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Marcia Maria de Souza Moretti

Produção de ligno-hemi-celulases por fermentação em estado sólido e avaliação dos efeitos da aplicação das enzimas na composição química e estrutura do bagaço e da palha de cana

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Orientador: Profª. Drª. Eleni Gomes
Co-orientador: Profº. Drº. Sebastião Taboga

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São José do Rio Preto
22 de novembro de 2013

"Sem Sonhos,

As perdas se tornam insuportáveis, as pedras no caminho se tornam montanhas,
Os fracassos se transformam em golpes fatais.
Mas se você tiver grandes sonhos...

Seus erros produzirão crescimento, seus desafios produzirão oportunidades,
Seus medos produzirão coragem. Por isso, nunca desista de seus sonhos!"

(Augusto Cury).

Dedico:

A meus pais, João e Nilce, pelo amor e dedicação, pelos ensinamentos e exemplos que serão levados comigo durante toda minha vida.

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Muito Obrigada!

RESUMO

No presente estudo, o fungo termofílico *Myceliophthora thermophila* M.7.7, foi cultivado em fermentação em estado sólido (FES), usando como fonte de carbono misturas de bagaço de cana, palha de cana e palha de milho, com farelo de trigo (w/w 1:1). As fontes de nutrientes minerais avaliadas foram substâncias químicas analíticas e fertilizantes agrícolas. Os efeitos das condições de fermentação, tais como temperatura, pH e umidade sobre a produção de xilanase, endoglucanase e β-glucosidase foram estudados. Além disso, foi realizado o prétratamento do bagaço e da palha de cana submetidos a 2 min de irradiação em micro-ondas com glicerol 70% em solução ácida, alcalina e água destilada. As frações sólidas resultantes do pré-tratamento foram utilizadas nas análises de fibras, TGA, DTG, DSC, FTIR e microscopia eletrônica de transmissão e as frações líquidas, para análises de açúcares e fenóis. Amostras de bagaço e de palha tratados e não tratados (controle) foram submetidas à hidrólise enzimática por 24 e 72 horas, a 55 °C, usando as soluções enzimáticas obtidas do cultivo de *M. thermophila* M.7.7. e comercial (Celluloclast 1.5L) com atividade de endoglucanase padronizadas a 60 U/g de substrato seco ou com relação à quantidade total de proteínas das soluções (5 mg/g de substrato seco). Ao extrato de *M. thermophila* M.7.7 foi feita uma suplementação com enzima β-glicosidase comercial, de modo a atingir a mesma atividade presente na enzima Celluloclast. As atividades máximas de xilanase (446,9 U/mL), endoglucanase (94,7 U/mL) e β-glicosidase (2,8 U/mL) foram obtidas no cultivo por FES com 70% de umidade, a 40 ºC, usando palha de milho e farelo de trigo e solução nutriente composta por fertilizantes agrícolas, com pH ajustado para 5,0. Os espectros de infravermelho e as análises térmicas mostraram que o prétratamento agiu principalmente sobre a lignina e a hemicelulose do bagaço de cana, enquanto que, a palha de cana sofreu menos alterações estruturais e químicas. Após 24 horas de hidrólise, os maiores rendimentos em açúcares redutores foram de 68,2 mg/g de bagaço e 74,0 mg/g de palha nas amostras de bagaço e palha hidrolisadas com a enzima produzida por *M. thermophila* M.7.7 e suplementada com β-glucosidase e com a Celluclast, respectivamente. Esses maiores rendimentos foram obtidos na hidrólise dos materiais tratados com micro-ondas imersos em glicerol acidificado com ácido sulfúrico. Em ensaios de hidrólise por 72 horas, utilizando-se esses mesmos materiais, os maiores rendimentos de açúcares redutores foram de 304,7 mg/g de palha e 240,9 mg/g de bagaço utilizando-se solução enzimática de *M. thermophila* M.7.7. com ou sem suplementação com β-glucosidase, respectivamente. Esses resultados indicaram que a suplementação com β-glucosidase não aumentou significantemente o rendimento da hidrólise.

Palavras-chave: *M. thermophila*, biomassa, micro-ondas, glicerol, hidrólise enzimática.

ABSTRACT

This study investigated the effect of non expensive carbon and nitrogen sources on enzyme production by Myceliophthora thermophila M.7.7 in solid-state fermentation. Three kinds of lignocellulosic waste (corn straw, sugarcane bagasse and sugarcane straw) and six nitrogen sources (urea, calcium nitrate, analytical ammonium sulphate, yeast extract, agricultural fertilizer NPK 20-05-20 and fertilizing grade ammonium sulphate) were tested. Some physical-chemical parameters of the fermentation, such as temperature, initial pH and moisture content of the substrate on enzyme production were evaluated. Furthermore, the pre-treatment of bagasse and sugar cane straw with microwave radiation in presence of glycerol diluted in water, sulphuric acid and sodium hydroxide on the chemical composition, fiber structure and the efficiency of subsequent enzymatic hydrolysis was investigated. Bagasse and cane straw were subjected to 2 min of irradiation and the solid fractions resulting were used in the analysis of fibers, TGA, DTG, DSC, FTIR, X-ray and transmission electron microscopy and liquid fractions used to detemine sugar and phenol contents. Samples of bagasse, treated cane straw and untreated cane straw (control) were submitted to enzyme hydrolysis for 24 to 72 h at 55 °C with enzymatic solutions obtained by the cultivation of M. thermophila M.7.7. and Celluloclast 1.5L based on 60 U of endoglucanase activity per g of dry substrate or on the total amount of protein solutions (5 mg protein/g of dry substrate). The enzyme solution from M. thermophila M.7.7 was supplemented with a commercial β-glucosidase in order to achieve the same activity present in Celluclast. The maximum xylanase activity (446.9 U/ml), endoglucanase (94.7 U/ml) and β-glucosidase (2.8 U/mL) were obtained in the cultivation by SSF with 70% humidity, 40 °C using corn stover and wheat bran and nutrient solution composed of agricultural fertilizers, with pH adjusted to 5.0. Infrared spectra and thermal analysis showed that the pretreatment had an effect mainly on lignin and hemicellulose from sugarcane bagasse, whereas sugar cane straw suffered lower strutural and chemical changes. In hydrolysis assays for 72 hours, using these same materials, the highest yield of reducing sugars was 304.7 mg/g of straw and 240.9 mg/g for bagasse when using enzyme solution of M. thermophila M.7.7 independent of supplementation with β-glucosidase or not. These results indicate that supplementation with β-glucosidase did not significantly increase the yield of hydrolysis.

Keywords : M. thermophila, biomass, microwaves, glycerol, enzymatic hydrolysis.

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Estrutura da Tese

A presente tese foi escrita de maneira a relacionar os conceitos que envolvem a produção de enzimas por fermentação em estado sólido usando o fungo termofílico *Myceliophthora thermophila*, além da aplicação dessas enzimas na sacarificação do bagaço e palha de cana de açúcar prétratados, sendo dividida em 3 capítulos e um apêndice:

- Capítulo I: Composto pela "revisão bibliográfica", abordando a apresentação do trabalho, informações a respeito das características dos materiais lignocelulósicos, o uso de resíduos na produção de enzimas por fungos termofílicos e o tratamento e hidrólise enzimática dos materiais lignocelulósicos;

- Capítulo II: Composto pelos objetivos gerais e específicos do presente trabalho;

- Capítulo III: Composto pela introdução, descreve a metodologia empregada na caracterização das amostras e nas reações em estudo referentes à primeira etapa do projeto, que teve como objetivo o aumento da produção de enzimas pelo fungo termofílico *M. thermophila*. Ainda contém a apresentação e discusão dos resultados obtidos, assim como a conclusão final do trabalho;

- Capítulo IV: Composto pela introdução, descreve a metodologia analítica empregada na caracterização das amostras referentes à segunta etapa do projeto, que teve como objetivo o pré-tratamento do bagaço e da palha de cana de açúcar, análises físico-químicas do material tratado e não tratado e hidrólise enzimática. Ainda contém a apresentação e discusão dos resultados obtidos, assim como a conclusão final do trabalho;

- Capítulo V: Composto pela conclusão dos resultados obtidos nos capítulos III e IV;

- Apêndice I: Composto pela apresentação de um trabalho iniciado durante o período do mestrado que teve seu término somente durante o doutorado.

INTRODUÇÃO

No Brasil há abundância de resíduos e subprodutos agrícolas e agroindustriais, dentre os quais destacam-se o bagaço e a palha de cana-de-açúcar. Esses resíduos lignocelulósicos são gerados em grande volume pela indústria sucroalcooleira. Especial atenção tem sido dada ao potencial uso do bagaço e da palha para a produção de etanol. Neste sentido, estudos têm sido desenvolvidos com a finalidade de se estabelecer tecnologias para viabilizar a obtenção deste produto a partir do bagaço e da palha de cana (CERQUEIRA LEITE *et al*., 2009).

Para que o etanol seja produzido a partir do bagaço e da palha de cana, as frações de celulose e hemicelulose desses resíduos devem inicialmente ser despolimerizadas, por hidrólise química ou enzimática, para a liberação de açúcares solúveis, os quais serão convertidos em etanol pelo micro-organismo fermentador. Cabe, porém, ressaltar que alguns obstáculos ainda devem ser superados para a eficiente conversão enzimática da lignocelulose em açúcares fermentescíveis.

Tal dificuldade está relacionada com a estrutura química desses materiais, que apresentam grande resistência à ação das enzimas hidrolíticas. Atualmente há uma extensa busca de processos para a hidrólise de materiais lignocelulósicos a açúcares fermentescíveis, porém, estes devem ser de baixo custo e, ao mesmo tempo, impedir a formação de compostos inibitórios para a fermentação alcoólica subseqüente. O uso de enzimas microbianas como celulases, xilanases e ligninases atende a essas exigências. Dentre essas hidrolases, as enzimas produzidas por fungos termofílicos apresentam importantes características, tais como superior termoestabilidade, atividade ótima a temperaturas elevadas e altas taxas de hidrólises (MERHEB-DINI *et al.,* 2009).

Em relação à obtenção de etanol a partir de materiais lignocelulósicos, observa-se que a íntima associação química e física entre a lignina e a celulose/hemicelulose na parede celular vegetal dificulta a degradação enzimática destes polissacarídeos. A lignina representa uma barreira de acesso aos polissacarídeos, protegendo-os da degradação enzimática (KRISHNA; CHOWDARY, 2005). Ademais, a cristalinidade da celulose também dificulta a ação das enzimas microbianas (GOULD, 1984). A fim de se amenizar as limitações à hidrólise enzimática do bagaço, prétratamentos físicos e/ou químicos são comumente empregados, visando a desestruturação da fibra, a parcial solubilização e/ou degradação da lignina e da hemicelulose, o decréscimo no grau de cristalinidade da celulose e o aumento da área superfícial, o que facilitaria acessibilidade e a ação das enzimas despolimerizantes (KRISHNA; CHOWDARY, 2005).

Desse modo, o presente projeto teve como objetivo estudar e melhorar a produção de celulases e xilanases pelo micro-organismo *M. thermophila* M.7.7, que em trabalhos anteriores

(MORETTI, 2010), se mostrou um promissor produtor de enzimas fibrolíticas, por meio de técnicas de fermentação em estado sólido, utilizando resíduos agro-industriais e fontes de nitrogênio de baixo custo. Subsequentemente, estas enzimas foram aplicadas na sacarificação do bagaço e da palha de cana-de-açúcar pré-tratados com irradiação de micro-ondas, usando solvente orgânico (glicerol). As análises químicas e estruturais das amostras de bagaço e palha de cana obtida após as etapas de tratamento e hidrólise enzimática também se mostraram viáveis dentro do contexto.

Capítulo 1

Revisão Bibliográfica

Biomassa lignocelulósica

O rápido consumo dos combustíveis fósseis e as perspectivas de esgotamento das fontes não renováveis têm motivado pesquisadores a propor fontes alternativas de combustíveis sustentáveis e renováveis (BASTOS, 2007; BIAN et al., 2013). A biomassa lignocelulósica tem sido apontada como a mais apropriada matéria prima para a produção de biocombustíveis, uma vez que, esta consiste de aproximadamente 75% de polissacarídeos (VAN DYK; PLETSCHKE, 2012). Fontes de lignocelulose incluem resíduos agroindustriais, tais como, palha de arroz, farelo de trigo (PALMAROLA-ANDRADOS et al., 2005), fibra de alfafa (SREENATH et al., 2001), palha de trigo (HONGZHANG, 2006), serragem de madeira, casca de arroz, palha de milho (SAHA et al., 2005) e bagaço e palha de cana-de-açúcar, sendo os dois últimos produzidos em grande quantidade no Brasil pelas indústrias de açúcar e álcool (MARTÍN et al., 2006; SUN, 2002).

A parede celular

De modo geral, a parede celular dos materiais lignocelulósicos apresenta aproximadamente 50-75 % de carboidratos na forma de celulose e hemicelulose, 20-30 % de lignina, além de pectina, proteínas, cinzas e uma pequena quantidade de extrativos. A composição dos materiais lignocelulósicos difere de uma espécie de planta para outra. Entretanto, a estrutura da parede celular entre as espécies é bem semelhante, sendo constituída por três camadas: lamela média, parede primária e parede secundária. A lamela média (LM) é uma camada intercelular, composta por substâncias pécticas, situada entre as paredes primárias de células vizinhas, consolidando a união dessas células (Figura 1) (MENON; RAO, 2012).

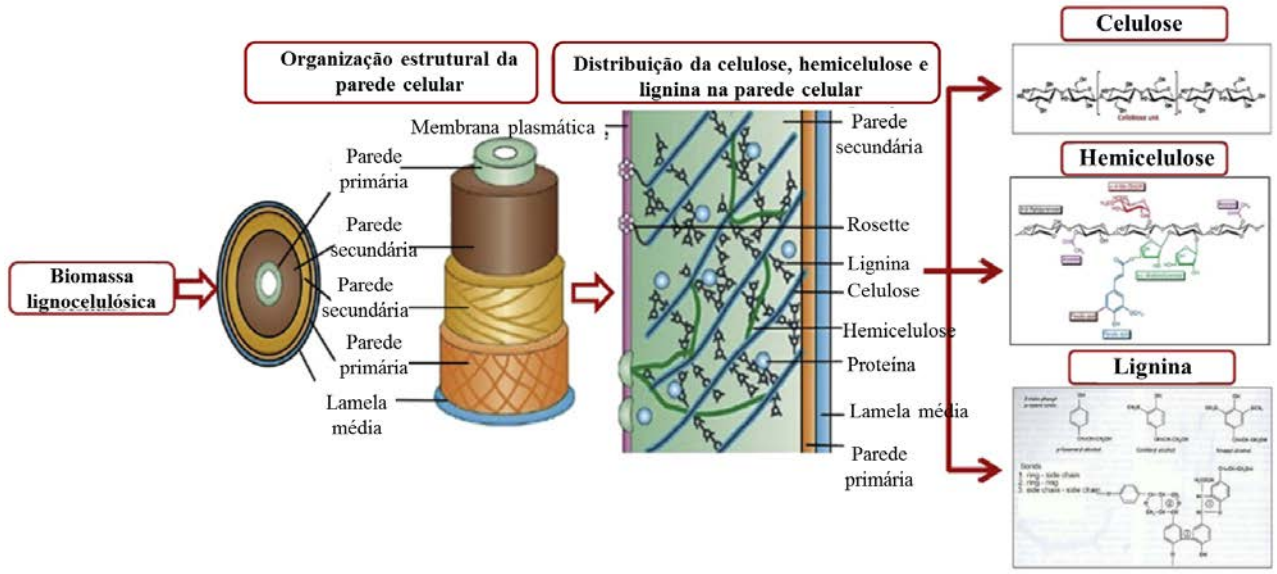

Figura 1: Ilustração esquemática da estrutura de lignocelulose (MENON; RAO, 2012).

A parede primária é mais externa, e é produzida durante o desenvolvimento da célula. Ela pode ser fina ou grossa, e pode apresentar camadas distintas, como em células epidérmicas. A parede primária apresenta fibrilas de celulose e sua matriz é composta, principalmente, por substâncias pécticas, hemicelulose e podendo também ser lignificada. Suas regiões mais delgadas recebem o nome de campos primários de pontoação que podem ser atravessados por filamentos citoplasmáticos (os plasmodesmos), que estabelecem comunicação entre o protoplasma de células adjacentes (Figura 2) (SRIVASTAVA, 2002).

Após o término da expansão celular da parede primária, a parede secundária é depositada na superfície interna da mesma, e ao se depositar, não cobre os campos primários de pontoação, resultando na formação de pontoação. A parede secundária é constituida principalmente de celulose e hemicelulose, além de quantidades variáveis de lignina, sendo esta mais rígida que a membrana primária. Além disso, ela pode apresentar três camadas, uma secundária fina (S1), uma relativamente extensa (S2), e novamente uma mais fina (S3). As variações na espessura total da parede secundária são principalmente devido às oscilações na espessura da camada central (S2) (Figura 2). Várias camadas de paredes secundárias são características de algumas células fibrosas (por exemplo, aqueles encontrados em tecidos de caule e muitas monocotiledôneas) (SRIVASTAVA, 2002).

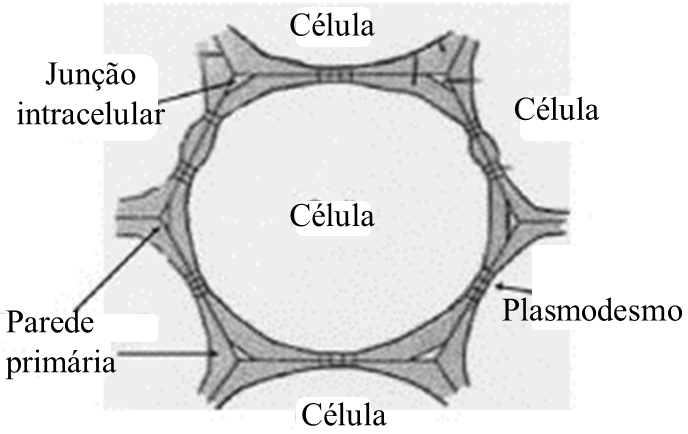

Figura 2: Esquema da parede celular da planta, mostrando as camadas da parede secundária e as regiões delgadas da parede primária e secundária que situam as membranas de pontoação com os plasmodesmos. (modificado de WALKER; BUTTERFIELD, 1996).

Celulose

A celulose é uma molecula formada por unidades de anidro-glicose ligadas covalentemente por um átomo de carbono C4 a um átomo de carbono C1 (ligações glicosídicas β-1,4) (LUNDQVIST et al., 2002). As extremidades da cadeia de celulose são quimicamente diferentes, uma possui um grupo D-glucopiranosil no qual o átomo de carbono anomérico está envolvido numa ligação glicosídica, enquanto, a outra extremidade possui um resíduo D-glucopiranose em que o átomo de carbono anomérico está livre (Figura 3) (PÉREZ; SAMAIN, 2010).

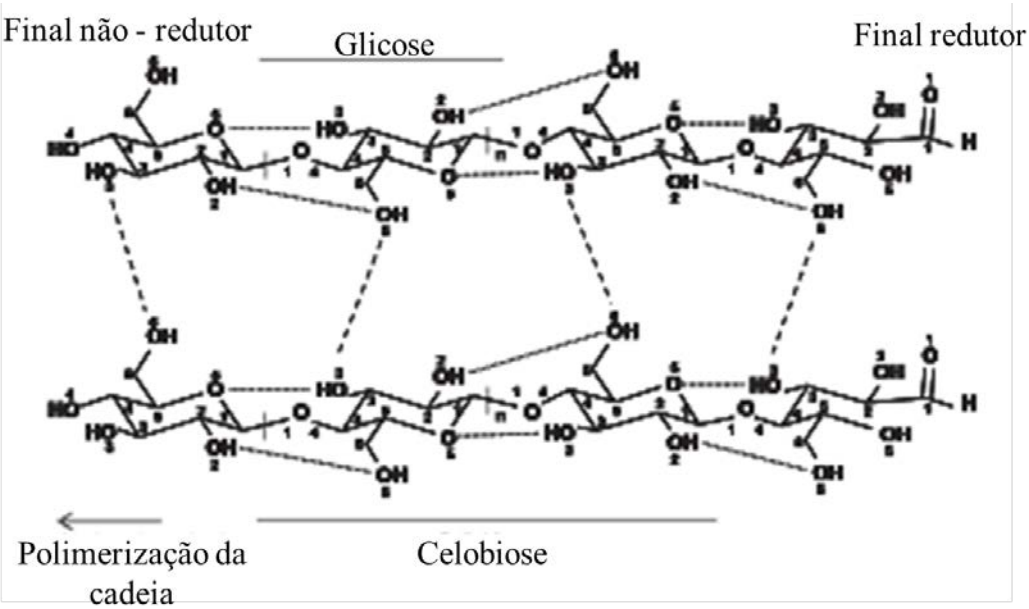

Figura 3: Estrutura representativa das cadeias de celulose (Modificado de FESTUCCI BUSELLI;OTONI; JOSHI, 2007).

A estrutura molecular da celulose contém um grande número de grupos hidroxil livres (três grupos OH por unidade glicose) que podem estar envolvidos em ligações de hidrogênio inter- e intra- molecular, que consequentemente dão origem a vários arranjos cristalinos (ROY et al., 2009, KRASSIG, 1985, PÉREZ; SAMAIN, 2010).

As fibras de celulose são constituídas por regiões cristalinas e amorfas, sendo as primeiras bastante coesas e de estrutura rígida, enquanto que as regiões amorfas são formadas por cadeias de celulose mais frouxamente organizadas (DA-SILVA et al., 1997). A celulose apresenta pelo menos cinco formas alomórficas (Iα, Iβ, II, III e IV), sendo a celulose I nativa, enquanto que as demais dependem que a primeira seja submetida a tratamento - químico e/ou térmico. Assim, ocorrerão alterações nas dimensões da célula unitária e, consequentemente, na sua estrutura cristalina, o que resulta em diferentes polimorfos da celulose (PÉREZ; SAMAIN, 2010). A celulose I consiste em duas fases: Iα a qual tem célula unitária triclínica contendo uma cadeia de celulose e Iβ que tem célula unitária monoclínica contendo duas cadeias paralelas (alomorfo Iβ é termodinamicamente mais estável) (Figura 4) (MOUBARIK; GRIMI; BOUSSETTA, 2013). As ligações de hidrogênio são diferentes para cada forma, refletindo diferentes propriedades físico-mecânicas.

A) B)

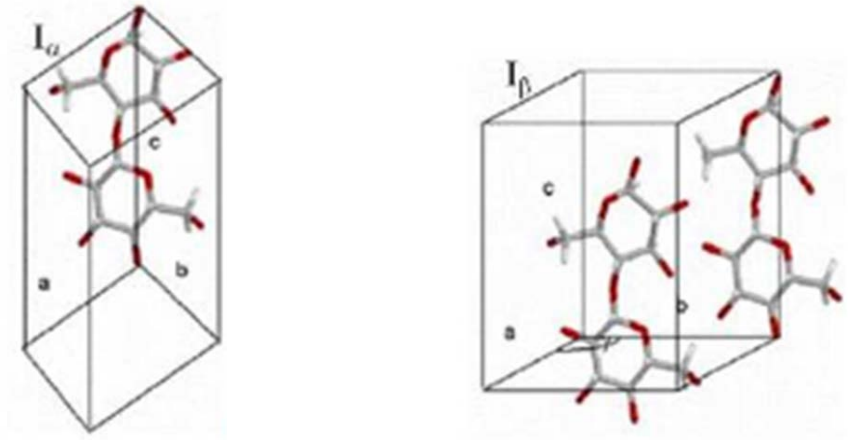

Figura 4: Representação da cela unitária das fases (A) Iα e (B) Iβ (KOYAMA et al., 1997).

As ligações de hidrogênio intramoleculares determinam o alinhamento das cadeias. Em ambas as formas cristalinas Iα e Iβ, as ligações entre o grupo hidroxila do átomo de carbono C-3 ao O-5 são relativamente fortes. Na celulose Iα outra ligação intracadeia é observada entre os grupos hidroxila de C-2 ao O-6 da molécula vizinha, no entanto, esta ligação é mais curta no alomorfo Iα (FESTUCCI-BUSELLI; OTONO; JOSHI, 2007). Além disso, na celulose Iα é observada a existência de ligações de hidrogénio intermoleculares entre o grupo hidroxila do

átomo de carbono C-6 ao O-3 pertencente à outra cadeia de celulose (Figura 5) (PÉREZ; SAMAIN, 2010).

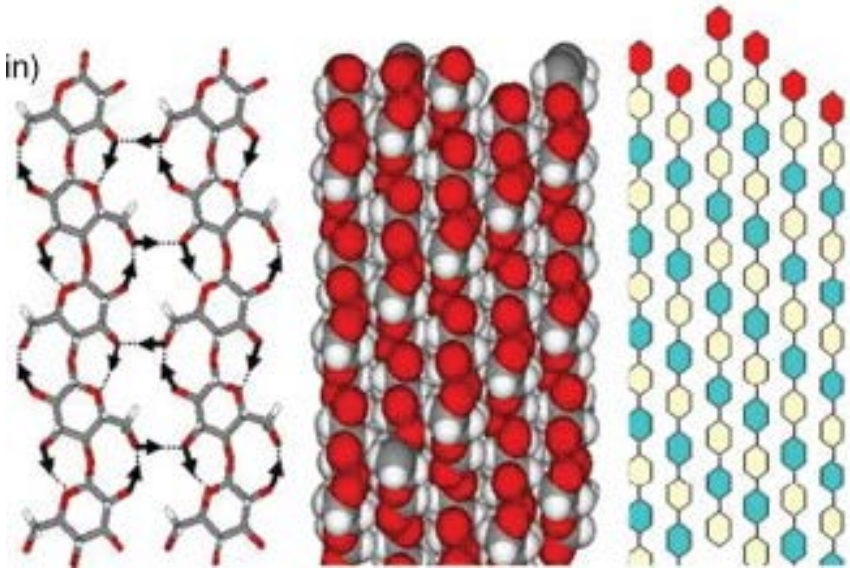

Figura 5: Detalhes da estrutura da celulose Iα (SIMON; SCHERAGA; MANLEY, 1998).

A estrutura cristalina da fibra de celulose Iβ, consiste de cadeias paralelas alinhadas lado a lado por ligações de hidrogênio em folhas planas (BIAN et al., 2013). Na estrutura cristalina da celulose Iβ, o oxigênio O-6 está fortemente unido por ligação de hidrogênio ao grupo hidroxila do atomo de carbono C-2 da molécula vizinha. Na celulose Iβ a ligação entre o grupo hidroxila do átomo de carbono C-3, e o anél de oxigênio O-5, é alternada entre os planos. Tal esquema múltiplo de ligação de hidrogênio explica as complexas bandas de estiramento (O-C) observadas nos espectros de infravermelho de celulose Iβ (PÉREZ; SAMAIN, 2010). Uma ligação intermolecular também existe entre o grupo hidroxila do átomo de carbono C-6 ao O-3 da cadeia adjacente, sendo esta ligação mais curta na celulose Iβ (Figura 6) (ROY et al., 2009; KRASSIG, 1993). Para Nishiyama e outros (2002) as ligações de hidrogênio intermoleculares fortes são inexistentes entre os planos regular e desemparelhado na celulose Iβ, sendo estes unidos por ligações fracas (C-H.....O). Mais tarde, o mesmo autor sugeriu a existência de mais interações fracas do tipo (C-H.....O) entre os planos da celulose Iβ comparado a celulose Iα (NISHIYAMA et al., 2003). Estes aspectos são responsáveis pela natureza linear e rígida da cadeia de celulose. Assim, a propriedade física e a reatividade química da celulose fibrosa não são apenas influenciadas pela constituição química da molécula celulose, mas são também determinadas pelo arranjo da cadeia de moléculas na fibra (ROY et al., 2009; KRASSIG, 1985).

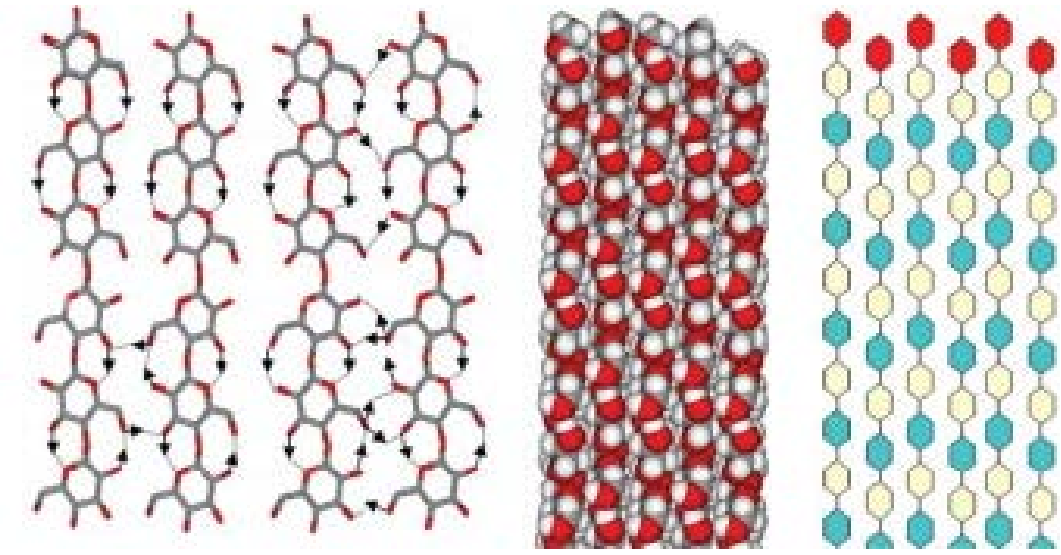

Figura 6: Detalhes da estrutura da celulose Iβ (KOYAMA, et al., 1997).

Das outras formas possíveis, a celulose II é o polimorfo majoritário na indústria de processamento de celulose e possui a estrutura mais estável entre os polimorfos. Ela é formada a partir de regeneração ou mercerização da celulose I. A mercerização envolve o aumento da intracristalinidade da celulose por meio do tratamento com solução alcalina (NaOH), seguido por lavagem e recristalização (PÉREZ; SAMAIN, 2010). A regeneração envolve a dissolução da celulose e sua posterior regeneração. Assim como na forma alomófica Iβ, a celulose II também apresenta ligações intra moleculares na posição C-3 do atomo de carbono, com o átomo de oxigênio O-5 da unidade de glicose vizinha. A grande diferença entre a celulose Iβ e II está relacionada às ligações de hidrogênio inter moleculares. Na Figura 7A observa-se que a celulose Iβ apresenta as ligações de hidrogênio entre os grupos hidroxila do átomo de carbono C-6 e O-3 pertencente à outra cadeia de celulose. Enquanto que, na celulose II as ligações de hidrogênio entre os grupos hidroxila do átomo de carbono C-6 estão associadas ao O-2 da cadeia adjacente (O'SULLIVAN, 1997). Este fato tem influência direta na estrutura do retículo cristalino como pode ser observado na Figura 7B.

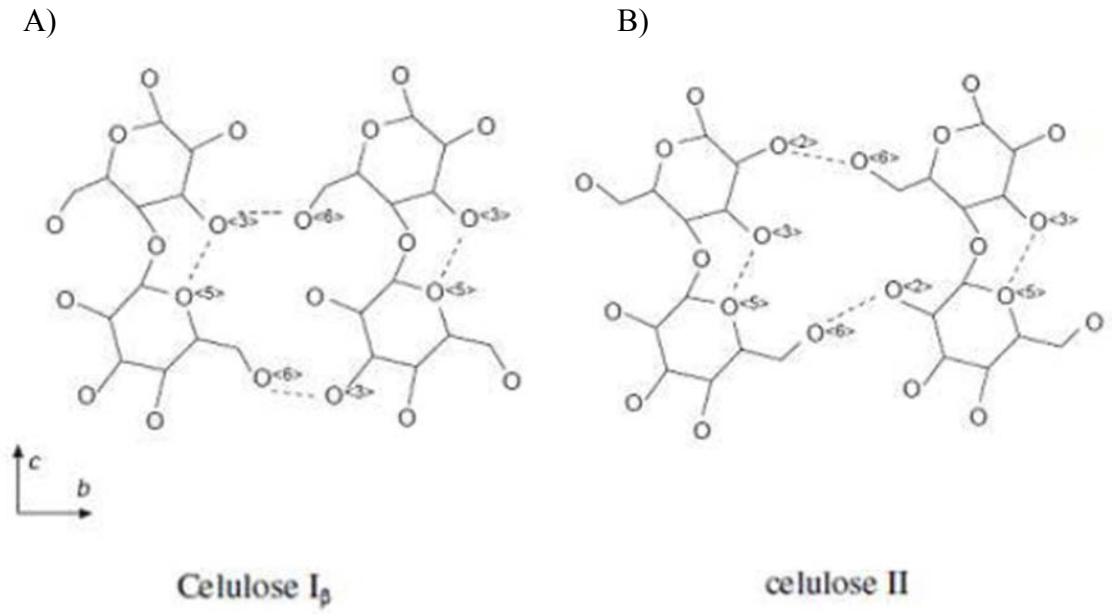

Figura 7: Distinção entre celulose Iβ e II a partir do padrão de ligações de hidrogênio (O'SULLIVAN, 1997).

O tratamento com amônia líquida ou com certas aminas, tais como 1,2-diaminoetano (etilenodiamina, EDA) permite a preparação da celulose III a partir da celulose I (que assume a forma de celulose III_I) ou celulose II (que assume a forma de celulose III_{II}). A celulose II_I tratada a altas temperaturas em glicerol é transformada em celulose IV. Nesta novamente existem dois tipos: celulose IV_I e IV_{II}, obtidas a partir da celulose III_I e $III_{II,}$ respectivamente. É conhecido que a celulose IV_I é a forma desordenada da celulose I. As condições que permitem a interconversão entre as formas polimórficas da celulose estão representadas na Figura 8.

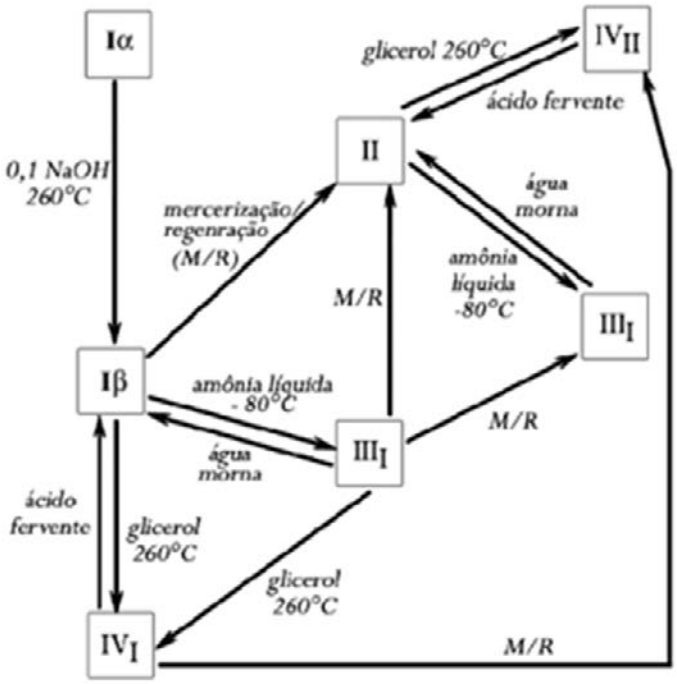

Figura 8: Representação das condições que permitem a interconversão entre as diferentes formas cristalinas da celulose **(**Adaptado de O'SULLIVAN, 1997).

As cadeias de celulose são empacotadas em microfibrilas com diâmetro entre 4-10 nm, visíveis em eletromicrografias. O conjunto de microfibrilas é denominado de macrofibrilas, aos quais são organizadas em lamelas para formar a estrutura fibrosa das várias camadas da parede celular vegetal. As microfibrilas de celulose são embebidas em uma matrix de hemicelulose, pectina e lignina. A lignina e hemicelulose são encontradas nos espaços entre as microfibrilas de celulose nas paredes célulares primária e secundária, bem como a lâmina média (CARLILE et al., 2002; VAN DYK; PLETSCHKE, 2012).

Hemicelulose

As hemiceluloses são heteropolissacarídeos de cadeia curta ramificada e constituída por várias unidades de monossacarídeos diferentes. Em média o grau de polimerização (DP) de hemiceluloses está por volta de 80-200. A hemicelulose geralmente está associada com vários outros componentes da parede celular, tais como celulose, proteínas da parede celular, lignina e outros compostos fenólicos por meio de ligações covalentes e hidrogênio, e por interações iônicas e hidrofóbicas (BALAT et al., 2008; PENG et al., 2012). A xilana é o principal componente da hemicelulose, cuja estrutura corresponde a um polímero de D-xilose unidos por ligações β-1,4. A xilana constitui cerca de 30-35% do peso seco total da parede celular das plantas, embora a quantidade de xilana possa diferir entre as plantas (VAN DYK; PLETSCHKE, 2012).

Hemicelulose de madeira dura (Hardwood): Em plantas lenhosas (madeira), a composição da xilana apresenta *O*-acetil-4-*O*-metilglucorono-β-D-xilana, e o conteúdo de glucoronoxilana pode variar de 15-30% do material seco, dependendo das espécies de madeira. A glucoronoxilana consiste de uma estrutura principal de ligações (1→4) de resíduos β-D-xilopiranosil. A maioria dos resíduos de xilose contém um grupo acetil em C-2 ou C-3 (aproximadamente 7:10 resíduos de acetil/unidades de xilose) (Figura 9). Os grupos acetil são responsáveis pela solubilidade da xilana em água, mas são removidos durante os pré-tratamentos alcalinos. A glucoronoxilana é substituída com resíduos glucorononosil e 4-*O*- metilglucoronosil por ligações α-(1→2).

A madeira dura também possui 2-5% de glucomanana, que é composto por unidades de β-glucoropiranose e β-manopiranose ligadas por ligações (1→4). Entretanto, em manana de madeira dura não ocorre a presença de galactose. Porém, em algumas plantas superiores são encontradas xiloglucanas presentes na parede celular primária. As xiloglucanas tem uma estrutura principal de β-(1→4)-glucopiranana celulósica com unidades de α-D-xilose presas em *O*-6. Na parede celular primária, as xiloglucanas são os principais polissacarídeos de interligação, interagindo com as microfibrilas de celulose por ligações de hidrogênio (PENG et al., 2012).

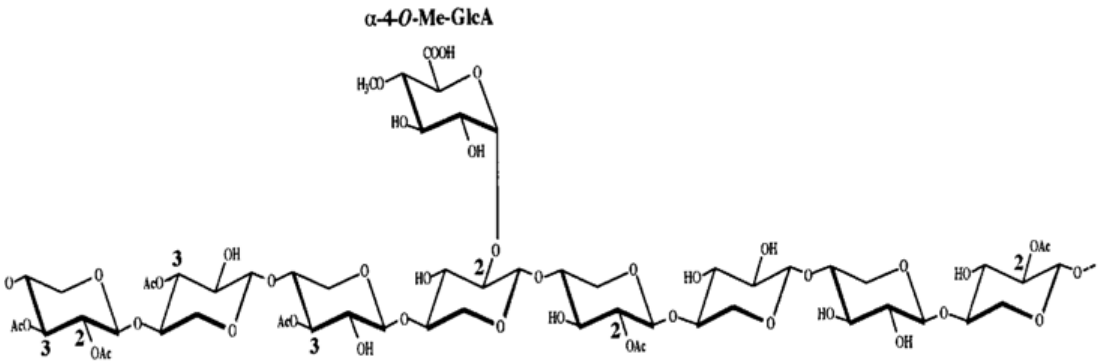

Figura 9. Esquema representativo da estrutura de *0*-acetil-4-*0*-metilglucuronoxilana presente em madeiras duras. Os números indicam os átomos de carbono aos quais ligam-se os grupos substituintes. Ac: grupo acetil; α-4-*0*-Me-GlcA: ácido α-4-*0*-metilglucurônico (SUNNA; ANTRANIKIAN, 1997).

Hemicelulose de madeira macia (Softwood): As hemiceluloses em madeira macia apresenta *O*-acetil-4-*O*-metilglucorono-β-D-xilana na composição da xilana. Além disso, as madeiras macias contém grande quantidade de arabinogalactona solúvel em água. Esta por sua

vez, possue uma estrutura principal de galactona que apresenta ligações β-(1→3) e os açúcares galactose β-(1→6) e arabinose β-(1→3 e 1→6) nos sítios de cadeia. A estrutura altamente ramificada é responsável pela baixa viscosidade e alta solubilidade desse polissacarídeo (Figura 10) (PENG et al., 2012).

As hemiceluloses de madeira sofwtood são galactoglucomananas acetiladas, que podem constituir até 10% da massa seca da madeira. Elas são compostas de uma estrutura principal de resíduos de β-D-glucopiranosil e β-D-manopiranosil unidos por ligações (1→4) com α-(1→6)-D-galactopiranosil. Uma importante característica estrutural são os gupos hidroxil nas posições C-2 e C-3 na unidade de cadeia principal, sendo parcialmente substituídos por grupos *O*-acetil, em média, um grupo para 3-4 unidades de hexose contendo esta substituição. Existem dois tipos de acetilgalactoglucomananas em madeiras macias, uma pobre em galactose (5-8% da madeira seca) e outra rica em galactose (10-15% da madeira seca), sendo a proporção de galactose:glicose:manose de 0,1:1:3 e 1:1:3, respectivamente.

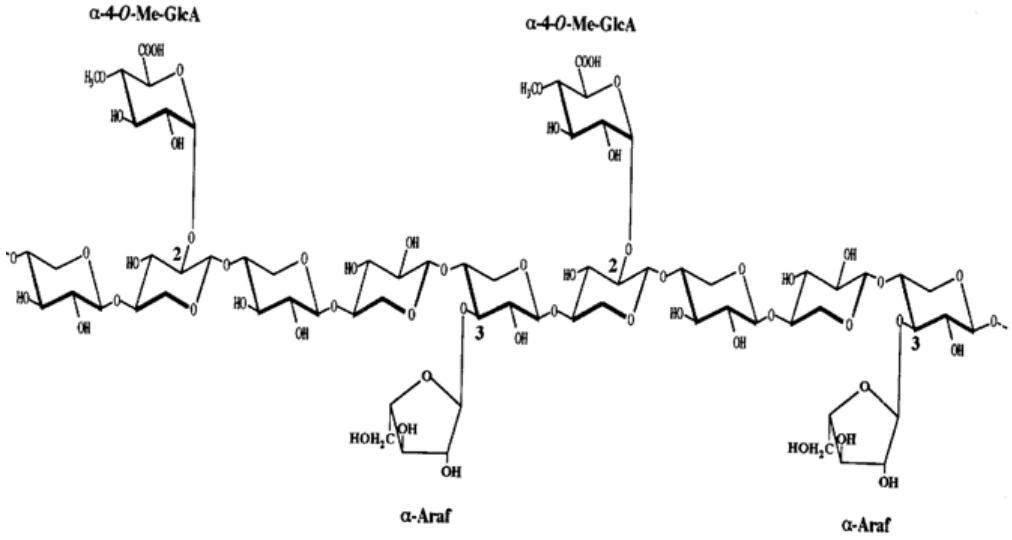

Figura 10. Estrutura de arabino 4-*0*-metilglucuronoxilana presente em madeiras macias. Os números indicam os átomos de carbono aos quais ligam-se os grupos substituintes. α-araf: arabinofuranose; α-4-*0*-Me-GlcA: ácido α-4-*0*-metilglucurônico (SUNNA; ANTRANIKIAN, 1997).

Hemicelulose de gramíneas: As arabinoxilanas são as principais hemiceluloses das gramíneas e neste polímero a estrutura linear principal de β-(1→4)-D-xilopiranosil é substituída por unidades de α-L-arabinofuranose nas posições 2-*O* e 3-*O* (Figura 11). Em materiais lignocelulósicos como: bagaço de cana-de-açúcar, farelo de trigo e bambu, as arabinoxilanas também podem ser substituídas por unidades de α-D-glucopiranosil urônico ou seus derivados 4-

O-metil. Considerando que as paredes celulares de gramíneas contém de 1-2% de grupos acetil (PENG et al., 2012).

De acordo com a quantidade de ácido glucurônico e arabinose, os tipos de arabinoxilana são classificados em arabinoglucuronoxilana e glucuronoarabinoxilana, respectivamente. A primeira é a hemicelulose dominante nas paredes celulares de gramas e cereais. (PENG et al., 2012). A estrutura principal da arabinoxilana em glucuronoarabinoxilana contém dez vezes menos ácido urônico que arabinose nos sítios de cadeia. O ácido ferulico e *p*-cumárico descritos em glucuronoarabinoxilana estão conectados a C-5 de unidades arabinosil. Além disso, o ácido ferulico também pode estar ligado às frações de hemicelulose e pectina e estes polissacarídeos são capazes de fazer ligações-cruzadas uns com outros, assim como, com compostos aromáticos poliméricos da lignina (VRIES; VISSER, 2001).

As β-(1→3, 1→4)-glucanas presentes em gramíneas consiste de uma cadeia de unidades glucopiranosil unidas por ligações (1→3) e (1→4). As ligações mistas das glucanas são dominadas por unidades celotriosil e celotetrasil unidas por ligações β-(1→3) embora, também ocorram segmentos de ligações β-(1→4) mais longas. A celulose também é constituida por β-D-glucana, que nesta está unida por ligações (1→4)-glucosídicas e, portanto, tem elevada rigidez (cristalino) e é insolúvel na maioria dos solventes. Porém, as ligações (1→3) existentes em β-(1→3, 1→4)-glucanos, torna as glucanas flexíveis e solúveis. A hemicelulose representa uma íntima conexão entre a lignina e as fibras de celulose, além de interagir covalentemente com a pectina (VRIES; VISSER, 2001).

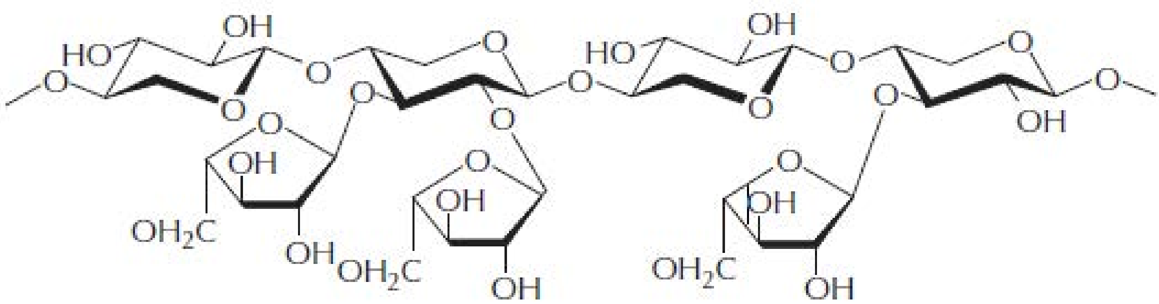

Figura 11: Estrutura primária da L-arabino-D-xilana solúvel em água (REN; SUN, 2010).

Lignina

A lignina é um dos polímeros mais abundante na natureza, constituindo-se de um polímero fenilpropanóide amorfo composto por unidades de álcoois *p*-cumarílico, coniferílico e sinapílico (Figura 12A) (BOURBONNAIS, PAICE, 1988; HIGUCHI, 2004).

Entre as funções biológicas da lignina destacam-se: dar rigidez à parede celular secundária das plantas vasculares; conferir hidrofobicidade à parede celular, o que permite maior eficiência no transporte de água pelos tecidos vasculares das plantas e dificulta o ataque microbiano aos tecidos do vegetal (Figura 12C) (ÖNNERUD, et al., 2002). A lignina é um polímero de difícil degradação, pois é interconectado por várias ligações C—C e éter, as quais não são hidrolisáveis sob condições biológicas. A estrutura predominante, perfazendo quase metade do seu total, é de éter com ligação β-*O*-4 (Figura 12B). A lignina é racêmica e, consequentemente, mesmo um simples dímero com ligação β-*O*-4, com dois carbonos assimétricos, possui quatro estereoisômeros. Como o número de isômeros aumenta geometricamente com o número de sub-unidades, a lignina forma uma estrutura bem complexa e sem unidades repetitivas definidas (HAMMEL, CULLEN, 2008).

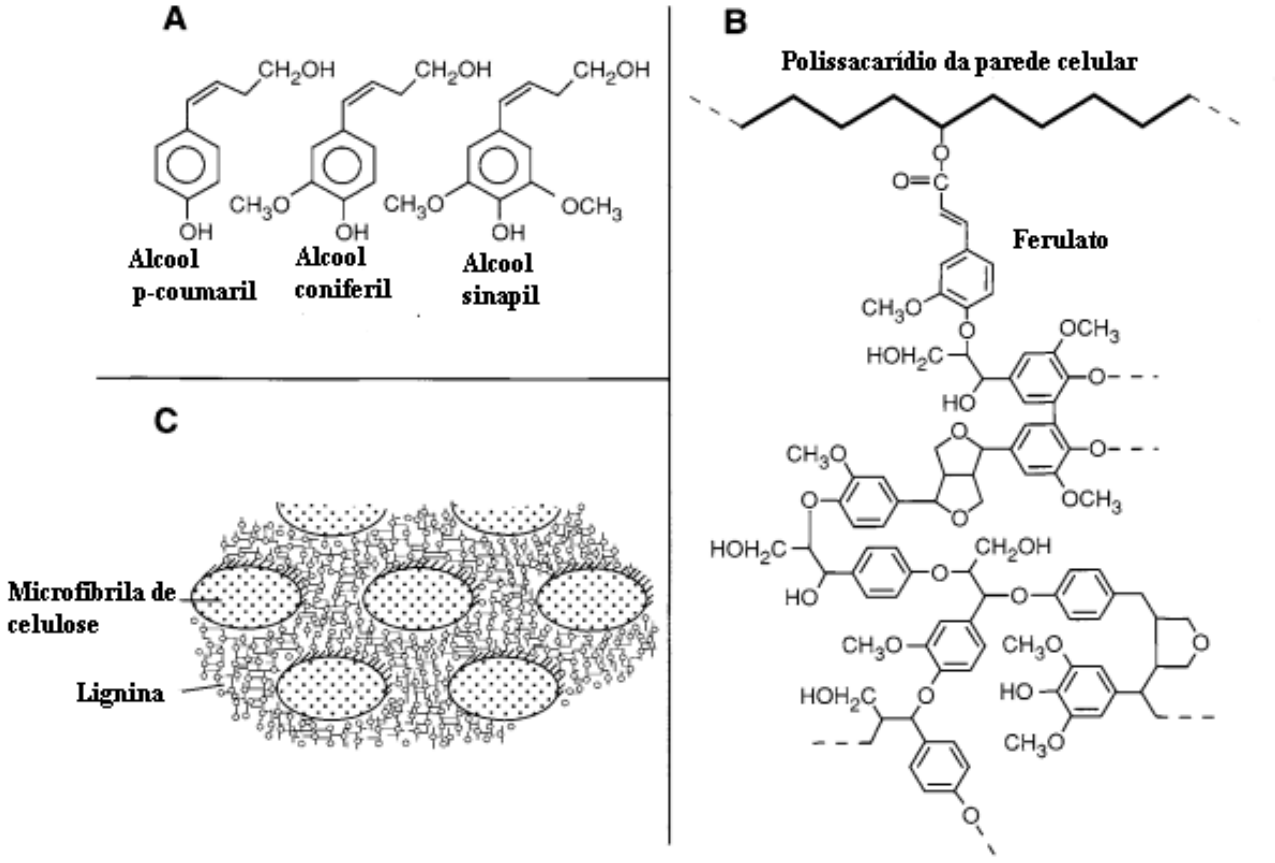

Figura 12. Estrutura da lignina, mostrando (A) os fenilpropanóides constituintes da lignina (B) os passos iniciais na lignificação (apenas uma pequena parte da molécula de lignina é mostrada) e o (C) desenho esquemático mostrando a relação de polissacarídeos e a lignina. (apenas as fibrilas de celulose são mostradas (adaptados de TUOR et al., 1995 e BRETT; WALDRON , 1996).

O material lignocelulósico pode ser convertido em combustível tanto por rotas termoquímicas quanto por rotas fermentativas. Os métodos termoquímicos envolvem a gaseificação de materiais celulósicos para produzir um gás de síntese (mistura de CO e H_2), que a partir de um novo processo pode ser cataliticamente convertido a vários tipos de combustíveis, tais como, metanol, etanol ou éter dimetil (EDM). Na rota fermentativa os carboidratos presentes nos materiais lignocelulósicos são fermentados a combustíveis, tais como, etanol, butanol e biogás, por meio de micro-organismos (GALBE; ZACCHI, 2012).

Conversão da lignocelulose a açúcares fermentescíveis usando fungos termofílicos e enzimas termoestáveis

Micro-organismos que crescem em altas temperaturas podem ser classificados como termotolerantes (temperatura ótima de crescimento em torno de 37ºC, mas tolerante a temperaturas mais altas), termofílicos (temperatura ótima de crescimento em torno de 45ºC) e hipertermofílicos (temperatura ótima de crescimento em torno de 80ºC) (MADIGAN et al., 2003). Poucas espécies de eucariotos conseguem crescer entre 45 e 50°C. Somente aproximadamente 30 espécies de fungos, entre as 50.000 descritas, crescem em temperaturas entre 40 e 45°C, embora existam algumas espécies com capacidade de crescer entre 60 a 62°C (MAHESHWARI et al., 2000).

Micro-organismos termofílicos são de grande interesse científico, pois servem como modelos para estudos de mecanismos fisiológicos adaptativos e evolutivos, além de apresentar um grande potencial para aplicações biotecnológicas, como a produção de enzimas termoestáveis as quais têm sido estudadas com vistas à sua aplicação em diversos processos industriais (MARTINS et al., 2002).

Enzimas produzidas por fungos termofílicos apresentam várias propriedades importantes. O uso de enzimas termofílicas e termoestáveis (termozimas) permite que os bioprocessos sejam realizados em temperaturas mais elevadas, conferindo a estes propriedades desejáveis, como maior fluidez do meio, operação em maiores concentrações de produtos e substratos, e diminuição do risco de contaminação por micro-organismos mesofílicos. Além de termo-estabilidade, as termozimas são geralmente mais resistentes a detergentes e a enzimas proteolíticas, além de serem estáveis em amplas faixas de pH, podendo ser usadas em diferentes tipos de materiais e processos (GOMES et al., 2007).

Celulases

A completa hidrólise da celulose a glicose requer a ação combinada de múltiplas enzimas com diferentes especificidades ao substrato (Figura 13) (ARO et al., 2005).

As *endo-(1,4)-β-D-glucanases* (EC 3.2.1.4) hidrolisam ligações internas (β-1,4), preferencialmente nas regiões amorfas que são mais suscetíveis, reduzindo o grau de polimerização deste substrato, produzindo terminais reduzidos e não-reduzidos. Sua atuação expõe as microfibrilas ao ataque subseqüente de outras enzimas, além de aumentar o número de oligossacarídeos com terminações susceptíveis ao ataque das exoglucanases (MAHESHWARI et al., 2000; MARTINS et al., 2008; FUKUDA et al., 2009).

As *exo-(1,4)-β-D-glucanases* (EC 3.2.1.91) tem ação de exoglucanase ao remover monômeros ou dímeros das porções terminais das cadeias. A avicelase possui um sistema de celulases formado por avicelase I, que se caracteriza por ser uma enzima rara que combina as atividades de exoglucanase e endoglucanase, e por avicelase II que representa um novo tipo de atividade celodextrinohidrolase (RIEDEL et al., 1997).

As *β-glucosidades* (EC 3.2.1.21) hidrolisam celobioses e, em alguns casos, outros oligossacarídeos curtos a glicose (MAHESHWARI et al., 2000; MARTINS et al., 2008; FUKUDA et al., 2009).

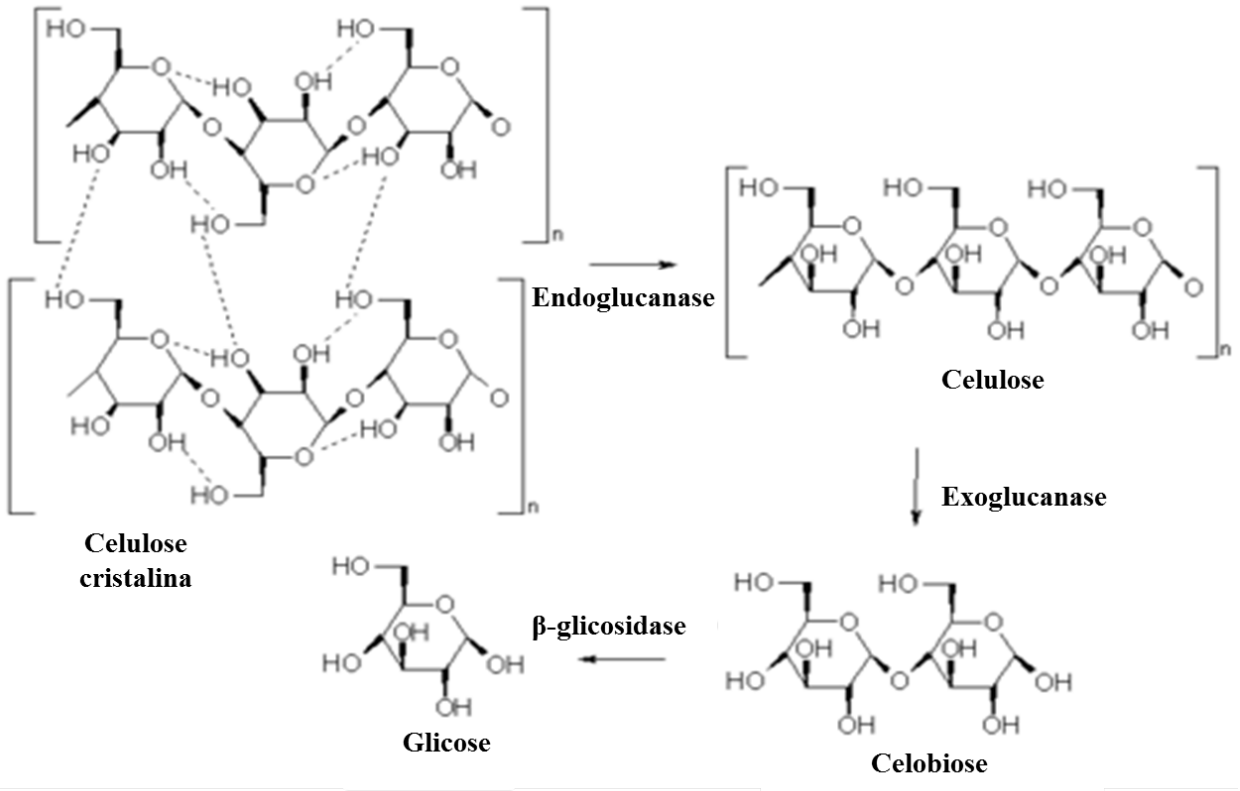

Figura 13: Representação esquemática de um sistema celulolítico.

Hemicelulases

Após a celulose, a hemicelulose é um dos mais abundantes polissacarídeos da natureza, sendo sua maior fração constituída por xilana. A completa degradação da hemicelulose requer ação sinergística de uma variedade de enzimas hidrolíticas, entre as quais se destacam as do complexo xilanolítico (Figura 14):

As *endo-1,4-β-D-xylanases* (EC 3.2.1.8) atacam internamente a estrutura polissacarídica, hidrolisando as ligações β-1,4 da molécula de xilana, resultando em um decréscimo no grau de polimerização do substrato.

As *β-xilosidases* (EC 3.2.1.37) hidrolisam xilooligossacarídios curtos a xilose.

As *α-L-arabinofuranosidases* (EC 3.2.1.55) hidrolisam os grupos α-L-arabinofuranosil terminais.

As *α-glucoronidases* (EC 3.2.1.1) são requeridas para a hidrólise das ligações α-1,2 glicosídicas entre xilose e ácido glicurônico ou sua ligação 4-*O*-metil-ester.

As *acetil xilana esterases* (EC 3.1.1.72) hidrolisam as ligações entre xilose e ácido acético e ácido ferrúlico.

As *feruloil esterases* (EC 3.1.1.73) (ácido ferúlico esterases, cinnamoil esterases, ácido cinnamico hidrolases, *p*-coumaroil esterases, hidroxicinnamoil esterases), apresentam um papel chave proporcionando um aumento na acessibilidade das enzimas hidrolíticas sobre as fibras de hemicelulose devido à remoção do ácido ferúlico das cadeias laterais e ligações cruzadas (PAPINUTTI; FORCHIASSI, 2007; WONG; SADDLER, 1988; WONG, 2006). A ferruloil esterase quebra as ligações éster entre os ácidos hidroxicinâmicos esterificados em arabinoxilana e certas pectinas presentes na parede celular da planta. Todas as enzimas (exceto a *p*-cumaril esterase de *A. awamori*) são ativas sobre ferrulato metil, que é um substrato sintético comumente usado nos ensaios de ferruloil esterase (VRIES et al., 1997).

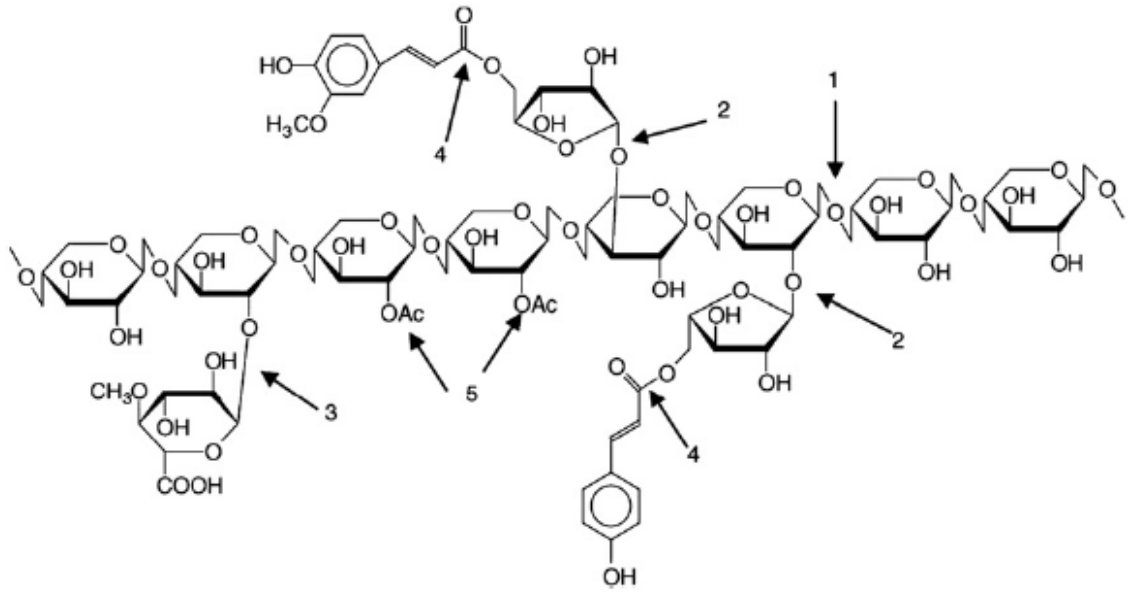

Figura 14: Estrutura da xilana e sítios de ações das enzimas do complexo hemicelulolítico. 1: endoxilanase, 2: α-L-arabinofuranosidases, 3: glucuronidases, 4: feruloil e coumaroil esterases, 5: xilana acetil esterase (CHÁVEZ et al., 2006).

Uso de resíduos agroindustriais como substratos na fermentação em estado sólido para produção de enzimas microbianas

Além do entendimento da ação enzimática sobre os substratos de difícil degradação, outro desafio a ser vencido pelos pesquisadores é a extensa busca por cepas produtoras de complexos celulolíticos e hemicelulolíticos muito ativos e estáveis, e principalmente, produtores dessas enzimas em processos eficientes e de baixo custo.

A produção de enzimas pelos micro-organismos difere de acordo com a natureza e a fisiologia dos mesmos. Por outro lado, as condições de cultivo (pH, temperatura, aeração, agitação, fontes de carbono e nitrogênio, tempo de incubação e tamanho das partículas dos substratos utilizados na fermentação) também influenciam no potencial de expressão e secreção das enzimas, alterando o rendimento na produção (LAKSHMI et al., 2009). Portanto, estudos envolvendo as condições de produção das enzimas por cada cepa em particular, podem modificar o perfil e quantidade dos complexos enzimáticos obtidos.

A fermentação em estado sólido (FES) é um processo interessante para produção de lignocelulases de maneira econômica, devido a possibilidade de usar resíduos agrícolas e agroindustriais como substrato para o crescimento microbiano (GAO et al., 2008; GRAMINHA et al., 2008). Apesar de alguns obstáculos, o cultivo de micro-organismos em FES oferece vantagens, tais como: (i) à alta produtividade; (ii) menor requerimento de espaço e energia; (iii)

problemas operacionais escassos; (iv) tecnologia simples e de baixo custo (PANDEY et al., 2000).

Os fungos, devido às suas características de reprodução e crescimento, adaptam-se a uma grande variedade de substratos, sendo decompositores naturais de material vegetal pois podem secretar uma grande variedade de enzimas, as quais têm sido produzidas e aplicadas industrialmente (GOMES et al., 1998). Assim, vários co-produtos agrícolas e agroindustriais são usados em processos de FES para a produção de celulases (GAO et al., 2008; KANG et al., 2004), xilanases (KANG et al., 2004), pectinases e ligninases (PAPINUTTI; FORCHIASSIN, 2007) e pectinases (COUTO; SANROMÁM, 2006), por fungos mesofílicos e termofílicos (Tabela 1).

Tabela 1. Produção de enzimas lignocelulolíticas por diferentes fungos, utilizando-se resíduos lignocelulósicos como substrato em FES.

Substrato(s)	Microrganismo(s)	Enzima(s)	Referência(s)
Farelo de soja	*Fomes sclerodemeus*	Endoglucanases Endoxilanases Lacase Poligalacturonase Manganês peroxidase	PAPINUTTI; FORCHIASSIN, 2007
Resíduos de palmeira	*Aspergillus terreus*	Xilanase	LAKSHMI et al., 2009
Palha de arroz	*Aspergillus fumigatus* *Myceliophthora* sp	Papel de filtro (FPase) Endoglucanase β-glucosidade β-xilosidase Xilanase	LIU et al., 2011 BADHAN et al., 2007
Bagaço de cana	*Penicillium janthinellum* *Trichoderma viride* *Aspergillus nidulans*	Papel de filtro (FPase) β-glucosidase Endoglucanase Xilanase	ADSUL et al., 2004 JABASINGH; NACHIYAR, 2011
Farelo de trigo	*Fomes sclerodemeus* *Aspergillus niger* *Trichoderma reesei* *Thermoascus aurantiacus* *Aureobasidium pullulans*	Endoglucanases Endoxilanases Lacase Poligalacturonase Manganês peroxidase Papel de filtro (FPase) β-glucosidade β-xilosidase Xilanase β-celobiohidrolase	PAPINUTTI; FORCHIASSIN, 2007 KANG et al., 2004 SUKUMARAN et al., 2008 HANIF et al., 2004 LEITE et al., 2008
Resíduos de Banana	*Pleurotus ostreatus* *Pleurotus sajor-caju*	Lacase Lignina peroxidase Endoglucanase Exoglucanase	REDDY et al., 2003

Prétratamento da biomassa lignocelulósica

O alvo da tecnologia de prétratamento é alterar ou remover impedimentos estruturais ou composicionais para melhorar as taxas de hidrólise e aumentar os rendimentos de açúcares fermentescíveis a partir da celulose ou hemicelulose (MOSIER et al., 2005). Assim, o prétratamento é necessário para alterar a estrutura da biomassa celulósica tornando-a mais acessível para as enzimas que atuam na conversão de carboidratos em açúcares fermentescíveis e para micro-organismos (PATEL; ONKARAPPA, 2007) (Figura 15). Um prétratamento eficiente deve reunir os seguintes requisitos (PHITSUWAN et al., 2013):

- Evitar a degradação de carboidratos: O principal foco da degradação da lignocelulose sempre foi aumentar a exposição das fibras de celulose. Por consequência, vários processos de prétratamento foram sugeridos, tais como, os tratamentos ácidos que visam a remoção da fração de hemicelulose. A função dos prétratamentos tem-se voltado para a máxima retenção da fração de polissacárido de modo a obter um melhor rendimento de açúcares totais.
- Ter custo efetivo: Alguns prétratamentos, tais como os químicos, necessitam que a biomassa lignocelulósica seja submetida à redução das partículas, sendo precedido por uma etapa de tratamento mecânico, o que gera um substancial custo de energia. Além disso, os prétratamentos que utilizam substâncias químicas devem oferecer a capacidade de reciclagem dos produtos utilizados.
- Alteração estrutural da lignina: Segundo VARNAI et al. (2010) a completa deslignificação da lignina não parece ser essencial no alcance de bons resultados de hidrólise. Alguns prétratamentos simplesmente alteram a localização da lignina, ou seja, não promovem a remoção dessa fração, e esse comportamento pode aumentar os rendimentos de hidrólise. Além disso, segundo ISHIZAWA et al. (2009) a digestibilidade da celulose na hidrólise pode ser reduzida quando o material lignocelulósico passou por um processo de severa deslignificação.
- Evitar a formação de co-produtos inibitórios: Várias condições de prétratamento geram compostos tóxicos que afetam a subsequente etapa de fermentação e hidrólise. A natureza e a concentração dos compostos tóxicos dependem da matéria prima, do prétratamento usado e das condições empregadas. De acordo com a origem, os produtos de degradação podem ser divididos em três grupos: derivados de furanos, tais como, 2-furaldeído e 5-hidroximetilfurfural; ácidos fracos, tais como, ácido acético e fórmico; além de compostos fenólicos. A degradação da lignina durante o pré-tratamento gera uma grande quantidade de compostos fenólicos, monômeros com diferentes grupos funcionais: aldeídos, cetonas e ácidos (TOMÁS-PEJÓ et al., 2010).

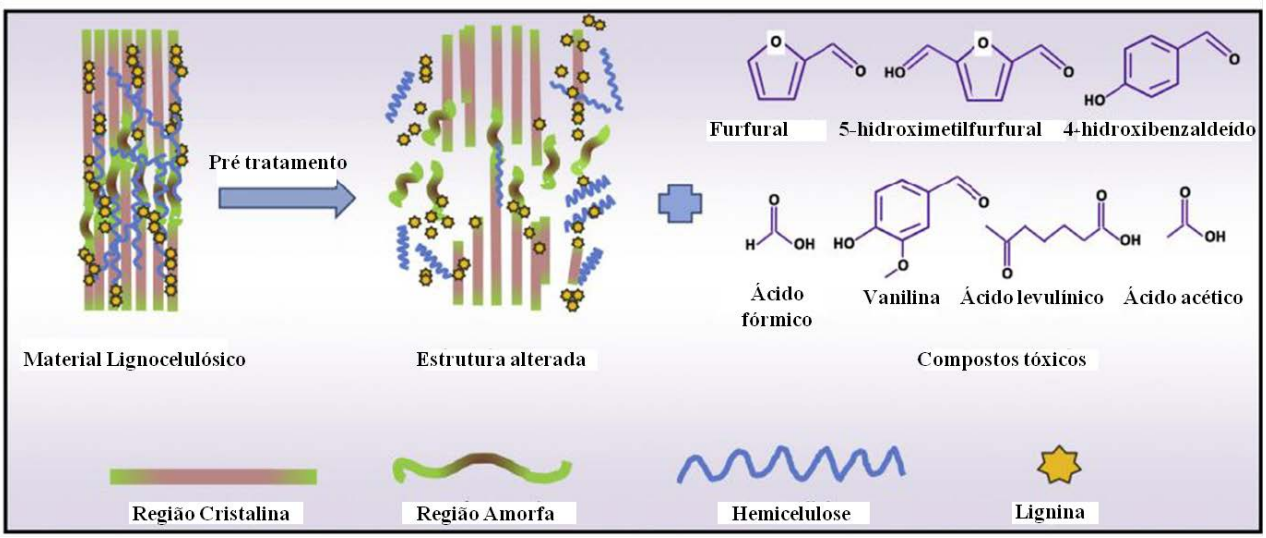

Figura 15: Representação esquemática da ação do pretratamento sobre o material lignocelulósico (PHITSUWAN et al., 2013).

Uma variedade de métodos biológicos, físicos, químicos e físico-químicos tem sido empregados em processos de prétratamentos de resíduos lignocelulósicos e sua eficácia técnica e econômica tem sido avaliada. O relativo sucesso de cada método depende da eficiência com que o material prétratado é transformado, e no caso de aplicações específicas tais como bioconversão, a dimensão para com a hidrólise enzimática é melhorada (RAMOS, 2003).

Prétratamento Biológico

O prétratamento biológico resulta na delignificação parcial da lignocelulose usando micro-organismos decompositores de lignina, tais como, fungos e bactérias (RAMOS, 2003). Até o momento as maiores eficiências na degradação da lignina foram obtidas por fungos de podridão branca, degradadores de lignina e fungos marrom, que atacam somente a celulose. O fungo de podridão branca mais eficiente até o momento é o *Phanerochaete chrysosporium,* o qual apresenta alta taxa de crescimento e elevada capacidade de biodegradação da lignina (MOOD et al., 2013). Entretando, o prétratamento biológico é um processo que somente se torna custo efetivo se aplicado em conjunto a outros métodos mecânicos ou físicos (RAMOS, 2003). Neste processo, o tamanho da partícula, o teor de umidade, o tempo de tratamento e a temperatura de incubação, podem afetar consideravelmente a degradação da lignina. Apesar do baixo consumo de energia, das brandas condições ambientais e de não envolver substâncias químicas, o prétratamento biológico ainda enfrenta alguns gargalos com relação a sua aplicação comercial. Isso se deve ao longo tempo de processamento, exigência de amplo espaço e a

necessidade de monitoramento contínuo do crescimento do micro-organismo (MOOD et al., 2013).

Prétratamento Químico

O prétratamento químico visa à solubilização da hemicelulose e lignina para exposição do componente de celulose à hidrólise acida ou enzimática. Uma ampla variedade de substâncias químicas tem sido sugerida na literatura, entre elas hidróxido de sódio, dióxido de sulfúrico, amônia aquosa, hidróxido de cálcio, carbonato de cálcio, ácido fosfórico, peróxido de hidrogênio alcalino, sais inorgânicos com propriedades ácidas, sais amônio, ácido acético, ácido fórmico, ácido sulfúrico, *n*-butilamina, *n*-propilamina e álcoois (metanol, etanol, butanol) na presença de um catalisador ácido ou alcalino (RAMOS, 2003).

Prétratamento ácido: O ácido sulfúrico é o reagente químico mais comumente utilizado para prétratar os polissacarídeos presentes na biomassa lignocelulósica, principalmente a hemicelulose. Prétratamento com ácido pode ser realizado sob baixa concentração de ácido e temperatura elevada ou sob concentração mais elevada de ácido e temperatura mais baixa (MOOD et al., 2013). Entretanto, a utilização de elevada concentração de ácido aumenta a formação de compostos inibitórios, além de problemas de corrosão dos equipamentos usados no processo. Sendo assim, o prétratamento com ácido diluído e temperatura elevada parece ser o método mais favorável para aplicação industrial quando comparado ao uso de ácido concentrado. Neste contexto, o prétratamento com ácido diluído tem sido apontado para produção de bioetanol em larga escala. Este método pode ser aplicado a altas temperaturas (180 ºC), por curto período de tempo, ou a baixas temperaturas (120 ºC), por um longo período de tempo (30-90 min). Dependendo da temperatura do processo, alguns compostos oriundos da degradação dos açúcares, tais como, furfural, 5-hidroximetilfurfural e compostos aromáticos provenientes da degradação da lignina são detectados. De qualquer forma, o prétratamento com ácido diluído gera menos produtos de degradação comparado ao uso de ácido concentrado (TOMÁS-PEJÓ et al., 2010).

Prétratamento alcalino: Os prétratamentos usando NaOH são processos químicos eficazes no tratamento do material lignocelulósico. O NaOH remove principalmente a lignina e hemicelulose presentes na biomassa, uma vez que, atua na clivagem das ligações ésteres promovendo o aumento da porosidade da biomassa (CAO et al., 2012). Além disso, este método também é conhecido por causar cavidades na fração de cellulose, uma vez que, as reações de saponificação que ocorrem, levam a ruptura das ligações cruzadas entre a hemicelulose e os demais componentes da lignocelulose, e por consequência, o aumento da porosidade da

biomassa. Comparado a outros pré-tratamentos, o alcalino é operado em temperaturas mais baixas, sendo também desnecessário o uso de reatores complexos. Por outro lado, apresenta a desvantagem no longo tempo de operação (horas ou dias) e a necessidade de neutralização da suspensão prétratada. Hidróxido de sódio, hidróxido de potássio, hidróxido de cálcio e peróxido alcalino são os produtos químicos mais comumente usados nos processos alcalinos (MOOD et al., 2013). Além disso, no caso do prétratamento realizado com peróxido alcalino não foram encontrados os compostos tóxicos, furfural e hidroximetilfurfural, que são prejudiciais para a levedura. Isso indica que é mais fácil para a levedura fermentar o hidrolisado proveniente de determinados pretratamentos alcalinos em comparação com o ácido diluído (TAHERZADEH; KARIMI, 2008).

Prétratamento com organosolvente: O solvente orgânico primeiramente impregna-se no tecido vegetal e faz com que haja solubilização dos fragmentos de lignina produzidos. Uma parte considerável de lignina é separada da polpa; a celulose liberada é facilmente hidrolisada pelas enzimas celulolíticas produzindo glicose (ARAQUE et al., 2008). O uso de solventes orgânicos em conjunto com catalisadores ácidos inorgânicos (HCl, H_2SO_4, etc) leva à quebra das ligações internas da lignina e da hemicelulose. Processos "organosolv" que empregam agentes deslignificantes como NaOH ou Na_2SO_3 também tem sido citados na literatura (RUZENE et al., 2007). O glicerol, um solvente orgânico de alto ponto de ebulição, é atualmente um dos principais subprodutos da indústria oleoquímica. Com o aumento do preço do petróleo e as preocupações com a geração de fontes de energia alternativas, tais indústrias tem prosperado, especialmente aquelas produtoras de biodiesel, elevando a geração de glicerol. Apesar do baixo custo deste produto, poucas pesquisas têm explorado seu uso no pré-tratamento de materiais lignocelulósicos, o que pode eficientemente promover a deslignificação com baixa degradação da celulose (KÜCÜK; DEMIRBAS, 1993; DEMIRBAS, 1998).

Prétratamento Físico

Os prétratamentos físicos, tais como, moagem, microondas e irradiação, também tem sido utilizados a fim de favorecer a hidrólise dos materiais lignocelulósicos. Contudo, a maior desvantagem destes métodos é o alto requerimento de energia. A moagem geralmente resulta na redução do tamanho da partícula do substrato (aumentando a área de superfície disponível) e diminuição da cristalinidade e do grau de polimerização da celulose (RAMOS, 2003).

Micro-ondas: A utilização de micro-ondas é um método alternativo para o aquecimento convencional. Comparado com o aquecimento por condução ou convecção, o qual é baseado na transferência superficial de calor, o método de aquecimento por micro-ondas usa a habilidade de

interação direta entre um objeto e um campo eletromagnético para gerar calor. Quando as micro-ondas são aplicadas à biomassa lignocelulósicos, elas seletivamente aquecem a parte mais polar, criando um ponto de aquecimento com os materiais não homogêneos. Acredita-se que esta característica resulta em um efeito de explosão entre as partículas, melhorando a desestruturação das porções recalcitrantes da lignocelulose. Além disso, o campo eletromagnético empregado pode criar efeitos não-termais que podem também facilitar a desintegração das estruturas mais cristalinas do material (DE LA HOZ et al., 2005).

O pré-tratamento com micro-ondas apresenta um resultado positivo na digestão da biomassa principalmente quando é associado a substâncias químicas. Aqueles que utilizam soluções ácidas hidrolisam as frações da hemicelulose, enquanto deixam as porções celulose e lignina intactas no sólido residual. Contudo, a utilização de soluções alcalinas tem mais efeito sobre os componentes de lignina e ambas celulose e hemicelulose permanecem intactas (GRAY et al., 2006).

Hidrólise enzimática da biomassa lignocelulósica

A complexidade dos substratos lignocelulósicos requer inúmeras enzimas trabalhando em sinergismo para promover a hidrólise. A hidrólise enzimática da biomassa lignocelulósica é realizada por enzimas altamente específicas, tais como, celulases, hemicelulases e ligninases (SUN; CHENG, 2002). Entretanto, durante o processo de hidrólise surgem alguns fatores que influenciam substancialmente o processo, sendo estes divididos em dois grupos: fatores relacionados às enzimas e aos substratos, embora esses estejam interligados durante o processo de hidrólise (ALVIRA et al., 2010). A variação da estrutura entre os substratos de diferentes fontes, os efeitos de diferentes tipos de prétratamentos e a dosagem e eficiência das enzimas usadas no processo, aumentam substancialmente a complexidade da hidrólise. (VAN DYK; PLESTCHKE, 2012).

A hidrólise enzimática do material lignocelulósico é um processo muito lento e a susceptibilidade do substrato às celulases depende de suas características estruturais tais como, cristalinidade e grau de polimerização da celulose, área de superfície e conteúdo de lignina (SUN; CHENG, 2002, ALVIRA et al., 2010).

- *Cristalinidade da celulose:* A celulose cristalina é considerada um fator determinante na taxa de hidrólise de substratos celulósicos. Entretanto, existem alguns complexos celulolíticos capazes de hidrolisar a celulose cristalina. Alguns prétratamentos melhoram as taxas de hidrólise em materiais lignocelulósicos; entretando, em alguns casos, estes

aumentam o índice de cristalinidade da fração de celulose. Este aumento tem sido apontado como sendo a remoção ou redução das frações amorfas da celulose após o tratamento.

- *Grau de polimerização da celulose*: Embora o papel do comprimento da cadeia de celulose não ser definitivamente conhecido, acredita-se que este afeta a hidrólise da celulose. E a despolimerização depende principalmente da natureza do substrato. Na hidrólise enzimática, a endoglucanase quebra ligações internas das cadeias de celulose, sendo responsável pela diminuição do grau de polimerização da celulose.
- *Área de superfície*: A acessibilidade das enzimas celulolíticas aos substratos é um dos principais fatores que influenciam a hidrólise enzimática. Alguns métodos de prétratamento são utilizados visando aumentar a superficie dos materiais lignocelulósicos ao ataque das enzimas.
- *Conteúdo de lignina*: A presença de lignina dificulta o acesso das celulases à celulose, agindo como uma barreira física, e assim, reduz a eficiência da hidrólise.

Durante a hidrólise enzimática dos substratos celulósicos surgem vários fatores que restringem a atividade catalítica das enzimas. Uma das dificuldades encontradas está relacionada com a reciclagem das celulases, pois as características de adsorção das celulases sobre os substratos lignocelulósicos, ainda é um processo não totalmente compreendido (LU et al., 2002). A degradação enzimática da celulose sólida é um processo complicado que se realiza a um limite de fase sólido-líquida, onde as enzimas são os componentes móveis. Quando as celulases agem *in vitro* sobre o substrato celulósico insolúvel, três processos ocorrem simultaneamente: (a) mudanças físicas e químicas no restante (ainda não solubilizado) da celulose em fase sólida, (b) hidrólise primária, envolvendo a liberação de intermediários solúveis da superfície das moléculas de celulose reagindo e (c) hidrólise secundária, envolvendo a hidrólise de intermediários solúveis para intermediários de baixo peso molecular, e por último para glicose (MOSIER et al., 2002).

Em geral, a taxa de hidrólise enzimática do material celulósico diminui rapidamente, sendo a degradação enzimática da celulose caracterizada por uma rápida fase inicial seguida por uma lenta fase secundária, que pode durar até que todo o substrato seja consumido. Isto tem sido explicado muito frequentemente pela rápida hidrólise da fração celulósica facilmente acessível, forte inibição pelo produto e lenta inativação de moléculas de enzima assimiladas adsorvidas (BALAT et al., 2008).

O investimento aplicado na hidrólise enzimática é baixo se comparado à hidrólise ácida, uma vez que é frequentemente conduzida sob condições moderadas (pH 5,0 e 45-70 ºC), sem problemas de corrosão. Além disso, os recentes métodos utilizando tecnologias modernas têm reduzido substancialmente os custos de produção das enzimas (KOVÁCS et al., 2009). Desse

modo, o presente trabalho teve por objetivo geral produzir enzimas do complexo hemicelulolítico usando fungo termofílico e utilizá-las para hidrolisar o bagaço e a palha de cana de açúcar antes e após tratamento com microondas e avaliar o nível de alterações químicas e morfológicas das fibras.

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Capítulo II

Objetivos

Conforme exposto, a recalcitrância do material lignocelulósico impõe barreiras ao aproveitamento dos açúcares retidos em sua estrutura. Desse modo, o presente projeto propôs estudar a produção de celulases, xilanases, ligninases, pelo microrganismo *Myceliophthora thermophila* M.7.7, que se mostradou um promissor produtor de enzimas fibrolíticas (Moretti 2010), por meio de técnicas de fermentação em estado sólido, utilizando resíduos agro-industriais de baixo custo avaliando-se o efeito de diversas condições físico-químicas no rendimento da produção das enzimas, buscando o desenvolvimento de métodos de produção de enzimas que sejam viáveis industrialmente. Propôs-se ainda, a aplicação das enzimas na sacarificação do bagaço e palha de cana-de-açúcar com e sem tratamento por micro-ondas. Com a finalidade de conhecer potencial de acesso das enzimas às fibras, foram realizados estudos de microscopia eletrônica do bagaço e palha pré-tratados e após serem submetidos à ação das enzimas. Com essas técnicas, pretendeu-se responder a questões que tem surgido ao longo das pesquisas do porque do baixo potencial de hidrólise do bagaço mesmo em presença de complexos ligno-celulolíticos altamente ativos.

Objetivos gerais

Desse modo, o presente trabalho teve por objetivo geral produzir enzimas do complexo hemicelulolítico usando fungo termofílico e utilizá-las para hidrolisar o bagaço e a palha de cana de açúcar antes e após tratamento com microondas e avaliar o nível de alterações químicas e morfológicas das fibras.

Objetivos específicos:

- o cultivo do fungo *Myceliophthora thermophila* M.7.7 por FES em diferentes condições físicoquímicas como, temperatura, umidade, fontes de nitrogênio e pH, e com variados substratos sólidos como fontes de carbono, visando maior rendimento na obtenção de celulases, xilanases, pectinases e ligninases;
- a aplicação de pré-tratamento físico (microondas) e hidrólise enzimática para desestruturação da parede celular do bagaço e da palha de cana de açúcar,
- a caracterização química do hidrolisado líquido obtido por tratamento do bagaço, de modo a se conhecer os componentes glicídicos, derivados dos polissacarídeos, e aromáticos derivados da lignina;
- a caracterização morfológica do material lignocelulósico tratado e não tratado por ambos os métodos usando técnica de microscopia eletrônica.

Capítulo III

Prodution of fibrolytic enzyme by Myceliophthora thermophila M.7.7. using inexpensive carbon sources and mineral nutrients

Production of fibrolytic enzyme by *Myceliophthora thermophila* M.7.7. using inexpensive carbon sources and mineral nutrients

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Abstract

This study investigated the effect of non expensive carbon and nitrogen sources on enzyme production by *Myceliophthora thermophila* M.7.7 in solid-state fermentation. Three kinds of lignocellulosic wastes (corn straw, sugarcane bagasse and sugarcane straw) and six nitrogen sources (urea, calcium nitrate, analytical ammonium sulphate, yeast extract, agricultural fertilizer NPK 20-05-20 and fertilizing grade ammonium sulphate) were tested. Some physical-chemical parameters of the fermentation, such as temperature, initial pH and moisture content of the substrate on enzyme production, were evaluated. The maximum activities of xylanase (446.9 U/ml), endoglucanase (94.7 U/ml) and β-glucosidase (2.8 U/ml) were observed in a mixture of corn straw and wheat bran (1:1 w/w) as the carbon source using fertilizer grade ammonium sulphate as the nitrogen source. This production occurred for an incubation time of 96 h, at 40 ºC, with initial moisture content of 70% and pH 5.0. These results have significant interest since they could be used for the future production of enzymes in a low-cost industrial process.

Keywords: *Myceliophthora thermophila*, solid-state fermentation, xylanase, endoglucanase, β-glucosidase.

1.Introduction

The hydrolysis of cellulose and hemicellulose present in plant cell walls into glucose and xylose requires the cooperative action of complex enzymes of cellulase and xylanase groups, respectively (Soni *et al*., 2010; Panagiotous *et al*., 2003). These enzymes have great application potential in several biotechnological processes such as the bioconversion of biomass wastes to fermentable sugars (Fang *et al.*, 2010; Pal *et al*., 2013).

Enzyme production by filamentous fungi is technologically attractive and has advantages over bacteria and yeasts due to their ability to grow on solid substrates and secrete a higher quantity of extracellular enzyme. These properties make possible the use of agro-industrial residues as substrates in solid state fermentation (SSF) allowing the production of low-cost enzymes (Singh *et al*., 2009; Gao *et al*., 2008; Jecu, 2000). In the fermentation process, it is also important to consider the cost of nitrogen and other macronutrient sources (Su *et al*., 2011) because, it is very important to find alternatives to reduce the cost of these components in the culture media. The fermentation parameters such as pH, temperature, moisture, aeration and incubation time influence the expression and secretion of the enzymes significantly affecting the targeted product (Lakshmia *et al*., 2009).

The heat released by the microbial activity during the fermentation process causes heating of the system thus requiring cooling of the bioreactor. The use of thermophilic fungus in SSF has been very promising since they can adapt to variations in temperature during the process and do not require cooling (Gomes *et al*., 2009).

This study aimed to evaluate the conditions of xylanase and cellulase production making them industrially viable in terms of cost and performance. The strategy used was to cultivate the thermophilic fungus *Myceliophthora thermophila* M.7.7 in SSF using inexpensive agro-industrial waste as carbon sources and commercial agricultural fertilizer as nutrients sources. In addition, the effect of various physical-chemical conditions was evaluated on enzyme production.

2.Materials and methods

Microorganism and effect of temperature on fungal growth

The strain *Myceliophthora thermophila* M.7.7 used in this study was isolated from decaying sugarcane bagasse piles (Moretti *et al*., 2012). The culture was maintained on Sabouraud agar under water and mineral oil, at room-temperature (25 ± 2 °C) and under 20% glycerol at -80 °C.

In order to investigate the performance of the strain at various temperatures, mycelia from pure cultures were spotted on agar plates and incubated at 37, 40, 45, 50 and 55 °C. The diameters of the colonies were measured at 12 h intervals. All experiments were performed in triplicates.

Enzyme production under Solid State Fermentation (SSF) on different agricultural wastes and mineral nutrients

The effect of lignocellulosic wastes (mixtures of corn straw, sugarcane bagasse or straw with wheat bran, w/w 1:1) on enzyme production was studied. The dried substrates were ground to particles of 3 mm and 5 g of the substrate mixture was placed in polypropylene bags (size 12x20 cm).

The nitrogen sources urea, calcium nitrate, ammonium sulphate, yeast extract, agricultural fertilizer NPK (20:05:20 -Heringer) and ammonium sulphate fertilizer grade were tested. Additional, nutrient solutions were composed of (g/L) 3.0 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.5 $CaCl_2$ and Tween 80 (1 ml/L) , pH 5.0 and of a solution formulated with agricultural fertilizer containing (g/L) 10.0 ammonium sulphate, 3.0 Mono-ammonium phosphate (MAP) (9% nitrogen and 48% phosphorus) and 2.0 potassium chloride (Heringer), 0.5 $MgSO_4.7H_2O$, 0.5 $CaCl_2$ and Tween 80 .

The substrates containing nutrient solution were autoclaved at 121 ºC for 30 min. The inoculum consisted of $2x10^7$ spores/g substrate and the final moisture content of the medium was 80%. After inoculation, the material was incubated at 45 ºC for 10 days and samples were collected at 48 h intervals. Crude enzyme solutions were obtained using suspensions of the fermented material in 100 mL distilled water, stirred for 30 min, filtered. The filtrate was centrifuged at 10000 × *g* for 15 min at 10 ºC and the supernatant liquid was used as crude enzyme.

Enzyme assays

Xylanase and endoglucanase activities were assayed in reaction mixtures containing 0.1 mL of crude enzyme and 0.9 mL of sodium acetate buffer solution at 0.1 M, pH 5.0 containing xylan (birchwood) (10.0 g/L) or carboxymethylcellulose (CMC) (40.0 g/L), which were then incubated at 60 ºC for 10 min. The reducing sugars released were measured by the DNS method (Miller, 1959) at 540 nm and expressed as xylose and glucose equivalents, respectively. The enzyme activity was defined in International Units (U), as the amount of enzyme required to release 1 µmol of product per 1 minute in the assay conditions.

The β-glucosidase activity was determined according to Leite *et al*., (2008) in a reaction mixture composed of 0.050 mL of crude enzyme solution, 0.250 mL of sodium acetate buffer (0.1 M; pH 5.0) and 0.250 mL of 4-nitrophenyl-β-Dglucopyranoside (4 mM), (PNPG, Sigma) incubated at 60 ºC, for 10 min. The reaction was stopped by the addition of 2.0 mL of Na_2CO_3 (2 M). One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per 1 minute in the assay conditions.

Effect of incubation temperature, initial pH and moisture on enzyme production

To evaluate the effect of incubation temperature, the two substrate and nutrient supplements which had high enzyme activity in the previous assay were used. The inoculated substrates were incubated at 40, 45, 50 ºC for 10 days. In all the experiments, the pH and moisture content were maintained as previously described.

The effect of initial pH of the media on enzyme production was evaluated at pH 5.0, 5.5, 6.0, using a substrate with 80% of moisture under a temperature that allowed high enzyme activity, for 10 days. The effect of substrate moisture was studied for 60, 70 and 80% using the best substrate and nutrient conditions. For the enzyme extraction, 10 mL of distilled water (1:10 w/v) per each 1 g of fermented material was added.

Evaluation of the amount of extraction solution and use of buffer and surfactant in the enzyme extraction

The fermented material was mixed and divided into five equal parts. Three were used to evaluate the proportion of extraction solution (1:10, 1:20 and 1:30 w/v) using distilled water and two of them were used to evaluate the type of extraction solution (Tween 80 at 0.2 mL/L and sodium acetate buffer 0.1M, pH 5.0) at 1:10 (w/v) proportions.

SDS-PAGE and zymogram analysis to activity detection of xylanase, endoglucanase and β-glucosidase

Polyacrylamide gel (SDS–PAGE) 10% (w/v) was used for detection of protein bands from crude enzyme solution as described by Laemmli (1970). The molar mass of proteins, under denaturizing conditions, was determined with reference standard proteins (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad from 6.5 to 200 kDa). Protein bands were stained with silver nitrate.

For zymogram activities, the samples of crude enzymes from *M. thermophila* M.7.7. were mixed in the loading buffer (2% SDS (w/v), 87% glycerol, 0.1 M Tris-HCl buffer pH 8.8 and

bromophenol blue). After electrophoretic running, the gels were incubated for 30 min at 60 ºC in solutions containing xylan or carboxymethylcellulose for xylanase and endoglucanase, respectively. After incubation, the gels were stained with 0.1% Congo red solution under gentle shaking for 15 min at 25 °C. Subsequently, the gels were immersed in 1M NaCl solution until the appearance of clear bands on the red background. For better resolution of the bands, 0.1 M HCl solution was added.

For the β-glucosidase zymogram, after running, the gel was incubated for 10 min at 25 ºC in 0.2 M acetate buffer pH 5.0. Subsequently, the gel was incubated for 1 h at 60 ºC in 0.2 M acetate buffer pH 5.0 containing 0.1% esculin and 0.03% ferric chloride until the appearance of dark bands when the gel was dipped in 10% glucose solution to stop the reaction.

3.Results and Discussion

Effect of temperature on fungal growth

Myceliophthora thermophila M.7.7 showed maximum growth rate (7.5 cm colony diameter) at 45 °C on a solid medium after 72 hours of incubation. Colony diameters of 6.4 cm and 5.9 cm were obtained at 40 °C and 37 °C, respectively. These results confirm the thermophilic profile of the fungus (Figure 1). This evaluation is necessary because the growth response of the fungus on a solid medium may differ from that in a liquid medium and provides substantial guidance for the solid state fermentation process. This methodology has been used by other authors showing consistent results (Martin *et al*., 2010; Silva *et al*., 2005).

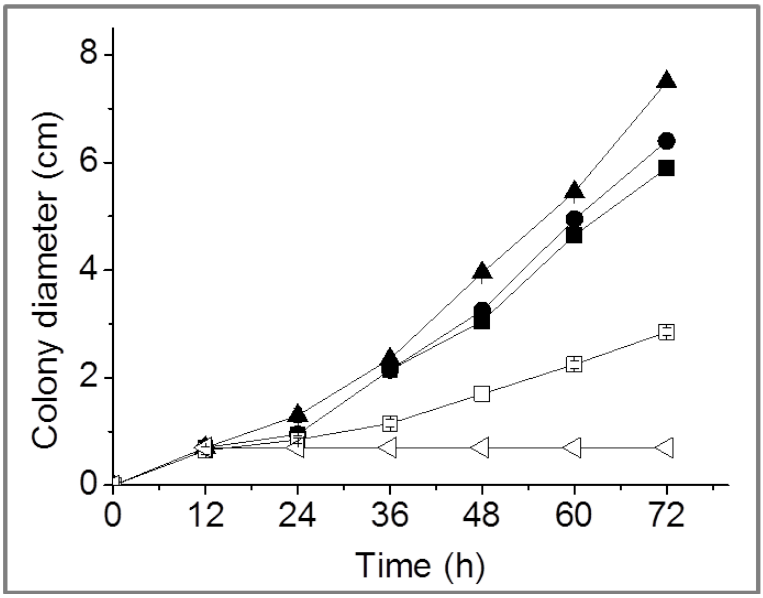

Figure 1: Growth curves of the fungus *Myceliophthora thermophila* M.7.7. In Petri dishes containing solid medium agar Saboroud, at different temperatures, for 72 h.

37ºC; 40ºC; 45ºC; 50ºC; 55 ºC.

Enzyme production using different agricultural wastes and mineral nutrients in Solid State Fermentation (SSF)

Corn straw with wheat bran was the best carbon source for xylanase and endoglucanase production by *M. thermophila* M.7.7 (120 and 40 U/mL respectively) (Figure 2C), while the mixture of sugar cane bagasse and wheat bran allowed the highest β- glucosidase production (Figure 2A). Badhan *et al*., (2007) obtained 62.0 U/ml of xylanase when using cultivated fungus *Myceliophthora* sp IMI 387099 in sugar cane bagasse but lower amounts of endoglucanase (0.7 U/ml) and β-glucosidase (0.2 U/ml).

In a medium with fertilizer grade ammonium sulphate and analytical ammonium sulphate, maximum xylanase and endoglucanase activity were observed (Figure 2A,B,C) and, when compared to other studies with fungus of the same genus, these values were higher. According to data from Badhan *et al*., (2007), the measured activities of xylanase, endoglucanase, and β-glucosidase were 90.0, 3.2 and 0.7 U/mL, respectively, when *Myceliophthora* sp IMI 387099 was cultivated on rice straw with addition of ammonium sulfate 0.3%.

The highest productions of β-glucosidase (4.1 and 3.5 U/mL) were obtained in the media containing yeast extract and urea, respectively. On the other hand, it is clear that the endoglucanase and xylanase production was negatively affected by supplementation with urea (Figure 2A,B,C). Similar behavior was observed by Kalogeris *et al*., (2003) when using cultivated thermophilic *Thermoascus aurantiacus*.

In this study, we can conclude that the corn straw with wheat bran was the best carbon source while ammonium sulfate was the most suitable nitrogen source for endoglucanase and xylanase production. Since there was no difference in enzyme production between the analytical grade and the fertilizer grade of the ammonium sulphate, we opted for the latter as the nitrogen source in the continuity of experiments considering the lower cost of input.

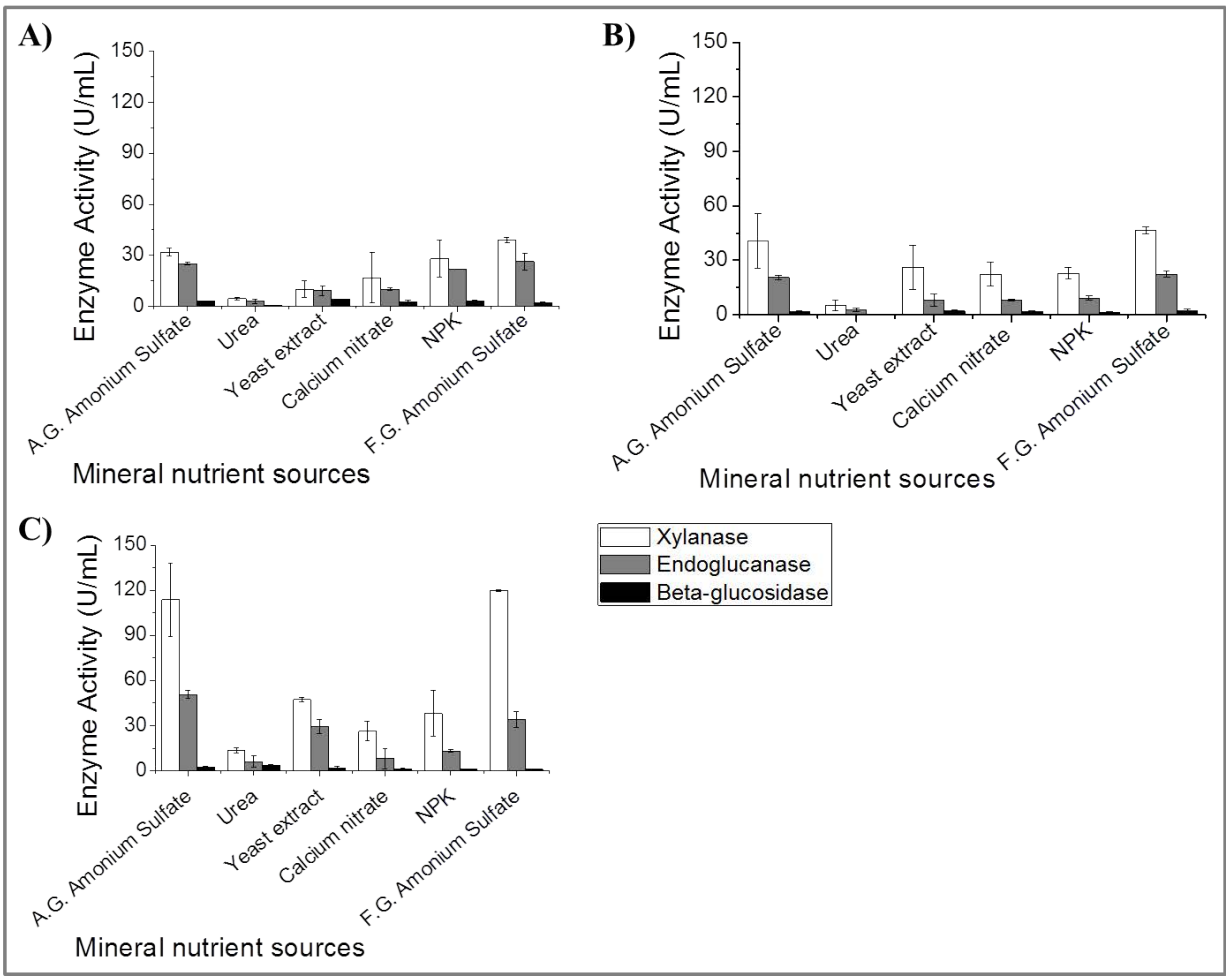

Figure 2: Maximum production of xylanase, endoglucanase and β-glucosidase by *M. thermophila* on **(A)** cane bagasse and wheat bran, **(B)** cane straw and wheat bran, **(C)** corn straw and wheat bran, and different mineral nutrient sources. The fermentations were carried out at 45 ºC, pH 5.0, 80% moisture along 240 h. Data are the averages of two assays. *Where: A.G.= analytical grade, F.G.=Fertilizer grade and NPK= 20:05:20.*

Evaluation of the amount of extraction solution and use of buffer and surfactant in the enzyme extraction

For enzyme extraction from the fermented solid substrate three different volumes of extraction solution were added. According to Figure 3 the ratio of fermented material to water of 1:10 resulted in the highest extraction of enzymes and there were no significant differences among the three volumes of extraction solution, with only slightly higher extraction occurring when Tween 80 was used. The specific activities (data not shown) confirm these results. Therefore, it was decided by using water as eluent extraction whereas the buffer and Tween 80 could interfere with the subsequent steps of the study of enzymes.

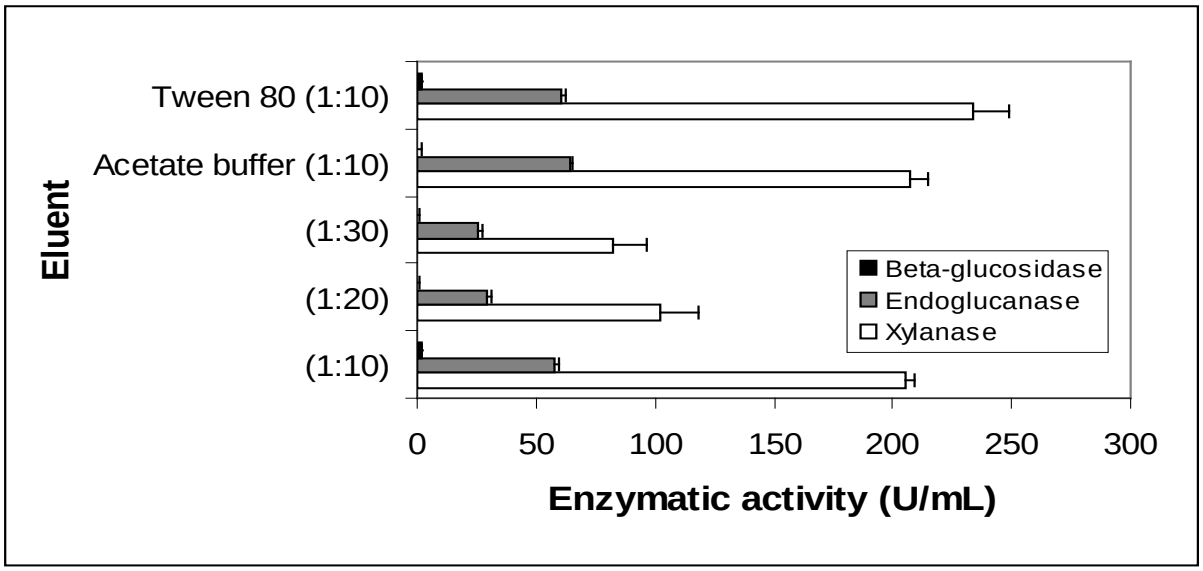

Figure 3: Effect of different eluents and their volume on the enzyme extraction: 1:10, 1:20, 1:30 (1 g of fermented material per volume of distilled water w/v), 1:10 sodium acetate buffer (0.1M, pH 5.0) and 1:10 Tween 80 (0.2 mL/L). The fermentation was carried out on corn straw and wheat bran (w/w 1:1) and a nutrient solution composed of analytical ammonium sulphate at 45 ºC, pH 5.0, 80% moisture and incubation for 96 h. Data are the averages of two assays.

Effect of incubation temperature on enzyme production

The maximum activity of xylanase (407.0 U/mL) was obtained when *M. thermophila* was grown at 40 °C (Figure 4A). When incubated at 45 °C and 50 °C there was a 50% reduction in the production of xylanase (Figure 4 B,C) while the endoglucanase production was little affected by temperature of incubation. The production of β-glucosidase peaked (3.6 U/mL) at 50 °C (Figure 4C). These data suggest that temperatures above 40ºC could be affecting the xylanase stability although the growth of fungus was higher at 45ºC and also indicated that cellulases were more thermostable then xylanase. Similar results were obtained by Roy *et al*., (1990) where there was a higher production of β-glucosidase (0.12 U / ml) when *M. thermophila* D14 was cultured at 50 °C.

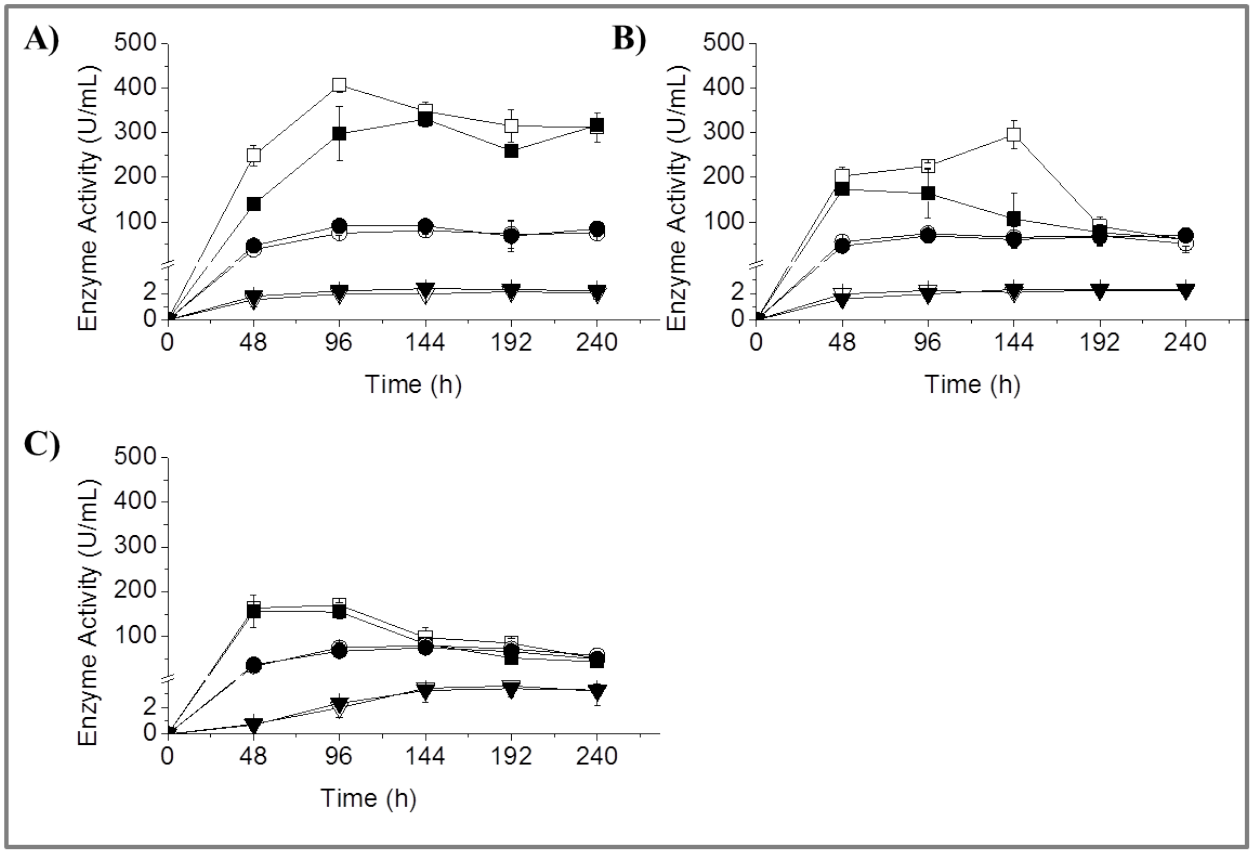

Figura 4: Effect of incubation temperature in SSF. At 40 °C **(A)** 45 °C **(B)** and 50 ºC **(C)** on production of xylanase (Square), endoglucanase (circle), and β-glucosidase (triangle) by *Myceliophthora thermophila* using a medium consisting of corn straw and wheat bran. The open symbol: analytical grade ammonium sulphate and fulled symbol: fertilizer grade ammonium sulphate.

Effect of initial pH and moisture on enzyme production

Figure 5A shows the effect of the pH of the culture medium on the production of cellulases and xylanases. There were no significant differences in the production of enzymes at the pH range tested, with less than 10% variation between the maximum and the minimum activity obtained in the different pHs tested, throughout the cultivation period of 240 h. These data corroborate other data reported in the literature such as those of Xiong *et al*., (2004) with xylanase production by *Trichoderma reesei* Rut C-30 , those of Shingh *et al*., (2009) with xylanase from *Coprinellus disseminatus* and those of Sohail *et al*., (2009) with endoglucanase from *Aspergillus niger* MS82.

The effect of substrate moisture on the production of enzymes by *M. thermophila* is shown in Figure 5B. The maximum xylanase activity (446.9 U/mL) was obtained on the substrate containing 70% moisture after 96 h of cultivation. When the moisture was 60% and 80%, there was a reduction in the xylanase activity (19% and 15%, respectively). The highest

endoglucanase (94.7 U/ml) and β-glucosidase (2.8 U/ml) activities were observed after 144 h and 240 h of fermentation respectively, with the same profile of xylanase, with a higher production at 70% moisture.

The strain *M. thermophila* M.7.7. showed itself to be quite stable under the effects of a wide range of pHs and moisture contents. This characteristic is very interesting for industrial application, since the control of pH and moisture during the solid-state fermentation are the most critical parameters to be controlled due to the heterogeneity and the consistency of the solid material normally used (Lonsane *et al*., 1985).

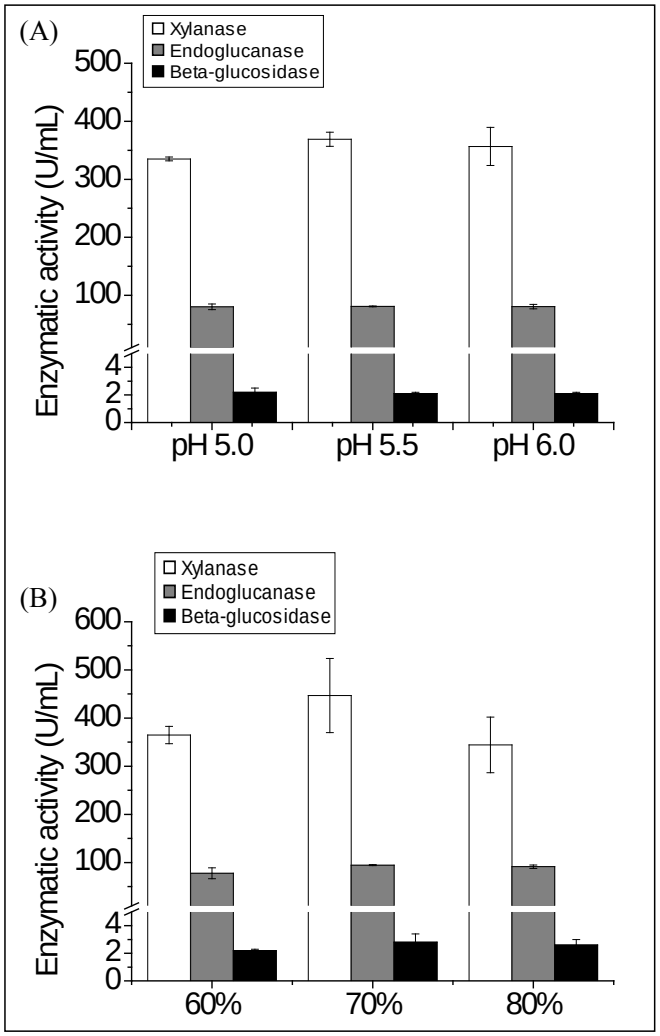

Figure 5: Xylanase, endoglucanase and β-glucosidase production by *M. thermophila* with different initial pH (A) and moisture content (B). The fermentation conditions were carried out on corn straw and wheat bran mixture (w/w 1:1) and a nutrient solution composed of fertilizer grade minerals and ammonium sulphate at 40 ºC, for 240 h. Data are the averages of two studies.

SDS-PAGE and zymogram analysis

The objective of this assay was to verify if the enzyme extract produced by *M. thermophila* M.7.7. on two culture media, using fertilizer grade (1) and analytical grade (2) ammonium sulphate exhibited similar profiles, since ions and metals contained in the first one could inhibit the expression or the activities of the enzymes.

As shown in Figure 6, line B revealed four isoform bands for endoglucanase, corresponding to about 38, 45, 97 and 166 kDa. For xylanase activity (line C) three active isoforms were observed with approximately 43, 60 and 100 kDa. β-glucosidase appeared in two bands corresponding to 50 and 200 kDa (line D). This similarity in the expression of enzymes in different culture media (1 and 2) shows that nutrient solutions formulated with fertilizer grade ammonium sulphate did not affect the enzymes expression or their activities.

The ability of *Myceliophthora thermophila* to produce xylanase and endoglucanse in media composed of inexpensive carbon sources and mineral nutrients has considerable commercial interest. Considering that the substitution of analytical chemical reagents for agricultural fertilizer in the cultivation of fungus reduces the cost of producing enzymes by approximately 99%. In addition, the fungus used in this study showed great stability in a wide range of temperatures, pHs and moisture contents providing a much easier approach to the fermentation process.

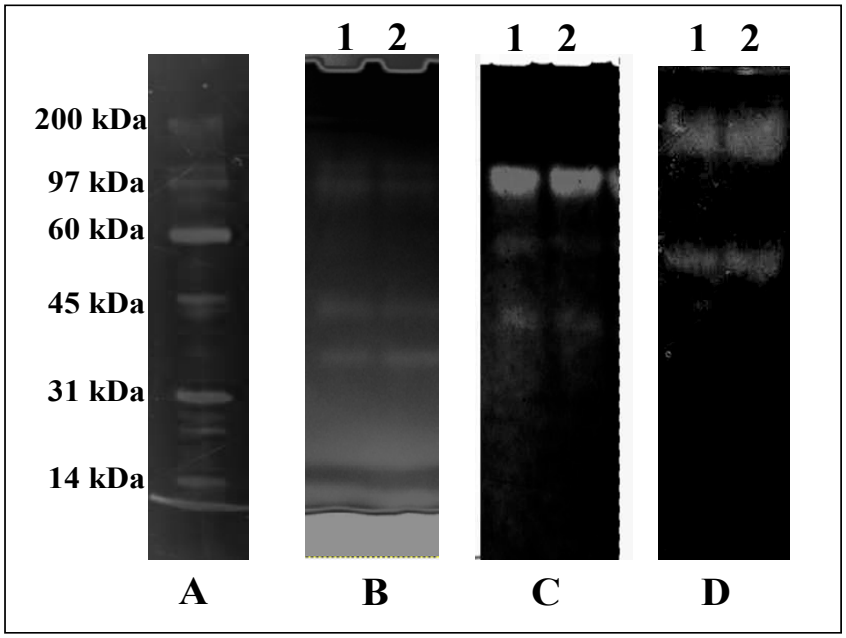

Figure 6: SDS-PAGE and zymogram analysis: (A) molecular weight markers, (B) crude endoglucanase, (C) crude xylanase, (D) crude β-glucosidase. The sample of crude enzymes from *M. thermophila* M.7.7. were obtained under SSF in: corn straw and wheat bran using two different nutrient solutions, fertilizer grade (1) and analytical grade (2) ammonium sulphate.

4.Conclusions

In this study, it was established that the best incubation time for enzyme production by the thermophilic fungus *M. thermophila* M.7.7 was 96 hours, at which time the maximum activity of xylanase (446.9 U / ml), endoglucanase (77.6 U / ml) and β-glucosidase (2.4 U / ml) was achieved. Corn straw and wheat bran (w/w 1:1) and fertilizer grade minerals can be successfully used as carbon and nitrogen sources. Temperature 40ºC, initial pH 5.0 and moisture content 70% afforded the highest enzyme production.

5.Acknowledgements

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Capítulo IV

Effect of pre-treatment on chemical composition, structure and enzymatic hydrolysis of bagasse and sugar cane straw

Effect of pre-treatment on chemical composition, structure and enzymatic hydrolysis of bagasse and sugar cane straw

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Abstract

The present work aimed to study the effect of pre-treatment of bagasse and sugar cane straw with microwave radiation in the presence of glycerol diluted in water, sulphuric acid and sodium hydroxide on the chemical composition, fiber structure and the efficiency of subsequent enzymatic hydrolysis. Bagasse and cane straw were subjected to 2 min of irradiation under the conditions mentioned and the solid fractions resulting were used in the analysis of fibers, TGA, DTG, DSC, FTIR, X-ray and transmission electron microscopy and the liquid fractions were used to determine sugar and phenol contents. Samples of bagasse and straw, treated and untreated (control) were submitted to enzymatic hydrolysis for 24 to 72 h at 55 °C with enzymatic solutions obtained by the cultivation of *M. thermophila* M.7.7. and Celluloclast 1.5L based on 60 U of endoglucanase activity per g of dry substrate or on the total amount of protein solutions (5 mg protein/g of dry substrate). The enzyme solution from *M. thermophila* M.7.7 was supplemented with a commercial β-glucosidase in order to achieve the same activity present in Celluloclast. Infrared spectra and thermal analysis showed that pre-treatment acted mainly on the lignin and hemicellulose of sugarcane bagasse, whereas in sugar cane straw lower structural and chemical changes were observed. After 24 hours of hydrolysis with the enzyme produced by *M. thermophila* M.7.7 supplemented with β-glucosidase, the highest yields of reducing sugars obtained from bagasse were 68.2 mg/g and with Celluloclast, were 74.0 mg/g from sugar cane straw using both the materials treated with microwave immersed in glycerol acidified with sulfuric acid. In hydrolysis assays for 72 h, using these same materials, the highest yield of reducing sugars was 304.7 mg/g for straw and 240.9 mg/g for bagasse when enzyme solution of *M. thermophila* M.7.7 was used, independent of supplementation with β-glucosidase or not,

indicating that supplementation with β-glucosidase did not significantly increase the yield of hydrolysis.

*Keywords***:** sugar cane bagasse, sugar cane straw, pre-treatment, microwave, saccharification

1.Introduction

The sugarcane bagasse and straw are the main by-products of the sugar and alcohol industry and are available in abundance in Brazil. This lignocellulose biomass is an important source of sugars for use in biotechnological processes for obtaining high added value products (Soares et al., 2011; Wanderley et al., 2013).

The conversion of lignocellulosic residues to ethanol is currently a topic of great interest around the world. Basically, this process consists of three steps: (i) pre-treatment of raw material for the reduction of lignin content and polysaccharide exposure; (ii) enzyme hydrolysis for the conversion of the polysaccharides into the monomers glucose and xylose; (iii) and fermenting the sugars to ethanol. The main technical and economic challenge in this process is the development of inexpensive pretreatment to improve the accessibility to the enzymes to cellulose without significant chemical changes (Mesa et al., 2011).

Several pre-treatment methods including physical, chemical, biological and combinations of these have been evaluated during recent decades, such as ionic liquid (Bian et al., 2013), hydrothermal (Ma et al., 2012), steam explosion, microwave radiation (Pang et al., 2013), organosolv (Mesa et al., 2011), in addition to acidic and alkaline treatments (Mesa et al., 2011).

The organosolv treatment is an effective technique in which lignin is extracted from lignocellulosic biomass thorugh the use of an organic solvent such as ethanol, ethylene glycol or glycerol. The polar structure of glycerol can easily penetrate the lignocellulosic material providing an effective reaction medium for the delignification as it can reach high temperatures at atmospheric pressure, reducing the energy consumption (Novo et al., 2011).

The microwave radiation is absorbed by the solution in a uniform way and provides an intense rotational movement of the water molecules generating heat by friction. Shocks caused by this rotational movement can be useful to promote the disintegration of hemicellulose-lignin complex (Chen et al. 2011). The pre-treatment of biomass with the application of microwaves in an alkaline medium reduced by 50% the time required to obtain the same amount of reducing sugars by enzymatic hydrolysis of rice straw when compared to the process without microwaves (Zhu et al. 2006).

This study aimed to evaluate the effects of the irradiation of sugar cane bagasse and sugar cane straw with microwave on media with aqueous glycerol, acid or alkaline solution at atmospheric pressure and evaluate the effects of these treatments on subsequent enzymatic hydrolysis of the material treated using an enzyme solution obtained by cultivation of *Myceliophthora thermophila* M.7.7 or commercial enzyme Celluloclast 1.5 L.

2.Material and Methods

Microwave treatment of sugar cane bagasse

The bagasse and straw were ground (particles 1 to 3 mm in size) and 5 g were immersed in 30 ml 70% solution of glycerol in water, sulphuric acid (0.02M) or sodium hydroxide (0.02M) for 20h and underwent microwave radiation (2450 MHz) for 2 min at 180°C. The assays were carried out in a 250 mL round flask. After the treatment, the material was mixed with 30 mL of distilled water, and was then stirred and filtered. The solid fraction was washed. One part was used for enzymatic hydrolysis, and the other part was used for dry-weight estimation. The filtrate was used for reducing sugar and for phenol derivative evaluation.

Chemical Analysis procedures

The qualitative determination of lignin from bagasse, treated cane straw and untreated cane straw was performed using the Wiesner test. 0.1 g of bagasse and sugar cane straw (size 3mm) were immersed in phloroglucinol reagent, which consisted of 1 % (w/v) phloroglucinol in 10.1 M hydrochloric acid-ethanol (25/75). In this test, the cell walls and tissues containing lignin become bright red-purple when treated with concentrated hydrochloric acid and phloroglucinol.

For chemical lignin determination, the dry bagasse samples (0.3 g) were shaken in 3.0 mL of sulphuric acid 72% (150 rpm; 30 °C; 30 min) and after, 84 mL of distilled water were added to the flask and the sample was autoclaved for 1 h (121 °C) and filtrated onto paper. Soluble lignin from this filtrate was evaluated directly at λ=240 nm, using gallic acid as the standard. Hydrolyzed cellulose and hemicellulose were quantified by the amount of sugar content released (ionic chromatograph). The material retained on the filter paper was dried at 105 °C for dry weight determination and burned (500 °C; 4 h) for ash quantification. These data were used to calculate the amount of insoluble lignin.

Glucose, xylose, galactose, arabinose, xylobiose and cellobiose were quantified using an ICS 5000 Dionex HPAEC-PAD ionic chromatograph with a CarboPac PA-1 anionic column. Solvent A (deionized water); B (500 mM NaOH) and C (300 mM sodium acetate with 150 mM NaOH). The elution (1.0 mL/min) was performed from 0 to 14 min in isocratic mode with 95.2%

A; 4.8% B and 0% C, and then changed to gradient mode for 60% A; 20% B and 20% C at 26 min. Total reducing sugars and phenolic compounds released were quantified according to the methods from Somogy-Nelson (Somogyi, 1952) and Folin-Ciocalteu (Singleton, 1999), respectively.

Glucose, cellobiose, cellotriose and cellotetraose were identified on silica gel plates (Merck) eluted with a mixture of n-butanol/acetic acid/ethyl ether/water (9:6:3:1), respectively. The revelations of bands were conducted using the mixture of 0.5 ml anisaldehyde, 9 mL of ethanol, 0.5 ml of concentrated sulfuric acid and 1 ml of acetic acid. The places sprayed with anisaldehyde solution were heated for 5-10 min at 105 ºC.

Physical analysis procedures

Fourier transformed infrared (FTIR) spectra were recorded in the range from 600 to 4000 cm^{-1} on a Perkin Elmer spectrophotometer Spectrum Two fitted with an ATR device. Approximately 0.1 g of dry bagasse was compressed at 5 ton/cm^2 to form flat discs and placed on the ATR crystal device with a pressure of 90 N/cm^2. All measurements were performed 5 times at different points on the disks for each sample and the final spectrum was generated using the mean of these measurements.

Thermal gravimetric (TG and DTG) curves were recorded in a Perkin-Elmer TGA-4000 thermogravimetric balance. About 8 mg of dried sugarcane bagasse was placed in a ceramic sample holder and heated at 50 °C/min under nitrogen atmosphere (20 mL/min) in the temperature range of 30-600 °C.

Differential Scanning Calorimeter (DSC) curves were recorded in a Perkin Elmer DSC-8000 calorimeter using sealed aluminum crucibles containing near to 2.0 mg of dry samples and an empty aluminum crucible at the reference side. Nitrogen was used as a purging gas (20 mL/min) in the temperature range of 100-275 °C at a heating rate of 25 °C/min.

X-ray diffractograms were obtained with a Rigaku miniflex diffractometer, operating at 30 kV and 15 mA. Scanning from values of 2º/min with angles of 3 to 70º (2 theta) and wavelength of x-rays 1.5418 Angstrom. The degree of crystallinity (CI) was calculated as the ratio of the intensity differences in the peak positions at 18º and 22º according to Equation (1),

$$IC = \frac{H_c}{H_a + H_c} \times 100 \quad (1)$$

Where H_a corresponds to the height referring to the amorphous phase (2 theta ~ 18º) and H_c corresponds to the height related to the crystalline phase (2 theta ~ 22º), both relative to the baseline (Browning, 1967).

Transmitted electron microscopy (TEM)

The fibers were fixed in 2.5% glutaraldehyde and 4% *p*-formaldehyde in a phosphate buffer (0.1 M, pH 7.3) for 24 h. The mixture was then washed with phosphate buffer and post-fixed in osmium tetroxide in a 1% phosphate buffer for 2 h. The material was washed with distilled water and block contrasted with 0.5% uranyl acetate in water for 1 h. The dehydration was performed in an ascending series of acetone solutions followed by saturating the product with a mixture of Araldite® and acetone (1:1) for 12 h. Finally, the block was submerged in pure Araldite® resin to finalize the hardening process. The semi-thin slices obtained from the contrasted blocks were stained with a mixture of methylene blue and blue II 1% borax (1:1) and examined under a light microscope to select blocks that exhibited the optimal characteristics for this study. Ultrathin sections were obtained from the selected blocks and were stained with a uranyl acetate saturated alcoholic solution, followed by staining with lead citrate. The materials were analyzed and photographed by TEM, using a model CM-100 (Philips).

Enzyme hydrolysis

Enzyme hydrolysis of untreated samples and treated samples were carried out in 50 ml flasks with rubber stoppers containing 2.5% dried substrate into a final reaction volume of 20 mL. The samples were incubated for 24 and 72 h at 55 °C using the reaction mixture containing acetate buffer (pH 5.0, 0.1 M) and enzyme solutions obtained by the cultivation of *M. thermophila* M.7.7 or commercial Celluclast 1.5L standardized based on endoglucanase activity (60 U / g of dry substrate) or on total protein (5 mg / g of dry substrate). In the other assay, the extract of *M. thermophila* M.7.7 was added with commercial β-glucosidase (Sigma) in order to achieve the same activity present in the Celluclast enzyme (Table 1).

Table 1: Enzyme activities of the enzyme solutions used in hydrolysis assays

	Enzyme	Activities of the enzyme solutions (U/g dry substrate)		
		Endoglucanase	Xylanase	β-glucosidase
Endoglucanase	*M. thermophila* M.7.7	60.0	435.6	0.35
	Celluclast 1.5L	60.0	9.4	0.15
Supplement	β-glucosidase Sigma	0.2	0.3	0.15
5 mg de protein	*M. thermophila* M.7.7	825.0	6050.0	5.1
	Celluclast 1.5L	451.2	71.0	1.2
Supplement	β-glucosidase Sigma	2.9	1.7	1.2

3.Results and Discussion

Total reducing sugar and phenolic compost released in the treatment of bagasse and cane straw.

The pre-treatment with microwave irradiation with 70 % glycerol in sulfuric acid (0.02 M) provided the highest release of reducing sugars in the eluent (10.4 mg/g from bagasse and 4.3 mg/g from straw) (Figure 1A, B). Similar results (10.9 mg/g dry biomass) were obtained by Linde et al (2008), with wheat straw treated with steam explosion and sulfuric acid as solvent but different from data reported by Hu and Wen (2008) with switchgrass in which the highest sugar release was obtained by treatment with microwaves in an alkaline medium.

The use of microwave irradiation with 70% glycerol in alkaline conditions resulted in a greater release of reducing sugars from straw (2.5 mg/g of dry straw) than from bagasse (Figure 3B).

The pretreatment with microwaves in the presence of 70% glycerol in an acidic medium released the highest quantity of phenolic compounds (19.0 mg from bagasse and 8.0 mg from straw) (Figure 1A, B). This result contradicts that established in the literature on the greater solubility of lignin in alkaline solution (Kim; Han, 2012, Hu; Wen, 2008, Kurakake, et al., 2001). However, most studies were performed using aqueous sodium hydroxide, while, in the present work, a mixture of alkali and glycerol was used. It is possible that in these conditions the formation of glycerate occurs, which could change the action of sodium hydroxide as the lignin solubilizing agent.

When considered the relation between sugar and phenol released the data showed that treatment with water afforded a proportion of 1.1 against 0.5 to 0.7 for them with H_2SO_4 and NaOH. These results is important because indicate the selective action of the treatment on the lignin without interfer with the sugar polysaccharides content.

A) B)

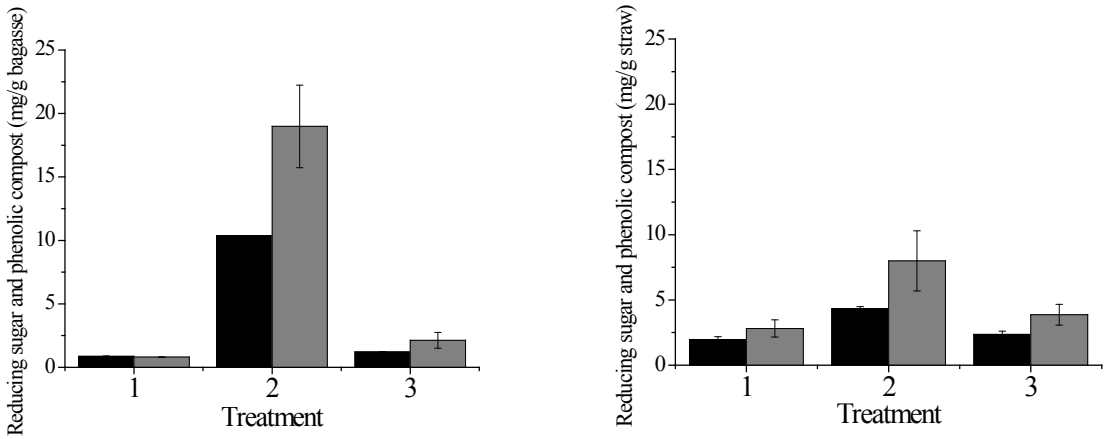

Figure 1: Reducing sugar (**black bar**) and phenolic compounds (**gray bar**) released from sugar cane bagasse **(A)** and sugar cane straw **(B)** after 2 min of microwave radiation.

*Where: Treatments - **(1)** = microwave and glycerol 70% in water, **(2)** = microwave and glycerol 70% in H_2SO_4 solution 0.02 M, **(3)** = microwave and glycerol 70% in NaOH solution 0.02 M.*

Qualitative analysis of lignin from bagasse and cane straw.

In the Wiesner test, a brilliant purple-red color results from the reaction of cinnamaldehyde groups, substituted with phloroglucinol in the presence of HCl. These reactive groups are present only in small amounts in lignins, but the test is very sensitive allowing detection of these compounds with good resolution level (Vance et al., 1980). Due to the sensitivity of the test, six repetitions were done for each untreated and treated sample so as to ensure a representative median.

The Wiesner test confirmed the results obtained by chemical analysis which showed the greatest loss of lignin in the microwave irradiation treated bagasse with 70% glycerol in sulfuric acid solution (Table 2). Moreover, this test was not conclusive for the straw because it didn't allow for detection of differences among the samples from different treatments (Table 2). According to Sarkanem and Hergert (1971), besides the reduction of cinnamaldehyde groups by loss of lignin, a weak staining could be due to the type of predominant compound in lignin and has been demonstrated that the syringyl lignins predominantly result in weak reactions with phloroglucinol.

Table 2: Intensity of the purple-red color of the fibers observed in straw and bagasse stained with phloroglucinol-HCl.

Treatments	Sugar cane bagasse	Sugar cane straw
Untreated	+++[a]	+
1	+++	+
2	+	+
3	+++	+

[a] = color intensity (+++) strong purple-red, (++) medium purple-red e (+) weak purple-red.

Where: Treatments - ***(1)*** *= microwave and glycerol 70% in water,* ***(2)*** *= microwave and glycerol 70% in H_2SO_4 solution 0.02 M,* ***(3)*** *= microwave and glycerol 70% in NaOH solution 0.02 M.*

Fiber composition analysis (lignin, xylan and cellulose) from bagasse and cane straw

The chemical analysis revealed that the bagasse was composed of 47% cellulose, 16% hemicellulose and 27% lignin and the straw presented 43% cellulose, 15% hemicellulose and 23% lignin (Table 3). After the treatment with microwaves and glycerol in H_2SO_4, the lignin content of the bagasse was reduced by 4% while for straw the highest decrease in lignin (3%) occurred by treatment with microwave and glycerol in NaOH. Data from Chen et al., (2012), with similar treatment to the present work, shows a 10% increase in the lignin fraction and reduction of 98% in the hemicellulose content.

Goshadrou et al. (2011) observed a 5.7% reduction in lignin fraction of sorghum bagasse pre-treated with 12% (w/v) NaOH solution while Pang et al (2013), when using a combined treatment with microwave and steam explosion, reported a 7.8% reduction in lignin fraction of corn stover. In the present work, the other treatments did not result in significant changes in the fraction of lignin in either lignocellulose material.

Table 3: Chemical composition of the fibers.

Treatement	Sugar cane bagasse (%)				Sugar cane straw (%)			
	Celullose	Xylan	Lignin	ashes	Cellulose	Xylan	Lignin	ashes
Untreated	47 ± 0.5	16 ± 0.1	27 ± 0.9	11	43 ± 0.8	15 ± 0.5	23 ± 0.6	11
1	45 ± 2.7	15 ± 0.6	25 ± 0.6	9	43 ± 0.2	15 ± 0.2	22 ± 2.4	13
2	48 ± 0.7	16 ± 0.2	23 ± 1.0	11	43 ± 0.1	15 ± 0.1	23 ± 0.4	10
3	45 ± 3.3	15 ± 0.2	26 ± 1.0	9	44 ± 0.9	15 ± 0.0	20 ± 2.0	12

Where: Treatments - ***(1)*** *= microwave and glycerol 70% in water,* ***(2)*** *= microwave and glycerol 70% in H_2SO_4 solution 0.02 M,* ***(3)*** *= microwave and glycerol 70% in NaOH solution 0.02 M.*

Analysis of the chemical structure of bagasse and cane straw using Attenuated Total Reflectance mode in a Fourier Transform Infrared Spectrometer (FTIR ATR)

The characteristic functional groups and absorption of infrared radiation of some compounds in the straw and bagasse are shown in Table 4. It can be observed that the fibrous fraction (cellulose, hemicellulose and lignin) showed characteristic absorption bands of the alkene, ester, aromatic, carbonyl and alcohol groups. In all samples a broad band near 3450 cm^{-1} was observed, characteristic of the stretching of the hydrogen from hydroxyl groups. Bands in the region between 3000-2800 cm^{-1} attributed to C-H of methyl and methylene groups were observed. In addition, bands representing the structure of lignin (near 1500 cm^{-1}) and polysaccharide (between 1300 cm-1) were also observed.

Table 4: Peak wavenumber (in cm^{-1}) of the main infrared absorption bands and their interpretation.

Wavenumber (cm^{-1})	Cellulose	Hemicellulose	Lignin	Assignment	Referência
3400	X		X	O−H stretching vibration	Moubarik et al., 2013, Guimarães et al., 2009
2925			X	C−H (groups CH_3 e CH_2) stretching vibration	Moubarik et al., 2013
2888		X		C−H symmetric stretching vibration	BIAN et al., 2012
2850	X		X	CH_2 symmetric stretching vibration	Guimarães et al., 2009, Hoi; Martincigh (2013)
1710		X	X	C=O stretching vibration	Hoi; Martincigh (2013), Cunha et al., 2011
1634			X	Carbonyl stretching of aromatic ring	Viera et al., 2007
1515-1600			X	C=C stretching of aromatic ring	Hoi; Martincigh (2013), Chen; Tu; Sheen 2011
1455		X	X	C−H bending	Hoi; Martincigh (2013)
1425-1460	X			CH_2 symmetric stretching	Mothé; Miranda (2009), Miranda, 2009
1315-1370	X			C−H bending	Gurgel et al., 2012, Hoi; Martincigh (2013)
1246	X	X		C−O−C stretching	Cunha et al., 2011, Mothé; Miranda (2009),
1203	X			O−H bending	Gurgel et al., 2012
1030-1162	X	X	X	C−O−C, C−O stretching	Guimarães et al., 2009, Chen; Ye; Sheen 2012, Hoi; Martincigh (2013)
895	X	X		Glycosidic linkages of glucose ring in cellulose	Hoi; Martincigh (2013), Sun et al., 2011
833			X	C−H in plane bending	Cunha et al., 2011

The samples of bagasse treated with microwave and glycerol in sulfuric acid and NaOH presented few alterations in the absorption spectra (Figure 2). The main alteration was observed in an absorption band at 1030 cm^{-1} attributed to the stretching of the C−O−C and C−O linkages present in the cellulose, hemicellulose and lignin (Guimarães et al, 2009). Since the ester linkages occur between lignin and hemicellulose residues, we can infer that the treatment resulted in a breaking of the association between lignin and polysaccharide. Cunha et al. (2011) reported the same changes in the sugar cane bagasse treated with acid and alkaline solutions.

The absorption spectra showed subtle changes in the band at 2925 cm^{-1}, which refer to the stretching vibration of C−H from CH_3 and CH_2 groups present in lignin, and the band at 2896 cm^{-1} attributed to C−H symmetric stretching vibration present in hemicelluloses was detected in treated samples but not in the control. According to Menon and Rao (2012), acidic and alkaline pre-treatment break the glycosidic bond of the side chain of hemiccellulose, promote structural alteration of lignin and partial decrystalization of the cellulose.

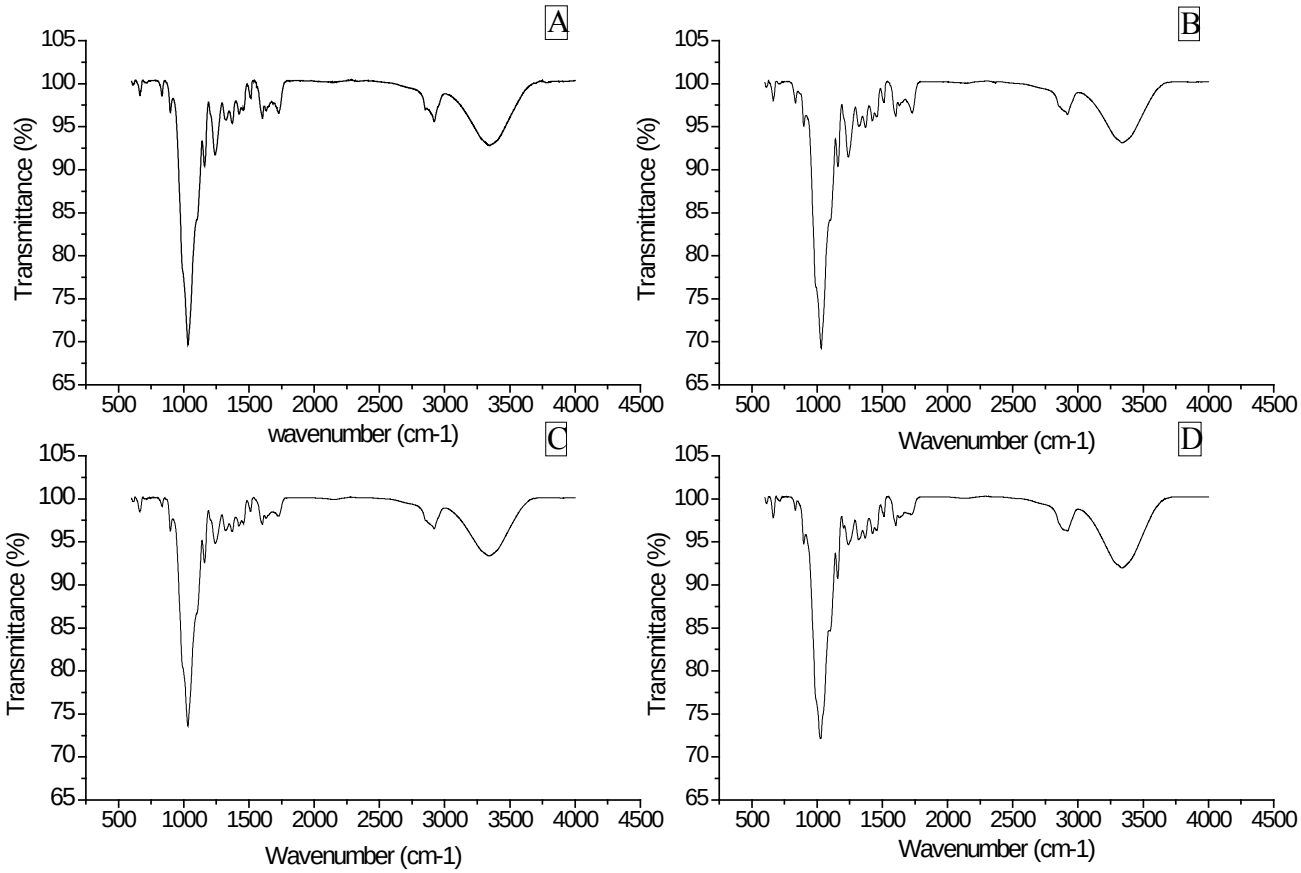

Figure 2: FTIR spectra of sugar cane bagasse after the treatments. *Where: **(A)** = control (untreated bagasse), **(B)** = microwave and glycerol 70% in water, **(C)** = microwave and glycerol 70% in H_2SO_4 solution 0.02 M, **(D)** = microwave and glycerol 70% in NaOH solution 0.02 M.*

Most alterations in the spectra of sugar cane straw were obtained at 1030 cm^{-1}. Unlike those observed with bagasse, this alteration was an intensification of the absorption mainly in the samples treated with glycerol and NaOH (Figure 3). These spectra refer to alterations on C−O−C and C−O linkages of the cellulose, hemicellulose and lignin. According to Sun et al. (2002), these results could be attributed to the formation of C−O−C linkages from the breaking of C=O bonds.

Another difference observed between straw and bagasse occurred at the 2850 cm^{-1} band attributed to the symmetric stretching of CH_2. Hoi and Martincigh (2013) reported that this band is found only in the leave and in this work it was sensitive all the treatments with increasing in the absorption.

Like those observed for bagasse, minor changes were detected at 1162, 1246 and 1370 cm^{-1} related to the C−O−C e C−O linkages.

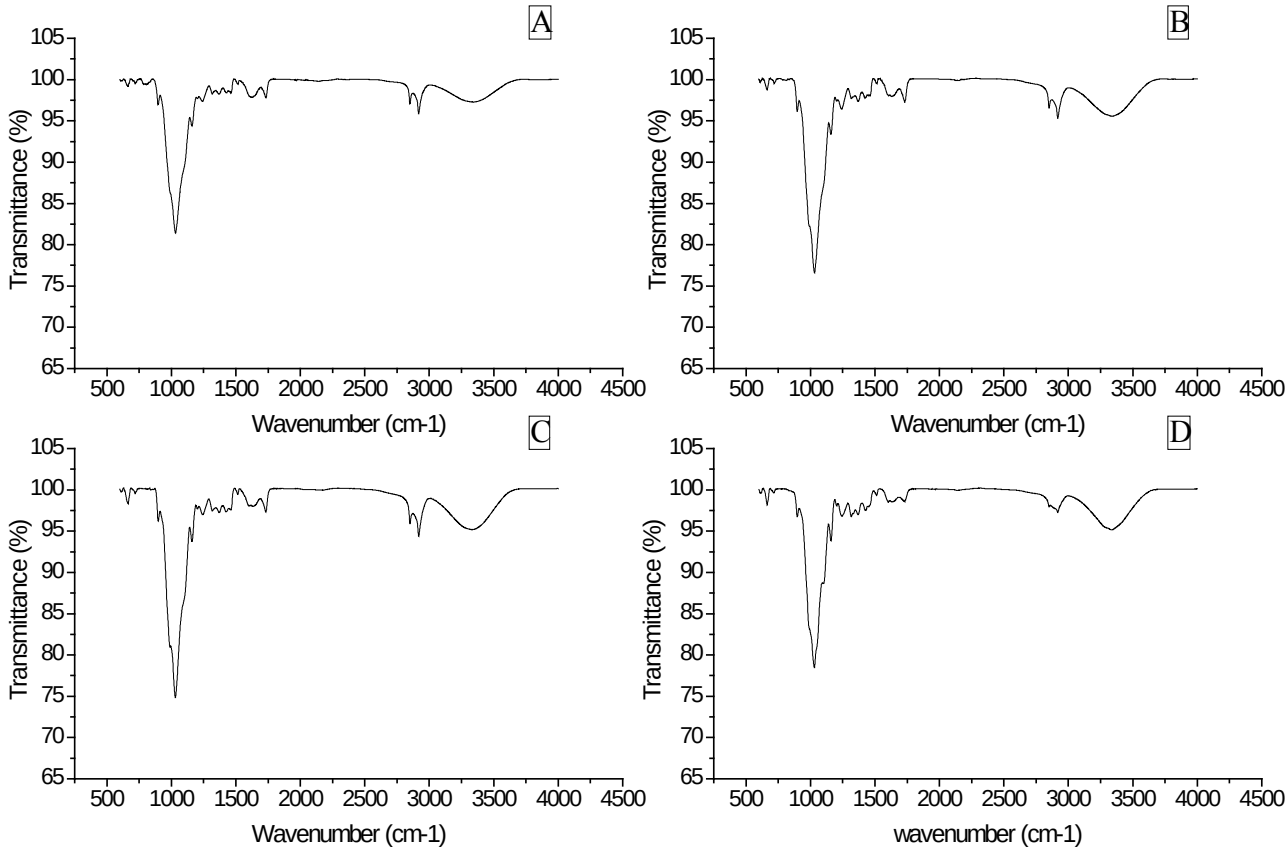

Figure 3: FTIR spectra of sugar cane straw after the treatments. *Where: (**A**) = control (untreated straw), **(B)** = microwave and glycerol 70% in water**, (C)** = microwave and glycerol 70% in NaOH solution 0.02 M, **(D)** = microwave and glycerol 70% in H_2SO_4 solution 0.02 M.*

Thermogravimetric analysis (TGA)

The TGA curves showed that the weight loss occurred only after 360 °C except for bagasse treated with sulfuric acid in glycerol which started around 400 ºC. The content of the ashes at 800 °C for bagasse was 11, 9, 9, and 11% for untreated bagasse, treated with microwave in aqueous glycerol solution, alkaline glycerol and acid glycerol solutions, respectively, and was 11, 13, 12 and 10% for straw in the same conditions (Figure 4A, B).

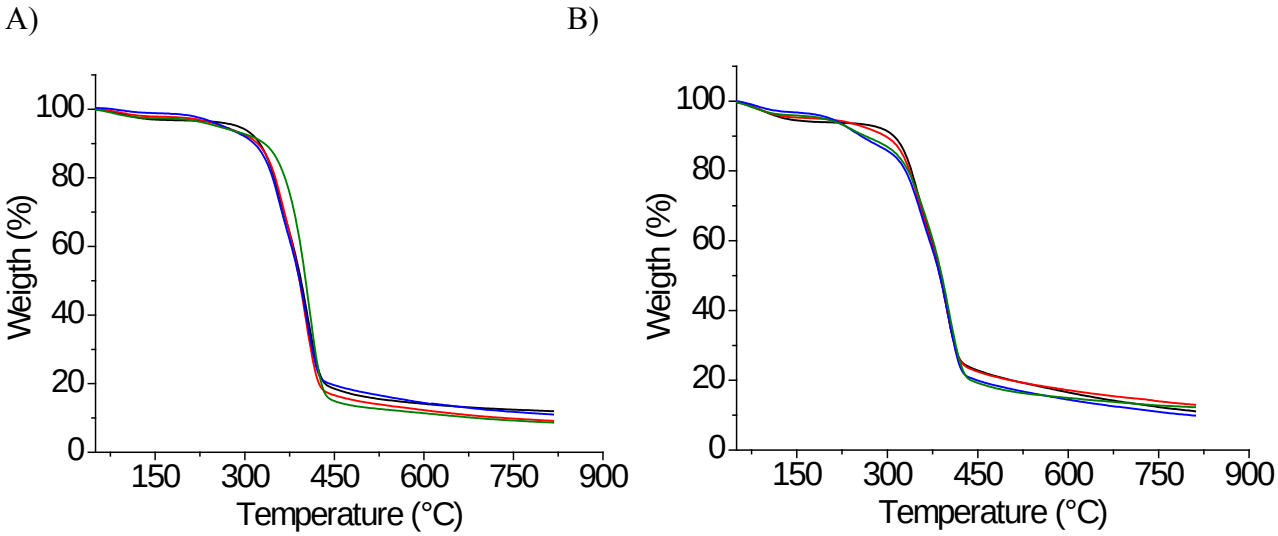

Figure 4: TG curve of sugar cane bagasse **(A)** and sugar cane straw **(B).** *Where:* ***(Black)*** *= control (untreated sample),* ***(Red)*** *= microwave and glycerol 70% in water,* ***(Green)*** *= microwave and glycerol 70% in H_2SO_4 solution 0.02 M,* ***(Blue)*** *= microwave and glycerol 70% in NaOH solution 0.02 M.*

The differential thermogravimetry DTG curves of samples of sugarcane bagasse and untreated straw showed two defined peaks at 350 and 400 °C. The first one can be attributed to the decomposition of hemicellulose and lignin, and the second corresponds to the cellulose decomposition (Cunha et al. 2,011, Hoi and Martincigh, 2013) (Figure 5A,B). Microwave treated sugarcane bagasse with 70% glycerol in sulfuric acid showed only one peak, which began at 300 °C and reached the maximum at 408 °C. The absence of the first peak (350 °C) is probably due to the degradation of lignin and hemicellulose fractions during the pretreatment. In addition, an increase in the intensity of the peak at 400 °C was observed, attributed to the cellulose fraction (Figure 5A). The DTG data corroborate those obtained by the FTIR and shown in Table 3, showing an increase in the fraction of cellulose and a reduction in lignin in the bagasse sample after treatment using microwave glycerol in an acid solution. A similar effect was observed in treatment with aqueous glycerol solutions with a reduction in the hemicellulose and lignin fractions.

For the sugar cane straw, the major changes in the DTG curves were observed in the samples treated with microwave glycerol in sulfuric acid and NaOH. Both curves showed a small peak around 230 °C which, according to Cunha et al (2011), is attributed to extraction residues. Furthermore, these curves showed a decrease in peak intensity near 350 °C, attributed to the degradation of hemicellulose and lignin. However, the peak near 400 °C, which refers to the portion of cellulose and lignin, did not show any significant change (Figure 5B).

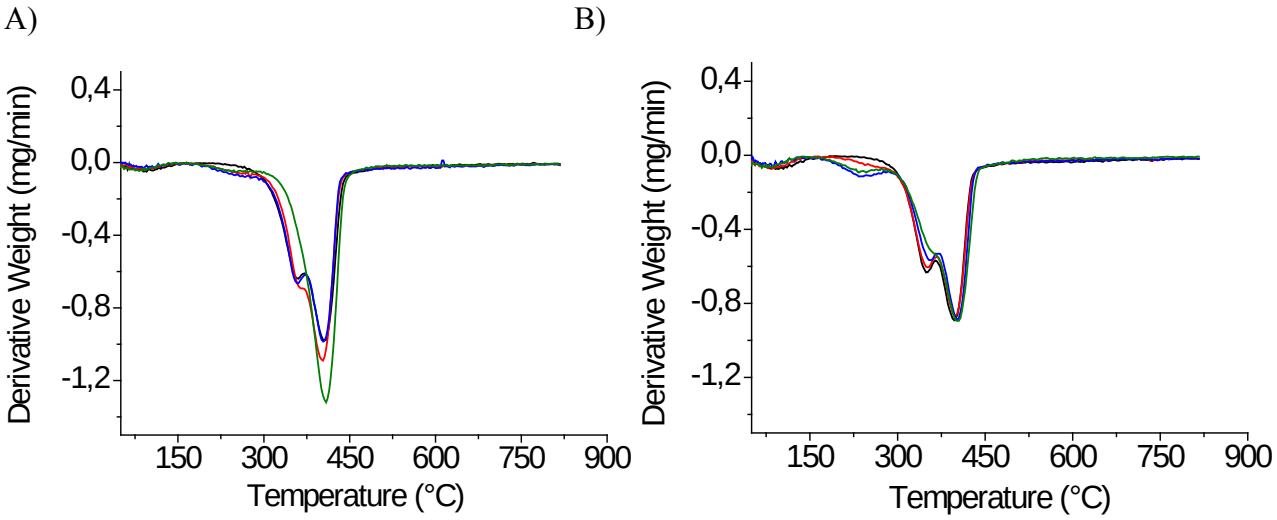

Figura 5: DTG curve of sugar cane bagasse **(A)** and sugar cane straw **(B).** *Where:* ***(Black)*** *= control (untreated sample),* ***(Red)*** *= microwave and glycerol 70% in water,* ***(Green)*** *= microwave and glycerol 70% in H_2SO_4 solution 0.02 M,* ***(Blue)*** *= microwave and glycerol 70% in NaOH solution 0.02 M.*

Differential scanning calorimetry (DSC)

The DSC thermograms of samples of cane bagasse and straw showed an endothermic peak near 100 °C which corresponds to the evaporation of moisture of the fibers (Figure 6), while the pyrolysis of lignin, hemicellulose and cellulose were exothermic. Hemicellulose is a branched amorphous structure and shows decomposition at low temperatures while the cellulose, which is a long polymer of glucose without branching and with hydrogen bonds, is more resistant to thermal breakdown. Moreover, the lignin due to the aromatic ring with different branches decomposes over a wider temperature range. Although the three components present structures and different points of thermal degradation when associated, they do not decompose as three independent reactions (Hoi and Martincigh, 2013).

The curves obtained from the fibers of bagasse (Figure 6A) showed that, after pretreatment, samples became more resistant to thermal degradation. The fibers of the non-treated bagasse

showed an exothermic peak at 200 °C, whereas, for the sample treated with microwave and 70% glycerol in distilled water, this peak was at 217 °C. Probably, the treatment removed the less ordered compounds which are more susceptible to thermal degradation and the fiber became more crystalline and thermo-resistant (Moretti et al. 2013). The sample treated with microwave and 70% glycerol in a sulfuric acid solution showed a less pronounced shift of the exothermic peak (205 °C), possibly due to the degradation of lignin (Table 3 and Figure 5) and the sample treated with microwave and 70% glycerol in a solution of sodium hydroxide kept the thermal degradation profile very similar to control residue (199 °C).

The DSC thermogram of the fiber of untreated straw showed an exothermic peak at 197 °C (Figure 6B) and after treatment with microwave and 70% glycerol in distilled water, the thermal decomposition occurred at 193 °C. These results corroborate those shown in Table 3 and Figures 4,5 that showed no change in chemical composition of this sample. Straw treated with microwave and glycerol in sulfuric acid and glycerol in sodium hydroxide showed exothermic peaks at 200 and 203 °C probably due to the decreasing of the lignin and hemicellulose fractions as shown in Table 2. The decreasing of these polymers increased the crystallinity of the fiber and it became more resistant to thermal degradation.

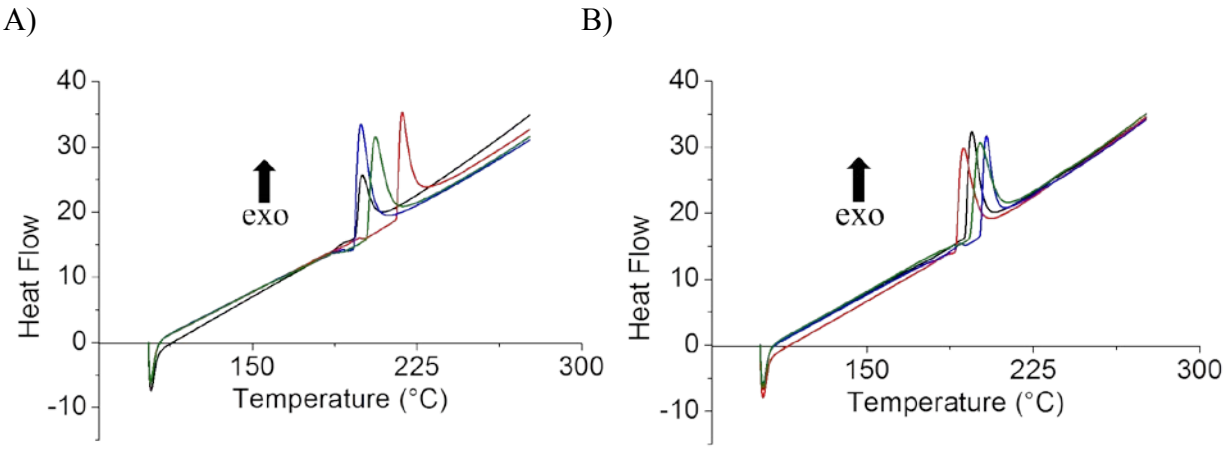

Figure 6: DSC curve of sugar cane bagasse **(A)** and sugar cane straw **(B).** *Where:* ***(Black)*** *= control (untreated sample),* ***(Red)*** *= microwave and glycerol 70% in water,* ***(Green)*** *= microwave and glycerol 70% in H_2SO_4 solution 0.02 M,* ***(Blue)*** *= microwave and glycerol 70% in NaOH solution 0.02 M.*

X-ray diffraction of sugarcane bagasse and straw

Aiming to confirm the indication of the increasing in the bagasse cristalinity and straw after treatments it was done X-ray diffration analysis of the samples wich results are in Table 5 and Figure 7.

Table 5. Crystallinity index of control and pre-treated fibers

Microwave treatment condition	H_c	$H_a + H_c$	Cristalinity (%)
Untreated bagasse	2398.3	3514.9	68.2
Bagasse - 1	2553.3	3731.6	68.4
Bagasse - 2	2401.6	3313.2	72.4
Untreated straw	2043.3	3069.9	66.5
Straw - 3	2093.3	3109.9	67.3

*Where: Treatments - **(1)** = microwave and glycerol 70% in water, **(2)** = microwave and glycerol 70% in H_2SO_4 solution 0.02 M, **(3)** = microwave and glycerol 70% in NaOH solution 0.02 M.*

Among the bagasse samples there were an increase in the cristalinity index for sample treated with microwave and glycerol in H_2SO_4 solution. Although it has been detected an increase in the thermo-resistance of the bagasse submitted to treatment with microwaves and glycerol water, the X-Ray data showed no increase in crystallinity, whereas for the bagasse treated with sulfuric acid, which also showed an increase in thermo-resistance, a considerable increase of crystallinity was detected. It is possible that in the treatment with water have been liberated compounds associated to the fibers and not inserted into the structure of the material, as it must have occurred with the samples in acid medium. This hypothesis is supported by the data about released phenols, decrease in the lignin and hemicellulose content and a slight increase in the proportion of cellulose in the sample after the treatment in acidic medium.

The increase in crystallinity index of treated straw in alkaline medium showed a slight increase, which is consitente with observed by TGA ,DTG, DSC and the chemical analysis.

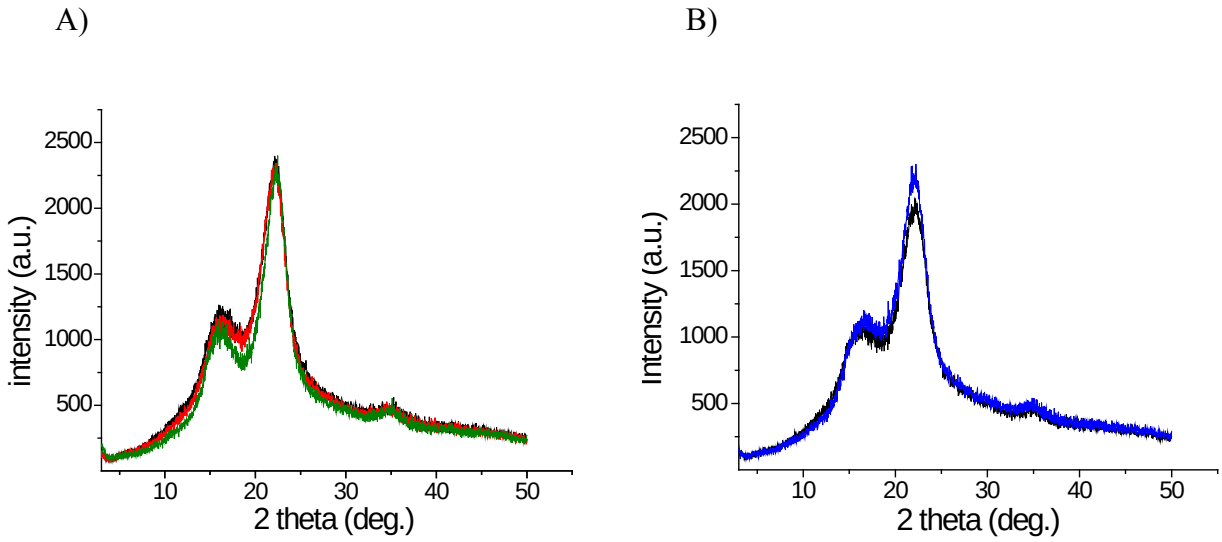

Figura 7: X-ray diffractogram of sugar cane bagasse **(A)** and sugar cane straw **(B).** *Where:* ***(Black)*** *= control (untreated sample),* ***(Red)*** *= microwave and glycerol 70% in water,* ***(Green)*** *= microwave and glycerol 70% in H_2SO_4 solution 0.02 M,* ***(Blue)*** *= microwave and glycerol 70% in NaOH solution 0.02 M.*

Ultrastructural analysis of bagasse and cane straw

In the untreated bagasse, a clear conservation of primary cell (PW) and secondary walls (SW) and the middle lamella (ML) (Figure 8a, c) in addition to plasmodesmata (PD) in the pit membrane (PM) (Figure 8 b) can be observed. In the samples treated with microwaves in 70% glycerol in distilled water the primary, secondary walls and middle lamella were preserved (Figure 8d, f) but there was a slight breakdown of the walls around the pit membrane (Figure 8e). In the bagasse treated in the presence of glycerol and sodium hydroxide, was observed a liquefaction of the secondary wall (Figures 8i), breakdown of pit membrane (Figure 8h) and change in electron density of the material and ruptures in the secondary membrane (Figure 8 j).

For the treated bagasse with 70% glycerol in a sulfuric acid solution, there was a loosening of the primary and secondary cell wall (Figures 8l, n). There was disruption of the hemicellulose in this region (Figure 8l). In some areas there was liquefaction of the cell walls (Figures 8 k), and the overall breakdown in the pit membrane (Figure 8 m).

In untreated straw, looser primary and secondary cell walls are clearly observed (Figure 8o). As in the bagasse, the pit membrane and plasmodesmata were well preserved (Figure 8p) When using the microwave irradiation with glycerol in distilled water the preservation of primary and secondary cell walls was observed (Figure 8q) but there was a slight disruption or rupture of the pit pontoação (Figures 8r). For the samples subjected to microwave treatment in 70% glycerol and sodium hydroxide, the liquefaction of the cell walls (Figures 8t) and a small disruption in plasmodesmata were found (Figure 8s) In the treatment using microwave irradiation with 70%

glycerol in sulfuric acid, very similar effects to those obtained with the pretreatment of sugarcane bagasse were observed. There was a loosening of cell walls (especially the secondary wall), so that it (the secondary wall) even becomes liquified (Figures 8u) and a breakdown in the pit membrane, as well as the absence of plasmodesmata (Figure 8v).

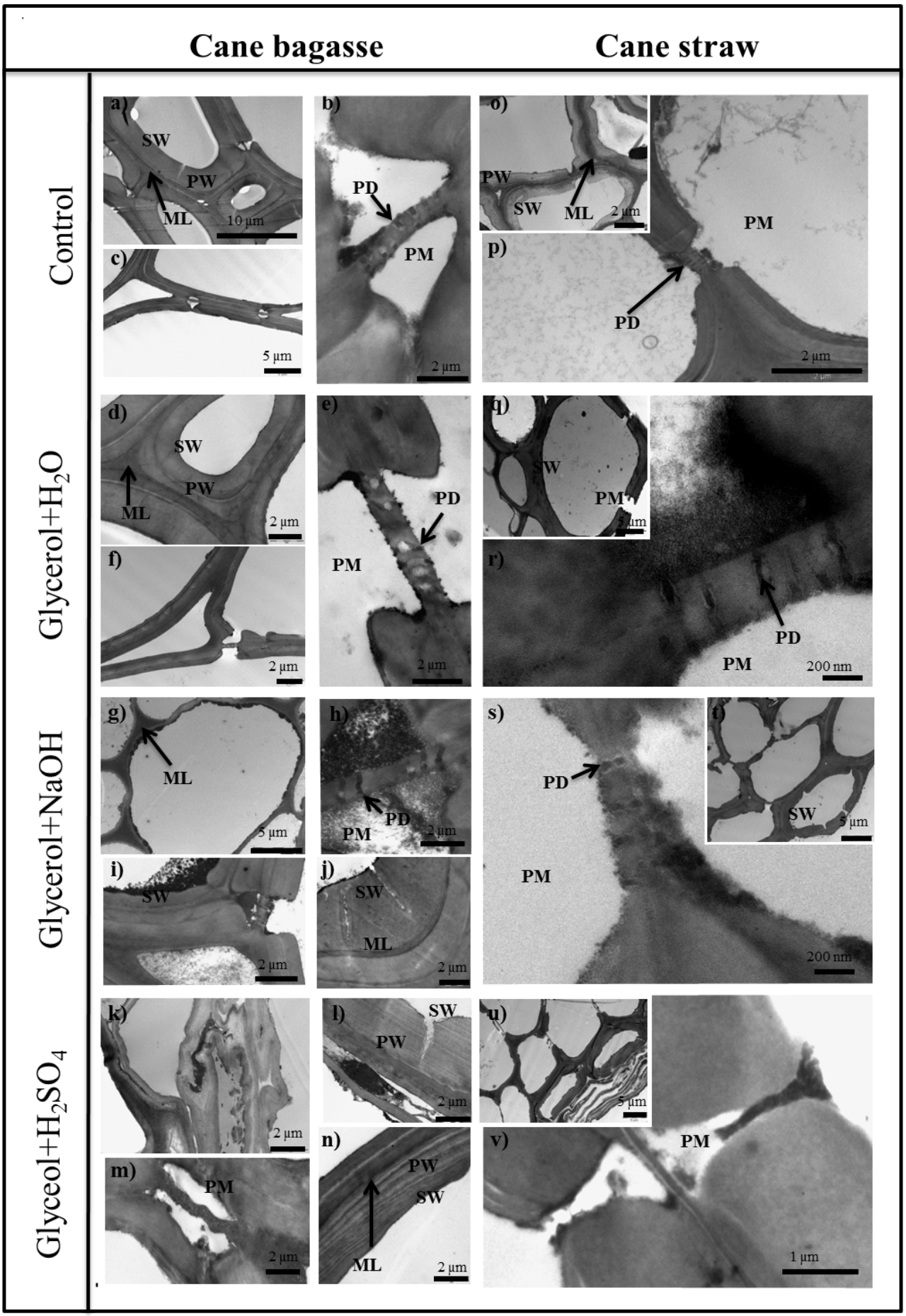

Figure 8: Transmission electron microscopy of sugar cane bagasse and straw untreated and treated.

Enzymatic hydrolyis of sugar cane bagases and straw

According to data from Figure 9A,B the treatment improved the enzyme hydrolysis of bagasse and straw. Samples of bagasse treated with microwaves in a medium with acidic glycerol solution yield 70 mg of reducing sugar from dry bagasse against 40 mg/g from untreated bagasse. These results corroborate data from the chemical and physical analyses which indicate a disintegration of fiber due to the treatment with microwaves mainly when acidic glycerol solution was used.

The bagasse hydrolysis was highest when the enzyme solution from *M. thermophila* M.7.7. supplemented with β-glucosidase was used, but for straw, the highest reducing sugar yield was afforded when using the Celluclast enzyme. These data indicate that β-glycosidase activity did not interfere in the hydrolysis of either material and that straw was more prone to the action of endoglucanase from celluclast than cellulose from the bagasse, behavior that was not observed for the enzyme from *M. thermophila*.

A) B)

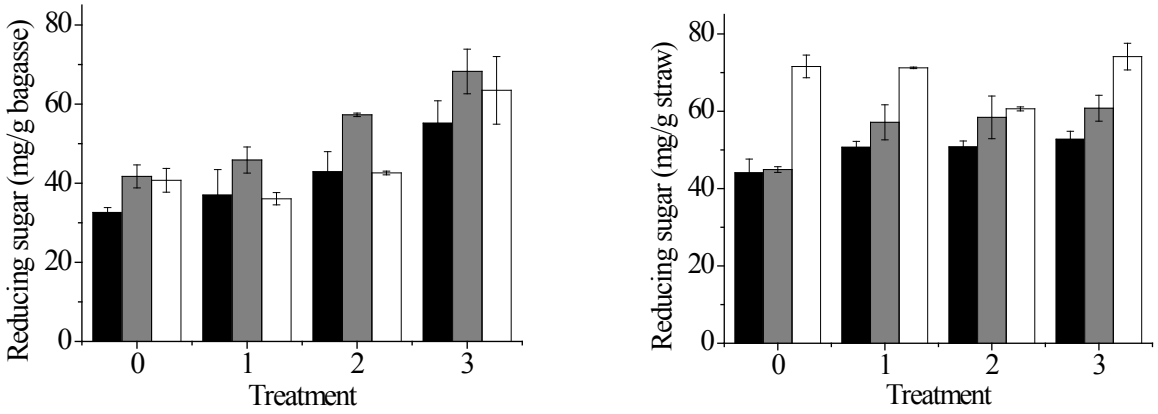

Figure 9: Enzyme hydrolysis of sugar cane bagasse **(A)** and sugar cane straw **(B)**, using 60 U of endoglucanase/g of substrate. *Where:* ***(Black bar)*** *= enzyme from M. thermophila M.7.7,* ***(Gray bar)*** *= enzyme from M. thermophila M.7.7. suplemented with β-glicosidase (Sigma),* ***(White bar)*** *= Celluclast 1.5L (Sigma),* ***(0)*** *= control (untreated sample),* ***(1)*** *= microwave and glycerol 70% in water,* ***(2)*** *= microwave and glycerol 70% in H_2SO_4 solution 0.02 M,* ***(3)*** *= microwave and glycerol 70% in NaOH solution 0.02 M.*

Data from Figure 10 show that the enzyme solution obtained by the culture of *M. thermophila* on lignocellulose material (sugar cane bagasse, corn straw and wheat bran) presented a high content of sugar and phenolic compounds, derived from hydrolysis of the substrate, which could be inhibiting the enzyme (Li et al., 2010).

Whereas other enzymes present in the enzyme solutions could be interfering with the results, we performed tests by taking, as a reference, the total protein of the enzyme solutions (Figure 10). The results showed that the released reducing sugar using an enzyme solution from *M. thermophila* M.7.7 was higher than that of the celluloclast enzyme confirming that other enzymes such as xylanase, avicelase and pectinase present in this solution (data not shown) contributed to the efficiency of hydrolysis.

The same results were observed for straw (Figure 11) with 257 mg of reducing sugar/g of substrate when an enzyme solution from *M. thermophila* was used and 196 mg for the commercial enzyme.

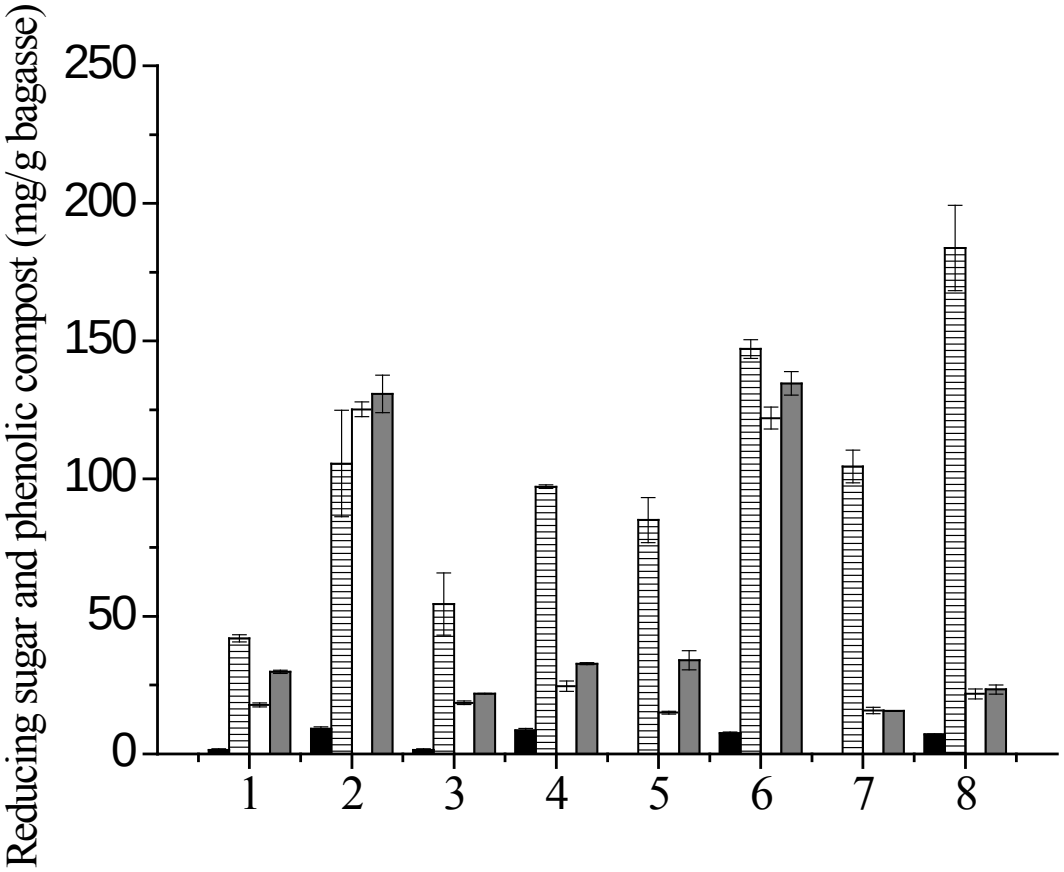

Figure 10: Enzyme hydrolysis of untreated bagasse (**1,2,3,4**) and treated in microwave and glycerol 70% in H_2SO_4 solution 0.02 M (**5,6,7,8**). *Where: (**Black bar**) = Reducing sugar in zero time, (**Striped bar**) = Reducing sugar after 72 h, (**White bar**) = phenolic compounds in zero time, (**Gray bar**) = phenolic compounds after 72 h, **(1,5)** = enzyme from M. thermophila M.7.7 using 60 U of endoglucanase/g of substrate, **(2,6)** enzyme from M. thermophila M.7.7 using 5 mg of protein/g of substrate, **(3,7)** Celluclast 1.5L (Sigma) using 60 U of endoglucanase/g of substrate, **(4,8)** Celluclast 1.5L (Sigma) using 5 mg of protein/g of substrate.*

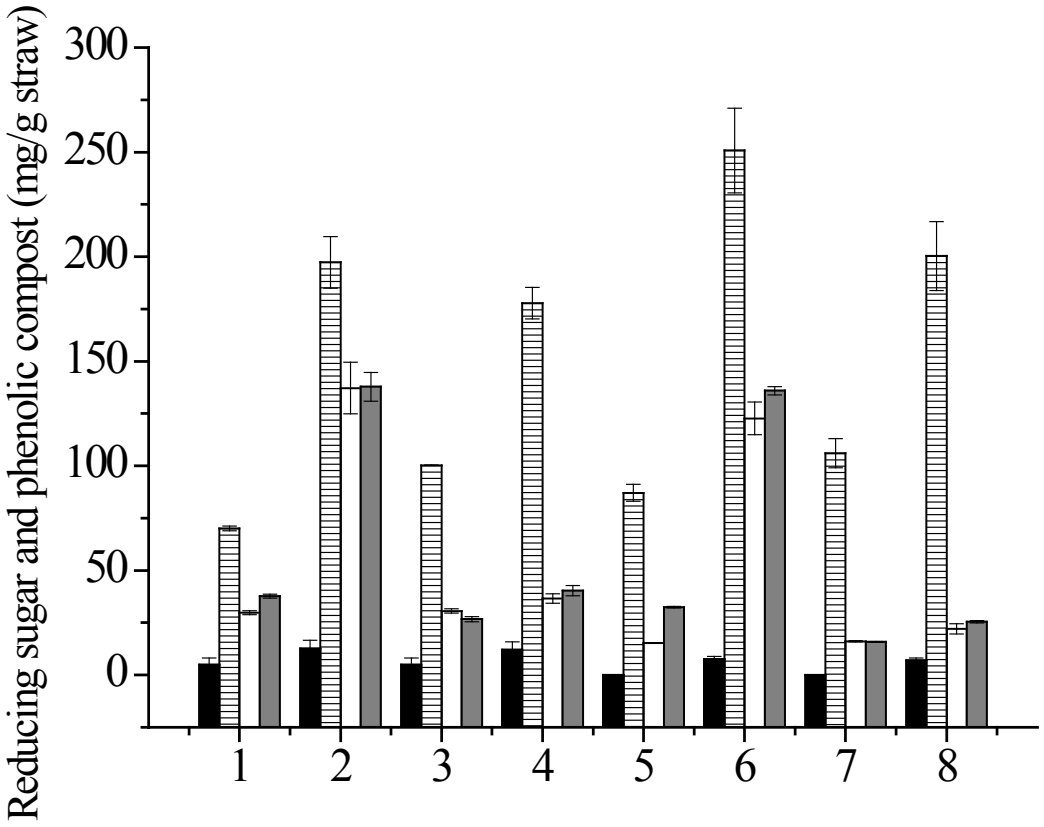

Figure 11: Enzyme hydrolysis of untreated straw (**1,2,3,4**) and treated in microwave and glycerol 70% in H_2SO_4 solution 0.02 M (**5,6,7,8**). *Where: (**Black bar**) = Reducing sugar in zero time, (**Striped bar**) = Reducing sugar after 72 h, (**White bar**) = phenolic compounds in zero time, (**Gray bar**) = phenolic compounds after 72 h, **(1,5)** = enzyme from M. thermophila M.7.7 using 60 U of endoglucanase/g of substrate, **(2,6)** enzyme from M. thermophila M.7.7 using 5 mg of protein/g of substrate, **(3,7)** Celluclast 1.5L (Sigma) using 60 U of endoglucanase/g of substrate, **(4,8)** Celluclast 1.5L (Sigma) using 5 mg of protein/g of substrate.*

The qualitative analysis by HPLC - Dionex showed that the treatment increased the hydrolysis of hemicellulose up to 150% (Table 6). As well as, did improve the cellulose hydrolysis suggesting that the accessibility of this polymer may be eased by lignin degradation, but was not efficient to decrease the crystallinity of the cellulose. The total bagasse hydrolysis was very low (between 3- 5%) although the total reducing sugar, determined by Somogy-Nelson's method, had been higher. This contradicts the findings and cannot be explained by the interference of the phenols present in the enzyme solutions, since a blank was done, or by the presence of oligosaccharides which were not detected by thin layer chromatography (Figure 12).

When the material used was sugar cane straw (Table 7), a different profile was observed with higher total hydrolysis and an increase in the breakdown of hemicellulose and decreasing of cellulose hydrolysis when the enzyme solution was supplemented with β-glycosidase. According to Bairoch (2000), β-D-glycosidases are able to hydrolyze β-D-galactosides, α-L-arabinosides, β-D-xylosides or β-D-fucosides.

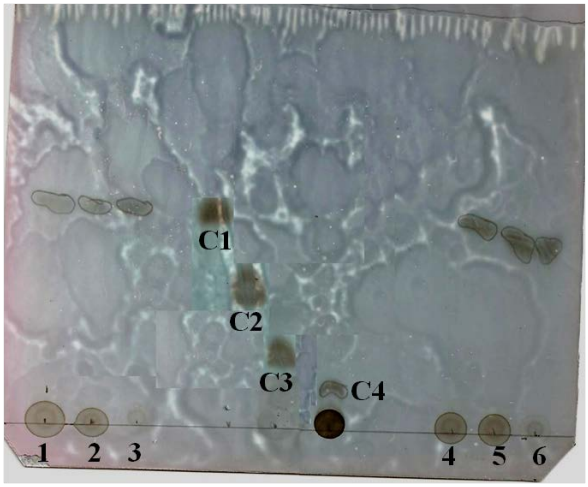

Figure 12: Thin-layer chromatography of hydrolized from bagasse **(lines 1,2,3)** and sugar cane straw **(lines 4,5,6)** treated with microwave and glycerol in acidic solution. ***(1,4)*** *= enzyme from M. thermophila M.7.7;* ***(2,5)*** *= enzyme from M. thermophila M.7.7. suplemented with β-glicosidase (Sigma),* ***(3,6)*** *= Celluclast 1.5L (Sigma),* ***(C1)*** *= glucose;* ***(C2)*** *= cellobiose;* ***(C3)*** *= cellotriose;* ***(C4)*** *= cellotetrose.*

Table 6. Carbohydrates released by enzyme hydrolysis of sugar cane bagasse treated with microwave radiation plus H_2SO_4.

Bagasse pre-treatment	Hemicellulose sugars (mg/g)			% Hemicellulose hydrolysis	Cellulose sugars (mg/g)	% Cellulose hydrolysis	Total bagasse hydrolysis (%)
	Arabinose	Galactose	Xylose		Glucose		
Enzyme from *Myceliophthora thermophila* M.7.7.							
Untreated (U/g)	0.3 ± 0.4	0.02 ± 0	2.2 ± 0.1	1.5	6.8 ± 1.4	1.5	1.0
Untreated (mg enzyme/g)	0.7 ± 0	0.04 ± 0	3.6 ± 0	2.5	21.0 ± 0.5	4.5	3.0
H_2SO_4 (U/g)	1.6 ± 0.1	0.4 ± 0	6.2 ± 0.6	5.0	27.0 ± 0.4	5.5	3.0
H_2SO_4 (mg enzyme/g)	2.7 ± 0.2	0.6 ± 0	6.8 ± 0.1	6.0	31.7 ± 6.3	6.5	4.0
Enzyme from *Myceliophthora thermophila* M.7.7. with β-glucosidase suplementation							
Untreated (U/g)	1.5 ± 0	0.2 ± 0	7.0 ± 0.1	5.5	12.9 ± 1.4	2.5	2.0
Untreated (mg enzyme/g)	2.7 ± 0.2	0.6 ± 0	6.8 ± 0.1	6.0	21.0 ± 0.5	4.5	3.0
H_2SO_4 (U/g)	1.0 ± 0	0.2 ± 0	15.4 ± 1.0	10.0	23.6 ± 2.5	5.0	4.0
H_2SO_4 (mg enzyme/g)	2.8 ± 0.2	1.0 ± 0.5	20.9 ± 0.3	15.5	16.4 ± 1.2	3.5	4.0
Commercial enzyme (Celluclast)							
Untreated (U/g)	0.2 ± 0	0.01 ± 0	1.6 ± 0.1	1.0	13.0 ± 2.4	3.0	1.5
Untreated (mg enzyme/g)	0.4 ± 0	0.07 ± 0	3.2 ± 0.1	2.0	21.6 ± 0.6	4.5	2.5
H_2SO_4 (U/g)	0.7 ± 0.1	nd	4.3 ± 0.2	2.5	19.4 ± 2.4	4.0	2.0
H_2SO_4 (mg enzyme/g)	0.2 ± 0	0.05 ± 0	9.3 ± 0.8	6.0	40.8 ± 6.0	8.5	5.0

Table 7. Carbohydrates released by enzyme hydrolysis of sugar cane straw treated with microwave radiation plus H_2SO_4.

Straw pre-treatment	Hemicellulose sugars (mg/g)			% Hemicellulose hydrolysis	Cellulose sugars (mg/g)	% Cellulose hydrolysis	Straw hydrolysis (%)
	Arabinose	Galactose	Xylose		Glucose		
Enzyme from *Myceliophthora thermophila* M.7.7.							
Untreated (U/g)	1.1 ± 0.1	0.1 ± 0	3.8 ± 0.3	3.0	26.1 ± 0.1	5.5	3.0
Untreated (mg enzyme/g)	3.0 ± 0.1	0.5 ± 0	8.3 ± 0.4	7.5	39.3 ± 2.0	8.5	5.0
H_2SO_4 (U/g)	1.3 ± 0	0.05 ± 0	2.1 ± 0.1	2.0	33.2 ± 1.7	7.0	3.5
H_2SO_4 (mg enzyme/g)	2.8 ± 0.1	0.4 ± 0	5.5 ± 0	5.5	48.0 ± 0.7	10.0	5.5
Enzyme from *Myceliophthora thermophila* M.7.7. with β-glucosidase suplementation							
Untreated (U/g)	1.5 ± 0.1	0.3 ± 0	3.5 ± 0.3	3.5	11.2 ± 0.3	1.8	0.5
Untreated (mg enzyme/g)	1.6 ± 0.1	0.7 ± 0.1	6.5 ± 0.5	5.5	18.2 ± 1.6	4.0	2.5
H_2SO_4 (U/g)	1.5 ± 0	0.2 ± 0	7.8 ± 0.2	6.0	24.0 ± 0.9	5.0	3.0
H_2SO_4 mg	1.6 ± 0	0.7 ± 0	25.9 ± 1.0	17.5	21.3 ± 1.0	4.5	5.0
Commercial enzyme (Celluclast)							
Untreated (U/g)	0.2 ± 0	0.03 ± 0	1.0 ± 0	1.0	24.3 ± 0.5	5.0	2.5
Untreated (mg enzyme/g)	0.6 ± 0	0.2 ± 0	3.1 ± 0	2.5	55.4 ± 1.1	12.0	6.0
H_2SO_4 (U/g)	0.1 ± 0	nd	1.3 ± 0.1	1.0	25.3 ± 3.1	5.0	2.5
H_2SO_4 (mg enzyme/g)	0.4 ± 0.1	0.1 ± 0	4.4 ± 0.5	3.0	64.2 ± 4.1	13.5	7.0

4.Conclusion

- Chemical and estructural differences were detected among samples the sugarcane bagasse and straw submitted to treatments with microwaves in acid and alkaline glycerol solutions;

- The treatment that most influence the structure of the bagasse was that in acid medium and resulted in the hydrolysis of lignin and increase the crystallinity of the fibers;

- The treatment of bagasse with microwave in glycerol and sulfuric acid also resulted in an increased of accessibility of enzymes to polymers with higher reducing sugar yield;

- Sugar cane straw showed a minor chemical and structural change than bagasse when submeted to microwave radiation in both acidic and alkaline conditions;

- Despite the smaller chemical - structural changes, the straw was more easily hydrolyzed by the enzyme preparations;

- There were differences in reducing sugars yield between the enzyme produced by *M. thermophila* and commercial enzyme Celluclast, when used the ratio protein/substrate as a reference for defining the amount of enzyme to be used. The preparation obtained from *M. thermophila* was considerably more efficient in the hydrolysis indicating that the presence of other enzymes besides the cellulolytic complex, has an important role in the lignocellulosic material saccharification.

5.Acknowledgements

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Capítulo V

Conclusões

Conclusões

- O fungo *M. thermophila* produziu altos valores de endoglucanase, xilanase e β-glucosidase usando fontes de carbono e nutrientes minerais de baixo custo;
- O fungo *M. thermophila* apresenta grande estabilidade na produção das enzimas quando submetido a diferentes temperatura, pH e umidade;
- Diferenças químicas e estruturais foram detectadas nas amostras de bagaço e palha de cana de açúcar submetida a tratamento com micro-ondas em soluções de glicerol ácidas e alcalinas;
- O tratamento que mais influenciou na estrutura do bagaço de cana foi com micro-ondas em solução de glicerol ácido e resultou na hidrólise da lignina e aumento da cristalinidade das fibras;
- O tratamento do bagaço com micro-ondas em glicerol e ácido sulfúrico também resultou no aumento da acessibilidade das enzimas aos polímeros, e consequentemente superiores rendimentos de açúcares redutores;
- A palha de cana de açúcar mostrou menores mudanças químicas e estruturais comparada ao bagaço quando submentida a radiação de micro-ondas em ambas as condições ácidas e alcalinas;
- Apesar de menores mudanças químicas e estruturais, a palha de cana foi mais facilmente hidrolisada pelo preparado enzimático;
- Não foram observadas diferenças no rendimento de açúcares redutores entre a enzima produzida por *M. thermophila* e a enzima comercial Celluclast, quando utilizado a taxa de proteína/substrato como referência para a definição da quantidade de enzima a ser utilizada. A preparação obtida a partir de *M. thermophila* foi consideravelmente mais eficaz na hidrólise, indicando a presença de outras enzimas, além do complexo de celulase.

Apêndice I

Pretreatment of sugarcane bagasse with microwaves irradiation and its effects on the structure and on enzymatic hydrolysis

Pretreatment of sugarcane bagasse with microwaves irradiation and its effects on the structure and on enzymatic hydrolysis

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Abstract

This paper refers to the new proposal of pre-treatment of sugarcane bagasse with microwave associated to glycerol, seeking greater release of fermentable sugars during enzymatic hydrolysis. The residue was subjected to microwave irradiation for 5 min with distilled water, phosphoric acid (pH 3.0) and glycerol (100%) before being enzymatically hydrolyzed using cellulase enzyme extract *Myceliophthora thermophila* M.7.7. and the commercial enzyme cocktail Celluclast 1.5 L. A variety of analyses including measurement of BET surface analysis, MET, TGA, DTG, DSC, ATR-FTIR and PAD-HPLC were used to facilitate the understanding of the physical and chemical characteristics of the solid fraction resulting from pre-treatment. Infrared spectra of untreated and treated bagasse in microwave irradiation and glycerol showed significant differences in the regions 1635, 1600 and 1510 related vibration of the aromatic ring, and the band at 1100 cm^{-1} is attributed to an overlap of C-O-H elongation of primary and secondary alcohols and at 980 cm^{-1} to stretching of glycosidic linkages C-O-C. The thermal analysis showed that the bagasse treated in a microwave irradiation and glycerol has higher thermal stability compared to the untreated bagasse. The experimental results indicated that 5.4 and 11.3% w/w of lignin and xylan fractions, respectively, are degraded after pretreatment of bagasse in microwave heating with glycerol. The highest yields of hydrolysis of hemicellulose (22.4%) and cellulose (40.2%) w/w were obtained in the reaction mixture containing the enzyme and Celluclast commercial cane bagasse treated in a microwave irradiation and glycerol after 24 h of incubation. The association of microwave and glycerol is a new alternative to deconstruction of lignocellulose structure.

Keywords: Bioenergy, cellulosic ethanol, enzymatic hydrolysis, sugarcane bagasse.

1. Introduction

In 2012, Brazil has been producing around 580 million tons of sugar cane and 174 million ton of bagasse, a solid waste resulting from juice extraction. A part of this material was burned for electricity generation and the remaining 16 million tons of dry bagasse have no direct use. Considering the high carbohydrate content of bagasse, it is an attractive raw material for cellulosic ethanol production where projected production of ethanol could be 3 billion liters per year [1]. However there is no economically feasible process to hydrolyze biomass for releasing sugar and this approach is a great challenge to modern science [2].

Several physical-chemical methods have been investigated for this purpose including steam and ammonia fiber explosion [3], hydrothermal method [4], peroxidation [5], acid hydrolysis with concentrated or diluted sulphuric, hydrochloric, phosphoric or paracetic acid [6,7,8,9], alkaline hydrolysis [10] the organosolv process [11,12], ionizing radiation [13], ultrasound [14] and microwave radiation [15], but the pretreatments need to be done in order to prevent sugar degradation and minimize the consequent formation of toxic derivatives.

There is no report about association of microwave and glycerol as a pretreatment of lignocellulosic material and it is a safe and efficient way for quickly heating sugarcane bagasse to 290 °C at atmospheric pressure and can be applied to the fibers in continuous or batch processes. The effect of microwave irradiation on the sugarcane bagasse impregnated with glycerol at atmospheric pressure was investigated using FTIR-ATR, BET surface analysis, thermal analysis (DSC, TG and DTG) and the analysis of its centesimal composition (lignin, cellulose, hemicellulose and ash content), as well as related to the sugarcane bagasse digestibility by hydrolytic enzymes from a newly isolated thermophilic fungus *Myceliophthora thermophila* M.7.7, and the commercial enzyme cocktail Celluclast 1.5 L.

2. Materials and Methods

2.1. Microwave treatment of sugarcane bagasse

Dried sugarcane bagasse was ground to obtain a powder of 1-3 mm mesh size and 10 g of this material was immersed in 30 mL of distilled water, dilute phosphoric acid (pH 3.0) or glycerol (100%) for 24 h and after, transferred to a 250 mL round-bottom flask inside the microwave oven and connected to a spinning reflux condenser on the top of the microwave oven. Samples were irradiated at 2450 MHz for 5 min. The temperature was measured using an infrared thermometer at the end of this process. After microwave irradiation, 30 mL of distilled water was added to the material, shacked and filtered. The liquid was used for determining the amount of reducing sugars and phenolic compounds. The solid material was dried at 60 °C and used in assays for fiber analysis and enzymatic hydrolysis.

2.2. Analytical chemical procedures

For lignin determination, the dry bagasse samples (0.3 g) were shaken in 3.0 mL of sulphuric acid 72% (150 rpm; 30 °C; 30 min) and, after 84 mL of distilled water was added to the flask, the sample was autoclaved for 1 h (121 °C) and filtrated on paper. Soluble lignin from this filtrate was evaluated directly at λ=240 nm, using gallic acid as the standard. Hydrolyzed cellulose and hemicellulose were quantified by the amount of sugar content released (ionic chromatograph). The material retained on the filter paper was dried at 105 °C for dry weight determination and burned (500 °C; 4 h) for ash quantification. These data were used to calculate the amount of insoluble lignin [16].

Glucose, xylose, galactose, arabinose, xylobiose and cellobiose were quantified using an ICS 5000 Dionex HPAEC-PAD ionic chromatograph with anionic column CarboPac PA-1. Solvent A (deionized water); B (500 mM NaOH) and C (300 mM sodium acetate with 150 mM NaOH). The elution (1.0 mL/min) was performed from 0 to 14 min in isocratic mode with 95.2% A; 4.8% B and 0% C, and then changed to gradient mode for 60% A; 20% B and 20% C at 26 min. Total reducing

103 sugars and phenolic compounds released were quantified according to the methods from Somogy-
104 Nelson [17] and Folin-Ciocalteu [18], respectively.
105
106 *2.3. Analytical physical procedures*
107 Fourier transformed infrared (FTIR) spectra were recorded in the range from 600 to 4000
108 cm^{-1} on a Perkin Elmer spectrophotometer Spectrum Two fitted with ATR device. Approximately
109 0.1 g of dry bagasse were compressed at 5 ton/cm^2 to form flat discs and placed on the ATR crystal
110 device with a pressure of 90 N/cm^2. All measurements were performed 5 times at different points of
111 the disks of each sample and the final spectrum was generated as a mean of them.
112 Thermal gravimetric (TG and DTG) curves were recorded in a Perkin-Elmer TGA-4000
113 thermogravimetric balance. About 8 mg of dried sugarcane bagasse was placed in a ceramic sample
114 holder and heated at 50 °C/min under nitrogen atmosphere (20 mL/min) in the temperature range of
115 30-600 °C.
116 Differential Scanning Calorimeter (DSC) curves were recorded in a Perkin Elmer DSC-8000
117 calorimeter using sealed aluminum crucibles containing near to 2.0 mg of dry samples and an empty
118 aluminum crucible at the reference side. Nitrogen was used as a purging gas (20 mL/min) in the
119 temperature range of 100-275 °C at a heating rate of 25 °C/min.
120 For BET surface area measurements, samples of dry bagasse were first outgassed at 200 °C
121 for 3 h and the isotherms of nitrogen adsorption-desorption were recorded at liquid nitrogen
122 temperature on a Gemini VII Surface Area and Porosity Analyzer (Micrometrics Instrument Co.).
123
124 *2.4. Transmitted electron microscopy (TEM)*
125 The fibers were fixed in 2.5% glutaraldehyde and 4% *p*-formaldehyde in a phosphate buffer
126 (0.1 M, pH 7.3) for 24 h. The mixture was then washed with phosphate buffer and post-fixed in
127 osmium tetroxide in a 1% phosphate buffer for 2 h. The material was washed with distilled water
128 and contrasted block with 0.5% uranyl acetate in water for 1 h. The dehydration was performed in

129 an ascending series of acetone solutions followed by saturating the product with a mixture of
130 Araldite® and acetone (1:1) for 12 h. Finally, the block is submerged in pure Araldite® resin to
131 finalize the hardening process. The semi-thin slices obtained from the contrasted blocks were
132 stained with a mixture of methylene blue and blue II 1% borax (1:1) and examined under a light
133 microscope to select blocks that exhibited the optimal characteristics for this study. Ultrathin
134 sections were obtained from the selected blocks and were stained with a uranyl acetate saturated
135 alcoholic solution, followed by staining with lead citrate. The materials were analyzed and
136 photographed by TEM, using a model CM-100 (Philips).
137
138 *2.5. Enzyme hydrolysis*
139 The samples of pre-treated bagasse (0.1 g) were transferred to glass flasks with rubber
140 stoppers containing 20 mL of a mixture of acetate buffer (pH 5.0, 0.1 M) and endoglucanase, β-
141 glucosidase and xylanase (47.0, 1.0 and 220.0 U/g dry bagasse, respectively) obtained by
142 *Myceliophthora thermophila* M.7.7 cultivation [19]. Commercial enzyme solution Celluclast 1.5 L
143 (Novozymes) was used in two different proportions: 47.0 U/g endoglucanase, 14.6 U/g β-
144 glucosidase and 7.3 U/g xylanase; 500.0 U/g endoglucanase, 156.6 U/g de β-glucosidase and 78.3
145 U/g xylanase. The reaction mixtures were incubated at 55 ºC for 24 h under agitation of 150 rpm,
146 filtered and the liquid used for qualitative and quantitative sugar analysis (Somogy-Nelson [17] and
147 ionic chromatography).
148
149 **3. Results and Discussion**
150 *3.1. Compositional analysis of pretreated solids and hydrolyzed*
151 The sugarcane bagasse (untreated dry bagasse) presented 46.9% of cellulose, 16.3% of
152 hemicellulose and 27.1% (w/w) of lignin (Table 1) and, after microwave pretreatment with glycerol,
153 the cellulose fraction had risen to 59.5 % (m/m) in contrast to a reduction in the xylan and the lignin
154 content (10.9, 15.8 %, respectively).

No significant changes in the polysaccharides and lignin were observed when replacing glycerol by water or aqueous H_3PO_4 (pH 3.0) in the microwave treatment. These data showed that the presence of glycerol was an overriding factor in the lignin hydrolysis. Pretreatment of lignicellulosic material in microwave irradiation (microwave intensity 680 W, irradiation time 24 min and substrate concentration 75 g/L) without chemicals, according to Ma et al. [20] didn't result in reduction of the lignin content. On the other hand, Chen et al. [21] observed an increase in the lignin fraction after pre-treatment of sugar cane bagasse with microwave irradiation associated with sulphuric acid 0.2 M (5 min of irradiation at 130 °C).

3.2. ATR-FTIR analysis

Chemical differences between untreated sugarcane bagasse and the bagasse irradiated with microwaves in the presence of glycerol were observed by ATR-FTIR (Fig. 2). There are attenuation bands attributed to lignin such as the stretching of the carbonyl group (1728 cm^{-1}), vibration of the aromatic ring (1635, 1600, 1510 cm^{-1}) [22, 23], syringyl group (1374 cm^{-1}, Sahoo et al. [24]) and in the C-H out of plane in the *p*-hydroxyphenyl propane units (833 cm^{-1}, Hoareau et al. [25]; Ciobanu et al. [26]). These alterations in the FTIR-ATR spectra are consistent with the lignin removal detected by chemical analysis (Table 1).

However, the biggest alteration was observed in the bands associated with cellulose and hemicellulose core structures such as those attributed to an overlap of C-O-H elongation of primary and secondary alcohols (at 980 cm^{-1}), C-O-C stretching of glycosidic linkages (at 1100 cm^{-1}), vibration of the ring C-O-C in hemicellulose (at 1050-1170 cm^{-1}) [27,28]. The band at 1170 cm^{-1} is typical of the arabinosyl side chains and its low intensity suggest that the hemicellulose was more sensitive to treatment than cellulose. These data are in accordance with Hendriks and Zeeman [29] and are corroborated by the chemical analysis which shows a decrease in the hemicellulose content (Table 1).

180 The absorption bands at 3440 and 2906 cm^{-1}, assigned as a stretching of the hydroxyl groups
181 present in cellulose, hemicellulose and lignin, and the axial deformation of C-H, respectively,
182 present in whole bagasse [30] did not show significant changes. This suggested that the general
183 structure was kept unchanged.
184 These data allow the inference that the microwave and glycerol treatment remove lignin and
185 hemicellulose preserving the cellulose structure.
186
187 *3.3. Thermal and surface area measurements*

188 The DSC curves of the control and bagasse treated with microwave and glycerol showed
189 similarities in the endothermic peak near 100 °C from evaporation water (Fig. 2). The exothermic
190 peak (T_{onset}) from treated bagasse in which extractives - part lignin and hemicellulose - were
191 removed, occurred at 203 °C versus the 195°C from the untreated bagasse. The energy absorbed in
192 the thermal degradation process (ΔH_{deg}) was reduced by 26.5% (from 103.7 ± 1.4 to 76.2 ± 4.1 J/g).
193 This suggests that increasing the proportion of crystalline cellulose resulted in the higher thermal
194 stability.
195 The TGA curve (Fig. 3A) shows that treated bagasse generated a higher amount of ashes
196 than untreated bagasse probably due to loss in volatile material during the treatment. The DTG
197 curves (Fig. 3B) shows different patterns of weight loss for the two materials. The control bagasse
198 lost mass at temperatures between 250 to 325 °C which refers to extractives that that have been
199 removed from the treated bagasse. The shoulder observed at 350 °C for the control does not occur
200 in the treated bagasse and is attributed to decomposition of lignin, hemicellulose and cellulose [30].
201 This data reinforce the hypothesis that hemicellulose and part of the lignin is removed by the
202 treatment. Three thermal events of weight loss during sugarcane bagasse heating have been
203 described by Mothé and Miranda [30]: (***i***) elimination of moisture, (***ii***) elimination of organic
204 extractives, such as fats, waxes, alkaloids, glycosides and terpenoids, and (***iii***) decomposition of
205 lignin, hemicellulose and cellulose. According to these authors the thermal degradation of these

206 polysaccharides occurs at temperatures very close to each other and is not possible to detect
207 separated peaks.

208 The surface area measurement showed that the bagasse surface did not change significantly
209 after irradiation with microwaves in the presence of glycerol (0.86 m^2/g for treated bagasse and 0.81
210 m^2/g for control) in discordance with Chen et al. [21] who observed an increase in surface area after
211 pre-treating sugar cane bagasse in microwave resulting from the fragmentation of bagasse at high
212 temperatures.

213

214 *3.4. Enzyme hydrolysis of pretreated cane bagasse*

215 Data from Fig. 4 show that the bagasse treated with glycerol and microwave was easily
216 hydrolyzed by enzyme solutions, releasing around 12 times more reducing sugar than the control
217 and the bagasse irradiated in presence of water or phosphoric acid. Using a commercial enzyme,
218 40% w/w hydrolyzation of the cellulose was obtained, similar to that reported by Chen et al. [15]
219 with sugar cane bagasse irradiated with microwave in the presence of 0.005M sulphuric acid. These
220 results could be attributed to lower lignin content of this material (Table 1).

221 It is important to note that, although the commercial enzyme and the enzyme solution
222 obtained from *M. thermophila* cultivation had the same level of endoglucanase activity, the first one
223 had a higher β-glucosidase activity (14 against 1 U/mL). Although had been used a quantity of 10
224 times of enzymes, there was no significant increase in the releasing of sugar.

225 The qualitative analysis of the sugar (Table 4) revealed that the major content of the sugar
226 was glucose but only 4% of cellulose was broken down by the enzyme solution from *M.*
227 *thermophila.* When commercial enzyme was used, 40% w/w of the cellulose from glycerol treated
228 bagasse was hydrolyzed. Since the endoglucanase activity was the same in both assays the lower
229 glucose released could be attributed to low β-glucosidase activity.

230

231

232 *3.5. Ultra structural analysis by transmission electronic microscopy (TEM)*

233 It was studied untreated and microwave and glycerol treated sugar cane. In the untreated
234 bagasse (Fig. 5A-D) it was possible observed preserved structures the primary wall (PW),
235 secondary wall (SW), middle lamella (ML), plasmodesmata (PD) and pit membrane (MP) in
236 contrast to treated bagasse in which was observed an important change in the electronic density
237 besides secondary wall loosing (Fig. 6A-C), flocculation and amorphous residues on the material
238 (Fig. 6A-B) and disruption of the pit membrane and plasmodesmata (Fig. 6D). Similar alteration
239 was observed by Chen et al. [21] using scanning electronic microscopy after treatment of bagasse
240 with microwave in acid and alkali conditions.

241 The changes observed in the secondary wall were consistent with the delignification
242 detected by chemical and physical analysis and could explain the facilitation of enzymatic
243 hydrolysis of the treated bagasse since the cellulose and part of hemicellulose were preserved
244 during the treatment.

245 As microwave treatment in the absence of glycerol (Table 1) and glycerol without
246 irradiation (data not shown) didn't cause any significant alteration in the bagasse, two explanations
247 are possible. One would be the higher temperature reached in the glycerol suspension (around 240
248 ºC in 2 min of treatment) as opposed to that observed for water or acid suspension (around 100 ºC),
249 another would be due to the highly polar polyalcohol structure that allows for easy penetration into
250 the lignocellulosic structure providing effective delignification.

251

252 **4. Conclusions**

253 The pre-treatment of sugarcane bagasse with microwave in presence of glycerol enabled the
254 removal of lignin, maintained the cellulose structure and improved enzyme hydrolysis. These
255 results indicate that it is a promising approach for saccharification of lignocellulosic material.

256

257

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Figure legends

Figure 1: FTIR-ATR spectra of bagasse under microwave and glycerol treatment. Dash line= untreated bagasse; continuous line= treated bagasse.

Figure 2: DSC Thermograms of bagasse under microwave and glycerol treatment. Dash line= untreated bagasse; continuous line= treated bagasse.

Figura 3: Thermograms (A) TG (B) DTG of bagasse under microwave and glycerol treatment. Dash line= untreated bagasse; continuous line= treated bagasse.

Figure 4: Enzymatic hydrolysis of untreated and microwave treated sugar cane bagasse. Reducing sugar from hydrolysis using (U/g) 47.0 of endoglucanase, 220.0 of xylanase and 1.0 of β-glucosidase from *Myceliophthora thermophila* M.7.7 ( ); 47.0 of endoglucanase, 7.3 of xylanase and 14.6 β-glucosidase ( ) or 500.0 endoglucanase and 156.6, 78.3 of xylanase of β-glucosidase ( ) of Celluclast 1.5 L (Novozymes) .

Figure 5: Electromicrography from TEM of untreated sugar cane bagasse. (A) general aspect of untreated bagasse (3400X- bar=5 μm); **(B)** preserved cell wall (5800X – bar = 5μm); **(C)** middle lamella (ML), primary wall (PW); secondary wall (SW). 13500X – bar = 2 μm; **(D)** pit membrane (MP); plasmodesmata (PD). 34000X – bar = 2 μm.

Figure 6: Electromicrography from TEM of microwave plus glycerol treated sugar cane bagasse. (A) general aspect. middle lamella (ML), primary wall (PW); secondary wall (SW). Observe floculation of secondary wall and electron density changes. 23000 X - bar = 200 nm; **(B)** loosing and disruption of secondary wall (thick arrow) and primary wall (thin arrow). 11000 X - bar – 500 nm; **(C)** middle lamella (ML); primary wall (PW); secondary wall (SW). 13000 X – bar= 500 nm; **(D)** disruption of membrane pit (MP) and plasmodesmata (PD). 18500 X – bar = 500 nm.

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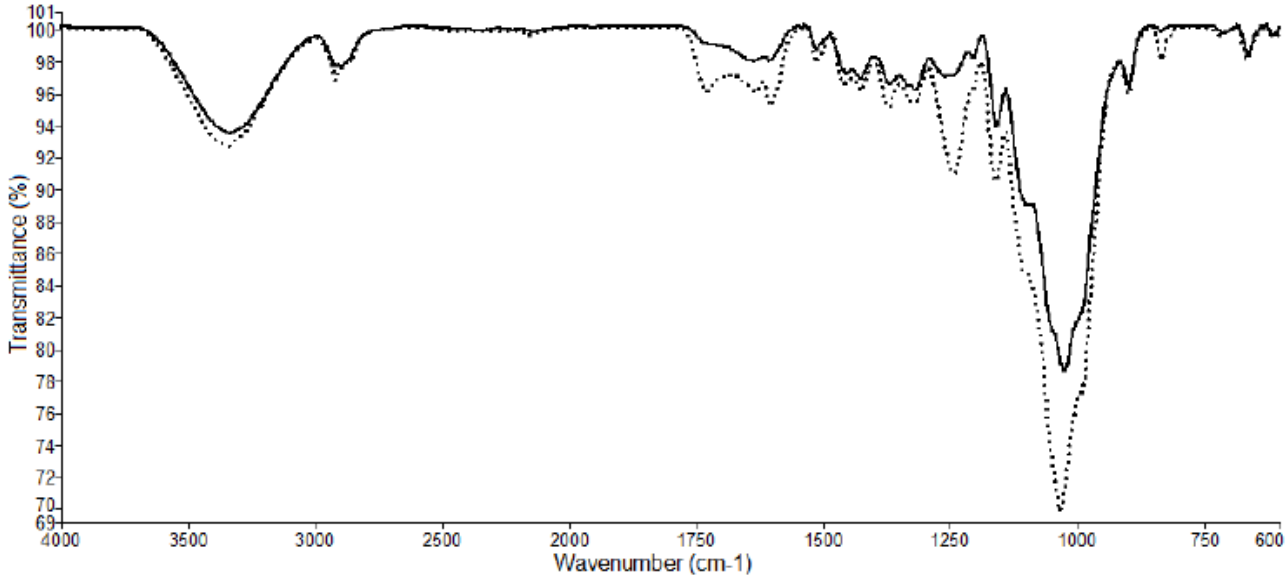
Transmittance (%)
101
100
98
96
94
92
90
88
86
84
82
80
78
76
74
72
70
69
4000
3500
3000
2500
2000
1750
1500
1250
1000
750
600
Wavenumber (cm-1)

391 **Figure 1**

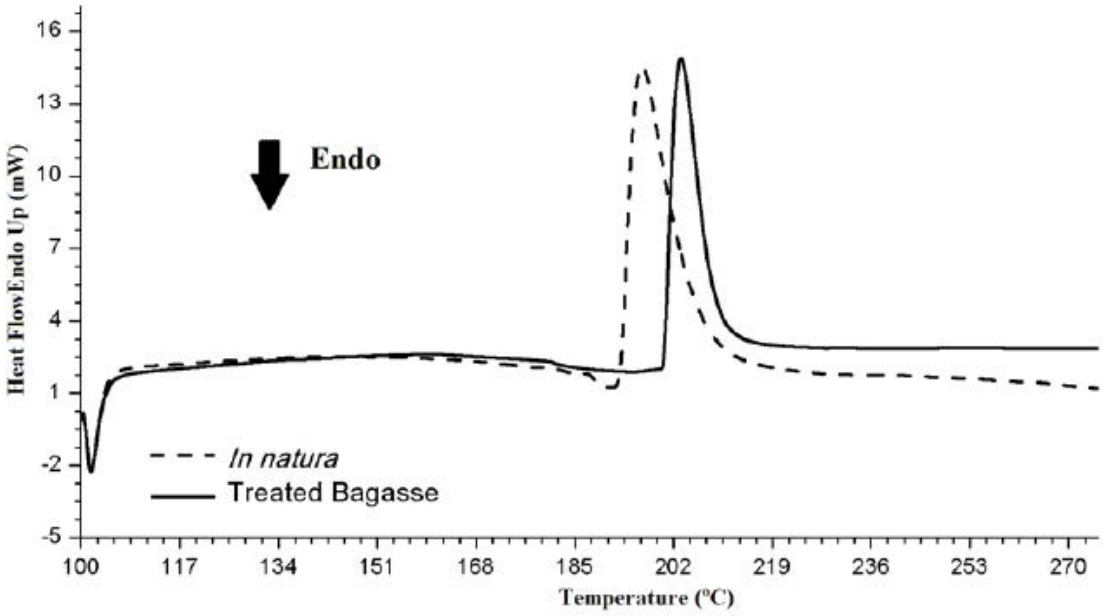
Endo
Heat FlowEndo Up (mW)
16
13
10
7
4
1
-2
-5
100
117
134
151
168
185
202
219
236
253
270
Temperature (°C)
In natura
Treated Bagasse

Figure 2

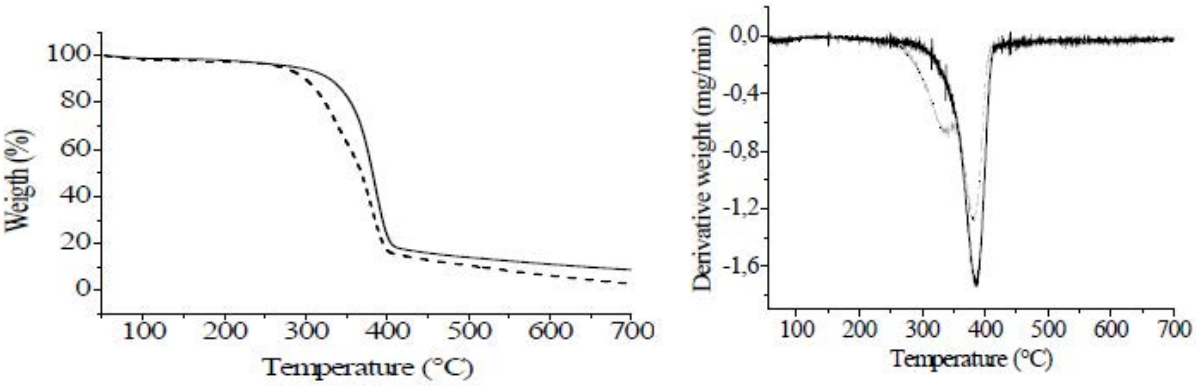
Weigth (%)
100
80
60
40
20
0
100 200 300 400 500 600 700
Temperature (°C)
Derivative weight (mg/min)
0,0
-0,4
-0,8
-1,2
-1,6
100 200 300 400 500 600 700
Temperature (°C)

Figura 3

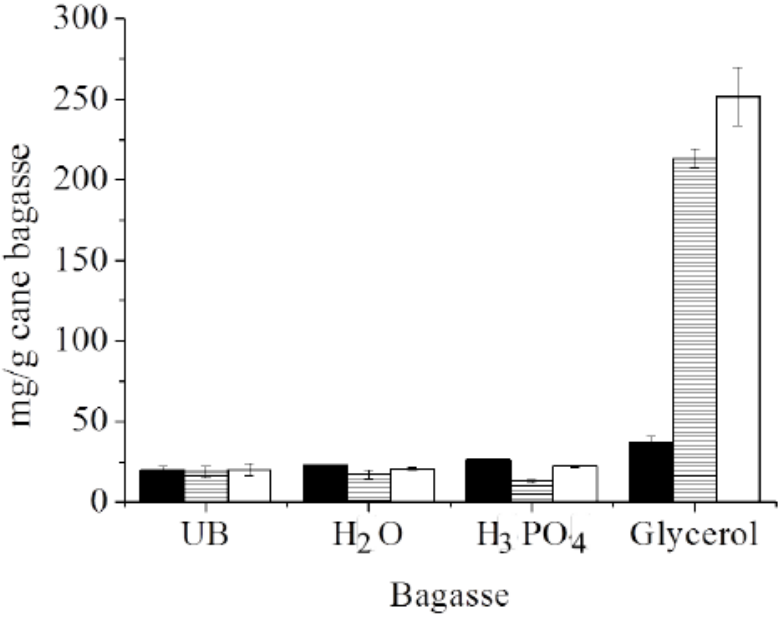
300
250
200
150
100
50
0
mg/g cane bagasse
UB
H2O
H3PO4
Glycerol
Bagasse

Figure 4

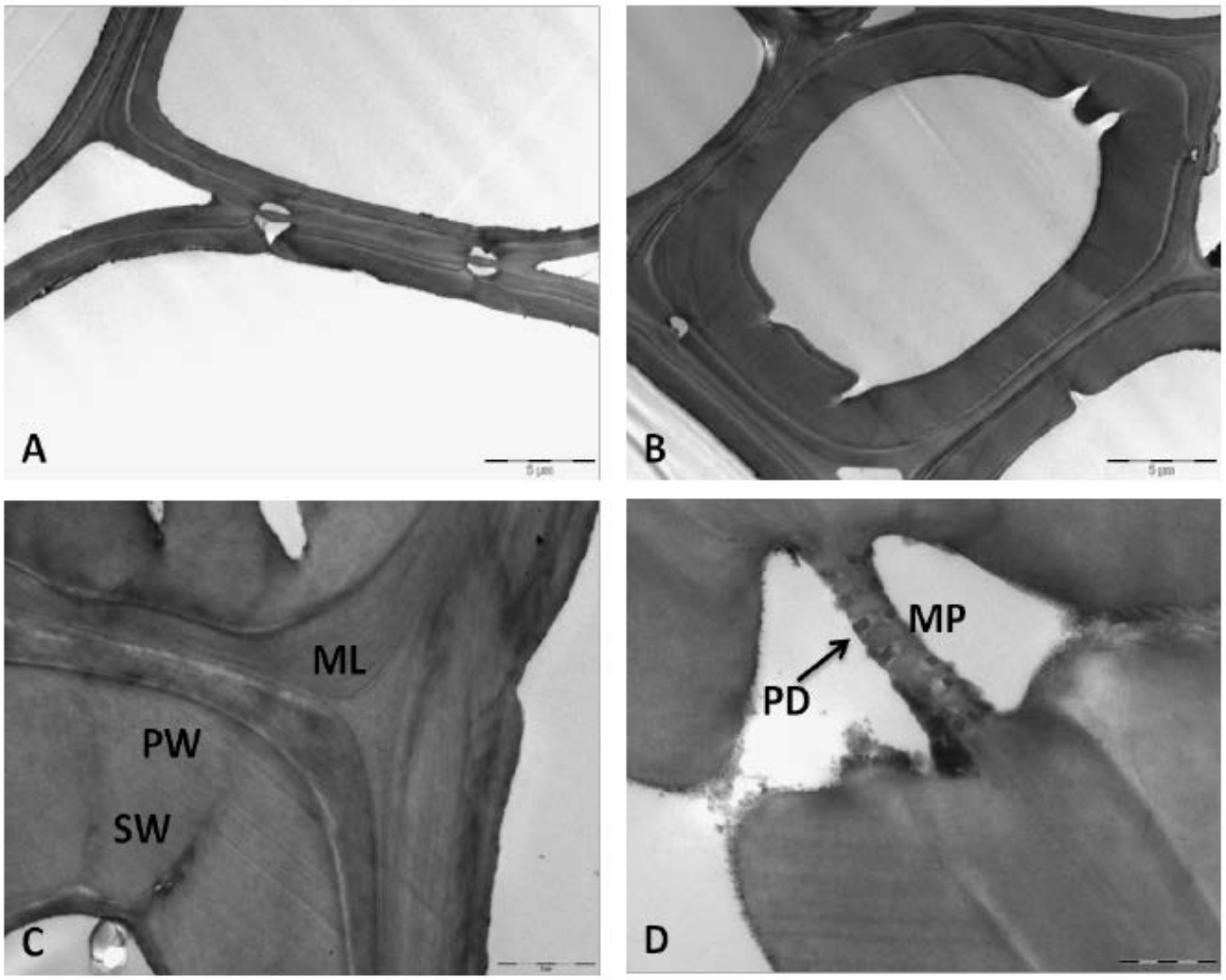

Figure 5

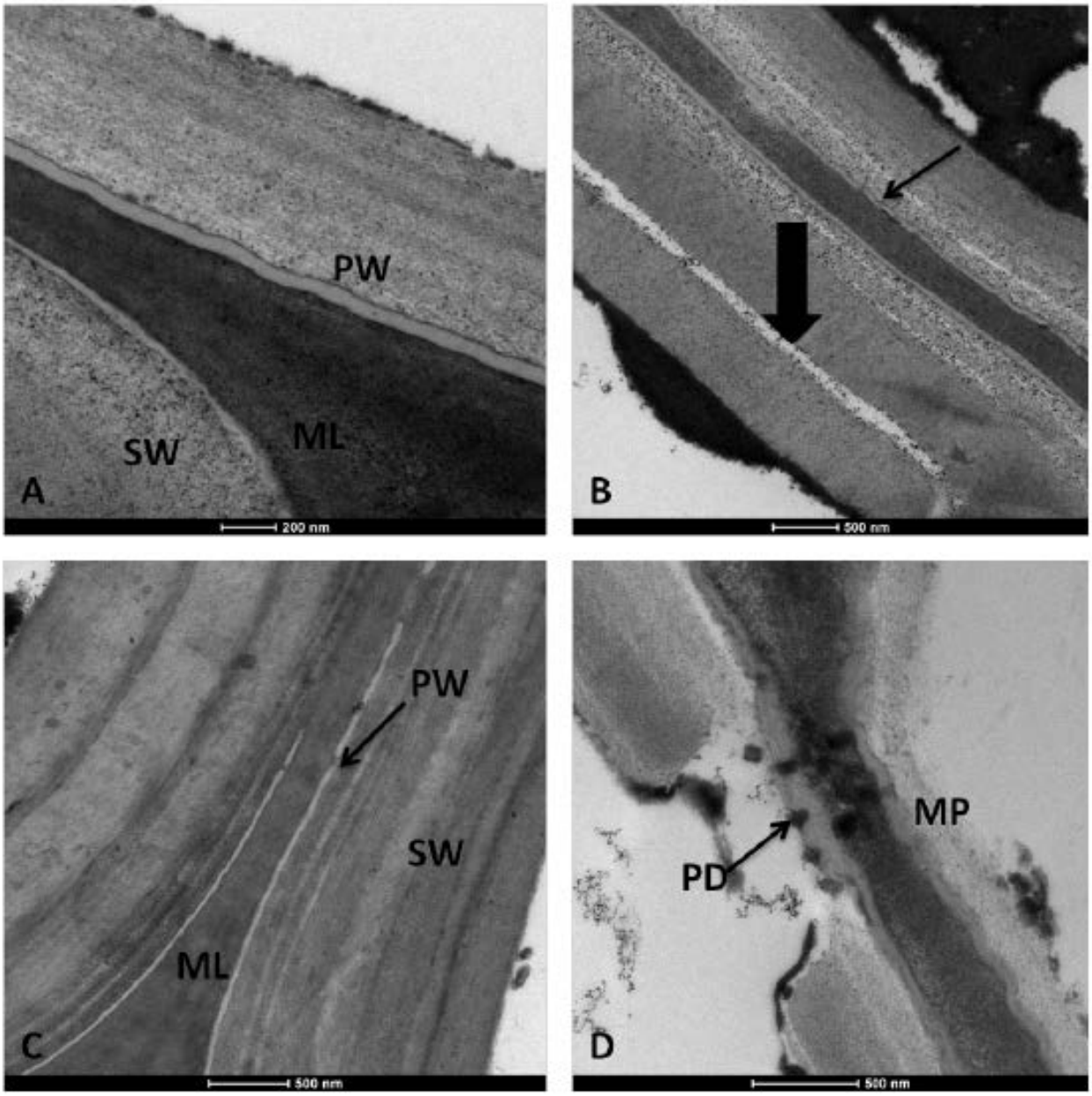

Figure 6

Table 1

Polysaccharides and lignin contents of control and pre-treated sugar cane bagasse and in the liquid fraction.

Sample	Solid fraction (%)*			Liquid fraction (%)*	
	cellulose	hemicellulose	lignin	total reducing sugar	total phenol
Untreated bagasse	46.9 ± 0.5	16.3 ± 0.1	27.1 ± 0.9	-	-
H_2O	46.5 ± 1.4	15.9 ± 1.1	23.5 ± 1.5	0.01 ± 0.0	0.06 ± 0.0
H_3PO_4	47.3 ± 0.5	16.4 ± 0.0	22.4 ± 1.1	0.01 ± 0.0	0.05 ± 0.0
Glycerol (100%)	59.5 ± 1.5	10.9 ± 2.1	15.8 ± 1.0	0.09 ± 0.0	1.40 ± 0.1

* (w/w)

Table 2
Carbohydrates released by enzyme hydrolysis of sugar cane bagasse

Enzyme from *Myceliophthora thermophila* M.7.7									
Pre-treatment	Hemicellulose sugars (mg/g)				% Hemicellulose hydrolysis [c]	Cellulose sugars (mg/g)		% Cellulose Hydrolysis [d]	Bagasse Hydrolysis (%) [e]
	Arabinose	Galactose	Xylobiose	Xylose		Cellobiose	Glucose		
Glycerol	2.4	nd*	10.7	5.1	16.7	0.6	23.4	4.0	4.2
H_3PO_4	2.2	0.2	9.1	4.4	9.6	0.5	20.8	4.5	3.7
H_2O	2.3	0.1	7.0	3.4	8.9	0.6	18.9	4.2	3.3
Bagasse control[a]	1.9	nd	6.7	3.5	7.5	0.5	17.9	3.9	3.0
Enzyme control[b]	1.0	1.2	2.2	0.6	--	2.8	6.2	--	--

Commercial enzyme (Celluclast)									
Pre-treatment	Hemicellulose sugars (mg/g)				% Hemicellulose hydrolysis [c]	Cellulose sugars (mg/g)		% Cellulose hydrolysis [d]	Bagasse hydrolysis (%) [e]
	Arabinose	Galactose	Xylobiose	Xylose		Cellobiose	Glucose		
Glycerol	nd	nd	4.2	20.3	22.4	2.0	237.5	40.2	26.4
H_3PO_4	0.2	nd	nd	1.6	1.1	0.5	21.0	4.5	2.3
H_2O	0.3	nd	0.9	1.8	1.3	0.9	22.6	4.7	2.5
Bagasse control[a]	0.4	nd	0.7	2.6	2.7	0.3	27.5	5.9	3.1
Enzyme control[b]	nd	nd	nd	nd	-	nd	4.5	-	-

*nd=not determined
[a] Bagasse control=not treated bagasse
[b] Enzyme control =sugar present in crude enzyme solution
[c] released sugar by residual hemicellulose content (w/w)
[d] released sugar by cellulose content (w/w)
[e] released sugar by dry bagasse (w/w)