled corn cob for support and as the main carbon source. The fermentations were carried out in 125-mL Erlenmeyer flasks containing 5 g (dry weight) of substrate moistened with 12 mL of culture media (final moisture of 70%) containing the following composition, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2. Furthermore, $MnCl_2$ (0.1 mg L^{-1}), H_3BO_3 (0.075 mg L^{-1}), Na_2MoO_4 (0.02 mg L^{-1}), $FeCl_3$ (1.0 mg L^{-1}) and $ZnSO_4$ (3.5 mg L^{-1}) also were added to the medium as trace elements. The flasks were autoclaved at 120 °C for 20 min and then inoculated with 3 mL of inoculum obtained as aforementioned. The flasks were maintained at 28 °C in a controlled temperature chamber and the enzymatic extractions were performed after 4, 8 and 12 days of fermentation. Enzymes secreted during SSF were extracted with sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate) under agitation of 150 rpm for 60 min at room temperature in an orbital rotary shaker. Solids were separated by filtration through a nylon cloth followed by centrifugation at 15000 x *g* for 10 min. The clarified supernatants were frozen and stored for subsequent enzymatic analysis. All experiments were carried out with three repetitions for each medium composition and for each incubation time.

4.3.3.2. Submerged fermentation (SmF)

SmF was carried out using Kraft pulp, wheat bran, milled corn cob and Avicel as carbon sources. The fermentations were carried out in 125-mL Erlenmeyer flasks containing 50 mL of medium with following composition, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25, yeast extract, 2 and carbon source, 10. Furthermore, $MnCl_2$ (0.1 mg L^{-1}), H_3BO_3 (0.075 mg L^{-1}), Na_2MoO_4 (0.02 mg L^{-1}), $FeCl_3$ (1.0 mg L^{-1}) and $ZnSO_4$ (3.5 mg L^{-1}) also were added to the medium as trace elements. The flasks were autoclaved at 120°C for 20 min and then inoculated with 3 milliliter of inoculum obtained as aforementioned. The flasks were conditioned in an orbital shaker at 28 °C and 150 rpm, and enzymatic extractions were performed after 4, 8 and 12 days of fermentation. The liquid extracts obtained were filtered through a cloth filter and immediately centrifuged at 15000 x g for 10 min to separate the solid fraction. The clarified supernatants were frozen and stored for subsequent enzymatic analysis. As was done in the SSF, all SmF experiments were carried out with three repetitions for each medium composition and for each incubation time.

4.3.4. Enzymatic assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50° C. All assays were performed in triplicate and the mean values calculated. Relative standard deviations of the measurements were below 5%. FPase and endoglucanase activities were determined using Whatman no. 1 filter paper and carboxymethilcellulose as substrates respectively, according to previously described standard conditions *[17]*. The total reducing sugar liberated during the enzymatic assays was quantified by the dinitrosalicylic acid (DNS) method *[18]* using glucose as a standard.

Xylanase activity was determined using xylan from birchwood (1% w/v at final concentration) as a substrate. The enzymatic reaction was initiated by the addition of 100 μL of the appropriate diluted enzyme solution to 400 μL xylan solution prepared in buffer. The reaction mixture was incubated for 30 min and the total reducing sugar released was determined by the DNS method using xylose as standard. β-Glucosidase activity was measured using ρPNGlc substrate. The mixture reaction contained 100 μL of the appropriately diluted enzyme solution, 125 μL of the synthetic substrate solution (1 mM at final concentration) and 275 μL of buffer. This reaction mixture was incubated for 30

min and terminated by the addition of 0.5 mL sodium carbonate solution (0.5 M). The values of absorbance were measured at 410 nm and the amount of ρ-nitrophenol released was estimated using a standard curve. Laccase activity was determined by monitoring the oxidation of ABTS in a reaction containing 100 μL of the appropriately diluted enzyme solution, 50 μL of substrate solution (1 mM at final concentration) and 355 μL of buffer. The reaction was carried out for 10 min and at the end of the incubation period the values of absorbance were immediately measured at 420 nm. The amount of oxidized ABTS produced was calculated using a molar extinction coefficient of 3.6 x 10^4 M^{-1} cm^{-1}. For all activities, one unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 μmol of the corresponding product (glucose equivalent, xylose, ρ-nitrophenol and oxidized ABTS) per minute under the assay condition used.

4.3.5. Determination of optimum pH

To determine the effects of pH on endoglucanase, β-glucosidase, FPase, xylanase and laccase activities the enzymatic assays were carried out at pH values ranging from 2.0-7.0 using a citric acid/sodium phosphate buffer *[19]*. The other assay conditions were as previously described.

4.3.6. Determination of optimum temperature

The effects of the temperature on endoglucanase, β-glucosidase, FPase, xylanase and laccase activities were evaluated within a range of 30-80°C at pH 5 according to the standardized methods.

4.4. Results and Discussion

4.4.1. Enzyme production as a function of the fungal strains.

In this study, the potential of six new fungal strains was investigated to produce enzymes for application in biorefinery processes. The microorganisms were obtained from eucalyptus plantations and among the strains evaluated there were three white-rot Basidiomycetes (*Pycnoporus sanguineus*, *Trametes* sp. J2 and *Trametes* sp. J5), two plant pathogenic Ascomycetes (*Chrysoporthe cubensis* and *Cylindrocladium pteridis*) and one unidentified Basidiomycete (J-129). The fungi were grown under SmF and SSF employing different carbon

sources and all results for xylanase, endoglucanase, FPase, β-glucosidase and laccase production are presented in Tables 2 (SSF) and 3 (SmF).

Table 2: Maximal enzymatic activity produced by wood-rot Basidiomycetes and plant pathogenic fungi cultured under SSF using corn cob, wheat bran and Kraft pulp as carbon sources.

		*Enzymatic Activity (U L^{-1})				
Strain	Substrate	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J-2	Corn cob	^{4}1300 ± 20	^{4}520± 20	^{4}37 ± 4	^{12}69 ± 5	^{4}584 ± 42
	Wheat bran	^{4}5487 ± 51	^{4}1945 ± 162	^{4}75 ± 2	^{8}519 ± 48	^{8}2444 ± 240
	Kraft pulp	^{12}744 ± 23	^{12}89 ± 9	^{8}34 ± 1	^{4}12 ± 1	^{4}4 ±0.5
Trametes sp. J-5	Corn cob	^{4}1626 ± 121	^{4}606 ± 48	^{4}68 ± 7	^{8}91 ± 13	^{4}4318 ± 2
	Wheat bran	^{4}4159 ± 297	^{4}716 ± 55	^{8}92 ± 4	^{8}409 ± 19	^{4}3169 ± 141
	Kraft pulp	^{8}533 ± 43	^{4}406 ± 18	^{4}45 ± 2	^{4}27 ± 1	^{4}12 ± 1
J-129	Corn cob	^{4}3903 ± 155	^{4}392 ± 14	^{4} 30 ± 2	^{12}99 ± 10	^{12}14 ± 3
	Wheat bran	^{4}3472 ± 81	^{8}585 ± 32	^{4}32 ± 3	^{8}373 ± 36	^{8}346 ± 14
	Kraft pulp	^{8}158 ± 12	^{4}68 ± 6	^{8}19 ± 4	^{4}19 ± 1	-
Pycnoporus sanguineus	Corn cob	^{4}865 ± 24	^{8}428 ± 21	^{8}24 ± 1	^{8}83 ± 8	^{4}823 ± 21
	Wheat bran	^{8}6049 ± 393	^{8}654 ± 35	^{4}56 ± 2	^{8}724 ± 49	^{8}11032 ± 391
	Kraft pulp	^{4}1151 ± 98	^{4}818 ± 61	^{12}136± 12	^{4}36 ± 4	^{4}61 ± 2
Chrysoporthe cubensis	Corn cob	^{4}27740 ± 987	^{4}471 ± 27	^{4}42 ± 5	^{4}219 ± 18	-
	Wheat bran	^{4}25004 ± 295	^{8}2335 ± 12	^{4}105 ± 8	^{4}1487 +13	^{4}19 ± 1
	Kraft pulp	^{4}10112 ± 82	^{4}513 ± 35	^{12}32 ± 7	^{4}39 ± 1	-
Cylindrocladium pteridis	Corn cob	^{4}16359 ± 810	^{12}229 ± 2	^{4}24 ± 3	^{12}106 ± 5	-
	Wheat bran	^{8}142519 ± 782	^{4}978 ± 86	^{8}66 ± 5	^{8}373 ± 27	-
	Kraft pulp	^{8}5387 ± 281	^{12}198 ± 8	^{4}44 ± 4	^{4}13 ± 1	-

*The superscripts indicate the fermentation time (in days) that provided the greatest activity.

It was noted that the production of a particular enzymatic activity was highly dependent on the fungal specie; however, all strains exhibited a quite diverse response when cultured on different substrates and culture condition. Firstly, the outstanding capacity of *C. pteridis* to produce xylanase activity was noted. This fungus was able to produce 142519 U L^{-1} when cultured on wheat bran under SSF (Table 2); nevertheless, considerable xylanase yields also were observed when *C. pteridis* was cultivated under SmF using wheat bran (85111 U L^{-1}) and Kraft pulp (38799 U L^{-1}) as substrates (Table 3).

Chrysoporthe cubensis showed the second best performance for xylanase production, and yields of 27740 and 25004 U L^{-1} were obtained for

cultivation on corn cob-SSF and wheat bran-SSF, respectively (Table 2). The xylanase yields observed for the Basidiomycete strains ranged from 132 to 8529 U L^{-1} (Tables 2 and 3), indicating that under appropriate cultivations conditions these species also can produce considerable amounts of xylanases.

Table 3: Maximal enzymatic activity produced by wood-rot Basidiomycetes and plant pathogenic fungi cultured under SmF using corn cob, wheat bran, Kraft pulp and Avicel as carbon sources.

		Enzymatic Activity U L^{-1}				
Fungi	Substrate	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J-2	Corn cob	^{12}5611 ± 196	^{12}597 ± 25	^{12}47 ± 4	^{12}118 ± 9	^{4}278 ± 11
	Wheat bran	^{8}1611± 61	^{8}138 ± 3	^{8}21 ± 1	^{8}33 ± 1	^{8}214 ± 25
	Kraft pulp	^{8}830 ± 63	^{12}325 ± 1	^{12}19 ± 1	^{12}30 ± 1	^{4}564 ± 33
	Avicel	^{12}223 ± 17	^{12}63 ± 2	-	-	^{12}320 ± 27
Trametes sp. J-5	Corn cob	^{12}4885 ± 312	^{12}658 ± 42	^{12}44 ± 5	^{12}77 ± 8	^{4}484 ± 39
	Wheat bran	^{8}2262 ± 45	^{8}174 ± 8	^{8}28 ± 2	^{8}59 ± 2	^{8}850 ± 16
	Kraft pulp	^{8}661 ±1	^{12}66 ± 7	^{8}6 ± 1	^{8}3 ± 1	^{8}833 ± 26
	Avicel	^{8}132 ± 4	^{12}42 ± 5	-	-	^{12}852 ± 56
J-129	Corn cob	^{8}8529 ± 385	^{12}669 ± 23	^{8}36 ± 2	^{12}407 ± 10	-
	Wheat bran	^{4}6844 ± 677	^{8}182 ± 17	^{4}21 ± 2	^{12}377 ± 31	^{12}75 ± 12
	Kraft pulp	^{4}1357 ± 58	^{12}369 ± 2	^{8}17 ± 2	^{12}180 ± 9	-
	Avicel	^{12}386 ± 5	^{12}160 ± 7	^{12}16 ± 3	^{12}70 ± 1	-
Pycnoporus sanguineus	Corn cob	^{8}1878 ± 78	^{8}890 ± 9	^{8}55 ± 5	^{8}114 ± 12	^{12}1430±108
	Wheat bran	^{8}931 ± 63	^{4}137 ± 11	^{8}20 ± 1	^{8}56 ± 6	^{8}2177 ± 216
	Kraft pulp	^{12}1217 ± 99	^{12}500 ± 14	^{12}35 ± 3	^{12}103 ± 7	^{12}2711 ± 127
	Avicel	^{12}165 ± 2	^{12}70 ± 8	-	-	-
Chrysoporthe cubensis	Corn cob	^{12}714 ± 49	^{12}120 ± 11	^{12}7 ± 0.8	^{12}82 ± 3	-
	Wheat bran	^{4}7190 ± 530	^{8}521 ± 16	^{4}37 ± 2	^{12}501 ± 36	^{12}54 ± 6
	Kraft pulp	^{12}6283 ± 502	^{8}393 ± 2	^{8}24 ± 2	^{12}70 ± 6	^{4}28 ± 4
	Avicel	^{12}514 ± 31	^{12}259 ± 18	^{12}15 ± 1	^{12}47 ± 2	^{12}15 ± 2
Cylindrocladium pteridis	Corn cob	^{12}7011 ± 685	^{12}239 ± 25	^{12}34 ± 2	^{12}89 ± 6	-
	Wheat bran	^{4}85111 ± 3579	^{12}214 ± 24	^{4}39 ± 3	^{8}185 ± 15	-
	Kraft pulp	^{8}38799 ± 2784	^{8}850 ± 32	^{12}30 ± 2	^{4}8 ± 3	-
	Avicel	^{8}6390 ± 512	^{12}90 ± 3	^{12}14 ± 1	^{12}43 ± 7	-

*The superscripts indicate the fermentation time (at days) that provided the highest activity

The greatest endoglucanase activities for SSF were observed in the cultivation of *C. cubensis* (2235 U L^{-1}), *Trametes* J2 (1945 U L^{-1}) and *C. pteridis* (978 U mL^{-1}) using wheat bran as carbon source (Table 2). On the other hand, at SmF, the best endoglucanase yields were observed when cultivating *P. Sanguineus* (890 U L^{-1}) and *C. pteridis* (850 U L^{-1}) on corn cob and Kraft pulp, respectively (Table 3).

The white-rot Basidiomycete *P. Sanguineus* presented the greatest ability to produce FPase activity, regardless of the cultivation system. When *P. sanguineus* was cultured under SmF with corn cob, a production of 55 U L^{-1} was observed (Table 3). Meanwhile, *P. sanguineus* cultivation under SSF resulted in a yield of 136 U L^{-1}; however, in this case, Kraft pulp was used as a substrate (Table 2). The highest FPase concentrations in both SSF and SmF systems were generally obtained for cultures of white-rot Basidiomycetes (*P. sanguineus, Trametes* sp. J2 and *Trametes* sp. J5), suggesting that these microorganisms have a great potential to produce an efficient cellulolytic complex for cellulose depolymerization. Nevertheless, a high FPase yield was also observed for cultures of *C. cubensis* on wheat bran-SSF (102 U L^{-1}) (Table 2) which suggests that this plant pathogenic fungus may also be a valuable source of cellulolytic enzymes.

A wide variation was observed for β-glucosidase production and titres ranging from 3 to 1487 U L^{-1} were encountered, including SSF and SmF experiments (Tables 2 and 3). *Chrysoporthe cubensis* presented the highest production of β-glucosidase activity, and yields of 501 and 1487 U L^{-1} were observed when the fungus was cultured using wheat bran as a carbon source for SmF and SSF, respectively. Appreciable productions of β-glucosidase were also observed in the enzymatic extracts of *Trametes* sp. J2, *Trametes* sp. J5, *P. sanguineus* and strain J-129 when they were cultured on wheat bran-SSF (Table 2).

As expected, high levels of laccase activity were found in enzymatic extracts from white-rot Basidiomycetes, whereas low or no laccase activity was detected for cultures of *C. cubensis*, *C. pteridis* and strain J-129. *Pycnoporus sanguineus* appeared to be the best laccase producer and titres of 11032 and 2711 U L^{-1} were observed when the fungus was cultured on wheat bran-SSF (Table 2) and Kraft pulp-SmF (Table 3), respectively. These yields were 2.5 and 3.2-fold higher than the maximum activity obtained by *Trametes* sp. J-5 (the

second best laccase producer) cultured under SSF and SmF respectively, which indicated the great potential of *P. sanguineus* to produce laccase activity.

The results reported here showed that the six strains evaluated have an outstanding potential to secrete enzymes of great interest for biotechnological processes. However, each fungal strain showed a specific ability to secrete different enzymatic activities. The white-rot Basidiomycetes *P. sanguineus*, *Trametes* sp. J2 and *Trametes* sp. J5 simultaneously produced considerable amounts of hydrolases and laccase (ligninase) under several fermentations conditions, and these results are in agreement with those reported for other species of white-rot Basidiomycete including *Cerrena maxima*, *Funalia trogii*, *Trametes pubescens*, *Trametes versicolor*, *Lentinus edodes* and *Pleurotus dryinus [20, 21]* . White-rot Basidiomycetes are the most frequent wood rotting organisms and they are characterized by their ability to degrade lignin, hemicelluloses and cellulose, often giving rise to a cellulose-enriched white material *[22]*. The particular capacity of white-rot Basidiomycetes to produce enzymes which degrade (or transform) all biomass components suggests that these microorganisms may have a central role in the future regarding enzyme production for use in biorefinery processes.

C. cubensis, *C. pteridis* and strain J-129 were not able to produce considerable ligninase activity. However, they showed an outstanding capacity to secrete cellulases and xylanases which suggests that these microorganisms may also be used as enzyme sources for application in several biorefinery processes such as biomass hydrolysis (cellulases and xylanase) and biobleaching of Kraft pulp (xylanases).

It was also observed that the ability of each fungal strain to produce a specific enzymatic activity was strongly influenced by the carbon source and the cultivation system. These results indicated that it is possible to induce the production of a certain enzymatic activity by the manipulation of culture conditions, thus obtaining an enzyme extract with characteristics desired for a given biotechnological process.

4.4.2. Enzyme production as a function of the carbon source

As it was indicated, the levels of extracellular enzyme activities produced during fermentation of different substrates varied with the fungal strain and fermentation conditions, but some general impressions might be noted

concerning the different carbon sources. First of all, it was clear that the best enzymatic productions were generally obtained when agricultural residues were used as substrate; however different responses were found depending of cultivation system. Considering SSF, all fungal strains produced greatest enzymatic activities when wheat bran was used as substrate. The only exceptions were laccase production by *Trametes* sp. J5 (highest activity on corn cob), endoglucanase and FPase production by *P. sanguineus* (highest activity on Kraft pulp) and xylanase production by *C. cubensis* and strain J-129 (highest activity on corn cob).

When SmF was considered, it was noted that for the Basidiomycete strains, corn cob was the best inducer of cellulase and xylanase production, whereas the highest laccase titres were obtained inn cultures with wheat bran or Kraft pulp. Contrarily, the plant pathogenic strains *C. cubensis* and *C. pteridis* produced the highest enzymatic activities under SmF when wheat bran was employed as substrate, where the only exception was the maximal endoglucanase production by *C. pteridis* which was found at cultivation on Kraft pulp. Still considering cultivation under SmF, it was noted for all fungal strains that the lowest enzymatic productions were found for cultivation using Avicel, which it suggests that this substrate was not a good inducer for lignocellulolytic enzyme production.

It is a well-established fact that culture conditions significantly affect the production of cellulases and hemicellulases. Carbohydrates or their derivatives induce most cellulolytic enzymes. Thus, the carbon source plays an important role in enzyme production *[23, 24]*. The results reported here suggest a close correlation between the complexity of the substrate and its properties to promote production and secretion of lignocellulolytic enzymes in the fungal strains investigated. Both, corn cob and wheat bran, are natural substrates consisting of not only cellulose, but also hemicellulose, lignin, proteins, starch, lipids and mineral *[25-27]*, unlike Kraft pulp and Avicel which are constituted almost exclusively of cellulose.

The diversity of wheat bran and corn cob composition suggests that these substrates can provide not only sugar to microorganisms, but also other nutrients such as amino acids, lipids and micronutrients which are essential for fungi growth and development. Moreover, corn cob and wheat bran are substrates rich in hemicellulose, mainly arabinoxylan *[25, 28]*, composts which

are easily digested and contribute as good sources of soluble sugars like arabinose and xylose, thus, promoting faster growing of the fungus with a concomitant higher enzyme production *[27]*.

However, it is necessary to emphasize that not all differences in enzyme production found for the different carbon sources can be attributed to the chemical composition of substrates used in this work. The substrates presented some differences in their morphology and particle size and these are aspects that also influence the development of the microorganisms and their capacity to secrete enzymes, mainly in SSF systems where the particle size of the substrate can affect both, aeration and specific area available for mycelia growth *[29]*. Therefore, additional studies need to be done to evaluate the effect of these variables over the enzyme production by six fungal strains studied here.

In this work, wheat bran and corn cob were the best inducers of lignocellulolytic enzymes and this can be considered advantageous because these agricultural residues are inexpensive and their use as substrates would certainly contribute to reduce the costs of enzymes production.

4.4.3. Enzyme production as a function of the fermentation system

Comparing the values of enzymatic activities obtained for the cultivations in SSF and SmF, it was noted that the responses were clearly influenced by the fungal strains and by the substrate used in each occasion, and this obviously interferer on a direct comparison between the results obtained in SSF and SmF. However, some general patterns were detected which may be quite useful in future studies involving the production of enzymes by the fungal strains investigated here.

Firstly, the enzymatic extracts obtained via SSF generally showed higher enzymatic activities (higher enzymatic concentrations) that those obtained under SmF. This is quite advantageous because concentrated extracts require fewer steps in downstream processing, thus reducing the operational costs for obtaining enzymatic extracts with adequate characteristics to be applied in biotechnological processes.

Secondly, despite of the higher enzymatic activities found in extracts obtained from SSF, it is worth noting that the enzyme productivity per gram of substrate in SmF was higher (data not shown). This implies that the availability

and the cost of obtaining of a particular lignocellulosic substrate also should be taken in account at the time of choosing the most appropriate procedure (SSF or SmF) for producing a specific enzymatic activity.

Lastly, it was observed that under SSF, the maximal activities were preferentially achieved on the fourth day of cultivation, unlike that observed under SmF where the maximal activities were generally detected after 8 and 12 days of fermentation. These results suggest that the fungal strains studied in this work could be cultured under SSF to obtain concentrated enzymatic extracts in short fermentation periods which would result in a lower processing cost.

The majority of reports on microbial production of cellulases, hemicellulases and ligninases utilize submerged fermentation technology (SmF) to obtain the enzymatic preparations *[5]*; however, several authors have suggested that the SSF is the most appropriated method for aerobic fungi cultivation because these growth conditions are similar to their natural habitats *[5, 21, 30]*. Moreover, other advantages are attributed to the use of SSF for cultivation of microorganisms and obtaining byproducts such as: low water demand, highly concentrated end product, lower catabolite repression, utilization of substrates insoluble in water, higher volume productivity, lower sterility demand and low energy demand for heating *[30]*. All these aspects associated with the use of an inexpensive raw material as substrate (e. g. agricultural residues, urban waste) make SSF a system strongly recommended for producing cellulases at lower prices than submerged cultures *[5, 30]*. Tengerdy et al. *[31]* estimated fermentation costs of cellulase production at US \$0.2 kg^{-1} in an *in situ* SSF, in contrast to US \$20 kg^{-1} in a stirred tank reactor.

However, the major advantages of SSF over SmF are still reported based on laboratorial experiments and there are severe engineering problems that prevent the establishment of SSF at the industrial scale. The main obstructions to SSF at large scale are due mainly to the build-up of gradients - of temperature, pH, moisture, substrate concentration or O_2 pressure - during cultivation, which are difficult to control under limited water availability *[30]*. Submerged fermentation is used in almost all industrial processes for production of enzymes because this method allows for better monitoring of growing conditions, thus ensuring more standardized and repeatable processes *[5]*. Nevertheless, several studies have been developed in recent years aiming

to overcome the limitations of SSF at the large scale and this suggest that SSF could gain a higher relevance in terms of industrial enzyme production since it presents a series of advantages over SmF.

The enzymatic productions obtained by *Trametes* sp. J2, *Trametes* sp. J5, *P. sanguineus*, *C. cubensis*, *C. pteridis* and strain J-129 cultured under different fermentation systems suggest that better yields could be obtained in the SSF system, however, further studies should be carried out to test new substrates and new culture conditions (humidity, temperature, C/N ratio) so that maximum production was achieved. Moreover, the problems concerning large scale production also should be investigated.

4.4.4. Partial characterization of enzymatic activities

Xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by the six different fungal strains were partially characterized in relation to optimal pH and temperature in order to determine the specific conditions for application of these enzymes in biotechnological processes. All enzymatic characterization experiments were carried out using crude extract obtained from cultivation on SSF during four days using wheat bran as a carbon source. This condition was standardized because it generally provided crude extracts with the highest enzymatic activities.

The values of pH that ensured maximal activities for xylanase, endoglucanase, FPase, β-glucosidase and laccase produced by the different fungal strains are shown in Table 4. The optimum pH for xylanase activities ranged from 4.0 (*C. cubensis*) to 6.0 (strains J-129); meanwhile, the maximum cellulolytic and β-glucosidase activities were found in the pH range of 3.0-5.5. Laccase activities were higher at low pH values and the maximal activities were observed in a range of 2.5-3.5. It is necessary to highlight that laccases are promiscuous enzymes which are able to oxidize a myriad of natural and synthetic substrates and it is widely reported that laccases tend to react differently with different substrates depending of pH *[32-34]*. Thus, the data reported here refers only to the synthetic substrate ABTS, requiring additional assays to determined the effect of pH on laccase activities to oxidize others substrates.

Table 4: Values of optimal pH found for xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by wood-rot Basidiomycetes and plant pathogenic fungi cultured under SSF, using wheat bran as a carbon source.

	Optimum pH				
Fungal Strain	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J2	5.0	3.5	5.0	4.5	3.0
Trametes sp. J5	5.0	4.0	5.0	4.5	2.5
J-129	6.0	4.5	5.5	5.0	3.5
Pycnoporus sanguineus	5.0	3.5	4.5	4.5	2.5
Chrysoporthe cubensis	4.0	4.0	4.5	4.5	2.5
Cylindrocladium pteridis	5.0	3.5	3.5	4.5	-

It was noted that the highest observed values for optimal pH were always associated with the enzymatic activities produced by fungal strain J-129. Furthermore, enzymes from strain J-129 were always more active than enzymes produced by another strains when incubated at higher pH values and this was particularly evident for xylanase and endoglucanase activities. Xylanases and endoglucanases from strain J-129 maintained 75 and 60 % of their maximal activities, respectively, when incubated at pH 7.5. On the other hand, xylanase and endoglucanase activities produced by the other fungal strains showed residual activities less than 15% when assayed at the same pH (data not shown). These results suggest that enzymes produced by strain J-129 have a particular potential to be applied in biotechnological processes that are carried out at neutral or slightly alkaline pH, such as biobleaching of Kraft pulp *[35, 36]* and biostoning *[37]*.

However, despite the particular characteristics shown by enzymes produced by strain J-129, it was observed that all others fungal strains secreted enzymes with maximal activities at acidic pH values and these results are in accordance with those reported for major fungal hydrolases *[34, 38-41]*. Acidic cellulases are usually more desirable for bioconversion, especially when acidic pretreatment of biomass is employed or when the enzymes makes up part of a cocktail with *T. reesei* enzymes whose optima activity is close to 5 *[5]*.

Table 5 presents the values of optimal temperatures for xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by the Basidiomycetes and plant pathogenic fungi investigated in this work. All enzymatic activities showed a maximal hydrolysis/oxidation rate when incubated at temperatures ranging from 50 to 60 °C. The only exceptions were

endoglucanase activity produced by the strains *Trametes* sp. J2 and *Trametes* sp. J5 which presented maximal actives at 70 and 65 °C, respectively.

Table 5: Values of optimal temperature found for xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by wood-rot Basidiomycetes and plant pathogenic fungi cultured under SSF, using wheat bran as a carbon source.

	Optimal Temperature (°C)				
Fungal Strain	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J2	60.0	70.0	60.0	60.0	55.0
Trametes sp. J5	55.0	65.0	60.0	50.0	55.0
J-129	55.0	60.0	50.0	60.0	60.0
Pycnoporus sanguineus	60.0	60.0	55.0	55.0	60.0
Chrysoporthe cubensis	55.0	55.0	50.0	55.0	55.0
Cylindrocladium pteridis	55.0	55.0	55.0	60.0	-

The incorporation of an enzymatic step in an industrial biotransformation process requires enzymes that showing high stability and high processivity at the conditions in which the process is usually carried out. For instance, alkaline and thermal stable xylanases are indicated for application in biobleaching processes since the conventional pulp bleaching is generally conducted under high temperature and pH. The values of optimal temperature found for hydrolytic and oxidizing activities suggest that the enzymatic extract produced by the six fungal strains has potential to be applied in biotransformation processes such as biomass saccharification, biobleaching, biostoning and others since a temperature range of 50-60 °C is usually employed in these processes *[5, 37, 42-44]*.

4.5. General remarks

In this work, six new fungal strains isolated from eucalyptus plantation were investigated in order to study their potential to produce enzymes of biotechnological interest for application in biorefinery processes. It was observed that the productions of xylanase, endoglucanase, FPase, β-glucosidase and laccase activities were strongly dependent on the fungal strain, carbon source employed and cultivation system, indicating that the exploration of cultivation conditions is a crucial step to evaluate and select new microorganisms with outstanding ability to secrete hydrolases and oxidases.

Among the strains studied, *P. sanguineus* produced the highest FPase activity, indicating that this microorganism could be a good enzyme supplier to processes of biomass saccharification. *Pycnoporus sanguineus* was also able to secrete large amounts of laccase, a multifunctional enzyme that due its capacity to oxidize different phenolic compounds, may be applied in different bioprocesses such as bioremediation, biomass saccharification, biobleaching and production of special papers *[4]*.

Chrysoporthe cubensis also produced significant amounts of cellulases, however its most prominent characteristic was the ability to produce β-glucosidase activity. β-Glucosidase is a key enzyme in enzymatic biomass saccharification processes because it prevents the natural inhibition of the process caused by cellobiose accumulation *[45]*. Currently, the main commercial extracts of cellulases employed in biomass saccharification are β-glucosidase deficient *[5, 46]*; thus enzymatic extracts produced by *C. cubensis* could be employed as a β-glucosidase supplement, boosting the saccharification processes.

Also deserving attention is the fungus *C. pteridis* which showed an unusual capacity to secrete xylanolytic activity when cultured under SSF using wheat bran as a support and this indicated that this microorganism has high potential to be an efficient producer of inexpensive xylanase.

Finally, the microorganisms investigated here showed to be promising for industrial application since they grow quickly under SSF or SmF using inexpensive agricultural residues and secreting large amounts of important industrial enzymes. Furthermore, the enzymatic activities obtained in this study presented properties that are frequently required for industrial application.

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5. Artigo 2 - Characterization of cellulolytic extract from *Pycnoporus sanguineous* PF-2 and its application in biomass saccharification

5.1. Abstract

The aim of this work was to evaluate the biochemical features of the white-rot fungi *Pycnoporus sanguineous* cellulolytic complex and its utilization to sugarcane bagasse hydrolysis. When cultivated under submerged fermentation using corn cobs as carbon source *P. sanguineous* produced high FPase, endoglucanase, β-glucosidase, xylanase, mannanase, α-galactosidase, α-arabinofuranosidase and polygalacturonase activities. The enzymatic extract was submitted to gel filtration chromatography and it was observed multiple forms of endoglucanase, β-glucosidase, xylanase and mannanase. The cellulase activities were characterized in relation to pH and temperature. β-Glucosidase and FPase activities were higher at 55 °C, pH 4.5 and endoglucanase activity was higher at 60 °C, in a pH range of 3.5-4.5. All cellulase activities were highly stable at 40 and 50 °C through 48 h of pre-incubation. Crude enzymatic extract from *P. sanguineous* was applied in a saccharification experiment using acid-treated and alkali-treated sugarcane bagasse as substrate and the hydrolysis yields were compared to that obtained by a commercial cellulase. The reactions were carried out at 50 °C for 72 h employing 10 FPase units per gram of substrate. Reducing sugar yields of 60.4 % and 64 % were reached when alkali-treated bagasse was hydrolyzed by *P. sanguineous* extract and commercial cellulase, respectively. Considering the glucose release *P. sanguineous* extract and commercial cellulase ensured yields of 22.6 and 36.5 %, respectively. The saccharification of acid-treated bagasse reached lower reducing sugar and glucose yields than saccharification of alkali-treated bagasse when *P. sanguineous* or commercial cellulase was employed. Present work showed that *P. sanguineous* has a great potential as an enzyme producer for biomass saccharification.

5.2. Introduction

The increased concern for the security of oil supplies and the negative impact of fossil fuels on the environment, particularly greenhouse gas

emissions, has put pressure on society to find renewable fuel alternatives. The most common renewable fuel produced today is ethanol from sugar or grain (starch); however, this raw material base will not be sufficient to meet alternative fuel demands. Thus, future large-scale use of ethanol will most certainly have to be based on production from lignocellulosic materials *[1]*. Lignocellulosic biomass materials such as agricultural residues (e.g., wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood) and dedicated crops (switchgrass, salix) are examples of potential raw materials for bioethanol production with great availability and low cost *[2]*. However, while fermentation of corn starch or sugarcane juice by *S. cerevisiae* is a well-established technology, conversion of lignocellulose biomass to ethanol is rather challenging. It involves a pretreatment process to release cellulose, hemicellulose and lignin from the lignocellulose matrix, enzymatic hydrolysis to produce reducing sugars and fermentation to convert sugar mixtures to ethanol *[3]*.

The enzymatic conversion of cellulose in reducing sugars is a process that requires the cooperative actions of three types of enzymes that act synergistically. Endoglucanases (E.C. 3.2.1.4) randomly attack cellulose chains exposing reducing and non-reducing ends of cellulose fibers. Then, cellobiohydrolases (E.C. 3.2.1.91) act on the reducing and non-reducing ends, releasing cellobiose units which by action of enzyme cellobiase/β-glucosidase (E.C. 3.2.1.21) are converted to glucose *[4]*. Moreover, hemicellulolytic enzymes such as xylanases (E.C. 3.2.1.8), mannanases (E.C. 3.2.1.78), β-xylosidases(E.C. 3.2.1.37), β-mannosidases (E.C. 3.2.1.25), α-arabinofuranosidases (E.C. 3.2.1.55), α-galactosidases (E.C. 3.2.1.22), acetyl xylan esterase (E.C. 3.2.1.72), feruloyl esterases (E.C. 3.1.1.73), p-coumaryl esterases (E.C. 3.2.1.73) and α-glucuronidase (E.C. 3.2.1.139) also play an important role in the cellulose depolymerization process. These enzymes hydrolyze and remove the hemicellulose fragments that coat the cellulose fibers, increasing the cellulose accessibility and spurring the action of cellulases *[5-7]*.

The major bottleneck in the production of second-generation bioethanol is the high cost of cellulolytic enzymes *[8]*. Large-scale application of cellulases to processes of lignocellulosic material degradation demand microorganisms with improved activity and productivity, as well as cellulases with better

resistance to product inhibition *[3]*. Enzymes for degradation of the polysaccharide part of biomass have been produced mostly by fungi belonging to genus *Trichoderma*. Fungi such as *Trichoderma reesei* and *Trichoderma viride* are able to secrete large amounts of endoglucanase and cellobiohydrolase enzymes; however, the amount of β-glucosidase secreted by *Trichoderma* species is very low and this leads to accumulation of cellobiose and thus an incomplete cellulose hydrolysis when cellulolytic extracts produced by these fungi are employed in saccharification processes *[3, 6, 9]*. Other important cellulase producing fungi, as those belonging to the genera *Penicillium* and *Aspergillus* are able to secrete large amounts of β-glucosidase. However the total cellulase activity (FPase) found in their enzymatic extracts is relatively low. As a result, production of glucose from waste cellulose is not commercially feasible yet *[3]*.

Based on these facts, there is a constant search for new cellulolytic microorganisms that are able to simultaneously produce cellulases in large quantities and in balanced proportions, so that it can be used for efficient saccharification of lignocellulosic biomass.

The white-rot Basidiomycetes are efficient decomposers of lignocellulose due their capability to synthesize the relevant hydrolytic enzymes (cellulases and hemicellulases) and the unique oxidative network of ligninolytic extracellular enzymes (e.g., laccase, manganese peroxidase, lignin peroxidase) which are responsible for degradation of the biomass components into low molecular weight compounds that can be assimilated by fungi *[10, 11]*. White-rot fungi species including *Pleurotus ostreatus [12], Ceriporiopsis subvermispora [13]* and *Trametes versicolor [14]* have been evaluated regarding their ability to produce ligninocellulolytic enzymes at different growth conditions and high titres of enzymes had been obtained. However, few reports are available concerning the use of enzymatic extracts produced by white-rot Basidiomycetes in biomass saccharification processes.

In the present study, the production of cellulases and hemicellulases by white-rot fungi *Pycnoporus sanguineus*, a well-known producer of ligninolytic enzymes, was examined in submerged fermentation using corn cob as carbon source. The produced cellulolytic complex was characterized in terms of pH, temperature and thermal stability. For the first time, an attempt was made to apply the cellulolytic complex from *P. sanguineus* in a process of biomass

saccharification using sugarcane bagasse as substrate. Saccharification assays using a commercial cellulases complex were carried out parallel in order to establish a comparison between performances. In addition, the effect of different sugarcane bagasse pretreatment methods on the saccharification yield also was analyzed.

5.3. Material and Methods

5.3.1. Materials

Substrates including ρ-nitrophenyl-β-D-glucopyranoside (pNPGlc), ρ-nitrophenyl-β-D-xylopyranoside (pNPXyl), ρ-nitrophenyl-β-D-mannopyranoside (pNPMan), ρ-nitrophenyl-β-D-galactopyranoside (pNPGal), ρ-nitrophenyl-α-D-arabinofuranoside (pNPAra), cellobiose, carboxymethilcellulose (CMC), xylan from birchwood, locust bean gum, polygalacturonic acid and also the chemical reagents monopotassium phosphate, ammonia nitrate, magnesium sulfate, calcium chloride, cuprum sulfate, sodium acetate, sodium carbonate, dinitrosalicylic acid (DNS) and potato dextrose agar (PDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Yeast extract was obtained from Himedia Laboratories Co. (Mumbai, Maharashtra, India). Chemical reagents including NaOH, $H_2SO_4$and potassium sodium tartrate were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil).The commercial cellulases complex, Multifect® CL, was purchased from Genencor International Inc. (Rochester, NY, USA). Milled corn cobs and sugarcane bagasse was obtained locally. All others reagents used in this study were of analytical grade.

5.3.2. Microorganism and enzymes production

The *Pycnoporus sanguineus* PF-2 used in this study was obtained from the mycological collection belonging to Laboratory of Forest Pathology of the Federal University of Viçosa, MG, Brazil. The fungus was maintained on PDA plates at 28 °C and subcultured periodically.

The enzymatic extract from *P. sanguineus* was obtained by cultivation of the fungus in submerged fermentation using milled corn cobs as carbon source. The medium used for the experiment had the following composition in grams per liter: KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25; NH_4NO_3, 1.0; $CaCl_2$, 1.0; yeast extract 3.0 and milled corn cobs, 20.0. Furthermore, the medium was supplemented with $MnCl_2$ (0.1 mg L^{-1}), H_3BO_3 (0.75 mg L^{-1}), Na_2MoO_4 (0.02 mg

L^{-1}), $FeCl_3$ (1.0 mg L^{-1}) and $ZnSO_4$ (3.5 mg L^{-1}) as trace elements. Erlenmeyers flasks containing 50 mL of the medium described above were inoculated with 5 mycelial plugs (7.0 mm of diameter) extracted from 5 day-old plates and cultivation was carried out for 120 h in an orbital shaker at 28 °C and 180 rpm. After this cultivation period, the liquid medium was filtered through a cloth filter and immediately centrifuged at 15000 x *g* for 10 min. The clarified supernatant was frozen and stored for subsequent enzymatic analysis.

5.3.3. Enzymatic assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50 °C. All assays were performed in triplicate and the mean values calculated. Relative standard deviations of the measurements were below 5 %. FPase and endoglucanase activities were determined using Whatman no. 1 filter paper and carboxymethilcellulose as substrates respectively, according to previously described standard conditions *[15]*. The total reducing sugars liberated during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method *[16]* using glucose as a standard.

Xylanase, mannanase and pectinase activities were determined using xylan from birchwood (1 % w/v final concentration), locust bean gum (0.4 % w/v) and polygalacturonic acid (0.2 % w/v) as substrates, respectively. The enzymatic reactions were initiated by the addition of 100 μL of the appropriate diluted enzyme solution to 400 μL of the polysaccharide substrate solution prepared in buffer. The reaction mixtures were incubated for 30 min and the total concentration of reducing sugar released was determined by the DNS method using xylose, mannose and galacturonic acid as standards.

Cellobiase activity was determined using cellobiose as substrate and the mixture reaction consisted of 100 μL of the appropriately diluted enzyme solution, 125 μL of cellobiose solution (2 mM at final concentration) and 275 μL of buffer. The reaction mixture was incubated for 30 min and 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). β-Glucosidase, β-xylosidase, β-mannosidase, α-galactosidase and α-arabinofuranosidase activities were measured using ρPNGlc, ρNPXyl, ρNPMan, ρNPGal and ρNPAra as substrates,

respectively. The mixture reactions contained appropriately 100 μL of the diluted enzyme solution, 125 μL of substrate synthetic solution (1 mM at final concentration) and 275 μL of buffer. The reaction mixture was incubated for 30 min and stopped by addition of 0.5 mL of the sodium carbonate solution (0.5 M). Absorbance of the reactions was measured at 410 nm and the amount of ρ-nitrophenol released was estimated using a standard curve. For all activities, one unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 μmol of the corresponding product (glucose, xylose, mannose, galacturonic acid and ρ-nitrophenol) per minute, under the assay condition used.

5.3.4. Analysis of the multienzymatic complex by gel filtration

The crude enzymatic extract produced by *P. sanguineus* using corn cobs as carbon source was submitted to gel filtration chromatography for analysis of the presence of multiples forms of cellulases and hemicellulases. A sample of the crude extract (8 mL) was loaded onto a Sephacryl S-200 column (90 cm x 2.5cm) equilibrated with sodium acetate buffer, 25 mM, pH 5 and eluted with the same buffer at a flow rate of 25 mL h^{-1}. Fractions of 3 mL were collected and the activities of endoglucanase, cellobiase, xylanase, mannanase, pectinase, β-glucosidase, β-xylosidase, α-galactosidase and α-arabinofuranosidase were determined along the elution. Each fraction was also analyzed for protein content by reading the samples at 280 nm.

5.3.5. Determination of optimum pH

To determine the effect of pH on endoglucanase, β-glucosidase and FPase activities, enzymatic assays were carried out at pH values ranging from 2.0-7.0 using citric acid/sodium phosphate buffer *[17]*. The other assays conditions were as aforementioned.

5.3.6. Determination of optimum temperature and thermal stability

The effect of the temperature on endoglucanase, β-glucosidase and FPase activities was evaluated in a range of 30-80 °C at pH 5 based the standardized methods described earlier. Thermal stability of endoglucanase, β-glucosidase and FPase activities were determined at different temperatures.

The enzymatic extract was diluted in sodium acetate buffer, 100 mM, pH 5 and maintained at 40, 50 or 60 °C for up to 48 h. At different time interval, aliquots were taken and the residual activities were measured using the standard assays. All data obtained during the pH and temperature characterization were expressed in terms of relative activity where the highest activity value obtained for each enzymatic activity was regarded as 100%.

5.3.7. Biomass pretreatment and biomass composition analysis

Milled sugarcane bagasse (particle size less than 1 mm) was submitted to both, alkaline and acid pretreatment before being employed in a saccharification experiment. The biomass was dried in an oven at 70 °C until reaching a constant weight. Sodium hydroxide or sulfuric acid at 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 120 °C for 60 min. The pretreated materials were separated into solid and liquid fraction using a Buchner funnel fitted 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 and acid treated sugarcane bagasse was determined using a modified Klason lignin method derived from the TAPPI Standard Method T222 om-98 *[18]*. Extractive-free biomass (0.3 g) was incubated at 30 °C with 3 mL of 72 % H_2SO_4 for 1 h with occasional mixing. The slurry was then transferred into a penicillin bottle containing 84 mL of deionized water and the flask was sealed with a rubber stopper and aluminum seal. The bottle was placed in an autoclave calibrated at 118 °C for 1 h and the slurry was then filtered through a medium coarseness sintered glass filter for gravimetric determination of acid-insoluble lignin. The concentration of the biomass sugars (arabinose, galactose, glucose, xylose, and mannose) in the filtrate was quantified using high-performance liquid chromatography (HPLC), while acid-soluble lignin was determined by absorption measurements at 205 nm *[19]*. The HPLC system DionexDX-300 (Dionex Co.- Sunnyvale, CA, USA) was equipped with a Carbopac PA1 column and a pulsed amperometric detector with a gold electrode. Prior to injection, samples were filtered through 0.45-mm HV filters and a volume of 20 µL was

loaded. The column was pre-equilibrated with a NaOH solution, 300 mM, and elution was carried out at a flow rate of 1.0 $mL.min^{-1}$ at room temperature.

5.3.8. Biomass saccharification

The crude enzymatic extract produced by *P. sanguineus* and the commercial cellulase (Multifect® CL) were applied in a biomass saccharification experiment. The *P. sanguineus* enzymatic extract was concentrated 5-fold before the experiment using an Amicon Ultrafiltration system (Millipore Co. – Billerica, MA, USA) and an YM-10 (Cut-off M_r 10,000 Da) membrane filter. Enzymatic saccharification of alkali and acid treated sugarcane bagasse was performed in 100 mL sample tubes at an initial solid concentration of 2 % dry matter (w/v) in 25 mL of 50 mM sodium acetate buffer at pH 4.5. Enzyme loading was 10 FPase units per gram of biomass and sodium azide (10 mM) and tetracycline (40 $\mu g.mL^{-1}$) were added to the reaction mixture to inhibit microbial contamination. The reaction was carried out in an orbital shaker at 250 rpm and 50 °C for 72 h. Samples (0.5 mL) were taken from the reaction mixture at different time intervals. These samples were immediately heated to 100 °C to denature the enzymes, cooled and then centrifuged for 5 min at 20000 x *g*. The supernatant was used for the determination of total reducing sugar and glucose released during the reaction. The total reducing sugar concentration was quantified by the DNS method *[16]*, while glucose concentration was estimated using a commercial analytical kit based on glucose oxidase and peroxidase. Results were calculated by averaging the values of the sample duplicates and from these average values subtracting value the respective controls which were carried out parallel, in which the active enzymatic extract was replaced by enzymatic samples boiled for 10 min. The cellulose hydrolysis rate and total carbohydrates (cellulose plus hemicellulose) hydrolysis rate were calculated using the following equation:

$$Glucose\ yield\ (\%) = \frac{Amount\ of\ glucose\ released\ (g) \times 0.9 \times 100}{Amount\ of\ cellulose\ in\ pretreated\ bagasse\ (g)}$$

$$Reducing\ sugar\ yield\ (\%) = \frac{Amount\ of\ reducing\ sugar\ released\ (g) \times 0.9 \times 100}{Amount\ of\ carbohydrate\ in\ pretreated\ bagasse\ (g)}$$

5.4. Results and Discussion

5.4.1. Enzyme production

Pycnoporus sanguineus was cultured under submerged fermentation using corn cob as carbon source. Cellulase and hemicellulase activities were measured after 120 h of fermentation and the activity values are shown in Table 1. Endoglucanase activity was the most prominent cellulolytic activity found in the crude extract (8.43 U mL^{-1}), nevertheless, considerable titres of FPase (0.25 U mL^{-1}) and β-glucosidase (0.25 U mL^{-1}) also were determined, suggesting that *P. sanguineus* is able to secret a complete cellulolytic enzymatic complex. With regards to hemicellulase production, xylanase and mannanase activity were the highest activities observed in the crude enzymatic extract from *P. sanguineus*. The fungus also produced α-galactosidase and α-arabinofuranosidase activities at high titres; however, low β-xylosidase and β-mannosidase activity were produced for the white-rot fungus. *Pycnoporus sanguineus* produced a significant amount of polygalacturonase (18.53 U mL^{-1}) during cultivation, revealing the great potential of this microorganism to produce pectinolytic enzymes.

Table 1: Cellulolytic and hemicellulolytic activities produced by *Pycnoporus sanguineus* cultured for 120 h at submerged fermentation using corn cob as carbon source.

Enzyme Group	Enzyme Activity	Activity (U mL^{-1})
Cellulases	FPase	0.25 ± 0.01
	Endoglucanase	8.43 ± 0.31
	β-Glucosidase	0.20 ± 0.0
	Cellobiase	0.06 ± 2.3
Hemicellulases	Xylanase	10.01 ± 0.35
	Mannanase	4.25 ± 0.27
	α-Galactosidase	0.95 ± 0.03
	β-Xylosidase	0.02 ± 0.001
	β-Mannosidase	0.01 ± 0.008
	α-Arabinofuranosidase	0.63 ± 0.01
Pectinase	Polygalacturonase	18.53 ± 0.42

Pycnoporus sanguineus has been studied extensively in recent years due its extraordinary capacity to produce laccases and others ligninases *[20, 21]*, however, there are few papers *[22, 23]* highlighting the potential of this fungus to produce cellulases and hemicellulases. The production of cellulases by *P. sanguineus* achieved in this work was comparable to those described for the fungi *Trichoderma reesei [24]* and *Penicillium funiculosum [25]* cultivated

under submerged condition employing steam pretreated spruce and pretreated sugar cane bagasse as carbon sources, respectively. *Trichoderma* and *Penicillium* are genus widely used for industrial production of cellulases *[3]* and this suggests that *P. sanguineus* also has potential to be used in the production of cellulases for biotechnological processes such as biomass saccharification. Furthermore, the crude enzymatic extract produced by *P. sanguineus* showed high xylanase, mannanase, α-galactosidase, α-arabinofuranosidase and pectinase activities and this is an appreciable feature since these so-called "accessory" enzymes can remove non-cellulosic polysaccharides that coat cellulose fibers, improving the action of cellulases during the biomass saccharification processes *[7]*.

5.4.2. Analysis of the multienzymatic complex by gel filtration

The crude enzymatic extract was submitted to gel filtration chromatography aiming to identify the presence of multiples forms of cellulases and hemicellulases. The chromatographic profiles are showed at Fig. 1 and it could be observed that the α-galactosidase, α-arabinofuranosidase (Fig. 1-A), β-xylosidase (Fig. 1-B) and polygalacturonase (Fig.1-D) activities were eluted in a single peak, suggesting that *P. sanguineus* produced a single form of each enzyme when cultured in submerged fermentation using corn cob as carbon source.

The profile for xylanase activity (Fig.1-B) showed that *P. sanguineus* was able to express at least three different enzymes with xylanolytic activity and it could also be inferred that they have an extremely diverse molecular mass since the peaks were separated by large volumes of elution. The endoglucanase (Fig.1-E) and mannanase (Fig.1-D) activities were separated in three and two peaks, respectively, revealing the presence of multiples forms of this enzyme in the crude enzymatic extract from *P. sanguineus*.

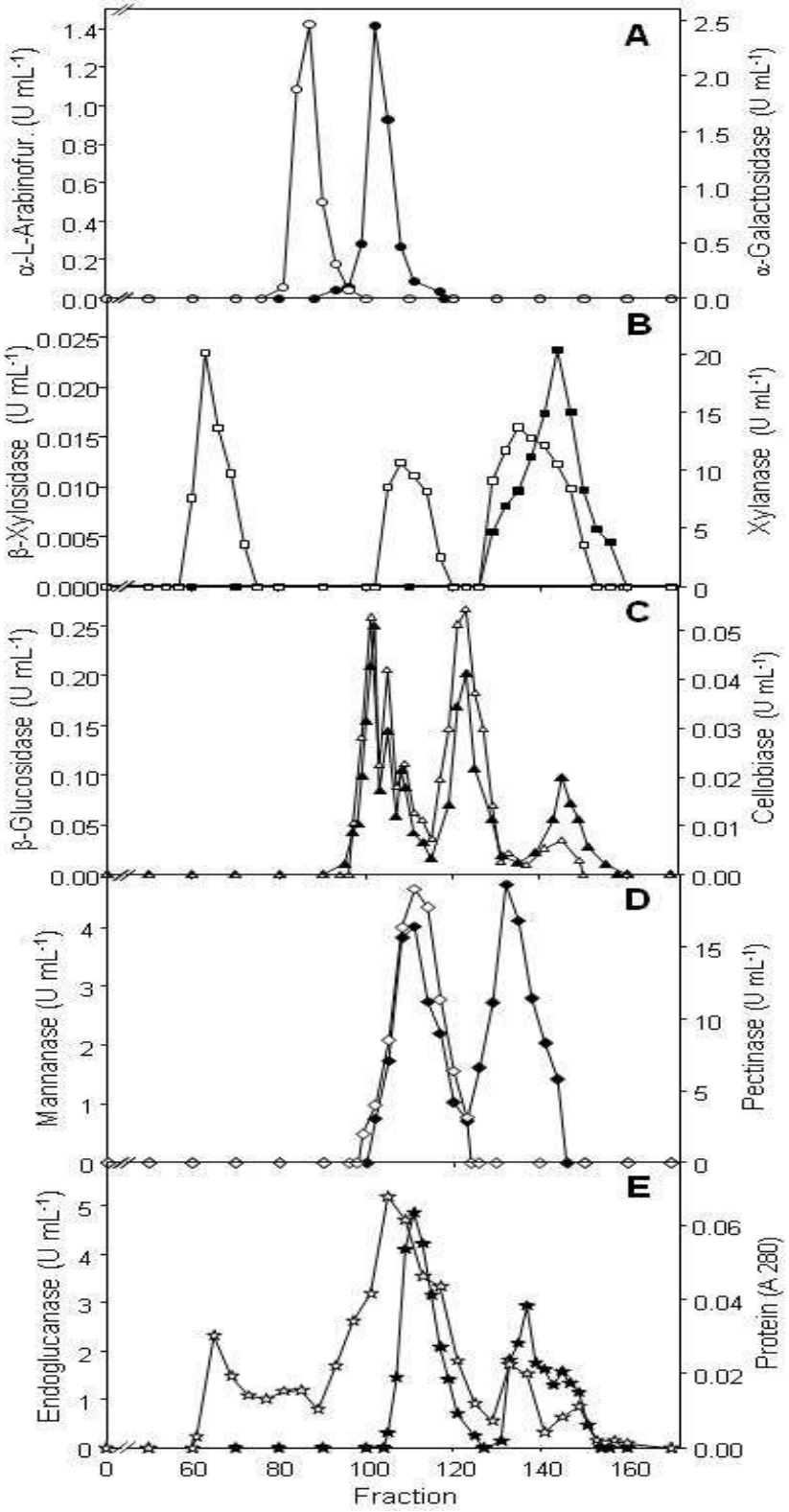

Figure 1: Sephacryl S-200 chromatograms of the crude enzymatic extract produced by *Pycnoporus sanguineus* cultured under submerged fermentation using corn cob as carbon source. Chromatogram A: α-arabinofuranosidase (●) and α-galactosidase (○) activities; Chromatogram B: Xylanase (□) and β-Xylosidase (■) activities; Chromatogram C: β-Glucosidase (▲) and Cellobiase (△) activities; Chromatogram D: Mannanase (◆) and polygalacturonase (◇) activities; Chromatogram E: Endoglucanase (★) activity and protein content (☆). β-Mannosidase activity was not detected during analysis of the elution profile.

As expected, the profiles for β-glucosidase and celobiase (Fig.1-C) were very similar, indicating that *P. sanguineus* secrete at least five different forms of enzymes with capacity to hydrolyze glucose based β-linked oligosaccharides, in natural and synthetic substrates. The secretion of multiples forms of cellulases and hemicellulases with different properties is a typical characteristic of saprophytic fungi *[11, 23, 26, 27]*. Several factors, including biotic and abiotic agents, are responsible to drive the expression of a specific enzyme or a specific group of enzymes; however, the carbon source is generally indicated as one of the most important factors. *Aspergillus terreus* expresses four forms of endoglucanase when cultured in solid fermentation using rice straw as carbon source; on the other hand, just two forms of endoglucanase are secreted by the fungus when it was cultured on wheat bran and sugarcane bagasse *[27]*. In the same way, it was previously verified that a different strain of *Pycnoporus sanguineus* (CEIBMD01) and *Bjerkandera adusta* (UAMH 8258) expressed different xylanase and endoglucanase profiles when cultured on different carbon sources *[23]*. This suggests that *P. sanguineus* could produce different enzymatic profiles if it was cultured under different conditions.

5.4.3. Effects of pH and temperature on cellulolytic activities from *Pycnoporus sanguineus*

The cellulolytic complex from *P. sanguineus* was partially characterized in order to establish suitable application for the enzymes in a process of biomass saccharification. The FPase, endoglucanase and β-glucosidase activities were characterized with regards to pH, temperature, and thermal stability.

The effect of pH on cellulolytic activities present in the crude extract is shown in Figure 2-A. The maximum endoglucanase activity was observed in a pH range of 3.5-4.0, while for FPase and β-glucosidase activities the maximum hydrolysis rate was detected at pH 4.5. All cellulolytic activities were sensitive to high values of pH and only endoglucanase activity was incipiently detected at pH 7.The FPase and β-glucosidase activities were also negatively affected when the reaction was carried out at lower pH values, however, a residual activity for endoglucanase of up to 40 % can be observed at pH 2.The relative activity of endoglucanase was higher than 60 % in the pH range of 2.5-5.5, nevertheless, it was noted that use of the cellulolytic extract from *P. sanguineus*

for biomass saccharification processes would be restricted to a pH range of 4.0-5.0 because FPase and β-glucosidase activities showed pronounced losses when incubated at a pH higher than 5 and lower than 4. Cellulases from *P. sanguineus* showed appreciable characteristics since acidic cellulases are usually more desirable for cellulose bioconversion due to the situations where acidic pretreatment of biomass is employed or when the enzymes make up part of the cocktail with *T. reesei* enzymes whose optima activity is near 5 *[28]*. Overall, the pH activity profile of cellulases from *P. sanguineus* showed features common to those reported for other cellulolytic fungal species *[11, 25, 29, 30]*.

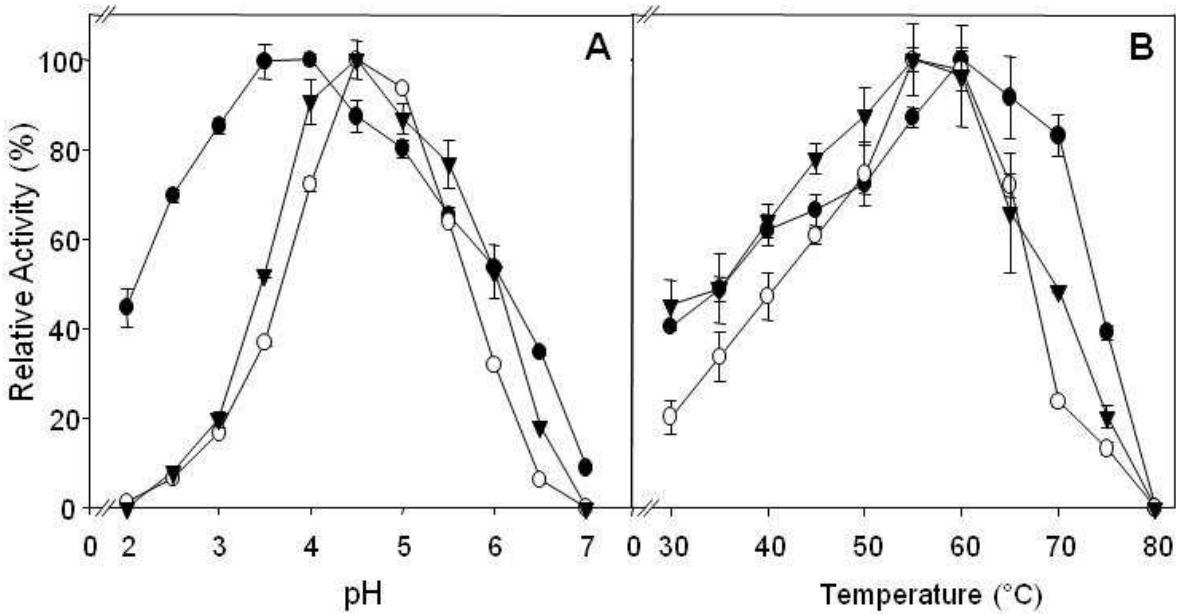

Figure 2: Effect of pH (A) and temperature (B) on endoglucanase (●), β-glucosidase (○) and FPase (▼) activities produced by *Pycnoporus sanguineus*.

The effect of temperature on cellulolytic activities from *P. sanguineus* was investigated in a range of 30-80 °C (Fig. 2-B). The optimum temperature for endoglucanase activity was 60 °C, while both FPase and β-glucosidase activities were maximum at 55 °C. Drastic losses of FPase and β-glucosidase activities were observed at temperatures greater than 60 °C; however, a residual activity of 83 % was observed for endoglucanase activity when the enzymatic reaction was carried out at 70 °C. These results show that endoglucanases from *P. sanguineus* have potential to be employed in industrial processes which require high temperatures such as those found in the textile industry *[29]*. The optimum temperatures found for cellulolytic activities from *P. sanguineus* indicate that the best condition to use its enzymatic extract in a

biomass saccharification process would be in a SHF (Separated Hydrolysis Fermentation) system where the temperature of hydrolysis generally employed is 50 °C. On the other hand, it can be observed that the cellulolytic complex from *P. sanguineus* could also be employed in a SSF (Simultaneous Saccharification Fermentation) system since the residual activities at 40 °C for endoglucanase, FPase and β-glucosidase were 62.02, 64.0 and 47.25 %, respectively. SSF can be carried out within a temperature range of 32-45 °C, depending on the thermotolerance of the microorganism involved in the fermentation *[31-33]* and this process is advantageous mainly because end product inhibition is minimized during the process and a lower enzyme loading is required *[33, 34]*.

Thermal stability of the cellulolytic complex produced by *P. sanguineus* was investigated over a 48 h period at three different temperatures. Profiles of the residual activities for three cellulolytic enzymes are presented in Fig. 3. All cellulase activities were highly stable at 40 °C and after 48 h of incubation the residual activities found for endoglucanase, FPase and β-glucosidase were 92.6, 94.17 and 87.35 %, respectively. Increasing the incubation temperature to 50 °C caused a slight decrease in the stability of cellulases, mainly β-glucosidase activity. However, it was clear that cellulases from *P. sanguineus* showed a great stability at 50 °C since the residual activities determined after 48 h of incubation were 76.22, 76.74 and 69.0 % for endoglucanase, FPase and β-glucosidase activities, respectively. Pronounced losses in activities were observed when cellulases from *P. sanguineus* were incubated at 60 °C and the half-lifes determined for endoglucanase, FPase and β-glucosidase were 17.37, 7.47 and 1.12 h respectively.

Enzymatic biomass saccharification is a process that requires a long time reaction. Generally, a reaction time between 24-72 h is used *[3, 35, 36]* and for this reason thermal stability is an essential feature for a cellulolytic complex to be employed in a biomass saccharification process. The high thermal stability found at 40 and 50 °C indicated that cellulases from *P. sanguineus* have adequate characteristics to be used in biomass conversion processes and also guarantee flexibility for use in both SSF and SHF systems.

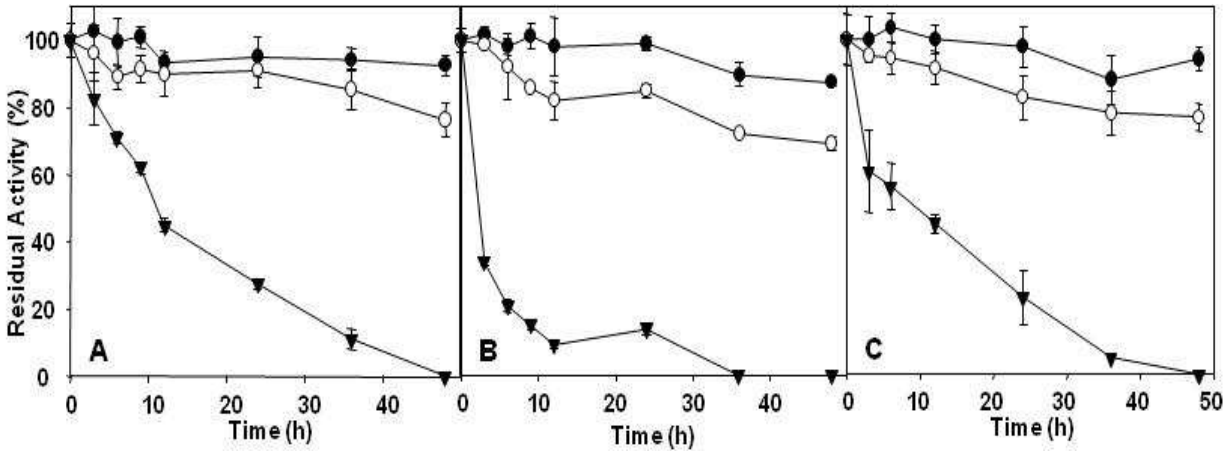

Figure 3: Thermal stability of cellulases produced by *P. sanguineus* at 40 °C (●), 50 °C (○) and 60 °C (▼). Endoglucanase activity (A); β-glucosidase activity (B); FPase (C) activity.

5.4.4. Biomass pretreatment and biomass composition analysis

In order to evaluate the performance of the cellulolytic complex produced by *P. sanguineus*, milled sugarcane bagasse was used as substrate in a biomass saccharification process. Sugarcane bagasse was selected as a substrate for the saccharification experiments because it is produced in large quantities in Brazil. The Annual Brazilian production of sugarcane bagasse is currently estimated at 186 million ton per year and this amount of biomass represent a potential to produce around 11.160 billion gallons of lignocellulosic ethanol per year *[37]*.

Prior to enzymatic hydrolysis, the raw material was subjected to dilute acid and alkaline pretreatment with the intent of evaluating the effect of different pretreatments on the enzymatic saccharification process. Table 2 summarizes the chemical compositions of raw and acid/alkaline pretreated sugarcane bagasse. The chemical composition of the raw sugarcane bagasse sample used in the present study was as follow: cellulose 45.4 %, hemicellulose 24.2 % (sum of xylans, galactans, mannans and arabinans) and lignin 22.0 %. Proteins, extractives and ashes were not measured in the experiments which would explain why the summations are less than 100 %.

The acid and alkaline pretreatments had different effects on chemical composition and weight loss of sugarcane bagasse, reflecting the effectiveness of each pretreatment process. Acid and alkaline pretreatments caused a weight loss of 54.9 and 53.54 %, respectively, indicating that both pretreatments resulted in an intense solubilization of biomass components. Values of biomass solubilization via acid or alkaline pretreatment have been published in a range

of 12.6 to 67.3 % and this discrepancy has been attributed to several factors such as characteristic of the biomass, type of chemical reagent, reagent concentration, temperature, residence time, biomass particle size and others *[38-41]*.

Table 2: Chemical composition of untreated, acid-treated and alkali-pretreated sugarcane bagasse.

	*Chemical Composition (%)					
Samples	Glucans	Xylans	Galactans	Mannans	Arabinans	Lignin
Raw material	45.4 ± 0.3	21.7 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	1.8 ± 0.0	22.0 ± 0.4
Acid-treated	58.8 ± 0.2	9.0 ± 0.1	n.d.	n.d.	0.6 ± 0.0	26.6 ± 0.4
Alkali-treated	51.5 ± 1.3	27.9 ± 0.3	n.d.	n.d.	2.8 ± 0.1	8.1 ± 0.3

*Values are indicated as percentage of dry matter

Figure 4 shows the percent recovery of the main components (cellulose, hemicellulose and lignin) of sugarcane bagasse after pretreatments. In both, acid and alkaline pretreatment, recovery of the cellulose fraction in the remaining solids was similar (58.38 and 53.97 %, respectively), but the removal of hemicellulose and lignin differed greatly. Pretreatment with sulfuric acid solubilized 82.14 % of the hemicellulose present in sugarcane bagasse; on another hand, a less pronounced removal of 45.55 % was observed in the lignin content. Differently, alkaline pretreatment with NaOH solubilized 82.5 % of lignin and 39.67 % of hemicellulose present in milled sugarcane bagasse. These data are in accordance with the literature and show that acid pretreatment preferentially removes hemicellulose while in alkaline pretreatments lignin is preferentially solubilized *[39, 40, 42]*.

The goal of the pretreatment process is to remove hemicellulose and lignin, reduce the crystallinity of cellulose and increase the porosity of the lignocellulosic material, thus facilitating access of cellulases to substrate. Furthermore, pretreatment also must meet the following requirement: improve the formation of sugars, avoid the loss of carbohydrates and avoid the formation of by-products that inhibit the subsequent hydrolysis and fermentation processes *[42]*.

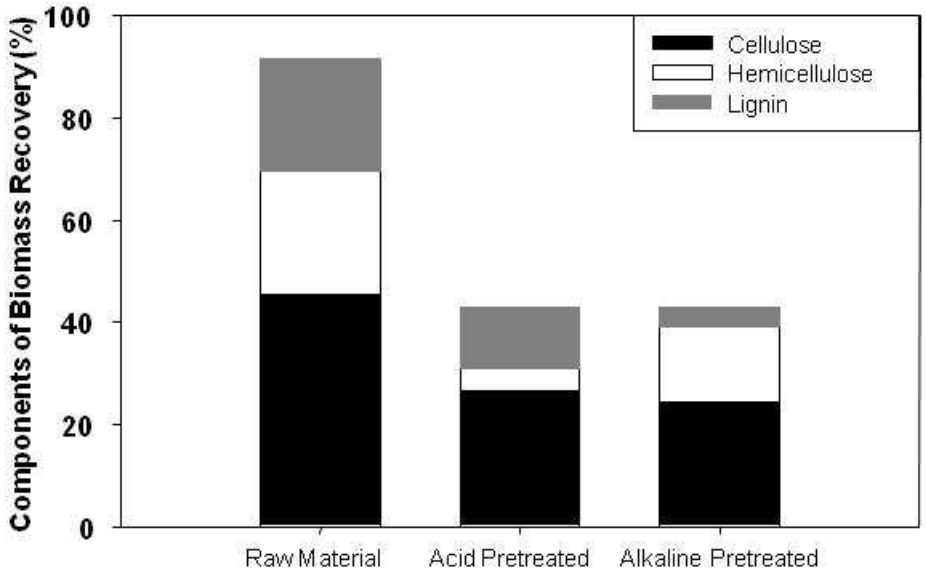

Figure 4: Effectiveness of the acid and alkaline pretreatments to solubilize the three main components (cellulose, hemicellulose and lignin) of sugarcane bagasse.

In this work, milled sugarcane bagasse was submitted to alkaline and acid pretreatment and the results obtained suggest that the alkaline pretreatment was more advantageous. Lignin removal plays a crucial role in enhancing enzymatic digestibility while hemicellulose removal is not necessary for high hydrolysis yield *[39]*. The low removal of hemicellulose also could guarantee a higher ethanol yield if a microorganism able to ferment hexoses and pentoses was used in the fermentation step. Furthermore, contrary to the acid pretreatment, the alkaline pretreatment produces low levels of inhibitor composts such as furfural and hidoxymetilfurfural which are inhibitors of the fermentation process*[43]*.

5.4.5. Biomass saccharification

Acid and alkali-treated sugarcane bagasse were submitted to enzymatic saccharification (10 FPase units per g substrate) using the enzymatic extract from *P. sanguineus*. Parallel saccharification assays were also carried out employing a commercial cellulase complex (Multifect® CL) aiming to establish a comparison between the performance of the *P. sanguineus* extract and a commercial product.

Prior to the saccharification assay, volumetric activities of cellulases and hemicellulases present in the *P. sanguineus* extract and Multifect® CL were determined in order to compare the enzymatic composition of both extracts. A direct comparison between volumetric activities found in both enzymatic

extracts did not permit a fair comparison because Multifect® CL is an industrial highly concentrated cellulase preparation which incorporates additives to guarantee the stability of a large quantity of protein in solution. Meanwhile, the enzymatic extract from *P. sanguineus* was produced in bench top fermenters and was concentrated only 5 times for this analysis. In order to establish a parameter to compare the different extracts, all values of enzymatic activities observed were indexed in relation to FPase activity, which represents the total cellulases activity of enzymatic complexes. FPase is the standard activity considered to determine enzyme loading in saccharification processes which justified the use of this parameter as a reference in this type of analysis *[44]*. The comparative results can be observed in Table 3.

Table 3: Comparative analysis of cellulase and hemicellulase activities present in the crude extract from *Pycnoporus sanguineus* and Multifect® CL. All activities are expressed in relation to total cellulase activity (FPase) found at each cellulolytic extract.

	Enzymatic activity (U)/FPase activity(U)	
Enzyme Activity	*P. sanguineus* extract	Multifect® CL
FPase	1.00	1.00
Endoglucanase	33.45	54.10
β-Glucosidase	1.01	0.85
Cellobiase	0.25	0.51
Xylanase	39.68	32.81
β-Xylosidase	0.06	n.d.
β-Mannosidase	0.03	n.d.
Mannanase	17.93	2.01
Pectinase	73.49	1.00
α-Galactosidase	3.75	n.d.
α-Arabinofuranosidase	2.48	n.d.

*n.d., not detected

At first glance, the results referring to the ratios β-glucosidase/FPase and cellobiase/FPase found in both enzymatic extracts stand out. β-Glucosidase and cellobiase, theoretically, represent the same enzymatic activity; however, while β-glucosidase activity is determined using synthetic substrate pNPGlc, cellobiase activity is determined using the natural substrate cellobiose. It was observed that the β-glucosidase/FPase ratio in the *P. sanguineus* extract (1.01) was higher than that found in the Multifect® CL extract (0.85). On the other hand, when cellobiose was considered as substrate an inverse behavior was observed because the cellobiase/FPase ratios determined for the *P.*

sanguineus extract and Multifect® CL were 0.25 and 0.51, respectively. This contradictory relationship can be explained by fact that p-nitrophenol is a good leaving group and the enzymes do not need to be specific to the β(1-4) linkage to hydrolyze this substrate *[44]*. This suggests that *P. sanguineus* produces enzymes that are able to hydrolyze pNPGlc but not all are specific to the natural substrate cellobiose. The results reported here also suggest that the determination of β-glucosidase activity using only pNPGlc as substrate may be not sufficient to evaluate the real potential of one cellulolytic extract to prevent the cellobiose accumulation during a biomass saccharification reaction.

Multifect® CL presented a higher endoglucanase/FPase ratio (54.1) than the *P. sanguineus* extract (33.45). Contrarily, it was observed that the *P. sanguineus* extract was richer than Multifect® CL in all hemicellulolytic activities and polygalacturonase activity. It has been reported that the presence of accessory enzymes (such as hemicellulases and pectinases) in a enzymatic saccharification reaction improves the cellulase performance because they can removing non-cellulosic polysaccharides that coat cellulose fibers, facilitating the access of cellulases to the substrate *[7, 41, 45]*. Furthermore, the presence of accessory enzyme ensures liberation of pentoses and hexoses (glucose, mannose and galactose) which can be converted to ethanol by appropriated microorganisms promoting an increase in the overall process yield *[1]*.

The *P. sanguineus* extract and Multifect® CL were applied for hydrolysis of acid and alkali-treated sugarcane bagasse and the percentage of total reducing sugars (Fig. 5-A) and glucose (Fig. 5-B) released after 72 h of reaction can be observed in figure 5. The crude enzymatic extract from *P. sanguineus* converted 60.4 % of biomass to reducing sugar after 72 h of reaction when using alkali-treated bagasse as substrate and this result was similar that found for Multifect® CL. However, considering the glucose released from alkali-treated bagasse, it was noted that the commercial cellulase resulted in a yield of 36.6 % whereas the *P. sanguineus* extract conferred a glucose yield of 22.6 %.

A large decrease in saccharification yield was observed when acid-treated bagasse was tested as substrate. After 72 h of reaction, reducing sugar yields of 13.8 and 35.1 % were observed for the *P. sanguineus* extract and Multifect® CL, respectively. As expected, the release of glucose was also negatively affected and the percentages of monosaccharides obtained at the

end of the assays (72 h) were 22.5 % for commercial cellulase and only 8.5 % for the crude enzymatic extract from *P. sanguineus*.

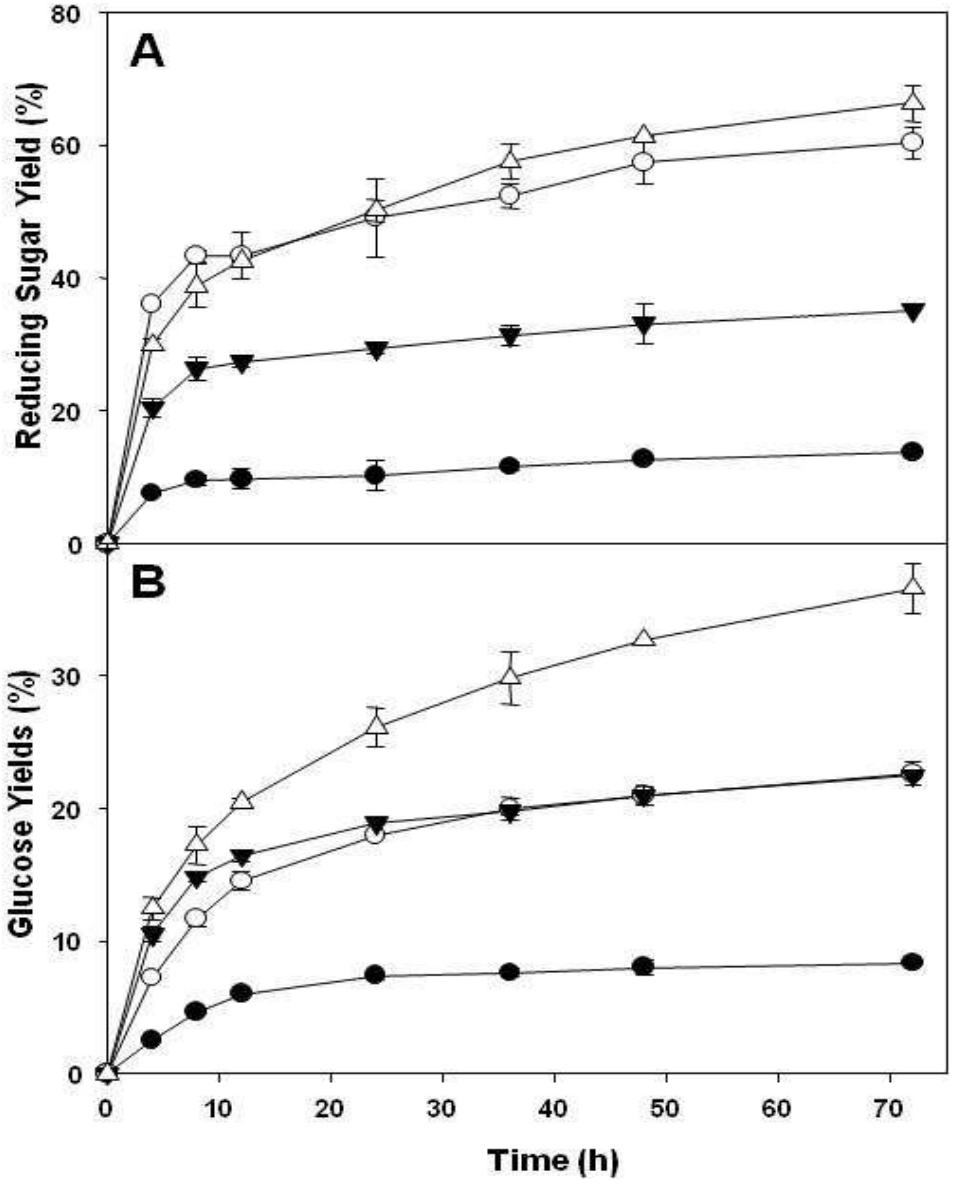

Figure 5: Time course for production of reducing sugar (A) and glucose (B) in the enzymatic saccharification of acid and alkali-treated sugarcane bagasse using the *Pycnoporus sanguineus* extract and commercial cellulase Multifect® CL. Alkali-treated bagasse + *P. sanguineus* extract (○); Acid-treated bagasse + *P. sanguineus* extract (●); Alkali-treated bagasse + Multifect® CL (△); Acid-treated bagasse + Multifect® CL (▼).

The contrast between the hydrolysis rates detected in sugarcane bagasse submitted to different pretreatment methods may be explained, in part, by the high content of lignin present in the acid pretreated biomass. Many studies have reported a close correlation between low lignin content and a high hydrolysis rate, although differences have been reported in the degree of lignin

removal needed *[39-41]*. Lignin limits the rate of hydrolysis by acting as a shield, preventing the digestible parts of the substrate to be hydrolyzed *[46]*. Furthermore, condensed lignin can absorb proteins from aqueous solutions and the removal of lignin should improve hydrolysis performance by reducing nonspecific adsorption of cellulases *[47]*.

Comparison between the *P. sanguineus* extract and Multifect® CL for saccharification of alkaline pretreated bagasse revealed that the *P. sanguineus* extract may be a good choice for the production of reducing sugars from lignocellulosic biomass since both enzymatic extracts showed a very similar performance. However, when comparing the glucose production for the same experiment, it was noted that after 72 h of reaction, Multifect® CL provided a glucose yield 13.9 % greater than that obtained with the *P. sanguineus* extract. This contrast in glucose production might be related to the specific characteristic of each enzymatic extract. It was observed that the cellobiase/FPase ratio for Multifect® CL and the *P. sanguineus* extract were 0.51 and 0.25, respectively (Table 3), and this may explain the lower glucose yield obtained by the cellulolytic extract from *P. sanguineus*.

A high cellobiase/FPase ratio is a decisive parameter to obtain maximal conversion of cellulose to glucose. Low cellobiase activity leads to accumulation of cellobiose which causes serious inhibition to the endoglucanase and cellobiohydrolase enzymes resulting in lower saccharification yields *[6]*. The vast majority of microbial cellulolytic extracts studied for saccharification processes, including that obtained from fungi of the *Trichoderma* genus, show an inappropriate cellobiase/FPase ratio and this deficiency is overcome by addition of extra cellobiase activity which is generally obtained from cultures of *Aspergillus* species *[6, 39, 41, 48]*.

In this work, the enzymatic extract was not supplemented with commercial cellobiase since the main objective of this study was evaluate the use of a single crude cellulolytic extract produced by *P. sanguineus* for the biomass saccharification process. The results obtained suggested that the *P. sanguineus* extract promoted an intense solubilization of alkaline treated sugarcane (high production of reducing sugar), however the glucose production could be improved by addition of extra cellobiase activity since the total glucose released was significantly lower than the theoretical yield. The same

observation would be appropriate for saccharification with the commercial Multifect® CL.

Curiously, it was also observed that the difference found for glucose production between the enzymatic treatments did not reflect the difference in reducing sugar production. How it observed in Table 3, the *P. sanguineus* extract was richer than Multifect® CL in polygalacturonase and hemicellulase activities and this may have resulted in a greater hydrolysis of the hemicellulose fraction present in alkali-treated bagasse hydrolyzed by the *P. sanguineus* extract leading to an equivalent production of reducing sugar in both enzymatic treatments, despite a smaller glucose production detected in the assay with the *P. sanguineus* extract.

Many works have reported the importance of hemicellulases and pectinases in the biomass saccharification process, stating that the presence of these enzymes in a cellulolytic complex improves the digestion of cell walls components due to increased accessibility *[7, 45]*. According to this viewpoint, *P. sanguineus* presents potential to be an efficient microbial factory for production of an enzymatic complex to be applied in saccharification processes since high titres of cellulase and hemicellulases were detected in its enzymatic extract.

In contrast to the results obtained when employing alkali-treated bagasse, a lower saccharification yield was observed for both enzymatic extracts when acid pretreated bagasse was used as substrate. The saccharification of acid pretreated bagasse by *P. sanguineus* presented yields 4.23 and 2.71 times smaller for reducing sugars and glucose production, respectively, when compared with saccharification yields obtained using alkaline pretreated bagasse. Low concentrations of reducing sugars and glucose were also observed after saccharification of acid pretreated bagasse by Multifect® CL where the percentage of reducing sugar and glucose obtained were respectively 1.97 and 1.62 times smaller when compared with alkaline pretreated bagasse hydrolysis. The results reported here showed that alkaline pretreatment seems be more suitable than acid pretreatment for application in saccharification of sugarcane bagasse because it guaranteed the higher production of reducing sugars and glucose, regardless of the enzymatic employed extract.

The results reported here indicated that the white-rot fungus *P. sanguineus* produces a cellulolytic complex with adequate characteristics for application in biomass saccharification and additional studies have been carried out at our laboratory in order to maximize its cellulase production which will allow for acquisition of a powerful enzymatic extract with a more balanced enzyme complex and higher titres of cellulases and hemicellulases.

5.5. Conclusion

This study showed that the white-rot fungus *P. sanguineus* was able to secret a wide range of cellulase and hemicellulase activities at high titres when cultured in liquid submerged fermentation using corn cob as carbon source. The cellulolytic complex produced by *P. sanguineus* showed desirable features to be applied to SHF and SSF systems such as high activities in a pH range of 4.0-5.0 and high thermal stability at 40 and 50 °C. Enzymatic hydrolysis of alkali-treated bagasse was carried out at 50 °C employing the crude enzymatic extract from *P. sanguineus*. After 72 h of reaction conversion yields of 60.4 and 22.6 % were obtained for reducing sugars and glucose, respectively. These saccharification rates were similar to those found for a commercial cellulase complex. The results hydrolysis suggests that *P. sanguineus* may be an efficient microbial factory for production of an enzymatic hemicellulolytic complex, representing an alternative for bioprocess based on cellulose saccharification.

5.6. References

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6. Artigo 3 - *Chrysoporthe cubensis*: a new factory of cellulases and hemicellulases for biomass saccharification.

6.1. Abstract

The objective of this work was to evaluate the biochemical features of the *Chrysoporthe cubensis* cellulolytic complex and its utilization to sugarcane bagasse hydrolysis. The fungus was cultivated under solid state fermentation using corn cob, wheat bran and Kraft pulp as carbon source. The highest endoglucanase (33.84 U g^{-1}), FPase (2.52 U g^{-1}), β-glucosidase (21.55 U g^{-1}) and xylanase (362.38 U g^{-1}) activities were obtained using wheat bran as carbon source. The cellulase activities were characterized in relation to pH and temperature. All enzymatic activities showed maximal hydrolysis rate at pH 4.0. Optimum temperatures for endoglucanase and FPase activities were 60 and 50 °C, respectively, while for xylanase and β-glucosidase activities was 55 °C. All enzymatic activities were highly stable at 40 and 50 °C through 48 h of pre-incubation. Crude cellulolytic extract from *C. cubensis* was applied in a saccharification experiment using alkali-treated sugarcane bagasse as substrate and the hydrolysis yields were compared to that obtained by a commercial cellulase. The reactions were carried out at 50 °C for 72 h employing 10 FPase units per gram of substrate. Reducing sugar yields of 89 % and 61.7 % were reached when alkali-treated bagasse was hydrolyzed by *C. cubensis* extract and commercial cellulase, respectively. Considering the glucose release, *C. cubensis* extract and commercial cellulase ensured yields of 52.7 and 36.5 %, respectively. Present work showed that cellulolytic extract from *C. cubensis* has a great potential to be used in biomass saccharification. In addition, due to its high β-glucosidase/FPase ratio, this enzymatic preparation can also be used as a supplement to poor β-glucosidase commercial cellulases.

6.2. Introduction

There is currently great interest in the degradation of lignocellulosic materials to monomeric sugars through the concerted action of cellulolytic enzymes, since sugars can serve as raw materials for production of valuable products such as ethanol, acid lactic, methane, hydrogen and others *[1, 2]*. Growing concerns over the potential consequences of a worldwide shortage of fossil fuels, the emission of greenhouse gases and air pollution by incomplete

combustion has resulted in an increased focus on the production of bioethanol from lignocellulosics and especially the possibility of using cellulases and hemicellulases to perform enzymatic hydrolysis of the lignocellulosic materials *[3-5]*.

The biotechnological conversion of cellulose into fermentable sugars requires a cooperative and synergistic action among three main enzyme types. Endoglucanases (E.C. 3.2.1.4) randomly attack cellulose chains generating reducing and non-reducing ends. Cellobiohydrolases (E.C. 3.2.1.91) act over the reducing and non-reducing ends, releasing cellobiose units which are converted to glucose by the action of β-glucosidase enzymes (E.C. 3.2.1.21) *[6]*. Moreover, hemicellulolytic enzymes such as xylanases (E.C. 3.2.1.8), mannanases (E.C. 3.2.1.78), β-xylosidases (E.C. 3.2.1.37), β-mannosidases (E.C. 3.2.1.25), α-Arabinofuranosidase (E.C. 3.2.1.55) and α-galactosidases (E.C. 3.2.1.22), also play an important role in the cellulose depolymerization process. These enzymes hydrolyze and remove the hemicellulose fragments that coat the cellulose fibers, increasing cellulose accessibility and boosting the action of cellulases *[1, 7, 8]*.

The major bottleneck to lignocellulosic bioethanol production is the high cost of the cellulolytic enzymes *[9]*. Large-scale application of cellulases for lignocellulosic material degradation processes demand microorganisms with improved activity and productivity as well as cellulases with better resistance to product inhibition *[10]*. Enzymes for degradation of the polysaccharide portion of biomass have been predominantly produced from fungi belonging to the genus *Trichoderma*. The fungi *Trichoderma reesei* and *Trichoderma viride* are able to secrete large amounts of endoglucanase and cellobiohydrolase enzymes; however, the amount of β-glucosidase secreted by *Trichoderma* species is very low and this may lead to accumulation of cellobiose and incomplete cellulose hydrolysis when cellulolytic extracts produced by these fungi are employed in saccharification processes *[10-12]*. Other important cellulase producing fungi, including those belonging to the genera *Penicillium* and *Aspergillus,* are able to secrete large amounts of β-glucosidase; however the total cellulase activity (FPase) found in their enzymatic extracts is relatively low. As a result, production of glucose from waste cellulose is not yet commercially feasible *[10]*. Considering this scenario, there is a constant search for new cellulolytic microorganisms that are able to produce cellulases in large quantities and in

balanced proportions, so that it can be used for efficient saccharification of lignocellulosic biomass.

Plant pathogenic fungi produce extracellular enzymes which can degrade the cell wall components of plants. These fungi do not only digest plant cell wall polymers to obtain important nutrients but also degrade the cell wall enabling cell penetration and spread through the plant tissue *[13]*. Plant pathogenic species such as *Fusarium graminearum [13]*, *Bipolaris sorokiniana [14]* and *Sclerotinia sclerotiorum [15]* have been evaluated with relation to secretion of hemicellulases, pectinases and cellulases, and it was observed that there is a close correlation between the capacity to secrete some hydrolases and the virulence of these microorganisms. However, despite the proven ability of plant pathogenic fungi to produce cell wall-degrading enzymes, few works report the utilization of these fungi in production of cellulases and hemicellulases destined for converting biomass in monomeric sugars.

In the present study, the production of cellulases and hemicellulases by *Chrysoporthe cubensis,* a well-documented pathogen of various tree species, was examined in solid state fermentation using different carbon sources. The produced cellulolytic complex was characterized in terms of pH, temperature and thermal stability. For the first time, an attempt was made to apply the cellulolytic complex from *C. cubensis* in a biomass saccharification process using sugarcane bagasse as substrate. Assays of saccharification using a commercial cellulases complex were carried out in parallel in order to establish a comparison between both extracts.

6.3. Material and Methods

6.3.1. Materials

Substrates including ρ-nitrophenyl-β-D-glucopyranoside (pNPGlc), ρ-nitrophenyl-β-D-xylopyranoside (pNPXyl), ρ-nitrophenyl-β-D-mannopyranoside (pNPMan), ρ-nitrophenyl-β-D-galactopyranoside (pNPGal), ρ-nitrophenyl-α-D-arabinofuranoside (pNPAra), cellobiose, carboxymethylcellulose (CMC), xylan from birchwood, locust bean gum, polygalacturonic acid and also the chemical reagents monopotassium phosphate, ammonia nitrate, magnesium sulfate, calcium chloride, cuprum sulfate, sodium acetate, sodium carbonate, dinitrosalicylic acid (DNS) and potato dextrose agar (PDA) were purchased from

Sigma Chemical Co. (St. Louis, MO, USA). Yeast extract was obtained from Himedia Laboratories Co. (Mumbai, Maharashtra, India). Chemical reagents including NaOH, H_2SO_4 and potassium sodium tartrate were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). A commercial cellulase complex, Multifect® CL, was purchased from Genencor International Inc. (Rochester, NY, USA). Kraft pulp was kindly supplied by the Pulp and Paper Laboratory of the Federal University of Viçosa, Viçosa, MG, Brazil. Sugarcane bagasse, corn cobs and wheat bran were obtained at the local market. All others reagents used in this study were of analytical grade.

6.3.2. Microorganism and inoculum preparation

The fungus *Chrysoporthe cubensis* LPF-1 used in this study was obtained from the mycological collection of the Forest Pathology Laboratory, Federal University of Viçosa, MG, Brazil. The fungus was maintained on PDA plates at 28 °C and subcultured periodically. The inoculum was prepared by growing the fungus under submerged fermentation in 250 mL Erlenmeyer flasks containing 100 mL of medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2.0. Each flask was inoculated with 10 agar plugs cut out of a 5 day-old colony of *C. cubensis* grown on PDA plates and incubated in a rotary shaker for 5 days, at 150 rpm and 28 °C. The culture obtained was aseptically homogenized with Polytron® and immediately used to inoculate the solid culture media. This inoculum preparation routine was employed for all experiment developed in this work.

6.3.3. Culture condition

In order to evaluate the effect of carbon sources on fungal growth and enzyme production, *C. cubensis* was cultured under solid state fermentation (SSF) using Kraft pulp, wheat bran and milled corn cob as support and the main carbon source. The fermentations were carried out in 125-mL Erlenmeyer flasks containing 5 g (dry weight) of substrate moistened with 12 mL of culture media (final moisture of 70 %) containing the following composition, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2. Furthermore, $MnCl_2$ (0.1 mg L^{-1}), H_3BO_3 (0.075 mg L^{-1}), Na_2MoO_4 (0.02 mg L^{-1}), $FeCl_3$ (1.0 mg L^{-1}) and $ZnSO_4$ (3.5 mg L^{-1}) also were added to the medium as trace elements. The flasks were autoclaved at 120 °C for 20 min and then inoculated with 3 mL

of inoculum obtained as aforementioned. The flasks were maintained at 30 °C in a controlled temperature chamber and the enzymatic extractions were performed after 4, 8 and 12 days of fermentation. Enzymes secreted during SSF were extracted with sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate), under agitation of 150 rpm for 60 min at room temperature. Solids were separated by filtration through nylon cloth followed by centrifugation at 15000 x *g* for 10 min; and the clarified supernatants were frozen and stored for subsequent enzymatic analysis. Experiments were carried out with three replicates for each medium composition and for each incubation time.

6.3.4. Enzymatic assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50 °C. All were performed in triplicate and the mean values calculated. Relative standard deviations of measurements were below 5 %. FPase and endoglucanase activities were determined using Whatman n°. 1 filter paper and carboxymethilcellulose as substrates respectively, according to previously described standard conditions *[16]*. The total reducing sugar liberated during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method *[17]* using glucose as a standard.

Xylanase, mannanase and pectinase activities were determined using xylan from birchwood (1 % w/v at final concentration), locust bean gum (0.4 % w/v) and poligalacturonic acid (0.2 % w/v) as substrates, respectively. The enzymatic reactions were initiated by the addition of 100 μL of the appropriately diluted enzyme solution to 400 μL of the polysaccharide substrate solution prepared in buffer. The reaction mixtures were incubated for 30 min and the total reducing sugar released was determined via the DNS method using xylose, mannose and galacturonic acid as standards. β-Glucosidase, β-xylosidase, β-mannosidase, α-galactosidase and α-arabinofuranosidase activities were measured using ρPNGlc, ρNPXyl, ρNPMan, ρNPGal and ρNPAra as substrates, respectively. The reaction mixtures contained 100 μL of the appropriately diluted enzyme solution, 125 μL of the synthetic substrate solution (1 mM at final concentration) and 275 μL of buffer. The reaction mixture was incubated for 30 min and stopped by addition of 0.5 mL sodium carbonate

solution (0.5 M). Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released was estimated by a standard curve. For all activities, one unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 μmol of the corresponding product (glucose equivalent, xylose, mannose, galacturonic acid and ρ-nitrophenol) per minute, under the assay condition used.

6.3.5. Determination of optimum pH

To determine the effect of pH on endoglucanase, β-glucosidase, FPase and xylanase activities, enzymatic assays were carried out at pH values ranging 2.0-7.0 using a citric acid/sodium phosphate buffer *[18]*. The other assays conditions were identical to those aforementioned.

6.3.6. Determination of optimum temperature and thermal stability

The effects of temperature on endoglucanase, β-glucosidase, FPase and xylanase activities were evaluated in the range of 30-80 °C at pH 5 according to the previously described standard methods. Thermal stability of cellulases and xylanase activities was determined at different temperatures. The enzymatic extract was diluted in sodium acetate buffer, 100 mM, pH 5 and maintained at 40, 50 or 60 °C for 48 h. At different time interval, aliquots were taken and the residual activities were measured using standard enzyme assay. All data obtaining during pH and temperature characterization were expressed in terms of relative activity where the highest activity value obtained for each enzymatic activity was considered as 100 %.

6.3.7. Biomass pretreatment and biomass composition analysis

Milled sugarcane bagasse (particle size less than 1 mm) was submitted to alkaline pretreatment prior to being employed in a saccharification experiment. The biomass was dried in an oven at 70 °C until the weight was constant. Sodium hydroxide 1 % was used to pretreat 25 g of milled sugarcane bagasse samples at a solid loading of 10 % (w/v); and treatments were performed in an autoclave at 120 °C for 60 min. The pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted 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 alkali-treated sugarcane bagasse was determined using a modified Klason lignin method derived from the TAPPI Standard Method T222 om-98 *[19]*. Extractive-free biomass (0.3 g) was incubated at 30 °C with 3 mL of 72 % H_2SO_4 for 1 h with occasional mixing. The slurry was then transferred into a penicillin bottle containing 84 mL of deionized water and the flask was sealed with a rubber stopper and aluminum seal. The bottle was placed in an autoclave calibrated at 118 °C for 1 h, then the slurry was filtered through a medium coarseness sintered glass filter for gravimetric determination of acid-insoluble lignin. Concentrations of biomass sugars (arabinose, galactose, glucose, xylose, and mannose) in the filtrate were quantified using high-performance liquid chromatography (HPLC), while acid-soluble lignin was determined by absorption measurements at 205 nm *[20]*. The HPLC system Dionex DX-300 (Dionex Co. - Sunnyvale, CA, USA) was equipped with a Carbopac PA1 column and a pulsed amperometric detector with a gold electrode. Prior to injection, samples were filtered through 0.45-mm HV filters and a volume of 20 µL was loaded into the chromatograph system. The column was pre-equilibrated with a NaOH solution, 300 mM, and elution was carried out at a flow rate of 1.0 mL min^{-1} at room temperature.

6.3.8. Biomass saccharification

The crude enzymatic extract produced by *C. cubensis* and commercial cellulase (Multifect® CL) were applied in a biomass saccharification experiment. The *C. cubensis* enzymatic extract was concentrated 5-fold before the experiment using an Amicon Ultrafiltration system (Millipore Co. – Billerica, MA, USA) and an YM-10 (Cut-off M_r 10,000 Da) membrane filter. Enzymatic saccharification of alkali-treated sugarcane bagasse was performed in 100 mL sample tubes at an initial solid concentration of 2 % dry matter (w/v) in 25 mL of 50 mM sodium acetate buffer at pH 4.5. Enzyme loading was specified as 10 FPase units per gram of biomass with the addition of sodium azide (10 mM) and tetracycline (40 $\mu g.mL^{-1}$) to the reaction mixture to inhibit microbial contamination. The reaction was carried out in an orbital shaker at 250 rpm and 50 °C for 72 h. Samples (0.5 mL) were taken from the reaction mixture at different time intervals. These samples were immediately heated to 100 °C to denature the enzymes, cooled and then centrifuged for 5 min at 15000 x *g*. The supernatant was used for the determination of total reducing sugar and glucose

released during the reaction. The total reducing sugar concentration was quantified via the dinitrosalicylic acid method *[17]*, while glucose concentration was estimated using a commercial analytical kit based on glucose oxidase and peroxidase (Quibasa Basic Chemical, Belo Horizonte, MG, Brazil). The results were calculated by averaging the values for samples duplicates and subtracting the average values for the respective controls which were carried out in parallel, in which the active enzymatic extract was replaced with enzymatic samples boiled for 10 min. The cellulose hydrolysis rate and total carbohydrates (cellulose plus hemicellulose) hydrolysis rate were calculated using the following equations:

$$Glucose\ yield\ (\%) = \frac{Amount\ of\ glucose\ released\ (g) \times 0.9 \times 100}{Amount\ of\ cellulose\ in\ pretreated\ bagasse\ (g)}$$

$$Reducing\ sugar\ yield\ (\%) = \frac{Amount\ of\ reducing\ sugar\ released\ (g) \times 0.9 \times 100}{Amount\ of\ carbohydrate\ in\ pretreated\ bagasse\ (g)}$$

6.4. Results and Discussion

6.4.1. Enzyme Production

Chrysoporthe cubensis is a well-documented pathogen of various tree species in tropical and sub-tropical areas of the world. On Eucalyptus (*Eucalyptus* spp.), the fungus causes serious damage, especially in commercial plantations of susceptible species or clones *[21]*. The fungus mainly attacks the steam region in eucalyptus plants resulting in the formation of well-defined deep lesion (cankers), causing losses in both, wood quality and volume productivity *[22]*. Pathogens such as *C. cubensis*, secrete numerous cell wall degrading enzymes which breach the plant cell wall and use it as a source of nutrients *[23]*.

In this work the potential of the plant pathogen *C. cubensis* to produce cellulases and hemicellulases enzymes for application in biotechnological processes was investigated for the first time. The fungus was grown under solid state fermentation using Kraft pulp, milled corn cob and wheat bran as lignocellulosic substrates and the production profiles of FPase, endoglucanase, β-glucosidase and xylanase activities are showed in Fig. 1. Greater cellulase activities were obtained when *C. cubensis* was cultured on wheat bran as a

substrate. The maximal endoglucanase activity (33.84 ± 0.13 U g^{-1}) was observed after 8 days of fermentation whereas the highest activities for FPase (2.52 ± 0.08 U g^{-1}) and β-glucosidase (21.55 ± 0.13 U g^{-1}) were detected at day four. Both Kraft pulp and corn cobs resulted in poor FPase, endoglucanase and β-glucosidase activities and these substrates were identified as weak inducers for cellulase production by *C. cubensis*. It was also observed that *C. cubensis* showed a significant capacity to produce xylanase activity and after four days of fermentation xylanase activities of 362.38 ± 3.02 and 304.8 ± 9.1 U g^{-1} were observed when the fungus was cultured on wheat bran and corn cob, respectively. On another hand, a marked decrease in xylanase production was observed for medium containing Kraft pulp.

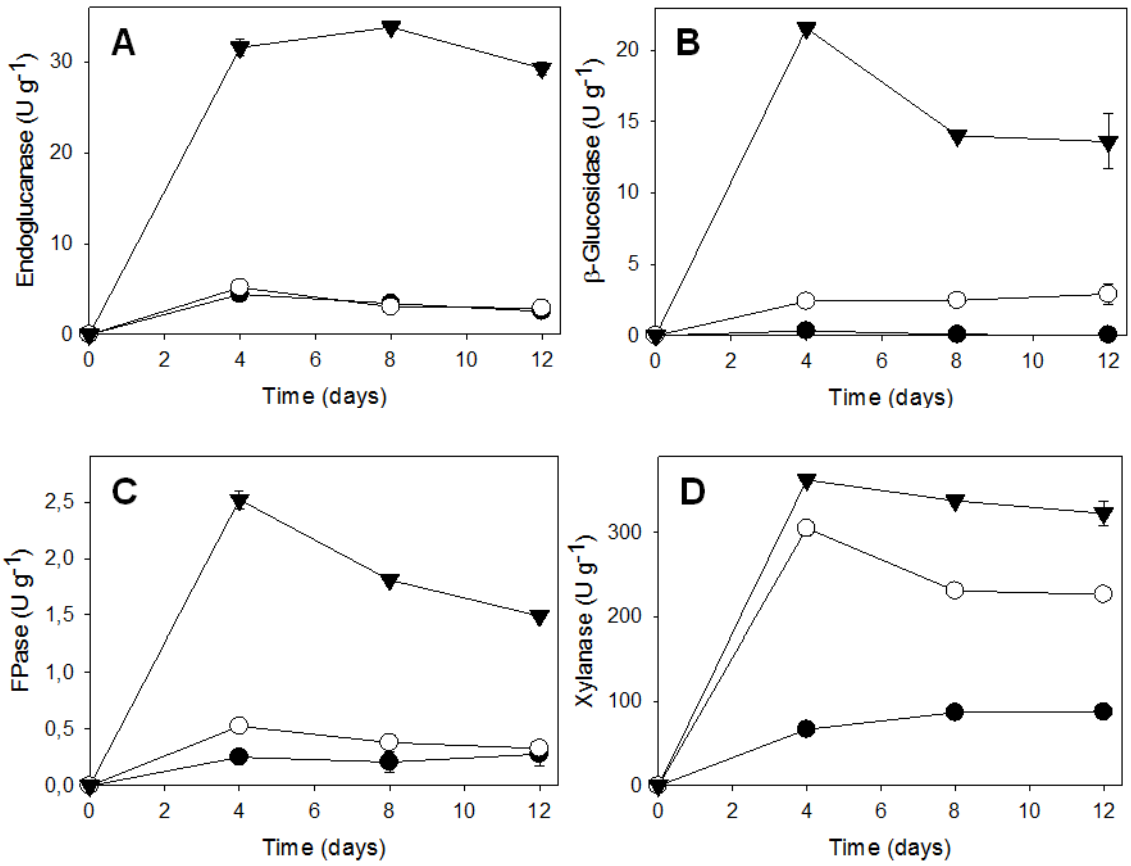

Figure 1: Time course production of endoglucanase (A), β-glucosidase (B), FPase (C) and xylanase (D) activities of the fungus *Chrysoporthe cubensis* cultured under solid state fermentation using wheat bran (▼), corn cobs (○) and Kraft pulp (●) as carbon source.

Chrysoporthe cubensis grown under solid state fermentation using wheat bran as a carbon source showed great potential to produce cellulolytic and xylanolytic activities. The enzymatic titres reached at this work were comparable to those obtained in SSF by well known cellulolytic species such as

Trichoderma spp., *Aspergillus* spp. and *Penicillium* spp., which are generally employed for industrial production of cellulases (Table 1). The majority of the reports on microbial production of cellulases utilize submerged fermentation (SmF); however, during the last two decades solid state fermentation has regained interest due its advantages over SmF which include high volumetric productivity, relatively higher concentration of the products, less effluent generation, requires for simple fermentation equipment, low risk of contamination and minimization of catabolic repression *[5, 24]*.

Table 1: Comparison of cellulases and xylanase activities production from different fungi cultured under solid state fermentation on lignocellulosic materials.

		Activity (U g^{-1})				
Fungi	Substrate	*Endo.	FPase	*β-gluc	*Xyl.	Ref.
Trichoderma reesei	Kinnow pulp + wheat bran	20.5	13.4	8.2	-	*[25]*
Aspergillus niger	Wheat bran	135.4	4.5	21.39	-	*[26]*
Trichoderma reesei	Wheat bran	-	3.8	-	-	*[27]*
Penicillium decumbens	Wheat bran + bagasse	-	3.8	-	-	*[28]*
Trichoderma reesei	Wheat bran + soy hulls	60.2	6.5	6.3	515.9	*[29]*
Aspergillus oryzae	Wheat bran + soy hulls	68.3	6.7	9.45	512.1	*[29]*
Aspergillus niger	Groundnut fodder	1.36	2.1	0.01	-	*[30]*
Penicillium decumbens	Rice bran	-	5.1	-	-	*[31]*
Trichoderma viride	Rice straw + rice bran	27.1	-	0.52	620.2	*[32]*
Chysoporte cubensis	Wheat bran	33.8	2.5	21.5	362.3	This work

*Endo: endoglucanase activity; *β-gluc: β-glucosidase activity; *Xyl: xylanase activity.

It is a well-established fact that culture conditions significantly affect the production of cellulases and hemicellulases by filamentous fungi. Carbohydrates or their derivatives induce most cellulolytic enzymes; thus the carbon source plays an important role in enzyme production *[1, 33]*. *Chrysoporthe cubensis* was cultured in a SSF system using three different carbon sources as substrate and it was clear that wheat bran induced the greatest production of cellulases in comparison to Kraft pulp and corn cobs. Several investigations with different cellulolytic microorganisms have emphasized the advantages of wheat bran (solely or mixed with other lignocellulosic materials) as substrate to obtaining high cellulase productions *[5, 29, 30, 34]*.

High contents of protein (13-19 %) and hemicellulose (higher than 30 %) combined with the low content of lignin (3-6 %) are outstanding characteristics of wheat bran that are generally identified as responsible for the high capacity of this substrate to induce the production of cellulases *[34-36]*. Due to its high protein content, wheat bran is a substrate with a well-balanced C/N ratio and this feature is crucial for a particular process to obtain specific bio-products from a SSF system *[29]*. The high content of hemicellulose along with minimal presence of lignin ensures high digestibility of the wheat bran, unlike substrates rich in crystalline cellulose and lignin. The hemicellulose is an accessible source of arabinose, xylose and hexoses and the availability of these readily metabolizable sugars favors fast growth of the microorganism with concomitant enzyme production. Moreover, it is generally believed that the initial defragmentation of hemicellulose from wheat bran generates oligosaccharides which play an important role in inducing synthesis of cell wall degrading enzymes *[29, 34]*.

Besides cellulase production, the fungus *C. cubensis* showed an elevated capacity to secrete xylanase enzymes. Wheat bran not only induced cellulolytic activities but was also the most effective for xylanase production. Although corn cobs resulted in low cellulase production, it was also a good inducer of xylanases and the activities obtained with this substrate were comparable to those obtained with wheat bran. It is widely reported that the presence of xylans in the substrate triggers the synthesis of xylanolytic enzymes by filamentous fungi such as *Trichoderma reesei* and *Aspergillus* spp. *[1, 33]* and a similar behavior was observed for *C. cubensis* since both wheat bran and corn cobs have a high concentration of xylans in their composition. Kraft pulp induced the lowest production of xylanases and this may be related to its low content of hemicellulose since the Kraft pulping is a process that promotes solubilization of hemicellulose present in wood chips.

Morgolles-Clark et al. *[37]* reported that there is considerable cross-talk at induction and expression of genes encoding different classes of enzymes. For instance, the same compounds may provoke expression of both, cellulases and hemicellulases. However, a diverse behavior of *C. cubensis* can be observed since when the fungus was cultured on corn cobs, xylanase production was high but cellulase secretion was poor. These results are particularly interesting because they reveal that *C. cubensis* may be a good

source for acquisition of cellulase-poor xylanase enzymatic extracts which have great potential to be applied in biobleaching processes in the paper industry *[38]*.

Because the focus of this work was evaluate the potential of the cellulolytic extract from *C. cubensis* for biomass saccharification processes, wheat bran was used as a substrate and a time fermentation of four days was established as standard conditions for production of the cellulolytic extract.

6.4.2. Effect of pH and temperature on cellulase and xylanase activities from *Chrysoporthe cubensis*

The cellulase and xylanase activities produced by *C. cubensis* under SSF using wheat bran as a carbon source were partially characterized in order to establish suitable application for the enzymes in a biomass saccharification process. FPase, endoglucanase, β-glucosidase and xylanase activities were investigated with regards to pH, temperature, and thermal stability.

The effect of pH on cellulolytic and xylanase activities present in the crude extract is shown in Fig. 2-A. Curiously, all enzymatic activities evaluated showed maximum activity when incubated at pH 4.0 and this similar behavior found for both, cellulases and xylanase activities can be considered an advantageous characteristic of the cellulolytic extract produced by *C. cubensis*. In other words, in a potential saccharification experiment, if the major enzymes responsible for biomass degradation present maximum activity at the same pH, then selection of this pH would ensure that no enzyme activity acts at sub-optimal conditions which would certainly accelerate the hydrolysis process.

All enzymatic activities were negatively affected when the assays were carried out at higher pH values. No β-glucosidase activity was detected at pH 7 while FPase and xylanase activities showed only 13.9 and 25.4 % of residual activity when incubated at the same pH. On another hand, endoglucanase activity was less sensible at high values of pH and a residual activity of 47.0 % was observed at pH 7. At the same way, lower pH values caused a decrease in all enzymatic activities. No FPase activity was detected at pH 2 and a marked decrease in β-glucosidase activity was also observed (16.6 % of residual activity). Meanwhile, endoglucanase and xylanase activities showed moderate stability at acidic pH values and the residual activities found for both enzymatic activities at pH 2 were 35.6 and 57.2 %, respectively. Overall, the pH activity

profile of cellulases and xylanase from *C. cubensis* presented features common to those reported for other cellulolytic fungal species *[39-42]*.

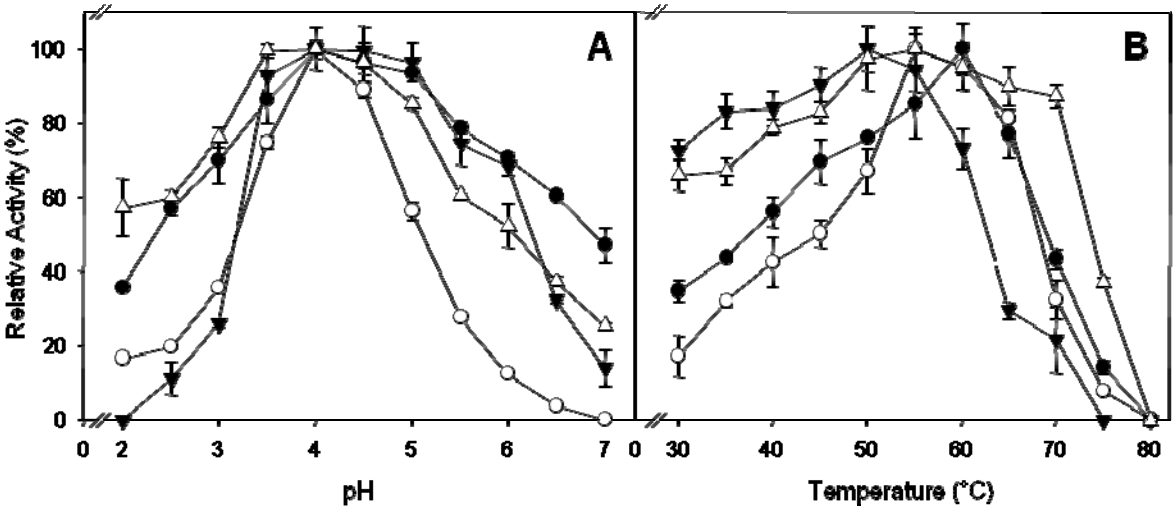

Figure 2: Effect of pH (A) and temperature (B) on endoglucanase (●), β-glucosidase (○), FPase (▼) and xylanase (△) activities produced by *Chrysoporthe cubensis* under SSF using wheat bran as the carbon source.

The effect of the temperature on cellulolytic and xylanase activities from *C. cubensis* was investigated in a range of 30-80 °C (Fig 2-B). The optimum temperature for endoglucanase and FPase activities were 60 and 50 °C respectively, while both, xylanase and β-glucosidase activities were maximum at 55 °C. Drastic losses of endoglucanase, β-glucosidase and FPase activities were observed at temperatures higher than 65°C. However, a residual xylanase activity of 87 % was observed when the enzymatic reaction was carried out at 70 °C, suggesting that xylanases from *C. cubensis* could have potential to be employed in industrial processes which require moderate-high temperatures such as those found in the textile and cellulose and paper industries *[43]*. When hydrolases from *C. cubensis* were assayed at 30 °C it was observed that both xylanase and mainly FPase activities maintained good hydrolysis performance, and residual activities of 66.0 and 72.8 % were observed, respectively. These results indicate that the enzymatic extract of *C. cubensis* presents a great potential for application in ethanol or other chemical production processes, especially if a complete cellulose hydrolysis at mild temperature condition was required.

Enzymatic saccharification with the objective of producing cellulosic bioethanol is a process highly affected by temperature, thus, it is essential that cellulases and hemicellulases present appropriate thermal properties for

application in this biotechnological process. In general, there are two operating modes for bioethanol production from lignocellulosic materials: separate hydrolysis and fermentation (SHF), and simultaneous saccharification and fermentation (SSF). In SHF the hydrolysis step is carried out at temperature that ensure the maximal enzymatic hydrolysis rate and for most cellulolytic extracts produced by filamentous fungi, including species from genera *Trichoderma*, *Penicillium* and *Aspergillus*, this temperature is in a range of 50-60 °C *[24, 40, 41, 44]*. The main advantage of SHF is a shorter reaction time for hydrolysis since the process is conducted at optimal operating condition. However, this process is limited because it is accepted that during enzymatic hydrolysis, cellulase activities (mainly endoglucanases and cellobiohydrolases) are severely inhibited by cellobiose and glucose which are the end products of the reaction *[11, 45]*.

In SSF, both, enzymatic hydrolysis and fermentation are carried out concomitantly, thus, the glucose released is readily fermented into ethanol thereby relieving inhibition of the cellulolytic system by the accumulation of products. However, the optimum conditions for enzymes and fermenting microorganisms are usually not the same, where cellulases have an optimum temperature between 50-60 °C and yeasts typically present optimal growth at roughly 32–37 °C. The operation of SSF is therefore at suboptimal conditions for enzymatic hydrolysis *[45]*.

Use of thermo tolerant yeasts is generally suggested as a good option to overcoming this incompatibility *[46, 47]*, however, the use of cellulolytic complexes that present high-performance hydrolysis at mid-range temperatures also would be an interesting alternative to solve this problem. Considering this fact, it was observed that the cellulolytic complex produced by *C. cubensis* shows outstanding potential to be applied in SSF because when FPase activity was analyzed in a temperature range of 30-40 °C it was noted that the cellulolytic complex maintained 72-84 % of its activity, compared to the maximum activity observed at 50 °C. These results show that even at low temperatures an intense cellulose hydrolysis was obtained, indicating that the cellulolytic complex from *C. cubensis* has appropriate characteristics to be applied in SSF, unlike commercial cellulase preparations which generally reach only 20 to 30 % of their maximal activity when incubated at 30 °C *[48]*.

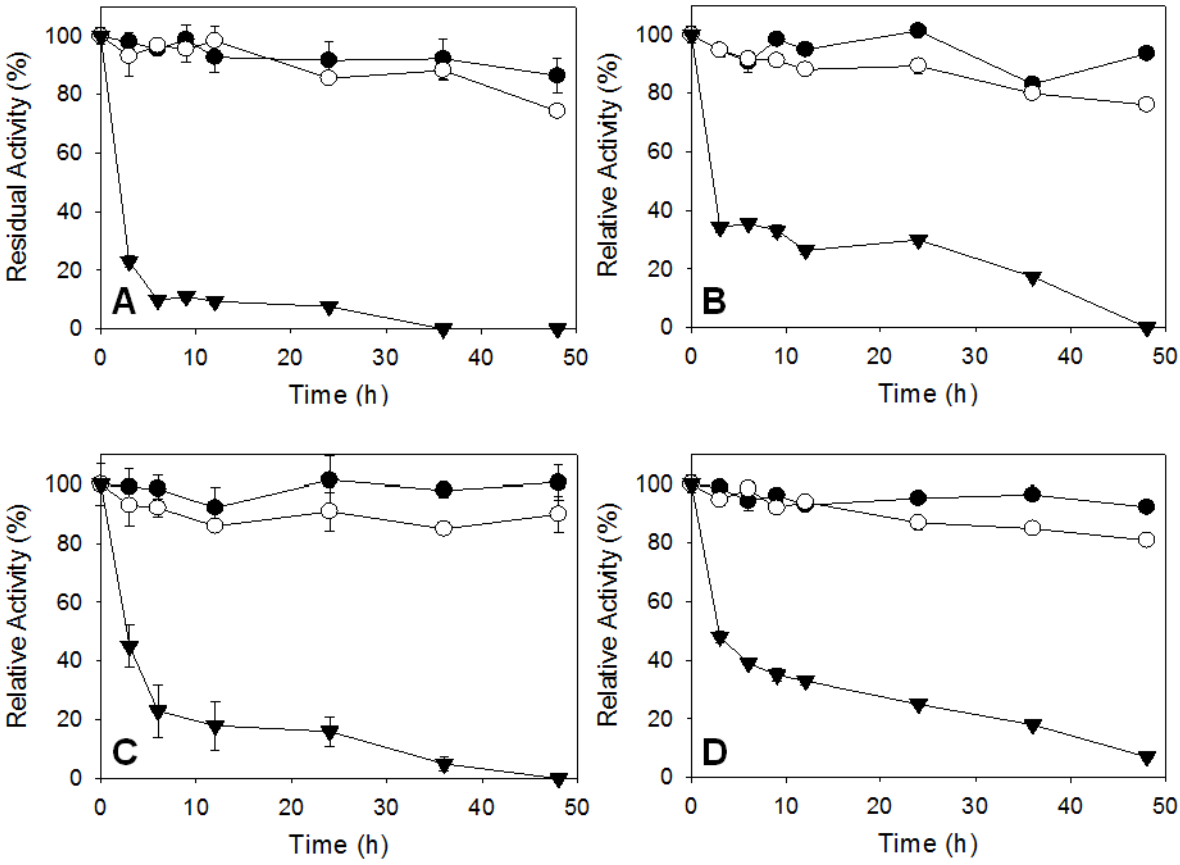

Figure 3: Thermal stability of cellulases and xylanase activities produced by *Chrysoporthe cubensis at* 40 °C (●), 50 °C (○) and 60 °C (▼). Endoglucanase activity (A); β-glucosidase activity (B); FPase activity (C) and Xylanase activity (D).

Thermal stability of the cellulases and xylanase produced *by C. cubensis* was performed for 48 h at three different temperatures. Profiles of the residual activities determined during the experiment are presented in Fig. 3. All enzymatic activities were highly stable at 40 °C and after 48 h of incubation the residual activities found for endoglucanase, FPase, β-glucosidase and xylanase were 86.4, 98.1, 93.6 and 92.3 % respectively. Increasing the incubation temperature to 50 °C resulted in a slightly decrease in the stability of cellulase and xylanase activities, however it was clear that all enzymatic activities also showed great stability at 50 °C since the residual activity values determined after 48 h of incubation were 74.3, 90.0, 76.1 and 81.2 % for endoglucanase, FPase, β-glucosidase and xylanase, respectively. Pronounced activity losses were observed when cellulases and hemicellulases from *C. cubensis* were incubated at 60 °C and at this temperature the half-lifes determined for endoglucanase, FPase, β-glucosidase and xylanase were 0.94, 2.17, 2.40 and 3.69 h, respectively.

Enzymatic biomass saccharification is a process that requires long reaction periods. Generally, a reaction time between 24-72 h is used *[10, 49, 50]* and for this reason thermal stability is an essential feature for a cellulolytic complex being employed in a biomass saccharification process. The high thermal stability encountered at 40 and 50 °C indicated that cellulases from *C. cubensis* have great potential to be used in biomass saccharification processes and also guarantee flexibility for use in both SSF and SHF systems.

6.4.3. Crude extract produced by *Chrysoporthe cubensis* and the commercial cellulase complex: a comparison between the enzymatic profiles

Enzymatic biomass degradation for the purpose of ethanol production is a process not only dependent of the presence and isolated action of endoglucanases, cellobiohydrolases and β-glucosidases; efficient biomass saccharification is a synergistic process that depends on a balanced proportion of different cellulases and can also be affected by the presence of hemicellulases and pectinases which in this context play the role of accessory enzymes.

In order to obtain more information on the enzymatic profile secreted by *C. cubensis*, a comparative study was performed. The crude enzymatic extract produced by *C. cubensis* under SSF using wheat bran as a carbon source was analyzed for measurement of cellulases, hemicellulases and pectinase activities. In addition to activities of FPase, endoglucanase, β-glucosidase and xylanase, the activity of other enzymes in the crude extract were also investigated, including: α-galactosidase, α-arabinofuranosidase, β-xylosidase, β-mannosidase, β-mannanase and pectinase. Multifect® CL, a commercial cellulases preparation obtained from cultures of *Trichoderma reesei* was analyzed in parallel to establish a comparison between the crude extract from *C. cubensis* and a commercial product.

A direct comparison between volumetric activities found in both enzymatic extracts did not permit a fair comparison because Multifect® CL is an industrial highly concentrated cellulase preparation which incorporates additives to guarantee the stability of a large quantity of protein in solution. Meanwhile, the enzymatic extract from *C. cubensis* was produced in bench top fermenters and it was concentrated only 5 times for this analysis. In order to establish a

parameter to compare the different extracts, all enzymatic activities observed were indexed in relation to FPase activity, which represents the total cellulase activity of the enzymatic complexes. FPase is the standard activity considered to determine the enzyme loading in saccharification processes which justifies the use of this parameter as a reference in this type of analysis *[51, 52]*. The comparative results can be observed in Table 2.

Table 2: Comparative analysis of cellulase and hemicellulase activities present in the crude extract from *Chrysoporthe cubensis* and Multifect® CL. All activity values are expressed in relation to total cellulase activity (FPase) found in each cellulolytic extract.

	Enzymatic activity (U)/FPase activity(U)	
Enzyme	*C. cubensis* extract	Multifect® CL
FPase	1.00	1.00
Endoglucanase	34.02	54.10
β-Glucosidase	35.74	0.85
Xylanase	390.74	32.81
β-Xylosidase	1.56	n.d.
β-Mannosidase	0.96	n.d.
Mannanase	27.82	2.01
Pectinase	48.22	1.00
α-Galactosidase	3.81	n.d.
α-Arabinofuranosidase	6.19	n.d.

*n.d., not detected

Particularly noteworthy was the high β-glucosidase/FPase ratio found in the crude *C. cubensis* extract. The β-glucosidase/FPase ratio in the *C. cubensis* extract was 42.04 fold higher than the value determined for the commercial extract Multifect® CL. This result showed that fungus *C. cubensis* presents a valuable characteristic because low levels of β-glucosidase are generally indicated as the main limitation for the use of cellulolytic extracts produced by *Trichoderma* species in biomass saccharification processes. A low β-glucosidase/FPase ratio leads to an accumulation of cellobiose during hydrolysis and this is an undesirable phenomenon because cellobiose is a strong inhibitor of endoglucanase and cellobiohydrolase enzymes. In other words, the accumulation of cellobiose prevents the complete hydrolysis of cellulose and reduces process yields *[29, 53]*. To overcoming this limitation, before being employed in a biomass saccharification process, the extracts produced by *Trichoderma* species are supplemented with enzymatic extracts

rich in β-glucosidase activities which are usually obtained from fungi cultures of the genera *Aspergillus* and *Penicillium [11, 52]*.

The commercial extract Multifect® CL showed an endoglucanase/FPase ratio 1.59-fold higher than the crude extract from *C. cubensis*. On another hand, it was clear that crude extract of *C. cubensis* was richer than Multifect® CL in all hemicellulolytic and pectinolytic activities. In fact, it was observed that *C. cubensis* was able to secrete a vast enzymatic arsenal for hemicellulose depolymerization, however xylanase, mannanase, pectinase and α-arabinofuranosidase presented the most prominent activities in the crude extract. The high levels of hemicellulases and pectinase in the *C. cubensis* crude extract can be considered advantageous because although hemicellulases and pectinases do not act directly on cellulose hydrolysis, it is widely reported that so-called “accessory” enzymes stimulate cellulose hydrolysis by removing non-cellulosic polysaccharides that coat cellulose fibers, facilitating the access of cellulases to the substrate *[7, 8, 11, 53]*. Furthermore, the presence of hemicellulases ensure the liberation of pentoses and others hexoses (e.g. galactose, mannose) which can be converted to ethanol by appropriate microorganisms, promoting an increase in the overall process yield *[54]*.

6.4.4. Biomass saccharification

Sugarcane bagasse was selected as a model substrate for saccharification experiments because it is a major feedstock produced in Brazil. The brazilian annual production of sugarcane bagasse is currently estimated at 186 million ton per year and this amount of biomass represents the potential to produce nearly 11.160 billion gallons of lignocellulosic ethanol per year *[55]*.

Lignocellulosic biomass cannot be saccharified by enzymes at high yields without a pretreatment procedure because the lignin and hemicellulose in the plant cell wall form a barrier to enzymes action *[3]*. In the present study, sugarcane bagasse was pretreated with 1 % NaOH prior to hydrolysis which was effective in fractionating mainly lignin components. The alkali-treated sugarcane bagasse consisted of 51.5 % cellulose, 30.7 % hemicellulose and 8.1 % lignin. When compared with the chemical composition of raw sugarcane bagasse (45.4 % cellulose, 24.2 % hemicellulose and 22.0 % lignin) the alkali pretreatment increased the proportion of cellulose and hemicellulose by 13 and

27 % respectively and decreased lignin by 63 %. These data are in accordance with literature which reports that alkaline pretreatment preferentially removes lignin *[3, 56, 57]*. The efficient removal of lignin is one of the most important requirements for effective enzymatic saccharification. Lignin limits the rate of hydrolysis by acting as a shield, preventing the digestible parts of the substrate to be hydrolyzed *[58]*. Furthermore, condensed lignin can absorb proteins from aqueous solutions and the removal of lignin should improve the hydrolysis performance by reducing nonspecific adsorption of cellulases *[59]*.

The *C. cubensis* crude extract and Multifect® CL were applied for hydrolysis of alkali-treated sugarcane bagasse aiming to compare the performance of both cellulolytic extracts and the time course of equivalent reducing sugar and glucose liberation during enzymatic hydrolysis for 72 h can be observed at Figure 4.

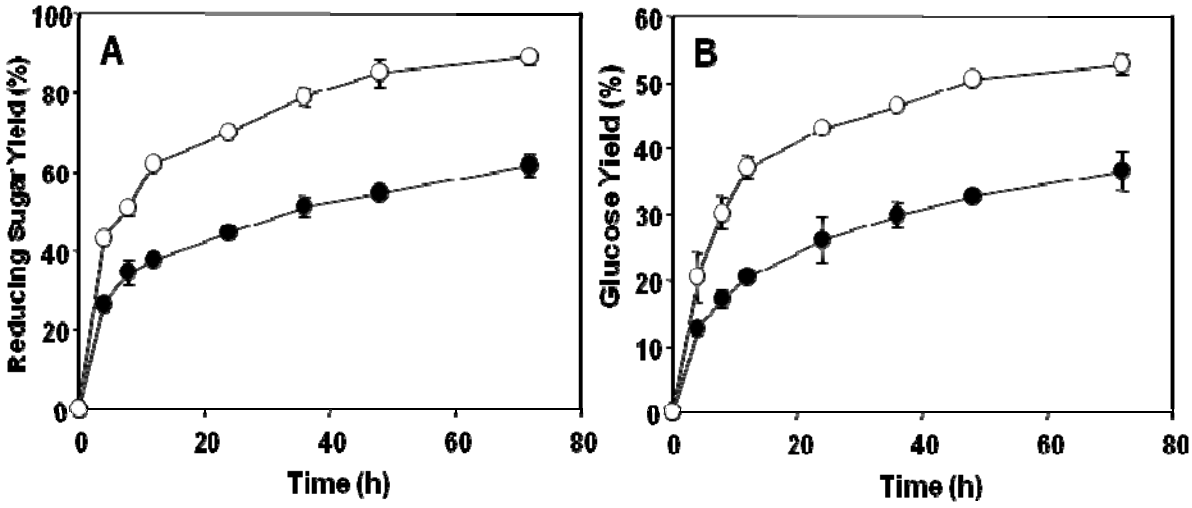

Figure 4: Time course production of reducing sugar (A) and glucose (B) for the enzymatic saccharification of alkali-treated sugarcane bagasse using the *Chrysoporthe cubensis* (○) extract and commercial cellulase Multifect® CL (●).

The same enzyme loading (10 FPase units/g of dry substrate) was used in both experiments; however, it was clear that the *C. cubensis* crude extract resulted in significantly greater sugar production compared with Multifect® CL. The glucose yield obtained after 72 h of reaction using the *C. cubensis* crude extract was 301.7 mg per gram of dry alkali-treated bagasse (corresponding to 52.7 % of glucans hydrolysis). On the other hand, a glucose production of 209.2 mg/g substrate (yield of 36.5 %) was observed in the saccharification reaction carried out with Multifect® CL. Considering the release of equivalent reducing sugars, the *C. cubensis* crude extract also was more efficient, achieving a

saccharification yield of 812.9 mg/g substrate (yield of 89.0 %) after 72 h of reaction. Meanwhile, a yield of 557.0 mg/g substrate (yield of 61.7 %) was observed for production of equivalent reducing sugar in the assay with Multifect CL®.

It is noteworthy that despite the same enzymatic loading (10 FPase U/g substrate) being used in both experiments, the *C. cubensis* extract showed to be much more efficient than Multifect® CL. These results suggest that the cellulolytic extract produced by *C. cubensis* presents the best balance between the different enzymatic activities involved in the biomass depolymerization process. The crude extract from *C. cubensis* presented a higher β-glucosidase/FPase ratio than Multifect CL (Table 2) and this can certainly be pointed out as one of the major reasons for the better saccharification performance encountered for the *C. cubensis* extract. Furthermore, the highest hemicellulase activities, mainly xylanase activity, present in the *C. cubensis* crude extract may also have contributed to enhance its saccharification performance in comparison with the commercial cellulase preparation.

The low β-glucosidase/FPase ratio (ranging of 0.5-1.0) detected for most commercial cellulase preparations obtained from *Trichoderma* species are generally indicated as the main limiting factor to obtain high rates of cellulose conversion *[51, 52]*. High β-glucosidase titres prevent the accumulation of cellobiose which inhibits endoglucanase and cellobiohydrolase enzymes leading to lower saccharification yields *[10]*. The crucial role of β-glucosidase activity for increasing cellulose conversion has been consistently reported and saccharification yields of roughly 90 % have been obtained through supplementation of commercial cellulases from *Trichoderma* spp. with β-glucosidase-rich extracts which are produced individually or obtained commercially *[10, 11, 53, 57]*.

In both enzymatic saccharification assays, a comparative analysis showed that the amount of reducing sugar released from alkali-treated sugarcane bagasse was always higher than the corresponding glucose production. This greater reducing sugar production may be due to the fact that beside cellulases, hemicellulases were also present in the cellulolytic extracts. Alkali-treated sugarcane bagasse contained 30.7 % hemicellulose in its composition and the action of the hemicellulases on this carbohydrate fraction probably boosted the release of equivalent reducing sugars, mainly xylose,

during saccharification. The *C. cubensis* crude extract was richer in hemicellulase activities than Multifect® CL; thus, the higher production of equivalent reducing sugar was expected in the assay with the *C. cubensis* extract.

Several reports have highlighted the importance of hemicellulases during the biomass saccharification process. Hemicellulases can remove fragments of hemicellulose that coat the cellulose fibers, thus increasing the accessibility of the cellulases to its substrate *[7, 8, 53]*. Furthermore, the action of hemicellulases release xylose, arabinose, galactose and mannose which are readily fermentable sugars and this can improve the overall production of ethanol from lignocellulosic biomass. However, a complete fermentation of sugar contained in lignocellulosic biomass requires a yeast that is capable of fermenting hexoses and pentoses *[60]*.

Saccharification studies have been published using different lignocellulosic materials and different source of cellulases *[10, 11, 50, 56, 61]*. However, establishment of a comparison between the results presented in this work and data found in literature may be inconclusive because the saccharification conditions employed in different reports are extremely diverse. The results obtained in this work strongly suggest that *C. cubensis* can be used in the future as a factory to produce enzymes for application in biomass conversion processes. Efforts are now being concentrated on optimizing the conditions for cellulase production by *C. cubensis*. Because this fungus showed an outstanding capacity to secrete β-glucosidase enzymes, attempts will be made to increase endoglucanase and cellobiohydrolase activities in the crude extract because these activities were probably the mostly limiting to cellulose saccharification. Different saccharification condition and the use of the cellulolytic extract from *C. cubensis* in simultaneous saccharification fermentations will also be tested. These experiments are underway.

6.5. Conclusion

The potential of the fungus C*. cubensis* to produce cellulases and hemicellulases was evaluated for the first time in this work. The fungus cultured under SSF using wheat bran as a carbon source was able to produce high FPase, endoglucanase, β-glucosidase and xylanase activities. Cellulase and xylanase activities produced by *C. cubensis* showed desirable features to be

applied in SHF and SSF systems such as high activities in acidic pH, high activity at temperatures in the range of 30-55 °C and high thermal stability at 40 and 50 °C. The cellulolytic extract produced by *C. cubensis* was applied in a saccharification test using alkali-treated sugarcane bagasse as substrate. The ability of the crude extract to hydrolyze lignocellulosic biomass was compared with a commercial cellulase extract and it was observed that the *C. cubensis* extract resulted in a higher production of glucose and equivalent reducing sugar. The cellulolytic extract from *C. cubensis* showed great potential to be applied in biomass saccharification processes with the objective of ethanol production.

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7. Conclusões gerais

Pycnoporus sanguineus, *Trametes* sp. J-2, *Trametes* sp. J-5, J-129, *Chrysoporthe cubensis* e *Cylindrocladium pteridis* apresentaram potencial para produção de enzimas de interesse biotecnológico, principalmente considerando-se os processos de conversão e transformação da biomassa vegetal.

Pycnoporus sanguineus e *Chrysoporthe cubensis* produziram extratos celulolíticos que permitiram a obtenção de rendimentos de sacarificação similares ou superiores àqueles alcançados pelo uso de uma preparação de celulases comercial. Tal fato revela o enorme potencial destes microrganismos para a produção de enzimas visando à conversão da biomassa.

Os resultados também evidenciaram o quão relevante os trabalhos de bioprospecção ainda são. Principalmente em um país tropical como o Brasil, cuja abundante diversidade biológica ainda encontra-se pouca explorada.