UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

PATRÍCIA FÉLIX ÁVILA

INFLUÊNCIA DE DIFERENTES PRÉ-TRATAMENTOS NA HIDRÓLISE ENZIMÁTICA DA BIOMASSA DA CANA-DE-AÇÚCAR PARA A PRODUÇÃO DE AÇÚCARES FERMENTESCÍVEIS E XILO-OLIGOSSACARÍDEOS

INFLUENCE OF DIFFERENT PRETREATMENTS IN ENZYMATIC HYDROLYSIS OF SUGARCANE BIOMASS FOR THE PRODUCTION OF FERMENTABLE SUGARS AND XILOOLISOSSACHARIDES

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Orientação: Profa. Dra. Rosana Goldbeck

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"Só se pode alcançar um grande êxito quando nos mantemos fiéis a nós mesmos. "

Friedrich Nietzsche

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RESUMO GERAL

Os resíduos agroindustriais lignocelulósicos tais como os provenientes do processamento da cana-de-açúcar podem ser uma fonte potencial de muitos compostos de interesse industrial, tais como compostos antioxidantes, xilo-oligossacarídeos (XOS), bem como fonte de polissacarídeos para a liberação de monossacarídeos fermentescíveis para a produção de biocombustíveis. Neste contexto um estudo exploratório comparativo entre a aplicação de diferentes pré-tratamentos como líquido iônico e ácido sulfúrico diluído, foi realizado de modo a analisar e entender as alterações da composição química e os efeitos das enzimas comerciais utilizadas no processo de hidrólise enzimática da biomassa de cana-de-açúcar (bagaço + palha) 50% (m/m). Planejamentos experimentais foram realizados a fim de determinar as concentrações enzimáticas ideais para produção de açúcares fermentescíveis (glicose e xilose) e XOS. A partir dos resultados encontrados, constatamos que a composição química da mistura de biomassa de cana-de-açúcar (bagaço + palha) após diferentes estratégias de pré-tratamento foram bastante similares. No entanto os pré-tratamentos com líquido iônico acetato de 1-etil-3-metil-imadazólio ([Emin][Ac]) (Iolitec®) proporcionaram maiores porcentagens de remoção de lignina total e, conseqüentemente, as biomassas pré-tratadas apresentaram valores de rendimentos de hidrólise enzimática ligeiramente maiores em glicose e xilose do que as amostras pré-tratadas com ácido sulfúrico diluído. Verificou-se também que as enzimas α-L-arabinofuranosidase (*Aspergillus ninger*), β-xilosidase (*Bacillus pumilus*) (Megazyme®), endo-1,4-xilanase (NS50030) e celulase (NS50013) (Novozyme®), nas concentrações estudadas (10 $mg/g_{substrato}$, 9 $mg/g_{substrato}$, 16 $mg/g_{substrato}$ e 22 $mg/g_{substrato}$) respectivamente, proporcionaram altos rendimentos em açúcares fermentescíveis para ambas as estratégias de pré-tratamento estudadas na hidrólise enzimática. Também foi constatado que a ausência da β-glicosidase (Novozyme®-NS50010) não afetou o processo, podendo nesta condição ser removida do meio reacional, diminuindo assim, os custos com as enzimas requeridas. Em relação a produção de XOS foi possível verificar que dentre as enzimas estudadas, apenas as enzimas α-L-arabinofuranosidase e endo-1,4-xilanase obtiveram efeito positivo e significativo em um intervalo de 90% de confiança para os dois tipos de substratos avaliados no planejamento Plackett-Burnan. A partir da realização do delinemamento central composto rotacional (DCCR) foi possível verificar valores de concentrações de XOS totais em torno de 251,12 mg/L e 229,17 mg/L para as amostras pré-tratadas com ácido sulfúrico diluído e com líquido iônico respectivamente. Todos modelos gerados se ajustaram bem aos dados experimentais com porcentagens de variação explicada (R^2) acima de 96%, demonstrando que as alternativas

empregadas são propriamente exeqvíveis para obtenção de concentrações enzimáticas ideais. O pré-tratamento com líquido iônico possibilitou uma redução em até 20% da concentração de endo-1,4-xilanase (NS50036) em relação a concentração empregada para a biomassa pré-tratada com ácido sulfúrico diluído. Por fim esse trabalho possibilitou obter informaçoes que proporcionaram o desenvolvimento técnico-científico da pesquisa brasileira além de cumprir com os requisitos de inovação com potencial aplicação industrial.
Palavras-chave: biomassa da cana-de-açúcar, pré-tratamento; líquido iônico, ácido sulfúrico diluído, hidrólise enzimática, xilo-oligossacarídeos, glicose, xilose.

ABSTRACT

Lignocellulosic agroindustrial wastes such from the processing of sugarcane may be a potential source of many compounds of industrial interest, such as antioxidant compounds, xylooligosaccharides (XOS), as well as source of polysaccharides for the release of monosaccharides for biofuels production. In this context, a comparative exploratory study among the application of different pretreatments as ionic liquid and dilute sulfuric acid was carried out in order to analyze and understand the changes of the chemical composition and the effects of the commercial enzymes used in the enzymatic hydrolysis process of the sugarcane biomass (bagasse + straw) 50% (w/w). Experimental design was performed to determine optimum enzyme concentrations for the production of fermentable sugars (glucose and xylose) and XOS. From the results found, we observed that the chemical composition of the sugarcane biomass mixture (bagasse + straw) after different pretreatment strategies presented similar values. On the other hand, pretreatments with 1-ethyl-3-methyl-imadazolium acetate ([Emin][Ac]) (Iolitec®) provided higher percentages of total lignin removal and hence pretreated samples slight higher yield values in glucose and xylose yields than the pretreated samples with dilute sulfuric acid. The enzymes α-L-arabinofuranosidase (*Aspergillus ninger*), β-xylosidase (*Bacillus pumilus*) (Megazyme®), endo-1,4-xylanase (NS50030) and cellulase (NS50013) (Novozyme®) in the study concentration range (10 mg/$g_{substrate}$, 9 mg/$g_{substrate}$, 16 mg/$g_{substrate}$ and 22 mg/$g_{substrate}$) respectively, provided high yields of fermentable sugars for both pretreatment strategies carried out in the enzymatic hydrolysis. In addition, the absence of β-glucosidase (Novozyme®-NS50010) did not affect the process, which could be removed from the reaction medium, thus reducing costs with required enzymes. Regarding theproduction of XOS, only the enzymes α-L-arabinofuranosidase and endo-1,4-xylanase presented a positive and significant effect in a 90% confidence interval for the two types of evaluated substrate in Plackett-Burnan planning. By conduction experiments central composite rotatable design (CCRD) was possible to verify values of total XOS concentrations of 251.12 mg/L and 229.17 mg/L for pretreated samples with diluted sulfuric acid and with ionic liquid respectively. All generated models fitted well to experimental data with percentages of explained variation (R^2) above 96%, demonstrating that the alternatives employed are properly feasible to obtain optimum enzymatic concentrations. The pretreatment with ionic liquid allowed a reduction of up to 20% in the concentration of endo-1,4-xylanase (NS50036) in relation to the concentration used for the pretreated biomass with dilute sulfuric acid. Finally, this work made it possible to obtain information that provided the technical-scientific

development of the Brazilian research, besides complying with the requirements of innovation with potential industrial application.

Keywords: sugarcane biomass, pretreatment, ionic liquid, dilute sulfuric acid, enzymatic hydrolysis, xylooligosaccharides, glucose, xylose.

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LISTA DE ABREVIATURAS E SIGLAS

2G - Etanol de segunda geração

AFEX- Explosão com amônia *(Ammonia Fiber Explosion)*

ANOVA - Análise de Variância

BG - β-glicosidase

BGU - Unidade de β-glicosidase *(β-glucosidase unit)*

CBH - Celobiohidrolase

CMC - Carboximetilcelulose

CMCase – Atividade de endoglucanase sobre o CMC

DCCR - Delineamento composto central rotacional

DNS - Ácido 3,5-dinitrosalicílico

DSA – Ácido sulfúrico diluído (*Dilute Sulfuric Acid*)

EG – Endoglucanase

([Emim][Ac]) - Acetato de 1-etil-3-metilimidazólio

FPase - Atividade de celulases total sobre o papel de filtro (*Fiter paper activity*)

FPU - Unidade de papel de filtro (*Filter paper unit*)

HPLC – Cromatografia líquida de alta eficiência *(High Performance Liquid Chromatography)*

IL – Líquido iônico (*Ionic Liquid*)

MPIL – Massa pré-tratada com liquido iônico (*Mass Pretreated with Ionic Liquid*)

MPSA- Massa pré-tratada com ácido sulfúrico *(Mass Pretreated with Sulfuric Acid)*

NREL- National Renewable Energy Laboratory

PAD- Detecção Amperométrica Pulsada (*Pulsed Amperometric Detection*)

TB – Banho termostático (*Thermostatic Bath*)

UB – Banho ultrassônico (*Ultrasonic Bath*)

XOS – Xilo-oligossacarídeos

X2- Xilobiose

X3- Xilotriose

X4- Xilotetraose

X5- Xilopentaose

X6- Xilohexaose

SUMÁRIO

CAPÍTULO I: INTRODUÇÃO GERAL E OBJETIVOS

1.1 INTRODUÇÃO GERAL

Grandes excedentes de subprodutos são gerados todos os anos a partir do processamento industrial de matérias-primas agrícolas. A maior parte destes subprodutos são utilizados como alimento para animais ou queimados como uma alternativa de geração de energia. No entanto, os mesmos podem ser uma fonte potencial de muitos compostos interessantes, tais como compostos antioxidantes, xilo-oligossacarídeos, bem como ser fonte de polissacarídeos para a liberação de monossacarídeos fermentsíveis para a produção de biocombustíveis e entre outras biomoléculas (AKPINAR, ERDOGAN, BONSTANCI, 2009).

Os xilo-oligossacarídeos (XOS), podem atribuir diversos efeitos benéficos para a saúde do ser humano, dentre os quais, a prevenção de cáries, a diminuição de níveis séricos de colesterol e o estímulo do crescimento de bactérias benéficas (probióticas) do trato intestinal (VAZQUEZ et al., 2000; HUTKINS et al; 2016). De acordo com Gibson et al. (2004), o seu efeito a saúde está relacionado com suas propriedades físico-químicas, por serem moderadamente doces, estáveis em uma ampla faixa de pH e temperatura e conferirem características organolépticas aos alimentos. Assim com o desenvolvimento de uma metodologia eficiente para a liberação de XOS a partir de materiais lignocelulósicos, como o bagaço e palha da cana-de-açúcar, espera-se impulsionar o desenvolvimento de diversos estudos relacionados às suas aplicações.

O desenvolvimento de novos processos economicamente rentáveis para o aproveitamento dos componentes da biomassa lignocelulósica, como no caso dos resíduos agrícolas (ex. palha e bagaço de cana-de-açúcar, palha de trigo e resíduos de milho, entre outros) para produção de etanol lignocelulósico, também conhecido como etanol de segunda geração, tem sido um campo de pesquisa bastante promissor, o qual vem exigindo grandes esforços da comunidade científica (SANTOS et al., 2012; ALVIRA et al., 2016). Já que esse combustível pode gerar benefícios tanto ambientais como econômicos, pois além de não competir pelo uso de terras destinadas a produção de alimentos, é um suprimento energético seguro e limpo (MACRELLI et al., 2012).

A biomassa da cana-de-açúcar, é uma matéria-prima barata, abundante e renovável que pode ser empregada para a produção sustentável de biocombustíveis, bioenergia e biomoléculas de alto valor agregado (GONÇALVES et al., 2012). O bagaço e a palha são os dois tipos de subprodutos fibrosos obtidos após o processamento da cana-de-açúcar. A proximidade às usinas, e a consequente redução de custos com transporte, contribui para que os mesmos sejam os materiais lignocelulósicos mais atraentes no Brasil (FERREIRA-LEITÃO

et al., 2010a). Para cada tonelada de cana processada são gerados cerca de 140 kg de palha e 140 kg de bagaço em base seca (HASSUANI et al., 2005). Dessa maneira, estima-se que o bagaço e a palha da cana-de-açúcar contêm dois terços da energia armazenada na planta (PIPPO et al., 2011).

A estreita associação e complexidade do complexo carboidrato-lignina da biomassa vegetal é um dos principais desafios na despolimerização visando a obtenção de açúcares fermetescíveis. Neste contexto, muito tem sido investido em tecnologia para tornar esse processo economicamente viável, como o desenvolvimento de técnicas de pré-tratamento eficiente e a otimização de coquetéis enzimáticos para a desconstrução da parede celular (BANERJEE et al., 2010a, BRANDT et al., 2013).

O pré-tratamento é uma importante etapa que antecede a hidrólise enzimática, visto que materiais lignocelulósicos nativos são altamente resistentes à hidrólise enzimática devido a presença da parede celular, que dificulta o acesso das enzimas ao substrato gerando rendimentos muito baixos de açúcares na hidrólise enzimática. Dessa forma, a etapa de pré-tratamento tem a função de desordenar a rígida estrutura da biomassa e tornar a celulose e a hemicelulose mais acessíveis às enzimas (SAHA et al., 2013). Assim ao longo dos anos muitas técnicas de pré-tratamento foram desenvolvidas com intuito de se obter melhores resultados de sacarificação na hidrólise enzimática, bem como gerar menos inibidores durante o processo como ácido acético, furfural, hidroximetilfurfural, entre outros (ISLAN et al., 2018).

O pré-tratamento com ácido diluído é o método que convencionalmente se aplica em processos de caráter industrial. O mesmo é um método muito atrativo industrialmente, por ser um produto de baixo custo de aquisição comparado às demais opções, bem como pela sua capacidade de não só solubilizar a hemicelulose, mas também convertê-la em açúcares fermentescíveis, o que elimina ou reduz a necessidade de se utilizar hemicelulases nos complexos enzimáticos durante a etapa de hidrólise enzimática (SAHA et al., 2005). Entretanto esse método possui diversos aspectos indesejáveis, dentre os quais: a ocorrência de corrosão nos equipamentos, o que implica altos investimentos em equipamentos resistentes à corrosão; demanda de um alto valor energético com o processo e com etapas de recuperação do ácido; liberação de efluentes tóxicos; mudanças bruscas no pH, além da geração de subprodutos que atuam como inibidores das etapas de sacarificação e fermentação (GALBE; ZACCHI, 2007).

Nesse contexto, o emprego de líquidos iônicos na etapa de pré-tratamento da biomassa lignocelulósica apresenta-se como uma interessante alternativa, pois além de não apresentar as desvantagens descritas para o pré-tratamento com ácido diluído, os mesmos vêm

despertando significante interesse principalmente devido à denominação desses reagentes como ambientalmente amigáveis, ou de caráter "verde". Por essas características, podem ser considerados potencialmente favoráveis na etapa de pré-tratamento da biomassa, pois substituem os solventes orgânicos tradicionais gerando menos inibidores como ácido acético, furfural e hidroximetilfurfural durante o processo (GHANDI, 2014). Entretanto, esse termo muitas vezes é utilizado de forma errônea, pois é muito importante ressaltar que os líquidos iônicos não são intrinsecamente "verdes", pois alguns são de fato extremamente tóxicos, porém eles podem ser projetados de forma a não serem tão nocivos ao meio ambiente, abrindo proporcionando o desenvolvimento de processos químicos sustentáveis (DA SILVA, 2013).

A hidrólise dos polissacarídeos contidos em materiais lignocelulósicos requer uma grande variedade de enzimas com diferentes especificidades. Atualmente as misturas enzimáticas disponíveis são complexas e parcialmente definidas, dentro de uma mistura de mais de 80 proteínas (NAGEDRAN et al.,2009). Estudos que se propõe a definir quais enzimas, e suas proporções, são importantes para hidrólise de compostos lignocelulósicos e poderiam resultar em um design racional de misturas mais eficientes e menos onerosas (GOLDBECK et al., 2014). Sabe-se que cada biomassa possuí especificidade peculiar a um dado tipo de pré-tratamento (explosão a vapor, líquidos iônicos, peróxido de hidrogênio), havendo, portanto, a possibilidade de diferentes combinações de pré-tratamento/biomassa (BANERJEE et al., 2010b).

Este trabalho encontra-se dividido em cinco capítulos, sendo que este primeiro capítulo I apresenta a introdução e os objetivos propostos nesta dissertação. No Capítulo II é apresentado uma revisão bibliográfica relacionada com a biomassa lignocelulósica e seus principais constituintes, abordando principalmente a biomassa da cana-de-açúcar e suas principais frações (palha e bagaço) como fonte de polissacarídeos para produção de etanol lignocelulósico; os tipos de pré-tratamentos empregados, discutindo principalmente sobre as estratégias com ácido diluído e com líquidos iônicos; atuação de celulases e hemicelulases na hidrólise enzimática; os desafios na elaboração de misturas enzimáticas, bem como a produção de XOS a partir dessa biomassa. Neste trabalho estudou-se a aplicação de estratégias de pré-tratamentos, como ácido diluído e liquido iônico, seguido de planejamentos experimentais de hidrólise enzimática com enzimas comerciais na mistura das frações da biomassa da cana-de-açúcar (palha + bagaço) com dois diferentes propósitos: o primeiro na obtenção de monossacarídeos para a produção de etanol lignocelulósico, apresentado no Capítulo III. Já como segundo objetivo deste trabalho, estudou-se a obtenção de XOS a partir da biomassa da

cana-de-açúcar (palha + bagaço), que está abordado no Capítulo IV. Este estudo partiu do conhecimento prévio dos efeitos das enzimas estudadas no Capítulo III, por meio de delineamentos (Plackett-Burman), que possibilitou a seleção das enzimas significativas para a produção de XOs para um estudo mais aprofundado de otimização através de planejamentos experimentais do tipo central rotacional (DCCR). Por fim, no Capítulo V, estão expostas as principais conclusões obtidas no decorrer deste trabalho, bem como sugestões para trabalhos futuros.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Avaliar a influência do pré-tratamento com líquido iônico (acetato de 1-etil-3-metilimidazólio), de modo a comparar com o pré-tratamento convencional com ácido sulfúrico diluído (H_2SO_4) na hidrólise enzimática da biomassa da cana-de-açúcar (bagaço + palha) visando a obtenção de monossacarídeos fermentescíveis (glicose e xilose), bem como de xilo-oligossacarídeos.

1.2.2 Objetivos Específicos

- Caracterizar quimicamente a biomassa lignocelulósica da cana-de-açúcar a ser estudada (bagaço + palha) antes e após os pré-tratamentos;

- Realizar pré-tratamentos com ácido sulfúrico diluído (H_2SO_4) e com o líquido iônico (acetato de 1-etil-3-metilimidazólio) na biomassa da cana de açúcar pré-tratada (bagaço + palha) visando monossacarídeos e xilo-oligossacarídeos;

- Realizar planejamento experimental empregando enzimas comerciais na mistura de biomassa de cana-de-açúcar empregada (bagaço + palha);

- Analisar os efeitos das enzimas na hidrólise da biomassa pré-tratada, utilizando o planejamento Plackett-Burman bem como quantificar a quantidade de açúcares liberados (xilose, glicose e xilo-oligossacarídeos);

- Determinar a faixa ótima de concentração proteica para cada enzima empregada durante a hidrólise enzimática da mistura das frações da biomassa de cana-de-açúcar visando a liberação de xilo-oligossacarídeos através de um DCCR (Delineamento Composto Central Rotacional).

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CAPÍTULO II: REVISÃO BIBLIOGRÁFICA

2.1 BIOMASSA LIGNOCELULÓSICA

A biomassa lignocelulósica é o principal recurso renovável disponível no meio ambiente, pois é apontada como um dos recursos naturais mais importantes para a produção de biocombustíveis e outros produtos com valor agregado (BRANDT et al., 2013). A mesma é constituída majoritariamente por celulose, hemicelulose e lignina (BALAT, 2011). Os polissacarídeos (celulose e hemicelulose) são recobertos por uma macromolécula (lignina), formando a microfibrila celulósica (Figura 2.1). Geralmente a distribuição de tais componentes varia entre 40-50% de celulose, 20-40% de hemicelulose e 18-35% de lignina (SUN et al., 2011). Entretanto o completo potencial dessa matéria prima ainda não foi totalmente explorado, em parte devido à formação de uma cadeia produtiva mundial baseada na produção de insumos pela indústria petroquímica, desenvolvida a partir do século XX. Outros fatores estão relacionados à natureza recalcitrante da biomassa e à dificuldade de modificar as propriedades de seus constituintes, tornando seu processamento dispendioso (DA SILVA, 2013).

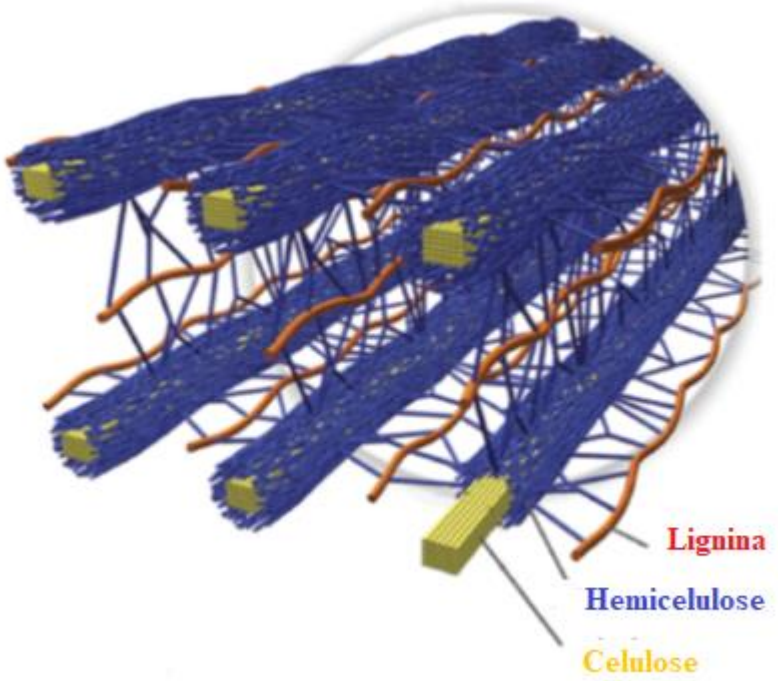

Figura 2.1. Estrutura da Biomassa Lignocelulósica (Adaptado de BRANDT et al., 2013).

De fato, a proposta de utilização de materiais lignocelulósicos para obtenção de etanol não é recente, pois há relatos que datam do final do século XIX possíveis tentativas de comercialização de etanol produzido através da hidrólise ácida da madeira, como na Alemanha em 1898 e nos Estados Unidos em 1910 (RAPIER, 2009; SHERRARD, 1945). Em meados da década de 70, houve um reestímulo a esta pesquisa, devido à crise mundial do petróleo. Assim

vários grupos de pesquisa estudaram a hidrólise ácida e enzimática da celulose e hemicelulose para a produção de etanol, inclusive no Brasil, entre os anos de 1973 e 1974 onde foi criado o programa Proálcool com objetivo de diminuir a dependência das importações de combustíveis automotores, que representava 80% da demanda de combustíveis e 47% do total das importações do país (KOHLHEPP, 2010). Devido ao programa a produção anual de etanol que estava em 600 milhões de litros, em 1974 passou para 10,6 bilhões em menos de uma década. Entretanto o aumento da produção interna de petróleo e a queda do preço do petróleo no cenário internacional, reduziu os interesses e subsídios do programa, o que tornou desinteressante o uso dessa matéria-prima, reconhecidamente recalcitrante, dessa forma muitos estudos foram retomados somente no início do século XXI (LEITE; LEAL, 2007; DA SILVA, 2013).

2.1.1. Composição Química de Materiais Lignocelulósicos

2.1.1.1 Celulose

A celulose é o composto mais abundante nos materiais lignocelulósicos, sendo um polímero linear formado exclusivamente por moléculas de glicose unidas por ligações glicosídicas β(1–4) (Figura 2.2).

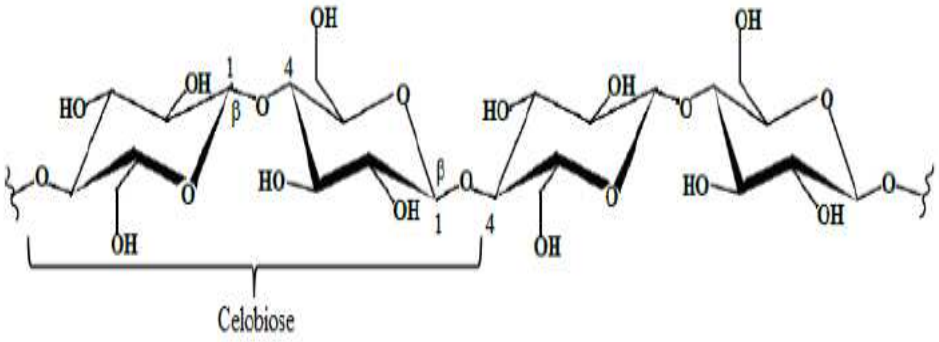

Figura 2.2. Estrutura molecular da celulose (Adaptado de FENGEL; WENGER,1984).

Moléculas de celulose são completamente lineares e têm forte tendência para formar ligações de hidrogênio intermoleculares, as quais ocorrem entre grupos OH de moléculas adjacentes de celulose, e intramoleculares quando ocorrem entre grupos OH de unidades glicosídicas adjacentes da mesma molécula de celulose (Figura 2.3b) (BRANDT et al., 2013). Essas ligações dificultam a livre rotação dos anéis em torno das ligações glicosídicas, resultando no enrijecimento da cadeia. O caráter linear observado nas cadeias de celulose permite que cadeias adjacentes se posicionem próximas uma das outras de forma que feixes de moléculas de celulose se agreguem na forma de microfibrilas (Figura 2.3a) na qual regiões

altamente ordenadas denominadas como cristalinas se alternam com regiões menos ordenadas denominadas como amorfas (Figura 2.4) (DUFRENSE, 2012).

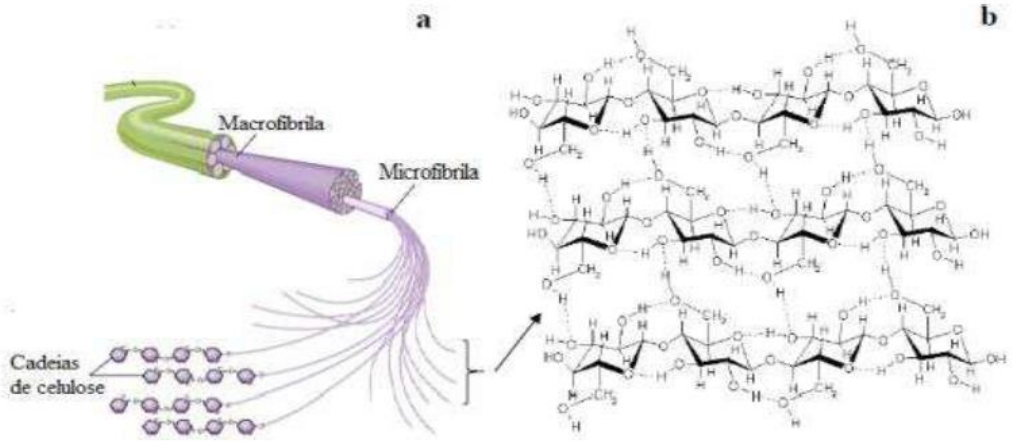

Figura 2.3. (a) Estrutura de cadeias de celulose em micro e macrofibrilas. (b) interações mediante ligações de hidrogênio e entre as cadeias (Adaptado de LINO, 2015).

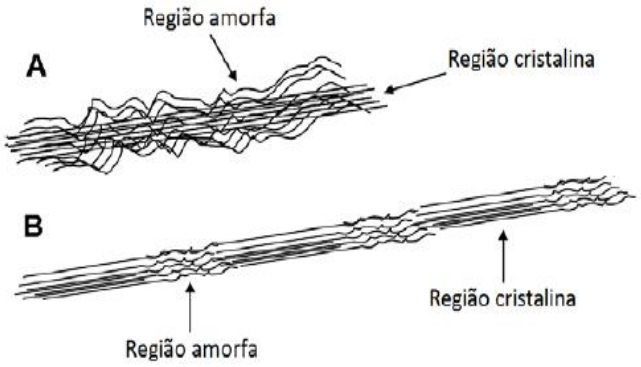

Figura 2.4. Diferentes distribuições celulose amorfo e cristalina (Adaptado de ANDERSEN, 2007).

As microfibrilas se unem formando fibrilas e estas formam as denominadas fibras celulósicas. Como consequência dessa estrutura fibrosa a celulose possui alta resistência à tração e é insolúvel na maioria dos solventes. (LINO, 2015).

Nos vegetais superiores, dois tipos de estrutura cristalina podem estar presentes, as celuloses I_α e I_β, em conjunto denominadas como celulose I, sendo o sistema I_β descrito como o mais representativo (FORTAN; RAGAUSKAS, 2012). No entanto, as proporções entre as formas I_α e I_β, o percentual de cristalinidade e o tamanho das fibras variam conforme a origem da celulose e da sua localização na parede celular (LANGAN et al., 2011).

Além da celulose I, outros alomorfos da estrutura cristalina podem ser obtidos após processamento mecânico, térmico ou químico da celulose (celulose II, III e IV). A celulose II,

um alomorfo comumente reportado na literatura, pode ser formada pela dissolução da celulose em um solvente compatível, seguida de uma etapa de recristalização por precipitação/regeneração ou pelo tratamento com solução aquosa de hidróxido de sódio (também chamado de mercerização) (O'SULLIVAN, 1997). Em uma solução alcalina, alguns átomos de hidrogênio dos grupos hidroxil da celulose são substituídos por átomos de sódio, sendo estabelecido um sistema iônico. Devido à osmose, a água tende a entrar no sistema e outras ligações de hidrogênio tendem a se romper e serem substituídas por ligações com o álcali. Quando a solução alcalina é removida, a pressão osmótica reduz e a as fibras de celulose, antes inchadas pelo tratamento, se contraem. Durante a contração, as ligações de hidrogênio são refeitas, mas em uma orientação diferente, formando a celulose II (DINAND et al., 2002).

Os alomorfos celulose IIII e celulose IIIII podem ser formados, em um processo reversível a partir da celulose I e II, respectivamente, pelo tratamento com amônia líquida (-80 °C) ou algumas aminas e a subsequente evaporação do excesso de amônia. Os alomorfos IVI e IVII podem ser preparados pelo aquecimento das celuloses IIII e IIIII, respectivamente, a 260 °C, na presença de glicerol (O'SULLIVAN, 1997).

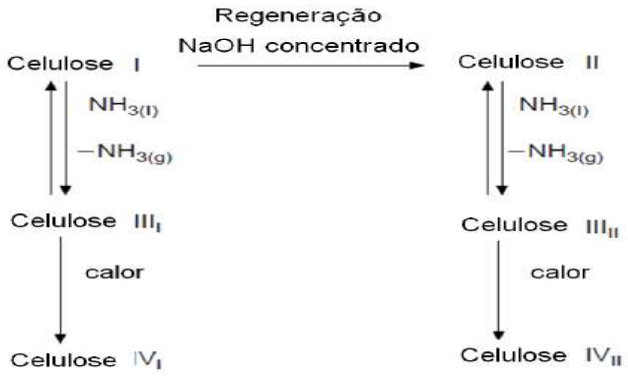

Figura 2.5. Interconversão dos polimorfos da celulose (Adaptado de O'SULLIVAN, 1997).

2.1.1.2 Hemicelulose

As hemiceluloses ou polioses são heteropolímeros ramificados, os quais são constituídos por uma variedade de polissacarídeos de baixo peso molecular, os mesmos estão associados com celulose e lignina (PENG et al., 2012). As unidades de açúcares que formam as polioses podem ser subdivididas em grupos, tais como: pentoses, hexoses, deoxihexoses e ácidos hexurônicos (Figura 2.6) (BRANDT, et al, 2013).

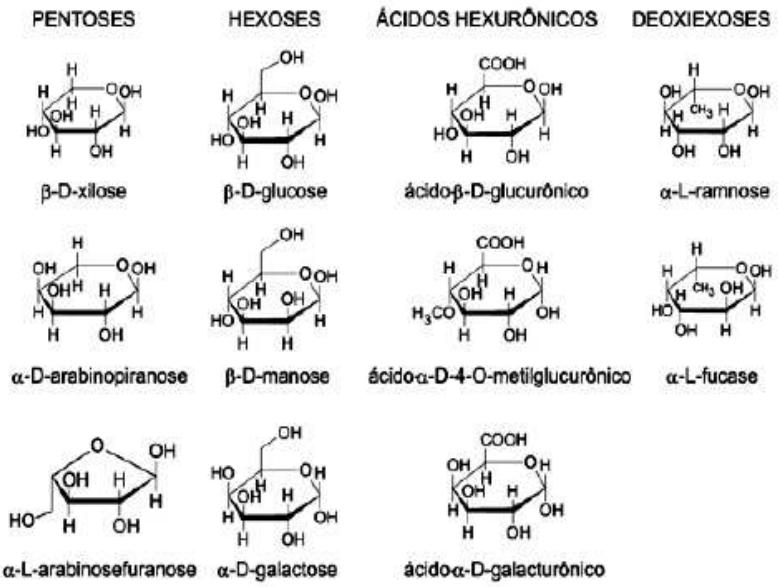

Figura 2.6. Estrutura química dos polissacarídeos que formam as hemiceluloses (Adaptado de FENGEL; WENGEL, 1984).

Ao contrário da celulose as hemiceluloses são amorfas e portanto mais solúvel e susceptível a hidrólise enzimática. A sua principal cadeia é constituída de um homopolímero de xilanas ou um heteropolímero de glucomananas, o qual é formado por unidades de arabinose, galactose e ácido 4-O-metilglucorônico que se ligam uns aos outros essencialmente por ligações glicosídicas β-(1→4), sendo também possível encontrar ligações glicosídicas β-(1→3), β-(1→6), α-(1→2), α-(1→3) e α-(1→6) nas ramificações (FENG et al., 2010).

Na parede celular, as hemiceluloses estão associadas a vários componentes, como celulose, proteínas, pectina e lignina (SUN et al., 2000). As hemiceluloses aderem fortemente à superfície das microfibrilas de celulose via ligações de hidrogênio, havendo relatos que essa adesão evitaria a formação de fibrilas maiores de celulose por agregação lateral (ALBERSHEIM et al., 2011). A formação de ligações covalentes entre a pectina e a xiloglucana também já foi reportada, entretanto os aspectos químicos envolvidos neste tipo de ligação ainda são desconhecidos (POPPER; FRY, 2005; BRETT et al., 2005). A formação de ligações de hidrogênio com a celulose, ligações covalentes com a lignina, denominadas como ligações do tipo éter e ligações do tipo éster entre as unidades acetil e ácidos hidroxicinâmicos principalmente em ácidos ferúlico e p-coumárico dificultam a liberação dos polímeros hemicelulósicos da matriz da parede celular (XU et al., 2005).

O percentual e estrutura química das hemiceluloses é bastante heterogêneo entre diferentes biomassas e diferentes componentes de uma única biomassa (caule, folhas, raízes,

casca) (PENG et al., 2012). As principais hemiceluloses são as xilanas e as glucomananas. As xilanas são as mais abundantes e estão disponíveis em grandes quantidades em resíduos florestais e agroindustriais. Em relação às biomassas madeireiras, as hemiceluloses contendo manana, como glucomananas e galactomananas são os principais componentes da hemicelulose de coníferas ou *softwoods* (*Pinus, Araucária*, entre outras), enquanto que em madeiras folhosas ou *hardwood*s (maioria das árvores brasileiras e o eucalipto) as glucuronoxilanas são mais representativas (GÍRIO et al., 2010).

Nas culturas agrícolas, como a da cana-de-açúcar o tipo de hemiceluloses mais abundante é a arabinoxilana, constituída por um esqueleto de resíduos de xilose ligado por ligações β-(1→4). Ligados a estes resíduos, nas posições C-2 e/ou C-3, estão resíduos de L-arabinose e ácido glucurônico (PENG et al., 2009). Além disso, os resíduos de xilose podem conter grupos *O*-acetil substituindo alguns dos grupos hidroxil. No bagaço e na palha de cana-de-açúcar, especificamente, o tipo de hemicelulose descrita como predominante é o 4-*O*-metil-glucuronoarabinoxilana (Figura 2.7) (PENG et al., 2009; BIAN et al., 2012).

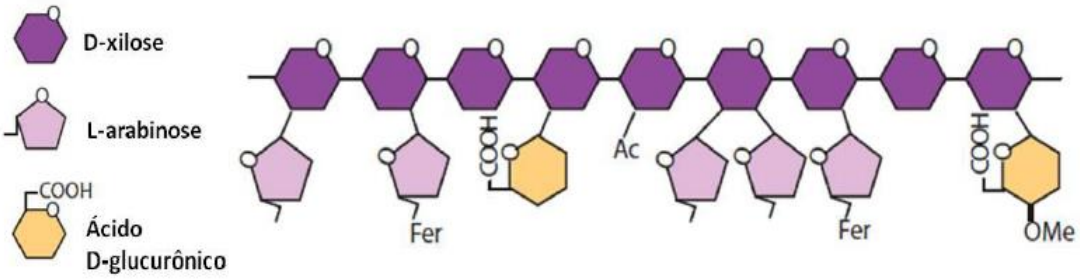

Figura 2.7. Estrutura esquemática da glucuronoarabinoxilana, descrita como predominante em monocotiledôneas. "Fer" representa a esterificação com ácido ferúlico (Adaptado de DA SILVA, 2013).

2.1.1.3 Lignina

Depois da celulose e hemicelulose, a lignina é a macromolécula mais abundante na biomassa lignocelulósica. A lignina não é homogeneamente distribuída na parede celular, estando ausente na parede celular primária, apresentando grande concentração na lamela média e na parede celular secundária (GELLERSTEDT;HENRIKSSON, 2008). Sua principal funcionalidade é conferir aos tecidos vegetais rigidez, impermeabilização, reforço estrutural, resistência ao ataque físico e microbiano (BRANDT et al., 2013).

A lignina é apontada como um dos principais fatores limitantes ao ataque enzimático da celulose (ZHU et al., 2008). De acordo com Brandt et al. (2013) a lignina nativa não só impede o acesso das enzimas aos polissacarídeos, como também possui alto poder de adsorvê-las, o que leva à necessidade de uma maior carga enzimática nos processos de hidrólise enzimática da biomassa lignocelulósica.

A lignina de monocotiledôneas, como a cana-de-açúcar é constituída pela polimerização de alcoóis cinamílicos (monolignóis), os quais estão agrupados três álcoois: coniferílico, *p*-coumarílico e sinapílico. A presença e estrutura desses compostos se diferem dependendo do tipo de planta. Na biomassa da cana de açúcar os três compostos estão presentes. Em coníferas a lignina é composta quase que exclusivamente pelo álcool coniferílico, apresentando pequenas quantidades de álcool *p*-coumarílico. Já em árvores folhosas (*hardwoods*), estão presentes os alcoóis coniferílico e sinapílico (Figura 2.8) (SARKANEN, 1971; FENGEL; WEGENER, 1984; SJÖSTRÖM, 1993).

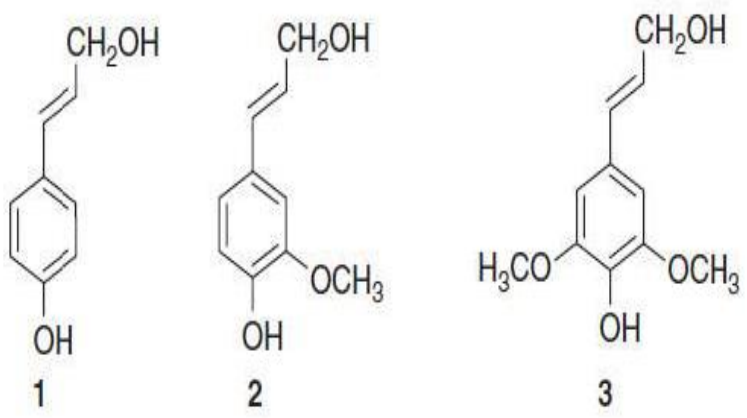

Figura 2.8. Precursores primários da lignina. (1) álcool *p*-coumarílico, (2) álcool coniferílico e (3) álcool sinapílico (Adaptado de GELLERSTEDT; HENRIKSSON, 2008).

2.1.2 Biomassa da Cana-de-açúcar

A cana-de-açúcar é uma planta proveniente do Sudeste Asiático pertencente ao gênero *Saccharum L.* A mesma é descrita como uma planta fina de formato cilíndrico, composta por um colmo e por folhas dispostas radialmente ao seu redor (Figura 2.9) (DA SILVA, 2013). Geralmente, a massa do colmo corresponde a 80% da massa da planta, por exemplo, Rodrigues et al. (1997) encontraram uma variação de 72,7 a 88,4% na proporção de massa dos colmos em relação a planta inteira, após avaliação de 11 variedades de cana-de-açúcar.

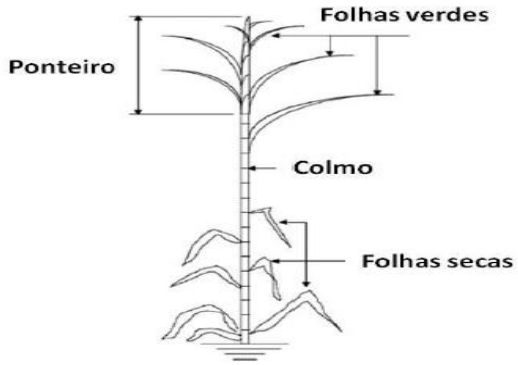

Figura 2.9. Cana de açúcar e denominação de suas diferentes partes (Adaptado de SEABRA et al., 2010).

A cana-de-açúcar foi introduzida no período colonial e se transformou em uma das principais culturas da economia brasileira (LINO, 2015). No Brasil cerca de 600 milhões de toneladas de subprodutos agroindustriais são produzidos anualmente, dentre esses diversos subprodutos agroindustriais, os provenientes do cultivo da cana-de-açúcar são os mais representativos (FERREIRA-LEITÃO et al., 2010a).

Os subprodutos fibrosos que podem ser obtidos após a colheita da cana-de-açúcar são denominados bagaço e palha. O bagaço é o material fibroso obtido após o processamento da cana-de-açúcar para a extração do caldo (DEEPCHAND, 1986). Já a palha, também conhecida como *trash*, corresponde às porções foliares e às ponteiras da cana, que, geralmente, é colhida juntamente com os colmos, mas deixados no próprio lugar de plantio para devolver ao solo parte dos nutrientes minerais imobilizados durante o crescimento (ALMEIDA, 2008).

O grande desenvolvimento da agroindústria canavieira, principalmente na produção de etanol combustível, tem atribuído grande incentivo no que se refere ao aproveitamento do bagaço produzido, por meio da introdução de novas e mais avançadas tecnologias agrícolas e industriais, bem como da recuperação total ou parcial da palha de cana-de-açúcar. Esse total aproveitamento da cana proporcionará coletar toda a cana-de-açúcar, como também otimizar o balanço energético da usina, de forma a aumentar a quantidade de biomassa disponível para ser convertida em etanol (SANTOS et al., 2012). Essas matérias-primas são abundantes, ricas em compostos lignocelulósicos e para sua aquisição demandam baixo investimento financeiro, o que é altamente vantajoso em processos de biorrefinaria visando à produção de etanol (CANILHA et al., 2010; WOLF, 2011).

O tecido vegetal da biomassa da cana de açúcar, como bagaço e palha bem como os resíduos de madeira apresentam os mesmos componentes químicos. Entretanto, as propriedades

físico-mecânicas, geométricas, térmicas e energéticas se diferem (SANTOS et al., 2012). A composição química do bagaço e da palha apresentam pequenas variações em função da variedade da cana empregada e da localização geográfica dos locais de cultivo (LINO, 2015).

O bagaço é o mais significativo subproduto da indústria sucroalcooleira em volume (97 milhões de toneladas) e a cada ano estima-se que gere um excesso de 12 a 15 milhões de toneladas (MACIEL, 2006). O Brasil deverá encerrar o ano/safra 2017/2018, com volume exportado de 28,69 milhões de toneladas, volume pouco maior que o total exportado durante o ano/safra 2016/2017 que foi de 28,93 mihões de toneladas (CONAB, 2017). De acordo com valores encontrados por Brienzo et al. (2009) o bagaço da cana sem extrativos apresentou em base seca valores de 42,4% de celulose, 25,2% de hemiceluloses, 19,6% de ligninas e 1,6% de cinzas. Os quais foram próximos aos valores reportados por Maciel (2006), 45, 35 e 15%, respectivamente, para a celulose, hemiceluloses e lignina.

A palha da cana-de-açúcar, sendo toda a parte aérea da planta menos os colmos industrializáveis, é constituída basicamente de celulose, hemicelulose e lignina, na proporção aproximada de 40, 30 e 25%, respectivamente. Trabalhos realizados por Silva (2009), com a palha da cana-de-açúcar in natura mostraram que o material apresenta 38% de celulose, 29% de hemicelulose e 24% de lignina. Aguilar et al. (1989), verificaram que a palha da cana apresenta um teor de cinzas entre duas e quatro vezes maior do que o bagaço da cana, variando em função do local de coleta do material, condições climáticas, estágio de desenvolvimento vegetativo e cultivar (PAES, 2005; CORTEZ, 2010).

2.2. PRÉ-TRATAMENTO

O pré-tratamento dos materiais lignocelulósicos é uma etapa crucial, a qual tem como propósito a desorganização da estrutura compacta da parede celular (Figura 2.10), possibilitando, liberação de açúcares fermentescíveis e maior acessibilidade das enzimas ao substrato durante a hidrólise enzimática (SHILL et al., 2011). Muitos tipos de pré-tratamento foram desenvolvidos nos últimos 30 anos, dentre eles processos biológicos, químicos e físico-químicos ou combinações deles, de forma a determinar a melhor maneira de disponibilizar açúcares fermentescíveis a partir da hidrólise da celulose e hemicelulose presente nos materiais lignocelulósicos, em termos de custo global, rendimento glicosídico e fermentabilidade do hidrolisado (ALVIRA et al., 2010).

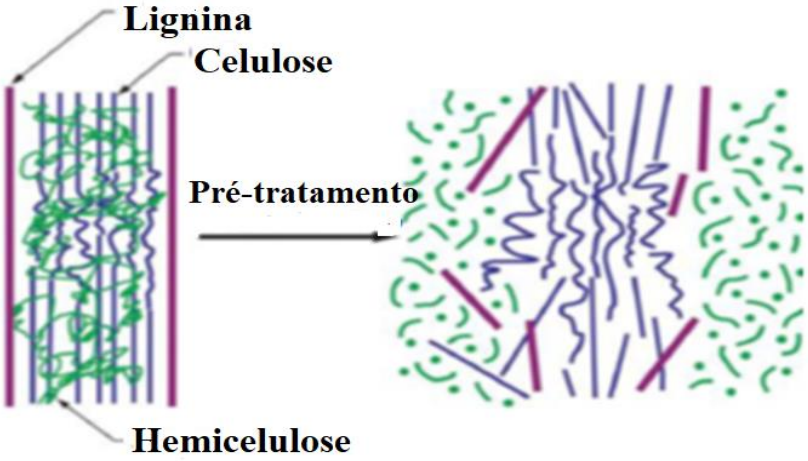

Figura 2.10. Desestruturação esquemática dos componentes da parede celular vegetal pela ação dos pré-tratamentos (Adaptado de BRANDT et al., 2013).

De acordo com Da Silva (2013) a susceptibilidade biológica da celulose está diretamente relacionada ao índice de cristalinidade, pois quando há um aumento na susceptibilidade o índice de cristalinidade diminui. A celulose é sacarificada rapidamente no caso em que o índice de cristalinidade é menor que 1,0. Diferentes métodos já foram reportados, dentre os quais: pré-tratamento com ácido diluído (MARTIN et al., 2007;CANILHA et al., 2011), pré-tratamento alcalino (PARK; KIM, 2012), explosão a vapor (RAMOS, 2003), explosão com amônia (ammonia fiber expansion - AFEX) (SENDICH et al., 2008), pré-tratamento hidrotérmico (SAHA et al., 2013), com líquidos iônicos (LI et al., 2010; DA SILVA et al., 2011;QIU et al., 2012; NINOMIYA et al., 2015a), percolação de amônia reciclada (ammonia recycled percolation- ARP) (KIM; LEE, 2005), microndas (LU et al., 2011), ultrassom (YACHMENEV et al., 2009) entre outros.

A eficiência de um determinado pré-tratamento está intimamente relacionada à acessibilidade ao ataque enzimático, como também à minimização da formação de co-produtos inibidores (FOSTON; RAGAUSKAS, 2012). Existem vários tipos de pré-tratamento que podem ser utilizados para aumentar a susceptibilidade da associação celulose-lignina e assim melhorar a eficiência da hidrólise enzimática. Os processos físicos, em geral, são utilizados para reduzir o grau de cristalinidade e polimerização, bem como aumentar a fração solúvel em água e reduzir o tamanho das partículas (DUNLAP et al.,1976). Em contrapartida os processos químicos têm recebido uma maior atenção, já que os pré-tratamentos físicos são relativamente ineficientes no aumento da digestibilidade da biomassa (FAN et al., 1982). Há relatos que os

pré-tratamentos mais eficazes são geralmente aqueles que empregam temperaturas e tempos de incubação mais elevados, indicando que pode haver uma temperatura efetiva de transição de lignina vítrea que deve ser excedida para solubilização eficiente da lignina (LI et al., 2010).

Devido a extensa variedade de materiais lignocelulósicos que diferem nas suas propriedades químicas e físicas, há uma interdependência entre o pré-tratamento e o tipo de substrato (FOSTON; RAGAUSKAS, 2012). Assim de acordo com Da Silva (2013), é evidente que diferentes pré-tratamentos terão impactos diversos nas propriedades físico-químicas da biomassa pré-tratada. Essas propriedades afetarão processos à montante, como o pré-acondicionamento do material, e à jusante, como a escolha das misturas enzimáticas, a seleção de microrganismos, o tratamento de resíduos e a produção de etanol. Consequentemente, a escolha do pré-tratamento tem um impacto econômico em várias etapas de uma biorrefinaria (LINO, 2015).

2.2.1.Pré-Tratamento com Ácido Diluído

O pré-tratamento com ácido diluído permite a desconstrução da estrutura do material lignocelulósico, e ao mesmo tempo a liberação de monómeros de açúcar, principalmente derivados da hemicelulose. Se o tratamento com ácido for realizado em condições brandas de concentração do ácido e temperatura, a fração de hemicelulose pode ser extraída sem afetar significativamente o teor de celulose. Ao contrário da celulose, a hemicelulose é amorfa e ramificada, sendo mais acessível aos agentes de hidrólise. Portanto, no pré-tratamento com ácido diluído, a hemicelulose é preferencialmente removida e hidrolisada (DA SILVA, 2013).

Este método utiliza quantidades mínimas de ácidos minerais (os mais utilizados são sulfúrico, nítrico e fosfórico) para auxiliar no processo de desestruturação da matriz lignocelulósica e hidrólise das hemiceluloses. Segundo Baudel (2006), esse pré-tratamento é realizado nas seguintes condições: concentração de ácido na faixa de 0,5 a 4% em massa em relação à biomassa, temperaturas variando entre 130 e 220ºC, tempo de residência de 2 a 30 minutos e uma carga de sólidos variando entre 10 e 40%. Com esse pré-tratamento, há relatos que cerca de 80-90% das hemiceluloses são removidas na fração líquida (PITARELO, 2007). Entretanto muitos autores já utilizaram temperaturas mais brandas, no caso 121°C, por exemplo Saha e Bothast (1999) no estudo de sacarificação da palha de milho, onde utilizaram o pré-tratamento com ácido diluído (0,5 % H_2SO_4, v/v) por 1 hora para separar a fração hemicelulósica, obtendo rendimentos satisfatórios na faixa de 85-100% do rendimento teórico. Entretanto, o processo demanda a utilização de uma matéria-prima com reduzido teor de cinzas

e impurezas devido ao efeito tamponante das mesmas, sendo necessária a lavagem da biomassa antes de ser submetida a esse pré-tratamento (CARDOSO et al., 2012).

As condições do processo são fatores determinantes para evitar reações indesejáveis, sendo que temperaturas abaixo de 150°C poderia promover uma diminuição na formação de compostos tóxicos derivados dos açúcares, no entanto podem reduzir os rendimentos na liberação de monossacarídeos durante a hidrólise. De fato, temperaturas elevadas, a partir de 160°C podem favorecer a hidrólise indesejada da fração celulósica e a formação de compostos tóxicos, como ácido acético, furfural e hidroximetilfurfural (SUN; CHENG, 2005; PAIVA, 1980).

As metodologias mais usadas e testadas são baseadas no ácido sulfúrico (GROHMANN et al., 1985; TORGET et al., 1992; NGUYEN et al., 2000). O pré-tratamento com ácido sulfúrico diluído tem sido relatado como um dos processos mais utilizados devido à sua alta eficiência (SUN; CHENG, 2005). No entanto, apresenta importantes inconvenientes relacionados à necessidade de uma etapa de neutralização, etapa que gera degradação de sais e de açúcares com a formação de coprodutos inibidores para as subsequentes etapas de sacarificação e fermentação (TAHERZADEH; KARIMI, 2007). A remoção de inibidores no hidrolisado aumenta consideravelmente o custo do processo e além disso gera um fluxo de resíduos. Os ácidos minerais são corrosivos para os equipamentos, exigindo o uso de materiais mais resistentes, e consequentemente um maior custo de manutenção. Os problemas ambientais causados por seus fluxos de resíduos e seu aspecto corrosivo, exigem a necessidade de outras opções para o pré-tratamento de materiais lignocelulósicos (DA SILVA et al., 2013).

Neste contexto os líquidos iônicos são reagentes com propriedades promissoras para o pré-tratamento da biomassa lignocelulósica, as quais serão abordadas com detalhes no tópico a seguir. Assim, podem ser uma alternativa para substituir os solventes orgânicos voláteis como o ácido sulfúrico, o qual foi utilizado na sua forma diluída no pré-tratamento da biomassa da cana-de-açúcar para um estudo comparativo com os resultados obtidos no pré-tratamento com líquido iônico realizado neste presente trabalho.

2.2.2. Pré-Tratamento com Líquidos Iônicos

Desde a organização do workshop "*Green Industrial Applications of Ionic Liquids*" (Creta, Grécia, 12-16 de abril de 2000), os líquidos iônicos (LIs) foram definidos como sais compostos apenas de espécies iônicas que apresentam uma temperatura de fusão inferior a 100 °C (SUN et al., 2011). Esse fato se justifica pela presença de grandes cátions orgânicos, onde ocorre redução na simetria dos íons, levando a uma dificuldade de empacotamento da estrutura

cristalina (ROSA, 2003). Além disso observa-se uma maior distância entre os íons, o que contribui para a redução das forças iônicas, diferente do que ocorre com os sais inorgânicos, os quais apresentam fortes interações iônicas, implicando em um alto ponto de fusão e ebulição (KROSSING et al., 2006). Os líquidos iônicos podem ser classificados como próticos e apróticos. Os primeiros tem como característica fundamental um próton disponível no cátion, enquanto os demais por convenção são considerados apróticos (GREAVES; DRUMMOND, 2015).

Os líquidos iônicos têm sido utilizados com sucesso na substituição de solventes orgânicos voláteis em diversos processos industriais pelo fato de apresentarem características muito interessantes e promissoras (LONG et al., 2013). Dentre elas a flexibilidade de produção, podendo ser produzidos de acordo com características físico-químicas desejadas por meio da combinação de diferentes cátions e aníons. Estes compostos são estáveis a uma ampla faixa de temperatura e possuem uma pressão de vapor não mensurável, o que os tornam mais seguros perante a exposição comparado aos solventes voláteis tradicionais (WLAZŁO; MARCINIAK, 2014).

Esses compostos podem ser utilizados com sucesso no pré-tratamento da biomassa lignocelulósica, pois são capazes de romper a estrutura da parede celular e fracionar parcialmente os seus componentes, alterando também a cristalinidade da celulose e sua estrutura (DA SILVA, 2013). Quando comparado a outros pré-tratamentos já descritos, os resultados provenientes do uso de LI são expressivos. Li et al. (2010) reportaram que foram necessárias apenas 12 horas de hidrólise enzimática do *switchgrass* pré-tratado com acetato 1-etil-3-metilimidazólio ([Emim][Ac]) para atingir valores superiores a 90% de rendimento, enquanto a hidrólise da mesma biomassa tratada com ácido diluído necessitou de 72 horas de sacarificação para atingir 80% de conversão.

O pré-tratamento da biomassa com líquidos iônicos é normalmente constituído por três etapas: dissolução, recuperação e lavagem. Primeiramente é realizado a dissolução total ou parcial da celulose, seguido pela recuperação da biomassa pré-tratada por meio da precipitação utilizando um anti-solvente,(geralmente a água.). Por fim, é realizada a lavagem do resíduo de modo a eliminar traços de líquidos iônicos que possam ser inibitórios às etapas subsequentes (LONG et al., 2013). Essa etapa tem sido aprimorada em alguns estudos, já que apresenta relevante importância para se obter melhores resultados. Por exemplo de Ninomiya et al. (2015b) estudaram a sacarificação do bagaço pré-tratado com líquidos iônicos constituídos por ions colino e imidazólio. Tal estudo possibilitou determinar o número mínimo de 5 lavagens

necessárias para se obter resultados satisfatórios no efeito inibitório na atividade das celulases e no crescimento das leveduras, de forma a garantir maior sustentabilidade ao processo, uma vez que água utilizada além de acrescer custos, é um recurso limitado.

Muitos trabalhos correlacionam a eficiência do pré-tratamento com LIs com diversos efeitos na estrutura e composição da biomassa pré-tratada capazes de aumentar a acessibilidade das enzimas, como: diminuição do percentual de lignina, diminuição do índice de cristalinidade da celulose, aumento do tamanho dos poros e aumento da área de superfície específica (BRANDT et al., 2013). Além de melhorar os rendimentos de sacarificação da celulose, alguns trabalhos também mostraram o aumento na eficiência de hidrólise da xilana do material pré-tratado (ZHAO et al., 2010; WU et al., 2011). No entanto, dependendo das condições de processo utilizadas, a extração de grande parte da fração hemicelulósica também foi reportada (LI et al., 2011). Diferentes estudos reportam que os LIs geralmente não degradam as cadeias de celulose ou reduzem o seu grau de polimerização e que a estrutura da lignina e da hemicelulose permanecem inalteradas após o pré-tratamento (ZHU et al.; 2006; SAMAYAM; SCHALL, 2010), havendo trabalhos que relatam também a possibilidade de processar a biomassa lignocelulósica com esses reagentes (FORT et al., 2007; XIE et al., 2007). Devido a grande solubilidade da lignina em certos LIs, após a recuperação dos polissacarídeos por adição do anti-solvente, verifica-se que grande parte da lignina permanece solubilizada no LI, ocorrendo portanto extração de grande parte da fração de lignina, que fica exposta ao solvente com a dissolução da celulose (FORT et al., 2007; LEE et al., 2009). Assim, a possibilidade de recuperar uma fração de lignina relativamente pura é uma oportunidade para a produção de produtos com alto valor agregado em adição à produção de etanol, o que pode favorecer a economia de uma biorrefinaria baseada no pré-tratamento com LIs. De fato, estudos apontam que a venda da lignina pode abaixar efetivamente o preço mínimo de venda do etanol a ponto de a lignina se tornar a maior fonte de renda de uma biorrefinaria baseada no uso de LIs (KLEIN-MARCUSCHAMER et al., 2011).

Apesar do grande crescimento na síntese de novos líquidos iônicos, a maior parte dos estudos ainda se baseia em líquidos iônicos com ânions de caráter básico como cloretos, acetatos, formatos e fosfatos e cátions com um núcleo metilimidazólico ou metilpiridínico com cadeias laterais alil-, etil- ou butil (GHANDI, 2014). De acordo com Sun et al. (2011) o acetato 1-etil-3-metilimidazólio ([Emim][Ac]) tem se mostrado vantajoso no pré-tratamento da biomassa lignocelulósica, devido ao seu baixo ponto de fusão, baixa viscosidade e menor

caráter corrosivo, dessa forma tem sido utilizado em grande parte dos estudos que envolvem a hidrólise da biomassa lignocelulósica. Sua estrutura pode ser observada na Figura 2.11.

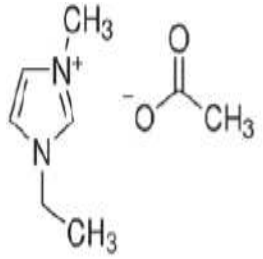

Figura 2.11. Estrutura química do acetato de 1-etil-3-metilimidazólio (Adaptado de DA SILVA, 2013).

Embora ainda exista uma certa carência de trabalhos relatando o pré-tratamento com LIs com a palha de cana-de-açúcar, há um número considerável de trabalhos relatando o pré-tratamento do bagaço da cana-de-açúcar com LIs. Quando comparado a outros pré-tratamentos já bem descritos, os resultados provenientes do uso de LI tais como ([Emim][Ac]) são expressivos. Por exemplo, Da Silva et al. (2011) foi o primeiro trabalho publicado a utilizar o ([Emim][Ac]) para o pré-tratamento do bagaço de cana-de-açúcar, o qual promoveu um aumento significativo nos rendimentos finais e nas taxas iniciais de sacarificação do bagaço. Entretanto em outros trabalhos como o de Yoon et al. (2012) foi reportado um rendimento relativamente baixo de 69,7% em açúcares redutores após a hidrólise enzimática (30 FPU/g de substrato) de bagaço pré-tratado com ([Emim][Ac]) por 15 minutos, a 145 °C utilizando um teor de sólidos de 14% durante o pré-tratamento. Os autores sugeriram que o pré-tratamento não foi tão eficiente devido ao alto teor de sólidos utilizado, o que impediu que o ([Emim][Ac]) propiciasse uma deslignificação efetiva, apesar do índice de cristalinidade ter sido reduzido. A Tabela 2.1 apresenta alguns resultados de sacarificação já reportados após o pré-tratamento de diversas biomassas com ([Emim][Ac]), evidenciando as diferentes condições de processo já utilizadas, com rendimentos (η) superiores a 90% em açúcares redutores após a hidrólise enzimática.

Tabela 2.1. Comparação dos rendimentos glicosídicos de sacarificação (η glicose) de biomassas pré-tratadas com ([Emin][Ac]), evidenciando as diferentes condições de processo já utilizadas (Adaptado de DA SILVA, 2013).

Condições de Pré-tratamento				**Condições de Hidrólise**			
Biomassa	**T (C°)**	**Tempo (h)**	**Teor de sólidos (%)**	**FPU/g celulose**	**Biomassa (%)**	**η glicose (%)**	**Ref.**
Celulose	110	0.5	2	NI	2.0	46	Ha et al., 2011
Switchgrass	160	3	3	NI	0.5	96	Li et al., 2010
Corn stover	160	3	3	NI	0.5	90	Le et al., 2009
Serragem de Bordo	130	1.5	5	NI	NI	95	Torr et al., 2012
Pinus radiata	120/150	0.5	5	20	1.5	93/81	Qiu et al., 2012
Bagaço de agave	120	0.5	5	30	1.0	87	Perez-Pimenta, 2013
Corn stover	160	3	15	NI	15	90	Wu et al., 2011
Serragem de Bordo	125	2	33	4.9	1.0	80	Wu et al., 2011
Populus nigra	125	2	33	4.9	1.0	72	Wu et al., 2011
Bambu	125	2	33	4.9	1.0	65	Ninomiya et al., 2013
Populus alba	160	3	10	NI	1.0	90	Kim et al., 2017
Bagaço de cana-de-açúcar	25*	1	20	40	3,3	99	Ninomiya et al., 2015b

NI- não informado. η = rendimento em açúcares redutores liberados; * uso de banho ultrassônico.

No entanto, ainda existem muitos desafios a serem atingidos antes que os LIs possam ser considerados uma opção real de pré-tratamento, desde seu alto custo e a necessidade de recuperação até o reciclo. Até o momento, a maior parte dos processos de pré-tratamento com LIs propôs métodos utilizando baixo teor de sólidos (em torno de 5%). Klein-Marcuschamer et al. (2011) apresentam que reduzir quantidade de LI é mais relevante que elevar a taxa de reciclo. A toxicidade de traços de LIs para enzimas e microrganismos fermentativos também precisa ser avaliada em detalhes, já que podem afetar negativamente o

desempenho hidrolítico (TURNER et al., 2003) e a produção de etanol (OUELLET et al., 2011). Apesar das atuais limitações, os LIs apresentam um grande potencial para o desenvolvimento de uma biorrefinaria baseada não somente na produção em larga escala de etanol, mas também de produtos de alto valor agregado (LI et al., 2010).

2.3. HIDRÓLISE ENZIMÁTICA

Após o pré-tratamento, a biomassa é sacarificada pela ação de enzimas altamente específicas que reduzem os complexos carboidratos em açúcares, evitando a degradação da glicose (BALAT, 2011). A hidrólise enzimática é preferida à hidrólises químicas, tais como ácida, requerer menos energia, não apresentar problemas de corrosão bem como a condução em condições brandas (pH: 4,8-6,0 e T: 45-50ºC) (SUN; CHANGE, 2002; SARKAR et al., 2012).

Na hidrólise enzimática ocorrem reações de múltiplos estágios em um sistema heterogêneo onde a celulose insolúvel é inicialmente hidrolisada na interface sólido-líquido pela ação sinérgica das celulases endoglucanases (EG) e exo-glicanases/celobiohidrolases (CBH). A degradação inicial é acompanhada da hidrólise na fase líquida dos produtos intermediários solúveis, dentre os quais estão incluídos celobiose e cadeias curtas de oligossacarídeos que são convertidos em glicose pelas β-glicosidases (BG) (ZHANG; LYND, 2004). Em geral, a hidrólise depende das características das enzimas tais como: adsorção da enzima no substrato, inibição competitiva ou não competitiva pelo produto final, sinergismo e limitações da transferência de massa que afetam o transporte da enzima para o substrato. A hidrólise é também afetada pelas características do substrato como: composição e distribuição dos componentes do substrato (lignina, hemicelulose, proteínas e gorduras), tamanho de partícula e a cristalinidade (BROWN et al.,2010).

Geralmente, em ensaios laboratoriais de hidrólise enzimática da biomassa, um dos parâmetros fundamentais a ser avaliado é a carga enzimática das celulases e a proporção entre as atividades celulolíticas. Para uma hidrólise eficiente, são adotadas as dosagens das atividades de FPase (unidade de papel filtro) e de β-glicosidase. A atividade FPase representa a atividade total de celulases na degradação de uma fita de papel de filtro (GHOSE, 1987). Devido ao papel de filtro apresentar um alto índice de cristalinidade, hipotetiza-se que a atividade mais expressiva para a degradação do papel seja a de celobioidrolase. Assim, ao se dosar as atividades FPase e β-glicosidase, é possível balancear as atividades necessárias para degradação total da celulose à glicose. De acordo com Da Silva (2013) alguns trabalhos relatam que para que ocorra a hidrólise completa da celulose uma relação de celobioidrolase:β-glicosidases de 1:1 é necessária. A maior parte dos estudos de hidrólise enzimática como o Rodrigues et al. (2015),

estabelece uma dosagem de 10-15 FPU (unidade de FPase – *Filter paper unit*) por grama de substrato a ser hidrolisado, entretanto alguns autores como Da Silva (2013) utilizam 20 FPU.

2.3.1. Celulases

Para que aconteça a hidrólise enzimática da cadeia de celulose à monômeros de glicose, é necessário a atuação de um complexo celulolítico, que abrange três classes de enzimas: exoglucanases/celobioidrolases, endoglucanases e β-glicosidases. Individualmente nenhuma enzima do complexo é capaz de hidrolisar a celulose de forma eficiente, necessitando, assim de uma ação sinérgica desse complexo (BÉGUIN; AUBERT, 1994). As exoglucanases atacam nas extremidades da cadeia de celulose, independente dos terminais serem ou não redutores, liberando como produto moléculas de celobiose (TEERI, 1997). Além disso, as mesmas são muito susceptíveis a degradação da porção cristalina da cadeia de celulose. Já as endoglucanases quebram as cadeias no seu interior possibilitando a redução do grau de polimerização, sendo mais propícias para hidrolisar as porções amorfas. Por fim, as β-glicosidases atuam na clivagem da molécula de celobiose em dois monômeros de glicose (WILSON, 2009, 2011) (Figura 2.12).

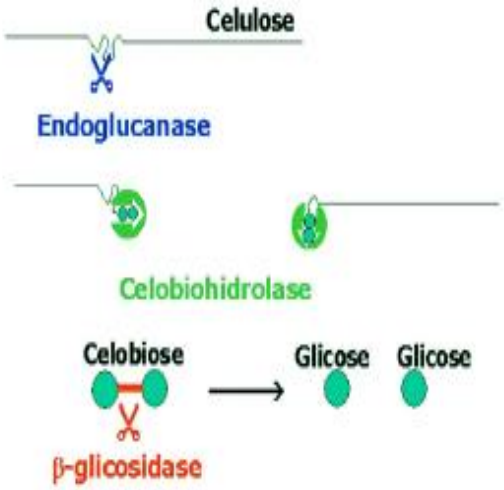

Figura 2.12. Estrutura esquemática do sistema celulolítico (Adaptado de SANTOS et al.,2012)

2.3.2 Hemicelulases

Como já descrito anteriormente, as hemiceluloses possuem uma composição mais abrangente que a celulose e, por isso, necessita de um maior número de enzimas para serem hidrolisada de forma eficiente. As enzimas que hidrolisam as hemiceluloses podem ser divididas em enzimas que atuam na cadeia principal e em enzimas que atuam nas ramificações, também denominadas de acessórias (BÉGUIN; AUBERT, 1994).

A xilana é um polímero de xilose, mas que na natureza, geralmente está associada com outros açúcares formando glucuronoxilanas, glucuronoarabinoxilanas, glucomananas,

arabinogalactanas, e galactoglucomananas (FENG et al., 2010). As principais enzimas para a hidrólise da xilana são as endoxilanases, que clivam ligações glicosídicas internas da cadeia principal da xilana, proporcionando a redução do grau de polimerização do substrato (KALOGERIS et al., 2001). Essas clivagens não ocorrem ao acaso, uma vez que as ligações a serem hidrolisadas dependem da natureza do substrato, como características de comprimento, do grau de ramificação e da presença de substituintes (LI et al., 2000). As β-xilosidases clivam pequenos xilo-oligossacarídeos em xilose, e são classificadas de acordo com sua afinidade relativa junto a xilobiose e xilo-oligossacarídeos maiores (BIELY, 1993). Analogamente, as principais enzimas para a degradação da manana são as endomananases e β-manosidases. A remoção dos grupos laterais da xilana ou manana aumenta o acesso das xilanases/manases à cadeia principal. Assim, enzimas acessórias como α-L-arabinofuranosidases, α-glucuronidase, feruloil esterase, α-galactosidase, acetil-xilana esterase e acetil-manana esterase são geralmente reportadas como importantes para melhorar a eficiência de hidrólise (JUHÁSZ et al., 2005).

As arabinases removem os resíduos de L-arabinose substituídos no C3 das unidades de xilose, podendo ser divididas em exo-α-L-arabinofuranosidases que degradam *p*-nitrofenil-α-L-arabinofuranosideos e arabinanas ramificadas, e endo-1,5-α-L-arabinases que hidrolisam somente oligômeros maiores que quatro arabinoses covalentemente ligadas (SQUINA et al., 2010). A presença de α-L-arabinofuranosidases para a hidrólise da biomassa da cana-de-açúcar é extremamente importante, visto que a hemicelulose da cana é uma arabinoxilana. As α-L-arabinofuranosidases apresentam especificidades diferentes, clivando ligações α-(1→2) ou α-(1→3), enquanto algumas são capazes de clivar resíduos com dupla substituição (SØRENSEN et al., 2007).

As feruloil esterases também apresentam papel de destaque por clivarem ligações entre a hemicellulose e a lignina (MEYER et al., 2009), bem como as ácido *p*-coumárico esterases que clivam ligações éster na xilana, entre arabinose e ácido *p*-coumárico (CREPIN et al., 2004). A Figura 2.13 ilustra a molécula de hemicelulose evidenciando ação de importantes enzimas na sua degradação.

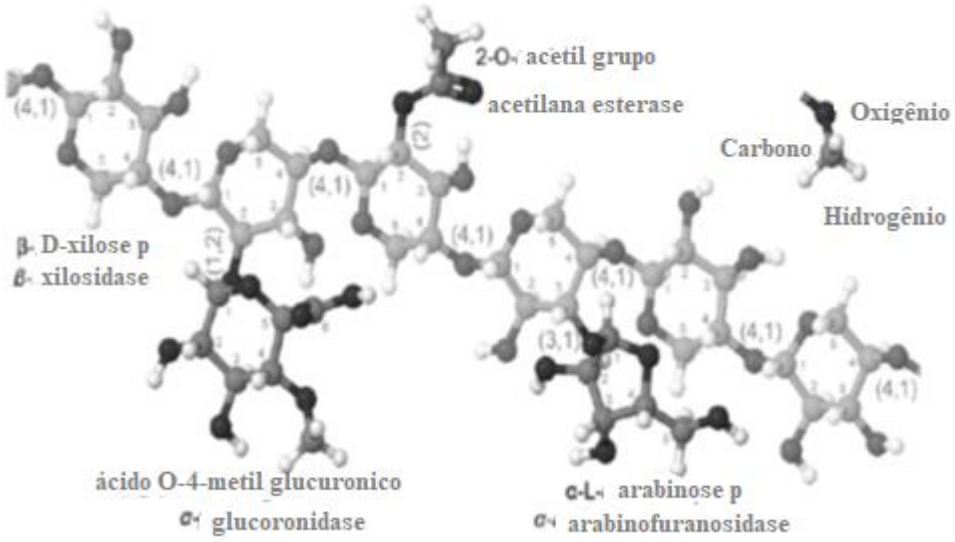

Figura 2.13. Estrutura da hemicelulose e respectivas enzimas que atuam na sua degradação (Adaptado de ZHANG et al., 2012).

Apesar da relevância, a presença e requerimento de hemicelulases é muitas vezes negligenciada em estudos que avaliam a eficiência de misturas enzimáticas para a hidrólise da biomassa. No entanto, mesmo em materiais pré-tratados onde a maior parte da hemicelulose tenha sido removida, existe ainda uma pequena proporção que está em associação com a celulose e a lignina, limitando o acesso das celulases à celulose (DA SILVA, 2013). Estudos demonstram que a adição de hemicelulases melhora o desempenho das celulases, aumentando a conversão da celulose em glicose, como já reportado em alguns estudos, tais como o de Gao et al. (2011) na hidrólise enzimática da palha de milho.

2.3.3. Misturas Enzimáticas

A produção de etanol de segunda geração economicamente viável está intimamente relacionada à redução de custos de produção, principalmente aqueles ligados a etapa de hidrólise enzimática e o aumento nas atividades específicas das enzimas empregadas. Todavia, para a produção de misturas enzimáticas com atividades específicas superiores às atualmente disponíveis comercialmente, requerem grandes esforços de pesquisa voltados para a produção seletiva das proteínas-chave identificadas, em quantidades suficientes para assegurar que o produto resultante seja economicamente viável (BANERJEE et al., 2010b). Pesquisas contínuas também são necessárias em relação: a bio-prospecção, a caracterização dos mecanismos de hidrólise, os efeitos de sinergia entre endoglucanases (EG), celobiohidrolases (CBH), β-

glicosidases (BG) e hemicelulases derivadas de múltiplos organismos, e a relativas tolerâncias a inibidores de enzimas, os quais continuam a oferecer alternativas potenciais para viabilidade econômica do processo (ZHANG et al., 2012).

A eficiência hidrolítica de um complexo multienzimático no processo de sacarificação da biomassa lignocelulósica depende tanto das propriedades das enzimas individuais quanto da sua relação nos coquetéis nos complexos multienzimáticos (GUSAKOV et al., 2007). Existem muitos estudos sobre hidrólise enzimática de diferentes biomassas pré-tratadas empregando misturas enzimáticas. Por exemplo, Barr et al. (2012) avaliaram Endoglucanases, celobiohidrolases, β-glucosidases, β-xilosidases, endoxilanases e esterases de acetilxilano para hidrólise de *Poplar e switchgrass* pré-tratadas com líquido iônico. Billard et al. (2012) estudaram a hidrólise da biomassa pré-tratada hidrotermicamente, empregando uma mistura de seis enzimas, incluindo quatro diferentes celulases, xiloglucanase e xilanase. Banerjee et al. (2010c) em estudos empregando a hidrólise enzimática em palha de milho, reportaram uma redução do número de enzimas requeridas nas misturas enzimáticas (11 componentes), enquanto que equivalentes atividades foram encontradas para misturas comerciais contendo mais de 80 enzimas derivadas de *T. reesei*. De acordo com os autores, o desenvolvimento de uma abordagem padronizada para avaliar a eficiência e desempenho das enzimas individuais e de misturas enzimáticas foi considerado crucial para maximizar suas atividades específicas para aplicações comerciais. Um estudo empregando o bagaço de cana-de-açúcar foi reportado por Goldbeck e colaboradores (2014), os quais desenvolveram um modelo enzimático para desconstrução da parede celular do bagaço da cana-de-açúcar, visando o entendimento do modo de atuação de hemicelulases purificadas na hidrólise da biomassa da cana de açúcar, onde mapeou sua acessibilidade e definiu quais as enzimas essências e secundárias para esta bioconversão.

No entanto, matérias primas com maiores quantidades de hemicelulose e lignina devem ser investigadas de forma experimental antes de eliminar enzimas ditas como "supérfluas" (ZHANG et al., 2012). Existem os desafios dispendiosos com a produção e purificação de proteínas específicas para a formulação de misturas enzimáticas, bem como o número de variáveis que afetam as condições ótimas de atuação dessas misturas tornam-se uma dispendiosa tarefa. A temperatura e pH após o tratamento prévio, a composição da biomassa lignocelulósica, a tolerância a inibidores, as proteínas acessórias necessárias, os efeitos sinérgicos e antagônicos entre as enzimas, as configurações dos reatores, e a produção eventual

de etanol são todos os possíveis fatores que devem ser considerados para a definição de uma mistura enzimática ideal com aplicações em escala industrial (ZHANG et al., 2012).

2.4. PRODUÇÃO DE XILO-OLIGOSSACARÍDEOS (XOS)

Os xilo-oligossacarídeos (XOS) são oligômeros de açúcar formados por unidades de xilose, que aparecem naturalmente em frutos, vegetais, leite e mel, sendo sua produção industrial obtida por meio de materiais lignocelulósicos (RAGAGNIN; DURRANT, 2008; SAMANTA et al., 2015). Os XOS podem ser utilizados em diferentes finalidades, dentre as quais destacam-se as aplicações nos alimentos (VÁZQUEZ et al., 2000) e na indústria farmacêutica (VÁZQUEZ et al., 2005). Os materiais lignocelulósicos utilizados para a produção dos XOS são provenientes de uma grande variedade de resíduos, como florestais (madeira de Eucalyptus) e agroindustriais, como sabugo de milho, amêndoas, oliva, cascas de arroz, cevada, aveia e bagaço de cana de açúcar (EVTUGUIM et al., 2003; MOURA et al., 2007; NABARLATZ et al., 2007; GOLDBECK et al., 2016).

Dentre as aplicações em alimentos, destaca-se o emprego como alimentos funcionais, atuando com prebióticos, os quais são definidos como ingredientes alimentares não digeríveis, com propriedades de estimular seletivamente o crescimento ou atividade de um ou um número limitado de bactérias no cólon, melhorando portanto a saúde do hospedeiro (ROBERFROID et al., 1999, GULÓN et al., 2008). Assim vários benefícios fisiológicos já foram relatados, dentre os quais: a redução do nível de colesterol, manutenção da saúde gastrointestinal, aumento da biodisponibilidade de cálcio, redução do risco de câncer de colón, efeitos citotóxicos em células de leucemia humana e efeito benéfico sobre diabetes mellitus tipo 2 (ANDO et al., 2004, SHEU et al., 2008).

Os materiais típicos para a produção dos XOS são provenientes de uma base rica em xilana, com algumas cadeias heterocíclicas de éter, devendo ser hidrolisada para gerar compostos degradados de cadeia longa (ENEYSKAYA et al., 2007; QUIÑONES et al., 2015). Assim os XOS podem ser disponibilizados como fonte de xilose para a obtenção de compostos de alto valor agregado tais como o xilitol (RIVAS et al., 2002), ésteres e éteres, os quais podem ser utilizados como compostos termoplásticos para filmes solúveis em água, revestimentos e cápsulas (NABARLATZ et al., 2007).

Pela característica dos XOS estarem ligados por ligações do tipo β (1→4) (Figura 2.14), os mesmos podem ser utilizados como importantes substratos ideais para atuação de xilanases. Embora a xilana seja o substrato natural para enzimas xilanolíticas, a sua estrutura e complexidade limita sua utilidade, dessa forma há estudos que visam compreender o modo de

atuação das enzimas, portanto tais componentes são utilizados para obter uma melhor compreensão do mecanismo de reação das enzimas xilanolíticas (AKPINAR, ERDOGAN, BONSTANCI, 2009).

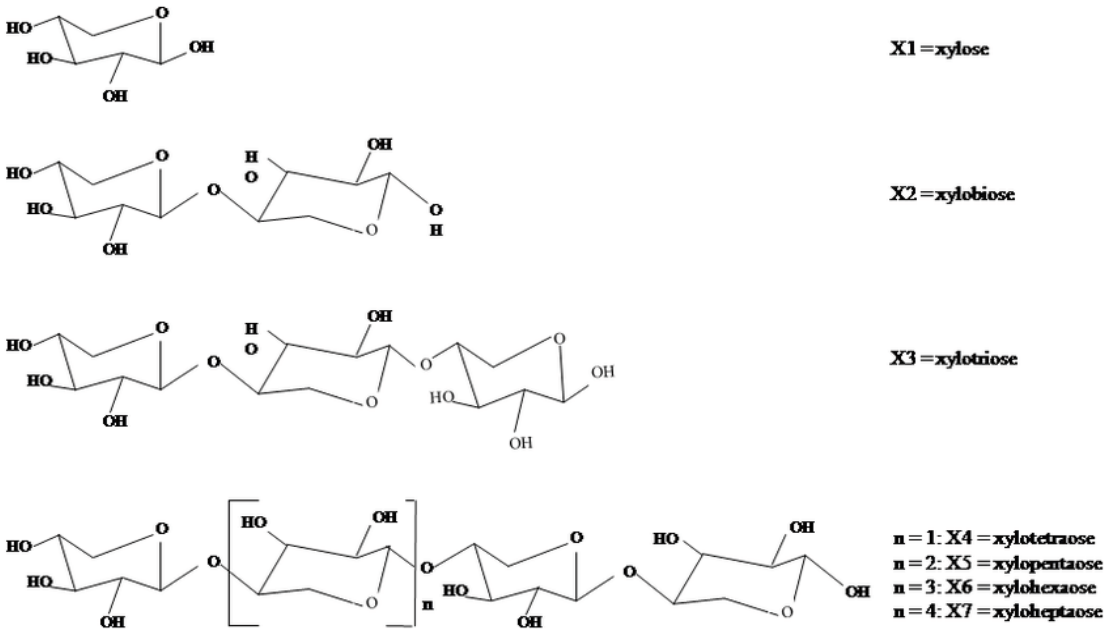

Figura 2.14. Estrutura esquemática das cadeias de xilo-oligossacarídeos (Adaptado de BRIENZO et al., 2016).

A composição e estrutura do XO dependem da fonte e de seu processo de produção. Os XOS podem ser obtidos por processos hidrolíticos: ácido, basico e enzimático (VAQUEZ et al., 2000), podendo ser produzidos por auto-hidrólise, ou por meio de hidrolisados enzimáticos de xilanos a partir de materiais lignocelulósicos (AKPINAR, ERDOGAN, BONSTANCI, 2009).

Para a produção direta de XOS a partir de materiais lignocelulósicos, frequentemente a fração xilana já é separada do complexo lignocelulósico no pré-tratamento (AKPINAR, ERDOGAN, BONSTANCI, 2009). Uma vez que a xilana tenha sido isolada ou degradada para a forma solúvel, a mesma estará susceptível à hidrólise (VÁSQUEZ et al., 2000; KOSUGI et al., 2002). Para produção de XOS por hidrólise enzimática, são necessários complexos enzimáticos contendo enzimas exo-xilanases e/ou β-xilosidases, para evitar a liberação de xilose. Estas enzimas podem ser diretamente adicionadas para a reação, imobilizadas ou produzidas in situ por microrganismos (BEG et al., 2001; IEMBO et al., 2001; REZENDE et al., 2002; XIONG et al., 2004; YOON et al., 2006). Por meio destes métodos,

longas cadeias de XOS podem ser produzidas, sendo preferíveis para o uso em alimentos as cadeias com grau de polimerização (GP) entre 2- 4 (LOO et al., 1999).

No processo de auto-hidrólise o material lignocelulósico é aquecido em uma solução aquosa e os íons hidroxônio provenientes da auto-ionização da água causam a despolimerização da xilana de forma a gerar xiloligômeros e xilose. (GARROTE et al., 2002). No entanto, os hidrolisados deste processo contêm uma variedade de componentes indesejáveis, tais como lignina solúvel e uma grande quantidade de monossacarídeos e produtos de desidratação, o que torna necessário uma intensa etapa de purificação (AKPINAR, ERDOGAN, BONSTANCI, 2009).

A hidrólise enzimática dos materiais lignocelulósicos é considerada um método eficiente para a produção de XOS pelo fato de ser seletiva e ao mesmo tempo controlada, bem como requerer condições brandas de pH e temperatura, minimizando portanto a geração de compostos que podem ser prejudiciais em etapas subsequentes. Na hidrólise enzimática, xilanases com diferentes especificidades de substrato podem produzir diferentes produtos. Assim o perfil dos XOS é dependente não apenas da enzima, mas também da fonte de xilano empregada. Dessa forma dependendo da fonte de xilano utilizada, a produção enzimática de XOS pode gerar heteroxilo-oligosacarídeos ramificados (VAN CRAYEVELD et al., 2008). Os quais dificultam a separação de XOS individuais por fracionamento cromatográfico. Diferentes métodos adequados à análise de oligossacarídeos, baseados peincipalmente na separação por cromatografia líquida de alta eficiencia (CLAE) ou cromatografia gasosa de oligossacarídeos pré-derivatizados (CHURMS, 1996; SUN et al., 2002).

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CAPÍTULO III: EVALUATION OF THE CHEMICAL COMPOSITION OF A MIXTURE OF SUGARCANE BAGASSE AND STRAW AFTER DIFFERENT PRETREATMENTS AND THEIR EFFECTS ON COMMERCIAL ENZYME COMBINATIONS FOR THE PRODUCTION OF FERMENTABLE SUGARS

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EVALUATION OF THE CHEMICAL COMPOSITION OF A MIXTURE OF SUGARCANE BAGASSE AND STRAW AFTER DIFFERENT PRETREATMENTS AND THEIR EFFECTS ON COMMERCIAL ENZYME COMBINATIONS FOR THE PRODUCTION OF FERMENTABLE SUGARS

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ABSTRACT

Replacement of fossil fuels with renewable sources has been studied as a strategy to mitigate the environmental impacts of energy production. This concern has encouraged the use of alternative sources of cost-competitive and sustainable biofuels, such as lignocellulosic biomass for second-generation (2G) ethanol production. As the enzymes required for biomass hydrolysis are costly, the development of less expensive enzyme mixtures for the hydrolysis of sugarcane biomass is important. The efficient conversion of biomass to fermentable sugars requires a critical pretreatment step to increase enzyme accessibility to the substrates during the hydrolysis step. Ionic liquids (ILs) are promising solvents for biomass pretreatment and can result in higher productivity and hydrolysis yield than conventional pretreatments, such as dilute sulfuric acid. The objective of this work was to evaluate changes in the chemical composition of a sugarcane biomass mixture composed of straw and bagasse in a 1:1 (w/w) ratio after pretreatment with the IL 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]) or with diluted sulfuric acid (DSA) and determine the effect of the pretreatments on the interaction between commercial enzymes for the production of fermentable sugars by enzymatic hydrolysis. Pretreatments with IL were more efficient in the removal of lignin than pretreatments with DSA. Cellulase and endo-1,4-xylanase had a higher contribution to the release of glucose and xylose for both pretreated samples. The highest values of glucose and xylose yields, 99.96% and 86.52%, respectively, were achieved with the enzymatic hydrolysis of the biomass pretreated with IL. Thus the pretreatment with ionic liquid has presented as a very promising alternative for the second generation ethanol production.

Keywords: Sugarcane biomass, enzymatic hydrolysis, ionic liquids, chemical composition.

3.1 INTRODUCTION

The scarcity of fossil fuel reserves, coupled with environmental damages, has led to a growing need for increased production and consumption renewable and sustainable fuels. Ethanol 2G is a liquid biofuel with the potential to replace partially the gasoline needed for transportation worldwide. Presently, ethanol is mainly produced from sugarcane juice (sucrose) in Brazil (HIRA; GUILEHERME; OLIVEIRA, 2009) and from corn (starch) in the USA (HETTINGA et al., 2009). However, it is well established that the widespread use of ethanol will require new sources of raw materials, such as wood and agricultural residues, that have the advantage of being widely available (HAHN-HAGERDAL et al., 2009). Even so, the cost of ethanol production from lignocellulosic biomass with current technologies is still very high (BORSE; SHETH, 2017).

These concerns have encouraged the exploration of cost-competitive and sustainable supplies of biofuel (CANILHA et al., 2012). Sugarcane bagasse and straw have attracted the interest of scientists in Brazil as potential sources for 2G ethanol production (DA SILVA et al., 2010), which can be explained not only by their chemical composition rich in polysaccharides, such as cellulose and hemicellulose, but also by the proximity of these by-products to ethanol production mills and the consequent reduction of transport costs (FERREIRA-LEITÃO et al., 2010a).

The production of ethanol from lignocellulosic materials requires four main steps: pretreatment of biomass, saccharification, fermentation, and distillation (MARGEOT et al., 2009). The lignocellulosic material must be pretreated to allow a higher conversion of cellulose and hemicellulose, which are consumed during enzymatic hydrolysis to produce glucose and xylose, sugars that are then fermented to produce ethanol (SUN; CHENG, 2002). The pretreatments act by disrupting the lignocellulosic matrix, reducing the amount of lignin and hemicellulose, and modifying the crystalline structure of cellulose to make it more susceptible to enzymatic attack (SILVERSTEIN et al., 2007). The yield of conversion of lignocellulosic materials into glucose and xylose is usually very low if the enzymatic hydrolysis is carried out without a preliminar pretreatment. Thus, pretreatments are applied when a high yield of conversion into glucose and xylose are required after the enzymatic hydrolysis of the solid fraction of the biomass (GUILHERME et al., 2015).

Many different strategies have been developed to convert polysaccharides into fermentable sugars. One strategy involves the conventional pretreatment with dilute acids, which promotes intense hemicellulose solubilization at high temperatures (CANILHA et al.,

2011). On the other hand, the utilization of such chemicals requires the use of acid-resistant equipment parts and a neutralization step for treating the effluent. Furthermore, the formation of inhibitors of saccharification and fermentation occurs when pretreating the biomass with acids (GALBE, 2007).

Recently, the use of ionic liquids (ILs) was shown to be efficient in the pretreatment of biomass as they were able to reduce the crystallinity of cellulose and partially remove hemicellulose and lignin while not generating degradation products that are inhibitory to enzymes and fermenting microorganisms (EGOROVA; ANANIKOV, 2018). ILs present the advantages of low volatility and are non-flammable and easily recyclable (GREMOS et al.,2011; FERRARA et al., 2017).

The lignocellulosic industry of the future will likely use many different pretreatment/biomass combinations, and therefore enzyme mixtures that can handle all of the different pretreatment/biomass combinations will be necessary (BANERJEE et al., 2010a). Although there is a considerable number of studies employing sugarcane bagasse biomass, there is a lack of studies using sugarcane straw and even less using the mixture of bagasse and straw as a substrate. Thus, the development of more efficient lignocellulose-degrading enzyme cocktails will require deeper and more precise knowledge regarding the specific enzymes that are involved in the hydrolysis of sugarcane biomass (BANERJEE et al., 2010d).

In the present work, we report the study and quantification of changes in the chemical composition of a mixture of the two main lignocellulosic fractions of sugarcane biomass, bagasse and straw, in a 1:1 (w/w) ratio. Changes in the chemical composition were caused by the different performance of the pretreatments applied, using dilute sulfuric acid (DSA) or the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]) using mild conditions. These conditions were employed as way to disorganize the structure of lignocellulosic biomass and the same time to preserve the source of hemicellulose in the biomasses studied. As the second step of this work was carried out an exploratory study to investigate the interaction of five different commercial enzymes using the Plackett-Burman design to produce xylose and glucose simultaneously through the pretreated biomass with commercial IL in comparison with the conventional pretreated biomass with dilute sulfuric acid. The performed pretreatments conditions associated with interaction of mixture of different enzymes in enzymatic hydrolysis assays, as well the biomass utilized became this study relevant and original. Because although there are many reports in literature using sugarcane biomass bagasse or straw, there is a lack of information yet about the use mixture of the two fractions

of this biomass, employing mild conditions with ionic liquids and dilute sulfuric acid in studies involving mixtures of cellulases and hemicellulases in response to these pretreatments.. IL was selected to be carried out in this study due to its low melting temperature, low viscosity, non-toxicity, and non-corrosivity, different from sulfuric acid, an extremely toxic and corrosive solvent (SAMAYAM; SCHALL, 2010).

3. 2 MATERIALS AND METHODS

3. 2.1 Sugarcane biomass

Sugarcane bagasse and straw were kindly supplied by Raízen, located in Piracicaba, São Paulo, Brazil, and by Dr. Paulo Graciano, from the Brazilian Bioethanol Science and Technology Laboratory (CTBE/CNPEM), Campinas, São Paulo. The moisture content of the raw materials was quantified according to the AOAC (1995) method. Sugarcane bagasse and straw contained 45% and 48% moisture (wet base), respectively. Both sugarcane biomass fractions were washed with water at room temperature to remove the excess of soil and dust (SZCZERBOWSKI et al., 2014). Samples were oven-dried at 80 °C to at least 10% moisture (dry base), then were ground in a cutting mill and sieved to retain particles in the range of 24–48 mesh. Sample humidity was kept at about 8–10% (dry base) for subsequent chemical characterization and application of pretreatments.

3.2.2 Dilute sulfuric acid pretreatment

A 1:1 (w/w) mixture of bagasse and straw was pretreated with two different concentrations of sulfuric acid, 0.5% (w/v) and 1% (w/v). The solid loading (10 wt %), temperature (100 °C), and residence time (30 minutes) were the same for the two experimental conditions. Initially, the biomass and the aqueous acid solution were added to a 250-mL Erlenmeyer flask immersed in a water bath with electrical heating. The residence time count initiated when the temperature reached the programmed value; after 30 minutes, the pretreatment was interrupted by immersing the flask in an ice bath, quenching the reaction. Then, the hemicellulose hydrolysates were separated from the solid fraction by vacuum filtration. The remaining solids were thoroughly washed with deionized water, oven-dried overnight at 105 °C, and stored in sealed bags at room temperature.

3.2.3. IL pretreatment

A 1:1 (w/w) mixture of bagasse and straw was pretreated with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]), purchased from (Iolitec®-Germany) (purity

98%) in two experimental conditions. The first pretreatment condition was carried out at 100°C, using a thermostated bath (TB). As an alternative source of energy, the second pretreatment condition was performed at 25°C using an ultrasonic bath (UB). The solid loading (5 wt %) and residence time (30 minutes) were the same for the two experimental conditions. Subsequently, deionized water was added to the IL solution in a 5:1 (v/w) ratio to recover the biomass (QIU et al., 2012). Then, the solids were washed repeatedly with deionized water until the wash solution was colorless to remove IL from the samples (NINOMIYA et al., 2015b). After the experiments, the IL/water mixture and the solid fraction were separated by vacuum filtration. The remaining solids were collected, oven-dried overnight at 105°C, and stored in sealed bags at room temperature.

3.2.4 Efficiency of pretreatments

The amount of solid fraction of the pretreated biomass was quantified after each pretreatment process using Equations (3.1) and (3.2). This value is necessary to evaluate the pretreatment process in relation to the reduction of lignin and hemicellulose (GUILHERME et al., 2015).

$$\text{Solid fraction } \left(\frac{\text{g}}{\text{g raw material}}\right)$$

$$\%\ \text{Recovered mass} = \left(\frac{\text{Final amount of insoluble material}}{\text{Initial amount of raw material}}\right) \times\ 100 \tag{3.1}$$

$$\%\ \text{Solubilization mass} = \left\{1 - \left(\frac{\text{Final amount of insoluble material}}{\text{Initial amount of raw material}}\right)\right\} \times\ 100 \tag{3.2}$$

3.2.5 Chemical composition analysis of sugarcane biomass

The mean values of the composition analysis of each sugarcane biomass fraction were obtained from three independent replicates. The total extractives content was determined only for the untreated biomass, whereas the determination of polysaccharide, ash, and lignin contents was performed for both types of samples (untreated free extractives samples and pretreated samples), described below in details. The ash content was determined by burning 1 g of sample in a tarred crucible for 6 hours, using a muffle furnace at 600 °C, until a constant weight was achieved, according to the NREL/TP-510-42622 procedure (SLUITER et al., 2005). Extractives were determined quantitatively in a Soxhlet apparatus, using 95% acetone followed by a hot-water extraction under reflux (SJÖSTRÖM, 2013).

The total lignin content was determined through hydrolysis with 72% (w/w) sulfuric acid, according to the analytical procedure recommended by NREL (SLUITER et al., 2008). Briefly, dried samples (300 mg) were hydrolyzed with 72% sulfuric acid (0.3 mL) at 30 °C for 60 minutes, followed by dilution to a final concentration of 4% by adding 84 mL of distilled water. The mixture was autoclaved at 121 °C for 60 minutes, cooled, and filtered through a porous bottom crucible (4–5 μm). The insoluble lignin content was quantified as the amount of insoluble solids obtained from the mass of insoluble ash. Soluble lignin was measured in an alkaline medium by spectrophotometric reading at 280 nm.

The holocellulose, α-cellulose, and hemicellulose contents were determined by treatment of the biomass with sodium chlorite under acidic conditions. The experimental procedure is an adaptation made by Da Silva et al. (2011) from the classical method described by Browning (1967) and Wise et al. (1946). This method was chosen for its feasibility, and its potential as an alternative method to the traditional method of Sluiter et al. (2008), which is an expensive method since it requires chromatographic techniques and therefore, a longer analysis time. Briefly, sodium chlorite (20 mg) was added to the extracted sample (500 mg) repeatedly (4 times) in 30 mL of a 7.5% acetic acid solution at 80°C for 60 minutes. The delignified product, holocellulose, was filtrated, washed with distilled water several times, oven-dried at 40°C for 24 hours, and weighed. Then, the holocellulose residue (200 mg) was added to a 17.5% NaOH aqueous solution (5 mL). The final residue, α-cellulose, was recovered by vacuum filtration and oven-dried at 40°C for 24 hours. The hemicellulose content was determined by the difference between the holocellulose and α-cellulose contents. Mean values of three replicates of the dry weight percentages of holocellulose, hemicellulose, α-cellulose, lignin, and ashes were submitted to the Tukey test at the 5% significance level using the software Statistica 8.0. (StatSoft Inc., Tulsa, OK, USA).

3. 2.6 Commercial enzymes and determination of protein concentration

Five enzymes were evaluated in an experimental design template: α-L-arabinofuranosidase (*Aspergillus ninger*) and β-xylosidase (*Bacillus pumilus*) from Megazyme® and endo-1,4-xylanase (NS50030), cellulase (NS50013), and β-glucosidase (NS50010) from Novozymes®. In addition, two commercial cellulase enzyme cocktails, Celluclast 1.5 L and Cellic® CTec2, both from Novozymes®, were used to compare with the enzymatic mixtures tested in the experimental design. The enzyme protein concentration was determined according to the method proposed by Lowry (1951), using bovine serum albumin (Sigma-Aldrich) as the standard.

3.2.7. Enzymatic hydrolysis

Enzymatic hydrolysis microassays were carried out in 1.5-mL Eppendorf tubes using a micropipette (Pipetman G P20G 2-20ul, Gilson). Plackett-Burman designs were applied, consisting of 12 experiments and three repetitions of the central point, totalizing 15 experiments, to assess the effects of the independent variables (commercial enzymes) on the degradation of two different substrates: a 1:1 (w/w) mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA) and a 1:1 (w/w) mixture of sugarcane bagasse and straw pretreated with the ionic liquid (MPIL). In the experimental design, the independent variables (factors) were the five commercial enzymes: α-L-arabinofuranosidase, β-xylosidase, endo-1,4-xylanase, cellulase , and β-glucosidase. Preliminary tests were performed and kinetic curves were generated to verify the minimum protein concentration of each enzyme required for the experiments (Appendix 1). The enzymes reference concentrations were set at 10 $mg/g_{substrate}$, 9 $mg/g_{substrate}$, 16 $mg/g_{substrate}$, 22 $mg/g_{substrate}$, and 7 $mg/g_{substrate}$ of α-L-arabinofuranosidase, β-xylosidase, endo-1,4-xylanase, cellulase, and β-glucosidase, respectively. The dependent variables (responses) was calculated based on the percentage of total cellulose and hemicellulose converted to free glucose and xylose, respectively. Two coded levels (-1 and +1) and the central point (0) of experimental design were defined: (-1) no enzyme addition; (+1) 100% of the enzyme reference concentration; and (0) 50% of the enzyme reference concentration. Analysis of the effects of each independent variable was conducted with the online software Protimiza Experimental Design (*http://experimental-design.protimiza.com.br/*). Experimental conditions were the same as described by Goldbeck et al. (2014) and Goldbeck et al. (2016): Reaction mixtures contained 20 mg of each substrate, a combination of commercial enzymes (experimental designs), and sodium phosphate buffer (0.1 M, pH 5.0) to complete a final volume of 1 mL. Samples were incubated at 50°C for 48 hours under agitation (1000 rpm). After this period, samples were centrifuged (12.000g for 15 minutes at 4°C), and the supernatant was collected for subsequent analyses.

Additional experiments were carried out on the pretreated biomass samples using two commercial enzyme cocktails separately, Celluclast® 1.5 L and Cellic® CTec2, under the same experimental conditions described above, but with an enzyme loading of 10 $FPU/g_{cellulose}$, in order to compare with the dependent variable results obtained in the Plackett-Burman designs.

3.2.8 Enzyme activity measurements

The FPase and CMCase activities were determined according to IUPAC recommendations with minor modifications conducted by Da Silva (2013). The reaction mixture consisted of 0.25 mL of a 2% (w/v) CMC solution in 50 mmol.L^{-1} sodium citrate buffer, pH 4.8 and 0.25 mL of the enzyme extract (diluted in sodium citrate buffer 50 mmol.L^{-1}, pH 4.8, when necessary). The reaction mixture was incubated at 50°C for 30 minutes. The enzymatic reaction was stopped by the immediate addition of 0.5 mL of DNS, then boiled for 5 minutes, cooled in an ice bath and diluted with 6.5 mL of distilled water before reading the absorbance at 540 nm. For determination of sugar concentration, a standard curve of glucose was developed according methodology proposed by Ghose (1987).

β-glucosidase, β-xylosidase, and α-L-arabinofuranosidase activities were determined according to the method described by Da Silva et al. (2010), using 100 µL of 10 mM ρ-nitrophenyl-β-D-glucopyranoside, ρ-nitrophenyl-b-D-xylopyranoside, and ρ-nitrophenyl-a-l-arabinofuranoside, respectively; 200 µL of 0.5 M sodium acetate buffer pH 5.0; 650 µL of Milli-Q water; and 50 µL of the appropriately diluted enzyme solution. After incubating the reaction mixture at 45°C for 10 minutes, the reaction was interrupted by the addition of 500 µL of 1M Na_2CO_3. The concentration of liberated ρ-nitrophenol was measured at 420 nm. One unit of β-glucosidase, β-xylosidase, or α-L-arabinofuranosidase was defined as the amount of enzyme that released 1 µmol of ρ-nitrophenol in 1 minute at 45°C. Endo-1,4-xylanase activity was assayed against 500 µL of 2% soluble xylan (from birchwood, Sigma-Aldrich) and 0.1 mL of appropriately diluted supernatant, which were mixed and reacted at 45 °C for 30 minutes in 50 mM of sodium acetate buffer, pH 5.0, in a total volume of 1 mL (DA SILVA et al., 2010). The resulting reducing sugars were analyzed using a DNS assay (MILLER, 1959). One unit of xylanase was defined as the amount of enzyme required to produce 1 µmol of reducing sugars in 1 minutes at 45°C.

3.2.9. Quantification of fermentable sugars

Supernatants of the enzymatic hydrolysis of the sugarcane mixtures were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPLC-PAD) to detected released monosaccharides (glucose and xylose). Separation was performed using a Dionex DX-500 (Sunnyvale, CA, EUA) instrument with a CarboPac PA1 column (4 mm × 250 mm), a CarboPac PA1 guard column (4 mm × 50 mm), and an electrochemical detector adopting a linear gradient elution with A (NaOH 50 mM), B (NaOAc 500 mM; NaOH 50 mM), and C (H2O). Concentrations of each monosaccharide were

calculated based on the construction of calibration curves with external standards (xylose and glucose) provided from Sigma-Aldrich. Percent theoretical conversions of cellulose into glucose and hemicellulose into xylose were calculated using the equations provided by NREL's LAP TP-510-43630, as described below:

$$\% \text{ Glucose yield} = \frac{0.9 \times [Glucose]}{f \times [Pretreated\ Biomass]} \times 100 \quad (3.3)$$

$$\% \text{ Xylose yield} = \frac{0.88 \times [Xylose]}{f \times [Pretreated\ Biomass]} \times 100 \quad (3.4)$$

where [Glucose], glucose concentration (g/L); [Xylose], xylose concentration (g/L); [Pretreated Biomass], dry pretreated biomass concentration at the beginning of enzymatic hydrolysis (g/L); and *f*, cellulose or hemicellulose fraction in the dry pretreated biomass (g/g).

3.3 RESULTS AND DISCUSSION

3.3.1 Chemical composition

Chemical characterization was performed on the mixture of sugarcane bagasse and straw and on these sugarcane fractions separately. The dry weight percentage values for each individual fraction were compared with the values obtained for the sugarcane mixture, since no information on its chemical characterization was available in the literature. The obtained data are summarized in Table 3.1.

Table 3.1. Chemical composition of untreated sugarcane biomass (dry weight basis).

Component	**Chemical composition (%)**		
	Bagasse	Straw	Bagasse + Straw
Holocellulose (α-cellulose + Hemicellulose)	74.6 ± 0.26	69.26 ± 0.32	72.24 ± 0.31
α-cellulose	44.32 ± 0.28	39.4 ± 0.23	41.99 ± 0.21
Hemicellulose	30.28 ± 0.54	29.86± 0.55	30.25 ± 0.52
Total Lignin (Klason Lignin+ Soluble Lignin)	23.85± 0.86	21.83 ± 0.91	23.74 ± 0.98
Klason Lignin	21.51± 0.34	18.25 ± 0.56	21.37 ± 0.43
Soluble Lignin	2.33 ± 0.52	2.58 ± 0.35	2.13 ± 0.55
Ashes	2.46 ± 0.11	4.05 ± 0.12	3.54 ± 0.14
Extratictives in acetone	2.48 ± 0.16	4.58 ± 0.21	2.97 ± 0.15

The holocellulose content of sugarcane bagasse (74.6 ± 0.26 %) was significantly higher than that of sugarcane straw (69.26 ± 0.32 %). Szczerbowski et al. (2014) also determined the chemical composition of these fractions of sugarcane biomass and found values of 79.71% of holocellulose and 45.06% of α-cellulose in the bagasse and 77.71% of holocellulose and 37.5%

of α-cellulose for the straw, relatively close results to those obtained in this study. Da Silva et al. (2011) also reported similar values for sugarcane bagasse, 74.7% of holocellulose and 46.0% of α-cellulose.

Sugarcane straw had a higher ash content (4.05%) than sugarcane bagasse (2.46%); the obtained value for the mixture of these two fractions was closer to that of bagasse. The obtained values are in agreement with those reported by Da Silva et al. (2010): 2.8% ash in sugarcane bagasse and 5.7% ash in sugarcane straw. However, an average ash content of 8.6% was found in another study by Vassilev et al. (2010) for different samples of sugarcane straw. Variable amounts of ash are usually associated with inorganic soil contaminations along with metal contaminations from the wear of industrial crushers, such as iron and titanium. Thus, the higher amounts of ash in straw samples reported by that authors were probably due to contamination with soil and dust. If the straw is washed before ash determination, as was performed in the present study, most of the silica present on the surface of the leaves is removed, resulting in a lower ash content. Comparative analyses based on literature data require detailed interpretation because the observed differences may be due to differences in sample preparation and handling (SZCZERBOWSKI et al., 2014)

Regarding total lignin, the content found in sugarcane bagasse (23.85%) is in agreement with that results verified by Ferreira-Leitão et al. (2010b), 23.6%. On the other hand, the authors found a considerably higher lignin content in sugarcane straw (36.1%), whereas, in the present study, sugarcane straw had 21.83% of total lignin. A similar lignin content in sugarcane straw (21.5%) was obtained in a study by Saad et al. (2008). This difference may be related to the heterogeneous character of sugarcane straw, which is composed of green and dry leaves (DA SILVA et al., 2011)

The obtained values for the mixture of the sugarcane biomass fractions (Table 3.1) were intermediate between those found for straw and bagasse. Thus, considering that the mixture is composed of a 1:1 ratio of each fraction, the values of the mixture are in agreement with those of the isolated fractions.

3.3.2 Influence of pretreatments on chemical composition

The pretreatment of lignocellulosic biomass results in changes in the chemical composition and physical structure of the substrates, including a redistribution of hemicellulose and lignin, increasing surface area and formation of pores (BRAGATTO et al., 2012). In this study, four different pretreatment strategies were carried out on a 1:1 (w/w) mixture of sugarcane straw and bagasse: ionic liquid ([Emim][Ac]) in a thermostated water bath at 100 °C

(IL in TB at 100 °C), ionic liquid ([Emim][Ac]) in an ultrasonic bath at 25 °C (IL in UB at 25 °C), and dilute sulfuric acid using concentrations of 0.5% (w/v) (DSA 0.5% w/v) and 1% (w/v) (DSA 1% w/v). Table 3.2 summarizes the four pretreatment strategies and the amounts of each major cell wall component of the sugarcane mixture before and after the pretreatments.

Table 3.2. Composition of the 1:1 (w/w) mixture of sugarcane bagasse and straw before and after the pretreatments (dry weight).

Pretreatment	Components (%)				
	Holocellulose	**α-cellulose**	**Hemicellulose**	**Lignin**	**Ashes**
DSA 0.5 % (w/v)	71.66 a	45.02 a	26.63 a	23.08 a	3.50 a
DSA 1.0 % (w/v)	68.50 b	39.67 b	28.83 b	23.02 a	3.44 a
IL in TB at 100°C	72.36 c	45.62 a	26.75 a	18.48 b	3.38 a
IL in UB at 25°C	72.76 c	44.27 a	28.48 b	18.77 b	3.23 a
Untreated	72.24 ac	41.99 c	30.25 d	23.74 c	3.54 a

Means followed by the same letter in the same column did not differ by the Tukey test at the 5% significance level. DSA 0.5 % (w/v) and DSA 1.0 % (w/v) correspond to pretreatment with dilute sulfuric acid at concentrations of 0.5 and 1% (w/v) and IL in TB at 100°C and IL in UB at 25°C correspond to pretreatment with ionic liquid in thermostatic bath at 100°C and ultrasonic bath at 25°C respectively.

Our results showed that, when compared with the untreated materials, both pretreatment strategies were able to remove lignin and hemicellulose as well as produce higher cellulose content. However, the pretreatment with dilute sulfuric acid 1% (w/v) led to a lower cellulose content when compared with the untreated biomass, with a reduction from 41.99% to 39.67% (Table 3.2). These results were expected because the amorphous hemicellulose in sugarcane, composed of acetylated glucuronoarabinoxylans, can be easily hydrolyzed by dilute acids at high temperatures (LAVARACK; GRI; RODMAN, 2002; NEUREITER et al., 2002; ADSUL et al., 2005). Cellulose, on the other hand, is known to be more recalcitrant toward dilute acid hydrolysis and, because of the surface-governed reaction mechanism, is expected to be substantially less hydrolyzed than hemicellulose (TORGET; KIN; LEE, 2000; ZHAO et al., 2009).

The pretreatments with IL removed more lignin and slightly less hemicellulose than the dilute acid pretreatments (Table 3.2), producing recovered materials with lower levels of residual lignin (18.48% vs. 23.02%, respectively) an slightly higher levels of residual hemicellulose (28.48% vs. 26.63%, respectively). The total cellulignin content after the pretreatments presented significant reduction when compared to the untreated mixture of sugarcane bagasse and straw. Regrading the ionic liquid pretreatment, total lignin decreased

from 23.74% to 18.48%; this result can be correlated with data shown in Figure 3.1 since the range of 47.30–50.25% of solubilized mass was attributed to lignin removal in the pretreatments with ([Emin][Ac]), whereas the lower range of 7.46–7.81% was observed in the pretreatments with dilute sulfuric acid. These results show that the pretreatments with the ionic liquid ([Emin][Ac]) resulted in a high degree of delignification. The obtained data are comparable to those reported by Qiu et al. (2012); the authors reported that 15.1% of the total mass was lost during pretreatment with IL and that 52.6% of this loss was attributed to lignin removal in the sugarcane bagasse pretreated with ([Emin][Ac]) under similar experimental conditions. Previous studies have indicated that ([Emin][Ac]) is effective in removing lignin (LEE et al., 2009; SAMAYAM; SCHALL, 2009; SHILL et al., 2011). Shill et al. (2011) highlighted that a complete delignification of biomass is difficult to achieve due to the location of lignin within the lignin-carbohydrate complex, the strong C–O–C and C–C bonds in its poly-ring structure, and its hydrophobicity (KIM et al., 2003).

As shown in Figure 3.1, the highest percentage of solubilized mass (11.12% w/w), and consequently the lowest percentage of recovered solids (88.88% w/w), can be verified when the pretreatment was performed with ([Emin][Ac]) in a TB at 100 °C. This extraction can generate porous structures in the cell wall and make the cellulose fibrils swell easily, resulting in a complete cellulose dissolution. After precipitation of the dissolved product, a material with rich cellulose content can be recovered as a consequence of the loss of water-soluble lignin and hemicellulose (DA SILVA et al., 2011). ([Emim][Ac]) has frequently been considered the most effective IL for cellulose dissolution and pretreatment for enzymatic hydrolysis (BRANDT et al., 2013). In actualy, anions act as hydrogen-bond acceptors that interact with the hydroxyl group of cellulose, thereby weakening its crystalline structure (JANESKO et al., 2011). Among the anions of imidazolium ILs, the acetate anion has been reported to have the highest hydrogen-bond basicity/value of β and the highest capability of reducing cellulose crystallinity (BRANDT et al., 2013).

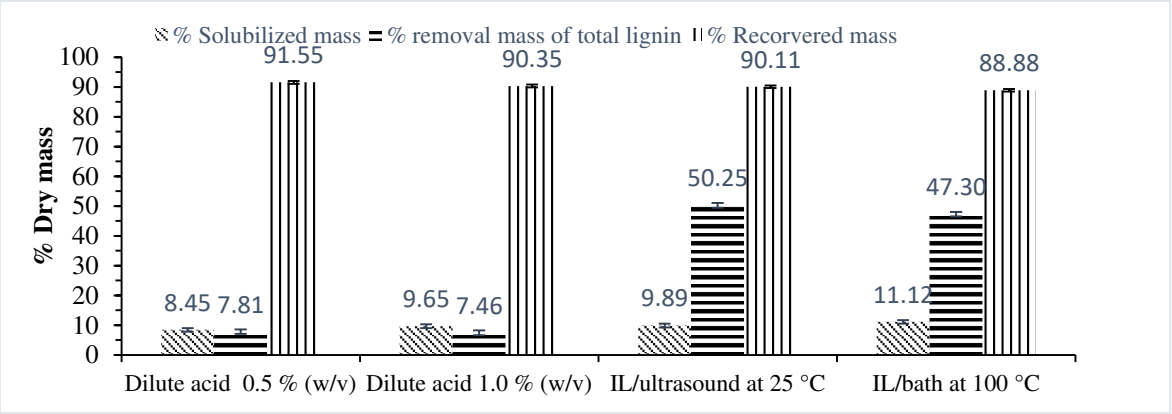

Figure 3.1. Changes in the content of lignin of the 1:1 (w/w) mixture of sugarcane bagasse and straw after different pretreatments and their respective percentage of recovered and solubilized mass.

In consequence of the similarity of chemical composition between pretreated biomasses analyzed, an exploratory study was carried out in order to evaluate the impact of each pretreatment on the effect of different combinations of commercial enzymes. Thus the pretreatment with IL in TB at 100 °C and the pretreatment with DSA 0.5% (w/v) were chosen to perform this study, as these pretreatments presented more similar results in relation to the changes in chemical composition of the analyzed biomass The dry weight percentages in the range of 39.67- 45.02% α-cellulose; 26.63-30.25% hemicellulose and 3.23-3.50% ashes did not differ by the Tukey test at the 5% significance level, as shown in Table 3.2. Although the use of an ultrasonic bath also has shown to be a feasible strategy of additional pretreatment since the loss of a considerable proportion of hemicellulose would be undesirable and could result in the low yield and high cost of sugars derived from biomass (LI et al., 2010).The similarity of the chemical composition of the sugarcane mixtures pretreated with IL in TB at 100 °C or DSA 0.5% (w/v) makes their analysis more appropriate for evaluating the performance and interaction of enzymes after pretreatments, which was the main objective of this work. In addition according to Li et al.(2010), the more effective pretreatments are generally those that employ higher temperatures and incubation times, indicating that there may be an effective lignin glass transition temperature that must be exceeded to efficiently solubilize lignin.

Table 3.3 presents the data on the percentage of glucose and xylose conversion obtained after enzymatic hydrolysis of the sugarcane mixtures pretreated with dilute sulfuric acid (MPSA) or with an ionic liquid (MPIL).

Table 3.3. Plackett-Burman experimental design matrix (P12 + 3 central points) for enzymatic hydrolysis evaluation of mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA) and mixture of sugarcane bagasse and straw pretreated with the ionic liquid (MPIL).

Experiment	X1	X2	X3	X4	X5	MPSA		MPIL	
						Xylose (%)	Glucose (%)	Xylose (%)	Glucose (%)
1	$22_{(+1)}$	$0_{(-1)}$	$7_{(+1)}$	$0_{(-1)}$	$0_{(-1)}$	19.66	19.66	18.59	80.89
2	$22_{(+1)}$	$16_{(+1)}$	$0_{(-1)}$	$9_{(+1)}$	$0_{(-1)}$	79.80	79.80	78.30	92.53
3	$0_{(-1)}$	$16_{(+1)}$	$7_{(+1)}$	$0_{(-1)}$	$10_{(+1)}$	45.11	45.11	51.48	11.54
4	$22_{(+1)}$	$0_{(-1)}$	$7_{(+1)}$	$9_{(+1)}$	$0_{(-1)}$	39.98	79.56	38.82	82.96
5	$22_{(+1)}$	$16_{(+1)}$	$0_{(-1)}$	$9_{(+1)}$	$10_{(+1)}$	84.10	93.66	86.52	99;92
6	$22_{(+1)}$	$16_{(1)}$	$7_{(+1)}$	$0_{(-1)}$	$10_{(+1)}$	56.34	56.34	61.35	91.54
7	$0_{(-1)}$	$16_{(1)}$	$7_{(+1)}$	$9_{(+1)}$	$0_{(-1)}$	77.66	77.66	79.61	12.53
8	$0_{(-1)}$	$0_{(-1)}$	$7_{(+1)}$	$9_{(+1)}$	$10_{(+1)}$	21.98	21.98	21.01	7.69
9	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$9_{(+1)}$	$10_{(+1)}$	22.97	22.97	25.66	8.78
10	$22_{(+1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$10_{(+1)}$	19.99	19.99	16.78	78.91
11	$0_{(-1)}$	$16_{(1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	20.65	20.65	23.69	9.57
12	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	0.00	0.00	0.00	0.00
13	$11_{(0)}$	$8_{(0)}$	$3.5_{(0)}$	$4.5_{(0)}$	$5_{(0)}$	38.33	38.33	40.30	71.02
14	$11_{(0)}$	$8_{(0)}$	$3.5_{(0)}$	$4.5_{(0)}$	$5_{(0)}$	44.12	44.12	42.27	76.35
15	$11_{(0)}$	$8_{(0)}$	$3.5_{(0)}$	$4.5_{(0)}$	$5_{(0)}$	40.15	40.15	38.49	70.33

Where: factors X1, X2, X3, X4 and X5 **($mg/g_{substrate}$)** correspond to enzymes (cellulase, endo-1,4-xylanase, and β-glycosidase, β-xylosidase and α-L-arabinofuranosidase) respectively, level –1 means no enzyme addition (0%), level +1 means 100% of the enzyme reference concentration, and level 0 means 50% of the enzyme reference concentration.

Table 3.4. Experimental and validation results enzymatic hydrolysis evaluation of mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA) and mixture of sugarcane bagasse and straw pretreated with the ionic liquid (MPIL).

Experiment	MPSA		MPIL	
	% Glucose	% Xylose	% Glucose	% Xylose
Plackett-Burman	93.66	84.10	99.92	86.52
Validation Result	91.89 ± 0,84	82.34 ± 0.67	97.89 ± 0.78	84.89 ± 0.76

The highest glucose yields achieved with MPIL and MPSA were 97.89 ± 0.78 and 84.89 ± 0.76, respectively. Banerjee et al.(2010d), taking a step toward the development of a more efficient enzyme cocktail for the conversion of biomass, also evaluated the effect of commercial enzymes (Accellerase 1000, Multifect Xylanase, and Spezyme CP, Novozyme 188) on the enzymatic hydrolysis of corn stover pretreated with ammonia-fiber expansion (AFEX). An enzyme loading of 15 mg of protein/g_{glucan} was employed for each enzyme, and,

after 48 h of hydrolysis at 50°C, provided yields of 53% and 41% of glucose and xylose, respectively. These values were significantly lower than the obtained present study. Thus, the pretreatments with IL and DSA were quite efficient to increase the accessibility of the enzymes to the sugarcane biomass. In addition, the studied mixture of sugarcane bagasse and straw was a very promising source of polysaccharides, which together contributed to achieving high conversion yields of fermentable sugars. On the other hand, the obtained glucose yield is in agreement with that obtained by Li et al. (2010) in experiments of enzymatic hydrolysis on switchgrass pretreated with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]).The authors reported significantly high saccharification kinetics, with cellulose digestibility reaching 96.0% within 24 hours. Although a longer hydrolysis time (48 hours) was used in the present work, a lower protein concentration of cellulase (22 mg protein/g substrate) was required, whereas, in the Li et al. (2010) study, a 50 mg protein/g substrate protein concentration was used. Thus, the synergistic effect between the other enzymes used in combination with cellulase contributed to achieve high yields of glucose and xylose while using a lower concentration of cellulase than in the cited study. For example, Da Silva et al. (2010) also reported a high yield of glucose (98.2%) with the enzymatic hydrolysis of sugarcane bagasse pretreated with ([Emim][Ac]), similarly to the results of this work. However, the authors achieved a considerably lower xylose yield (60%) than that obtained in this work (86.52%). The authors explain that this relatively low yield of xylose could be correlated with the considerable removal/degradation of xylan during the pretreatment. Therefore, the yields of glucose and xylose obtained in this work are consistent with the results of the chemical composition of the pretreated biomasses since only a slight loss of hemicellulose occurred during these pretreatment reactions.

Regarding the enzymatic hydrolysis of biomass pretreated with dilute acids, Zhao et al. (2009) reported a glucose yield of 84% after 48 hours when using an enzyme loading of 84 FPU/g_{glucan} in Cattails pretreated with 0.5% sulfuric acid, a result relatively similar to the obtained in this study after the enzymatic hydrolysis of MPSA. On the other hand, Canilha et al. (2011) reported the low efficiency of cellulose saccharification (30.0%) in sugarcane bagasse pretreated with 0.5% sulfuric acid, which can be explained by the high solid loading employed (15% wt), whereas only 2% wt was used in this work. Yoon et al. (2012) also reported low yields of reducing sugars after the enzymatic hydrolysis of sugarcane bagasse pretreated with ([Emim][Ac]). The authors explained that the low yield was due to the high solids content, which prevented ([Emim][Ac]) from performing an effective delignification.

In order to compare the results, MPSA and MPIL were hydrolyzed using two different commercial cellulolytic cocktails of enzymes, Celluclast® 1.5 and Cellic® CTec2, under the same experimental conditions employed in the enzymatic hydrolysis experimental design, with an enzyme loading of 10 FPU/$g_{cellulose}$, as performed by Rodrigues et al. (2015). The obtained enzymatic hydrolysis yields of glucose and xylose for both pretreated biomasses, MPSA and MPIL, are shown in Table 3.5.

Table 3.5. Yields of glucose and xylose obtained after enzymatic hydrolysis using different commercial cellulolytic cocktails for enzymatic hydrolysis evaluation of mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA) and mixture of sugarcane bagasse and straw pretreated with the ionic liquid (MPIL).

Enzyme	MPSA		MPIL	
	Glucose (%)	**Xylose (%)**	**Glucose (%)**	**Xylose (%)**
Cellic® CTec2	61.05	12.56	75.16	12.61
Celluclast® 1.5	33.63	3.88	42.98	4.65

The enzyme preparations differed significantly in their ability to convert cellulose and hemicellulose, Cellic® CTec2 being more efficient than Celluclast® 1.5 for both monosaccharides. Thus, we observed that when using an enzyme loading of 10 FPU/$g_{cellulose}$, Cellic® CTec2 was able to convert 61.05% of glucose after 48 hours of hydrolysis, whereas when using Celluclast® 1.5, the glucose yield reached only 33.63%. Rodrigues et al. (2015) also reported higher values of conversion to glucose using Cellic® CTec2 than Celluclast® 1.5 for the hydrolysis of hydrothermally pretreated wheat straw biomass. The authors found values of glucose conversion of 50% after 48 hours using Cellic® CTec2, whereas, for Celluclast® 1.5, a conversion of 20% was observed, results that are in agreement with those obtained in this work.

The yields of glucose and xylose obtained with the cellulolytic cocktails for both pretreated biomasses were significantly lower than those obtained in the experimental design (experiments nos. 1, 2, 5, 6, and 10), showing that most of the enzyme combinations proposed in the Plackett-Burman design were very efficient in hydrolyzing the studied biomass mixtures.

3.3.3 Effect of commercial enzyme mixtures

The conversion of lignocellulosic biomass into liquid fuels and other chemicals can be achieved using multi-enzymatic systems. In this study, the combinatorial effects of five

commercial enzymes, α-L-arabinofuranosidase, β-xylosidase, endo-1,4-xylanas, cellulase, and β-glucosidase, were assessed using the Plackett-Burman experimental design strategy to analyze synergistic and/or antagonistic effects of enzyme interactions for biomass degradation (RODRIGUES; IEMMA, 2014).

Based on the Pareto chart analysis ($p < 0.1$) (Figure 3.2 and Figure 3.3), all significant effects were positive as well there was no difference in the significant effects on the biomass samples in response to the pretreatments with IL and DSA. This result can be correlated with the slight difference in the chemical composition of the sugarcane mixtures after the pretreatments. Since the rate of enzymatic hydrolysis and reducing sugars yield is directly affected by biomass structure and composition, this affirmation is also valid for hemicellulases and cellulases, as was demonstrated for hemicellulases in different xylan sources in a previous study by Goldbeck et al. (2014). Also, Rodrigues et al. (2015) highlighted that the concept of accessory enzymes has evolved over time since most enzymes in enzymatic cocktails are considered essential to increase sugar yield during biomass saccharification. The previous studies have shown that supplementation of cellulase mixtures with hemicellulases can improve the rate and yield of cellulose conversion because the removal of hemicellulose exposes the cellulose fibrils and increases substrate accessibility. Furthermore, synergism between the enzymes is a widely observed phenomenon in biomass hydrolysis and depends on several factors, including the nature of the substrate and the source of enzymes. Literature data show that these enzymes contribute synergistically to the release of glucose (WANG et al., 2011).

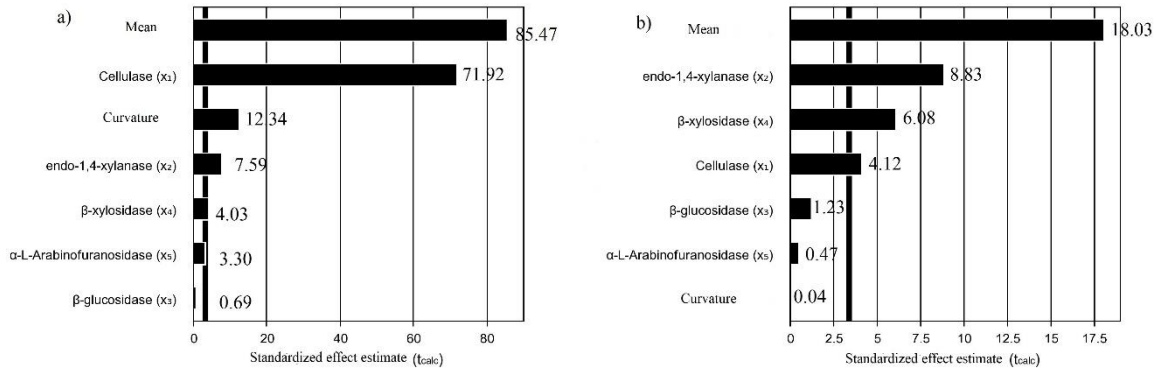

Figure 3.2. Pareto chart of standardized effects ($p < 0.1$) of (a) glucose and (b) xylose released after enzymatic hydrolysis of the mixture of sugarcane bagasse and straw pretreated with 0.5% (w/v) sulfuric acid.

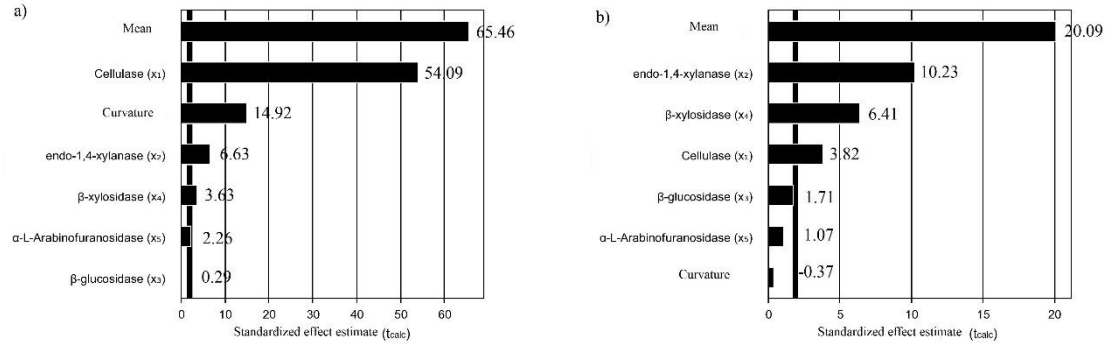

Figure 3.3. Pareto chart of standardized effects ($p < 0.1$) of (a) glucose and (b) xylose released after enzymatic hydrolysis of the mixture of sugarcane bagasse and straw pretreated with ([Emin][Ac]).

Cellulase and endo-1,4-xylanase presented a greater contribution for obtaining higher yields of glucose and xylose, respectively, as well as slowing synergistic effect on the release of both monosaccharides. This great effect shown by cellulase can be attributed to the fact that this enzyme is actually a cellulosic complex since cellulase formulations contain a broad spectrum of enzymes that work synergistically to convert cellulose to simpler sugars, namely, endoglucanases, cellobiohydrolases, and β-glucosidases (RODRIGUES et al., 2015). In relation to β-glucosidase, a synergistic effect on the release of glucose was expected, however this enzyme had no effect on hydrolysis since it is responsible for hydrolyzing cellobiose to produce glucose (SUN; CHENG, 2002). This fact can be explained by the activity already present in cellulase, which was characterized with an enzyme loading of 43 BGU/$g_{cellulose}$ that was enough to achieve a high cellulose conversion in this study. A supplementary enzyme loading was added through β-glucosidase, corresponding to 52 BGU/$g_{cellulose}$, which had no effect in the enzymatic hydrolysis process. Thus, it is assumed that β-glucosidase saturation occurred when an extra enzyme loading was added. β-xylosidase together with endo-1,4-xylanase had a great synergistic effect on the release of xylose, as expected, since both enzymes act on the main chain of xylan, being directly responsible for the release of xylose monosaccharides (DUFF, 1996).

3.4 CONCLUSION

The chemical compositions of the mixtures of sugarcane bagasse and straw after different pretreatment strategies were similar in relation to their major components. However, the pretreatment with IL led to a higher removal of lignin and, consequently, slightly higher yields of glucose and xylose when compared to the pretreatment with dilute sulfuric acid. The use of α-L-arabinofuranosidase, β-xylosidase, endo-1,4-xylanase, and cellulase, in the studied

protein concentrations for the enzymatic hydrolysis of sugarcane biomass, provided a high yield of fermentable sugars with both pretreatment strategies. Moreover, β-glucosidase did not affect the hydrolysis process when using the evaluated protein concentration of cellulase, thus in this condition it can be removed from the reaction to reduce enzyme costs.

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CAPÍTULO IV: OPTIMIZATION OF COMMERCIAL MIXTURE ENZYMES CONCENTRATIONS FOR XYLOOLIGOSACCHARIDES PRODUCTION BY HYDROLYSIS OF SUGARCANE BIOMASS PRETREATED WITH IONIC LIQUID OR DILUTE ACID

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ABSTRACT

Xylooligosaccharides (XOS) are non-digestible food ingredients with prebiotic properties for selectively promoting the growth of probiotics, thus providing many health benefits. The production of XOS by enzymatic hydrolysis of lignocellulosic biomass is limited mainly by the high cost of enzyme production. The objective of this study was to select and optimize the concentration of five different commercial enzymes for the production of XOS with degree of polymerization of 2–6 by enzymatic hydrolysis of a mixture of sugarcane bagasse and straw (1:1) (m/m), pretreated with ionic liquid or sulfuric acid diluted to 0.5% (m/v). In the Plackett–Burman experiments, the factors studied were five commercial enzymes: α-L-arabinofuranosidase and β-xylosidase from Megazyme® and endo-1,4-xylanase (NS50030), cellulase (NS50013), and β-glucosidase (NS50010) from Novozymes®. The levels studied were (+1) corresponding to the presence in range concentration of 10 $mg/g_{substrate}$, 9 $mg/g_{substrate}$, 16 $mg/g_{substrate}$ and 22 $mg/g_{substrate}$, respectively; (-1) the absence of enzyme, and (0) the half that concentration. However only the enzymes endo-1,4-xylanase (NS50030, Novozyme®) and α-L-arabinofuranosidase (Megazyme®) presented a positive and significant effect at the 5% significance level. Using a rotatable central composite design, an increase in the production of XOS of 45% and 62% was achieved for the mixtures pretreated with ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]) and dilute acid, respectively, in relation to the highest concentration obtained in the Plackett–Burman experiments. The use of ionic liquid pretreatment was economically advantageous for requiring a lower enzyme concentration in the hydrolysis step: biomass pretreated with ionic liquid required up to 20% less of the optimum concentration of endo-1,4-xylanase to yield the same amount of XOS in relation to biomass

pretreated with dilute acid, thus representing a possible alternative to reduce the cost with the used enzyme mixtures.

Keywords: xylooligosaccharides, enzymatic hydrolysis, ionic liquids, dilute sulfuric acid

4.1 INTRODUCTION

Lignocellulosic biomass, such as sugarcane biomass (bagasse and straw), is a cheap, abundant, and renewable raw material that can be employed for sustainable production of biofuel, bioenergy, and several value-added biomolecules (JAYAPAL et al., 2013; GONÇALVES et al., 2012; DAMÁSIO et al., 2012). Due to the lignocellulosic nature of plant wastes, they can be fractionated into cellulose, hemicellulose, and lignin, which, can be used as renewable sources for the production of value-added products (ZILLIOX, DEBEIRE, 1998; AGRUPIS, MAEKAWA, 1999). As xylan is the main component of the hemicellulosic fraction of plant wastes, lignocellulosic biomass could be an appropriate starting material for the production of xylose, xylitol, and xylooligosaccharides.

Xylooligosaccharides (XOS) are sugar oligomers produced during the hydrolysis of xylan, a heteropolysaccharide with a homopolymeric backbone of xylose units (SAHA, 2003). XOS are used in pharmaceutical industries and are incorporated into many food products. As food ingredients, XOS have an acceptable taste and are non-cariogenic low-calorie additives that can be used in weight-loss diets (VÁZQUEZ et al., 2000). In food processing, XOS show advantages over inulin in terms of heat and acid resistance, allowing their use in low-pH juices and carbonated drinks (MODLER, 1994). One of the most important applications of XOS is in functional foods, as these compounds are stable over a wide range of pH and temperature and promote beneficial health effects for their selective metabolism by bifidobacteria, increased production of volatile fatty acids, and anti-gastric ulcer activity (VÁZQUEZ et al., 2000).

XOS production by autohydrolysis or hydrothermal processing of lignocellulosic biomass results in hydrolysates containing a large variety of undesirable components, such as xylose and lignin, demanding a complex purification step (ZHU et al., 2006). Thus, enzymatic hydrolysis is generally preferred by food industries as it does not produce undesirable by-products neither requires special equipment operating at high-temperature and high-pressure conditions, in contrast to autohydrolysis (AKPINAR, ERDOGAN, BOSTANCI, 2009). XOS can be produced by endoxylanases, which hydrolyze the β-1,4-glycosidic linkages in xylan. For XOS production, the enzyme complex should have low exoxylanase or β-xylosidase activity.

Enzymes with high exoxylanase or β-xylosidase activity produce high amounts of xylose, which causes inhibitory effects on XOS production (VAZQUEZ et al., 2002). Furthermore, endo-1,4-β-xylanases must have feasible activity under the hydrolysis conditions. Actually, knowledge about the xylanolytic activity, optimum pH and temperature, thermal stability, and kinetic parameters of a commercial xylanase preparation is pivotal to achieve relevant XOS yield as well as feasible large-scale production (ACHARY, PRAPULLA, 2009).

Currently, the food industry spends high time and money to produce XOS, and, therefore, their market value is costly. The high cost of enzymes is a hindrance to XOS production and largely contributes to the final cost of the process. A better understanding of which enzymes, in which proportions, are important for lignocellulosic degradation, leading to the rational design of more efficient, and thus less expensive, enzyme mixtures (BANERJEE et al., 2010c). Due to different pretreatment conditions (e.g., steam, hot water, ionic liquids, dilute acid, AFEX™, or alkaline peroxide), there is a large number of possible pretreatment/substrate combinations. Thus, it is necessary to know which enzymes present significant effect on XOS production in order to optimize the process, decrease the cost, and achieve high final yields (BANERJEE et al., 2010b).

This study investigated the effect of hemicellulases and cellulases on a mixture of sugarcane bagasse and straw pretreated with ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]) provided from Iolitec® (purity 98%) or dilute sulfuric acid (95-98%) provided from Sinth®. Plackett–Burman experimental designs were employed to verify the synergistic/antagonistic effects of hemicellulases and cellulases in the production of XOS. Two types of sugarcane mixtures were tested: sugarcane mixture pretreated with dilute sulfuric acid (MPSA) and sugarcane mixture pretreated with ionic liquid (MPIL). Five commercial enzymes were evaluated: α-L-arabinofuranosidase, β-xylosidase, endo-1,4-xylanase, cellulase, and β-glucosidase. The enzymes with significant effects on XOS production were evaluated using a central composite rotational design (CCRD) to define their optimal enzyme concentrations.

4.2 MATERIAL AND METHODS

4.2.1. Raw materials

Sugarcane bagasse was kindly supplied by Raízen, Piracicaba, SP, and sugarcane straw by Dr. Paulo Graciano, from the Brazilian Bioethanol Science and Technology Laboratory (CTBE/CNPEM), Campinas, SP, Brazil. The moisture content of the raw materials

was quantified according to AOAC (1995) method. Sugarcane bagasse and straw contained 45% and 48% moisture (wet base), respectively. Both sugarcane fractions were washed with water at room temperature to remove excess soil and dust (SZCZERBOWSKI et al., 2014). Samples were oven-dried at 80°C to at least 10% moisture (dry base), ground in a cutting mill, and sieved to retain particles in the range of 24–48 mesh.

4.2.2. Pretreatments

The sugarcane mixtures were pretreated with 0.5% (w/v) dilute sulfuric acid or the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]), acquired from Iolitec® (Germany) (Purity 98%). Pretreatments were performed in 250 mL Erlenmeyer flasks placed in an thermostatic bath at 100°C for 30 min. Biomass at a solid loading of 5 wt% and 10 wt% was used for the pretreatments with dilute sulfuric acid (GÓMEZ-RUEDA, 2010) and ionic liquid (QIU et al., 2012), respectively. Solids were washed repeatedly with deionized water, until the wash solution was colorless, to remove any remaining ionic liquid or acid from the samples (NINOMIYA et al., 2015b). After the experiments, the solid fraction was separated by vacuum filtration, and the remaining solids were collected, oven-dried at 105°C overnight, and stored in sealed bags at room temperature.

4.2.3. Chemical composition determination

The holocellulose, α-cellulose, and hemicellulose contents were measured by treatment of the biomass with sodium chlorite under acidic conditions. The experimental procedure used in this study was proposed by Da Silva et al. (2011) as an adaption from the classical method described by Browning (1967) and Wise et al. (1946). This method was chosen for its feasibility, and its potential as an alternative method to the traditional method of Sluiter et al. (2008), which is an expensive method since it requires chromatographic techniques and therefore, a longer analysis time. Ash content was determined by burning 1 g of sample in a tarred crucible for 6 h using a muffle furnace at 600°C until constant weight, according to the NREL/TP-510-42622 procedure (SLUITER et al., 2005). Total lignin content was determined by hydrolysis with 72% (w/w) sulfuric acid according to the analytical procedure recommended by NREL (SLUITER et al., 2008). The composition analysis results of both sugarcane mixtures were obtained by the mean of three independent replicates.

4.2.4. Commercial enzymes and determination of enzyme concentration

The enzymes used in the experimental design were α-L-arabinofuranosidase and β-xylosidase from Megazyme® and endo-1,4-xylanase (NS50030), cellulase (NS50013), and β-

glucosidase (NS50010) from Novozymes®. The enzyme concentration was determined according to the method proposed by Lowry (1951) using bovine serum albumin (Sigma-Aldrich) as a standard.

4.2.5. Enzymatic hydrolysis and experimental designs

The present study used a Plackett–Burman design to analyze synergistic/antagonistic effects of commercial hemicellulolytic and cellulolytic enzymes on biomass degradation. Enzymatic micro-assays were carried out in 1.5 mL Eppendorf tubes using a 2–20 μL micropipette (Pipetman G P20G, Gilson). The Plackett–Burman design consisted of 12 experiments and 3 repetitions of the central point, totaling 15 experiments, in order to assess the effects of the independent variables on the degradation of MPSA and MPIL for XOS release (RODRIGUES; IEMMA, 2014). The independent variables used in the experimental design were the five commercial enzymes: α-L-arabinofuranosidase, β-xylosidase, endo-1,4-xylanase, cellulase, and β-glucosidase. Preliminary tests were performed and kinetic curves were generated to verify the minimum concentration of each enzyme required for the experiments (Appendix 1). The enzyme concentrations were set at 10 $mg/g_{substrate}$, 9 $mg/g_{substrate}$, 16 $mg/g_{substrate}$, 22 $mg/g_{substrate}$, and 7 $mg/g_{substrate}$ for α-L-arabinofuranosidase, β-xylosidase, endo-1,4-xylanase, cellulase, and β-glucosidase, respectively. The process response (dependent variable) was the concentration of free XOS released from the hydrolysates. Three levels of experimental design were defined: −1, no enzyme addition; +1, 100% of enzyme concentration; and 0, 50% of enzyme concentration (central point). Analysis of the effects of each independent variable was conducted with the aid of the online software Protimiza Experimental Design (http://experimental-design.protimiza.com.br/). The reaction mixtures contained 20 mg of substrate (MPIL or MPSA), a combination of commercial enzymes as described by Goldbeck et al. (2014, 2016), and sodium phosphate buffer (0.1 M, pH 5.0) to complete the final volume of 1 mL. Samples were incubated at 50°C for 48 hours under agitation (1000 rpm). After this period, samples were centrifuged (12.000g for 15 minutes at 4°C), and the supernatant was collected for subsequent analysis. After performing the experiments, the enzymes that presented significant effects were further evaluated using a central composite rotatable design (CCRD) to define their optimal concentration range. A CCRD was elaborated with two variables (α-L-arabinofuranosidase and endo-1,4-xylanase) by using the online software Protimiza Experimental Design (*http://experimental-design.protimiza.com.br/*). The α-L-

arabinofuranosidase concentration range varied from 3 to 17 mg/$g_{substrate}$ and the endo-1,4-xylanase concentration range varied from 5 to 27 mg/$g_{substrate}$, including the axial points (± 1.41) (Table 4.1). These levels were chosen in order to increase the response (positive effect), but in the same time to decrease the enzymatic cost. Thus the superior levels used in the Plackett-Burman design (+1) were keep in the level (0) in the CCRD and the previously studied range was relocated both to the left to try to reduce the required enzyme concentration and also to the right in order to increase the concentration of XOS produced.

Table 4.1. Levels and variables used in the central composite rotatable design (CCRD)

Variable (mg enzyme/g substrate)	**-1.41**	**-1**	**0**	**1**	**1.41**
α-L-arabinofuranosidase	3	5.03	10	14.96	17
endo-1,4-xylanase	5	8.3	16	23.69	27

4.2.6. Enzyme activity assays

Filter paper activity (FPase) and carboxymethyl cellulase (CMCase) activity were determined according to IUPAC recommendations (GHOSE, 1987). β-glucosidase, β-xylosidase, and α-L-arabinofuranosidase activities were determined according to the method conducted by Da Silva et al. (2010), using 100 µL of 10 mM ρ-nitrophenyl-β-D-glucopyranoside, ρ-nitrophenyl-b-D-xylopyranoside, and ρ-nitrophenyl-a-l-arabinofuranoside, respectively, 200 µL of 0.5 M sodium acetate buffer pH 5.0, 650 µL of Milli-Q water, and 50 µL of the appropriately diluted enzyme solution. After incubating the reaction mixture at 45°C for 10 min, the reaction was stopped by adding 500 µL of 1 M Na_2CO_3. The concentration of liberated ρ-nitrophenol was measured at 420 nm. One unit of β-glucosidase, β-xylosidase, and α-L-arabinofuranosidase was defined as the amount of enzyme that released 1 µmol of ρ-nitrophenol at 45°C in 1 min. Endo-1,4-xylanase activity was assayed against 500 µL of 2% soluble xylan from Birchwood (Sigma–Aldrich) and 0.1 mL of appropriately diluted supernatant, which were mixed and reacted at 45°C for 30 minutes in 50 mM of sodium acetate buffer pH 5.0, in a total volume of 1 mL (DA SILVA et al., 2011). The resulting reducing sugars were analyzed using a dinitrosalicylic acid (DNS) assay (MILLER, 1959). One unit of xylanase was defined as the amount of enzyme required to produce 1 µmol of reducing sugars in 1 minute at 45°C.

4.2.7. Xylose and XOS quantification

Xylose and XOS quantification was performed by high performance anion exchange (HPLC–PAD) using the supernatants that resulted from the enzymatic hydrolysis of the sugarcane mixtures. Chromatographic analysis was performed on a Dionex DX-500 (Sunnyvale, CA, EUA) with a CarboPac PA100 column (4 mm × 250 mm), a CarboPac PA100 guard column (4 mm × 50 mm), and an electrochemical detector, adopting a linear gradient of A (NaOH 50 mM) and B (NaOAc 500 mM; NaOH 50 mM). The integrated peak areas were adjusted based on standards purchased from Megazyme® (Bray, County Wicklow, Ireland): xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

4.3 RESULTS AND DISCUSSION

4.3.1. Compositional analysis of sugarcane mixtures submitted to different pretreatments

Pretreatment of the lignocellulosic biomass results in changes to the chemical composition and physical structure of the plant material, such as redistribution of hemicellulose and lignin fractions, increase in surface area, and formation of pores (BRAGATO et al., 2012). The rate of enzymatic hemicellulose conversion is also directly affected by biomass structure and composition (VASCONCELOS et al., 2013). The chemical compositions of the two substrates used in this study are shown in Table 4.2. Although α-cellulose was the major constituent of MPIL and MPSA and the percentage of hemicellulose was similar between them, MPIL had a lower dry weight percentage of lignin (18.48 ± 0.23%) in comparison with MPSA (23.08 ± 0.26%).

Table 4.2. Chemical composition of the sugarcane mixture pretreated with 0.5 % (w/v) dilute sulfuric acid (MPSA) and sugarcane mixture pretreated with ionic liquid (MPIL).

Composition	MPSA	MPIL
α-Cellulose	45.02 ± 0.35	45.62 ± 0.25
Hemicellulose	26.63 ± 0.56	26.75 ± 0.66
Total lignin	23.08 ± 0.62	18.48 ± 0.58
Ashes	3.50 ± 0.31	3.38 ± 0.43

These results indicate that ionic liquid pretreatment can produce a substrate with a similar composition to that obtained by dilute sulfuric acid pretreatment while preserving the

proportions of cellulose and hemicellulose and yielding relatively lower lignin content. The pretreatments employed in this study preserved the hemicellulose and cellulose contents of the sugarcane biomass mixtures. Acids are commonly used in the industrial pretreatment of biomass to solubilize its hemicellulosic fraction. Cost-effective hydrolysis and utilization of soluble sugar oligomers formed by the reaction is an integral process of biofuel production (KIM, KREKE, LADISCH, 2013). In relation to ionic liquid pretreatment, recent studies have shown that ([Emim][Ac]) can effectively solubilize biomass (SARATH et al., 2008) and provide polysaccharides with lower lignin concentrations (SINGH et al., 2009). In the present study, ionic liquid pretreatment generated a product that was efficiently hydrolyzed using a mixture of commercial cellulase and hemicellulase. Furthermore, several recent studies reported the complete dissolution and partial delignification of sugarcane, softwood, and hardwood with various ionic liquids (LEE et al., 2009; SUN et al., 2009; TAN et al., 2009). Although ionic liquid pretreatment is more expensive than dilute acid pretreatment, it was selected in the present study because it generates a lower amount of enzyme inhibitors and promotes a highly selective delignification, justifying its use in this present work.

4.3.2. Experimental design (Plackett–Burman)

The conversion of lignocellulosic biomass into liquid fuels and other chemical products can be achieved through the use of multi-enzymatic systems. In this study, the combinatorial effects of five different commercial enzymes, including three hemicellulolytic enzymes (α-L-arabinofuranosidase, β-xylosidase, and endo-1,4-xylanase) and two cellulolytic enzymes (cellulase and β-glucosidase), were evaluated by using Plackett–Burman designs to analyze the synergistic/antagonistic effects of the enzymes on the release of XOS from MPSA and MPIL. Actually, there are no reports of significant effects of cellulases on the release of XOS. However, cellulases were included in this study to perform an exploratory investigation of the performance of different commercial enzymes on the evaluated biomass and to ascertain possible synergistic or antagonistic effects with hemicellulases, thus providing a comprehensive view of the evaluated process. The carbohydrate profile and total XOS production (X2–X6) obtained in the Plackett–Burman experiments with different enzyme combinations are presented in Table 4.3.

Table 4.3. Plackett–Burman design matrix (P12 + 3 central points) for evaluating the enzymatic hydrolysis of mixture of sugarcane bagasse and straw pretreated with ionic liquid (MPIL) and with dilute sulfuric acid (MPSA).

						MPIL		MPSA	
Experiment	**V1**	**V2**	**V3**	**V4**	**V5**	**Hemicellulose conversion (%)**	**XOS (X2-X6) (mg/L)**	**Hemicellulose conversion (%)**	**XOS (X2-X6) (mg/L)**
1	$22_{(1)}$	$0_{(-1)}$	$7_{(+1)}$	$0_{(-1)}$	$0_{(-1)}$	19.01	19.50	20.00	16.60
2	$22_{(+1)}$	$16_{(+1)}$	$0_{(-1)}$	$9_{(+1)}$	$0_{(-1)}$	79.98	79.00	81.00	57.40
3	$0_{(-1)}$	$16_{(+1)}$	$7_{(+1)}$	$0_{(-1)}$	$10_{(+1)}$	55.05	168.00	48.46	157.20
4	$22_{(+1)}$	$0_{(-1)}$	$7_{(+1)}$	$9_{(+1)}$	$0_{(-1)}$	39.04	10.20	40.44	21.60
5	$22_{(+1)}$	$16_{(+1)}$	$0_{(-1)}$	$9_{(+1)}$	$10_{(+1)}$	88.59	97.40	86.12	94.50
6	$22_{(+1)}$	$16_{(1)}$	$7_{(+1)}$	$0_{(-1)}$	$10_{(+1)}$	64.33	140.42	59.12	130.20
7	$0_{(-1)}$	$16_{(1)}$	$7_{(+1)}$	$9_{(+1)}$	$0_{(-1)}$	81.36	82.60	79.14	69.50
8	$0_{(-1)}$	$0_{(-1)}$	$7_{(+1)}$	$9_{(+1)}$	$10_{(+1)}$	21.84	39.20	22.78	38.95
9	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$9_{(+1)}$	$10_{(+1)}$	27.26	75.40	24.45	69.34
10	$22_{(+1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$10_{(+1)}$	17.34	26.40	20.28	13.60
11	$0_{(-1)}$	$16_{(1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	25.10	66.20	22.22	73.50
12	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	$0_{(-1)}$	0.00	0.00	0.00	0.00
13	$11_{(0)}$	$8_{(0)}$	$3.5_{(0)}$	$4.5_{(0)}$	$5_{(0)}$	41.59	60.89	39.55	57.00
14	$11_{(0)}$	$8_{(0)}$	$3.5_{(0)}$	$4.5_{(0)}$	$5_{(0)}$	43.48	56.81	45.23	52.00
15	$11_{(0)}$	$8_{(0)}$	$3.5_{(0)}$	$4.5_{(0)}$	$5_{(0)}$	39.71	57.58	41.31	54.20

Where: V1, V2, V3, V4, and V5 ($mg/g_{substrate}$) correspond to cellulase, endo-1,4-xylanase, β-glucosidase, β-xylosidase, and α-L-arabinofuranosidase, respectively; level −1 represents no enzyme addition (0%); level +1 represents 100% of enzyme concentration; and level 0 represents 50% of enzyme concentration. XOS corresponds to the concentration of xylooligosaccharides: (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

The cost and hydrolytic efficiency of the enzymes are important factors that restrict the commercialization of lignocellulosic biomass products (ALVIRA et al., 2001), and experimental designs constitute an attractive method that reduce the spent time in the optimization of enzyme mixtures (SUWANNARANGSEE et al., 2012). Regarding the lignocellulosic biomass mixtures evaluated in this study, MPIL presented the highest percentage of hemicellulose conversion (88.59%), achieved in experiment 5 (Table 4.3), suggesting that a lignocellulosic substrate with lower lignin content may increase substrate-enzyme interaction and the subsequent saccharification efficiency. However, the highest XOS yield was not obtained in experiment 5. This fact can be explained by the presence of β-xylosidase, which has been reported as an antagonistic effect on the release of XOS, responsible for cleaving X2 molecules into two X1 molecules. This result was similar to that of MPSA (Table 4.3), as the pretreated biomass presented similar hemicellulose content. On the

other hand, when the enzymes endo-1,4-xylanase and α-L-arabinofuranosidase were added in the absence of β-xylosidase, experiments 3 and 6, XOS yields were considerably higher for MPSA and MPIL in comparison to experiment 5, as shown in Table 4.3. This indicates that endo-1,4-xylanase and α-L-arabinofuranosidase can act synergistically on MPSA and MPIL, favoring the release of XOS. Although a higher XOS production occurred in experiments 3 and 6, the amount of XOS produced was still low. Taking this into account, a new study was conducted aiming the optimization of the enzyme concentration range using the response surface method based on CCRD, which will be presented below. Furthermore, experiments 13, 14, and 15 (central points) presented similar results, demonstrating the reproducibility of the performed experiments.

Exploratory studies on XOS production are highly important as XOS are considered an alternative to add value to biofuel production from lignocellulosic biomass, such as sugarcane bagasse and straw. XOS are some of the major soluble products of hemicellulosic hydrolysates generated after biomass pretreatment and can act as enzyme inhibitors in the step of cellulose hydrolysis required for second-generation ethanol production. However, XOS have excellent prebiotic properties and are one of the few nutraceutical products that can be produced from lignocellulosic biomass. As lignocellulosic biomass is cheap and abundant, the production of XOS from agricultural wastes offers a great opportunity for food and pharmaceutical industries (SAMANTA et al., 2015). XOS promote several beneficial health effects for their immunomodulatory, anti-inflammatory, and antioxidant activities, and have been considered in the treatment of various diseases.

Different from chemical hydrolysis and autohydrolysis processes, which generate XOS with a high degree of polymerization, enzymatic hydrolysis is a desirable method for XOS production because of its high specificity and minimum production of undesirable by-products (JAIN, KUMAR, SATYANARAYANA, 2014). Previous studies have been conducted on XOS production by enzymatic hydrolysis of lignocellulosic biomasses. For example, Brienzo et al. (2010) reported XOS yields of 2.8–6.4 mg/mL from the enzymatic hydrolysis of pretreated sugarcane bagasse, and Samanta et al. (2012) reported 2.8 mg/mL of XOS produced from natural grass. These yields are considerably higher than those achieved in this study, but it is important to note that both studies performed a previous extraction of the xylan fraction from the hemicellulose fraction, which contributed significantly to their higher yields.

MPSA and MPIL resulted in effective XOS production, with varied concentrations of X2–X6. However, ionic liquid pretreatment favored the release of X3, while dilute sulfuric

acid pretreatment favored X2 production (Figure 4.1). This fact can be explained by the mechanism of structural modification of the biomass that occurs with each pretreatment. In acid-catalyzed pretreatments, the hemicellulose layer is partly hydrolyzed, whereas in pretreatments with ionic liquids, the dissolution of hemicellulose occurs through the penetration of ions into the extensive network of hydrogen interactions between cellulose chains (BRANDT et al., 2013). Thus, results indicate that after pretreatment with dilute sulfuric acid, hemicellulose chains are partly depolymerized, which contributes to generating XOS with a lower degree of polymerization (DP). This is desirable because XOS with low DP, such as X2 (DP 2) and X3 (DP 3), are consumed faster by probiotic bacteria and have increased prebiotic activity (VAN LOO et al., 1999; REDDY; KRISHNAN, 2016). Tests performed in humans showed that XOS intake had a beneficial effect on intestinal flora and that X2 was not excreted in feces or urine after 24 hours of ingestion. XOS were not hydrolyzed by saliva, pancreatin, or gastric juice, suggesting the use of XOS by intestinal bacteria (OKAZAKI et al., 1991).

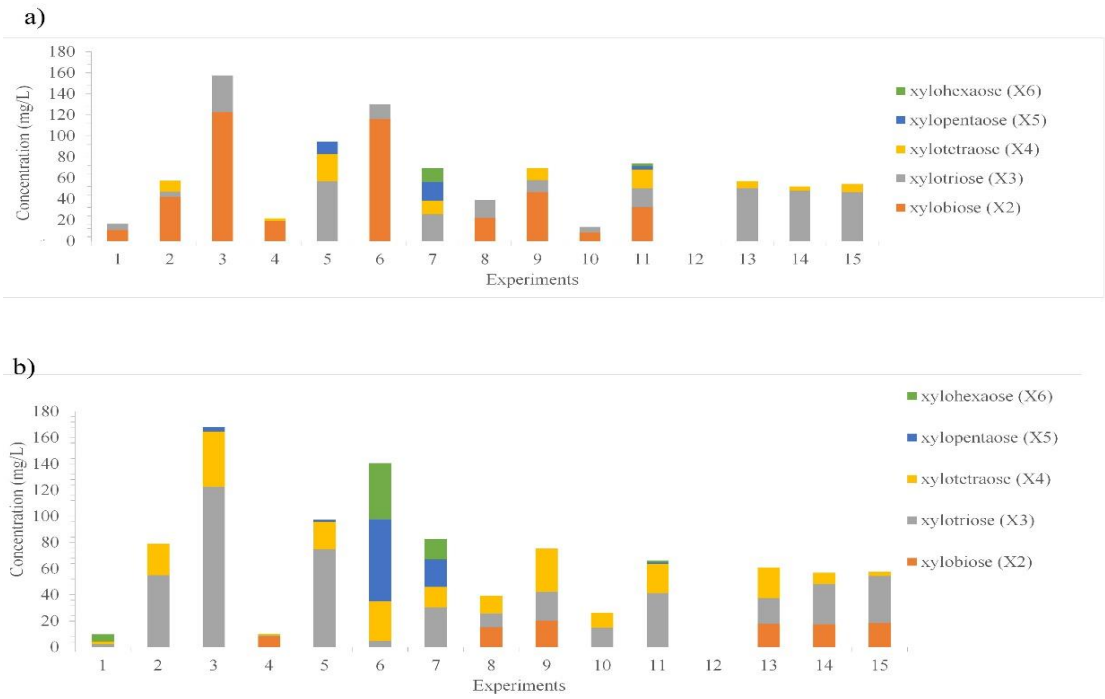

Figure 4.1. HPLC-PAD analysis of xylooligosacharides (XOS) released from (a) mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA) (b) mixture of sugarcane bagasse and straw pretreated with the ionic liquid (MPIL). Concentrations were calculated using a standard curve of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

MPIL presented the highest conversion of hemicellulose (88.59%) and a slightly higher release of total XOS (168 mg/L) in relation to MPSA (157 mg/L) (Table 4.3). The highest yield of XOS and highest conversion of hemicellulose did not occur in the same experiment, which can be explained by the different enzyme combinations used in the Plackett–Burman design. The experiments that showed higher values of hemicellulose conversion for both biomass were those in which β-xylosidase was present. β-xylosidase had a positive and significant effect on the release of X1, which, synergistically with the other enzymes, contributed to a high conversion of hemicellulose. However, when β-xylosidase was not present, XOS yield increased, although not enough to increase hemicellulose conversion, as the concentrations of XOS were lower than those of X1 in both pretreated biomasses. Analysis of the carbohydrate profiles indicated that X2 and X4 were the major components of XOS produced from MPSA and MPIL, along with smaller concentrations of X5 and X6. This same carbohydrate profile was observed by Manisseri and Gudipati (2010) in the hydrolysis of wheat bran using purified xylanases and by Goldbeck et al. (2014, 2016) in the hydrolysis of sugarcane bagasse by purified hemicellulases. Thus, it is possible to consider that enzymatic hydrolysis is a desirable method for XOS production because of its specificity of delignification, minimum production of undesirable by-products, and ability to yield low-DP XOS; as reported in the literature, XOS with a low DP (2–4) are preferred for food-related applications (VAN LOO et al., 1999).

4.3.4. Effect of commercial enzyme combinations and CCRD

Based on the Pareto Chart (Figure 4.2), only α-L-arabinofuranosidase and endo-1,4-xylanase present a positive and significant effect ($p < 0.1$) on the release of total XOS from MPIL and MPSA. In addition, both enzymes showed positive effects on the conversion of hemicellulose and release of X1. These results corroborate those obtained by De Paiva (2013) who also observed synergistic effects between α-L-arabinofuranosidase and endo-1,4-xylanase on rye arabinoxylan and sugarcane bagasse. Similarly to this study, the author verified that the carbohydrate profile obtained from the hydrolysis of rye were similar to those from sugarcane bagasse, releasing mainly X2, X3, and X4. In contrast, Goldbeck et al. (2014, 2016), evaluating the mode of action of six hemicellulolytic enzymes on sugarcane bagasse and wheat arabinoxylan, verified that only the endo-1,4-xylanase of the GH11 family (*Penicillium funiculosum*) showed a positive and significant effect on XOS production. The authors also reported that the studied GH51 (*Bacillus subtilis*) and GH54 (*Aspergillus niger*) α-L-arabinofuranosidases did not have the expected effect on XOS production because the enzymes

were not in their ideal conditions. However, De Paiva (2013) found that GH11 endo-1,4-xylanase and GH54 arabinofuranosidase, secreted by a fungal expression system of *Aspergillus nidulans*, present synergistic effect on lignocellulosic biomass degradation for XOS production. According to the author, XOS branched with arabinose were preferentially degraded when the enzymes were added to sugarcane bagasse, as observed in this study for sugarcane bagasse and straw. Thus, the synergistic effect that resulted in higher XOS release is correlated with the mode of action of these enzymes, especially in XOS branched with arabinose residues, facilitating enzyme access to arabinoxylan chains and leading to higher XOS production.

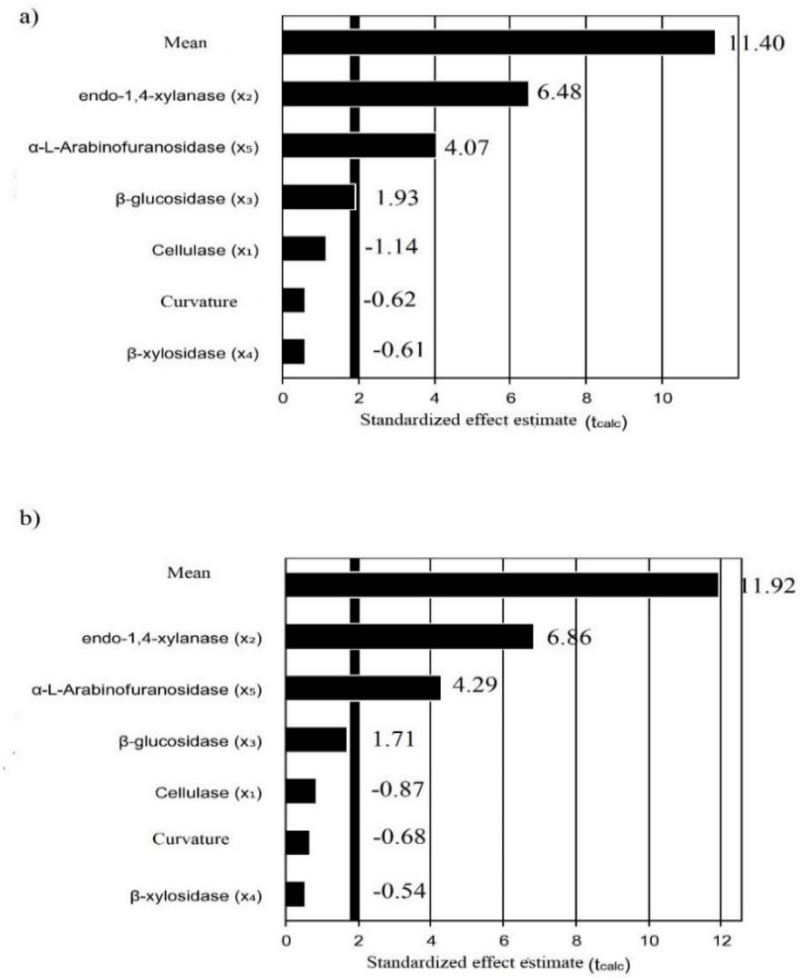

Figure 4.2. Pareto chart of standardized effects ($p < 0.1$) on total XOS concentration (a) mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA) (b) mixture of sugarcane bagasse and straw pretreated with the ionic liquid (MPIL).

CCRD included only two independent variables, α-L-arabinofuranosidase and endo-1,4-xylanase enzyme concentrations, which were the only positive and significant variables in the Plackett–Burman experiments, as previously discussed (Figure 4.2), according to the re-established study range presented in Table 4.1. The response variables were concentration of total XOS released after 48 hours of hydrolysis and the conversion of hemicellulose. The CCRD matrix (Tables 4.4 and 4.5) corresponds to an experimental design with 3 central points and 4 axial points, totaling 11 experiments.

Table 4.4. Central composite rotatable design (CCRD) matrix used to determine the optimal enzyme concentrations for mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA).

Experiment	V1	V2	Carbohydrate profile (%)							XOS (mg/L)
			X1	X2	X3	X4	X5	X6	Total	
1	$5.03_{(-1)}$	$8.3_{(-1)}$	8.96	12.68	3.41	3.17	3.98	3.37	26.62	125.21
2	$14.96_{(+1)}$	$8.3_{(-1)}$	11.09	17.21	2.73	2.92	4.62	4.03	31.51	148.32
3	$5.03_{(-1)}$	$23.69_{(+1)}$	11.73	29.30	2.70	3.29	4.67	3.77	43.74	205.21
4	$14.96_{(+1)}$	$23.69_{(+1)}$	38.40	36.86	4.81	4.15	3.32	4.44	53.59	251.12
5	$3_{(-1.41)}$	$16_{(0)}$	14.08	20.24	3.13	1.68	2.30	2.61	29.96	140.13
6	$17_{(+1.41)}$	$16_{(0)}$	19.42	27.00	3.98	2.84	3.53	3.91	41.26	193.22
7	$10_{(0)}$	$5_{(-1.41)}$	7.96	9.57	4.41	1.95	3.13	4.51	23.57	110.21
8	$10_{(0)}$	$27_{(+1.41)}$	40.07	38.52	3.34	2.66	3.65	4.87	53.05	249.15
9	$10_{(0)}$	$16_{(0)}$	18.37	33.40	3.79	2.26	2.89	1.85	44.20	207.32
10	$10_{(0)}$	$16_{(0)}$	18.63	34.94	2.42	2.06	3.56	2.65	45.63	214.19
11	$10_{(0)}$	$16_{(0)}$	18.31	32.98	3.32	1.87	3.11	2.04	43.32	203.21

Where: V1 and V2 **($mg/g_{substrate}$)** correspond to α-L-arabinofuranosidase and endo-1,4-xylanase, respectively. XOS corresponds to the xylooligosaccharides: xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

Table 4.4 shows that the maximum release of total XOS for MPSA (251.12 mg/L), and consequently the highest hemicellulose conversion (53.59%), was achieved in experiment 4. These results were similar to those found in experiment 8 (249.15 mg/L) (53.05% of hemicellulose conversion), in which the concentration of α-L-arabinofuranosidase was avaluated at the central point; this demonstrates that the reduction in enzyme concentration from experiment 4 to 8 did not significantly affect the response variable, indicating a possible reduction in the production cost of XOS. XOS have the advantage to be obtained from highly available low-cost sources, such as forestry and agro-industrial wastes, as well as have properties similar or superior to those of other prebiotic compounds, such as fructooligosaccharides and isomaltooligosaccharides (MENEZES, DURRANT, 2008). These results were similar to MPIL (Table 4.5), which also yielded a higher concentration of total

XOS (229.17 mg/L) achieving a higher hemicellulose conversion (48.64%) in experiment 4. However, differently from MPSA, MPIL experiment 6 (226.12 mg/L) (48.08%) showed similar values to experiment 4, which were also relatively close to the values obtained in the central points. Goldbeck et al. (2016) also obtained satisfactory results (65% of hemicellulose conversion) using CCRD to optimize the conversion of hemicellulose to XOS from sugarcane bagasse.

Table 4.5. Central composite rotatable design (CCRD) matrix used to determine the optimal enzyme concentration for mixture of sugarcane bagasse and straw pretreated with the ionic liquid (MPIL).

Experiment	V1	V2	Carbohydrate profile (%)							XOS (mg/L)
			X1	X2	X3	X4	X5	X6	Total	
1	$5.03_{(-1)}$	$8.3_{(-1)}$	11.14	4.38	6.12	3.16	1.84	3.35	18.85	89.13
2	$14.96_{(+1)}$	$8.3_{(-1)}$	13.29	4.79	7.84	4.88	4.60	4.01	26.12	123.34
3	$5.03_{(-1)}$	$23.69_{(+1)}$	13.94	10.21	12.88	2.62	3.71	3.75	35.17	166.31
4	$14.96_{(+1)}$	$23.69_{(+1)}$	42.24	15.33	19.15	4.13	4.79	5.24	48.64	229.17
5	$3_{(-1.41)}$	$16_{(0)}$	15.86	8.75	12.18	1.94	2.66	2.60	28.12	132.16
6	$17_{(+1.41)}$	$16_{(0)}$	18.54	11.11	23.96	4.96	3.94	4.11	48.08	226.12
7	$10_{(0)}$	$5_{(-1.41)}$	8.60	5.53	7.27	1.94	1.86	4.49	20.08	95.10
8	$10_{(0)}$	$27_{(1.41)}$	43.95	10.71	23.75	4.34	3.64	4.85	47.29	223.25
9	$10_{(0)}$	$16_{(0)}$	20.85	15.87	18.56	4.59	3.73	3.54	46.29	218.21
10	$10_{(0)}$	$16_{(0)}$	22.00	16.60	18.47	4.18	3.54	3.91	46.70	220.34
11	$10_{(0)}$	$16_{(0)}$	20.77	15.71	18.31	3.99	3.94	4.15	46.10	217.13

Where: V1 and V2 **($mg/g_{substrate}$)** correspond to α-L-arabinofuranosidase and endo-1,4-xylanase, respectively. XOS corresponds to the xylooligosaccharides: xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

Models that reflect the release of XOS for each pretreated sample were generated, where the coefficients were evaluated at ($p < 0.05$), and for both only the coefficient x1.x2 was not significant, and therefore the models were reparametrized. The reparametrized 2nd order models for pretreated samples with dilute sulfuric acid and ionic liquid are represented by equations 4.1 and 4.2, respectively.

$$Y_1 = 208.24 + 18.01\ x_1 - 18.46\ x_1^2 + 47.41\ x_2 - 11.96\ x_2^2 \quad (4.1)$$

$$Y_2 = 218.56 + 28.74\ x_1 - 24\ x_1^2 + 45.53\ x_2 - 33.98\ x_2^2 \quad (4.2)$$

In order to verify the validity of the regression coefficients and the mathematical models obtained, a variance analysis (ANOVA) was performed for each pretreated biomass (Tables 4.6 and 4.7). For both samples, the calculated F value for the regression, considering all the enzymes studied, was much higher than the tabulated F value (approximately 20 times higher for MPSA and 10 times higher for MPIL). In addition, percent of variance (R^2) explained

by the models was higher than 96%. Thus, the values predicted by our models fit well with the experimental values and validated the CCRD experiments. Figures 4.3 and 4.4 represent the contour curves and surface response plots generated in the CCRD for the optimum range of enzyme concentrations for XOS production after 48 hours of enzymatic hydrolysis of MPSA and MPIL, respectively.

Table 4.6. ANOVA of total XOS production from mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA), showing significant differences ($p < 0.05$) between different concentrations of α-L-arabinofuranosidase and endo-1,4-xylanase.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_{cal}/F_{Tab}	R^2
Regression	22766.3	4	5691.6	19.10	98.31
Residual	392.0	6	65.3		
Total	23158.9	10			

Table 4.7. ANOVA of total XOS production from mixture of sugarcane bagasse and straw pretreated with ionic liquid (MPIL), showing significant differences ($p < 0.05$) between different concentrations of α-L-arabinofuranosidase and endo-1,4-xylanase.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_{cal}/F_{Tab}	R^2
Regression	30928.7	4	7732.2	10.59	96.95
Residual	960.8	6	160.1		
Total	31889.5	10			

Experimental design is a statistically convenient technique for planning experiments in the field of bioprocesses, including the optimization of enzyme mixtures for biomass hydrolysis (RODRIGUES, IEMMA, 2014). According to the surface response plots generated in CCRD (Figure 4.3), the optimal conditions for the release of XOS were observed between the central points and the level +1 for MPSA (254.52 mg/L) and MPIL (241.41 mg/mL) using 12.5 mg α-L-arabinofuranosidase for both pretreated biomasses and 25.0 and 20 mg of 1,4-endo-xylanase for MPSA and MPIL, respectively. Comparing these results with those from Table 4.3 (Plackett–Burman design), it was possible to verify that the total concentrations of XOS were significantly higher than those obtained using the Plackett–Burman design. This showed that a small increase in the enzyme concentration of the two studied enzymes, approximately 20% for α-L-arabinofuranosidase and 25% for 1,4-endo-xylanase, resulting in an increase in XOS production of approximately 62% for MPSA and 45% for MPIL, in comparison to the highest values reached in the Plackett–Burman experiments. Thus, both pretreatments were considered interesting alternatives for XOS

production. Even though dilute sulfuric acid pretreatment provided greater XOS yield, ionic liquid pretreatment was economically advantageous in relation to enzyme concentrations required in the hydrolysis step, showing that up to 20% less of the optimum concentration of endo-1,4-xylanase needed for MPSA hydrolysis can generate a similar XOS yield in MPIL hydrolysis, thus representing a possible alternative for reducing the costs with enzyme mixtures. Several studies such as (LE et al., 2009; WU et al., 2011; DA SILVA et al., 2011; NYNOMIA et a., 2015) correlated the efficiency of ionic liquid pretreatments with different effects on the structure and composition of the pretreated biomass that are capable of increasing pore size, specific surface area, and enzyme accessibility and decreasing lignin content. This contributes significantly to increase xylan hydrolysis efficiency of the pretreated material (ZHAO et al., 2010; WU et al., 2011).

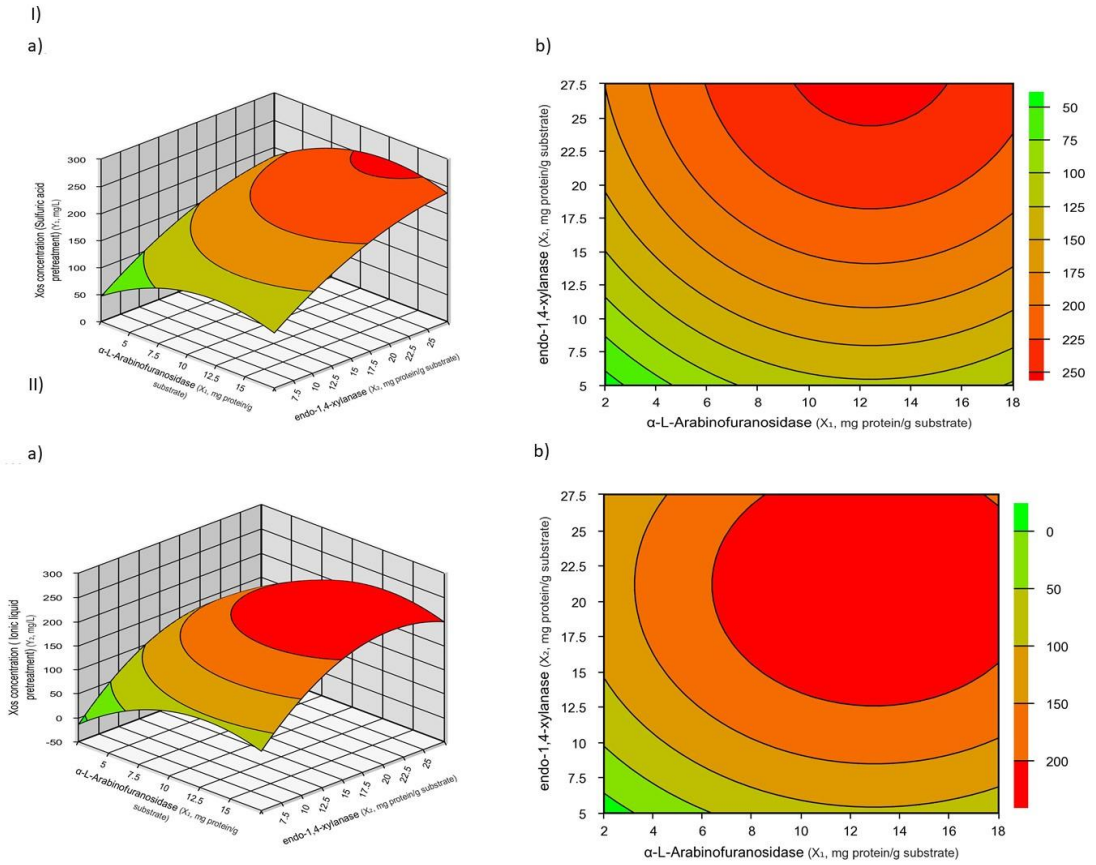

Figure 4.3. Surface response plot (a) and contour curves (b) generated in the CCRD, illustrating the optimum enzyme concentration range of α-L-arabinofuranosidase and endo-1,4-xylanase for XOS released from (I) mixture of sugarcane bagasse and straw pretreated with dilute sulfuric acid (MPSA) and (II) mixture of sugarcane bagasse and straw pretreated with ionic liquid (MPIL).

Validation of models was performed using experimental tests in triplicate under the same conditions, as presented in Table 4.8. The concentration of XOS was within the expected range, and the predicted results were close to experimental results, thus demonstrating that our strategy for developing more efficient enzyme mixtures for XOS production was successful.

Table 4.8. Predicted and experimental values of xylooligosaccharides concentration released after the enzymatic hydrolysis of pretreated samples with dilute sulfuric acid (MPSA) and with ionic liquid (MPIL).

Pretreated biomass	Commercial enzyme	Enzyme concentration (mg/g substrate)	Predicted value (mg/L)	Experimental value (mg/L)
MPSA	α-L-arabinofuranosidase endo-1,4-xylanase	12.5 25.0	254.52	261.23 ± 0.75
MPIL	α-L-arabinofuranosidase endo-1,4-xylanase	12.5 20.0	241.41	249.54 ± 0.61

4.4 CONCLUSION

Among the enzymes evaluated, only α-L-arabinofuranosidase and endo-1,4-xylanase had a significant effect on XOS production. Our results of the CCRD experiments showed that both pretreatments are potential alternatives for XOS production by enzymatic hydrolysis of a mixture of sugarcane bagasse and straw. As well as that it was possible to verify an increase in XOS production of approximately 62% for MPSA and 45% for MPIL, in comparison to the highest values reached in the Plackett–Burman experiments. Even though dilute acid pretreatment provided greater XOS yields, ionic liquid pretreatment was economically advantageous in relation to the enzyme concentrations required for the hydrolysis step, representing a possible alternative to reduce the cost with enzyme mixtures. This demonstrates that our strategy for increasing the efficiency and reducing the costs with enzyme mixtures for XOS production was successful.

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CAPÍTULO V: CONCLUSÕES GERAIS E SUGESTÕES PARA TRABALHOS FUTUROS

5.1 CONCLUSÕES GERAIS

A partir do estudo exploratório e comparativo para a aplicação de diferentes pré-tratamentos, como líquido iônico e ácido sulfúrico diluído foi possível estudar a alteração da composição química e os efeitos das enzimas comerciais empregadas na hidrólise enzimática da Biomassa da cana-de-açúcar. Através deste estudo foi possível obter informações que proporcionaram o desenvolvimento técnico-científico da pesquisa brasileira, bem como cumprir com os requisitos de inovação com potencial aplicação industrial. De forma sucinta, os principais resultados e conclusões desse estudo encontram-se listados abaixo:

- As estratégias empregando ([Emin][Ac]) à temperatura de 100°C e ácido sulfúrico diluído à 0,5% (m/v) foram selecionadas para serem aplicadas na biomassa para os estudos de hidrólise enzimática por terem apresentado maior similaridade na composição química após os pré-tratamentos realizados.
- Todas as estratégias de pré-tratamento com líquido iônico apresentaram maior remoção de lignina e, consequentemente, rendimentos ligeiramente mais elevados em glicose e xilose quando comparados com o pré-tratamento com ácido sulfúrico diluído.
- O uso da α-L-arabinofuranosidase (Megazyme®), β-xilosidase (Megazyme®), endo-1,4-xilanase (NS50036) Novozyme® e celulase (NS50030) Novozyme® na hidrólise enzimática da Biomassa da cana-de-açúcar (palha e bagaço), proporcionaram altos rendimentos em açúcares fermentescíveis (glicose e xilose) para ambas as estratégias de pré-tratamento. Além disso, a β-glicosidase (NS50010) não apresentou efeito significativo, portanto nesta condição, ela pode ser removida da reação para reduzir os eventuais custos com as enzimas empregadas.
- Em relação a produção de xilo-oligossacarídeos apenas as enzimas α-L-arabinofuranosidase (Megazyme®) e endo-1,4-xilanase apresentaram efeitos positivos e significativos na liberação de XOS.
- O DCCR foi uma alternativa bem sucedida para a produção de XOS, uma vez que proporcionou um aumento considerável na produção de XOS em comparação aos resultados obtidos no planejamento Plackett-Burman, gerando modelos que se ajustaram muito bem aos dados experimentais obtidos.
- O pré-tratamento com líquido iônico se mostrou mais vantajoso economicamente em relação a concentração de enzimas, pois demandou menor concentração proteica de enzima por grama de substrato possibilitando uma redução em até 20% da concentração de endo-

1,4-xilanase (NS50036) empregada para a biomassa pré-tratada com ácido sulfúrico diluído a 0.5 % (m/v) para uma produção semelhante em XOS.

- Diante do exposto o líquido iônico ([Emin][Ac]) se apresentou como uma potencial alternativa para substituição de solventes tradicionais em diversos processos industriais, apresentando potencial para diminuir a dependência da indústria química de compostos orgânicos voláteis.

5.2 TRABALHOS FUTUROS

A aplicação de diferentes pré-tratamentos para o processamento da biomassa lignocelulósica, bem como o desenvolvimento de misturas enzimáticas menos onerosas e mais eficientes para produção de bioprodutos de alto valor agregado é uma área em expansão e com muitos aspectos a serem explorados. Os xilo-oligossacarídeos produzidos a partir da hidrólise enzimática de resíduos agroindustriais são relatados por apresentar diversos benefícios à saúde, como o estímulo do crescimento de bactérias benéficas (probióticas) no trato digestivo, atuando assim como potenciais produtos prebióticos na saúde humana. No entanto, o alto custo das enzimas, bem como as etapas de purificação necessárias para sua produção a partir da parecede celular lignocelulósica dificultam o desenvolvimento de um bioproduto economicamente viável. Assim como continuidade deste trabalho, projetos futuros estão sendo planejados visando avaliar outros parâmetros e opções que não fizeram parte deste estudo, como:

- Realizar análises da microestrutura das biomassas após os diferentes tratamentos;
- Realizar um estudo de separação e purificação dos xilo-oligossacarídeos obtidos utilizando membranas de nanofiltração;
- Investigar o seu potencial prebiótico, por meio de testes “in vitro” de modo a investigar a resistência à hidrólise de enzimas do trato digestivo; estimulo do crescimento de bactérias consideradas benéficas de maneira a comprovar as suas atividades prebióticas.
- Realização de testes “in vivo” de modo a confirmar o potencial prebiótico dos oligossacarídeos produzidos.

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APÊNDICE I (*Appendix 1*): CURVAS DE DETERMINAÇÃO DAS CONCENTRAÇÕES ENZIMÁTICAS

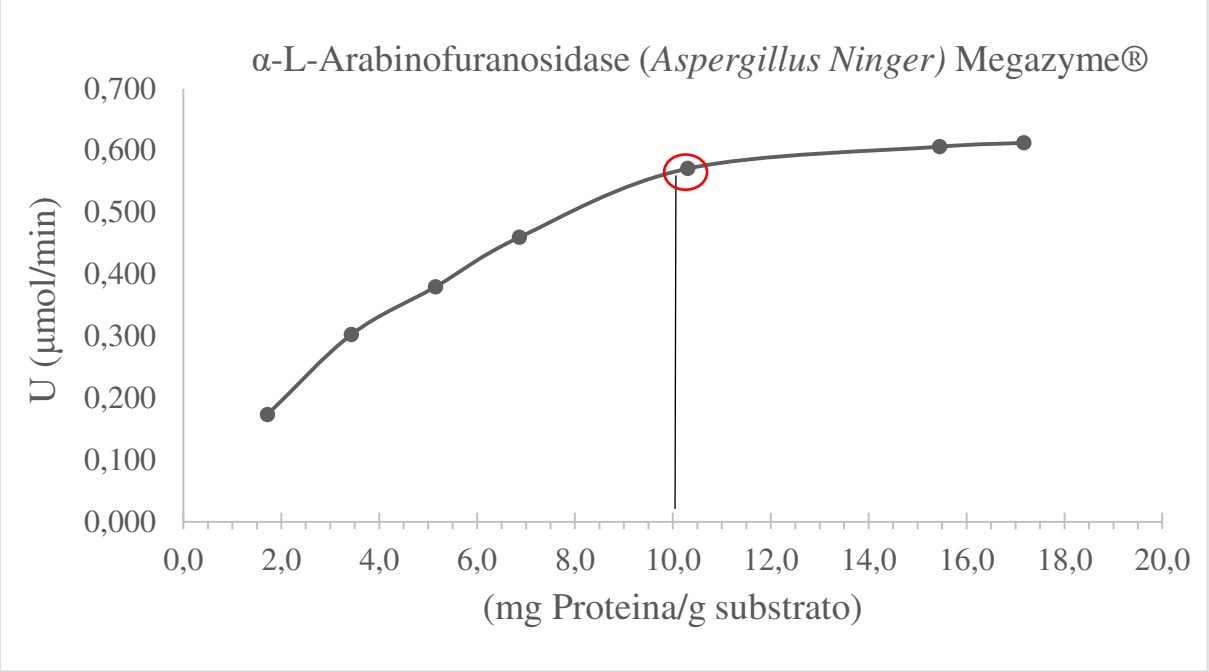

Figura 1. Curva de determinação da concentração proteica da α-L-arabinofuranosidase (*Aspergillus Ninger*) Megazyme® utilizando a matriz sólida de bagaço + palha de cana-de-açúcar 50% (m/m).

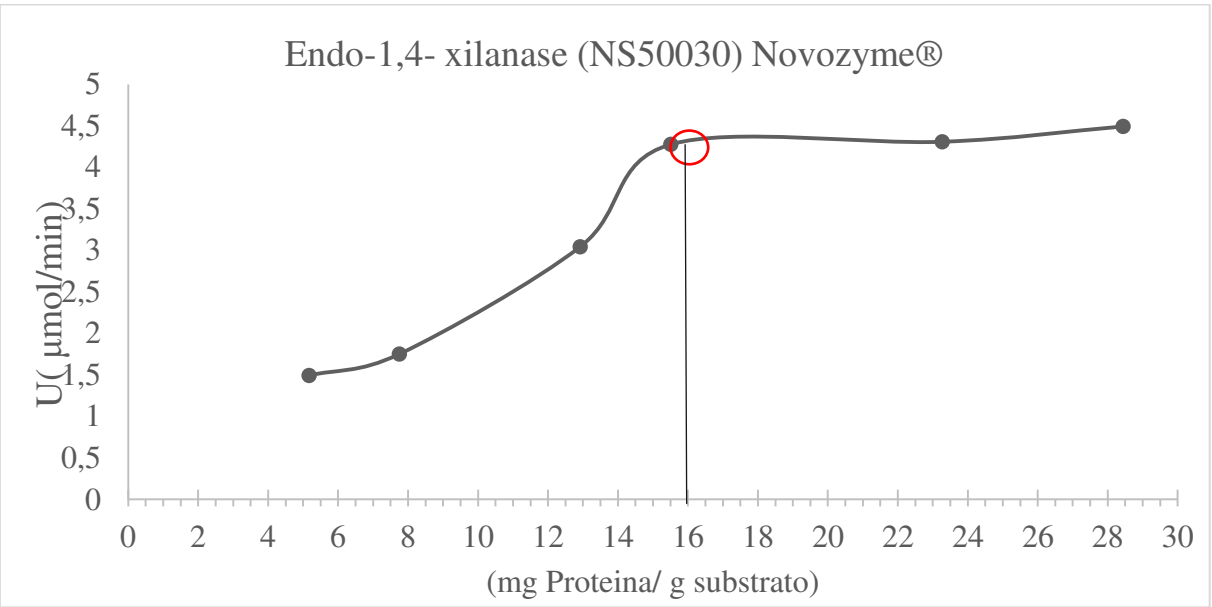

Figura 2. Curva de determinação da concentração proteica da Endo-1,4-xilanase (NS50030) Novozyme® utilizando a matriz sólida de bagaço + palha de cana-de-açúcar 50% (m/m).

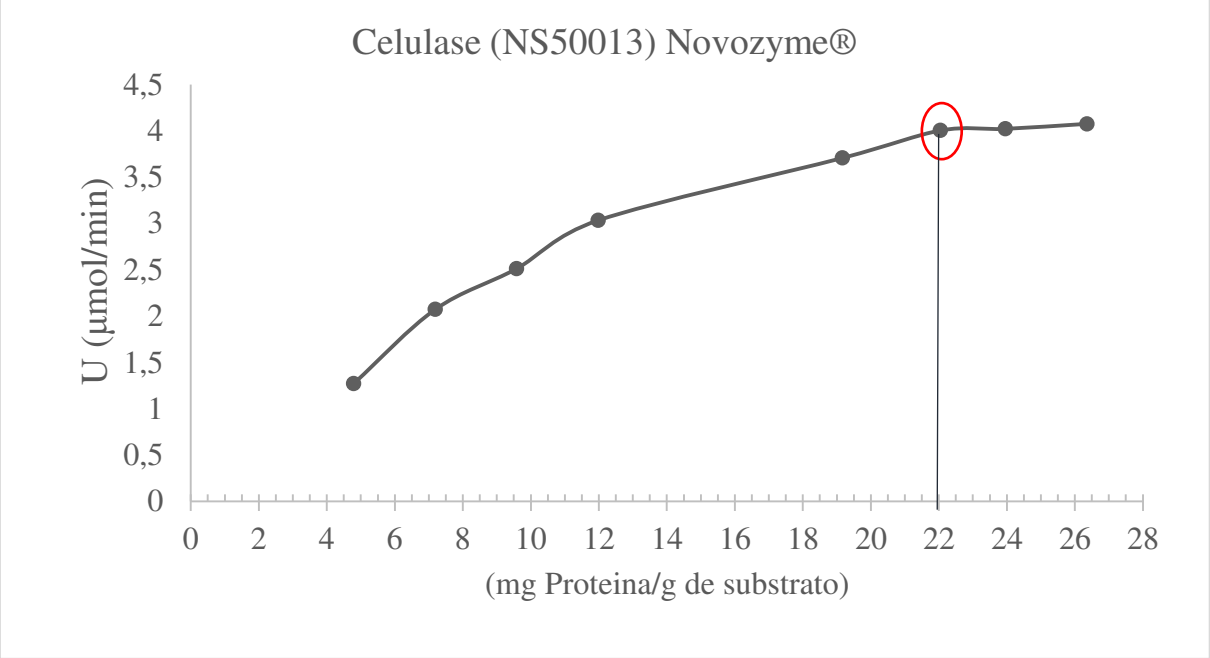

Figura 3. Curva de determinação da concentração proteica da Celulase (NS50013) Novozyme® utilizando a matriz sólida de bagaço + palha de cana-de-açúcar 50% (m/m).

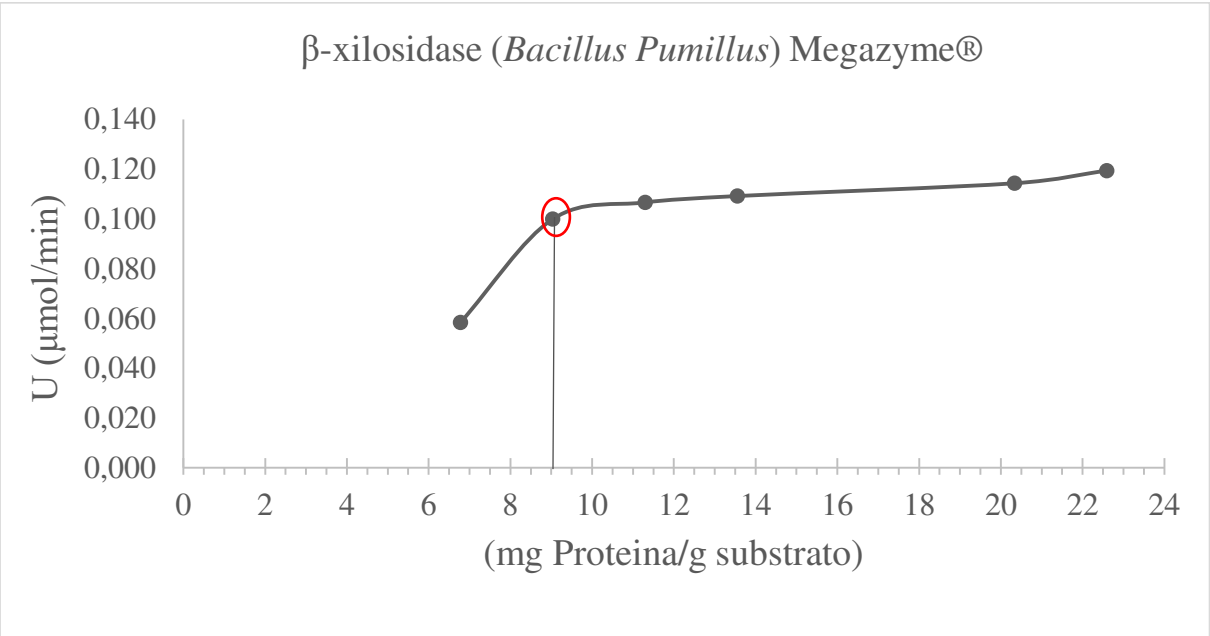

Figura 4. Curva de determinação da concentração proteica da β-xilosidase (*Bacillus Pumillus*) Megazyme® utilizando a matriz sólida de bagaço + palha de cana-de-açúcar 50% (m/m).

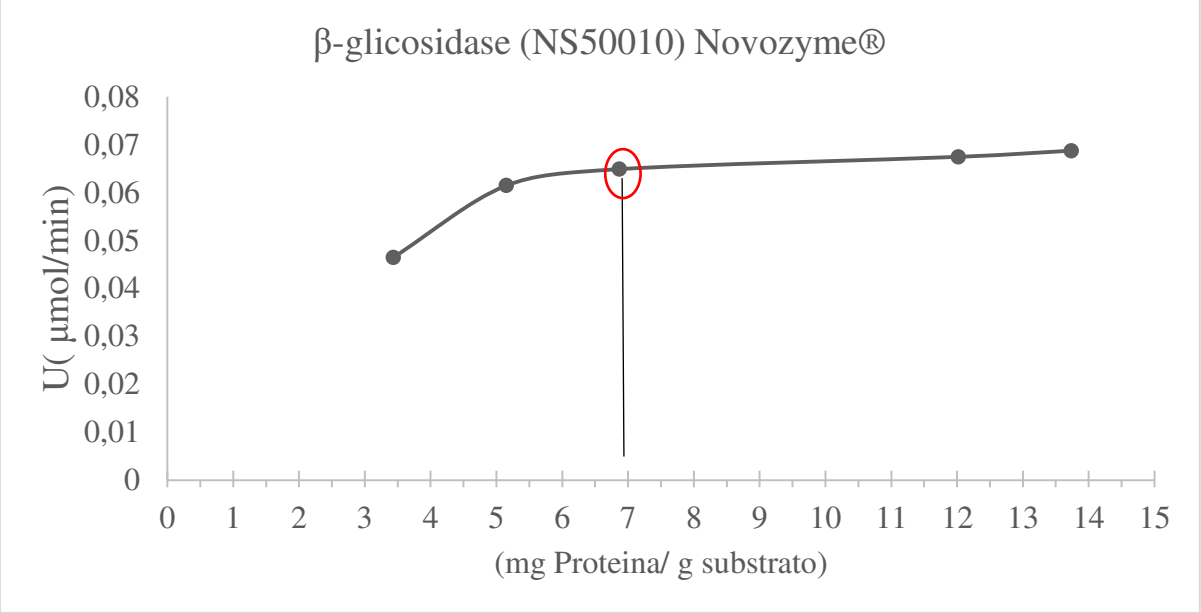

Figura 5. Curva de determinação da concentração proteica da β-glicosidase (NS50010) Novozyme® utilizando a matriz sólida de bagaço + palha de cana-de-açúcar 50% (m/m).