BIOCONVERSÃO DE AÇÚCARES PROVENIENTES DE BIOMASSAS HIDROLISADAS A ETANOL E PRÉ-TRATAMENTOS DE MATERIAIS LIGNOCELULÓSICOS COM LÍQUIDO IÔNICO

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PROGRAMA DE PÓS GRADUAÇÃO EM ENGENHARIA QUÍMICA

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Tese de doutorado submetida ao Programa de Pós Graduação em Engenharia Química da UFRGS como um dos requisitos para a obtenção do grau de Doutor em Engenharia Química.

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A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado intitulada *"Bioconversão de açúcares provenientes de biomassas hidrolisadas a etanol e pré-tratamentos de materiais lignocelulósicos com líquido iônico"*, elaborada por Fernanda da Cunha Pereira, como requisito parcial para obtenção do grau de Doutor em Engenharia Química.

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Agradecimentos

RESUMO

Bioconversão de açúcares provenientes de biomassas hidrolisadas a etanol e pré-tratamentos de materiais lignocelulósicos com líquido iônico.

A necessidade de modificar a matriz energética, fortemente dependente de combustíveis fósseis, está impulsionando estudos que visam buscar alternativas mais sustentáveis. Uma alternativa promissora é a utilização de processos biotecnológicos para a conversão de resíduos lignocelulósicos agroindustriais ricos em celulose (casca de soja, casca de arroz, bagaço de cana-de-açúcar, entre outros) a açúcares fermentescíveis para uma posterior fermentação a etanol de segunda geração. Neste contexto, o presente trabalho tem por objetivo estudar a capacidade de conversão dos açúcares provenientes de biomassas hidrolisadas por diferentes leveduras e avaliar a utilização de líquidos iônicos na dissolução de materiais lignocelulósicos. Desta forma, a capacidade de bioconversão dos açúcares contidos no hidrolisado de casca de arroz (RHH) e em meio sintético a etanol foi avaliada utilizando a levedura *Candida shehatae* e sua co-cultura com *Saccharomyces cerevisiae*. Este estudo foi realizado em agitador orbital e biorreator. Nos experimentos utilizando co-cultura e agitador orbital o rendimento de etanol ($Y_{P/S}$) foi de 0,42 e 0,51 g g^{-1} em meio sintético simulando a composição do hidrolisado e em RHH, respectivamente. Utilizando culturas puras de *C. shehatae* nas mesmas condições, o rendimento de etanol foi de 0,40 g g^{-1}. Determinadas leveduras tem seu metabolismo alterado com a variação de oxigenação do meio. Com o intuito de realizar esta avaliação, experimentos em anaerobiose e com limitação de oxigênio foram realizados em biorreatores. Nestas condições e utilizando as leveduras em co-cultura foi possível obter rendimentos de etanol similares (0,50-0,51g g^{-1}) em meio sintético, enquanto que em RHH, rendimentos de 0,48 e 0,44 g g^{-1} foram obtidos, respectivamente. Assim como o RHH, o hidrolisado de casca de soja também pode ser uma alternativa para este desenvolvimento. Foi investigada a capacidade de uma linhagem recentemente isolada de *Candida guilliermondii* converter hexoses e pentoses a partir de hidrolisado ácido/enzimático de casca de soja em etanol. As condições operacionais e suplementação do meio de cultivo foram otimizados utilizando planejamentos experimentais estatísticos (Plackett-Burman e CCD). Os resultados demonstraram que a *C. guilliermondii* BL 13 foi capaz de crescer em hidrolisado não-suplementado, não-detoxificado, e as melhores condições de cultivo foram 28 °C, pH 5 e um tamanho de inóculo de 10^9 UFC ml^{-1}. A produtividade do etanol atingiu um máximo de 1,4 g h^{-1} L^{-1}e o rendimento de 0,41 g g^{-1}. Para que os materiais lignocelulósicos sejam utilizados em processos destinados à produção de etanol, uma etapa de pré-tratamento é necessária a fim de desorganizar a estrutura deste material facilitando a sacarificação dos açúcares. Com esta finalidade e pensando na utilização de recursos renováveis, foram utilizados no pré-tratamento de casca de soja líquido iônico (LI) de acetato de 1-butil-3-metilimidazólio ([bmim][Ac]). Desta forma, a fim de otimizar o processo de dissolução da celulose, os efeitos da temperatura, tempo de incubação, da relação sólido/líquido (biomassa/IL) e a concentração de líquido iônico (mistura de IL-água) foram avaliadas utilizando um planejamento composto central (CCD). A biomassa regenerada foi analisada por espectroscopia de infravermelho com transformada de Fourier (FTIR). A eficiência do pré-tratamento foi quantificada através da glicose liberada após hidrólise enzimática utilizando um complexo celulolítico

produzido por *Penicillium echinulatum* em comparação a casca de soja não tratada (matriz celulolítica). O hidrolisado do ponto ótimo foi utilizado em processo fermentativo com *C. shehatae* HM 52,2. As condições ótimas foram de 75 °C, 165 minutos de tempo de incubação, 57 % (fração de massa) de [bmim] [Ac] e 12,5 % de carga sólida. As análises de FTIR demonstraram que a casca de soja teve uma perda na cristalinidade de sua estrutura. A utilização do complexo enzimático no processo de hidrólise da biomassa conseguiu liberar 92 % da glicose da matriz celulósica e foi obtido uma conversão de glicose em etanol com rendimento de 0,31 (g g^{-1}). As alterações na estrutura da biomassa regenerada após tratamento com os líquidos iônicos cloreto de 1-butil-3-metilimidazólio ([bmim][Cl]) ou acetato de 1-butil-3-metilimidazólio ([bmim][Ac]) também foram estudadas. A caracterização foi realizada por uma combinação de análise termogravimétrica (TGA), análise de espectroscopia de absorção no infravermelho por Transformada de Fourier (FTIR), difracção de raios X (XRD) e por microscopia electrônica de varredura (MEV). Os tratamentos foram estudados utilizando diferentes tempos de tratamento (2 h e 6 h), diferentes temperaturas (75 °C e 100 °C) e variações na concentração de LI (50 % e 100 %). Os resultados sugerem que a casca de soja apresenta uma maior perda da cristalinidade do que a casca de arroz. A alta recalcitrância da casca de arroz devido a sílica presente pode explicar a pequena redução na cristalinidade desta biomassa. Observou-se que a dissolução da biomassa lignocelulósica sofreu influencia do tipo de biomassa e LI utilizado, temperatura, tempo e proporção LI-água.

ABSTRACT

The necessity of changing energy matrix, which is highly dependent on fossil fuel, it is booking studies wich seek more sustainable alternatives. One promising alternative is the use of biotechnological processes from the conversion of lignocellulosic agroindustrial waste rich in cellulose (soybean hulls, rice hull, bagasse, etc.) to fermentable sugars for subsequent fermentation second-generation ethanol. In this context, the present work has the goal to study the capacity of the conversion sugars coming from biomass hydrolyze by different yeasts and evaluates the use of ionic liquids on the dissolution of lignocellulosic materials.Thus, the capacity of the sugars bioconversion contained in the rice hull hidrolisate (RHH) and in synthetic medium for ethanol was evaluated using the yeast *Candida shehatae* and their co-culture with *Saccharomyces cerevisiae*. This study was carried in an orbital shaker and bioreactor. In co-culture experiments using orbital shaker the ethanol yields ($Y_{P/S}$) was 0.42 and 0.51 g g^{-1} in synthetic medium simulating the sugar composition of RHH and in RHH, respectively. By using pure cultures of *C. shehatae* in the same conditions the ethanol yield was 0.40 g g^{-1}. Certain yeasts have their altered metabolism by varying the oxygenation of the medium. In order to accomplish this evaluation experiments under anaerobic conditions and with limited oxygen were performed in bioreactors. In those conditions and by using the yeast in co-culture, it was possible to obtain similar ethanol yields of (0.50 to 0.51 g g^{-1}) in the synthetic medium, while in RHH, yields of 0.48 and 0.44 g g^{-1} were obtained respectively.The ability of a newly isolated strain of *Candida guilliermondii* BL13converting hexoses and pentoses from the soybean hull hydrolysate acid / enzyme in ethanol was investigated. The operating conditions and supplementation of the culture medium were optimized using statistical experimental design (Plackett-Burman and CCD). The results bring to us the conclusion that *C. guilliermondii* BL 13 was able to grow on non-supplemented and non-detoxified hydrolyzate, and best culture conditions were 28 °C, pH 5 and inoculum size of 10^9 CFU ml^{-1}. The ethanol productivity reached a maximum of 1.4 g L^{-1} h^{-1}, and the yield of 0.41 g g^{-1}. For that lignocellulosic materials was used in processes for the production of ethanol, a pretreatment step is required in order to disrupt the structure of this material facilitating the saccharification of sugar. With this purpose, and considering the use of renewable resources were used in the pretreatment of soybean hulls with ionic liquid (IL) 1-butyl-3-methylimidazolium acetate ([bmim][Ac]). Thus, in order to optimize the dissolution of the cellulose process, the effects of temperature, incubation time, the solid / liquid ratio (biomass / IL), and the concentration of ionic liquid (IL-water mixture) were evaluated using a central composite design (CCD). The regenerated biomass was analyzed by Fourier transform infrared (FTIR) analysis. The effectiveness of the pretreatment was measured by glucose released after enzymatic hydrolysis using a cellulolytic complex produced by *Penicillium echinulatum* in untreated soybean hulls comparison (cellulolsic matrix). The hydrolyzate was used in optimum fermentation with *C. shehatae* HM 52.2. Optimal conditions were 75 °C, 165 minutes of incubation time, 57 % (mass fraction) of [bmim][Ac] and 12.5 % of solid. The FTIR analysis showed that soybean hulls had a loss in the crystallinity of its structure. The use of the enzyme complex in the biomass hydrolysis process achieved 92 % of the glucose release the cellulosic matrix was obtained and an ethanol conversion of glucose to yield 0.31 (g g^{-1}).The changes in the structure of the regenerated biomass after treatment with ionic liquids 1-butyl-3-methylimidazolium chloride ([bmim][CI]) or 1-butyl-3-methylimidazolium acetate ([bmim][Ac]) were also studied. The characterization was

done by a combination of thermogravimetric analysis (TGA), Fourier transform infrared (FTIR) analysis, X-ray diffraction (XRD) and scanning electron microscopy (SEM). The treatments were studied using different treatment times (2h and 6h), different temperatures (75 °C and 100 °C) and different IL concentration (50 % and 100 %). The results suggest that soybean hull has a higher loss of crystallinity than rice hull. The high recalcitrance of rice hull, due to silica present, may explain the low decrystallization this biomass. It was observed that the dissolution of the lignocellulosic biomass has undergone the influence of the type of biomass used and IL, temperature, time and ratio IL-water.

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TABELA DE ABREVIATURAS

Q_P	Produtividade volumétrica
$Y_{P/S}$	Coeficiente de produtividade
A_{LIG}	Absorbância de todos os compostos
G	Glicose
X	Xilose
A	Arabinose
DO	Densidade óptica
CLAE	Cromatografia líquida de alta eficiência
UFC	Unidades Formadoras de Colônia
rpm	Rotações por minuto
g	Aceleração da gravidade
vvm	Volume de ar/ volume de meio/minuto
HMF	Hidroximetilfurfural
Sc-Cs	Consórcio *S. cerevisiae* e *C. shehatae*
YM	meio extrato levedura e extrato de malte (Yeast Malt)
AEM	Meio ágar extrato de malte
EM	Meio estrato de malte
RHH	Hidrolisado de casca de arroz
SHH	Hidrolisado de casca de soja
RLA	Resíduo lignocelulósico agroindustrial
RID	Detector de infravermelho
LI	Líquido iônico
[bmim][Cl]	Cloreto de 1-butil-3-metilimidazólio
[bmim][Ac]	Acetato 1-butil-3-metilimidazólio

Capítulo 1

Introdução

Cada vez mais a demanda energética mundial vem aumentando devido ao crescimento populacional a cada ano. Esse consumo é fortemente dependente de combustíveis fósseis que contribuem para o aumento da poluição e acúmulo de gases na atmosfera. Tendo em vista este cenário mundial, a busca por fontes energéticas sustentáveis e renováveis tem atraído a atenção de todos.

O etanol é um combustível que tem suas emissões de dióxido de carbono após a combustão absorvidas naturalmente pelo meio ambiente. A utilização de etanol de segunda geração produzido biotecnologicamente a partir de materiais lignocelulósicos ricos em celulose e hemicelulose é uma alternativa. Esses materiais são, em sua maioria, resíduos lignocelulósicos agroindustriais (RLAs) como cascas, palhas e bagaços. A alta produtividade da agroindústria pode gerar problemas ambientais devido ao acúmulo na natureza dos resíduos gerados pelo beneficiamento das culturas produzidas (arroz, soja, milho, entre outros), não sendo destinados de forma correta e eficaz. Vale lembrar que apenas uma pequena parcela desses RLAs são subaproveitados no beneficiamento de rações animais e adubos ecológicos, ocorrendo a perda de potenciais recursos energéticos. Desta forma, a produção biotecnológica de "*commodities*" a partir de hidrolisados de RLAs é uma alternativa promissora, não só para agregar valor aos RLAs, mas também como uma alternativa mais econômica e limpa de produção de etanol de segunda geração (*Bioetanol*).

Inúmeras publicações têm abordado processos biotecnológicos utilizando os resíduos lignocelulósicos agroindustriais devido a possibilidade de converter a celulose e a hemicelulose em produtos de alto valor agregado como etanol e xilitol. Estas publicações geralmente referem-se ao aproveitamento de resíduos localmente produzidos. Publicações chinesas utilizam em suas pesquisas subprodutos do processamento de arroz, os norte-americanos do milho e os brasileiros, em sua grande maioria, do bagaço de cana-de-açúcar. Tendo em vista que o Rio Grande do Sul não participa ativamente do eixo de produção de cana-de-açúcar, mas é um dos maiores

produtores de soja e arroz, por exemplo, os resíduos gerados do beneficiamento destas culturas são de grande interesse para os processos biotecnológicos.

A utilização dos RLAs requer que estes passem por uma etapa de pré-tratamento para que os açúcares fermentescíveis sejam liberados da matriz lignocelulósica e, desta forma, possam ser bioconvertidos. Os pré-tratamentos podem ser realizados através de hidrólise química ou enzimática, sendo que a técnica mais difundida é a hidrólise ácida diluída. Através dela ocorre a liberação das pentoses (xilose e arabinose) e hexoses (manose, galactose e outros) constituintes da hemicelulose e celulose. O hidrolisado formado a partir deste pré-tratamento é constituído de diferentes proporções dos açúcares, já que cada tipo de RLAs possui uma composição de celulose e hemicelulose. Dependendo das condições de fermentação (aeração do meio, agitação, pH, entre outros) destes hidrolisados lignocelulósicos os produtos formados podem ter diferentes proporções (etanol e/ou xilitol).

Entretanto, este pré-tratamento pode gerar a formação de inibidores do processo de fermentação dos açúcares a etanol como os furaldeídos, ácidos e outros, além da grande quantidade de energia requerida para sua realização. Portanto, são necessárias pesquisas de novas tecnologias que permitam o aproveitamento dos açúcares contidos nos materiais lignocelulósicos, de forma integral, com menor geração de inibidores e de poluentes. Uma alternativa às hidrólises química seria a dissolução da biomassa lignocelulósica com líquidos iônicos (LIs) que demonstram ser um eficiente solvente dos materiais lignocelulósicos, permitindo a fácil recuperação da celulose com a adição de um antissolvente. Este processo resulta em um produto (celulose regenerada) que pode ser hidrolisado usando uma mistura de celulase comercial. A dissolução completa ou deslignificação parcial de vários RLAs vem sendo estudada, mas diferentes performances são obtidas frente a diferentes resíduos. Entretanto, os mecanismos de ação dos LIs sobre os RLAs, as tecnologias a serem empregadas, as alterações promovidas no substrato e o impacto destas alterações na subsequente hidrólise enzimática e na fermentação ainda não estão bem esclarecidas. Desta forma, é de fundamental importância o desenvolvimento de pesquisas que contribuam para a elucidação destas questões, agregando conhecimento no desenvolvimento e aplicação de novas tecnologias de aproveitamento de RLAs e sua bioconversão a etanol e demais produtos de alto valor agregado.

Tendo em vista o que foi relatado acima, este trabalho tem por **objetivo geral** ampliar os conhecimentos sobre a produção biotecnológica de etanol e xilitol a partir de

resíduos agroindustriais lignocelulósicos (casca de soja e de arroz), assim como avaliar a utilização de novas tecnologias no pré-tratamento destes.

Sendo os **objetivos específicos** apresentados abaixo:

1. estudar as cinéticas de crescimento, formação de produto e consumo de substrato em agitador orbital e biorreator, utilizando diferentes meios de cultivo e microrganismos fermentadores de pentoses e hexoses;

2. estudar a necessidade de suplementação dos hidrolisados e otimizar as condições de cultivo (pH, temperatura e inóculo);

3. avaliar a utilização de líquidos iônicos como pré-tratamento dos materiais lignocelulósicos,

4. caracterizar os materiais tratados com líquido iônico por análise termogravimétrica (TGA), espectroscopia de absorção no infravermelho (FTIR), difração de raios-X (XDR) e a microscopia eletrônica de varredura (MEV).

Capítulo 2

Revisão Bibliográfica

2. 1. Biomassa

A matriz energética mundial esta fortemente dependente de combustíveis fósseis. Entretanto, sua utilização vem sofrendo continua pressão social devido à diminuição das reservas, instabilidade de preços e impactos ambientais associados à emissão antropogênica de dióxido de carbono. A busca por combustíveis alternativos, como o etanol, gerou um grande interesse naqueles vindos de biomassa vegetal. Duas classes de carboidratos compõem a biomassa, a primeira é formada por açúcares de reserva, como o amido e a sacarose, que são amplamente utilizadas pelas indústrias alimentícias e de bioconversão a etanol (etanol de primeira geração). A segunda classe é composta pelas partes estruturais da planta, é o caso da lignocelulose, que é formada por açúcares mais complexos e de difícil acesso responsáveis pela produção do etanol de segunda geração, o qual tem despertado grande interesse (OCTAVE e THOMAS, 2009).

A biomassa lignocelulósica surgiu como uma fonte alternativa ambientalmente sustentável para insumos químicos, novos materiais e combustíveis. Essa está presente em grandes quantidades em resíduos de atividades agrícolas, florestais e da indústria de papel. Por conter açúcares fermentescíveis necessários para a biotransformação em biocombustíveis e insumos de química fina com alto valor agregado, a biomassa lignocelulósica foi reconhecida como potencial fonte sustentável de energia (LEE *et al*., 2009).

2.1.1. Materiais lignocelulósicos

Em estimativa feita por Kim e Yum (2006), são gerados 1,5 trilhões de toneladas de biomassa lignocelulósica por ano, tornando essa uma fonte inesgotável de matéria-prima para formação de bioprodutos, como o etanol e o xilitol. Esse enorme acúmulo de resíduos lignocelulósicos provém, em grande parte, do processamento de culturas agrícolas. Nesse âmbito, merecem destaque as significativas quantidades de resíduos de plantas remanescentes e inutilizadas após as colheitas de sementes e grãos, bem como os resíduos oriundos do manufaturamento em indústrias.

O Brasil produz uma diversidade de grãos e culturas que, consequentemente, geram uma vasta quantidade de resíduos agroindustriais que podem vir a ser bioconvertidos a etanol. Este país é o segundo maior produtor mundial de soja (95 milhões de toneladas) e está entre os dez maiores em produção de arroz (12 milhões de tonelada) (CONAB, 2015). Com relação à soja, o Rio Grande do Sul é o terceiro maior produtor do país, com cerca de 14 milhões de toneladas e o maior produtor de arroz, com cerca de 8 milhões de toneladas (CONAB, 2015).

A casca do grão de soja representa o maior subproduto das indústrias processadoras dessa semente e constitui por volta de 8 % de todo o grão (GNANASAMBANDAN e PROCTOR, 1999). Segundo o Departamento de Agricultura dos Estados Unidos-USDA, a safra mundial de soja 2014/2105 esta estimada em aproximadamente 318,6 milhões de toneladas (USDA, 2015). Considerando esta safra como exemplo, foram geradas naquele ano em todo o mundo aproximadamente 25,5 milhões de toneladas de casca de soja, o que torna esse resíduo lignocelulósico atrativo e interessante para gerar produtos com valor agregado maior, utilizando processos biotecnológicos.

Com relação ao arroz, tanto a casca quanto a palha do arroz, que são resíduos lignocelulósicos agroindustriais, têm sido objeto de estudos da conversão dessa biomassa a etanol de segunda geração. Cerca de 20% do peso seco do arroz é casca, e pode servir como um substrato de baixo custo para a produção de etanol combustível nos EUA e em outros países produtores de arroz como o Brasil (SAHA, 2005). Dados divulgados pela FAS/USDA indicam que a produção mundial de arroz, para a safra 2014/2015 deverá ser de 476 milhões de toneladas (USDA, 2015). Desta forma, aproximadamente 95,2 milhões de toneladas de casca são gerados em todo o mundo,

tornando esse resíduo lignocelulósico um dos mais expressivos em volume e uma alternativa interessante para bioconversão em produtos de alto valor agregado já que sua utilização como ração animal não é muito utilizada, pois é de difícil digestibilidade por animais, possui baixa densidade, grande quantidade de cinzas/sílica como componentes e características abrasivas (SAHA e COTTA, 2008).

2.1.2 Composição dos materiais lignocelulósicos

Os materiais lignocelulósicos possuem características de composição, estrutura molecular e propriedades físico-químicas tais que resultam numa notável resistência à hidrólise. Esta propriedade de "recalcitrância hidrolítica" vem dificultando o desenvolvimento de tecnologias de custo efetivo para a conversão de materiais lignocelulósicos a açúcares fermentescíveis, que, simultaneamente, seja energeticamente viável e ecologicamente correta (LI *et al*., 2008; LEE *et al*., 2009). A lignocelulose é um material orgânico renovável e é o principal componente estrutural celular de todas as plantas. Essa biomassa vegetal lignocelulósica é composta por três componentes principais: celulose (40 – 50 %), hemicelulose (20-40 %) e lignina (20-30 %), mas também estão presentes na composição extrativos e compostos inorgânicos. A composição varia largamente dependendo da espécie da planta, idade, tempo de colheita e condição ou estágio de crescimento (JEFFRIES e JIN, 2000). Os três tipos de polímeros principais (celulose, hemicelulose e lignina) estão fortemente entrelaçados e quimicamente ligados por forças não covalentes e ligações covalentes cruzadas (PÉREZ *et al.,* 2002). A Figura 1 ilustra a composição dos materiais lignocelulósicos.
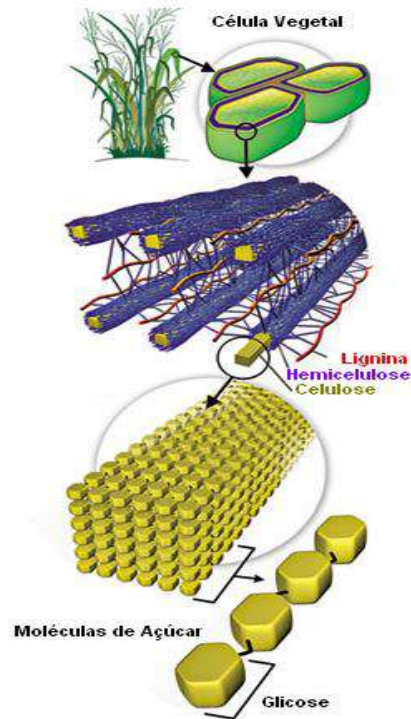
Figura 1: Ilustração da composição dos materiais lignocelulósicos (DEANGELIS *et al*., 2010).

A celulose (Figura 2) é um polímero linear semicristalino, que é composto por subunidades de D-glicose, ligadas através de ligações glicosídicas β-1, 4, formando o dímero de celobiose. Estes formam longas cadeias (ou fibrilas elementares), ligadas entre si por ligações de hidrogênio e forças de van der Waals. Celulose geralmente está presente na forma cristalina e uma pequena quantidade não organizada que se encontra na forma amorfa. A parte amorfa da celulose é mais suscetível à degradação enzimática (PÉREZ *et al*., 2002).
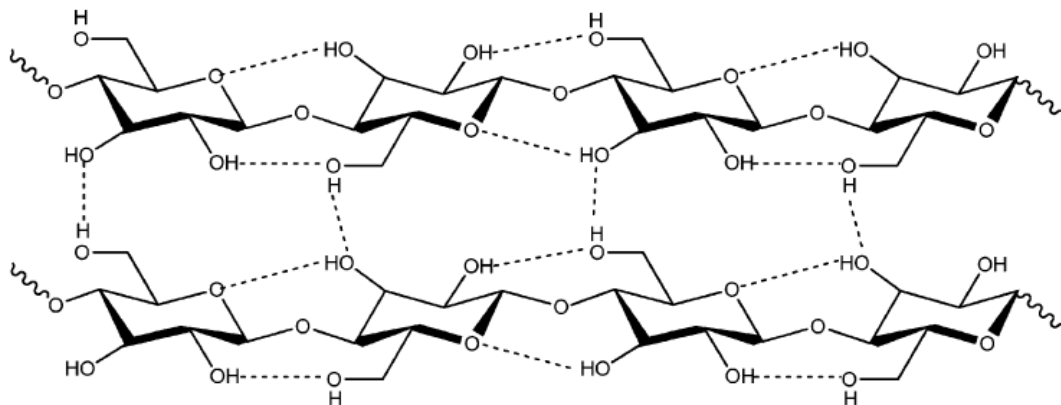
Figura 2: Estrutura da celulose demonstrando as ligações de hidrogênio intermolecular e intramolecular (WANG *et al*., 2012).

A Figura 3 ilustra a hemicelulose que é um polissacarídeo com menor massa molar do que a celulose. Esta é formada a partir de D-xilose, D-manose, D-galactose, D-glicose, L-arabinose, 4-O-metil-glucurônico, D-galacturônico e ácidos D-glucurônico. Os açúcares são unidos por ligações glicosídicas β-1,4 e, por vezes, β-1,3. A principal diferença entre celulose e hemicelulose é que a hemicelulose tem ramos com pequenas cadeias laterais que são constituídas de diferentes açúcares e a celulose consiste de oligômeros facilmente hidrolisáveis (SÁNCHEZ, 2009).
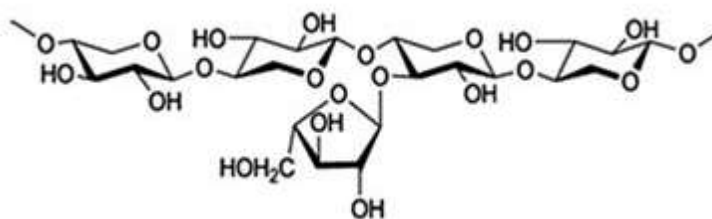
Figura 3: Estrutura da hemicelulose (ZHANG, 2013).

A lignina está ligada à hemicelulose e celulose, formando um selo físico que é uma barreira impenetrável na parede celular das plantas. Ela está presente na parede celular para dar apoio estrutural, impermeabilidade e resistência contra o ataque microbiano e estresse oxidativo. Constitui um macromolécula amorfa, não solúvel em água e opticamente inativa, formada a partir de unidades de fenil-propano unidas por ligações não-hidrolisáveis. Este polímero é formado através da geração de radicais livres, que são liberados na desidrogenação mediados pela peroxidase de três diferentes álcoois fenil propanóico: guaiacil, siringil e ρ-hidroxifenil (SÁNCHEZ, 2009). A lignocelulose foi sendo selecionada durante a evolução para ser o elemento chave da estrutura da planta e consequentemente ser resistente aos estresses bióticos e abióticos. Porém, essa característica acaba sendo um problema na indústria processadora dessa biomassa (OCTAVE e THOMAS, 2009).

Da mesma forma, fazem parte dos materiais lignocelulósicos, mas em pequena proporção, os extrativos que se consistem de ceras, gorduras, resinas, taninos, óleos essenciais, compostos fenólicos, terpenos, ácidos alifáticos, alcoóis e substâncias inorgânicas (KUHAD e SINGH, 1993).

Ter o conhecimento da estrutura e da composição da biomassa lignocelulósica é fundamental para a utilização de pré-tratamentos e/ou tratamentos que possam

aumentar a disponibilização dos açúcares fermentescíveis constituintes da celulose e hemicelulose para posterior bioconversão destes.

2.1.3 Pré-tratamentos de materiais lignocelulósicos

Processos que visam à bioconversão da biomassa lignocelulósica através da fermentação dos açúcares constituintes deste material requerem um pré-tratamento (Figura 4) para melhorar a acessibilidade aos polissacarídeos da parede celular. A cristalinidade natural da lignocelulose e a sua resistência à hidrólise são os maiores obstáculos na produção de açúcares fermentescíveis de forma economicamente viável (KUMAR *et al*., 2008). Os materiais lignocelulósicos podem ser tratados por processos físicos, químicos e/ou biológicos. Os tratamentos físicos incluem diversos tipos de moagem, já os tratamentos químicos e físicos incluem explosão a vapor, extração por água quente, explosão de fibra com amônia, hidrólise alcalina, hidrólise ácida diluída, entre outros (SUN e CHENG, 2002). Ainda existem os tratamentos biológicos realizados por fungos que podem reduzir o grau de polimerização da celulose ou hidrólisar parcialmente a hemicelulose (TAHERZADEH e KARIMI, 2008). Estes tratamentos provocam efeitos como o aumento na porosidade dos materiais, redução na cristalinidade da celulose, depolimerização parcial da celulose, solubilização da hemicelulose e/ou lignina e a modificação da estrutura da lignina, de forma a aumentar sua susceptibilidade à hidrólise enzimática ou liberar os açúcares fermentescíveis (MARGEOT *et al*., 2009).
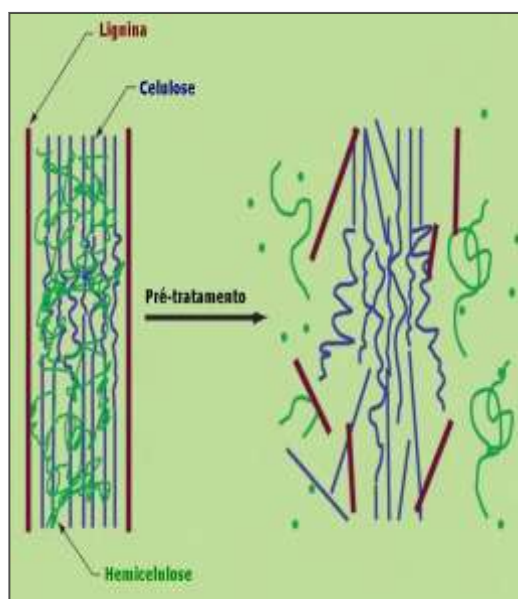
Figura 4: Ilustração da modificação estrutural da biomassa lignocelulósica após pré-tratamento (adaptado de MOSIER *et al*., 2005).

Num processo típico de pré-tratamento por hidrólises para a produção de etanol de segunda geração, a hemicelulose é depolimerizada em pentoses (xilose, predominantemente), enquanto que a celulose é convertida em hexoses, basicamente, glicose (CHEN *et al*., 2012). Dentre estas técnicas, o pré-tratamento com ácido sulfúrico diluído tem se mostrado como o principal pré-tratamento empregado e está em desenvolvimento comercial (LI *et al*., 2010).

Pré-tratamentos realizados com ácido diluído e temperaturas moderadas são considerados eficazes em relação custo-benefício e atuam causando o afrouxamento da parede celular da matriz através da degradação de hemicelulose (SIVAKUMAN *et al*., 2008). Um fator importante a ser considerado durante a hidrólise ácida diluída é que, dependendo das condições empregadas, compostos secundários dos açúcares e da lignina podem ser gerados, dificultando a hidrólise enzimática e inibindo o crescimento de microrganismos fermentadores que poderão ser utilizados posteriormente a esta etapa (MUSSATO e ROBERTO, 2004; SCHNEIDERMAN *et al*., 2015). Segundo Palmqvist

e Hahan-Hagerdal (2000), quando altas temperaturas e pressões são utilizadas no pré-tratamento, xilose e glicose podem ser degradadas em furfural e hidroximetilfurfural (HMF), respectivamente, que são posteriormente degradados em ácido fórmico e levulínico. Além desses compostos, outras substâncias tóxicas aos microrganismos podem ser formadas durante a hidrólise, como compostos fenólicos que são gerados a partir da quebra parcial da lignina, ácidos siríngico, vanílico, palmítico, entre outros.

Alternativamente aos processos físicos e químicos aplicados pode-se empregar enzimas como catalisadores da hidrólise dos materiais lignocelulósicos a monossacarídeos. A hidrólise enzimática, que utiliza celulases, é bastante eficiente na conversão da celulose pura, no entanto este processo aplicado a um resíduo sem pré-tratamento apresenta baixas velocidades de reação, principalmente devido à complexidade e heterogeneidade da parede celular das microfibrilas, além das enzimas representarem um alto custo para o processo (MONIRUZZAMAN *et al*., 2010).

As celulases são utilizadas para hidrolisar celulose a glicose (Figura 5), seletivamente, sob condições brandas (30–70 ^{o}C). Este complexo enzimático celulolítico, que combina diferentes enzimas, cada qual possuindo uma tarefa específica na hidrólise da celulose, é produzido por uma grande variedade de microrganismos, incluindo fungos e bactérias. Uma das enzimas que compõe este complexo são as endoglicanases. Elas agem internamente nas regiões amorfas da cadeia de celulose, clivando ligações glicosídicas, reduzindo o grau de polimerização. Já as exoglicanases degradam a cadeia de celulose a partir de seus terminais redutores ou não redutores, podendo clivar desde regiões amorfas às de alta cristalinidade, liberando celobiose que é hidrolisada a glicose pela β-glicosidase (HAYES, 2009).

Existem vários fatores que podem influenciar tanto o rendimento de açúcares, quanto a velocidade da hidrólise. No que concerne às características da biomassa,

fatores como a cristalinidade da celulose, seu grau de polimerização, conteúdo de hemicelulose, aumento de tamanho de partícula podem afetar negativamente a hidrólise enzimática, de forma que tais fatores devem ser considerados na escolha de um pré-tratamento apropriado. A lignina também é empecilho à hidrólise, não só pelo conteúdo absoluto, mas também por sua composição particular e suas associações com polissacarídeos (HAYES, 2009). Assim, métodos de pré-tratamento mais eficientes e economicamente viáveis se fazem necessários. Deseja-se, sobretudo que seja empregado sob condições físico-químicas mais brandas, baixo aporte energético e que se façam uso de solventes totalmente recicláveis. Uma alternativa altamente viável e bastante promissora é o uso de líquidos iônicos (LIs) (ZAVREL *et al*., 2009).
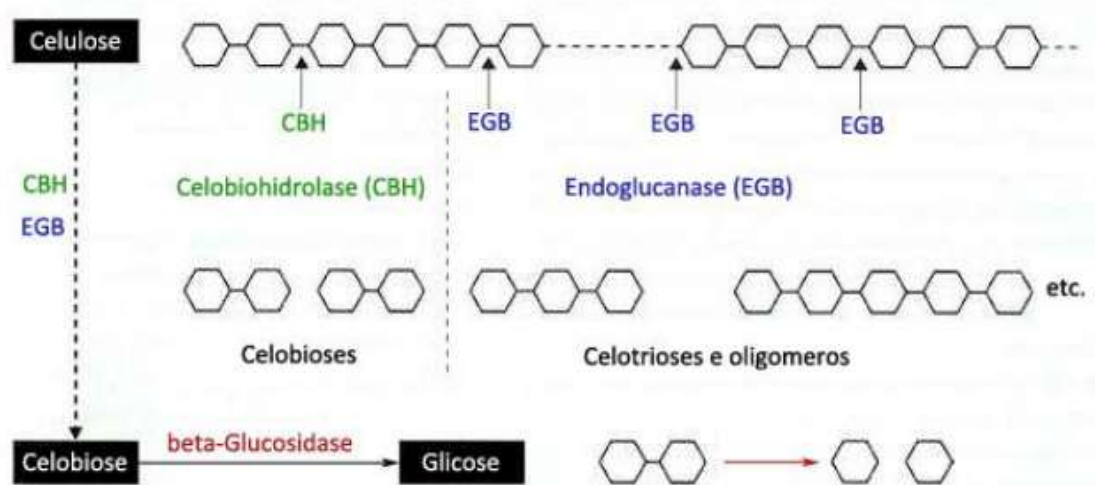
Figura 5: Representação esquemática da ação catalítica de um complexo enzimático celulolítico (celulase) sobre a celulose com geração de glicose (OGEDA, T.L., 2011).

2.1.4 Pré-tratamento com Líquidos Iônicos

Tem se desenvolvido um crescente interesse no uso de LIs como solventes na dissolução de biomassa com uma fácil recuperação da celulose em consequência da adição de um antissolvente (LI *et al*., 2010). Líquidos iônicos são estruturas salinas, compostas por cátions orgânicos e ânions usualmente inorgânicos e que são normalmente líquidos numa ampla faixa de temperatura, que, em muitos representantes da classe, inclui a temperatura ambiente. Estes possuem características únicas como a pressão de vapor extremamente baixa, não flamabilidade e estabilidades térmica, química e eletroquímica. Em diversos processos onde encontram aplicabilidade, características físicas, tais como capacidade de dissolver compostos polares e apolares orgânicos, inorgânicos e poliméricos, foram exploradas com vantagem (LEE *et al*., 2009). Também verificou-se que, em muitos processos, os LIs podem ser reciclados inúmeras vezes, sem perda de propriedades e da eficiência de recuperação de açúcares (ZHU *et al*., 2006; SEOUD *et al*., 2007 and HAYES, 2009; AUXENFANS *et al*., 2014).

Alguns LIs são capazes de dissolver a celulose; este fato pode ser vantajoso para uma posterior hidrólise enzimática, pois a celulose reconstituída (regenerada) após o processo de dissolução apresenta um nível de cristalinidade inferior ao da celulose inicial, facilitando o acesso de celulases às cadeias polissacarídicas (LEE *et al*., 2009). A matriz lignocelulósica é dissolvida, rompendo as associações complexas existentes entre lignina, hemicelulose e celulose. Uma celulose relativamente pura pode então ser precipitada pela adição de água, etanol ou acetona, enquanto que os outros componentes permanecem em solução. Esta celulose regenerada pode ter um grau de polimerização similar à de partida, mas o grau de cristalinidade pode ser manipulado durante o processo de regeneração (HAYES, 2009).

A celulose é significativamente solúvel em alguns LIs hidrofílicos, como, cloreto de 1-butil-3-metil imidazólio ([bmim][Cl]) e cloreto de 1-alil-3-metil imidazólio ([Amim][Cl]). Sugere-se que os íons cloreto participem do processo de dissolução, rompendo as ligações de hidrogênio da celulose e que cadeias alquílicas mais longas, caso presentes na estrutura do cátion, reduzam a eficiência da dissolução (LEE *et al*., 2009). Dadi e colaboradores (2007), sugerem que os ânions e cátions são responsáveis pela dissolução da celulose (Figura 6). O grupo hidroxila livre da celulose é atacado e desprotonado pelo ânion do LI e o cátion interage com os átomos de oxigênio da hidroxila. Assim, as ligações de hidrogênio da celulose são interrompidas e substituídas por ligações de hidrogênio entre o ânion do IL e a hidroxila da celulose e, desta forma, a celulose cristalina é desorganizada devido ao processo de dissolução (DADI *et al*., 2007; SHI *et al*., 2014).
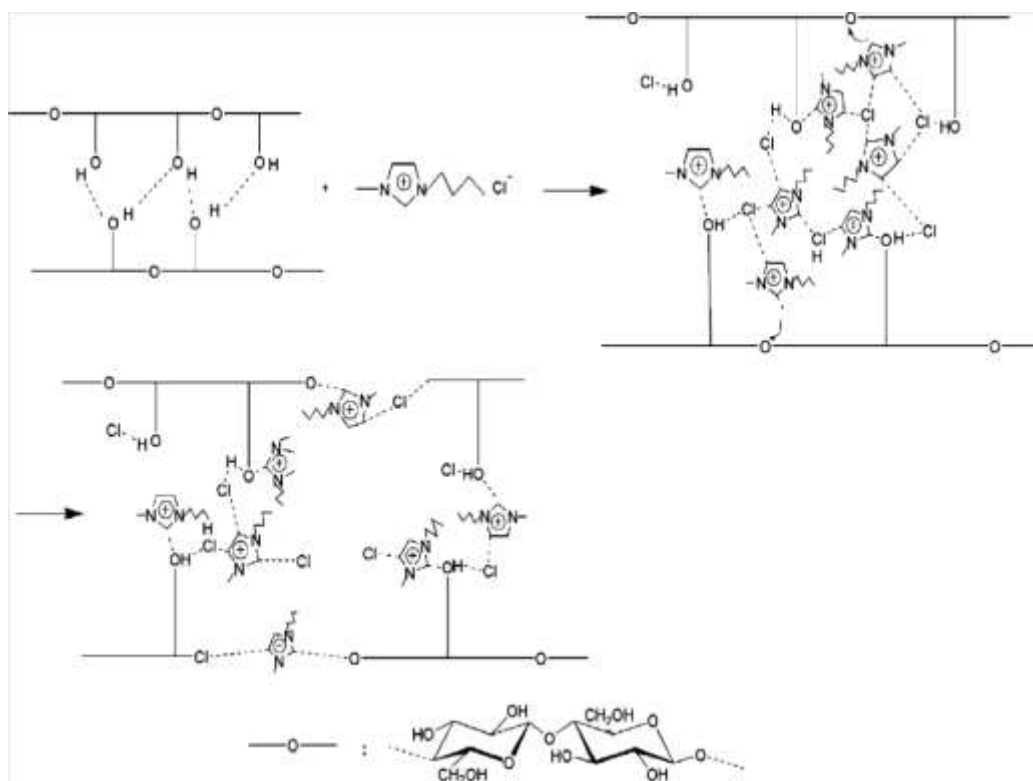
Figura 6: Mecanismo de dissolução da celulose por [bmim][Cl] (LIU *et al*., 2011).

Pesquisadores relatam a dissolução dos mais variados tipos de biomassas lignocelulósicas, dentre estes, madeira (SUN *et al.*,2009), palha de trigo (FU e MAZZA, 2011), folhas de palmeira de óleo (TAN e LEE, 2011), bagaço de cana-de-açúcar (YOON *et al.*, 2012; QIU *et al.*, 2014), palha de milho (WU *et al.*, 2011), palha de arroz (POORNEJAD *et al.*, 2013), gramíneas (SHI *et al.*, 2014) entre outros.

Alguns representantes dos LIs fazem parte do seleto grupo de solventes capazes de dissolver celulose sob condições experimentais moderadas. Em particular, haletos de 1,3-dialquil imidazólio (Figura 6) dissolvem fibras de madeira de pinheiro, e esse processo pode ser utilizado para separar lignina (ZHU *et al.*, 2006; LI *et al.*, 2007; FORT *et al.*, 2007, ZENG *et al.*, 2014).

R^1, R^2 = grupo alquila

X = Cl, Br, etc
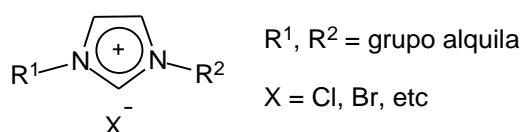
Figura 7: Exemplos de líquidos iônicos derivados do cátion 1,3-dialquil imidazólio.

Resultados significativos na dissolução de biomassa lignocelulósica foram verificados quando utilizados os líquidos iônicos cloreto de 1-butil-3-metilimidazólio (TAN e LEE, 2011) e acetato 1-butil-3-metilimidazólio (POORNEJAD *et al.*, 2013). As estruturas desses líquidos iônicos estão representadas na Figura 8.
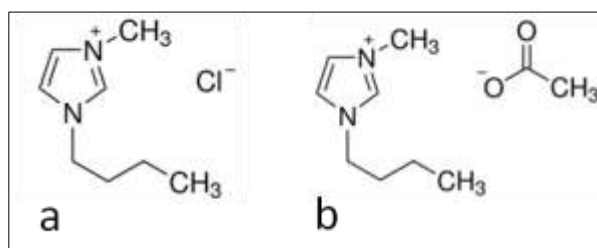
Figura 8: Estrutura molecular dos líquidos iônicos: a) cloreto de 1-butil-3-metilimidazólio e b) acetato 1-butil-3-metilimidazólio.

A utilização dos LIs pode-se se resumir da seguinte forma, a matriz lignocelulósica é dissolvida, rompendo as associações complexas existentes entre lignina, hemicelulose e celulose. Este processo pode gerar uma celulose regenerada com menor grau de cristalinidade. Isto significa que o emprego de LIs pode ser um pré-tratamento eficaz, rendendo celulose mais adequada à hidrólise enzimática (HAYES, 2009). A hidrólise enzimática da celulose regenerada a partir do LI é mais rápida quando a comparada à celulose não tratada, isso ocorre porque a celulose tratada com o LI é mais porosa e possui um menor grau de cristalinidade ou contém mais partes amorfas que o polímero não tratado, o que aumenta a eficácia da ação enzimática (MONIRUZZAMAN *et al.,* 2010). Outro ponto positivo na utilização de LIs no pré-tratamento de materiais lignocelulósicos é que não é gerado produtos degradados tóxicos para enzimas ou microrganismos de processos fermentativos (DADI *et al*., 2007; LEE *et al*., 2009).

2.2. Bioconversão de hidrolisados lignocelulósicos

Microrganismos que fermentem tanto as hexoses (glicose, manose e galactose), quanto pentoses (xilose e arabinose) na presença de compostos inibitórios, incluindo ácidos fracos, furaldeídos e fenóis, são necessários para a fermentação de hidrolisados lignocelulósicos a etanol (MARGEOT *et al*., 2009). Isso se deve a necessidade de converter eficientemente todos os açúcares presentes nos hidrolisados lignocelulósicos a etanol, com a finalidade de maximizar a rentabilidade de um processo industrial e melhorar a competitividade de custos da produção de bioetanol (KUHAD *et al*., 2011).

A levedura *Saccharomyces cerevisiae* é microrganismo mais utilizado no processo de fermentação alcoólica. Esta levedura converte facilmente glicose à etanol e gás carbônico (CO_2), apresentando uma alta tolerância à etanol quando comparada com outras (SÁNCHEZ e CARDONA, 2008). Porém a sua utilização na produção de etanol de segunda geração, sobre hidrolisados que contém pentoses e hexoses não é eficiente, já que essa levedura não consegue converter pentoses a etanol. Várias pesquisas objetivam o desenvolvimento de cepas modificadas de *S. cerevisiae* que consigam utilizar as pentoses presentes nos hidrolisados, mas estas cepas tem-se apresentado demasiadamente sensíveis aos hidrolisados (HAHN – HAGERDAL *et al*., 2007).

Um dos gargalos da produção de etanol de segunda geração através de processos fermentativos, que utilizem materiais lignocelulósicos, é a conversão das pentoses a etanol. A principal pentose liberada nos hidrolisados de biomassas lignocelulósicas é a xilose. Sendo que, o conhecimento sobre espécies que fermentam pentoses ainda é limitado. Dentre as principais espécies que fermentam xilose, pode-se citar: *Pachysolen tannophilus* (CHENG *et al*., 2008); *Pichia stipitis* (AGBOGBO *et al*., 2006; CANILHA *et al*., 2010), *Candida guilliermondii* (SCHIRMER-MICHEL *et al*., 2008), C*andida shehatae* (CHANDEL *et al*., 2007), entre outros.

Outras duas leveduras foram descritas como promissoras em relação a fermentação de xilose: *Spathaspora passalidarum*, levedura isolada do intestino de besouros que se alimentam de madeira (NGUYEN *et al*., 2006), e *Spathaspora arborariae*, levedura isolada a partir da madeira em decomposição de duas diferentes localidades do Brasil (CADETE *et al.,* 2009). Estas apresentaram um grande potencial com relação a produção biotecnológica de etanol de segunda geração a partir de biomassa lignocelulósica (DA CUNHA-PEREIRA *et al*., 2011).

Um dos fatores que afeta significantemente a utilização dos carboidratos disponíveis e sua eventual conversão a etanol é aeração do cultivo (SAHA e WOODWARD, 1997). A limitação de oxigênio pode estar associada ao acúmulo de etanol no meio. Sob condições aeróbias, grande parte da xilose metabolizada é convertida a xilitol, que é o principal co-produto formado nos cultivos de xilose sob condições limitadas de oxigênio, comprometendo assim, a produção de etanol. Inúmeras causas são atribuídas para a ineficiente incorporação das moléculas derivadas da metabolização da xilose, na rota bioquímica central, que culmina na formação de etanol. A causa mais importante consiste no desequilíbrio redox entre os co-fatores das enzimas xilose redutase e xilitol desidrogenase, o qual ocorre sob condições limitadas de oxigênio, necessárias para que o substrato seja convertido em etanol e não a biomassa.

Outra causa consiste nas limitações metabólicas que ocorrem a partir do xilitol desidrogenase, incluindo a etapa de conversão de xilulose a xilulose-5-fosfato catalisada pela xiluloquinase (WALFRIDSSON *et al*., 1995). Além disso, quando a concentração de xilose atinge determinado nível e a oxigenação aumenta, alguns microrganismos preferem utilizar o etanol produzido como fonte de carbono, isso foi demonstrado em microrganismos como *Pachysolen tannophilus* (MALESZKA e SCHNEIDER, 1982)*, Candida tropicalis* e *Candida guilliermondii* (SCHIRMER-MICHEL *et al*., 2008)*, Sphataspora arborariae*, *C. shehatae* (CHANDEL *et al*., 2007; DA CUNHA-PEREIRA *et al*., 2011).

Não é apenas a oxigenação do meio, espécie e linhagem dos microrganismos que interferem na produção de etanol. Outros fatores como pH, nutrientes minerais e orgânicos, temperatura, pressão osmótica, concentração do microrganismo, também afetam os rendimentos obtidos (GUTIERREZ *et al*., 1991; ALVEZ, 1994). Autores

sugerem que a suplementação do meio de cultivo confere um aumento na concentração de etanol (SREEKUMAR *et al*., 1999; DANESI *et al*., 2006).

Co-fermentações têm sido bastante utilizadas tanto na forma livre como imobilizada, com o objetivo de utilizar as pentoses e as hexoses presentes em hidrolisados lignocelulósicos. Meios de fermentação com misturas de açúcares têm tido sucesso na conversão a etanol utilizando leveduras geneticamente modificadas e bactérias. Entretanto, a utilização de dois microrganismos simultaneamente em um cultivo (co-cultura) também vem sendo objetivos de estudos (DA CUNHA PEREIRA *et al*., 2011; HICKERT *et al*, 2013; ASHOOR *et al*., 2015). Neste caso, a finalidade é combinar um microrganismo fermentador de pentoses e outro de glicose ao mesmo tempo para a produção de etanol. A utilização das co-culturas podem ser livres, imobilizadas e com reciclo de células, e vem sendo amplamente testadas (FU e PEIRIS, 2008; FU *et al*., 2009; DA CUNHA-PEREIRA *et al*., 2011; ASHOOR *et al*., 2015).

Outro produto que tem despertado a atenção em cultivos que hidrolisados de materiais lignocelulósicos é o xilitol. Este é um álcool de açúcar contendo cinco átomos de carbono, que tem sido altamente valorizado pelas indústrias de alimentos e farmacêutica devido ao seu poder adoçante (MUSSATTO *et al*., 2006). O valor de mercado do xilitol esta crescendo e possui aplicações em pastas de dente, flúor e gomas de mascar, bem como em alimentos para fins dietéticos (MARTINÉZ *et al*., 2012).

As leveduras são consideradas as melhores produtoras de xilitol. Sendo que as leveduras do gênero *Candida* estão entre as maiores produtoras (*C. shehatae*, *C. guilliermondii*, *C. tropicalis, C. parapsilosis, C. biodinii e C. mogii*) (WINKELHAUSEN e KUZMANOVA, 1998), além de novas leveduras isoladas como a *Sphatapora arborariae* (DA CUNHA-PEREIRA *et al*., 2011). A produção de xilitol por essas leveduras é possível porque elas possuem a enzima xilose-redutase, a qual, em

presença do co-fator NADPH, catalisa a redução da xilose a xilitol como primeiro passo no metabolismo da xilose (WINKELHAUSEN e KUZMANOVA, 1998). Logo após, ocorre a oxidação do xilitol a D-xilulose mediante xilitol-desidrogenase na presença do co-fator NAD^+.

Em cultivos que utilizam quantidades limitadas de oxigênio pode ocorrer tanto a formação de xilitol quanto de etanol. Nessas leveduras, a redução da xilose implica na obtenção de NAD^+, recuperando, independentemente, da cadeia respiratória, o co-fator da xilitol desidrogenase, permitindo assim a fermentação de xilose a etanol (ROBERTO *et al*., 1999).

Capítulo 3

Materiais e Métodos

3. 2. Microrganismos, manutenção e inóculo

As leveduras utilizadas nos experimentos foram *Saccharomyces cerevisiae* (ICV d953), uma cepa comercial utilizada na fermentação de vinho que isolada a partir de uvas Syrah da região do Vale do Ródano na França; *Candida shehatae* (HM 52.2), isolada de madeira podre da Mata Atlâncida da cidade de Nova Friburgo no Brasil e *Candida guilliermondii* (BL 13), isolada de cascas de arroz podre. Estas cepas pertencentes à coleção do Bioteclab. Os estoques de cultura destes microrganismos foram mantidos em placas de Petri e tubos de ensaio contendo meio de cultura ágar extrato de malte (AEM) composto, em g L^{-1}: extrato de levedura, 3; extrato de malte, 3; peptona, 5; glicose, 10 e ágar, 20; e em microtubos contendo 20 % de glicerol e 80 % de meio de cultura EM composto, em g L^{-1}: extrato de levedura, 3; extrato de malte, 3; peptona, 5 e glicose, 10. As culturas crescidas em meio sólido foram mantidas a 4 ^{o}C e as culturas líquidas contendo glicerol em frezeer (-18 ^{o}C).

Os pré-inóculos foram preparados através do cultivo de cada espécie em meio EM, separadamente, em frasco Erlenmeyer de 500 mL contendo 150 mL de meio. As células foram cultivadas em agitador orbital (Marconi, MA 830) a 180 rpm, 30 °C por 24 horas. Em seguida, as células foram recolhidas por centrifugação a 3000 × *g* por 20 minutos. O *pellet* formado foi lavado com solução salina e ressuspenso diretamente no mesmo meio que foi utilizado na fermentação. Os inóculos dos cultivos isolados e dos

consórcios foram padronizados para que a concentração celular da suspensão atingisse densidade óptica (DO_{600}) igual a 1. O volume de inóculo perfazia 10 % do volume total de cultivo em todos os frascos utilizados nos experimentos.

3. 2. Líquidos iônicos

Para a síntese do cloreto de 1-butil-3-metilimidazólio, utilizou-se um balão de fundo redondo não volumétrico de 2 L composto de três tubuladuras. Este foi acoplado em um banho de aquecimento de óleo, um adaptador para entrada de nitrogênio, um adaptador de termômetro interno, um agitador mecânico e um condensador de refluxo. O frasco foi purgado com nitrogênio e carregado com 151,5 g de destilado de N-metilimidazole, 100 mL de acetonitrila e 220 g de 1-clorobutano foi levado a um refluxo suave (75 - 80 °C de temperatura interna). A solução foi aquecida sob refluxo durante 48 horas e, em seguida, arrefecida até à temperatura ambiente. Após, o material volátil foi removido da solução amarela resultante sob pressão reduzida. O óleo amarelo claro remanescente foi re-dissolvido em acetonitrila e adicionado gota a gota em uma solução agitada de 1000 mL de acetato de etila. Para promover a cristalização do sal, um cristal cloreto de 1-butil-3-metilimidazólio foi adicionado como agente nuclente. O sal de imidazólio começa a cristalizar quase imediatamente e o frasco foi mantido arrefecido a -30 °C durante 2 horas. A solução sobrenadante foi removido por meio de filtração e o sólido branco resultante foi seco sob pressão reduzida a 30 °C durante 6 horas, resultando o cloreto de 1-butil-3-metilimidazólio (DUPONT *et al.,* 2002).

O [bmim][Cl] foi utilizado para a elaboração do [bmim][Ac] em que, utiliza-se uma resina de troca aniônica (Amberlite IRA-400) (Figura 9) . A resina de troca aniônica foi previamente preparada com uma solução de NaOH 1 mol L^{-1} e o processo monitorado com

uma solução aquosa de AgNO₃. Quando a troca de ânions cloreto por hidróxido estava completa, o precipitado de AgCl não foi mais observado. Uma solução aquosa de [bmim][Cl] a 0,1 mol L^{-1} foi preparada e passada através de uma coluna contendo a resina, efetuando-se assim a troca dos ânions cloreto e metanossulfonato por ânions hidroxila. Na solução obtida foi adicionado ácido acético e, após agitação, a solução foi concentrada em rota-evaporador e, posteriormente, seca no vácuo com agitação vigorosa e aquecimento a 80 °C por 24 horas (WU *et al.,* 2008, SIMON, 2013).
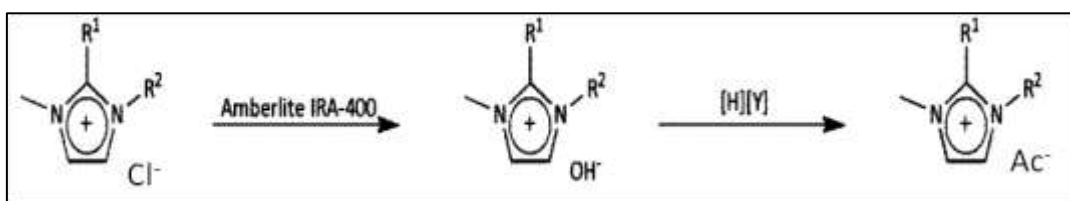
Figura 9: Esquema da síntese do [bmim][Ac] (Adaptado de SIMON, 2013).

3. 3. Obtenção do hidrolisado ácido diluído de casca de arroz (RHH) e de soja (SHH)

A casca de arroz foi hidrolisada em autoclave utilizando frascos Erlenmeyers de 2L. A relação sólido:líquido (casca de arroz moída:ácido sulfúrico 1 %) foi de 1:10. A hidrólise foi conduzida a 121°C durante 60 minutos (CUNHA-PEREIA *et al.*, 2011. Já o hidrolisado de casca de soja utiliza uma relação sólido:líquido de 1:8,8 e tempo de reação de 40 min (CASSALES *et al.*, 2011).

Os hidrolisados resultantes foram resfriados em banho-maria e filtrados. O sobrenadante foi estocado em refrigerador a 4 °C para posterior concentração e tratamento. Os hidrolisados foram concentrados para aumentar a concentração dos

açúcares em aproximadamente 2,8 vezes para o RHH e 4 vezes para o SHH, utilizando concentrador a 70 °C, sob vácuo.

3. 4. Hidrolisado enzimático da casca de soja

A fração sólida proveniente da hidrólise ácida diluída da casca de soja foi lavada com água até esta obter pH neutro. A hidrólise enzimática da celulose foi realizada em proporção sólido:líquido de 1:20, utilizando tampão de fosfato-citrato (pH 4,8). Foi utilizado um complexo enzimático celulolítico proveniente de culturas de *Penicillium echinulatum* S1M29 na proporção de 15 FPU (unidade de filtro de papel) g^{-1} de biomassa seca. O meio foi incubado em agitador orbital a 50°C, 120 rpm, durante 72 h. O sobrenadante foi concentrado em concentrador a 70 °C, sob vácuo a fim de aumentar a concentração dos açúcares (HICKERT *et al.,* 2013).

3. 5. Hidrolisado ácido-enzimático de casca de soja

O hidrolisado ácido-enzimático da casca de soja é uma mistura na proporção de 1:1 de cada um dos dois hidrolisados da casca de soja descritos nos itens 3.3 e 3.4 (HICKERT *et al.,* 2013).

3. 6. Pré-tratamento de casca de soja e de arroz com líquidos iônicos

Em um tubo de polipropileno foram adicionados líquido iônico ou uma mistura de água-líquido iônico e casca de soja ou de arroz. Os tubos foram incubados num banho de óleo a diferentes temperaturas e tempos de incubação. Todos os experimentos foram realizados sob pressão atmosférica normal. Após o pré-tratamento, 10 mL de

água deionizada foram adicionados às amostras, a fim de servir como um anti-solvente na mistura e para precipitar a celulose. As misturas resultantes foram brevemente centrifugadas a 7000 × g, e o sobrenadante foi removido. O precipitado (biomassa contendo celulose regenerada tratada) foi lavada com 10 mL de água (anti-solvente) e centrifugadas a 7000 × g. Este procedimento foi repetido quatro vezes. A biomassa tratada foi seca em estufa (75 °C, 24 h) no mesmo tubo do pré-tratamento.

Num primeiro momento resolveu-se estudar os efeitos da temperatura, tempo de incubação, carga de sólido (razão casca de soja/LI) e concentração de líquido iônico (mistura LI-água) no pré-tratamento de casca de soja utilizando [bmim][Ac] através de um planejamento composto central (Tabela 1 do terceiro artigo). A quantificação foi realizada através dos açúcares liberados após hidrólise enzimática. A hidrólise enzimática da biomassa pré-tratada com LI foi realizada utilizando o complexo enzimático celulolítico derivado de culturas de *P. echinulatum* S1M29. No tubo de polipropileno contendo a biomassa pré-tratada seca, a enzima foi adicionada a uma proporção líquido-sólido de 1:80 de biomassa tratada seca e o pH foi ajustado utilizando um tampão citrato-fosfato (pH 4,8). Foi utilizado 60 FPU g^{-1} de biomassa seca e a mistura (enzima mais a biomassa pré-tratada) foi incubada num agitador rotativo a 120 rpm, 50 °C durante 48 h. Para a fermentação, o hidrolisado foi centrifugada; o sobrenadante foi, então, recuperado e concentrado.

Um estudo foi realizado, para investigar a modificação das estruturas das cascas de soja e arroz quando tratadas com os líquidos iônicos [bmim][Cl] e [bmim][Ac] (Tabela 1 do quarto artigo). A celulose regenerada foi caracterizada por análise termogravimétrica (TGA), espectroscopia de absorção no infravermelho (FTIR), difração de raios-X (XDR) e microscopia eletrônica de varredura (MEV).

3. 7. Estudo da cinética de conversão de açúcares em etanol.

Foram realizados experimentos sob diferentes condições de cultivo para avaliar o consumo de açúcares fermentescíveis, produção de etanol e de outros produtos de fermentação.

A fim de selecionar o microrganismo e/ou consórcio (*S. cerevisiae* e/ou *C. shehatae*) com maior potencial de produção de etanol a partir da conversão de glicose, xilose e arabinose foram realizados ensaios utilizando meio semi-sintético e hidrolisado de casca de arroz (concentrado) tanto em agitador orbital quanto em biorreator. O meio semissintético ($G_{20}X_{20}A_{10}$) era composto por (g L^{-1}): extrato de levedura, 3; peptona, 5; xilose, 20; glicose, 20 e arabinose, 10. Os ensaios foram conduzidos em frascos Erlenmeyers de 2L contendo 450 mL de meio semi-sintético $G_{20}X_{20}A_{10}$, em agitador orbital (Marconi, MA 830) a 180 rpm, 28 °C por 108 horas. Os mesmos parâmetros de cultivo foram empregados no ensaio utilizando RHH como meio de cultivo, no entanto o período de incubação chegou a 240 horas. Os ensaios conduzidos em biorreatores de cultivo submerso com capacidade total para 2 L (Biostat B model, B. Braun Biotech International, Alemanha) utilizaram 1,5 L de meio semissintético e RHH em condições limitadas de oxigênio (180 rpm e 0,33 vvm) a temperatura de 28°C e pH 5. O meio semissintético para simular a composição do RHH era composto em (g L^{-1}) por: extrato de levedura, 3; peptona, 5; xilose, 15; glicose, 30 e arabinose, 5. O tempo de cultivo foi de 228 horas.

Cultivos com *C. guilliermoundii* foram realizados em frascos Erlenmeyers de 250 mL contendo 60 mL de hidrolisado em agitador orbital a 180 rpm por 72 h. Primeiramente, foi utilizado o delineamento estatístico Plackett-Burman (PBD, Plackett-Burman Design) para identificar os nutrientes que possuem efeito significativo

sobre a produção de etanol quando o hidrolisado de casca de soja era suplementado. Foram avaliados milhocina, extrato de levedura, peptona e Tween 80. Cada variável independente foi avaliada quanto a presença ou ausência destes nos cultivos (Tabela 1 do segundo artigo). Após a análises dos resultados da suplementação, seguiu-se com um planejamento experimental relativo a otimização das condições de cultivo de *C. guilliermoundii* em SHH. As variáveis independentes de estudo foram: temperatura, pH e concentração de inóculo (Tabela 2 do segundo artigo). As condições de máxima produção de etanol estabelecidas pelo planejamento fatorial foram utilizadas em cultivo de *C. guilliermoundii* sobre o hidrolisado ácido-enzimático de casca de soja.

C. shehatae foi utilizada na fermentação do hidrolisado enzimático da condição otimizada do pré-tratamento de casca de soja com [bmim][Ac]. Foram utilizados frascos Erlenmeyers de 125 ml contendo 25 ml do hidrolisado e agitador orbital a 30 °C, 180 rpm por 48h.

Alíquotas foram coletadas para determinação da concentração celular (peso seco e UFC – unidades formadoras de colônia) e quantificação de glicose, xilose, arabinose, xilitol e etanol, através de análise cromatográfica em Cromatografia Líquida de Alta Eficiência (CLAE).

3. 8. Métodos Analíticos

3.8.1 Determinação da composição da casca de arroz e de soja

A casca de arroz utilizada nos experimentos foi cedida pela Nelson Wendt & Cia Ltda (Pelotas, RS) e a casca de soja foi doada pela SOLAE (Esteio, RS). As cascas

foram moídas em partículas menores que 1 mm de diâmetro, ensacada e armazenada a temperatura ambiente até a realização das hidrólises.

A análise das cascas foi realizada de acordo com a metodologia descrita por Dunnig e Dallas (1949), que se fundamenta na sacarificação quantitativa dos polissacarídeos de diferentes matérias-primas vegetais. A umidade da casca foi determinada em balança de infravermelho (OHAUS) por 30 minutos a 105 °C. Aproximadamente 2 g de casca (massa seca) foram pesadas e transferidas para um béquer ao qual foram adicionados 10 mL de ácido sulfúrico 72 % (massa/massa). A mistura foi agitada continuamente com bastão de vidro e climatizada em banho-maria a 50 °C por 7 minutos. Nessa etapa, denominada hidrólise principal, ocorreu o rompimento das fibras de celulose em oligômeros. Após completar o tempo da hidrólise, a reação foi interrompida com adição de 275 mL de água destilada e o conteúdo foi transferido para um frasco de Erlenmeyer de 500 mL. Em uma etapa de pós-hidrólise, a suspensão foi autoclavada a 121 °C por 15 minutos. Após resfriamento, a suspensão foi filtrada em cadinho Gooch com placa porosa de vidro sinterizado, transferida quantitativamente para um balão volumétrico de 500 mL e o volume foi completado com água destilada.

O resíduo sólido, (após várias lavagens) foi seco em estufa a 105 °C e pesado até massa constante. A diferença entre a massa seca do resíduo e a massa seca inicial da amostra foi utilizada para determinar a porcentagem de lignina insolúvel presente nos materiais lignocelulósicos. A lignina isolúvel foi determinada com a fração líquida utilizando espectrofotômetro UV em 280 nm. A fração líquida obtida foi analisada por CLAE para determinar as concentrações dos componentes do material lignocelulósico (ácido glucurônico, celobiose, glicose, xilose, arabinose e ácido acético) utilizados para o cálculo da porcentagem de celulose e hemicelulose das cascas.

Para determinação das cinzas, 1 g de casca (massa seca) foi submetida a um tratamento térmico em mufla a 600 °C, durante 4 horas, até combustão total da matéria orgânica. Em seguida, esta foi pesada até obter-se massa constante (SILVA e QUEIROZ, 2005). O teor de proteína foi determinado conforme metodologia descrita por HORWIITZ (1975), utilizando o método de Kjeldahl, empregando-se fator de 6,25 para casca de arroz.

3.8.2 Determinação da concentração celular

As concentrações celulares foram determinadas por turbidimetria em espectrofotômetro (Ultrospec 3100 pro) no comprimento de onda de 600 nm, por gravimetria através da determinação de biomassa seca, utilizando estufa (Fanem 315 SE) a 75 °C até obtenção de peso constante.

3.8.3 Determinação da concentração de substrato e produtos

Os teores de glicose, xilose, arabinose, etanol e xilitol foram determinados por Cromatografia Líquida de Alta Eficiência CLAE, em equipamento Shimadzu, com auxílio de detector de índice de refração (RID), empregando-se coluna Bio-Rad Aminex HPX-87H a 45ºC, utilizando como fase móvel H_2SO_4 (5 mM) a um fluxo de 0,6 mL min^{-1}. As amostras foram filtradas em filtro "*Sep-Pack*" C18 (Millipore) ou em membrana de acetato de celulose (0,2 μm). A concentração das substâncias foi determinada através de curvas padrão de calibração preparadas com padrões de grau analítico, previamente seco sob sílica e vácuo.

3.8.4 Determinação da concentração de furfural e hidroximetilfurfural

As concentrações de furfural e hidroximetilfurfural (HMF) foram determinadas por CLAE, sob as seguintes condições: coluna Nucleosil C-18 mantida a 25 ^{o}C; detector ultravioleta/visível SPD-M20A a 276nm, tendo acetonitrila/água (2:8) com 1% de ácido acético como eluente em fluxo de 1,1 mL minuto^{-1}. O volume de amostra injetada foi de 20 μL. As amostras foram previamente filtradas em membrana de acetato de celulose ME25 com 0,45 μL e D13 mm. A concentração das substâncias foi determinada através de curvas padrão de calibração preparadas com padrões de grau analítico, previamente seco sob sílica e vácuo.

3.8.5 Determinação dos parâmetros

Para avaliar a cinética dos cultivos alguns parâmetros foram adotados.

$$Y_{P/S} = \frac{(P - P_0)}{(S_0 - S)} \qquad (1)$$

$$Q_p = \frac{(P - P_0)}{tempo} \qquad (2)$$

Onde:

$Y_{P/S}$: coeficiente rendimento de etanol (g etanol por g açúcar total consumido)

P : quantidade de produto final (g L^{-1})

P_0 : quantidade de produto inicial (g L^{-1})

S : quantidade de substrato final (g L^{-1})

S_0 : quantidade de substrato inicial (g L^{-1})

Q_P : coeficiente de produtividade volumétrica (g h^{-1} L^{-1})

Tempo : tempo na máxima conversão (h)

No pré-tratamento com casca de soja e [bmim][Ac], o rendimento de recuperação de glicose foi calculado como sendo a razão entre a glicose liberada pela hidrólise enzimática da biomassa pré-tratada com o líquido iônico e a quantidade total de glicose na matriz celulósica inicial (casca de soja não tratada).

3.8.6 Determinação de pH e Pressão Osmótica

O pH foi determinado em pHmetro (PHTEK, PHS 3B) e a pressão osmótica foi determinada em osmômetro (VAPRO 5520).

3.8.7 Análises estatísticas

A análise dos resultados obtidos nos planejamentos experimentais foram realizadas por meio do programa "Statistica" versão 10. A análise estatística da variância ANOVA foi utilizada. Os resultados foram expressos através de tabelas e gráficos.

3.8.8 Análise Termogravimétrica (TGA)

A análise termogravimétrica das amostras foi realizada sob atmosfera de nitrogênio em um aparelho Shimadzu modelo TGA-50 existente no LAPMA Instituto de Física-UFRGS. As amostras foram aquecidas até 600 °C a uma taxa de 10 °C min^{-1}.

3.8.9 Análise de Espectroscopia de absorção no infravermelho (FTIR)

As amostras foram analisadas utilizando espectroscopia na região do infravermelho com transformada de Fourier (FTIR). Os espectros foram obtidos em um equipamento Prestigie 21 em modo ATR, com uma resolução de 4 cm^{-1} e 32 scans. As análises foram realizadas na Central Analítica do Instituto de Química-UFRGS.

3.8.10 Difração de Raios X (DRX)

As análises de difração de raios X das amostras em pó foram realizadas no Instituto de Física-UFRGS, em um difratômetro modelo D500 da Siemens, usando Cu-Kα (λ= 0,15418 nm) como fonte de radiação.

3.8.11 Microscopia Eletrônica de Varredura (MEV)

Os materiais foram analisados por microscopia eletrônica de varredura (MEV) em um equipamento Jeol, modelo JEOL JSM6060, operando a 18 kV e com ampliação de 500 X. Estas foram realizadas no Centro de Microscopia Eletrônica-UFRGS.

Capítulo 4

Resultados

Neste trabalho os resultados estão apresentados na forma de artigos científicos submetidos à publicação. Os artigos constituintes desse capítulo estão apresentados nos itens I, II, III e IV respectivamente, de acordo com as normas exigidas pelos periódicos. Em cada um são apresentados introdução ao assunto abordado, materiais e métodos detalhados, resultados, discussões e referências bibliográficas.

4.1. Resultados I

'Ethanogenic fermentation of co-cultures of *Candida shehatae* HM 52.2 and *Saccharomyces cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate'.

Este artigo consiste no estudo da capacidade de *Candida shehatae, Saccharomyces cerevisiae*, ou a combinação destas duas leveduras em converter todo o açúcar presente no hidrolisado de casca de arroz (RHH) a etanol. Foram realizados testes em meio sintético simulando o hidrolisado, e estudos sobre a oxigenação do meio. Este artigo foi publicado na revista Bioresource Technology.

Ethanogenic fermentation of co-cultures of *Candida shehatae* HM 52.2 and *Saccharomyces cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate.

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ABSTRACT

The ability of *Candida shehatae*, *Saccharomyces cerevisiae*, or the combination of these two yeasts in converting the mixed sugar composition of rice hull hydrolysate (RHH) as substrate for ethanol production is presented. In shake flask experiments, co-cultures showed ethanol yields ($Y_{P/S}$) of 0.42 and 0.51 in synthetic medium simulating the sugar composition of RHH and in RHH, respectively, with both glucose and xylose being completely depleted, while pure cultures of *C. shehatae* produced slightly lower ethanol yields (0.40). Experiments were scaled-up to bioreactors, in which anaerobiosis and oxygen limitation conditions were tested. Bioreactor co-cultures produced similar ethanol yields in both conditions (0.50-0.51) in synthetic medium, while in RHH, yields of 0.48 and 0.44 were obtained, respectively. The results showed near-theoretical yields of ethanol. Results suggest the feasibility of co-cultures of *C. shehatae*, a newly isolated strain, and *S. cerevisiae* in RHH as substrate for second-generation ethanol production.

Keywords: Bioethanol; fermentation; rice hull hydrolysate; *Candida shehatae*; *Saccharomyces cerevisiae*.

1. INTRODUCTION

Agriculture residues, forest products and other lignocellulosic biomass are the most abundant and low cost renewable resources for ethanol and energy production. Since biomass-derived energy is part of the global carbon cycle, the use of fuel ethanol can significantly reduce the net carbon dioxide emissions if technology develops to a point where bioethanol could economically replaces fossil fuels (LI et al., 2009). In a typical bioconversion process to produce second-generation bioethanol, the hemicellulose is chained-down to pentoses (predominantly xylose), while cellulose is converted to hexoses, basically glucose, by hydrolyses pretreatments (CHEN et al., 2012). Rice hull (RH) is one of the almost abundant lignocellulosic waste materials in the world, accounting for more than 120 million metric tons generated per year (YU et al., 2009). Although rice hull finds utilization as fuel in industrial boilers, its high content in ashes and huge amounts produced represents serious technological and environmental concerns. Therefore, it could be postulated its use as substrate for ethanogenic fermentation.

Ideally, ethanol production from lignocellulosic hydrolysates would require that microorganisms ferment both hexoses and the pentoses in the presence of the inhibitory compounds produced during hydrolysis. These inhibitory compounds are weak acids, such as acetic acid, furaldehydes and phenolic compounds, mainly furfural and hydroxymethylfurfural, resulting from several complex reactions during the physicochemical treatment of biomass (MUSSATTO and ROBERTO, 2006).

Therefore, fermentation processes would be economically viable only if both hexose and pentose sugars present in the hydrolysates are converted to ethanol. *S. cerevisiae*, which is used for industrial ethanol production, has several advantages due to its high ethanol productivity, high tolerance to ethanol and high inhibitor tolerance. However, it cannot utilize xylose, the predominant pentose sugar of biomass hydrolysates (MATSUSHIKA et al., 2009). On the other hand, yeasts like *Scheffersomyces* (*Pichia*) *stipitis, Pachysolen tannophilus* (FU and PEIRIS, 2008), and *Meyerozyma* (*Candida*) *guilliermondii* (MUSSATTO et al., 2005), have been reported as xylose converters, but showing low tolerance to inhibitors; requiring a small and well-controlled supply of oxygen for maximal ethanol production; and being sensitive to ethanol (MATSUSHIKA et al., 2009).

One possibility to circumvent these problems is the use of co-cultures of different yeasts, capable of both hexoses and pentoses metabolisms. Successful co-culturing methods have been described to improve the efficiency of lignocellulosic biomass fermentation by *Spathaspora arborariae* and *S. cerevisiae*; immobilized *Zymomonas mobilis* and free-cell *S. stipitis*; among others (CUNHA-PEREIRA et al., 2011; FU and PEIRIS, 2008).

In this context, the aims of this research were to investigate the use of rice hull hydrolysate (RHH) as substrate for ethanol production and the kinetics of glucose, xylose and arabinose consumption by *C. shehatae* and the co-cultures of *C. shehatae* and *S. cerevisiae* in the presence of the inhibitory compounds: acetic acid, furfural and hydroxymethylfurfural. The *C. shehatae* HM 52.2 strain has been recently isolated and never tested in bioprocesses before. Oxygen limited conditions were compared against anaerobiosis using synthetic medium and concentrate RHH in shaker and bioreactor cultivations following sugar consumption, cell growth, and ethanol productivity.

2. Materials and Methods

2.1. Chemical characterization of rice hull

Rice hull (RH) was obtained from a local rice mill (State of Rio Grande do Sul, Brazil, centroid geo-coordinates at $30^o51'04"S$ and $51^o48'44"W$; 39 m above sea level) as dried material and processed without any further treatments before hydrolysis, which is described bellow. Unless otherwise stated, all chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA). For analytical characterization of RH, it was submitted to quantitative acid hydrolysis with 72 % (mass fraction) sulphuric acid solution, in a solid-liquid proportion of 1:10. Monosaccharides and acetic acid liberated by hydrolysis were determined by HPLC in order to estimate (after corrections for stoichiometry and sugar decomposition) the contents of cellulose (as glucan), hemicelluloses (as xylan and arabinan), and acetyl groups. The acid soluble lignin was determined by UV-spectrophotometry (see item 2.6.). Protein was determined as total nitrogen content by the Kjeldahl method, using the N $\times$ 6.25 conversion factor. Ashes were determined by weight difference before and after incineration of the soybean hull sample in a muffle furnace at 600 ^{o}C for 4 h (SILVA and QUEIROZ, 2005). The mineral content was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). All determinations were carried out in triplicate.

2.2. Microorganisms, cell maintenance, and materials

The strains used in this study were *Saccharomyces cerevisiae* ICV D254 (Lalvin, Institut Coopératif du Vin, France), a commercial wild-type strain isolated from Syrah grapes from the Rhône Valley region, in France, used for wine fermentation, and *Candida shehatae* HM 52.2, a recently isolated yeast strain from rotting wood, isolated as follows. Rotting wood samples were collected at the Private Natural Heritage Reserve of Bello & Kerida, an area of Atlantic Rain Forest ecosystem located in the city of Nova Friburgo, Rio de Janeiro, Brazil (centroid geo-coordinates at $22^o17'14''$S and $42^o32'01''$W; 858 m above sea level). The local climate in this ecological reserve is altitudinal tropical, with cold and dry winter and fresh and rainy summers, with annual mean temperatures around 16°C. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. One gram of each wood sample was placed in flasks with 20 mL sterile xylan (yeast nitrogen base 0.67 %, xylan 1 %, chloramphenicol 0.02 %; pH 5.0 ± 0.2) medium. The flasks were incubated at 25 °C on an incubator shaker (New Brunswick, USA) at 150 rpm for 3 to 10 days. When growth was detected, 0.5 mL of the cultures was then transferred separately to tubes containing 5 mL sterile xylan and the tubes were incubated as described above. One loopful of culture from each tube was streaked on yeast extract-malt extract agar (YM, glucose 1 %, yeast extract 0.3 %, malt extract 0.3 %, peptone 0.5 %, agar 2 % and chloramphenicol 0.02%) (CADETE et al., 2012). The yeast was identified based on the sequencing of the D1/D2 variable domains and internal transcribed spacer (ITS) of the large-subunit rRNA gene as described by CADETE et al., 2012. *Candida shehatae* HM 52.2 was never tested in bioprocesses before. Yeasts were kept frozen at -20 °C in stock cultures of 20 % (volume fraction) glycerol and 80

% of culture medium containing (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, and glucose, 5.

2.3. Inocula preparations

Inocula for all cultivations were prepared by cultivating the yeasts in synthetic medium according to compositions described below in 500 mL Erlenmeyer flasks filled with 150 mL of medium. Cultivations were carried out in an orbital shaker (Marconi MA 830, Brazil) at 180 rpm, 30 °C for 24 h. Late exponential-phase cells were collected by centrifugation at 3 000 g for 10 min, the pellets were washed with sterile distilled water and resuspended directly into the medium to be used as inoculum (10 % volume fraction) for the cultivations, always with cell concentrations of 1.0 OD (600 nm), corresponding to cell dry weights of 2.3 g L^{-1} of *C. shehatae* and 2.9 g L^{-1} of *S. cerevisiae*.

2.4. Media composition and cultivation conditions in orbital shaker

The microorganisms, either isolated or in consortium, were cultivated in synthetic medium and in rice hull hydrolysate (RHH). For shaker flasks cultivations the synthetic medium ($G_{20}X_{20}A_{10}$) had the following composition (in g L^{-1}): yeast extract, 3; peptone, 5; glucose, 20; xylose, 20; and arabinose 10; pH adjusted to 5 with 1 M HCl. Sugars were always autoclaved separately from yeast extract and peptone in order to avoid caramelization and other reactions. For the cultivations, RHH was obtained by the diluted acid hydrolysis of rice hull in autoclave (121° C, 60 min, solid-liquid ratio of 1:10, 1 % volume fraction of sulphuric acid). The liquid fraction was recovered by

filtration and the pH was adjusted to 5 with solid drops of sodium hydroxide. The hydrolysate was vacuum-concentrated at 70 ^{o}C in order to increase its sugar and protein concentrations to the following final amounts (in g L^{-1}): glucose, 35; xylose, 13; arabinose, 4; and protein 5. The amount of toxic compounds (or inhibitors of microbial growth), formed during hydrolysis, in the final RHH was determined to be (in g L^{-1}): HMF, 0.07; furfural, 0.01; acetic acid, 1.6. Neither detoxification nor supplementation was made to the RHH. Cultures were carried out in 2 L Erlenmeyer flasks containing 450 mL of either G$_{30}$X$_{15}$A$_5$ or RHH in an orbital shaker at 180 rpm, 30 °C for 108 and 240 h, simulating a oxygen limited condition (CUNHA-PEREIRA et al., 2011). Samples were collected at stipulated points for determination of biomass by cell counting (CFU) or cell dry weight and quantification of sugars, xylitol, ethanol, and acetic acid. All experiments were conducted in triplicates.

2.5. Co-cultures in bioreactor

Experiments were carried out in fully equipped 2 L bioreactors (model Biostat B, Braun Biotech International, Germany) with RHH or synthetic medium (G$_{30}$X$_{15}$A$_5$) with the following composition (in g L^{-1}): yeast extract, 3; peptone, 5; glucose, 30; xylose, 15; and arabinose 5; pH adjusted to 5 with 1 M HCl. The different amounts of sugars in the synthetic medium used in the bioreactor were intended to better simulate the RHH composition. For each experiment, a 75 mL seed culture of each strain (OD = 1.0), totaling 150 mL of inoculum, was added into 1 500 mL of medium. The pH of the cultures were controlled and maintained at 5 by automatically adding 1 M solutions of NaOH or HCl. The oxygen-controlled experiments were run using an aeration rate of 0.33 vvm, controlled by a needle valve and with a rotameter. Temperature and agitation speed were maintained at 30 °C and

180 rpm, respectively, in all bioreactor experiments. The total cultivation time was 228 h. Samples were collected at stipulated points for determination of biomass by either colony forming units (CFU) or cell dry weight (CDW) and the quantification of sugars, xylitol, and ethanol. All experiments were performed in duplicate.

2.6. Analytical methods

Hydrolysed samples were analysed by HPLC. Glucose, xylose, arabinose, and acetic acid concentrations were determined with a refractive index (RI) (Shimadzu) detector and a Bio-Rad HPX-87H (300 × 7.8 mm) column at 45 ∘C, using 0.005 M sulphuric acid as eluent, flow rate of 0.6 mL/min and sample volumes of 20 μL. Furfural and hydroxymethylfurfural were determined with a UV detector (at 276 nm) and a Nucleosil C18 5-μm pore size (250 × 4.6 mm) column at room temperature, using acetonitrile–water (2:8) containing 10 g L-1 acetic acid as eluent, flow rate of 1.1 mL min-1 and sample volumes of 20 μL. Samples were centrifuged, washed twice with cold distilled water and dried up in pre-weighted plastic tubes at 80 oC to a constant weight (SCHIMER-MICHEL et al., 2008), Alternatively, biomass was estimated as viable cells, using CFU (colony forming units) plated in yeast morphology agar (YMA) medium. Soluble lignin (SL) was estimated by UV spectrophotometry at 280 nm. The pH of hydrolyzed samples were raised to 12 with 6 M NaOH and this solution was diluted with distilled water in order to obtain an absorbance reading not exceeding 1 unit of absorbance. The osmotic pressure of RHH was measured by placing 30 μL samples into the chamber of an osmometer (VAPRO 5520).

2.7 Kinetic parameters calculation

The yields of ethanol production ($Y_{P/S}$, g g^{-1}) was defined as the ratio between the amount of ethanol produced and total sugars consumed present in medium up to the moment xylitol started to appear in the medium; for xylitol, conversion yields ($Y_{X/X}$, g g^{-1}) calculation was the ratio between xylitol produced and xylose consumed.

3. RESULTS AND DISCUSSION

3.1. Rice hull composition

The chemical composition of rice hull varies depending on the processing technology, plant genetics, soil, and growth conditions, among other factors. The composition (% mass fraction, dry weight) of the rice hull used in this work was determined to be: cellobiose, 0.4; glucose, 34.1 xylose 12.7; arabinose 1.3; acetic acid 1.3; HMF 0.3; furfural 0.9; insoluble lignin 21.9; soluble lignin 6.1; extractives 3.1; ashes 15.9; proteins (N×6.45) 2.0. This composition is rich in sugars to be fermented into ethanol, but also contain high quantities of lignin (28 %) and − in contrast with other agro residues - ashes (15.7 %). Other raw materials such as brewers spent grain, sugarcane bagasse and wheat straw have around 28 %, 24 % and 24 % lignin, respectively (MUSSATTO and ROBERTO, 2006; LASER et al., 2002; MIELENZ et al., 2009). One of the possible problems caused by the high lignin content of lignocellulosic residues is related to the pre-treatment of dilute acid hydrolysis, which can result in the appearance of phenolic compounds, from the partial degradation of the polymer (ALMEIDA et al., 2007). The broad composition of RH is shown in Table 1, compared to other lignocellulosic materials. The content of fermentable sugars in RH exceeds 45 %, matching the values found for brewers spent grain (46 %), and soybean

hull (48 %) suggesting that RH is a very promising substrate for the bioconversions. The composition of RH sugars found in this work is similar with data reported by other authors, the differences among values being explained by the natural variations of plant origin, classification, and processing technologies.

3.2. Shake flask cultures of *C. shehatae* and its co-cultures with *S. cerevisiae* in $G_{20}X_{20}A_{10}$ and RHH

The kinetics of *C. shehatae* HM 52.2 cultivation in $G_{20}X_{20}A_{10}$ and in RHH is shown in Figures 1A and 1B, respectively, while the kinetic parameters (in comparison with bioreactor cultivations) are shown in Table 2. *C. shehatae* HM 52.2 was able to metabolize both glucose and xylose, showing that this is yeast has the enzymes for xylose transport and metabolism, but is carbon catabolite repressed (CCR) in the presence of glucose (Figure 1A). Somewhat contrasting, KASTNER et al., 1999 reported that glucose did not completely repress xylose utilization by *C. shehatae* strain ATCC 22984, since both glucose and xylose were simultaneously consumed during the fermentations. In general, glucose, mannose, and xylose share the same, unspecific, transporters and the active transport systems are repressed by both glucose and high substrate concentrations in *S. stipitis* and *C. shehatae* (GÍRIO et al., 2010).

The low production of xylitol in RHH could be explained by the presence of furanic toxics in the medium, the osmotic pressure or the combination of these factors. Furfural can work as external electron acceptors regenerating NAD^+, a cofactor of xylitol dehydrogenase that converts xylitol to xylulose, which is finally fermented to ethanol (WAHLBOM and HAHN-HÄGERDAL, 2002), rerouting the metabolism away from xylitol. RHH had a high osmotic pressure (1539 mOsm kg^{-1}) implying a low

solubility for oxygen. Very restricted aeration conditions favour the accumulation of NADH, which can inhibit the activity of NADPH-dependent xylose reductase, thus modifying the preference dependence cofactor NADPH to NADH. This modification results in the formation of NAD^+ by reducing the xylose, recovering xylitol dehydrogenase cofactor, thus diverting the fermentation of xylose to ethanol (WINKELHAUSEN and KUZMANOVA, 1998; SCHIRMER-MICHEL et al., 2008).

Arabinose was metabolized in a later phase, when both glucose and xylose were exhausted, a similar metabolic profile observed for other *Candida* species, as reported by SCHIRMER-MICHEL et al., 2008.

The observed ethanol yields ($Y_{P/S}$) was 0.40 g g^{-1}, while xylitol yields was 0.45 g g^{-1}. Comparatively, YADAV et al., 2011, reported that co-culture with *S. stipitis* NCIM 3498 and *C. shehatae* NCIM 3501 in shake flasks with varying concentrations of xylose (1 to 6 %), at 30 °C, 150 rpm for 48 h obtained yields of 0.40 g g^{-1} for the highest xylose concentration. CHANDEL et al., 2011, investigated the metabolism of *S. stipitis* on synthetic medium to simulate wild-sugarcane bagasse hydrolysate with a complex mixture of sugars and toxic compounds, including the furan derivatives and acetic acid. They reported ethanol yields 0.44 g g^{-1} for this yeast, while *S. cerevisiae* VS3 (control) achieved yields of only 0.22 g g^{-1}.

C. shehatae was able to grow in the RHH, where the presence of furan derivatives (0.23 g L^{-1}) and acetic acid (1.3 g L^{-1}) could disrupt its metabolism (Figure 1B). Most of glucose and xylose, and a smaller amount of arabinose were consumed, with ethanol and xylitol yields of 0.40 g g^{-1} and 0.16 g g^{-1} obtained, respectively. Apparently, the presence of toxic compounds did not affect ethanol production, but was strongly negative for xylose conversion into xylitol. Similar results were reported by MUSSATTO et al., 2005, for *C. guilliermondii* grown on hydrolysed brewers spent

grain without detoxification. SAMPAIO et al., 1997, tested the influence of toxic compounds on xylose-to-xylitol bioconversion by *D. hansenii* UFV-170, with a set of experiments performed on semi-synthetic medium. They reported that xylitol and arabitol productions were negatively affected by furfural, not dependable to its concentration. WAHLBOM and HAHN-HÄGERDAL 2002, reported that during xylose fermentation, xylitol excretion decreased after addition of furfural, possibly because NADH was oxidized to NAD+ during its reduction to furfuryl alcohol, suggesting that furfural present in lignocellulosic hydrolysates could be beneficial for xylose fermentation to ethanol. These authors then postulated that HMF, which requires NADPH for reduction, did not affect xylitol excretion.

Using similar substrate as in this work (rice straw and hulls), SILVA et al., (2012), used non-detoxified rice straw hydrolysate in shaker cultures of *S. stipitis* reporting ethanol yields of 0.37 g g^{-1}, while SAHA et al., (2005) obtained ethanol yields of 0.43 g g^{-1} under micro-aerobiosis cultivation of a recombinant ethanogenic *Escherichia coli* (FBR 5) strain in RHH.

In order to understand the conversion kinetics of both xylose and glucose into ethanol by co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* in $G_{20}X_{20}A_{10}$ and RHH, shaker flask cultivations were set up and results are shown in Figures 1C and 1D, respectively. In the co-culture, the same CCR profile observed for cultures of *C. shehatae* was in place, with xylose being consumed after the complete depletion of glucose. Interestingly, arabinose was not metabolized in the co-cultures. Several yeasts can utilize arabinose as a carbon and energy sources, but most of them are unable to ferment it into ethanol (WISSELINK, et al., 2007). ROBERTO et al., 1994, investigated the metabolism of *C. guilliermondii* in three different synthetic media containing xylose, glucose, and arabinose made up to simulate the compositions of sugarcane

bagasse and rice straw hydrolysates and reported that arabinose was poorly metabolized in all simulations. MUSSATTO et al., 2006, suggested that many microorganisms, including ethanogenic yeasts, are able to regenerate co-factors necessary for the conversion of arabinose to xylulose, therefore producing xylitol from this pentose. In this work, values for $Y_{P/S}$ (ethanol) are similar in both cultivations. However, $Y_{X/X}$ (xylitol) was higher in the *C. shehatae* cultivation than in the co-culture of yeasts, suggesting that arabinose was also partially converted into xylitol (Table 2). This remarkable behaviour concerning the metabolism of *C. shehatae* towards arabinose was similarly observed for other yeasts, suggesting the existence of CCR related to this sugar. MUSSATTO et al., 2006 reported that in cultures of *C. guilliermondii* FTI 20037 a concentration of xylose two times higher, or glucose ten times higher, than that of arabinose were enough to completely repress the uptake of the later, by inhibiting the action of enzymes involved on its metabolism. The kinetics shown in Figures 1 A and C for *C. shehatae* strongly support the same behaviour for this yeast. Surprisingly, higher yields of ethanol were obtained in RHH (0.51 g g^{-1}) with the co-culture than in the synthetic medium (0.42 g g^{-1}) and for the isolated cultures of *C. shehatae* or *S. cerevisiae*, seem above. Similar behaviour was observed for *S. stipitis* grown in either synthetic medium with only glucose or xylose, and in rice straw hydrolysate (RSH with $G_{17}X_{32}$), with higher ethanol yields in the RSH (CHEN et al., 2012). CHANDEL et al., 2011, studied the co-cultures of *S. stipitis* NCIM 3498 and thermotolerant *S. cerevisiae*-VS3 in both sugarcane bagasse hydrolysate and synthetic medium, with ethanol yields of 0.48 g g^{-1} and 0.49 g g^{-1}, respectively. Figures 1C and 1D also show that small amounts of xylitol were produced in both media, with yields of 0.20 and 0.13 g g^{-1} in synthetic medium and RHH respectively. The low production of xylitol most certainly is reflecting that culture conditions, especially oxygen concentration throughout

cultivation, were not optimized to xylose conversion into xylitol, which requires tight controls, not possible to attain in shaker flask.

3.3. Kinetics of bioreactor cultivations of co-cultures of *S. cerevisiae* and *C. shehatae* under anaerobiosis and oxygen limitation conditions

The efficiency of a bioprocess is affected by medium composition and operational conditions used. The oxygen supply is one of the most important environmental factors in xylose fermentation by yeasts, affecting both the rates and the yields of xylitol and ethanol accumulation (DU PREEZ, 1994). In this research, two oxygen conditions (anaerobiosis and oxygen limitation) were analysed in bioreactor co-cultures of *S. cerevisiae* and *C. shehatae* growing in $G_{30}X_{15}A_5$ and in RHH as shown in Figure 2. Kinetic parameters are presented in Table 3 in comparison with the other cultivations. Under oxygen limitation, all sugars were metabolized in a CCR-positive profile, including arabinose, which was not used by cells in shaker co-cultures. Carbon catabolite repression can limit the industrial application of co-cultures with xylose-fermenting yeasts, because ethanol produced from glucose may inhibit xylose fermentation (CHEN, 2011). In RHH, ethanol yields were 0.44 g g^{-1} and 0.48 g g^{-1} under oxygen limitation and anaerobiosis, respectively, while in synthetic medium these values were up to 0.51 g g^{-1} and 0.50 g g^{-1}. These results compare well with other reports on the literature. For instance, FU and PEIRIS 2008, reported overall ethanol yields of 0.33 g g^{-1} for the total amount of sugars (0.49 g g^{-1} in the glucose fermentation stage, and 0.17 g g^{-1} in xylose fermentation stage) by a co-culture of *Pachysolen tannophilus* and *Zymomonas mobilis*, using a synthetic medium with 60 g L^{-1} glucose and 40 g L^{-1} xylose as carbon sources, and different conditions of aeration in the glucose

and xylose fermentation stages. GUTIÉRREZ-RIVERA et al., 2011, reported ethanol yields of 0.46 g g^{-1}, under oxygen limitation, and 0.20 g g^{-1} under anaerobic conditions for bioreactor co-cultures of *S. cerevisiae* and *S. stipitis* in synthetic medium containing glucose and xylose.

Concerning xylitol, yields of 0.24 g g^{-1} in synthetic medium and 0.11 g g^{-1} in RHH under oxygen limitation were obtained, while xylitol was not detected under anaerobiosis (Table 2). Xylose is reduced to xylitol using - preferentially or exclusively - NADPH, which is then oxidized to xylulose in a strictly NAD$^+$-dependent manner. The two steps use different redox factors, leading to the accumulation of NADH that cannot be recycled under anaerobiosis, thus inducing the accumulation of xylitol (HOU et al., 2009). PENG et al., 2012, investigated the metabolism of recombinant *S. cerevisiae* BSPX021 expressing xylose reductase–xylitol dehydrogenase (XR–XDH), in oxygen-limited shake flask cultivation with glucose and xylose. The authors reported xylitol yields of 0.27 g g^{-1}, similar to values obtained in this work in synthetic medium. WINKELHAUSEN et al., 2004, growing *C. boidini* in synthetic medium free of inhibitory compounds, and with five times higher xylose concentrations than used in this work, reported xylitol yields of 0.16 g g^{-1}. SCHIRMER-MICHEL et al., 2008 using soybean hull hydrolysate, with *C. guilliermondii* under oxygen limitation, reported xylitol yields of 0.22 g g^{-1}, with the formation of glycerol as a by-product (4.5 g L^{-1}). In this work, only 1.15 g L^{-1} of glycerol was detected. VAN MARIS et al., 2007, demonstrated that under anaerobic conditions, reoxidation of excess NADH could be accomplished via the production of compounds that are more reduced than xylose, such as xylitol and/or glycerol. Glycerol production is a well-known redox sink during hexose fermentation, especially under anaerobic conditions. Since there was low

glycerol formation, it might be suggested that NADH was preferentially shuttled into xylitol formation in the co-culture used in this work.

4. CONCLUSION

It was demonstrated the possibility of using RHH as a substrate for ethanol production by co-cultures of *S. cerevisiae* and *C. shehatae*, which proved to be an efficient converter of hexoses and pentoses to ethanol. The co-culture was effective to simultaneously convert glucose and xylose, maximizing substrate utilization rates, increasing ethanol yields and production rates. Bioconversion of hexoses and pentoses can be influenced by the rate of oxygenation and furanic inhibitors in the medium. Further studies are granted in order to optimize cultures of *C. shehatae* in co-cultures with other ethanogenic microorganisms, under different oxygen conditions, especially on lignocellulosic hydrolysates.

ACKNOWLEDGEMENTS

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Table 1. Comparison of rice hulls broad composition used in this work with other residues and other compositions of the same reported in the literature.

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Brewers spent grain	17.0	28.0	23.0	MUSSATTO and ROBERTO, 2006
Soybean hull	38.4	10.2	2.8	MIELENZ et al., 2009
Rice hull	35	12	15	SAHA et al., 2005
Rice hull	34	13	29	This work

Table 2. Kinetic parameters obtained for pure cultures of *C. shehatae* HM 52.2, and for the co-cultivations of *C. shehatae* HM 52.2 and *S. cerevisiae* ICV 254D in synthetic medium and rice hull hydrolysate (RHH).

Yeast	Conditions		$G_{20}X_{20}A_{10}$		$G_{30}X_{15}A_5$		RHH	
			$Y_{P/S}$ (g g^{-1})	$Y_{X/X}$ (g g^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/X}$ (g g^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/X}$ (g g^{-1})
C. shehatae	Orbital shaker		0.40	0.45	-	-	0.40	0.13
S. cerevisiae + *C. shehatae*			0.42	0.20	-	-	0.51	0.13
S. cerevisiae + *C. shehatae*	Bioreactor	Anaerobic	-	-	0.50	0	0.48	0
		Oxygen Limitation	-	-	0.51	0.24	0.44	0.11

$Y_{P/S}$: ethanol coefficient yield (g ethanol per g total sugar consumed);
$Y_{X/X}$: xylitol coefficient yield (g xylitol per g xylose consumed).
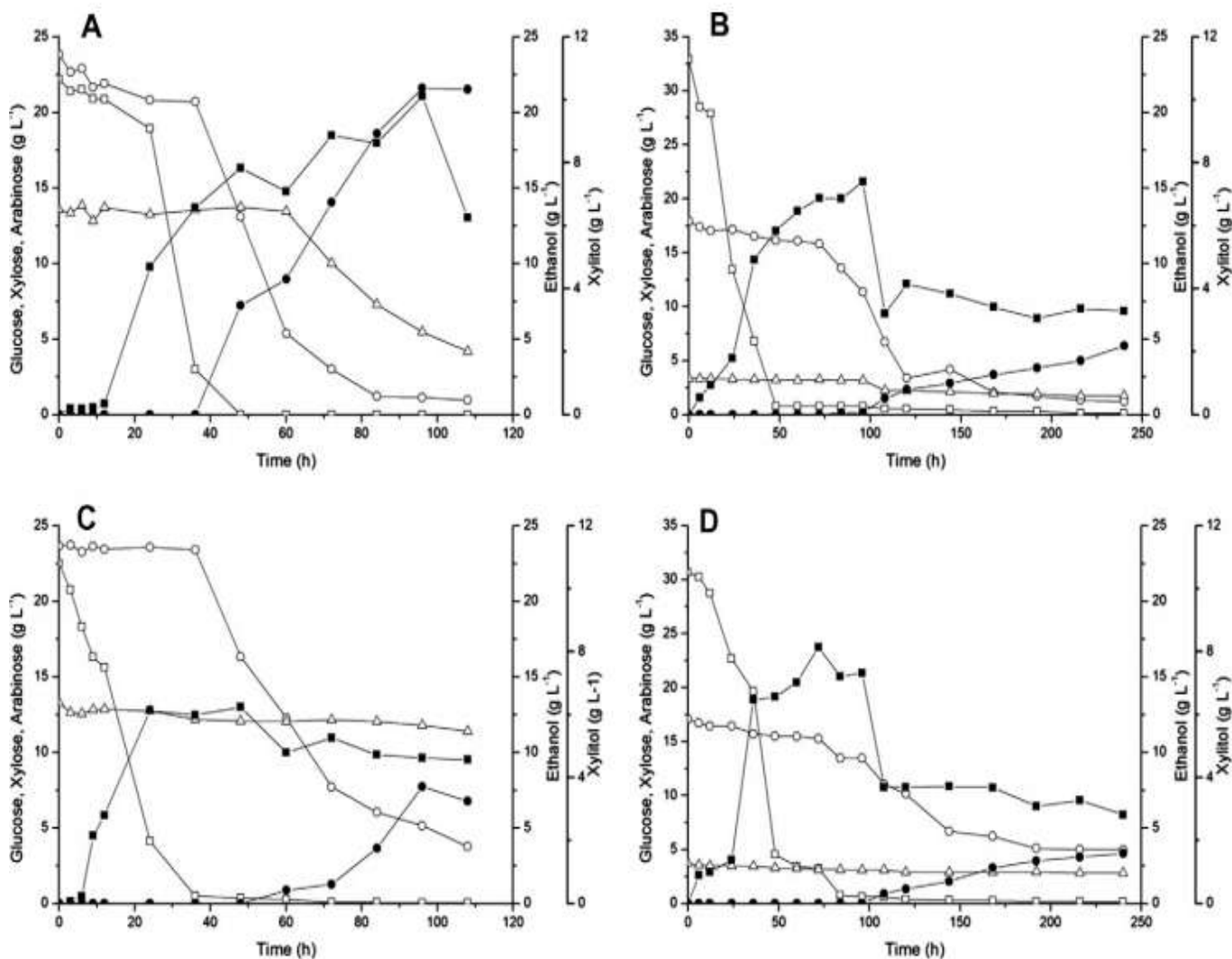
Figure 1. Shake flask kinetics of substrate consumption, ethanol and xylitol production: (A) *C. shehatae* HM 52.2 cultivated in synthetic medium, and (B) in rice hull hydrolysate (RHH); (C) Co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* HM 52.2 cultivated in synthetic medium, and (D) in rice hull hydrolysate (RHH). Glucose (□); xylose (○); arabinose (△); ethanol (▪); and xylitol (●). Results represent the mean of triplicates.
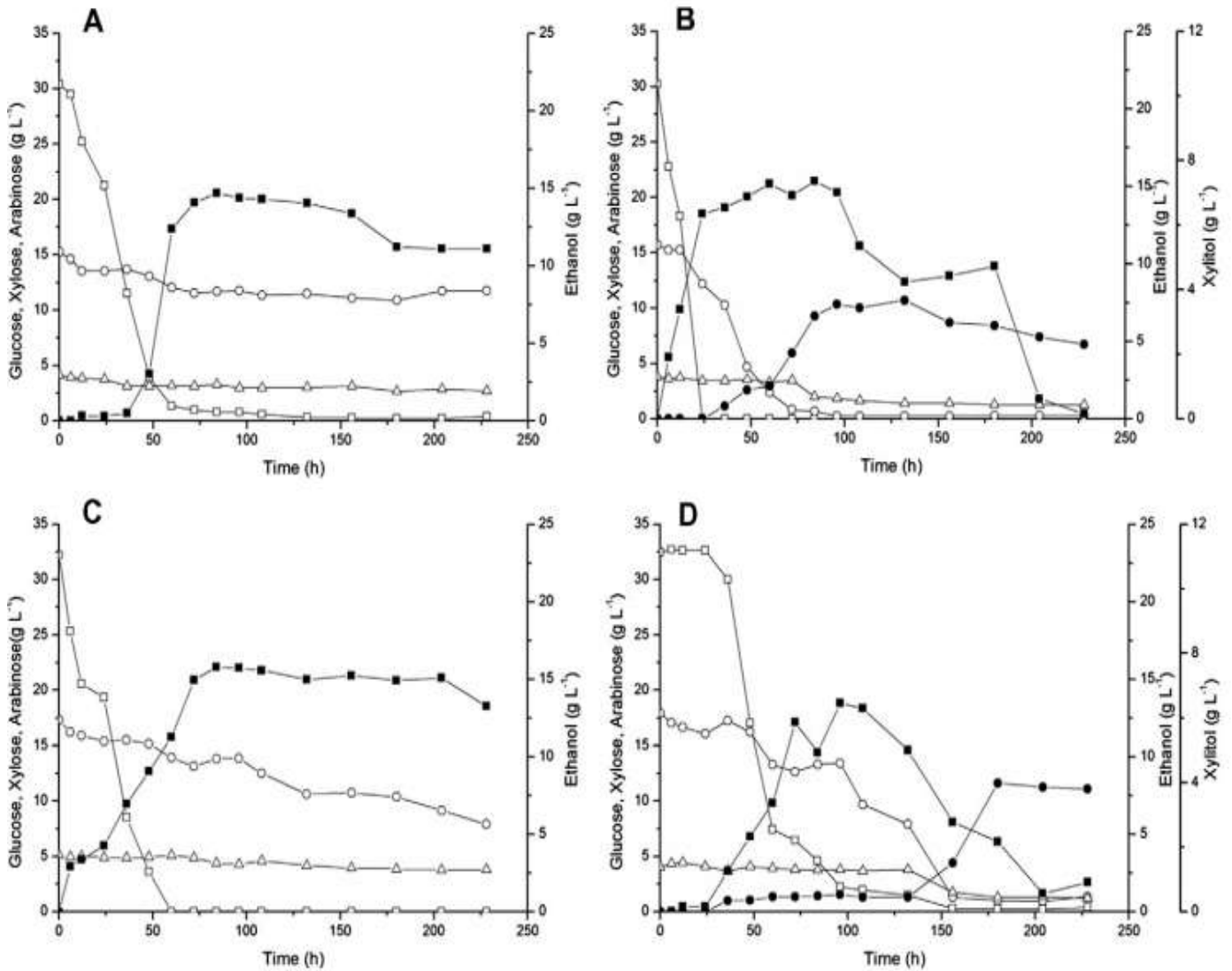
Figure 2. Bioreactor kinetics of substrate consumption, ethanol and xylitol production of co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* HM 52.2 cultivated in (A) synthetic medium under anaerobiosis, and (B) under oxygen limitation; (C) in rice hull hydrolysate (RHH) under anaerobiosis, and (D) under oxygen limitation. Glucose (□); xylose (○); arabinose (△); ethanol (▪); and xylitol (●). Results represent the mean of duplicates.

4.2. Resultados II

'Bioconversion of sugars liberated from soybean hull hydrolysates into fuel ethanol by *Candida guilliermondii* BL 13.'

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Este artigo consiste no estudo da otimização da formulação do meio de cultivo e condições operacionais deste através de Plackett-Burman e planejamento fatorial composto central, respectivamente. A partir dos dados obtidos nos planejamentos relatados acima, também foi avaliada a capacidade da *Candida guilliermondii* BL 13 converter os açúcares contidos no hidrolisado ácido-enzimático de casca de soja em etanol. Este artigo foi submetido à revista Biochemical Engineering Journal.

Fermentation of hexoses and pentoses from hydrolyzed soybean hull into ethanol by *Candida guilliermondii* BL 13.

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Abstract

We investigated the ability of a recently isolated strain of *Candida guilliermondii* to convert hexoses and pentoses obtained from acid-enzymatic soybean hull hydrolysates into ethanol. Operational conditions and media formulation were optimized to ethanol production using statistical experimental designs (Plackett-Burman and CCD). Results showed that *C. guilliermondii* BL 13 was capable of growing in non-supplemented, non-detoxified hydrolysates, and the best culture conditions were determined to be 28 °C, pH 5.0, and 10^9 CFU mL^{-1} inoculum size, respectively. Ethanol productivity peaked at 1.4 g L^{-1} h^{-1} and yields of 0.41 g g^{-1}, about 80.4 % of the expected theoretical yields, were observed. Under the experimental conditions of this work, small amounts of xylitol were also produced. These results suggest that *C. guilliermondii* BL13 is potentially useful for applications in second-generation ethanol production from lignocellulosic biomass.

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1. INTRODUCTION

Lignocellulose biomass materials represent an abundant and renewable source of carbohydrates that can be used for the production of chemical specialties and biofuels of high-added value through biochemical processes, being a promising alternative to oil-based products [1]. Although the production of ethanol from sugary substrates such as sugarcane, in Brazil, or maize starch in the USA, is economically sound and have been the major sources of ethanol, the great bulk of biomass consists of cellulose, hemicellulose, and lignin, the main components of agro-industrial residues and their byproducts [2]. The use of these residues for the production of ethanol might become a promising technology, which could contribute to reduce the negative environmental impacts of fossil fuels and the competition of food and fuel for arable land [3]. To these aims, however, compatible costs of biomass preparation (i. e., the liberation of sugars from the lignocellulosic material), and the use of yeast strains capable of converting both hexoses and pentoses into ethanol, are yet to be met by research.

Soybean (*Glycine max*) is the worldwide most cultivated plant, with the United States of America and Brazil as the main producers, representing 35 % and 27 % of the global production share, respectively [4]. Grain hulls represent the major by-product of the soybean processing industry, representing approximately 8 % to 10 % (mass fraction) of the whole seed [5]. The insoluble carbohydrate fraction of soybean hull cell walls consists of 27 % of hemicellulose [6]. In a typical hydrolysis process, the hemicellulose is depolymerized into pentoses (predominantly xylose), whereas cellulose is breakdown into hexoses, mainly glucose [7].

Considering that hemicellulose is the second most abundant polymer in lignocellulosic materials, the conversion of biomass to ethanol only becomes economically viable if both pentoses and hexoses are converted into this alcohol. This conversion depends on the ability of microorganisms to ferment the different sugars resulting from hydrolysis. Pentoses cannot be fermented by wild-type strains of *Saccharomyces cerevisiae*, the most extensively used microorganism for ethanol production, whereas yeasts such as *Scheffersomyces (Pichia)*, *Candida*, and *Pachysolen* have been used for the xylose conversion [8, 9]. Alternatively, other alcohols such as xylitol and butanol can be biotechnologically produced by some of these yeasts. Xylitol is a natural five-carbon alcohol that has been highly valued for food and pharmaceutical applications because of its sweetening power, as a dental cavities reducer, and as insulin-independent carbohydrate source for diabetics-patients support [10]. However, the production of xylitol by yeasts is highly depended on several parameters, such as the type of biomass used, the hydrolysis conditions, and fermentation conditions such as pH, substrate concentration, size of inoculum, and medium composition [11].

Based on these considerations, the present work aimed at the evaluating the biotechnological bioconversion of sugars liberated from soybean hull hydrolysate (SHH) into fuel ethanol by a recently isolated strain of *Candida guilliermondii* (also known as *Meyerozyma guilliermondii*), which has never been used in bioprocesses before. Initially, a Plackett-Burman design was used to evaluate the effect of SHH nutrients supplementation (peptone, yeast extract, corn steep liquor, and Tween 80) on ethanol production. Following that, a central composite design (CCD) was elaborated in order to improve the fermentation conditions (temperature, pH, and inoculum size), and the results were analyzed using the response surface methodology. Xylitol, as secondary by-product of the fermentation, was also analyzed, but its production was not optimized.

To our knowledge, this is the first report on the literature describing the optimization of fermentation conditions for ethanol production using a *C. guilliermondii* strain on a lignocellulosic biomass hydrolysate.

2. MATERIALS AND METHODS

2.1. Microorganism and cell maintenance

Candida guilliermondii BL 13 was used in this research. This yeast was isolated from environment-discharged piles of rotten rice hulls. The isolated strain was identified comparing the ITS1 and ITS4 amplicon sequences with GenBank databases (access number JQ425356.1). Stock cultures were maintained on YM agar containing (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; agar, 20. For long-term stocks storage, cells were kept frozen at -20 °C in 20 % glycerol (volume fraction) and 80 % of culture medium.

2.2. Soybean hull hydrolysate preparation

The Solae Company kindly supplied the soybean hulls (Esteio, Brazil). The hulls were milled to a particle size less than 1 mm in diameter and the hydrolysate (SHH) was obtained by acid diluted hydrolysis of this material in an autoclave (Phoenix, Brazil) at 122 °C, in a solution of 1 % (volume fraction) concentrated sulfuric acid, solid-liquid proportion (mass fraction) of 1.0:8.8 during 40 min reaction time. This procedure was based on a previously published experimental design [6]. After the hydrolysis, the liquid and solid fractions were separated by filtration and the liquid part was vacuum-concentrated at 60 °C in order to increase its final sugar concentration. The pH was adjusted to 5.0 with NaOH. The solid fraction was washed with tap water to neutral pH.

Enzymatic hydrolysis of cellulose present in this solid fraction was performed using a cellulolytic enzyme complex produced by *Penicillium echinulatum* strain S1M29, which was obtained from the mutant strain 9A02S1 [12]. The enzymatic hydrolysis was carried out at a solid-liquid ratio (mass fraction, dry matter) of 1:20 in citrate phosphate buffer (pH 4.8) and 15 FPU g^{-1} dry matter of enzyme, incubated on an orbital shaker at 120 rpm, 50 ℃ for 72 h [13]. Both fractions of hydrolysates were mixed, autoclaved at 0.5 atm for 30 min and then vacuum-concentrated at 70 °C in order to increase the sugar concentration to the following final values (in g L^{-1}): glucose, 42; xylose, 15; arabinose, 6; and cellobiose, 7. The amount of toxic compounds (inhibitors of microbial growth), formed during hydrolysis, in the final soybean hull acid-enzymatic hydrolysate (SH-AEH), was determined by HPLC analysis and had the following composition (in g L^{-1}): HMF, 0.58; furfural, 0.08; acetic acid, 2.1. The pH was adjusted to 5.0 with solid pastilles of NaOH. Both hydrolysates (SHH and SH-AEH) were used in the fermentations without any detoxification (removal of furans and acetic acid), apart from the loss during the final sterilization in the autoclave.

2.3. Inocula preparation and fermentation conditions in orbital shaker

Pre-inocula were prepared by seeding a loopful of yeast cells (one isolated colony) from plates into 250 mL Erlenmeyer flasks containing 50 mL of synthetic medium composed of (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10. Cultivations were carried out in an orbital shaker (Marconi MA 830, Brazil) at 180 rpm, 28 °C for 24 h and cells were subsequently recovered by centrifugation (3 000 *g*, 15 min). The cell pellet was washed with sterile distilled water, resuspended in culture medium and inoculated into culture flasks (10 % volume fraction).

The experiments using SHH as substrate were carried out in 250 mL Erlenmeyer flasks stoppered using cotton-wool plugs, containing 60 mL of SHH and incubated in an orbital shaker at 180 rpm for 72 h, with the temperature varying according to the CCD. Samples were collected during cultivation to determine biomass, ethanol, glycerol, and residual sugars concentrations in the broth. The experiments using SH-AEH as substrate were carried in 500 mL Erlenmeyer flasks, filled with 120 mL of this hydrolysate, and incubated in an orbital shaker under the best conditions obtained in the CCD (2.5).

2.4. Plackett-Burman design

Plackett-Burman (PB) design was used to screen and evaluate the effects of four complex nutrients (peptone, yeast extract, corn steep liquor, and Tween 80) on the bioconversion of SHH into ethanol by *C. guilliermondii* BL 13. The PB design consisted of eight fermentations plus three replicates at central point to evaluate the reproducibility of the experimental procedure. The variables were chosen because they would represent the addition of a nitrogen source (peptone); growth cofactors (yeast extract and corn steep liquor, which is an inexpensive nutrient source); and a source of unsaturated fatty acids (tween 80) in order to avoid physiological impairments due to oxygen limitations. For each variable, the presence (+1) and absence (-1) levels of the component were tested (Table 1). Student's t-test was performed to determine the significance of each variable. Significant positive effects were considered when the reported p-values were lower than 0.05.

2.5. Central composite design

A 2^3 full factorial central composite design (CCD), with four replicates at the central point and six axial points resulting in a total of eighteen experiments, was used to investigate the fermentation conditions of temperature, pH, and inoculum size for ethanol production by *C. guilliermondii* BL 13 on SHH. The levels of the independent variables of the CCD are presented in Table 2, and were chosen based on the literature [14, 15, 16]. Experimental data were analyzed by the response surface regression procedure. This method is based upon the use of a polynomial model represented by equation (1), to calculate the predicted response, which includes all interaction terms:

$$Y = \beta_0 + \sum \beta_i X_i + \sum_{i \neq j} \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \tag{1}$$

Where Y is the response variable, b_0 the constant, β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the coded level of the independent variables. The above quadratic equation was used to plot surfaces for all variables.

2.6. Fermentation using acid-enzymatic SHH

In order to improve the conversion of total sugars present in the soybean hull into ethanol, an acid-enzymatic hydrolysate (SH-AEH) was used for fermentations under the conditions that were optimized in the CCD. The experimental procedure was identical as the cultivations described in 2.3, except for the medium, which was SH-AEH (composition defined in 2.2). These experiments were conducted in triplicates.

2.7. Analytical methods

Glucose, xylose, arabinose, ethanol, xylitol, and glycerol concentrations were determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 x 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 µL. Furfural and HMF were determined by HPLC using an UV detector (at 276 nm) and a Nucleosil C18 column (250 x 4.6 mm) at 24 °C, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1}.

Biomass was estimated as viable cells, using CFU (colony forming units) plated in yeast morphology agar (YMA) medium. Results were statistically evaluated using the Statistica 7.0 software.

2.8. Calculation of fermentation parameters

Ethanol conversion yields ($Y_{P/S}$) was calculated as the ratio between the highest ethanol concentration produced and the sugar consumed (difference between the initial and residual sugar concentrations). Ethanol productivity (Q_P) was defined as the ratio between final ethanol concentration and total fermentation time.

3. RESULTS AND DISCUSSION

3.1. Analysis of supplementation requirements using PB design

The components yeast extract, corn steep liquor, peptone, and Tween 80 were screened at the confidence level of 90 % based on their effects. All coefficients

exhibited high *p*-values for ethanol yield and productivity (Table 3), implying that they had no significant effects on ethanol production, productivity, and yields.

Therefore, these results suggest that essential nutrients are already present in SHH as this substrate is rich in nitrogen and has several minerals in its chemical composition [6], fulfilling the requirements of the yeast metabolism. Thus, the addition of these nutrients could be eliminated for the subsequent experiments, at the same time showing that the use of SHH is interesting for fermentation processes because it does not require expensive supplementations.

3.2. Optimization of fermentation conditions

A central composite design was carried out to evaluate the effect of temperature, pH, and inoculum size on ethanol productivity and yields during the fermentation of SHH by *C. guilliermondii* BL 13.The models for ethanol productivity and yields were predicted using the analysis of variance (ANOVA) aiming at the lowest *p*-values for the regression model, shown in Table 4. First, a quadratic model was estimated and the less significant values were removed in order to obtain the lowest *p*-values for the regression model. The data provided by the model equations indicates that 74.20 % (R^2=0.7420) of the ethanol productivity, and 74.22% (R^2=0.7422) of ethanol yields can be explained by the model, showing that the model provides good predictions of the experimental results. It was observed that the model is highly significant, both for ethanol productivity (*p*=0.003) and for yields (*p*=0.000004).

After removing the less significant effects, the model for ethanol productivity was found to be linear, thus the effect of inoculum size can be easily investigated. The model suggests that, within the chosen range, ethanol productivity is independent of pH

and temperature. Figure 1 shows the response surfaces described by the model (Table 4) to estimate ethanol productivity based on the independent variables temperature (x_2) and inoculum size (x_3).

Laopaiboon et al. [17], studied the effects of initial cell concentrations (10^6, 10^7 and 10^8 CFU mL^{-1}) on ethanol production in batch fermentations of *S. cerevisiae* in sweet sorghum juice. They reported that, although the final ethanol concentration would remain the same, there was a faster substrate consumption and increased ethanol productivity proportionally to higher initial cell concentrations.

Figure 2 show the response surfaces obtained for ethanol yields described by the model shown in Table 4. *C. guilliermondii* BL 13 produced higher yields at pH below 4.5. The individual effects of the initial pH have been well documented in the literature for some strains of *C. guilliermondii* for xylitol production [18, 19], but not for ethanol. Reports in the literature describing the use of *C. guilliermondii* to produce ethanol on soybean hull hydrolysate as substrate are scarce, making difficult to compare our results against those of other researchers. Comparatively, regarding the variable temperature, Phisalaphong et al. [20], studied the effect of it on ethanol fermentation by *S. cerevisiae* M30. They reported that increased ethanol yields were obtained when temperatures were increased from until the optimal of 30 ^{o}C, with higher values having a negative effect on cell metabolism and ethanol production.

The interaction between temperature and inoculum concentration suggests that, at low inoculum size, higher temperatures have higher positive response and, inversely, increasing inoculum size, lower temperatures are significantly better for ethanol yields. For instance, Silva and Roberto [14] studied the combined effects of initial xylose concentration and inoculum size on xylitol production by *C. guilliermondii growing* in rice straw hydrolysate. They reported that the optimum xylose concentration and

inoculum size to be 82 and 3 g L^{-1}, respectively. The authors did not comment on ethanol production. Powchinda et al. [21], have demonstrated that for *S. cerevisiae*, up to a critical amount of cells, the increase in inoculum size increases ethanol yields because there is a better utilization of sugars by yeast cells. However, high cell densities can adversely affect mass and energy transfer in culture broths and increase cell-to-cell interactions, negatively affecting metabolism and ethanol production [22, 23].

In this work, the highest ethanol productivity would be dependent of inoculum size (1 x 10^9 CFU mL^{-1}), independently of the pH and temperature, whereas best ethanol yields would be achieved for pH and inocula sizes around their minimal values and temperature between 28 °C and 29 °C. Although larger inoculum sizes would increase ethanol productivity, they would negatively affect ethanol yields, probably because of more energy being channeled for cells maintenance. Similar results were reported by Yamada et al. [24], which evaluated the effect of the initial inoculum concentration on ethanol production from brown rice hydrolysate by cultures of *S. cerevisiae*.

In order to validate the models predicted by the CCD, experiments were carried out in triplicates under the conditions representing the observed maximal response for ethanol productivity, which was 0.24 g L^{-1} h^{-1}, using inoculum size of 1 x 10^9 CFU mL^{-1}, pH 5.0 and temperature of 28 °C. An experimental mean value of 0.21 g L^{-1} h^{-1} ethanol productivity was obtained, close enough to the observed value in the CCD, validating the response model.

Xylitol production by *C. guilliermondii* BL 13 has been reported in several works [10, 14, 25]. However, there are few reports concerning ethanol production by this yeast and none of them performed the optimization of fermentation conditions. This fact reflects the metabolic preference of this yeast to ferment xylose to xylitol instead of ethanol under specific conditions of oxygen limitation. Nevertheless, in this work we

were able to show moderately high ethanol productivities (0.21 g L^{-1} h^{-1}) by *C. guilliermondii* BL 13. Our results compared well with another report for this yeast, for example, *C. guilliermondii* strain NRRL Y-2075 presented ethanol productivities of 0.12 g L^{-1} h^{-1} when cultivated on non-detoxified concentrated SHH containing 0.8 g L^{-1} of glucose and 16 g L^{-1} of xylose on shaker flasks [26].

3.3. Ethanol production under optimal conditions in SH-AEH

Since the results of *C. guilliermondii* BL 13 fermentation of SHH were interesting from the point of view of ethanol production, we decided to test this yeast in a richer medium, containing sugars from the hemicellulose and cellulose fractions of soybean hull, which was named SH-AEH, obtained by the enzymatic and acid hydrolysis of this material (section 2.2). The kinetics of sugar consumption, ethanol, and xylitol production for *C. guilliermondii* BL 13 on SH-AEH are shown in Figure 3. Fermentation of SH-AEH proceeded vigorously during the first 12 h with all glucose consumed with a corresponding ethanol production of 16.8 g L^{-1} and a high ethanol productivity (1.4 g L^{-1} h^{-1}) with yields of 0.41 g g^{-1}. For this cultivation, the xylitol productivity was 0.05 g L^{-1} h^{-1} with yields of conversion of 0.46 g g^{-1}. Because conditions in shaker culture as conducted in this work were under a limited supply of oxygen, it would be expected that produced ethanol should be consumed along with xylose in a diauxic kinetics after glucose depletion, fact clearly observable in Figure 3. Similar fermentation profiles were reported for other yeast species cultivated in biomass hydrolysates [15, 26, 27]. Using *C. guilliermondii* strain FTI 20037 in supplemented enzymatic hydrolysate of the sugarcane bagasse, which was de-lignified and pretreated

by hydrothermal processing, Silva et al. [28] reported an ethanol production of 20.5 g L^{-1} in 28 h, in shaker cultures growing at 200 rpm and 30 ºC.

Roberto et al. [25], evaluated xylitol production by *C. guilliermondii* strain FTI 20037 in rice straw hydrolysate. High initial cell density did not show a positive effect in this bioconversion, and increasing the initial cell density from 0.67 g L^{-1} to 2.41 g L^{-1} had a detrimental effect in the rate of xylose utilization and xylitol accumulation, resulting in xylitol yields of 0.47 g g^{-1} and 51 % efficiency at 72 h of cultivation. These results are consistent with those found in the present work, where xylose was slowly converted into xylitol, even after all glucose had been metabolized, resulting in yields of 0.46 g g^{-1} and conversion efficiency of 50 % at 72 h of culture.

In Table 5, we present a general comparison of data for ethanol production obtained in this work and data obtained using several other agro industrial byproducts or residues, including starchy and sugary biomass. Comparing the experiments of this work with those using starch, sweet sorghum, brown rice, and cassava pulp [17, 24, 33], the results are promising, considering that SH-AEH was not supplemented [17, 24, 33], and because genetically modified or adapted yeast strains were not used [17, 24, 33] in the fermentation. Results for SH-AEH, compared with other lignocellulosic biomass such as corn stalk and stover, soybean hull, rice straw, and sugarcane bagasse [26, 28, 30, 31, 32], show better ethanol volumetric productivity (1.4 g L^{-1} h^{-1}), even when pretreatment and enzymatic hydrolysis were used in combination with inhibitors-adapted or ethanol-tolerant strains (productivities not higher than 0.89 g L^{-1} h^{-1}) [30, 31].

3. CONCLUSION

It was shown the possibility of using SHH and SH-AEH as substrates for ethanol production without the addition of any nutrients, as demonstrated by PB design. Ethanol productivity and yield when using SHH could be improved by optimizing temperature, pH, and inoculum size using CCD. *C. guilliermondii* BL 13 proved to be an efficient converter of hexoses and pentoses to ethanol and, to a lesser extent, xylitol. Using enzymatic hydrolysate under the optimal conditions resulted in higher ethanol productivities. Further studies are granted in order to optimize cultures of *C. guilliermondii* BL 13 in co-cultures with other ethanogenic microorganisms, under different oxygen conditions, especially on lignocellulosic hydrolysates. Results obtained in the present study are promising in terms of product yield and volumetric ethanol productivity for further scaling-up studies of such a process.

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Table 1. Levels of the real and the codified values of independent variables tested in the

Plackett-Burman design.

Independent variables (g L^{-1})	Range of levels		
	-1	0	1
Yeast Extract	0	2.0	4.0
Corn Steep Liquor	0	2.0	4.0
Peptone	0	2.5	5.0
Tween 80	0	0.2	0.4

Table 2. Levels of the real and the codified values of independent variables used in the

central composite design.

Independent Variables	Symbols	-1.68	Range of levels			
			-1	0	1	1.68
pH	x_1	4.16	4.5	5.0	5.5	5.84
Temperature (°C)	x_2	23	25	28	31	33
Inoculum (Log CFU mL^{-1})	x_3	5	6	7	8	9

Table 3. Plackett-Burman effect estimates values of ethanol productivity (Q_P) and yield ($Y_{P/S}$).

Response variable	Factor	Effect	*p*-value
Q_P	Mean/Interaction	0.1373	0.0000
	Yeast Extract	0.0144	0.2887
	Corn Steep Liquor	-0.0036	0.7830
	Peptone	-0.0015	0.9087
	Tween 80	-0.0110	0.4081
$Y_{P/S}$	Mean/Interaction	0.3697	0.0000
	Yeast Extract	0.0483	0.2734
	Corn Steep Liquor	-0.0675	0.1429
	Peptone	0.0125	0.7657
	Tween 80	-0.0181	0.6669

Table 4. Regression coefficients estimated by means of the ANOVA for ethanol productivity in function of temperature (T), pH and inoculum size (I).

Response variable	Source	Coefficient	p-value	Regression		
				p-valeu	F	R^2
Q_P	Mean/Interaction	0.1361	0.0000	0.000004	46.014	0.7420
	Linear I	0.0519	0.000004			
$Y_{P/S}$	Mean/Interaction	0.3281	0.0000	0.003	6.9086	0.7422
	Linear pH	-0.0100	0.1097			
	Linear T	0.0106	0.0952			
	Quadratic T	-0.0264	0.0009			
	Quadratic I	-0.0117	0.0195			
	T x I	-0.0187	0.0297			

Table 5. Comparison of several feedstocks used in fermentation to obtain ethanol.

Feedstock	Pretreatment	Strains	Ethanol concentration (g L^{-1})	Y$_{P/S}$ (g g^{-1})	Q$_P$ (g L^{-1} h^{-1})	Reference
Starch/Sugar						
Sweet sorghum	-	*S. cerevisiae*	100	0.42	1.67	Laopaiboon et al., [17]
Brown rice	-	*S. cerevisiae*	28.8	0.43	1.2	Yamada et al., [24]
Cassava pulp (5 %)	Hydrothermal reaction and enzymatic hydrolysis	*S. cerevisiae* displaying *Rhizopus oryzae* glucoamylase	18.6	0.50	0.77	Kosugi et al., [33]
Lignocellulose						
Soybean hull	Acid and enzymatic hydrolysis	*C. guilliermondii*	16.8	0.41	1.4	This work
Corn stalk	Steam exploded and enzymatic hydrolysis	*P. stipitis*	42.15	0.45	0.89	Yang et al., [31]
Corn stover	Steam exploded and enzymatic hydrolysis	*S. cerevisiae*	43.21	0.47	0.72	Li et al., [30]
Soybean hull	Acid hydrolysis	*C. guilliermondii*	5.78	0.53	0.24	Schirmer-Michel et al., [26]
Rice straw	Acid hydrolysis	*P. stipitis*	18.7	0.37	0.39	Silva et al.,[29]
Sugarcane bagasse	OAFEX and Enzymatic hydrolysis	*C. shehatae* *S. cerevisiae*	4.83 6.6	0.28 0.46	0.20 0.47	Chandel et al., [32]
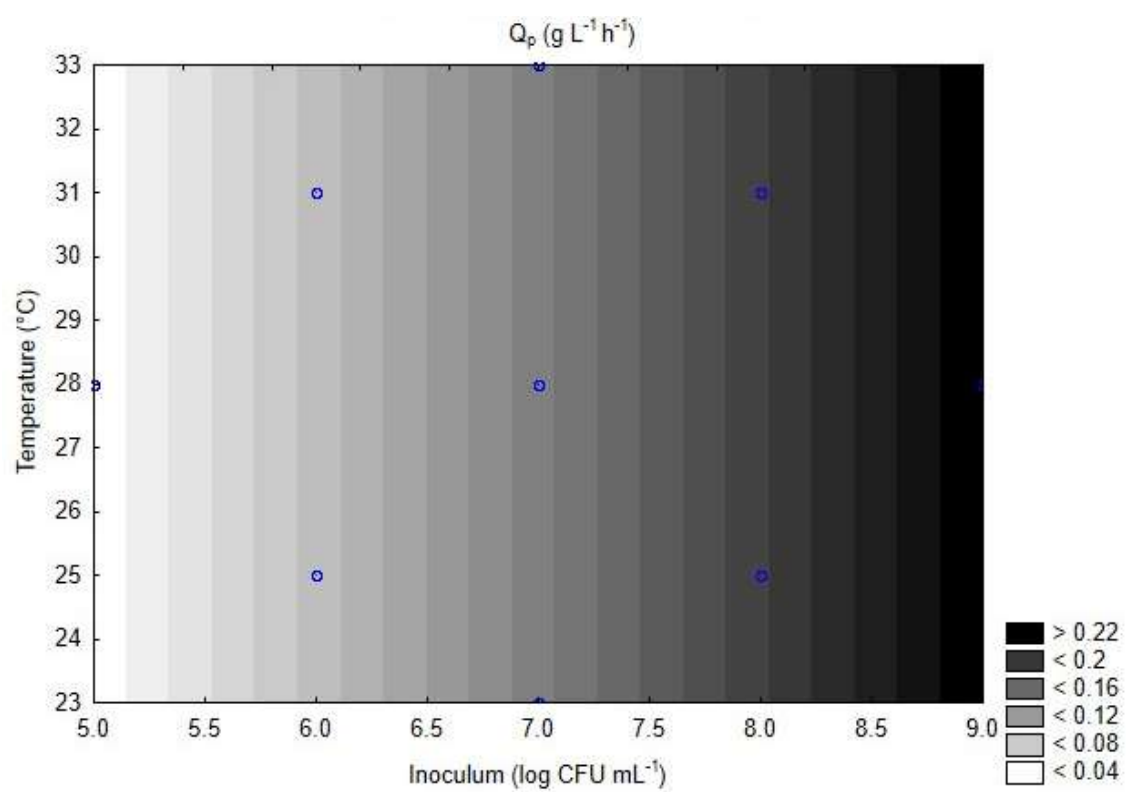
Figure 1. Response surface plots for ethanol productivity (Q_P, g L^{-1} h^{-1}) as function of inoculum size and temperature.
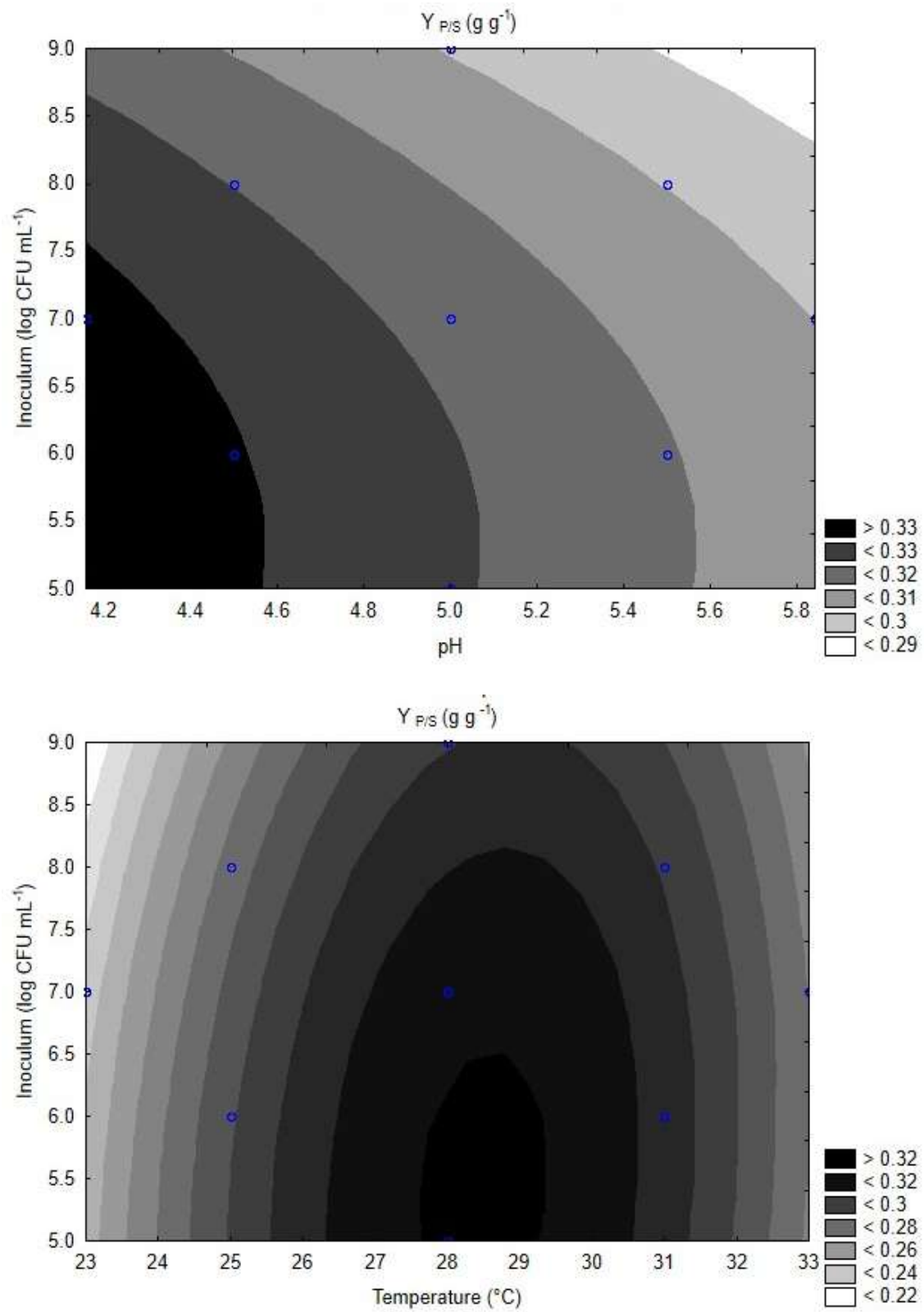
Figure 2. . Response surface plots for ethanol yields ($Y_{P/S}$, g g^{-1}) as function of (A) inoculum size and pH and temperature a (B) inoculum size and temperature.
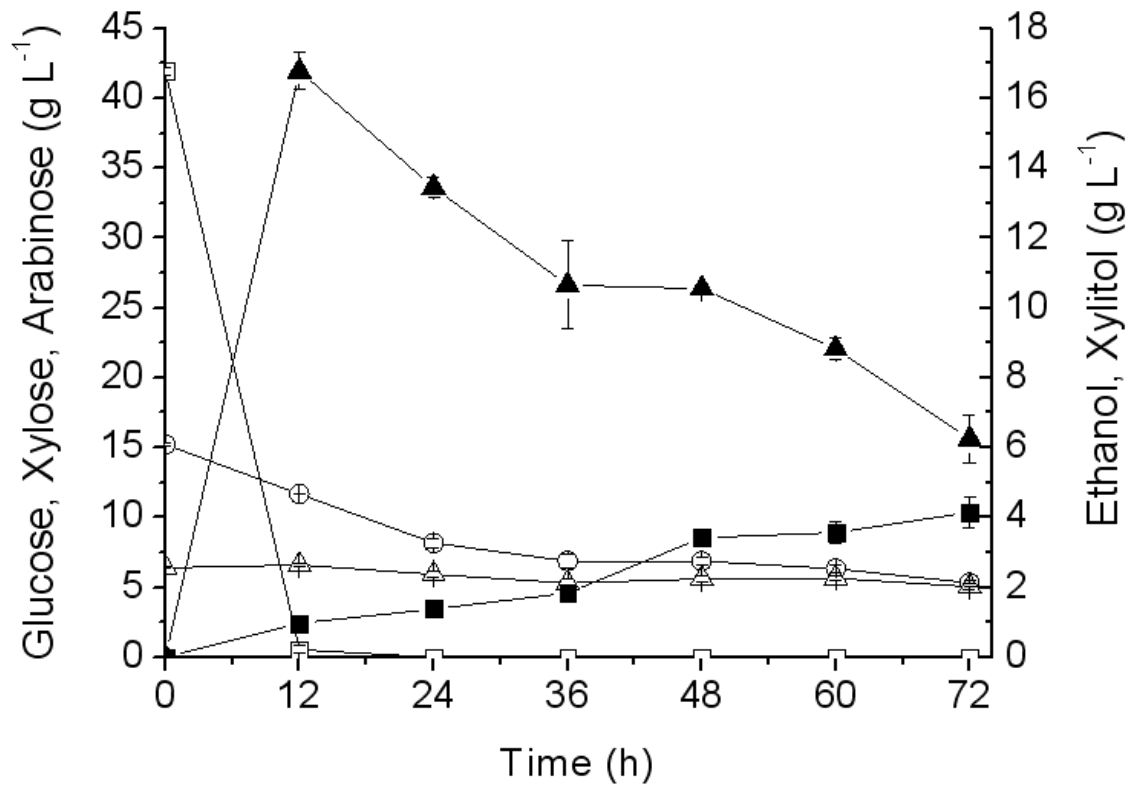
Figure 3. Kinetics of substrate consumption, ethanol and xylitol production by *C. guilliermondii* BL13 cultivated on soybean hull acid-enzymatic hydrolysate. Experiments were carried out in orbital shaker at 28 °C, pH 5.0, 180 rpm. Glucose (□); xylose (○); arabinose (Δ); ethanol (▲); xylitol (■). Results are the mean of triplicates.

4.3. Resultados III

'Liberation of fermentable sugars from soybean hull biomass using ionic liquid 1-butyl-3-methylimidazolium acetate'

Este artigo consiste no estudo da otimização do pré-tratamento de casca de soja utilizando o líquido iônico acetato de 1-butil-3-metilimidazólio ([bmim][Ac]). Neste avaliou-se os efeitos da temperatura, tempo de incubação, carga de sólido (razão casca de soja/LI) e concentração de líquido iônico (mistura LI-água) no pré-tratamento de casca de soja utilizando [bmim][Ac] através de um planejamento composto central. Este artigo foi submetido à revista Biotechnology Progress.

Liberation of fermentable sugars from soybean hull biomass using ionic liquid 1-butyl-3-methylimidazolium acetate and their bioconversion to ethanol

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Abstract

Optimized hydrolysis of lignocellulosic waste biomass is essential to achieve the liberation of sugars to be used in fermentation process. Ionic liquids, a new class of solvents, have been tested in the pretreatment of cellulosic materials to improve the subsequent enzymatic hydrolysis of the biomass. Optimized application of ionic liquids on biomass is important to advance the use of this technology. In this research, we investigated the effects of using 1-butyl-3-methylimidazolium acetate ([bmim][Ac]) on the decomposition of soybean hull, an abundant cellulosic industrial waste. Reaction aspects of temperature, incubation time, ionic liquid concentration, and solid load were optimized before carrying out the enzymatic hydrolysis of this residue to liberate fermentable glucose. Optimal conditions were found to be 75 °C, 165 min incubation time, 57 % (mass fraction) of [bmim][Ac], and 12.5 % solid loading. Pretreated soybean

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hull lost its crystallinity, which eased enzymatic hydrolysis, confirmed by Fourier Transform Infrared (FTIR) analysis. The enzymatic hydrolysis of the biomass using an enzyme complex from *Penicillium echinulatum* liberated 92 % of glucose from the cellulose matrix. The hydrolysate was free of any toxic compounds, such as hydroxymethylfurfural and furfural. The obtained hydrolysate was tested for fermentation using *Candida shehatae* HM 52.2, which was able to convert glucose to ethanol at yields of 0.31. These results suggest the possible use of ionic liquids for the pretreatment of some lignocellulosic waste materials, avoiding the formation of toxic compounds, to be used in second-generation ethanol production and other fermentation processes.

Introduction

Lignocellulosic biomass, in special waste materials from agro-industrial activities, are abundant renewable sources of sugars to be used in the production of biofuels and other bio-products.[1,2] Most common biomass are agricultural and agro-industrial residues, forestry wastes, waste paper, and other energy crops[3]. These materials are formed of cellulose, a crystalline polymer of glucose; hemicellulose, which is a complex polymer of xylose and other sugars; and lignin, a complex phenolic compound.[4]

Soybean is one of the most cultivated crops in the world, with reported annual productions of 285 million tons, of which, around 10 % (mass fraction) is of seed hulls, a final by-product of processing industries.[5] Soybean hull is a lignocellulosic biomass that could find use in biotechnological process, with the advantage of possessing small

amounts of lignin, which eases its saccharification.[6] However, the pretreatment of soybean hull, and other lignocellulosic biomass in general, is required in order to separate lignin and hemicellulose from cellulose, to reduce cellulose crystallinity, and to increase the porosity of the materials, allowing the hydrolysis process to be efficient.[7,8] Some pretreatment methods have demonstrated important drawbacks during the preparation of hydrolysates, such as the generation of toxic compounds (hydroxymethylfurfural, furfural, and aldehydes), which significantly reduce the recovery of sugars during enzymatic hydrolysis. These toxic compounds also disrupt microbial metabolism during the fermentation of released sugars into ethanol and other products.[7] Furthermore, most pretreatment methods are not environmentally sound and safe because they require high-energy inputs, whereas toxic and hazardous wastes are produced that must be properly treated and discharged. Therefore, more efficient pretreatment methods are sought demanding lower energy inputs, using mild physico-chemical conditions, and that could use fully recyclable solvents.[9] Although cellulose cannot be solubilized in water and in many organic solvents, it can be dissolved in certain classes of ionic liquids (ILs).[10]

There has been a growing interest in the use of ILs as solvents for the dissolution of biomass to recover cellulose.[11] Ionic liquids, which are salts characteristically composed of large organic cations and small inorganic anions, are liquids in a wide range of temperatures. ILs are a class of interesting chemicals because they have extremely low vapor pressure, non-flammability, and they are thermally and chemically stable. Therefore, ILs can, in principle, be recycled into the process indefinitely, although technologies to this end have yet to be advanced.[12,13] The scale-up use of IL in biorefineries will depend on the development of efficient recycling processes.[14] For instance, Wu et al.[15] demonstrated that saccharification yields of corn stovers were

unaffected when 1-ethyl-3-methylimidizolium acetate ([emim][Ac]) was used and recycled 10 times. Auxenfans et al. [16] showed the feasibility of recovering and reusing [emim][Ac] up to 7 times in the efficient pretreatment of softwood and hardwood sawdusts, always obtaining similar yields of glucose.

Already tested in chemical processes, ILs exhibited excellent physical characteristics, including the ability to solubilize polar and non-polar organic, inorganic, and polymeric compounds.[3] Some reports on the literature have shown the use of ILs to dissolve cellulose, especially using 1-butyl-3-methylimidazolium chloride.[9,10,12,17] This IL is solid at room temperature, presenting high viscosity when melted, and the chloride ion is corrosive, facts that complicate its processing. On the other hand, ionic liquids in which the anion is acetate, present lower viscosities and melting points, suggesting their possible use for biomass pretreatments.[18]

Brandt et al.[19] demonstrated that the acetate anion-ILs is the most effective for the dissolution of cellulose because of the high β parameter value of the [AC] anion (1.201), compared to [Cl] anion (0.83), or to [DMP] anion (1.118). Solute and substrate hydrogen bonding interactions between biomass and IL are defined by the solvent properties. The Kamlet–Taft parameters separate these properties into polarizability (π*), hydrogen bond donor capacity (α), and hydrogen bond acceptor capacity (β).[20] Shi et al.[21] observed a positive linear correlation between β values and glucose yields when pretreating switch grass with [emim][Ac]–water mixtures. Sun et al.[22] showed that higher β values are associated with greater lignin removal and higher sugar yields when treating switch grass with [emim][Lys].

The dissolution of lignocellulosic biomass using ILs can be explained by the hydrogen bond basicity of the IL anion, β Kamlet-Taft parameter. High hydrogen bond contributes for the high dissolution capacities of ILs because it weakens the inter and

intramolecular hydrogen-binding network of the polymeric chains.[15,19,23] Several other parameters might interfere of the ability of ILs to dissolve lignocellulosic biomass, namely the type and structure of the biomass, the IL/biomass ratio during incubation, temperature and time of incubation, and the water content of the solution mixture.[24]

In this context, the aims of this research were to optimize processing conditions for the pretreatment of soybean hull using the ionic liquid [bmim][Ac]. The effects of temperature, incubation time, solid load (biomass/IL ratio), and ionic liquid concentration (mixture IL-water) were evaluated using central composite design (CCD). In addition, the regenerated biomass was also analyzed by Fourier transform infrared spectroscopy (FTIR). The pretreated biomass was subsequently hydrolyzed using a cellulolytic complex produced by *Penicillium echinulatum*. Sugar liberation was quantified, and this hydrolysate was used to produce ethanol by *Candida shehatae* HM 52.2, an yeast strain *that is capable of converting pentoses and hexoses into ethanol.[25,26]*.

Materials and Methods

Biomass and its composition analysis

Soybean hulls were kindly supplied by The Solae Company (Esteio, RS, Brazil). The material was milled to particle sizes smaller than 1 mm in diameter and stored until further use or analysis.

The composition of soybean hull was determined by submitting the biomass to concentrated sulfuric acid hydrolysis, following methodology described by Dunnig and Dallas.[27] The obtained monosaccharides were quantified by HPLC (item 2.8), and the acid-soluble lignin was estimated by UV spectrophotometry at 280 nm. All determinations were carried out in triplicates.

Ionic Liquid preparation

In order to obtain the ionic liquid 1-butyl-3-methylimidazolium acetate ([bmim][Ac]), we first synthesized 1-butyl-3-methylimidazolium chloride ([bmim][Cl]), which is a precursor of ILs with carboxylate anions, following procedures described in the literature and characterized by ^{1}H NMR (Varian Inova 300 MHz, Agilent 500 MHz).[28,29] Briefly, an anion exchange resin (Amberlite IRA-400 (OH), Supelco) was packed into a column and washed using 1 mol L^{-1} NaOH. The process was monitored by titration using 0.1 mol L^{-1} $AgNO_3$, until AgCl stopped being formed (complete exchange of chloride anions by hydroxide). The column was then ready to receive the aqueous solution of [bmim][Cl] (0.1 mol L^{-1}), which was passed through it in order to exchange chloride anions by hydroxyl. Acetic acid was added to the obtained solution of [bmim], which was then concentrated on a rotary evaporator at 70° C. The final purified [bmim][Ac] was dried under vacuum and vigorous stirring, at 80° C for 48 h. Description of the obtained ILs. [bmim][Ac]: colorless viscous liquid; ^{1}H RMN (500 MHz - $CDCl_3$) d ppm 0.97 (t, J = 7.4 Hz, $3H^9$); 1.33-1.41 (m, $2H^8$); 1.83-1.89 (m, $2H^7$); 1.97 (s, $3H^{12}$); 4.05 (s, $3H^{10}$); 4.29 (t, J = 7.4 Hz, $2H^6$), 7.24 (s, $1H^5$); 7.30 (s, $1H^4$); 11.08 (s, $1H^2$). [bmim][Cl]: white solid; ^{1}H RMN (300 MHz - D_2O) d ppm 0.89 (t, J = 7.4 Hz, $3H^9$); 1.22-1.35 (m, $2H^8$); 1.77-1.87 (m, $2H^7$); 3.86 (s, $3H^{10}$); 4.17 (t, J = 7.2 Hz, $2H^6$); 7.40 (d, J = 2.0 Hz, $1H^5$); 7.45 (d, J = 2.0 Hz, $1H^4$).

Enzymatic complex and yeast strain used in the fermentation

The enzymatic complex used in this study was produced and extracted from cultures of *Penicillium echinulatum* strain S1M29 (DSM 18942), isolated, described,

and characterized by Dillon et al.[30] *This* cellulase complex has been described as an efficient enzymatic preparation for the hydrolysis of lignocellulosic biomass.[30]

Candida shehatae HM 52.2 was used in the fermentation of the liberated sugars. This strain was isolated from rotting wood. This strain was chosen because it can metabolize C-5 and C-6 sugars; it has been previously used for soybean hull acid hydrolysate fermentation, producing good conversions of glucose to ethanol.[26] The yeast was kept frozen at −20 °C in stock cultures of 20 % glycerol (volume fraction) and 80 % of culture medium, which contained (in $g L^{-1}$): yeast extract, 3; malt extract, 3; peptone, and glucose, 5.

Ionic liquid pretreatment of soybean hull and its subsequent enzymatic hydrolysis

The soybean hull pretreatment using [bmim][Ac] was conducted as follows. In a polypropylene tube, 1 g of IL–water mixture (various concentrations of IL) was added to different amounts of soybean hull samples. The tubes were incubated in an oil bath under various temperatures and incubation times, as shown in Table 1, according to the experimental design described below. All experiments were performed under normal atmospheric pressure. After this procedure, 10 mL of deionized water were added to samples serving as anti-solvent in the mixture and to precipitate the cellulose. The resulting mixtures were briefly centrifuged at 7 000 *g*, and the supernatant was removed. The precipitate (treated biomass containing regenerated cellulose) was washed with 10 mL of water (anti-solvent) and centrifuged at 7 000 *g*, procedure that was repeated four times. The treated biomass was then oven-dried (75 °C, 24 h) in the same tube and used for the enzymatic hydrolysis.

The enzymatic hydrolysis of the IL-pretreated biomass was performed using the cellulolytic enzyme complex derived from cultures of *P. echinulatum* strain S1M29.[30]

Enzyme was added to the polypropylene tube containing the dried pretreated biomass, observing a solid-liquid proportion of 1:80 of the dried biomass and the pH adjusted using citrate-phosphate buffer (pH 4.8). The enzymatic solution was standardized to produce an activity of 60 FPU (filter paper unit) g^{-1} dried biomass and the mixture (enzyme plus pretreated biomass) was incubated on a rotatory shaker at 120 rpm, 50 ºC for 48 h. After completion of the reaction, the hydrolysate was centrifuged and the supernatant was then recovered and concentrated prior to be used in the fermentation (see below).

Central composite design

In order to investigate the best conditions of pretreatment of the lignocellulosic material using the IL, a 2^4 full factorial central composite design (CCD) was employed, composed of four replicates at the central point, resulting in 28 experiments. The independent variables were: temperature (X_1), incubation time (X_2), ionic liquid concentration (X_3), and solid (biomass) load (X_4). The levels of the independent variables of the CCD are presented in Table 1. Experimental data were analyzed by multiple regressions using the polynomial model represented by equation (1).

$$Y = \beta_0 + \sum \beta_i X_i + \sum_{i \neq j} \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \qquad (1)$$

Where Y is the response variable, β_0 the independent coefficient and β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the coded variables.

Fourier transform infrared (FTIR) analysis

The FTIR spectra of samples of the soybean hull, untreated and pretreated (regenerated biomass), were determined by Fourier transform infrared (FTIR)

spectroscopy with a Varian 640-IR spectrometer. Samples were analyzed using ATR technique. The spectra were obtained at room temperature with 32 accumulative scans and 4 cm^{-1} of resolution.

Fermentation of liberated sugars

Fermentation of the liberated sugars from hydrolyzed soybean hull was carried out in order to demonstrate that this process would be suitable for treating lignocellulosic biomass intended for second-generation ethanol production. The hydrolysate, obtained as described in 2.4, was vacuum-evaporated at 70 °C to concentrate glucose to 21 g L^{-1}, and was autoclaved at 1 atm for 15 min. The pre-inoculum of *C. shehatae* HM 52.2 was prepared by seeding a loopful of yeast cells (one isolated colony) from plates into 125 mL Erlenmeyer flasks containing 25 mL of synthetic medium composed of (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10. Cultivations were carried out in an orbital shaker at 180 rpm, 30 °C until culture reached mid-exponential growth phase. Cells were then centrifuged at 3 000 *g* for 10 min, the formed pellets were washed with sterile distilled water and resuspended directly into the medium to be used in the fermentation. A 10 % volume of this cell suspension, showing a cell concentration of optical density 1 (OD, 600 nm), was inoculated into the culture flasks. The fermentation was performed in 125 mL Erlenmeyer flasks containing 25 mL of medium (the hydrolysate without any supplementation), which were incubated on rotary shaker at 30 °C, 180 rpm, for 48 h. Biomass was estimated as viable cells, expressed as CFU (colony forming units) plated in yeast morphology agar (YMA) medium.

Analytical methods

Sugars and alcohols concentrations were determined using a HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 x 7.8 mm), using 5 mM sulfuric acid as eluent, at 45 °C, flow rate of 0.6 mL min^{-1}, and sample volumes of 20 μL. Furfural and HMF were determined by HPLC with a UV detector (at 276 nm), using a Nucleosil C18 column (250 x 4.6 mm) at room temperature, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1}, and sample volumes of 20 μL.

Results of the analyses were statistically evaluated using the Statistica ver. 10.0 software.

Calculation of parameters

The yields of glucose recovery from biomass were calculated as being the ratio between the liberated glucose by the enzymatic hydrolysis of the pretreated biomass with ionic liquid and the total amount of glucose in the original cellulosic matrix (untreated soybean hull).

Ethanol yields ($Y_{P/S}$) were calculated as the ratio between the highest ethanol concentration obtained and the amount of sugar consumed (difference between the initial and residual sugar concentrations).

Results and Discussion

Composition of soybean hull

Determination of the composition of the raw soybean hull was necessary in order to calculate the amount of glucose expected to be recovered after the enzymatic hydrolysis of the IL-pretreated material and to compare its composition with other

lignocellulosic biomass. Results of these analyses are shown in Table 2. Results show that soybean hull has a high content of glucose (39 %), followed by xylose (16.5 %), and arabinose (5.3 %). In contrast, this biomass has a very low amount of lignin, as compared to other agro-residues, including sugarcane bagasse, one of the most studied lignocellulosic materials, suggesting a very interesting composition for bioconversion of fermentable sugars into ethanol. Lignin is known to be an obstacle for the efficient bioconversion of polysaccharides from biomass to sugars for fermentation into fuels.[32] Pretreatments that use high temperatures and pressures, phenolic compounds are generated, caused by the partial degradation of lignin.[33] This can lead to the formation of toxic phenolic compounds that show inhibitory action over microbial physiology.[32]

Optimization of the pretreatment conditions using [bmim][Ac]

This is the first report on the possible outcomes of pretreating soybean hull using ILs in order to replace other physico-chemical treatments intended to breakdown the lignocellulosic matrix and to obtain fermentable sugars. Table 1 presents the planned CCD, whereas in Table 3 and in Figure 1 (A to D) are presented the results of the experimental design. The variables were chosen based upon our previous experience on biomass hydrolysis: the temperature of reaction, the time of incubation of biomass in IL, the concentration of [bmim][Ac], and the amount of solid in the reaction. The response was the amount of glucose that could be recovered after the enzymatic hydrolysis of the pretreated biomass. The experimental data were approximated using a quadratic regression model. The non-significant effects were removed from the final model in order to obtain the lowest p-values for the reduced regression model (Table 2). The model was highly significant ($p = 0.0004$), indicating that 73.2 % ($R^2=0.732$) of glucose recovery can be explained by the model, showing that it provides good predictions of

the experimental results. Response surfaces generated from the regression model, for each independent variable, are shown in the Figure 1 A to D.

Literature reports have shown that the increase in temperature would accelerate the rates of lignocellulosic biomass breakdown by ionic liquids.[1,23,36] The mechanism that best explains this effect is the instability caused by temperature on the hydrogen bonds in the cellulose structure.[9] However, high temperatures might result in degradation of carbohydrates, and could modify lignin properties, turning more difficult and costly the recovery of sugars.[9,36] Another concern in using high temperatures is related to energy input, which is a disadvantage for a sustainable and cost-effective process. In our study, the best range of temperature to recover glucose from IL-pretreated soybean hull was found to be between 60 °C and 80 °C (Fig. 1 A and C).

Results depicted in Figure 1 A show the interaction between incubation time and temperature for an efficient pretreatment. At lower temperatures, longer times are required for the pretreatment of soybean hull with [bmim][Ac]. Results suggest that glucose recovery increases with time, up to around 165 min, from where no further gains in sugar liberation are observed (Figures 1 A and B). Poornejad et al.[37] tested [bmim][Ac] for the pretreatment of rice straw and observed that an increase in the incubation time from 60 min to 180 min did not significantly increase glucose recovery (97 % and 99 %, respectively). Tan et al.[34] used [bmim][Cl] for the pretreatment of palm oil frond and reported that glucose recover increased from 26.2 % to 96.7 % as temperature went from 60 °C to 100 °C, under the incubation time of 15 min tested in their work. Yoon et al.[36] pretreated sugarcane bagasse with [emim][Ac] and showed yields of 54.6 % to 66 % of reducing sugars for incubation times increasing from 15 min to 45 min, respectively, at the lower temperature tested (120 °C). However, the authors reported that prolonged incubation times decreased the yields of reducing sugars

at higher temperatures (> 135 °C).[36] The authors observed the formation of coagulates under the combination of long time-high temperatures. Qiu et al.[38] observed the same coagulation process when pretreated cane bagasse with [emim][Ac] at high temperatures (>120°C) and long residence time (>40 min). The increased amount of hydrogen bonds between cellulose and ionic liquid is the probable cause of the coagulation.[38] These coagulates make more difficult the regeneration of the cellulose and may contain residual ionic liquid, even after washing the pretreated biomass. IL residue may cause enzymatic inactivation during the hydrolysis step.[36,39] Although the combination of long incubation time and high temperature during the pretreatment of biomass using ILs will generally enhance the dissolution of them, it will also degrade sugars or the IL being used.[23,34]

The ideal amount of IL to be used in the treatment is of fundamental importance because of the high costs of these chemicals. Another advantage for using the smallest amount of aqueous-IL solutions in the pretreatment of lignocellulosic biomass is the reduced viscosity of solutions, which eases the process operation and the recycling of the mixture.[1,40] The results of the CCD in this work, showed that for [bmim][Ac] and soybean hull, highest glucose recovery was achieved using 55 % to 60 % of [bmim][Ac] in the solution (Figures 1 B and D). Swatloski et al.[17] reported that water impaired the efficiency of IL to solubilize cellulose. In the mixture IL-water, water molecules would compete with IL anions to form hydrogen bonds with cellulose, promoting only a partial dissolution of lignocellulosic biomass, thus the right amount of water must be determined for each system. [17,41] Miscanthus chips were treated with [bmim][MeSO$_4$] using 20 % (mass fraction) of water and the results showed that biomass presented high digestibility after treatment, solubilizing around 80 % of glucose after 8 h at 120 °C.[35] However, when [bmim][MeSO$_4$] was used pure (residual water of only 0.3 %), it was

not possible to enzymatically digest the biomass.[35] Fu and Mazza[40] evaluated the use [emim][Ac], either pure or in aqueous solutions, for the treatment of triticale and wheat straws. The authors reported that the highest cellulose hydrolysis was achieved using 50 % [emim][Ac]-water solution at 150°C, 90 min (sugar yields of 81 % were obtained, compared to only 67 % when the pure IL was used).[40] Similarly, in the optimization of wheat straw treatment using [emim][Ac], 71.4 % of fermentable sugars were recovered when 50.5 % IL solution was employed at 158°C for 3.6 h.[1]

The solid/liquid ratio of IL-mediated treatment of biomass is an important reaction factor related to the access of IL to the cellulose molecules. This will define the cellulose regeneration efficiency, the enzymatic reaction of the treated biomass, the recovery of glucose, and other parameters used during the pretreatment.[24] In the present work, results suggest that the optimum solid amount in the reaction should be between 11 % and 14 % (Figures 1 C and D).

Wu et al.[15] pretreated corn stover with [emim][Ac] at 125 °C for 1 h using a solid/liquid ratio of 33 % (mass fraction) resulting in glucose yields of approximately 80 %. The authors postulated that, for this solid/liquid ratio, the molar ratio of ionic liquid to glucose molecules in the cellulose matrix (6:1) is equivalent to the maximum number of reacting hydrogen bonds. The authors suggested that it is possible to disrupt the crystalline structure of cellulose, which is mediated by sufficient acetate anions forming new hydrogen bonds with each glucose molecule in the cellulose matrix.[15]

Summing-up, we found that the optimum processing condition for glucose recovery from soybean hull was determined to be 75 °C, 165 min of incubation, IL concentration of 57 % (mass fraction), and solid load of 12.5 %. The predicted glucose recovery by the CCD was 98.48 % at these optima conditions and the experimental validation obtained was of 91.7 %, demonstrating good agreement with the model.

Although in our work the aim was to optimize conditions for the recovery of glucose, using cellulolytic enzyme complex form *P. echinulatum* strain S1M29 under the optimum conditions of the treatment, it was possible to recover approximately 76.5 % of the xylose present in the soybean hull biomass (calculated in same way as described for glucose). This is an interesting result, which could be further explored in future works aiming at C-5 recovery for fermentation, especially considering that xylose is the second most abundant sugar in soybean hull.

In Table 4 is presented a comparison of sugar recovery (either glucose or total sugars, sometimes not specified by authors), obtained in pretreatments of different sources of biomass, using ILs and commercial enzymes. Our results compare very well even with those obtained using higher amounts of ILs, showing that it is possible to obtain good yields of sugar (91.7 %) using aqueous solutions of IL and low temperatures. For instance, Fu and Mazza[1], employed CCD to optimize the pretreatment conditions of wheat straw with ionic liquid [emim][Ac] to obtain fermentable sugars. The solid load was fixed at 5 % (mass fraction), and the authors varied temperature (130–170 °C), reaction time (0.5–5.5 h), and the concentration of ionic liquid (0–100 %). The optimum pretreatment conditions obtained were 158 °C, [emim][Ac] concentration of 49.5 %, and reaction time of 3.6 h, allowing for 71.4 % recovery of fermentable sugars present in the wheat straw matrix after enzymatic hydrolysis. Tan et al.[34] optimized the pretreatment of palm biomass using [bmim][Cl], studying the effects of temperature (60-100 °C), reaction time (15-60 min), and solid load (2-10 %), and found optima conditions to be 80 °C, 15 min, and 10 % of solid loading, recovering 100 % of glucose present in lignocellulosic matrix.[34] Yoon et al.[36] employed CCD to optimize the pretreatment of sugarcane bagasse, considered by many researchers one of the most promising and abundant lignocellulosic biomass, using 1-ethyl-3-

methylimidazolium acetate. Similar to other studies, the authors investigated the effects of temperature (120–160 °C), reaction time (15–45 min), and solid load (3–15 %). The optimized condition for the pretreatment was defined as 145 °C, 15 min, and 14 % of solid loading, with sugar yields of 69.7 % after enzymatic hydrolysis. Finally, rice straw was pretreated with [bmim][Ac] at 120 °C for 1, 3, and 5 h with solid loads of 5 %.[37] After pretreatment for 3 h, followed by 72 h of enzymatic hydrolysis, 99 % of glucose was recovered from the biomass matrix.[37]

Our work was the only one where the effect of the four operating conditions (temperature-X_1, incubation time-X_2, IL concentration-X_3, and solid load-X_4) and their interactions was investigated. Their correlations generated the reduced model represented by equation 2, with data shown in Table 3. Concerning the ionic liquid used, [bmim][Ac], it compared well for the recovery of glucose against other ILs, rendering similar yields (see above discussion and Table 4).

$$\begin{aligned} \% \text{ yield of glucose recovery} &= 95.82 + 4.56X_1 - 3.38X_1^2 \\ &+ 6.25X_2 + 4.04X_3 - 4.33X_3^2 + 4.24X_4 - 4.56X_4^2 - 6.38X_1X_2 \end{aligned} \tag{2}$$

Comparison of pretreatment using [bmim][Ac] and other physico-chemical pretreatments

Because most physico-chemical pretreatments of lignocellulosic biomass are based on the use of acids, alkalis, pressure, and heat, we found the need to compare the IL pretreatment with them. Li et al.[11] compared the efficiency of two switchgrass pretreatment technologies, diluted acid hydrolysis or dissolution using [emim][AC]. The cellulose treated with IL, after dissolution and precipitation with the anti-solvent, exhibited reduced crystallinity, increased surface area, and decreased lignin content,

when compared to the diluted acid-pretreatment. In special, there was no observable change in the cellulose crystallinity in the acid-treated sample. The IL treated sample showed a structural change from cellulose-I to cellulose-II that allowed for higher performances of enzymatic hydrolysis.[11]

Cassales et al.[33] pretreated soybean hull with diluted acid in autoclave (153 °C; 1.7 % H_2SO_4 (mass fraction), reaction time of 60 min), obtaining only 8 % of glucose liberation, at the same time generating approximately 10 g dm^{-3} of toxic compounds (HMF, furfural, and acetic acid), which are inhibitors of microbial growth. The formation of toxic compounds during acid/thermal pretreatments of lignocellulosic biomass is indeed an important drawback concerning the use of the liberated sugars in fermentation process. For instance, in another research concerning this problem, Hickert et al.[26] cultivated *S. cerevisiae* and *C. shehatae* in soybean hull that had been thermally and acid pretreated prior to enzymatic hydrolysis, reporting that after 24 h of cultivation the yeasts lost their viability and around 50 % of glucose present in the medium was not metabolized. The authors reported that the toxic compounds present in the medium inhibited yeast growth and sugar consumption. In contrast, in the present research, we carried out the HPLC analysis of the hydrolyzed soybean hull pretreated with [bmim][Ac] and we found no traces of any toxic compounds.

FTIR analysis

The FTIR spectra of samples of regenerated biomass after the pretreatment using [bmim][Ac], and the untreated soybean hull (control) are shown in Figure 2. This analysis was applied to verify whether there were changes in the chemical structures of soybean hull after the treatment with IL. The ATR FTIR spectroscopy in the region of 600–4000 cm^{-1} is commonly used to study the fine structural characteristics of cellulose.

The peak at 3340 cm^{-1} is related to -OH stretching vibration, whereas the one at 2909 cm^{-1} is related with -CH$_2$ and -CH$_3$ asymmetric and symmetric stretching vibrations. The presence of such bands is characteristic of lignocellulosic materials, confirming the presence of hemicellulose, cellulose, and lignin.[42] The peak at 1640 cm^{-1} in the treated sample is associated with the adsorbed water. Partial removal of hemicelluloses is associated with the reduction of intensity of this peak in the treated soybean hull.[43]

The intensity of the peak at 1540 cm^{-1} for the regenerated biomass practically disappears, thus revealing the removal of lignin after the treatment using [bmim][Ac]. In addition, the peak at 1424 cm^{-1} for the untreated sample is caused by -CH$_2$ distortion of -CH$_2$-OH groups of cellulose. The reduction of this band shows that the intra-molecular hydrogen bond at -CH$_2$-OH is broken, thus decreasing the crystallinity of cellulose.[44] The absorbance at 897 cm^{-1} appeared more intensely in the treated biomass, showing the formation of amorphous cellulose in the regenerated biomass.[36] Amorphous cellulose is characterized by high porosity and irregular structure, which becomes more susceptible to hydrolysis by enzyme, therefore, contributing to greater glucose recovery.[45]

The lateral order index (LOI) was employed in order to interpret the changes in crystallinity of soybean hull cellulose, both untreated and pretreated. The LOI is the ratio of absorbance bands around 1429 cm^{-1} and 893 cm^{-1}, which is the direct correlation between the typical amorphous and crystalline regions of cellulose.[7,37,46] In this work, the crystallinity decreased by approximately 50 %, as shown by the value reduction of LOI from 1.53 for the untreated material and 0.78 for the soybean hull pretreated with [bmim][Ac]. The results obtained from the FTIR analyses confirms that pretreatment with [bmim][Ac] has the capacity to disrupt the structure of soybean hull biomass.

Ethanol production using the glucose obtained under the optima conditions of pretreatment using [bmim][Ac]

Although the objective of this work was to evaluate the possibility of using [bmim][Ac] on soybean hull for its subsequent enzymatic hydrolysis, we found important to test the fermentability of the recovered sugars. In Figure 3, is presented the kinetics of biomass growth, sugars consumption, and products formation of *C. shehatae* HM 52.2 cultivation in the medium prepared with the enzymatic hydrolysate of soybean hull after pretreatment with [bmim][Ac]. This was not an optimized fermentation procedure. *C. shehatae* HM 52.2 was chosen because in a previous work it proved to be capable of metabolizing C-5 and C-6 sugars, which were present in the final hydrolysate.

Under the cultivation conditions used in this work, a limited supply of oxygen was given to the yeast culture, allowing the metabolism of both glucose and xylose by *C. shehatae* HM 52.2, because xylose is only used by cells under microaerophilic conditions. Fermentation proceeded vigorously between 12 and 24 h; all glucose was consumed with a corresponding ethanol production of 6 ± 0.3 g L^{-1}, representing yields of 0.31 ± 0.01 (60.8 % of the maximum theoretical ethanol yield from glucose). Xylose and ethanol were also consumed in a diauxic growth, with small amounts xylitol being produced (yields of 0.12 ± 0.03). Viability of cells was not affected throughout the cultivation (results not shown). Although ideal conditions for ethanol production were not employed, the results showed good production yields, which obviously must be optimized for scaling up of the fermentation process. Nevertheless, it appears that [bmim][Ac]-treated soybean hull biomass is an excellent alternative to the traditional physico-chemical treatments most commonly in place.

Conclusions

The present study shows that soybean hull – and possibly many other sources of lignocellulosic biomass - can be pretreated with [bmim][Ac], as an alternative technology for hydrolysis and subsequent fermentation of liberated sugars. Pretreatment conditions such as incubation temperature and time, the concentration of IL, and the solid load, are important factors to be investigated and should be taken into account when performing IL-treatments of biomass. Almost the total amount of glucose contained in the untreated cellulosic matrix was recovered after the enzymatic treatment, along with a considerable amount of xylose, suggesting that IL-treatment could also be investigated for C-5 sugars recovery. The hydrolysate proved adequate to *C. shehatae* HM 52.2 fermentation, with no loss of cell viability on the account of the absence of any toxic compounds. In conclusion, results obtained in the present study are promising in terms of preparing hydrolysates for second-generation ethanol production or other bioproducts obtained by fermentation.

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Table 1. Levels of real and codified values of independent variables used in the central composite design to define the effects of the [bmim][Ac] pretreatment on soybean hull biomass.

Independent variables	Symbols	Range of levels				
		-2	-1	0	1	2
Temperature (°C)	x_1	30	46.5	63	79.5	96
Incubation Time (min)	x_2	30	112.5	195	277.5	360
IL concentration (%)	x_3	0	25	50	75	100
Solid load (%)	x_4	2	6.5	11	16.5	20

Table 2. Soybean hull chemical composition defined in this work and comparison with other sources of biomass, usually used for second-generation ethanol.

Lignocellulosic Biomass	Glucose (%)	Xylose (%)	Arabinose (%)	Lignin (%)	Reference
Wheat straw	33.69	16.69	1.5	18.38	1
Corn stover	35.3[#]	24.2	NI	17.3	15
Sugarcane bagasse	40	24	NI	19	31
Oil palm fronds	23.5	NI	NI	18.46	34
Miscanthus	43.6	18.3	3.4	26.5	35
Soybean hull	39.7*	19.6	5.9	9.1	33
Soybean hull	39.4*	16.5	5.3	9.0	This work

NI = not informed; * Sugars were liberated following the total acid hydrolysis of the biomass matrix. See M&M for details. [#] Expressed as cellulose.

Table 3. Regression coefficients of the coded variables estimated by ANOVA for glucose recovery as a function of: temperature (T), incubation time (t), IL concentration (IL), and solid load (S).

Response variable	Source	Coefficient	p-value	Regression		
				p-value	F	R^2
Glucose Recovery Yield	Mean/Interaction	95.8216	0.0000	0.0004	6.4888	0.7321
	Linear T	4.5558	0.0180			
	Quadratic T	-3.3855	0.0614			
	Linear t	6.2542	0.0021			
	Linear IL	4.0392	0.0332			
	Quadratic IL	-4.3330	0.0198			
	Linear S	4.2383	0.0263			
	Quadratic S	-4.5605	0.0149			
	T x t	-6.3825	0.0080			

Table 4. Comparison of some results for the pretreatment of lignocellulosic biomass using ionic liquids.

Lignocellulosic Biomass	Temperature (°C)	Incubation time (min)	IL conc. (%)	Solid load (%)	Sugar yields (%)	Ionic liquid	Enzyme (origin and load)	Reference
Soybean hull	75	165	57	12.5	91.7*	[bmim] [Ac]	Enzyme complex from *P. echinulatum*: 60 FPU g^{-1} dried biomass	This work
Wheat straw	158	216	49.5	5	71.4**	[emim] [Ac]	Celluclast 1.5l: 60 FPU g^{-1} cellulose Novozym 188: 64 pNPGU g^{-1} cellulose	1
Corn stover	125	60	100	33	80*	[emim] [Ac]	Cellulase from *T. reesei*: 49 FPU g^{-1} dried biomass	15

|								
							Novozym 188: 432	
							IU g^{-1} dried biomass	
Palm	80	15	100	14	100*	[bmim]	Celluclast 1.5l: 60	34
						[Cl]	FPU g^{-1} substrate	
							Novozym 188: 200	
							CBU	
							g^{-1} substrate	
Sugarcane	145	15	100	14	69.7**	[emim]	Cellulase Onozuka	36
bagasse						[Ac]	R-10: 30 FPU g^{-1}	
							substrate	
Rice straw	120	180	100	5	99*	[bmim]	Celluclast 1.5l: 20	37
						[Ac]	FPU g^{-1} cellulose	
							Novozym 188: 30 IU	
							g^{-1} β-glucosidase	

*sugar = glucose
**sugar = total sugars

Figures Captions
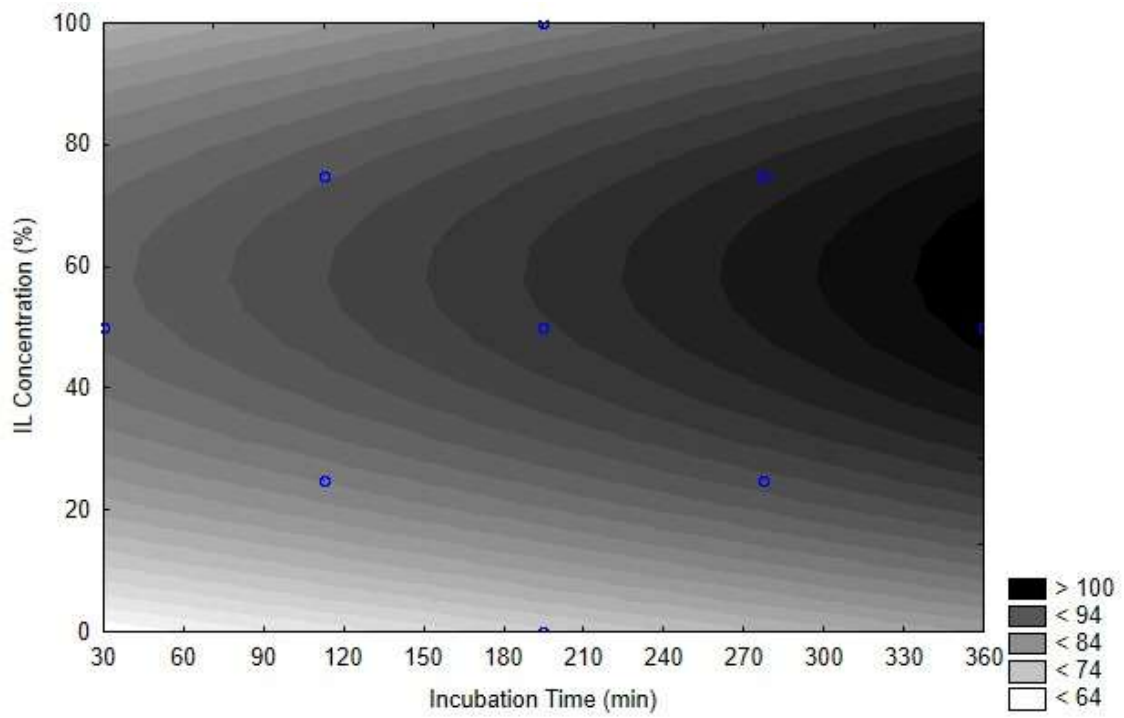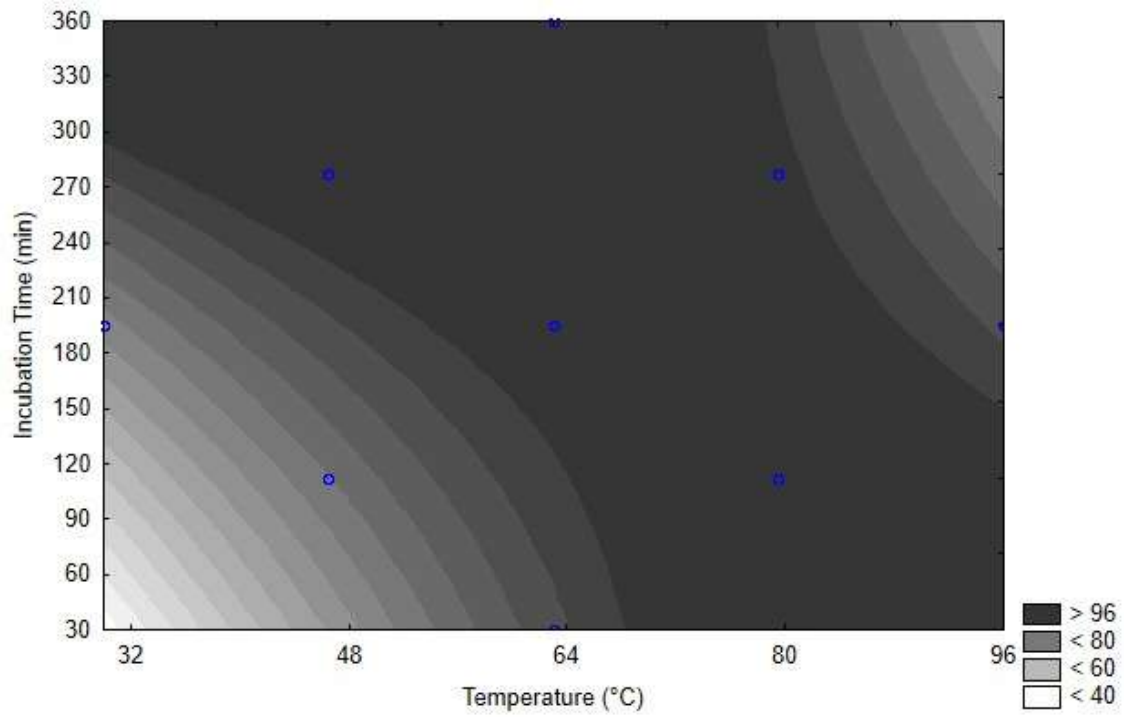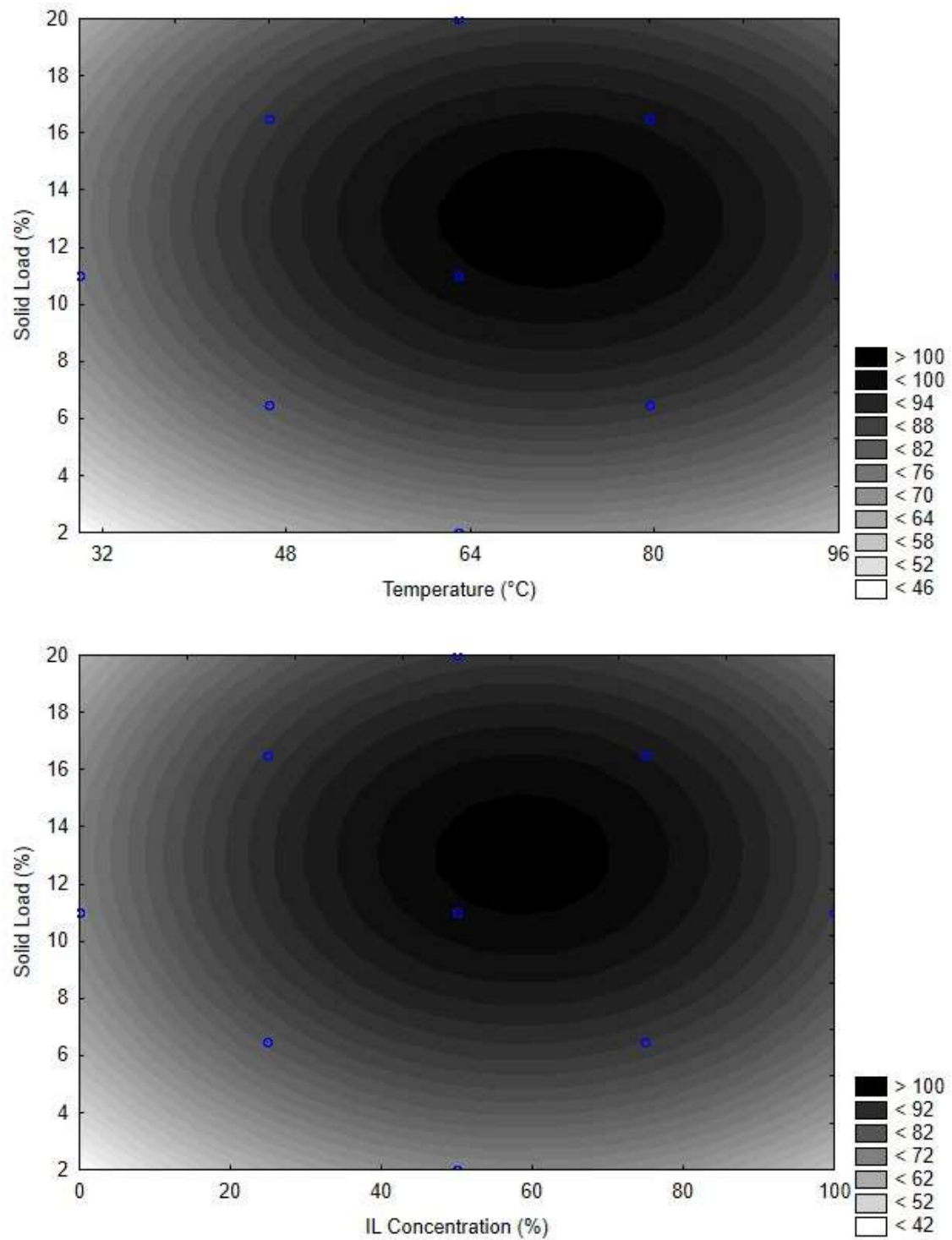
Figure 1. Response surfaces generated by the regression model for glucose yields (%) after enzymatic hydrolysis in relation to the independent variables: (A) Incubation time x Temperature, (B) IL concentration x Incubation time, (C) Solid load x Temperature, and (D) IL Concentration x Solid load. In the plots, the two factors that are not compared are always represented at the central point of the design.
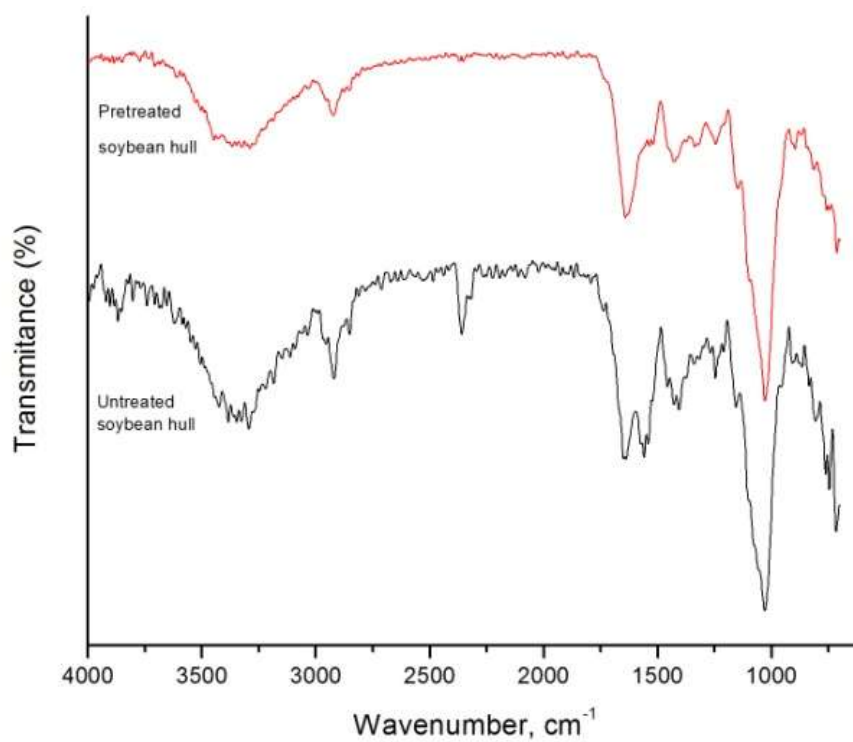
Figure 2. FTIR spectra of untreated soybean hull (control) and the pretreated biomass using [bmim][Ac].
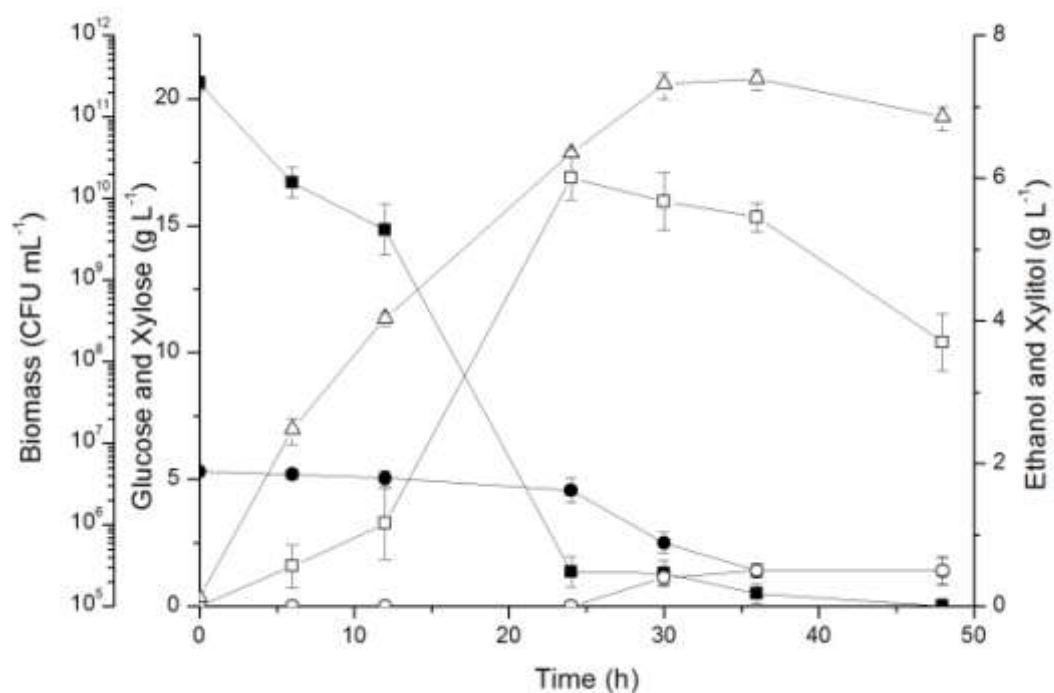
Figure 3. Kinetics of substrate consumption, ethanol, and xylitol production by *C. shehatae* HM 52.2 cultivated using the hydrolyzed medium of IL-pretreated soybean hull. Experiments were carried out in an orbital shaker at 30 °C, pH 5.0, 180 rpm. Biomass (Δ); glucose (■); xylose (●); ethanol (□); xylitol (○). Results are the mean of duplicates.

4.4.Resultados IV

'Characterization of the soybean and rice hulls after treatment with ionic liquids.'

Este artigo consiste no estudo da modificação da estrutura da biomassa regenerada (casca de soja e de arroz) após tratamento utilizando os líquidos iônicos: cloreto de 1-butil-3-metilimidazólio ([bmim][Cl]) e acetato de 1-butil-3-metilimidazólio ([bmim][Ac]). Para avaliara as alteraçãos foram utilizadas as análises de termogravimétrica (TGA), Espectroscopia de Absorção no Infravermelho por Transformada de Fourier (FTIR), difracção de raios X (XRD) e por microscopia electrônica de varredura (MEV).

Characterization of the soybean and rice hulls after treatment with ionic liquids.

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Abstract

We investigated the changes in the physical structure of cellulose recovered from soybean and rice hulls treated with the ionic liquids 1-butyl-3-methylimidazolium chloride ([bmim][Cl]), and 1-butyl-3-methylimidazolium acetate ([bmim][Ac]). The characterization was carried out by a combination of thermogravimetric analysis (TGA), Fourier transform infrared analysis (FTIR), X-ray diffraction (XRD), and scanning electron microscopy (SEM). Regenerated cellulose from soybean hull showed loss of crystallinity and higher structural disruption for both ionic liquid treatments compared against the untreated material. Rice hull, in contrast, showed only a partial structural disruption when treated with [bmim][Ac] and almost no disruption with [bmim][Cl], recalcitrance that might be a consequence of the high content of silica in its composition.

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1. Introduction

Lignocellulosic biomass derived from agricultural wastes, grasses, and trees are abundant and renewable feedstocks for the production of biofuels and chemicals. These materials are mainly constituted by polymeric carbohydrates, cellulose and hemicellulose, and by lignin (Zhang and Zhao, 2010). Hemicellulose is relatively amorphous in its structure (Lee et al., 2009) and is composed of heterogeneous polymers of pentoses, hexoses, and uronic acids, representing around 15 to 35 % of lignocellulosic biomass (Gírio et al., 2010). Lignin, comprising 15 to 35 % of the lignocellulosic matrix (Ek et al., 2009) is an complex aromatic polymer of phenylpropanoid units acting as cement binding cellulose and hemicellulose (Chandra et al., 2007). However, the most abundant component of lignocellulosic biomass is cellulose, representing approximately 35 to 50 % of plant dry weight (Lynd et al., 1999). Cellulose is crystalline in its structure and is composed exclusively of glucose units, the most important sugar for ethanol fermentation (Lee et al., 2009).

In order to reduce the lignocellulosic biomass recalcitrance, chemical and physical pretreatments are required to open its structure by dislocating and removing lignin, depolymerizing hemicellulose, and decrease the crystallinity of cellulose. However, cellulose cannot be dissolved in water or the majority of conventional organic solvents because of the extensive network of inter and intra-molecular hydrogen bonds (Swatloski et al. 2002, Murakami et al. 2007), representing a major challenge to its application in bioprocess, and pressing research to solve this problem.

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One promising new technology that has been devised to use as cellulose solvent is the use of ionic liquids (ILs), which are salts, typically composed of large organic cations and small inorganic anions that are liquids through a wide range of temperatures. Considered environmentally safe solvents, ILs show low vapor pressures, high thermal stabilities, relative nontoxicity and ease of recycling (Zhu et al., 2006; El Seoud et al., 2007 and Hayes, 2009). Some reports have shown the interesting results using ILs for the dissolution of cellulose from wood and other agro-residues (Fort et al., 2007; Wei et al., 2011; Yoon et al., 2012). Among the many possible ILs to be used, good results have been obtained using imidazolium-based ILs with short side chains because of their reduced viscosity and melting point (Dadi et al., 2006). In relation to anions of the ILs, the most promising are chloride and acetate because these have the ability to break down the hydrogen bonds of cellulose (Pu et al.2007, Fort et al., 2007).

The use of the ILs for the pretreatment of lignocellulosic materials can reduce the crystallinity of cellulose and partially remove hemicellulose and lignin, at the same time not generating degradation products, such as hydroxymethylfurfural and furfural, which inhibit enzymes or the growth of fermenting microorganisms (Dadi et al., 2007; Lee et al., 2009).

In the context of these considerations, the aim of this study was to determine the effects of application of two ILs commonly used for lignocellulosic biomass treatments, 1-butyl-3-methylimidazolium chloride ([bmim][Cl]), and 1-butyl-3-methylimidazolium acetate ([bmim][Ac]), on soybean and rice hulls, which are abundant lignocellulosic agro-residues, for the possible liberation of sugars for fermentation. The regenerated cellulose was characterized by infrared spectrometric analyzer, thermogravimetric analysis, X-ray diffractometer, and scanning electron microscopy and their properties were discussed.

2. Materials and Methods

2.1 Biomass and compositional analysis

Soybean hulls were kindly supplied by The Solae Company (Esteio, RS, Brazil), whereas rice hulls were obtained from a local rice mill (Pelotas, RS, Brazil). The hulls, processed without any further treatments, were milled to a particle size < 1 mm in diameter and stored at room temperature until further use or analysis.

2.2 Ionic Liquid preparation

We firstly synthesized [bmim][Cl], which is a precursor of ILs with carboxylate anions ([bmim][Ac]), following procedures described in the literature and characterized by ^{1}H NMR (Varian Inova 300 MHz, Agilent 500 MHz) (Dupont et al., 2002, Wu et al., 2008).

Description of the ILs used: a) [bmim][Ac], colorless viscous liquid. ^{1}H RMN (500 MHz - $CDCl_3$) d ppm 0.97 (t, J = 7.4 Hz, 3H[9]); 1.33-1.41 (m, 2H[8]); 1.83-1.89 (m, 2H[7]); 1.97 (s, 3H[12]); 4.05 (s, 3H[10]); 4.29 (t, J = 7.4 Hz, 2H[6]), 7.24 (s, 1H[5]); 7.30 (s, 1H[4]); 11.08 (s, 1H[2]); b) [bmim][Cl], white solid. ^{1}H RMN (300 MHz - D_2O) d ppm 0.89 (t, J = 7.4 Hz, 3H[9]); 1.22-1.35 (m, 2H[8]); 1.77-1.87 (m, 2H[7]); 3.86 (s, 3H[10]); 4.17 (t, J = 7.2 Hz, 2H[6]); 7.40 (d, J = 2.0 Hz, 1H[5]); 7.45 (d, J = 2.0 Hz, 1H[4]).

2.3 Ionic liquid pretreatment of soybean hull

In a polypropylene tube, 2 g of IL or IL–water mixture (1 g of each) were mixed with 240 mg of the lignocellulosic biomass sample. The samples were incubated in an oil bath at 75°C or 100°C and different incubation times (2 or 6 h), as shown in details in Table 1. All experiments were performed under atmospheric pressure. After

pretreatment, 10 mL of deionized water was added to samples to serve as an anti-solvent in the mixture and to regenerate the cellulose. The resulting mixture was briefly centrifuged (7000 g), and the supernatant was removed. The precipitate (treated biomass containing regenerated cellulose) was washed with the same amount of anti-solvent and subsequently centrifuged four times (7000 g). The treated biomass was oven-dried at 75 °C for 24 h.

2.4 Thermogravimetric analysis

The samples were submitted to thermogravimetric analysis (TGA), using a Shimadzu TGA-50. The samples were heated up to 600 °C at a rate of 10 °C min^{-1} and a flow rate of 50 ml min^{-1} of N$_2$.

2.5 Fourier transform infrared (FTIR) analysis

The FTIR spectra of samples were obtained using a Shimadzu FTIR, model Prestigie 21, in a ATR mode, with a resolution of 4 cm^{-1} and 32 cumulative scans.

2.6 X-ray powder diffraction (XDR) analysis

In order to determine the transformation in the crystallinity of treated cellulose, XRD studies were carried out using a Siemens D-500 powder diffractometer, equipped with Soller slits and a graphite monochromator in the secondary beam. Data were collected with CuKα radiation, with a wavelength of 0.15418 nm.

2.7 Scanning electron microscopy (SEM)

The materials were analyzed by scanning electron microscopy (SEM) in a Jeol equipment, model JEOL JSM6060, operating at 18 kV and with magnification of 500 times.

3. Results and discussion

3.1 Thermogravimetric analysis

We used thermogravimetric analysis (TGA) in order to get information on the weight loss of samples as a function of temperature, thus defining the thermal decomposition profiles of untreated and regenerated cellulose. This analysis is important to check whether the pretreatment of soybean and rice hulls with the ILs affected the thermal stability of the lignocellulosic materials. The TGA results are shown in Figure 1 (soybean hull) and Figure 2 (rice hull). Observing the curves, these can be divided into three principal different regions, independent of treatment or biomass (Alemdar and Sain, 2008; Wei et al., 2012). Analyzing the results of Figure 1, results show the thermal stability of the lignocellulosic biomass decreases after treatment and regeneration with water. The first part of the curve goes up to approximately 200 °C, showing a slight decline of the sample weight due to the release of unbound water with the drying procedure. The second stage is between 200 °C and 380 °C, in which a great decrease in weight is caused by the thermal decomposition of hemicellulose and cellulose. The regenerate samples exhibit a decreased temperature of decomposition compared to the original sample, clearly indicating a loss of crystallinity of the cellulose (Swatloski et al., 2002; Fort et al., 2007;). Above 380 °C, the samples went to the final stage, associated with thermal decomposition of lignin, with weight loss not as significant as compared to the second stage.

The results suggest that IL treatment of samples decreased the thermal stability of cellulose. Kin et al., 2001, reported that the modification of the thermal behavior of cellulose is caused by the adherence of the anion group of IL into the surface of cellulose. The increased surface area of treated cellulose also decreases the thermal stability because there is a bigger area exposed to heat (Lu and Hsieh, 2010).

The TGA results for rice hulls are shown in Figure 2. This biomass undergone the same three stages observed for soybean hulls. However, after treatment with [bmim][CI] and cellulose regeneration (Figure 2 B and D), we could not observe a decrease in decomposition temperature compared with the untreated biomass. When rice hull is pretreated using [bmim][Ac] at 100 °C for 6 h (Figure 2 C), there was a small decrease in decomposition temperature of 380 °C to 370 °C, which may be related to the decrease in crystallinity of the biomass.

3.2 Fourier transform infrared (FTIR) analysis

In Figure 3 are shown the spectra of regenerated cellulose of soybean hull after treatment with IL and its corresponding untreated sample. The structure of regenerated biomasses suffered chemical and structural changes compared with the untreated biomass. For these analysis, ATR FTIR spectroscopy in the region of $600 - 4\,000$ cm^{-1} was used, which is commonly used to study the fine structural characteristics of cellulose (Poornejad et al., 2013). The peak around of $1\,640$ cm^{-1} is assigned to the absorbed water (Sun et al., 2004; Morán et al., 2008). However, the reduction of the intensity of this peak in the treated biomass is due to the partial removal of hemicellulose (Alemdar and Sain, 2008). In the spectra of untreated samples, the peaks in the region between 1420-1540 cm^{-1} represents the aromatic C-C stretch of aromatic rings of lignin (Sun et al., 2005; Xiao et al., 2001). In the curves treated samples, the

intensity of these peaks decreased, attributed to the partial removal of lignin (Alemdar and Sain, 2008).

The peak in approximately 1025 cm^{-1} corresponds to C–O–C pyranose ring skeletal vibration at cellulose/hemicellulose. The emergence a small sharp peak in 895 cm^{-1} is an indicative of a glycosidic C-H deformation with ring vibration contribution, a characteristic of β-glycosidic linkages between glucose in cellulose (Wei et al., 2012). This peak also indicates that the regenerated cellulose is more amorphous and disordered than the original material. The narrowing of peak in 1025 cm^{-1} and the emergence of another in 895 cm^{-1} for the treated samples are indicating that the regenerated material is richer in carbohydrates (Singh et al., 2009; Sun et al., 2009). Therefore, comparing the FTIR spectra of untreated and treated samples with [bmim][Ac] and [bmim][Cl], it can be seen that inter and intra-molecular hydrogen bonds of cellulose were significantly broken by the treatment with IL.

Comparing the types of ILs used (Figure 3) and the reduction of the peaks between 1 420-1 540 cm^{-1} related with lignin, we noted that the [bmim][Ac] (Figure 3 A and C) could remove lignin, whereas [bmim][Cl] (Figure 3 B and D) only partially removes this component. When using [bmim][Ac] during longer incubation time (Figure 3 A), it was observed a peak at 1550 cm^{-1} representing the C-C in-plane stretching vibration of imidazolium cation of IL, which was entrapped in the regenerated cellulose structure after treatment (Liu et al., 2012).

The same analysis was performed for rice hulls. For the conditions used in this study, there were no significant differences in the spectra (data not shown) indicative of any decrease in crystallinity.

3.3 X-ray powder diffraction (XDR) analysis

In Figure 4 are shown the XRD patterns of the samples treated with ILs compared to the untreated controls. For all controls it was verified the diffraction peaks on about 15 and 22°, typical of cellulose type I (Mansikkamäki et al., 2005). For the soybean hull, the first peak at 16.6° corresponds to the (110) planes, and the second peak at 22.5° corresponds to the (002) plane of cellulose I. The peak around 16.6°reduced after treatment when [bmim][Ac] was used (Figure 4 A and C), and was absent in treatments with [bmim][Cl] (Figure 4 B and D), whereas peak (002) became wider and less intense. Results suggest that treatment with IL can reduce the cellulose crystallinity in soybean hull. It was observed that the reduction of peak 16.6° increased with the temperature and with the amount of IL used. However, increasing time of treatment did not show any significant modifications.

Dadi et al. 2007, suggested that anion and cation are responsible for the cellulose dissolution. The free hydroxyl group of the cellulose is attacked and deprotonated by the anion of IL, while the cation interacts with the hydroxyl oxygen atoms. Thus, hydrogen bonds in cellulose are disrupted and replaced by hydrogen bonding between the anion of ILs and cellulose hydroxyl, thus cellulose crystallinity is destroyed because of dissolution.

The XRD analysis has shown the same results of FTIR. The modification in the patterns for rice hull (data no shown) was not as pronounced as for soybean hull (reduction of peak around 16°), indicating that there was no expressive change in the crystallinity of the cellulose of this biomass.

3.4 Scanning electron microscopy (SEM)

We carried out the SEM analysis in order to detect possible changes in the surface of the biomass related to different treatments with the ILs. The SEM images of the soybean hull and rice hull samples are represented in Figures 5 and 6, respectively. Swanson et al. 1985, reported that the soybean hull consists of cuticle, epidermis (palisade cells), hypodermis (hourglass cells), and parenchymal cells.

The cells of the palisade layer are presented with a stretched position perpendicular to the surface of the hull. The hypodermis is made up of a layer of uniform cells, appearing continuously and showing a non-uniform cell thickness, with the presence of large intercellular spaces. This structure can be seen in the pictures of the untreated samples of soybean hull (Figure 5 A). In contrast, the regenerated samples were clearly modified by both treatments with [bmim][Ac] and [bmim][Cl]. Destructuring was proportional with time treatment. The changes were both in the morphology of samples, as well as the crystallinity, which decreased as observed in the X-ray diffraction analysis. The SEM images suggest that the [bmim][Cl] produced a more porous surface of soybean hulls. Tan et al. 2011, has assigned these alterations to the breakdown of biomass natural structures during the dissolution of cellulose in presence of IL, causing a reduction in crystallinity. Other studies have also reported that the structure of the biomass became more porous and loses crystallinity after IL treatment (Tan et al., 2011; Yoon et al., 2012).

Figure 6 shows the SEM images for treated and untreated rice hulls treated with [bmim][Ac] and [bmim][Cl]. Rice hull is a material with a complex composition, having silica and organic material combined, resulting in a fibrous and very recalcitrant material. The silica is concentrated on protuberances and trichomes of the epidermis (Liou et al., 2004). The protuberances and silica is clearly visible in the SEM images of

untreated biomass (Figure 6 A). Although the results of other analysis (TGA, FTIR and XDR) did not show significant changes in the structure of treated rice hull compared to the untreated biomass, the SEM analysis shows that rice hull treated with [bmim][Ac] had a higher degree of disruption than the material treated using [bmim][Cl] in 6 h (Figures 6 D and E, respectively). Ang et al. 2011, suggested that the breaking of the biomass surface might be caused by the solvating action of ILs, in which the lignocellulosic matrix is swelled and dissolved.

4. **Conclusion**

The results obtained in this study suggest that ILs can be used in the treatment of lignocellulosic biomass such as soybean and rice hulls. This is an alternative technology, leaving the surface of the materials exposed to subsequently apply the enzymatic hydrolysis to obtain fermentable sugars. The type of IL used it's the biomass will differ with respect to disruption of their structures. Soybean hulls were more easily decrystalized for anyone of the ILs used when compared with rice hulls, which was modified more intensely by [bmim][Ac] than by [bmim][Cl]. Dissolution of the lignocellulosic biomass was influenced by the type of IL used, the ratio of ionic liquid, temperature, and time of treatment. Results obtained in the present study grant further studies in order to optimize the use of this technology for the conversion of soybean and rice hulls into value-added products.

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Table 1. Lignocellulosic biomass used in this research and treatment conditions.

Lignocellulosic Biomass	Treatment		
	Ionic Liquid	Temperature (°C)	Time (h)
Soybean Hull	50% [bmim][Ac]+50% water	75	2
	100%[bmim][Ac]	75	and
	100%[bmim][Ac]	100	6
	50% [bmim][Cl]+50% water	75	2
	100%[bmim][Cl]	75	and
	100%[bmim][Cl]	100	6
Rice Hull	50% [bmim][Ac]+50% water	75	2
	100%[bmim][Ac]	75	and
	100%[bmim][Ac]	100	6
	50% [bmim][Cl]+50% water	75	2
	100%[bmim][Cl]	75	and
	100%[bmim][Cl]	100	6

Figures Captions
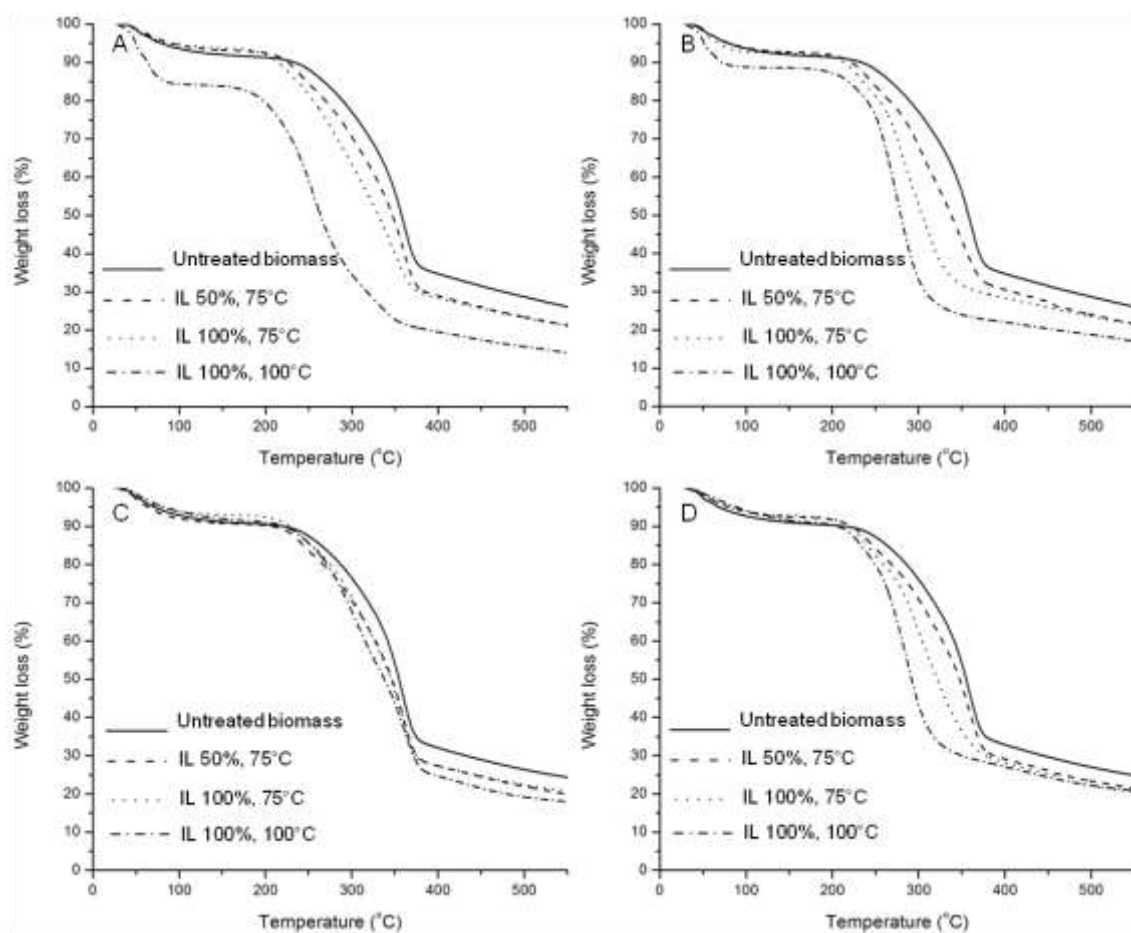
Fig.1 TGA thermograms of soybean hull treated with [bmim][Ac] (A and C), and [bmim][Cl] (B and D), for 2 h (A and B), and 6 h (C and D)
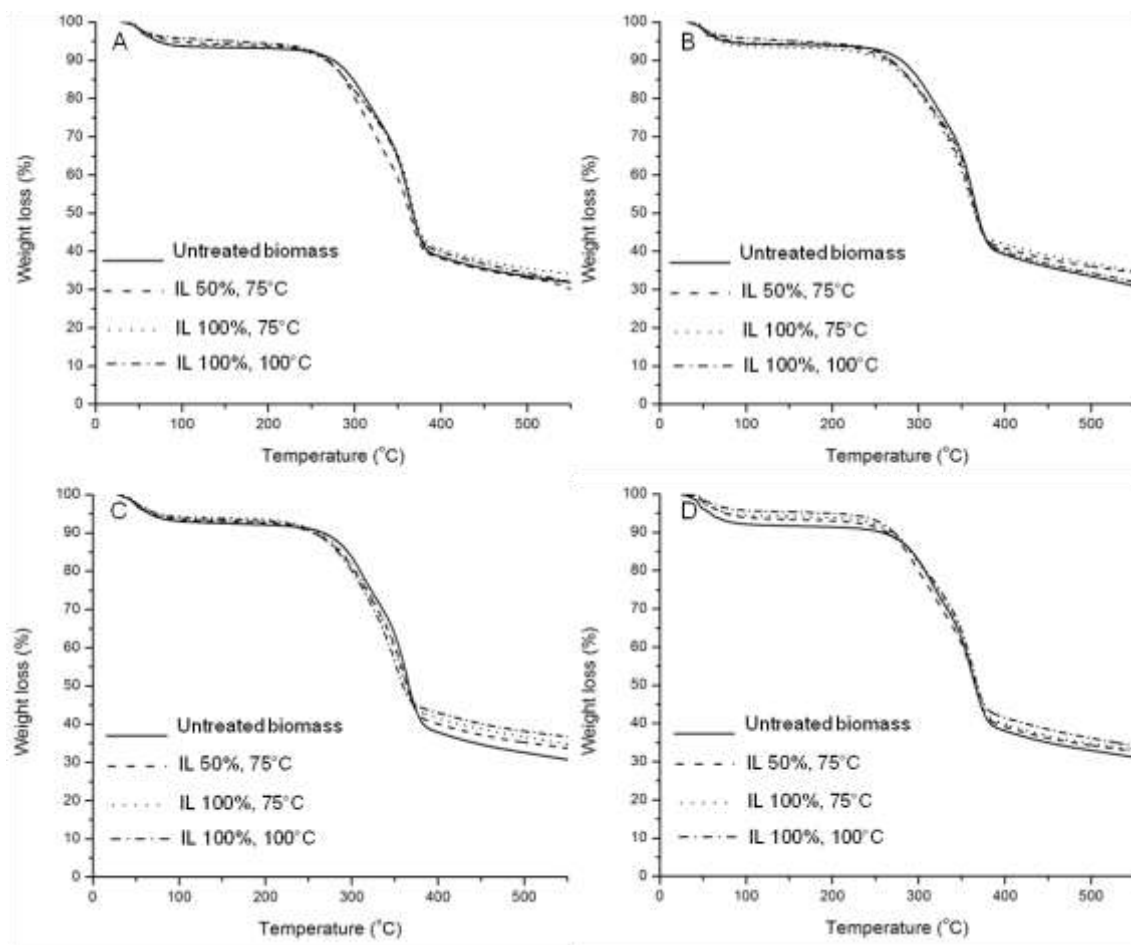
Fig 2 TGA thermograms of rice hull treated with [bmim][Ac] (A and C), and [bmim][Cl] (B and D), for 2 h (A and B), and 6 h (C and D)

Fig. 3 FTIR spectra of soybean hull treated with [bmim][Ac] (A and C), and [bmim][Cl] (B and D), for 2 h (A and B), and 6 h (C and D)
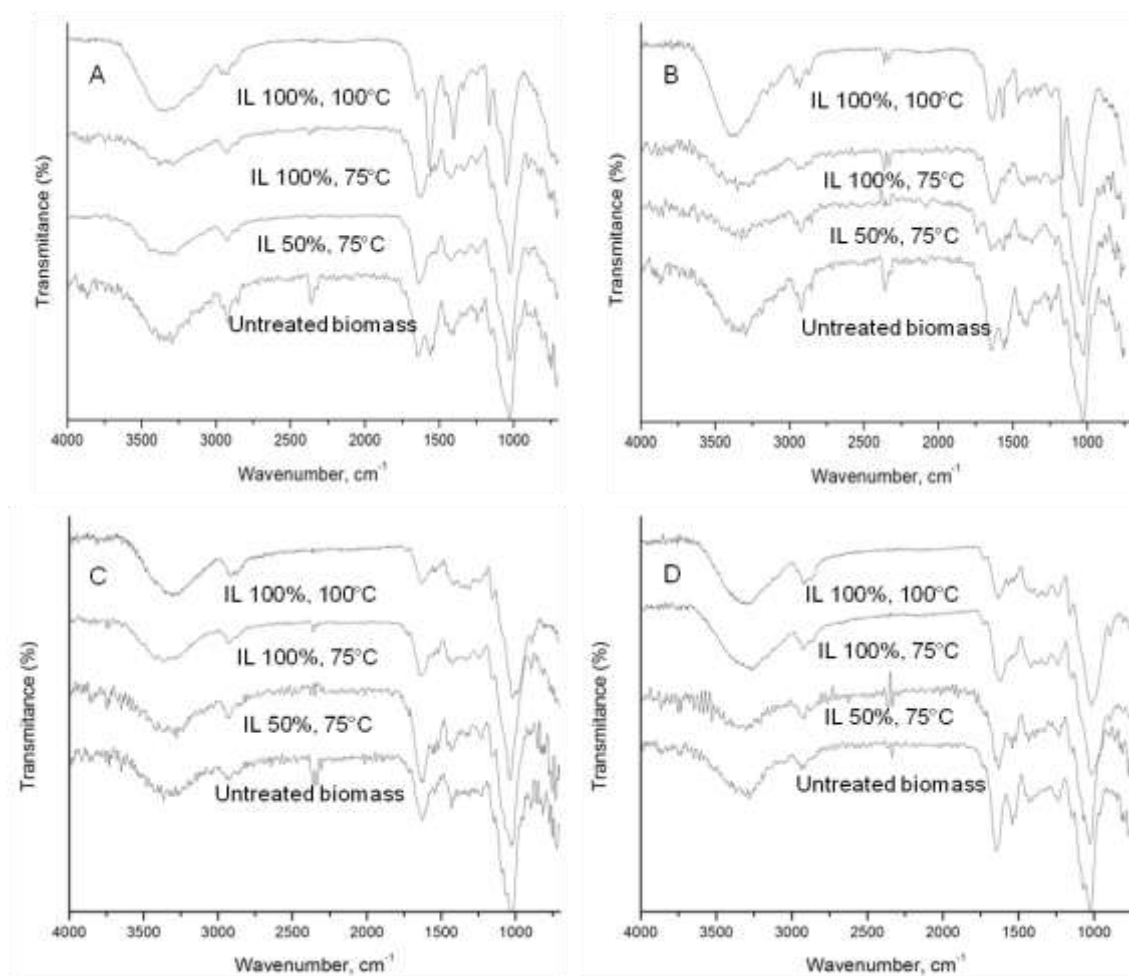
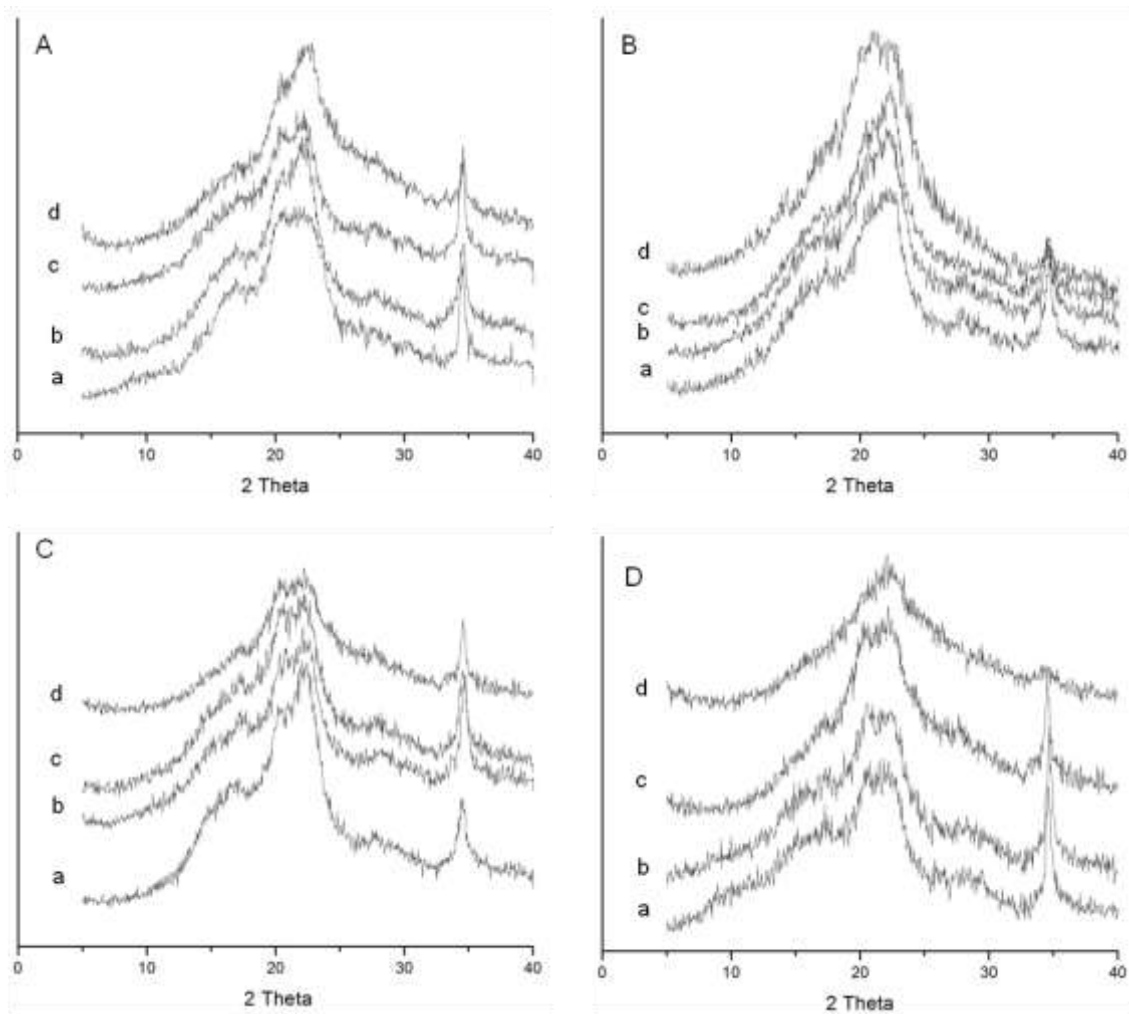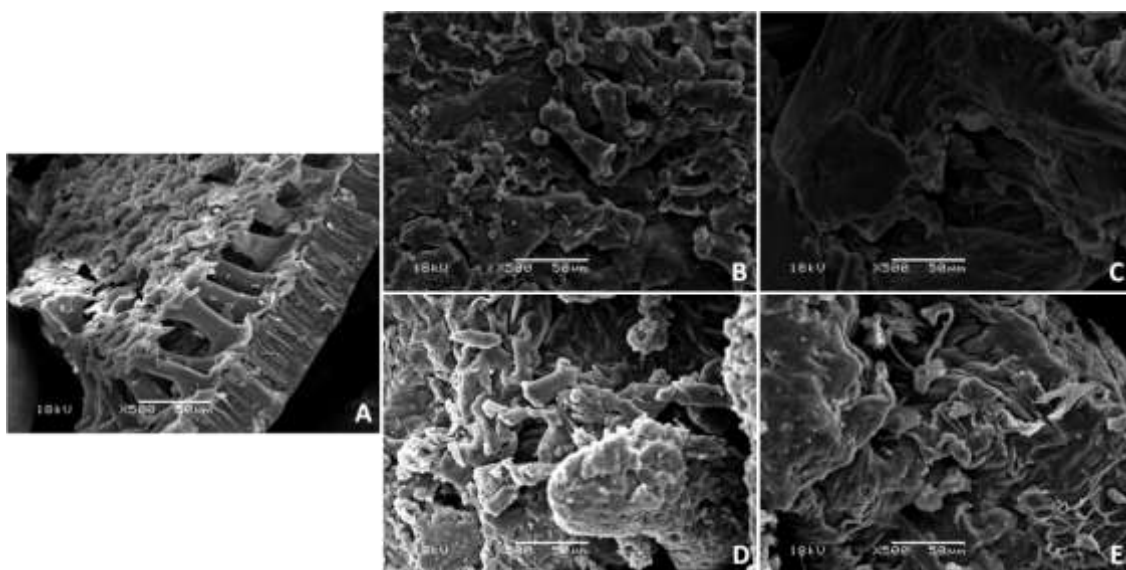**Fig.4** X-ray diffraction analysis of soybean hull treated with [bmim][Ac] (A and C), and [bmim][Cl] (B and D), for 2 h (A and B), and 6 h (C and D). Legend: a) untreated biomass, b) IL 50 % and 75°C, c) IL 100 % and 75°C, and d) IL 100 % and 100°C.

Fig. 5. SEM comparison of soybean hull before and after treatment with IL (500 times magnification). a) untreated soybean hull outer surface, b) 100 °C, 2 h treated with [bmim][Ac], c) 100 °C, 6 h treated with [bmim][Ac], d) 100 °C, 2 h treated with [bmim][Cl], e) 100 °C, 6 h treated with [bmim][Cl].
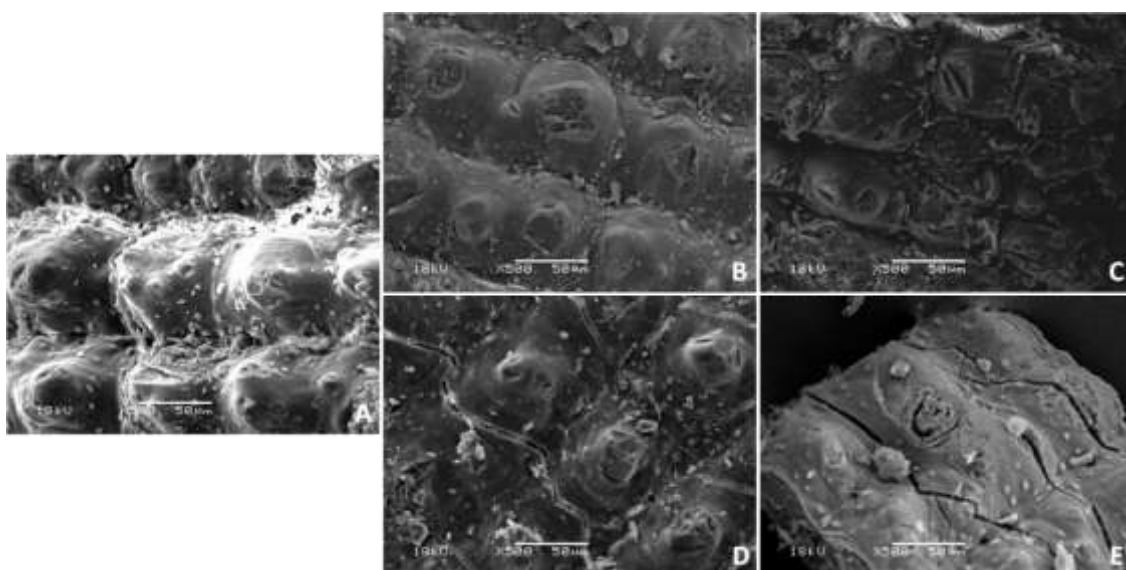
Fig. 6. SEM comparison of rice hull before and after treatment with IL (600 times magnification). a) untreated rice hull outer surface, b) 100 °C, 2 h pretreated with [bmim][Ac], c) 100 °C, 6 h treated with [bmim][Ac], d) 100 °C, 2 h treated with [bmim][Cl], e) 100 °C, 6 h treated with [bmim][Cl].

Capítulo 5

5.1 Considerações Finais

Cada vez mais alternativas ambientalmente corretas, sustentáveis e renováveis vem sendo buscadas como opção a utilização de combustíveis fósseis e o etanol, por considerar os fatores enumerados acima, vem se destacando com uma opção. Entretanto, a produção de etanol de primeira geração, a partir da cana-de-açúcar, encontra alguns entraves na sua produção ou devido a entre safra da cana ou até mesmo pela alta do preço do açúcar no mercado internacional o qual leva a produção de etanol ser desviada para açúcar.

O Rio Grande do Sul destaca-se por ser um dos maiores produtores de soja e arroz do Brasil. O beneficiamento destes grãos gera uma grande quantidade de resíduos e estes são predominantemente cascas que são materiais lignocelulósicos. Os materiais lignocelulósicos são formados principalmente por celulose, hemicelulose e lignina. A celulose e a hemicelulose são polímeros formados de monossacarídeos principalmente glicose e xilose, respectivamente, os quais são substratos essenciais para processos fermentativos que visam a produção de etanol e/ou xilitol.

Tendo em vista estas considerações, a produção de etanol a partir de resíduos lignocelulósicos agroindustriais tem se mostrado uma alternativa bastante promissora, já que aumenta a disponibilidade de recursos energéticos, diminui a poluição e o acúmulo de CO_2 no ar, além de agregar valor a estes materiais.

No presente trabalho, diferentes tecnologias foram utilizadas visando a utilização de biomassa lignocelulósica para produção de etanol. Primeiramente

testaram-se dois meios de cultura, hidrolisado de casca de arroz e meio sintético simulando o hidrolisado, utilizados na conversão destes a etanol por leveduras *C. shehatae* e *S. cerevisiae*. O uso de co-culturas destas leveduras apresentou altos rendimentos de etanol, tanto em frascos mantidos sob agitação quanto em biorreatores, sendo maiores que aqueles obtidos com o uso de um microrganismo isolado, aproximando-se do rendimento teórico (0,51). Os resultados encontrados, nesta primeira parte do trabalho, sugerem a possibilidade de utilização do hidrolisado de casca de arroz como substrato para a produção de etanol de segunda geração.

A casca de soja também foi contemplada em outra etapa do trabalho. Através da utilização de recursos estatísticos, foi verificado se era necessária a suplementação do hidrolisado de casca de soja, assim como as condições ótimas para bioconversão deste a etanol. A levedura *C. guillermoundii* BL13, recentemente isolada, foi testada em hidrolisado ácido/enzimático de casca de soja a fim de avaliar a sua capacidade de converter as hexoses e pentoses contidas neste em etanol. Os resultados indicam que não há necessidade de suplementação do hidrolisado e que, em condições ótimas reveladas pelo planejamento experimental, *C. guillermoundii* BL13 consegue uma alta produtividade de etanol sem a necessidade de detoxificação do meio. Assim, esta levedura tem grande potencial para ser utilizada em processos biotecnológicos que visem a produção de etanol.

Para que tanto a casca de soja ou de arroz seja utilizada nos processos de bioconversão de materiais lignocelulósicos, uma etapa de pré-tratamento, a fim de desorganizar a estrutura destes materiais facilitando a sacarificação dos açúcares, é necessária. Tendo em vista a utilização de novas tecnologias, os líquidos iônicos cloreto de 1-butil-3-metilimidazólio ([bmim][Cl]) e acetato de 1-butil-3-metilimidazólio ([bmim][Ac]) foram utilizados na dissolução da celulose contidas nos materiais

lignocelulósicos. Condições de pré-tratamento tais como temperatura, tempo de incubação, concentração de LI e a carga sólida, são fatores importantes a serem investigados quando se realiza pré-tratamento de biomassas com LIs. Primeiramente, através de recursos estatísticos, foram avaliadas e otimizadas as condições de pré-tratamento de casca de soja com [bmim][Ac]. A biomassa regenerada foi então submetida a hidrólise enzimática utilizando um complexo enzimático celolulítico de *Penicillium echinulatum* strain S1M29A. A otimização foi baseada na condição em que foi possível obter uma maior quantidade de glicose liberada após a hidrólise enzimática.

Os resultados indicam que a utilização de líquido iônico aumenta a disponibilidade da celulose e da hemicelulose facilitando a sacarificação pois, na condição otimizada, foi possível recuperar quase que a totalidade da glicose contida na casca de soja original e uma quantidade significativa de xilose. Outro fator interessante, foi que o ponto otimizado leva uma combinação de LI-água, o que reduz a quantidade de LI utilizado no pré-tratamento. Através da análise de Espectroscopia de Absorção no Infravermelho por Transformada de Fourier (FTIR), foi possível observar que a casca de soja pré-tratada dininuiu a cristalinidade facilitando a hidrólise enzimática. Resultados demonstraram que o hidrolisado enzimático foi livre de quaisquer compostos tóxicos, tais como hidroximethilfurfural e furfural. O hidrolisado foi utilizado no cultivo *C. shehatae* HM 52.2 e mostrou-se adequada para processos fermentativos devido a ausência de compostos tóxicos. Estes indicam a possibilidade da utilização de líquidos iónicos para o pré-tratamento de materiais lignocelulósicos, evitando a formação de compostos tóxicos, sendo utilizado na produção de etanol de segunda geração e outros produtos de fermentação.

Em outro estudo, foram investigadas as alterações na estrutura da celulose regenerada a partir das cascas de soja e de arroz tratado com os líquidos iónicos cloreto

de 1-butil-3-metilimidazólio ([bmim][Cl]) ou acetato de 1-butil-3-metilimidazólio ([bmim][Ac]). A caracterização foi realizada por uma combinação de análise termogravimétrica (TGA), análise de Espectroscopia de Absorção no Infravermelho por Transformada de Fourier (FTIR), difracção de raios X (XRD) e por microscopia electrônica de varredura (MEV). Após o tratamento, a celulose regenerada a partir de casca de soja apresentou uma diminuição na cristalinidade e maior perturbação estrutural para ambos os tratamentos líquidos iônicos em comparação com o material original (não tratado). Entretanto, a casca de arroz mostrou apenas uma ruptura estrutural parcial quando tratados com [bmim][AC] e quase nenhuma com [bmim][Cl]. O alto teor de sílica na composição da casca de arroz pode ser o fator que impediu uma maior desorganização nesta biomassa. Observou-se que o tipo de LI utilizado e biomassa podem diferir no que diz respeito as perturbações sofridas nas estruturas das biomassas. Os resultados obtidos neste estudo sugerem portanto que LIs podem ser utilizados no tratamento de biomassa lignocelulósica como as cascas soja e de arroz.

Desta forma, verifica-se que este estudo tem relevância já que foi possível utilizar resíduos agroindustrias na produção de etanol de segunda geração. A utilização de co-cultura de microrganismos mostrou-se com uma alternatia eficiente para o aumento do rendimento de etanol. Foi possível verificar que, mesmo sem a suplementação ou detoxificação dos hidrolisados utilizados como meio de cultivo, os microganismos utilizados apresentaram uma alta conversão dos açúcares presentes nos hidrolisados. Outro fato importante deste trabalho é utilização de uma nova tecnologia no pré-tratamento da biomassa lignocelulósica – líquidos iônicos. Esta é uma tecnologia alternativa, que proporciona uma maior exposição da biomassa para que posteriormente seja aplicada uma hidrólise enzimática para obtenção de açúcares fermentáveis. Os resultados sugerem que os líquidos iónicos podem ser utilizados no pré-tratamento de

RLAs com a finalidade de produzir de etanol e outros produtos de valor agregado adivindos do processo fermentativo.

5.2 Conclusões

A produção de etanol a partir de resíduos lignocelulósicos agroindustriais tem se mostrado uma alternativa bastante promissora.

A casca de soja é uma fonte de material lignocelulósicos com grande potencial a serem utilizados em bioprocessos.

Os líquidos iônicos aumentam a disponibilidade da celulose e hemicelulose o que aumenta a eficiência de uma posterior hidrólise enzimática.

5.3 Perspectivas

O desenvolvimento de tecnologia(s) que utilizem os materiais lignocelulósicos como alternativa para produção de biocombustíveis ou bioprodutos esta cada vez mais sendo pesquisada.

Uma alternativa para o maior aproveitamento dos açúcares disponíveis no hidrolisados é buscar ou desenvolver (através de manipulação genética) microrganismos capazes de converter pentose e hexoses a etanol e/ou xilitol de maneira eficiente. A utilização da hidrólise enzimática posterior a hidrolise ácida utilizando uma mistura de enzimas poderia aumentar a disponibilidade dos acúcares fermentescíveis e assim aumentar a produtividade de bioprodutos.

Com relação aos tratamentos com os líquidos iônicos, deve-se buscar desenvolver métodos para a reutilização dos líquidos iônicos nos pré-tratamentos afim de investigar quantos reciclos eles podem ser utilizados sem comprometimento da eficiência tratamento.

Realizar estudo, tanto de leveduras quanto de enzimas, para verificar a toxicidade dos líquido iônicos para estas. Buscar LIs que possam ser utilizados em conjunto com as enzimas (imobilizadas ou livres) sem desativá-las.

Realizar estudos com diferentes estratégias (sequência) nos pré-tratamentos (novos e já utilizados no laboratório), visando a deslignificação das biomassas e até mesmo a remoção da sílica da casca de arroz para propiciar um maior ataque enzimático na estrutura da biomassa.

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