UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
ESCOLA DE ENGENHARIA
DEPARTAMENTO DE ENGENHARIA QUÍMICA
PROGRAMA DE PÓS GRADUAÇÃO EM ENGENHARIA QUÍMICA

BIOCONVERSÃO DE HIDROLISADOS DE CASCA DE ARROZ E SOJA A ETANOL E XILITOL POR LEVEDURAS

Lílian Raquel Hickert

Química Industrial.

Microbiologia Agrícola e do Ambiente, Msc.

Porto Alegre, junho de 2014.

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

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À Comissão Examinadora, abaixo assinada, aprova a Tese "Bioconversão de hidrolisados de casca de arroz e soja em etanol e xilitol por leveduras", elaborada por Lílian Raquel Hickert, como requisito parcial para obtenção do Grau de Doutor em Engenharia Química.

Comissão Examinadora:

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Profª. Drª. Liliana Amaral Féris

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Profª. Drª. Marlí Camassola

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Profº. Drº. Júlio Xandro Heck

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'El mundo era tan reciente que muchas cosa carecían de nombre, u para nombrarlas había que señalarlas con el dedo.'

'Cuando llovió durante cuatro años, once meses y dos días en Macondo. ' – **Gabriel Garcia Márquez -** Cien años de Soledad

À minha família, que sempre está comigo, me apoiando. Aos meus pais, que sempre me incentivam. Ao Leo, meu marido, que começou a acompanhar tudo quando ainda era mestrado, pela ajuda, apoio e inóculos no sábado à tarde. E principalmente pela calma e incentivo de sempre.

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RESUMO

Os resíduos lignocelulósicos agroindustriais, como a casca de arroz e a casca de soja, são fontes abundantes e de baixo custo na produção biotecnológica de compostos de alto valor agregado como etanol e xilitol, por figurarem como fontes de celulose e hemicelulose. No presente trabalho será estudada a capacidade de conversão dos açúcares provenientes destes resíduos por diferentes leveduras ampliando os conhecimentos sobre a produção biotecnológica de alcoóis. A capacidade de *Candida shehatae, Saccharomyces cerevisiae,* e a co-cultura destas duas leveduras na conversão do açúcar presente no hidrolisado de casca de arroz (RHH) utilizado como substrato para a produção de etanol foi estudada. Em experimentos em agitador orbital, as co-culturas dessas leveduras apresentaram rendimentos de etanol ($Y_{P/S}$) de 0,42 e 0,51 em meio sintético simulando a composição do hidrolisado e em RHH, respectivamente. Ao analisar a produção de etanol com culturas puras de *C. shehatae* o rendimento de etanol foi ligeiramente inferior (0,40). Visando analisar o metabolismo das leveduras sob condições de anaerobiose e de limitação de oxigênio, foram realizados experimentos em biorreatores, onde a utilização de co-culturas produziu rendimentos de etanol similares em ambas condições (0,50-0,51) em meio sintético, enquanto que em RHH, rendimentos de 0,48 e 0,44 foram obtidos, respectivamente. Novas estratégias de produção de etanol a partir de hidrolisado de casca de arroz também foram testadas, como a sacarificação e co-fermentação simultânea por *S. cerevisiae, Spathaspora arborariae* e pela combinação destas leveduras. Nas culturas sob limitação de oxigênio, *S. cerevisiae* foi capaz de metabolizar a glicose presente RHH, resultando em um rendimento de etanol ($Y_{P/S}$) de 0,45. A co-cultura de *S. cerevisiae* e *S. arborariae* foi capaz de metabolizar pentoses e hexoses presentes em RHH, obtendo $Y_{P/S}$ de 0,48 g g^{-1} e rendimento de xilitol ($Y_{X/X}$) de 0,39 g g^{-1} e com o uso de sacarificação e co-fermentação simultânea produziu-se 14,5 e 3 g L^{-1} de etanol e xilitol, respectivamente. No hidrolisado de casca de soja (SHH), testou-se a capacidade das celulases provenientes do fungo *Penicillium echinulatum* S1M29, em aumentar a quantidade de açúcares no meio de hidrolisado. O rendimento de sacarificação foi de 72 %, quando foi utilizado 15 FPU g^{-1} de matéria seca, incubado num agitador orbital a 120 rpm, 50 ºC durante 96 h. Após a sacarificação, a capacidade das células imobilizadas de *S. cerevisiae, C. shehatae, S. arborariae,* ou a combinação de *C. shehatae, S. arborariae* com *S. cerevisiae,* para a conversão de açúcares presentes em SHH como substrato para a produção de etanol foi estudada. Os melhores coeficientes de rendimento de etanol ($Y_{P/S}$) foram de 0,45, 0,47 e 0,38, utilizando culturas puras de *S. cerevisiae*, *C. shehatae*, e *S. arborariae* respectivamente, e $Y_{P/S}$ de 0,48 e 0,40 g g^{-1}, para co-culturas de *S. cerevisiae* e *C. shehatae* ou *S. arborariae*, respectivamente. As leveduras com os melhores rendimentos de etanol (*S. cerevisiae* e *C. shehatae*) tiveram seu metabolismo testado em biorreatores imobilizados. Estas culturas em biorreatores produziram um rendimento do etanol de 0,49, para *S. cerevisiae* e 0,41 g g^{-1} usando *C. shehatae*. Visando a melhora do processo de fermentação do hidrolisado de casca de soja (HCS), realizaram-se experimentos estatísticos (Plackett-Burman e CCD), para diferentes condições operacionais e formulações do meio. Com o Plackett-Burman testou-se os efeitos da suplementação com quatro nutrientes (peptona, extrato de levedura, milhocina e Tween 80). Através do planejamento fatorial composto central (CCD) com quatro repetições no ponto central e seis pontos axiais, analisou-se os efeitos das condições de fermentação (temperatura, pH e tamanho do inóculo) para a produção de etanol por *C* .

guilliermondii. Os resultados demonstraram que nenhuma suplementação do meio foi necessária, sendo *C. guilliermondii* capaz de crescer em hidrolisado não-suplementado e não-desintoxicado. As melhores condições de cultura foram determinadas pelo CCD como sendo de 28 °C, pH 5.0, e 10^9 UFC ml^{-1} de tamanho do inóculo, respectivamente. O coeficiente de produtividade de etanol atingiu um máximo de 1,4 g L^{-1} h^{-1} cerca de 80 % do rendimento teórico esperado, resultando em um coeficiente de rendimento de etanol ($Y_{P/S}$) de 0,41 g g^{-1}.

Palavras chave: resíduos agroindustriais; bioetanol; xilitol; co-culturas; sacarificação enzimática.

ABSTRACT

The lignocellulosic agroindustrial residues such as rice hull and soybean hull are abundant and inexpensive wastes and can be used in biotechnological production of high value-added compounds such as ethanol and xylitol, like sources of cellulose and hemicellulose. In this paper was tested the ability of converting sugars from these wastes by different yeasts, using the knowledge about the biotechnological production of alcohols. 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. New technologies to produce ethanol from RHH were tested, with the simultaneous saccharification and co-fermentation by *S. cerevisiae*, *Spathaspora arborariae* and the combination of these yeasts. In bioreactor cultures under oxygen limitation, *S. cerevisiae* was capable of metabolizing glucose from RHH, which contained small amounts of acetic acid, furfural, and hydroxymethylfurfural, achieving ethanol yields of 0.45. In the co-culture of *S. cerevisiae* and *S. arborariae* pentoses and hexoses from RHH, were converted to ethanol and xylitol, with yields of 0.48 and 0.39, and using simultaneous saccharification and co-fermentation with both yeasts produced ethanol and xylitol to final concentrations of 14.5 g L^{-1} and 3 g L^{-1}, respectively. In soybean hull hydrolysate (SHH), was studied the ability of cellulase from *Penicillium echinulatum* S1M29, to increase the amount of sugars in the hydrolysate medium. The saccharification yield was 72 % using 15 FPU g^{-1} dry matter on orbital shaker at 120 rpm, 50 °C for 96 h. After saccharification, the ability of immobilized cells of *S. cerevisiae, C. shehatae*, *S. arborariae*, or a combination of *C. shehatae*, *S. arborariae* with *S. cerevisiae* for the conversion of sugars present in SHH as a substrate for ethanol production was studied. In shaker cultivations, the bioconversion of SHH into ethanol showed yields ($Y_{P/S}$) of 0.43, 0.47, and 0.38, in cultures of *S. cerevisiae, C. shehatae*, and *S.arborariae*, respectively. Co-cultures of *S. cerevisiae* and *C. shehatae* or *S. cerevisiae* and *S. arborariae*, produced $Y_{P/S}$ of 0.48 and 0.40, respectively. *S. cerevisiae* and *C.shehatae* were immobilized in Ca-alginate and cultivated in bioreactors to analyse the possibility of scaling up this process. Immobilized-cell cultures showed yields of 0.45 and 0.38, respectively. Aiming to improve the fermentation of soybean hull hydrolysate (HCS), operational conditions and medium formulation were optimized using statistical experimental designs (Plackett-Burman and CCD). Plackett-Burman was used to analysate the effects of supplementation with four nutrients (peptone, yeast extract, corn steep liquor and Tween 80). Using factorial central composite design (CCD) with four replications at the center point and six axial points, was examined the effects of fermentation conditions (temperature, pH, and inoculum size) for ethanol production by *Candida guilliermondii* BL13. Results showed that *C. guilliermondii* was capable of growing in non-supplemented, non-detoxified hydrolysate, and the best culture conditions were determined to be 28 °C, pH 5.0, and 109 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 % of expected theoretical yields, were observed.

Keywords: agroindustrial waste; bioethanol; xylitol; cocultures; enzymatic saccharification. .

Capítulo 1

Introdução

A preocupação com a exaustão das reservas de combustíveis fósseis, a flutuação no preço do petróleo e as mudanças climáticas cada vez mais evidentes estão levando à busca por fontes de energia renováveis, tais como os biocombustíveis. Neste contexto, o etanol de 2ª geração (produzido a partir de materiais lignocelulósico) recebe grande atenção, devido aos baixos custos da matéria-prima e à possibilidade do uso deste etanol em mistura com a gasolina.

Grandes quantidades de resíduos lignocelulósicos são acumuladas no meio ambiente decorrentes da produção agrícola e do beneficiamento de cereais. Apenas destes resíduos, estima-se que 491 milhões de litros de etanol por ano poderiam ser produzidos. A conversão de resíduos agroindustriais mediante processos biotecnológicos permite, também, o desenvolvimento de tecnologia e ciência em países em desenvolvimento, onde este tipo de material é mais abundante justamente pelo fato de esses países apresentarem grandes produções agrícolas, matérias-primas e baixas produções de materiais manufaturados, cujo valor agregado é superior.

Os resíduos lignocelulósicos agroindustriais são compostos principalmente por polímeros conhecidos, celulose e hemicelulose, e passíveis de conversão a etanol, através de hidrólise química e/ou posterior uso de bioprocesso. Uma visão geral das publicações envolvendo estes resíduos aponta um aproveitamento regional de produtos, verificando-se produções científicas empregando subprodutos do processamento do milho por grupos norte-americanos; e do bagaço de cana-de-açúcar no Brasil. Porém, no Brasil, além da cultura da cana que se concentra principalmente na região sudeste do país, há a cultura de milho, de soja, de trigo, de algodão, entre outras. O estado do Rio Grande do Sul (RS), por exemplo, é um dos maiores estados produtores da cultura da soja e do arroz.

A fim de viabilizar economicamente a produção de etanol a partir de resíduos lignocelulósicos, o conceito de biorrefinaria torna-se importante. A biorrefinaria visa aproveitar as sinergias entre a produção de biocombustíveis e outros bioprodutos de alto valor agregado, aumentando a diversidade econômica do processo. A fermentação destes hidrolisados lignocelulósicos em produtos com diferentes proporções de um ou de outro, como no caso do etanol e/ou xilitol, requer a presença de microrganismos que fermentem todos os açúcares presentes no meio.

O objetivo principal de desenvolvimento desta tese foi contribuir para o desenvolvimento da tecnologia na produção de biocombustíveis de segunda geração através do estudo da bioconversão dos açúcares provenientes do hidrolisado de casca de arroz e soja a etanol e xilitol através do uso de diferentes tecnologias de bioprocesso.

Os objetivos específicos foram:

Estudar a composição química da casca de arroz e da casca de soja;

Testar a hidrólise ácida da casca de arroz, em autoclave, visando aumento na liberação de açúcares;

Avaliar a produção de etanol, xilitol e biomassa e o consumo de açúcares em hidrolisados de casca de soja e arroz por leveduras;

Aperfeiçoar e estudar os parâmetros das cinéticas de crescimento, formação de produto e consumo de substrato em agitador orbital, utilizando diferentes microrganismos fermentadores de glicose e xilose;

Testar esferas de alginato de cálcio como suporte para imobilização de(s) microrganismo(s), bem como sua resistência ao hidrolisado ácido e enzimático em hidrolisado de casca de soja;

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

Avaliar a utilização de hidrólise enzimática e fermentação em separado, hidrólise e fermentação simultânea, com o uso de culturas de células livres, co-culturas e testes com culturas imobilizadas.

Capítulo 2

Revisão Bibliográfica

A revisão bibliográfica desta tese visa introduzir assuntos de grande importância no cenário de conversão da biomassa lignocelulósica, especificamente casca de arroz e de casca de soja, a etanol e xilitol por leveduras.

2. 1. Biomassa

Com a crescente instabilidade dos preços do petróleo do Oriente Médio, muitos países decidiram optar por uma política energética baseada na utilização de biocombustíveis. Isso inicia uma pressão para a produção de tecnologias que fortaleçam a produção de etanol de 2ª geração. Para demonstrar o crescimento do interesse nesta área de pesquisa, diversas empresas estão implementando, apenas nos Estados Unidos da América (EUA) com plantas piloto, a utilização de biomassa celulósica para a produção de etanol de segunda geração, e diversas outras já estão com tecnologia e processos consolidados e em pleno funcionamento (Tabela 1) (BASTOS, 2012).

No Brasil, também há iniciativas da Petrobrás Biocombustíveis que desde 2004 vem evoluindo de forma consistente na tecnologia de conversão do bagaço da cana-de-açúcar a etanol, através de parcerias com instituições científicas e empresas de tecnologia nacionais e internacionais, permitindo a produção de 80 mil litros de etanol de segunda geração em uma planta de demonstração, permitindo um aumento na produção de até 40 % sem necessidade de ampliar a área plantada com canavial.

Tabela 3. Empresas de etanol de segunda geração em funcionamento nos EUA, tecnologia utilizada e capacidade anual (BASTOS, 2012).

Empresa	Tecnologia de conversão	Matéria-prima	Produto primário	Capacidade biocombustível (gal/ano)	Escala	Localização
Solazyme Inc.	Algas	Algas	Lipídios de algas	300.000	Piloto	Riverside, Pennsylvania
Sapphire Energy Inc.	Algas	Algas	Lipídios de algas	1.000.000	Demonstração	Columbus, NewMexico
Algenol Biofuels Inc.	Algas	Algas	Etanol	100.000	Piloto	Fort Meyers, Flórida
Lignol	Bioquímica	Recursos florestais	Etanol	2.500.000	Demonstração	Femdale, Washington
Pacific Biogasol	Bioquímica	Plantas energéticas, resíduos agrícolas	Etanol	2.700.000	Demonstração	Boardman, Oregon
Amyris Biotechnologies Inc.	Bioquímica	Plantas energéticas	Diesel renovável	1.370	Piloto	Emeryville, Califórnia
Logos Technologies	Bioquímica	Plantas energéticas, recursos florestais, resíduos agrícolas	Etanol	50.000	Piloto	Visalia, Califórnia
Abengoa	Bioquímica	Plantas energéticas, recursos florestais, resíduos agrícolas	Etanol	15.000.000	Comercial	Kugoton, Kansas
POET	Bioquímica	Resíduos agrícolas	Etanol	25.000.000	Comercial	Emmetsbrug, Iowa
ICM Inc.	Bioquímica	Plantas energéticas	Etanol	345.000	Piloto	St. Joseph, Missouri
Mascoma	Bioquímica	Recursos florestais	Etanol	40.000.000	Comercial	Kinross, Michigan
American Process Inc. (API)	Bioquímica	Recursos florestais	Etanol	894.000	Piloto	Alpena, Michigan
Archer Daniels Midland (ADM)	Bioquímica	Resíduos agrícolas	Etanol	25.800	Piloto	Decatur, Illinois
Bluefire LLC	Bioquímica	Recursos florestais, MSW	Etanol	19.000.000	Comercial	Fulton, Mississippi
Myriant	Bioquímica	Plantas energéticas	Bioprodutos	0	Demonstração	Lake Providence, Louisiana
Verenium	Bioquímica	Plantas energéticas, resíduos agrícolas	Etanol	1.400.000	Demonstração	Jennings, Louisiana
RSA	Bioquímica	Recursos florestais	Biobutanol	1.500.000	Demonstração	Old Town, Maine

Além da Petrobrás, desde 2006, o Centro de Tecnologia Canavieira (CTC) vem estudando a produção de etanol a partir do bagaço e da palha da cana, com apoio do BNDES-FINEP. Este projeto está sendo desenvolvido por meio de parceria com as empresas Novozymes e Andritz, sendo que a primeira planta deve estar funcionando em 2014 em escala semi-industrial, e a comercial, até 2018. Além disso, o Laboratório Nacional de Ciência e Energia (CTBE), com sede em Campinas-SP, vem estudando processos de obtenção de etanol celulósico, energia elétrica e coprodutos derivados da química verde de forma a aproveitar integralmente a cana-de-açúcar. Tais tecnologias visam explorar a integração com a produção atual de etanol e obedecer a critérios de viabilidade tecno-econômica e de sustentabilidade da cadeia produtiva.

No campo privado, a empresa Raízen - oriunda da união da Shell com a Cosan, com apoio da canadense Iogen e da americana Codexis, que fabricará, aproximadamente, 40 milhões de litros de etanol de segunda geração, por ano, sem precisar aumentar a área cultivada, a partir do ano de 2014. A Raízen deu início à construção de sua primeira planta industrial para a produção do biocombustível em escala comercial, localizada na unidade Costa Pinto, em Piracicaba (SP), sendo o início da sua operação está previsto para o ano-safra 2014/2015. Este bioetanol será gerado a partir dos coprodutos do processo de produção de etanol atual, incrementando a produção do combustível, inclusive no período de entressafra.

O Brasil tem atraído a atenção de empresas multinacionais, como é o caso da TMO Renewables, uma empresa britânica especializada em combustíveis renováveis. A companhia anunciou, em meados de 2013, o início de uma *joint venture* com a Usina Santa Maria para erguer a primeira fábrica de produção de bioetanol do país, que será erguida em São Paulo, tendo como matéria-prima restos de cana-de-açúcar. A produção está prevista para iniciar ainda em 2014. Enquanto a Usina Santa Maria será responsável

por erguer a planta em si, a TMO entrará com a tecnologia necessária para a fabricação do bioetanol.

O termo biocombustível é atribuído a qualquer combustível alternativo que deriva de materiais orgânicos, tais como culturas alimentícias energéticas (milho, açúcar, trigo, cana, beterraba, mandioca, entre outros), resíduos de colheita (palha de arroz, casca de arroz, palha de milho, espigas de milho) ou resíduos de biomassa (resíduos de alimentos, resíduos do gado, desperdício de papel, resíduos de construção derivados de madeira e outros) (DAS NEVES et al., 2007). No entanto, o uso de culturas alimentares para produção de bioetanol pode provocar conflito com a produção de alimentos (SOCCOL et al., 2010). Já a produção de etanol a partir de resíduos de colheita, ou de resíduos de biomassa busca evitar este conflito. Esta biomassa, devido as grandes quantidades de resíduos disponíveis, tem potencial para se tornar significativa fonte de matéria-prima (GIL et al., 2010).

A biomassa vegetal é composta por duas classes, a primeira é formada por açúcares de reserva como o amido e a sacarose, que é amplamente utilizada pelas indústrias alimentícias e de bioconversão a etanol. 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 (OCTAVE e THOMAS, 2009).

2.1.1. Materiais lignocelulósicos

O complexo lignocelulósico é o biopolímero mais abundante na Terra (SÁNCHEZ e CARDONA, 2008). A biomassa lignocelulósica representa uma matéria-prima abundante para produção de etanol, não concorrendo com o uso alimentício, como a cana e o milho. Estes materiais são amplamente disponíveis a baixo custo e

renováveis sendo considerados ideais para a produção de etanol (JONNSON et al., 2007). Estima-se que a biomassa lignocelulósica compreenda cerca 50 % da biomassa do mundo (CLAASSEN et al., 1999). Uma estimativa feita por KIM e YUM (2006) relata que são gerados 1,5 trilhões de toneladas de biomassa lignocelulósica por ano, tornando-a uma fonte inesgotável de matéria-prima para formação de bioprodutos, como o etanol e o xilitol. Este 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 processamento em indústrias.

Um grande número de estudos para o desenvolvimento de uma produção de etanol de segunda geração em grande escala tem sido realizado em todo o mundo (SIVAKUMAN et al., 2008). No entanto, o principal fator limitante é o grau de complexidade inerente ao tratamento desta matéria-prima. Isto está relacionado com a natureza e composição da biomassa lignocelulósica. Dois dos principais polímeros da biomassa devem ser quebrados em açúcares fermentescíveis a fim de serem convertidos em etanol ou outro subproduto (SÁNCHEZ e CARDONA, 2008). A biomassa bruta é composta principalmente de celulose, hemicelulose, lignina e proteínas. Estes polímeros estão associados uns aos outros em uma matriz heterogênica, em diferentes graus de polimerização, variando a composição relativa de acordo com o tipo, a espécie e fonte da biomassa.

A celulose é o principal constituinte da parede celular da planta conferindo suporte estrutural e está também presente em bactérias, fungos e algas. A celulose é um homopolímero extremamente ordenado de celobiose, o qual é dímero de glicose, que

possui em sua extensão cerca de 10.000 unidades glicosídicas formando uma cadeia rígida em forma de fibras (CHANDEL et al., 2011), demostrado na Figura 1.

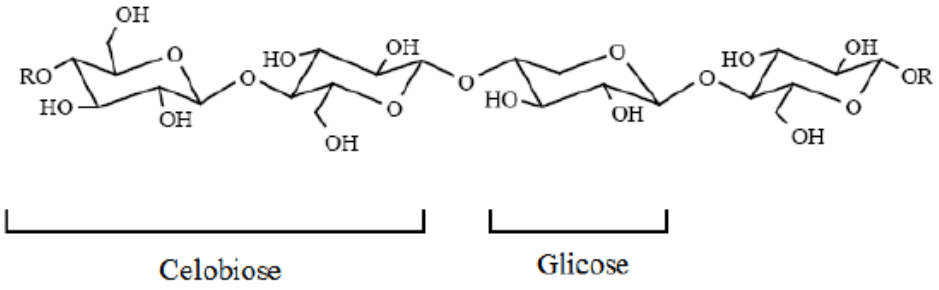


Figura 1. Estrutura da celulose, parte central da cadeia molecular (adaptado de FENGEL e WEGENER, 1989).

A hemicelulose por sua vez é um heteropolímero menor, com grau de polimerização entre 100 e 200 e possui muitas ramificações contendo diferentes carboidratos como a xilose, arabinose, manose, galactose, glicose, assim como ácidos urônicos (Figura 2). Dependendo da predominância do tipo de açúcar, as hemiceluloses podem ser chamadas de arabino-xilanas, mananas, glucanas ou galactanas. Esses açúcares contêm cinco (pentoses) ou seis (hexoses) carbonos em sua estrutura e são unidos por ligações glicosídicas do tipo 1-3, 1-4 e 1-6, quase sempre acetiladas, formando uma estrutura fraca e hidrofílica que serve como uma conexão entre a lignina e as fibras de celulose, além de conferir rigidez ao complexo celulose-hemicelulose-lignina (CHANDEL et al., 2011; STAMBUK et al., 2008).

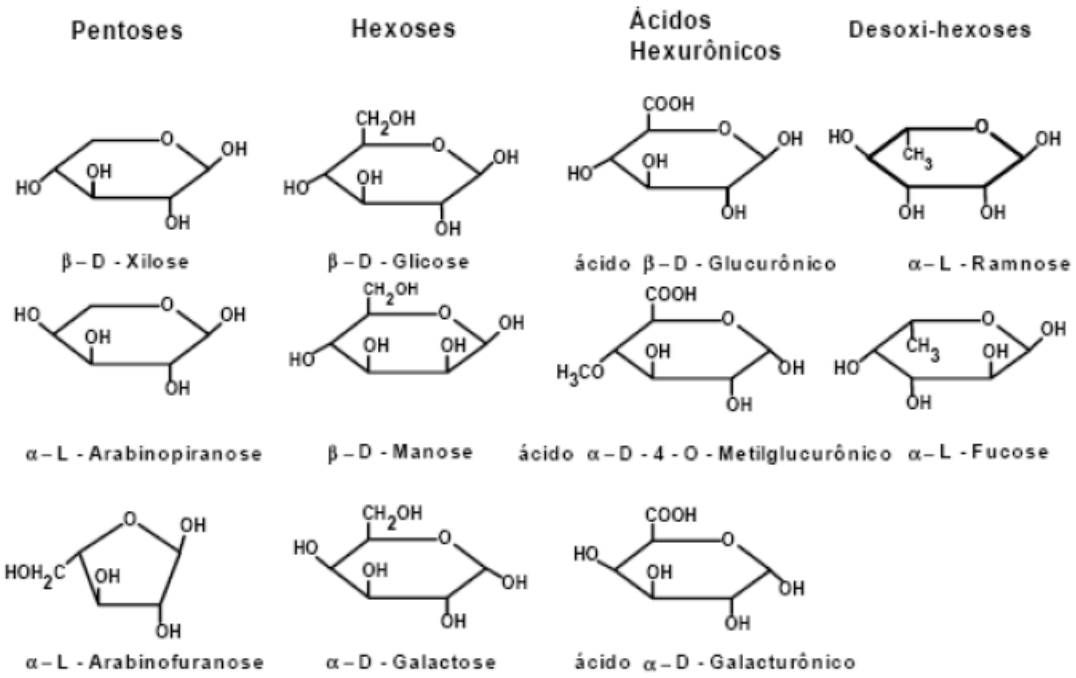


Figura 2. Estrutura dos monossacarídeos que formam as hemiceluloses (adaptado de FENGEL e WEGENER, 1989).

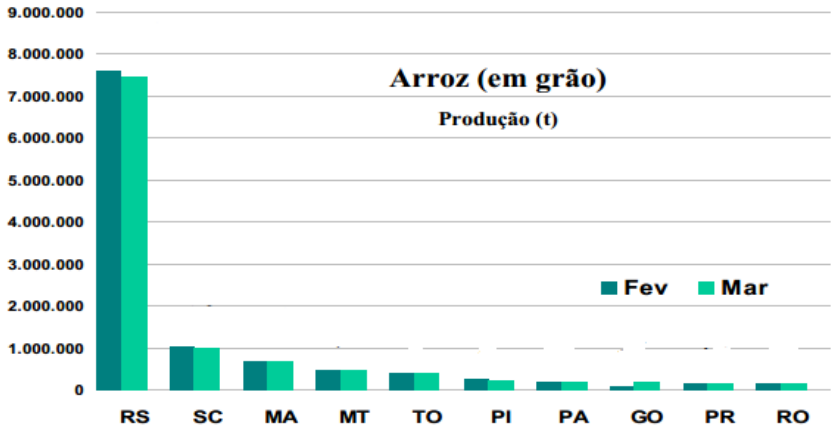
A lignina, que também faz parte da composição dos materiais lignocelulósicos, é uma macromolécula tridimensional composta basicamente por unidades de fenilpropano, que se processa por via radicalar a partir da reação de três diferentes álcoois cinamílicos precursores (guaiacil, siringil e p-hidroxifenil). Na parede celular, a lignina está associada às polioses através de interações físicas e ligações covalentes. O fato de a lignina envolver as células funcionando como uma "cola" dificulta a biodegradação, protege a planta contra o ataque de microrganismos e confere coesão à estrutura interna além de resistência ao esforço mecânico (FENGEL e WEGENER, 1989; READING et al., 2003).

A Tabela 2 traz a composição de diferentes materiais lignocelulósicos utilizados na produção de etanol de segunda geração.

Tabela 2. Composição de diferentes materiais lignocelulósicos.

Material lignocelulósico	Celulose (%)	Hemicelulose (%)	Lignina (%)	Bibliografia
Bagaço de malte	17	28	23	MUSSATTO e ROBERTO, 2006
Casca de soja	38	10	3	MIELENZ et al., 2009
Casca de arroz	35	12	15	SAHA et al., 2005
Palha de arroz	35	22	12	YADAV et al., 2011
Palha de trigo	34	24	18	GUERRA-RODRIGUÉZ et al., 2012
Bagaço de cana	39	26	24	LASER et al., 2002

Entender a estrutura do material lignocelulósico de interesse e sua composição é um fator determinante para o sucesso da pesquisa de etanol de segunda geração. O Brasil tem uma diversidade grande em resíduos agroindustriais que podem ver convertidos a etanol de 2ª geração. Por exemplo, somente para a produção de etanol a partir de cana-de-açúcar são esmagadas 287,6 milhões de toneladas de cana-de-açúcar para produção de 22.857,6 bilhões de litros de etanol, 17,2 % menor que a produção da safra 2010/11, sendo o estado de São Paulo o maior produtor (MAPA/CONAB, 2012). A cada ano são produzidas toneladas de resíduos oriundos dessa cultura, o chamado bagaço de cana, o qual é massivamente estudado para bioconversão a etanol por grupos de pesquisa do sudeste brasileiro. Estados como o Rio Grande do Sul (RS), não participam do mercado alcooleiro e não produzem bagaço de cana com grande expressão. Em compensação, o RS é um dos maiores produtores de soja e arroz do Brasil e, consequentemente, de resíduos provenientes do processamento desse grão. A produção de arroz no país foi de 15 milhões de toneladas, sendo o RS, o principal estado produtor, com 63,3 % da produção nacional (IBGE, 2012). A Figura 3 nos mostra a proporção em comparação com outros estados brasileiros.

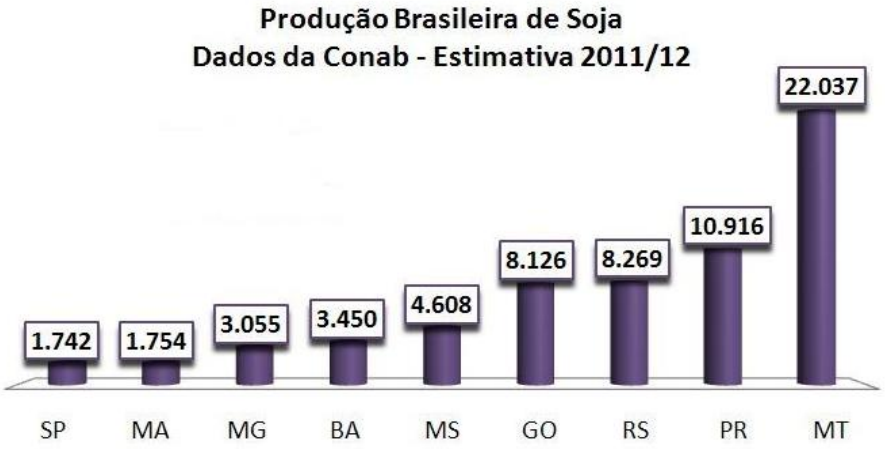


Fonte: IBGE - Produção Agrícola Arroz, 2012.

Figura 3. Produção de arroz em toneladas nos meses de fevereiro e março de 2012 nos diferentes estados brasileiros.

A casca de arroz representa 20 % (fração de massa) do arroz colhido, sendo um dos mais abundantes resíduos lignocelulósicos, representando mais de 120 milhões de toneladas geradas por ano (YU et al., 2009). A casca de arroz torna-se, neste contexto, um resíduo muito abundante e de baixo valor agregado pelo seu baixo valor como ração animal, pois é de difícil digestibilidade por animais, possui baixa densidade, grande quantidade de cinzas/sílica como componentes e características abrasivas (SAHA e COTTA, 2008).

Quanto à produção de soja, o Brasil é o segundo maior produtor e exportador de soja no mundo, sendo superado apenas pelos EUA. A produção anual é de aproximadamente 68 milhões de toneladas. O Rio Grande do Sul é o terceiro maior produtor de soja no Brasil, perfazendo 20 % da produção de soja, ficando atrás do estado de Mato Grosso e Paraná (Figura 4) (MAPA/CONAB, 2012).



Fonte: MAPA/CONAB – Acompanhamento da safra 2011/2012.

Figura 4. Produção de soja em toneladas nos principais estados plantadores do grão.

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). Considerando como exemplo a produção mundial de soja em 2008, de 210,6 milhões de toneladas (EMBRAPA, 2009), foram geradas naquele ano em todo o mundo, aproximadamente, 17 milhões de toneladas de casca de soja, o que torna esse resíduo lignocelulósico um dos mais expressivos em volume e uma alternativa interessante para bioconversão do seu hidrolisado a produtos de alto valor agregado.

2.1.1.1. Pré-tratamentos de materiais lignocelulósicos

A fim de liberar os açúcares fermentescíveis presentes na biomassa lignocelulósica, que são fundamentais para a bioconversão em etanol e outros produtos de química fina, é necessário hidrolisar este material, quer através da utilização de

produtos químicos (particularmente eficaz sobre a fração de hemicelulose), enzimas (principalmente para a hidrólise da celulose), ou uma combinação de ambas as abordagens (SCHIRMER-MICHEL et al., 2008). Há uma série de características essenciais para que o pré-tratamento da biomassa lignocelulósica se torne eficaz, como por exemplo, ter um baixo custo capital e ser operacional. Deve ser eficaz a uma vasta gama de diferentes materiais lignocelulósicos e deve resultar na recuperação da maioria dos açúcares presentes nos mesmos. A necessidade de preparação / manipulação ou etapas de pré-condicionamento, como redução de tamanho, deve ser minimizado (AGBOR et al., 2011).

A conversão de qualquer biomassa lignocelulósica a etanol segue uma metodologia semelhante, que inclui um pré-tratamento para melhorar a acessibilidade dos polissacarídeos da parede celular de hidrólise, podendo ser seguido ou não por uma hidrólise enzimática dos polissacarídeos em açúcares componentes e, finalmente, a fermentação dos açúcares monoméricos em etanol, como demonstrado na Figura 5 (SIVAKUMAN et al., 2008). Num processo típico de pré-tratamento por hidrólises para a produção de etanol 2ª geração, a hemicelulose é despolimerizada em pentoses (xilose e arabinose, predominantemente), enquanto que a celulose é convertida em hexoses, basicamente em glicose e manose (CHEN et al., 2012).

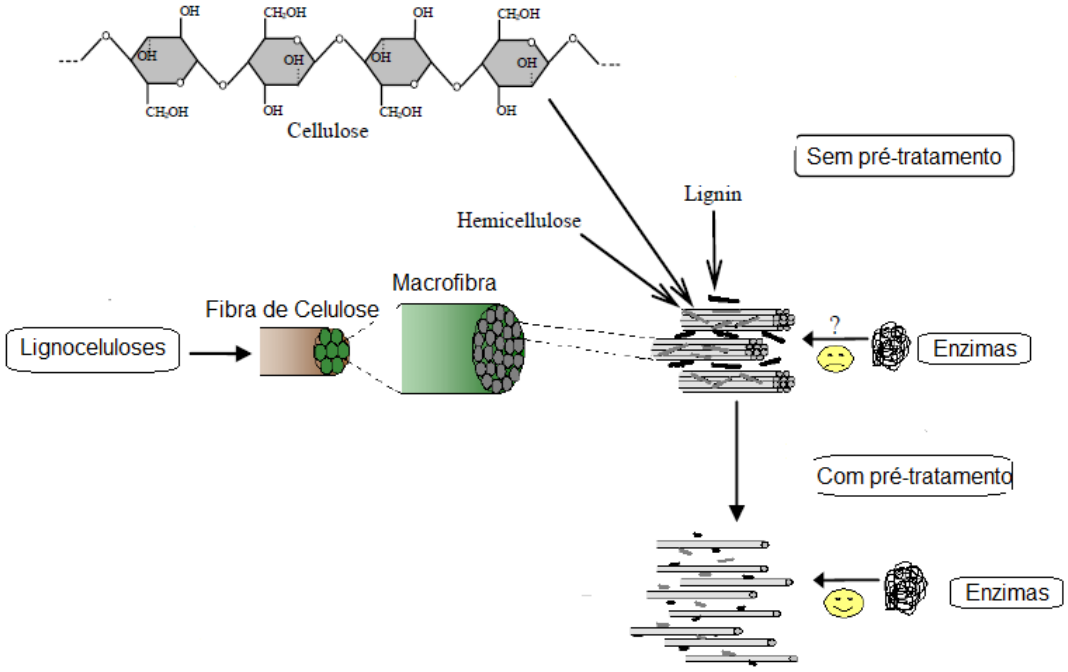


Figura 5. Estrutura do material lignocelulósico e o efeito do pré-tratamento sobre a acessibilidade enzimática (Adaptado de TAHERZADEH e KARIMI, 2008).

Os pré-tratamentos se fazem necessários, pois provocam efeitos como o aumento na acessibilidade na área de superfície, redução na cristalização da celulose, despolimerização parcial da celulose, solubilização da hemicelulose e/ou lignina e a modificação da estrutura da lignina (MARGEOT et al., 2009).

Diferentes métodos de pré-tratamento estão sendo testados ao longo dos últimos anos, porém ainda constata-se uma necessidade de desenvolver alternativas tecnológicas eficientes em termos de custo global e competitividade econômica. O pré-tratamento é atualmente uma das etapas mais caras na tecnologia de etanol de segunda geração, aumentando os custos operacionais. Ele é, contudo, essencial para garantir bons rendimentos finais de açúcares. Por exemplo, sem tratamento enzimático prévio geralmente menos de 20 % dos açúcares é disponibilizado para posterior fermentação, e

com o uso de hidrólise enzimática este rendimento pode subir até cerca de 90 % (HAYES et al., 2009).

Segundo TAHERZADEH e KARIMI, 2008, um eficaz e econômico pré-tratamento deve atender aos seguintes requisitos: (a) produção de fibras celulósicas reativos para ataque enzimático, (b) evitar a destruição da hemicelulose e celulose, (c) evitar a formação de possíveis inibidores de enzimas hidrolíticas e microorganismos que fermentadores, (d) minimizar a energia gasta, (e) reduzir o custo na redução de tamanho de matérias-primas, (f) redução do custo de material usado na construção de reatores de pré-tratamento, (g) produzir poucos resíduos, (h) o consumo de pouca ou nenhuma química ou utilizar um produto químico barato.

Os pré-tratamentos podem ser divididos em três principais grupos: químicos, físicos e/ou biológicos.

2.1.1.2. Pré-tratamentos químicos

Os pré-tratamentos químicos caracterizam-se pela combinação de alguns produtos químicos com a matéria prima, ou então com a utilização de apenas um, porém aliado às altas temperaturas e pressões para torná-lo realmente efetivo.

Entre os vários pré-tratamentos químicos existentes está o pré-tratamento por ozonólise, onde o ozônio é utilizado para degradar a lignina e a hemicelulose dos materiais lignocelulósicos. Este possui como vantagens: (1) efetivamente remove a lignina; (2) não produz resíduos tóxicos para bioprocessos; e (3) as reações são

realizadas em temperatura e pressão ambientes (VIDAL e MOLINIER, 1988). No entanto, uma grande quantidade de ozônio é necessária, tornando o processo inviável economicamente.

Vários solventes, tais como etanol e metanol também podem ser aplicados para remover lignina em processos conhecidos como *organosolv*, mas os custos com os solventes e com a recuperação dos produtos finais o torna inviável. No processo de organosolv, um solvente orgânico misturado com catalisadores ácidos inorgânicos (HCl ou H_2SO_4) é utilizado para quebrar a lignina e desmembrar a hemicelulose (SUN e CHENG, 2002).

O pré-tratamento alcalino é usado frequentemente para aumentar a digestibilidade de materiais lignocelulósicos. Este processo foi desenvolvido originalmente na indústria de papel e celulose nos processos de polpação formar papel de fibra longa, sendo indicado, especialmente quando se trabalha com palhas ou cascas, devido ao alto conteúdo de lignina presentes nestas (MOSIER et al, 2005). A desvantagem deste processo está relacionada com o preço de soda cáustica e a dificuldade da sua recuperação (SUN e CHENG, 2002). Além disso, na maior parte dos métodos de deslignificação, parte da hemicelulose também é hidrolisada, com isso, a deslignificação não mostra efeito exclusivo na lignina (HOSSEINI et al., 2013). Uma alternativa conhecida também como pré-tratamento alcalino é a utilização simultânea de uma base com o peróxido. A deslignificação de materiais lignocelulósicos com peróxido de hidrogênio depende fortemente do pH, uma vez que a sua dissociação ocorre em valores de pH cerca de 11,5. Esta dissociação resulta na formação de radicais altamente reativos, que agem com a molécula de lignina e conduz à sua solubilização e oxidação. Algumas variações deste processo envolvem duas fases, a primeira utilizando soda cáustica e a segunda o peróxido. A deslignificação oxidativa com peróxido ocorre a

baixas temperaturas (25 – 40ºC), e, como regra geral, os resíduos gerados têm uma baixa carga poluente (HAMMELINK et al., 2005).

Dentre os pré-tratamentos químicos, o realizado com ácido diluído e temperaturas intermediárias é considerado um dos mais eficazes em relação custo-benefício, e atua causando 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, inibindo o crescimento de microrganismos fermentadores que serão utilizados posteriormente a esta etapa (MUSSATO e ROBERTO, 2004). Segundo PALMQVIST e HAHN-HÄGERDAL (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 (Figura 6). 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, como ácidos siríngico, vanílico, palmítico.

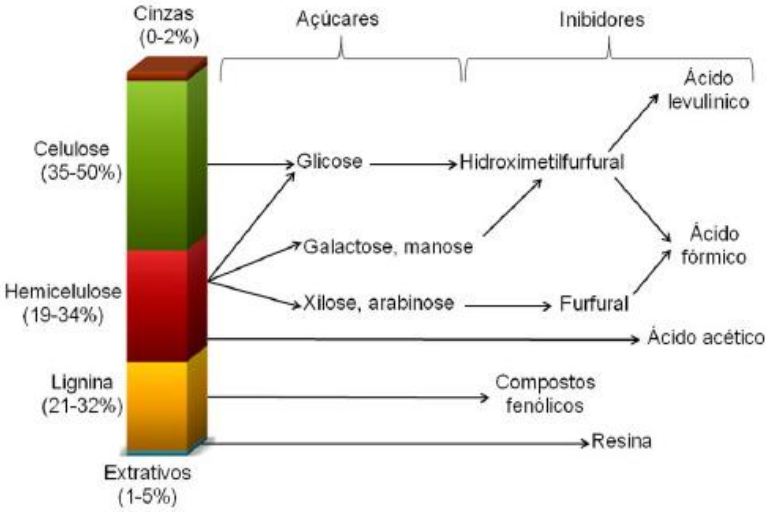


Figura 6. Formação dos principais inibidores do processo fermentativo (Adaptado de PALMQVIST e HAHN-HÄGERDAL, 2000).

2.1.1.3. Pré-tratamentos físicos-químicos

Os tratamentos físicos podem ser desde diferentes tipos de moagem, até tratamentos físicos associados aos químicos, onde se incluem a explosão a vapor, extração por água líquida quente (LHW) e explosão de fibra com amônia (AFEX) (SUN e CHENG, 2002).

No processo de pré-tratamento por explosão a vapor o material lignocelulósico é sujeito a tratamento a vapor a temperaturas variando de 180, 200 ºC ou superiores, por alguns minutos, etapa posterior ou não à impregnação por catalisadores como ácido sulfúrico e dióxido de enxofre. Na ausência de catalisadores, ocorrem reações de autohidrólise, nas quais o ácido acético proveniente da biomassa age como catalisador das reações de hidrólise da hemicelulose por meio da diminuição do pH do meio (CARRASCO et al., 2010).

A AFEX (*Ammonia Fiber Explosion*) é um tipo de pré-tratamento físico-químico em que os materiais lignocelulósicos, são expostos à amônia líquida em alta pressão e temperatura por um período de tempo e em seguida, a pressão é reduzida

rapidamente. Em um processo típico AFEX, a dosagem de amoníaco líquido é 1-2 kg de amônia/kg de biomassa seca, a temperatura de 90 º C, e tempo de residência de 30 minutos. AFEX não é capaz de solubilizar significativamente a hemicelulose na comparação com a pré-tratamento ácido.

2.1.1.4. Pré-tratamentos biológicos

Além desses processos, ainda existe o tratamento de deslignificação biológica e a própria hidrólise enzimática da celulose, conhecidos como tratamentos biológicos, que necessitam de maior tempo de processo e possuem alto custo de produção (SUN e CHENG, 2002). O pré-tratamento biológico com enzimas, que vem sendo muito utilizado para quebra dos polissacarídeos e liberação de glicoses principalmente, é um método seguro e ambientalmente correto, com grandes porcentagens de conversão (YU et al., 2009).

O tratamento enzimático gera rendimentos mais elevados de glicose no meio, além de formação reduzida de compostos tóxicos, quando comparado à hidrólise ácida. No entanto, se as enzimas celulolíticas são adicionadas à celulose nativa, a conversão da celulose em açúcar será extremamente lenta, uma vez que a celulose é bem protegida pela matriz de hemicelulose e lignina. Portanto, o pré-tratamento da matéria-prima é igualmente necessário para expor a celulose ou modificar os poros do material, e permitir que as enzimas penetrem nas fibras e hidrolisem a celulose em açúcares monoméricos (GALBE e ZACCHI, 2002). Neste caso, o pré-tratamento utilizando ácido diluído pode solubilizar eficazmente hemicelulose em açúcares monoméricos (arabinose, galactose, glucose, manose e xilose) e oligômeros solúveis, melhorando assim a conversão de celulose pelo uso de enzimas (CHANDEL et al., 2011).

ARANTES e SADDLER, 2010, esquematizaram o mecanismo de como as enzimas (celulases) atuam na hidrólise enzimática. Inicialmente, a celulase precisa estar adsorvida na superfície da celulose insolúvel, pós-hidrólise ácida branda, quando a parte inacessível do substrato é estruturalmente afrouxada, aumentando a desordem molecular e expondo as cadeias de celulose enterradas dentro das microfibrilas (Figura 7-A). Uma vez que a rede de celulose é acessível às enzimas, a ação sinérgica de endo e exo-glucanases promove a fragmentação de moléculas acessíveis aos celo-oligossacarídeos (moléculas de celulose com um grau de polimerização menor que 6 unidades) (Figura 7-B), que são rapidamente hidrolisados, principalmente a celobiose (Figura 7-C). A celobiose é o produto da hidrólise da celulose primária. Em sistemas comerciais de celulase, uma fonte extrínseca de β-glucosidase é geralmente adicionada para hidrolisar a celobiose para glicose (Figura 7-D), aumentando a eficiência de conversão enzimática.

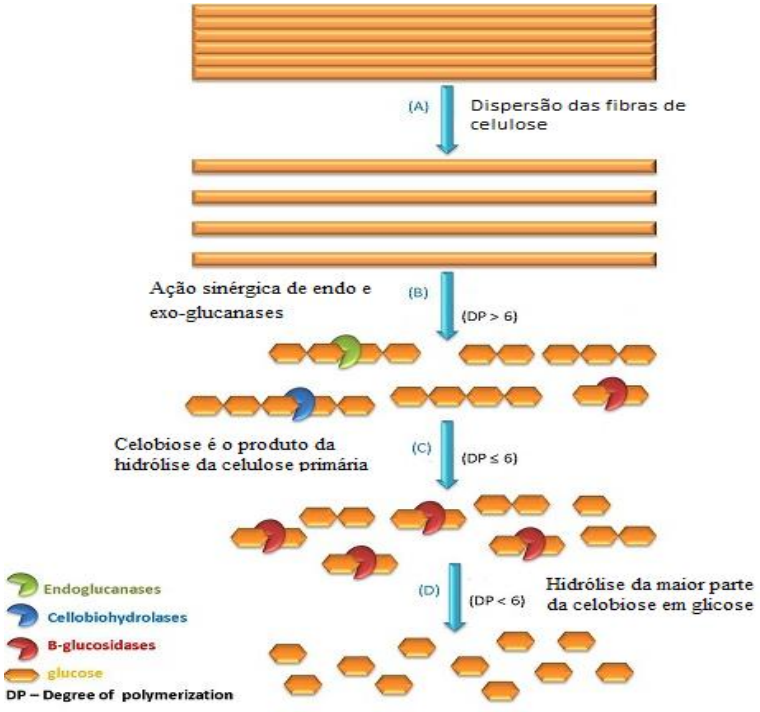


Figura 7. Mecanismo para despolimeração da celulose por celulases (Adaptado de ARANTES e SADDLER, 2010).

O custo da hidrólise, em particular para o processo enzimático, é um fator importante. A produção de etanol requer uma hidrólise completa da celulose e da

hemicelulose com um mínimo de degradação de açúcar, seguido por uma eficiente fermentação de todos os açúcares contidos na biomassa. No curto prazo, alguns co-produtos são susceptíveis de serem utilizados para a produção de calor combustível ou em eletricidade, no entanto, a longo prazo, a tecnologia de bioetanol será a base para o desenvolvimento sustentável na produção de *commodities* químicas e materiais das futuras biorrefinarias. Uma alta viabilidade econômica pode ser alcançada quando o calor gerado (energia) pelas queimas dos sólidos resultantes da hidrólise for combinado com o processo intensivo de hidrólise e fermentação em uma integração do sistema (por exemplo, pré-tratamento, destilação, evaporação) (HAHN-HAGERDAL et al., 2006).

2.2. Bioconversão de hidrolisados lignocelulósicos a etanol

A fermentação de hidrolisados lignocelulósicos a etanol requer a presença de 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 (MARGEOT et al., 2009). Uma eficiente conversão de todos os açúcares presentes nos hidrolisados lignocelulósicos a etanol é um pré-requisito para maximizar a rentabilidade de um processo industrial e melhorar a competitividade de custos da produção de bioetanol (FU et al., 2009; KUHAD et al., 2011).

A conversão de hexoses geralmente é feita pela levedura *Saccharomyces cerevisiae* que é um dos microrganismos mais utilizados na produção de etanol de milho, melaço e cana-de-açúcar, devido à sua alta eficiência na fermentação de hexoses e tolerância superior ao etanol e ao baixo pH. Além disso, possui alta tolerância aos

compostos inibitórios presentes na biomassa lignocelulósica pré-tratada (KLINKE et al., 2004). No entanto, devido à falta de uma via metabólica da xilose em xilulose, *S. cerevisiae* não pode utilizar xilose, apenas se as cepas forem reconstruídas por engenharia metabólica. A fim de superar essa limitação, várias tentativas de desenvolver linhagens recombinantes de *S. cerevisiae* com base na sua capacidade para fermentar xilose em etanol têm sido relatados na literatura (MATSUSHIKA et al., 2009).

Para tornar o processo de produção de etanol de 2ª geração viável economicamente, a bioconversão das hemiceluloses em açúcares fermentescíveis é essencial. O método mais promissor para a hidrólise de polissacarídeos em açúcares monoméricos é através da utilização de enzimas, isto é, as celulases e hemicelulases.

A conversão microbiana a etanol das pentoses, que representam 25 – 40 % dos açúcares totais presentes na biomassa, é identificada como um dos principais desafios da pesquisa para a tecnologia do etanol de segunda geração (FROMANGER et al., 2010). O açúcar xilose (pentose) é o principal monossacarídeo liberado a partir de hidrolisados de plantas. Muitas leveduras são conhecidas por metabolisar xilose, incluindo gêneros como *Candida*, *Scheffersomyces (Pichia)*, *Hansenula*, *Debaryomyces*, *Schawannyomyces*, entre outras. A Figura 8 representa a via metabólica da fermentação de xilose a etanol por microrganismos.

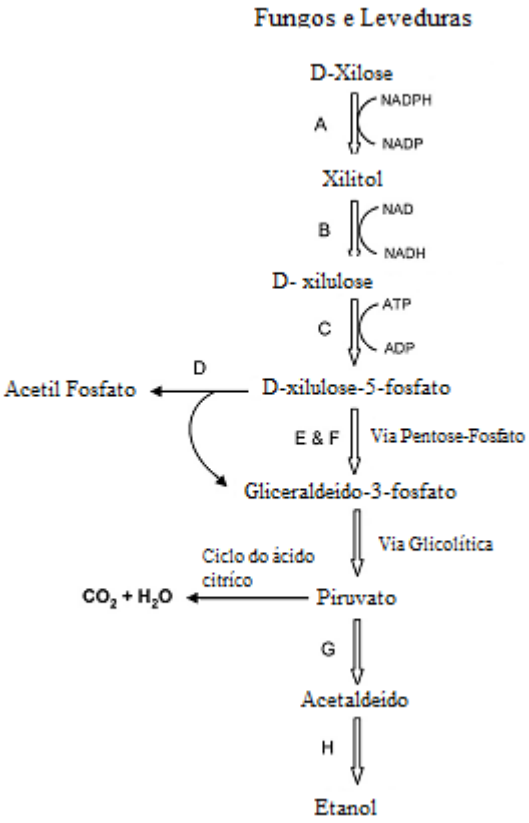


Figura 8. Diagrama esquemático do metabolismo de microrganismos fermentadores de xilose a etanol (adaptado de KUHAD et al., 2011).

As espécies de leveduras conhecidas como melhores fermentadoras de xilose a etanol são *Scheffersomyces (Pichia) stipitis*, *Pachisolen tannophilus* e *Candida shehatae*, porém têm um rendimento relativamente baixo e baixa tolerância aos inibidores (TIAN et al., 2009). Alguns fatores podem afetar significantemente a taxa de utilização da xilose disponível e sua eventual conversão a etanol. Uma das mais importantes é aeração do cultivo (SAHA e WOODWARD, 1997). O processo de fermentação das pentoses não deve ocorrer em altos níveis de oxigenação, devido a esta provocar a síntese de alta massa celular (biomassa), baixo rendimento de etanol e consumo mais elevado de energia (KUHAD et al., 2011). 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. 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 ao invés da xilose, isso foi demonstrado em microrganismos como *Sphataspora arborariae*, *C. shehatae* e *Candida guilliermondii* (DA CUNHA PEREIRA et al., 2011; SCHIRMER-MICHEL et al., 2008).

A outra abordagem visando o aproveitamento total dos açúcares presentes no hidrolisado é a utilização de dois microrganismos ao mesmo tempo, o que é chamado de co-cultura (dois microrganismos são cultivados em conjunto e simultaneamente no mesmo meio). A utilização de co-culturas para a produção de etanol parece ter vantagens em relação ao uso de uma única cultura desde que haja ação sinérgica das vias metabólicas das estirpes (CHEN, 2011). A idéia central ao se usar co-cultura para a produção de etanol é combinar uma levedura fermentadora de pentoses e um microrganismo fermentador de glicose simultaneamente. Estas co-culturas podem ser livres, ou imobilizadas, e vem sendo amplamente testadas em ambas as formas (DA CUNHA-PEREIRA et al., 2011; FU et al., 2009; FU e PEIRIS, 2008).

2.3. Bioconversão de hidrolisados lignocelulósicos a xilitol

Uma alternativa de formação de produtos de alto valor agregado através de hidrolisados lignocelulósicos dentro do conceito de utilização total da biomassa é a produção de xilitol. O xilitol é um álcool de açúcar natural cinco átomos de carbono, que tem sido altamente valorizado pelas indústrias de alimentos e farmacêutica devido ao seu poder adoçante (como a sacarose) (MUSSATTO et al., 2006). O valor de mercado do xilitol é atualmente 340 milhões de dólares com aplicações em pastas de

dente, flúor e gomas de mascar, como bem como em alimentos para fins dietéticos (MARTINÉZ et al., 2012).

No entanto, a produção de xilitol por leveduras é influenciada por vários fatores, incluindo a concentração inicial de células no meio, as condições de cultura como temperatura, pH, concentração de xilose, fornecimento de oxigênio, presença de outros açúcares e presença de compostos inibidores. Por isso é de importância fundamental compreender a influência desses fatores sobre a xilose e sua bioconversão para o xilitol para o desenvolvimento de um sistema com tecnologia eficiente para a produção em larga escala por meio biotecnológico (MUSSATTO et al., 2006).

Em geral, dentre os microrganismos, 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 (*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^+ (Figura 9).

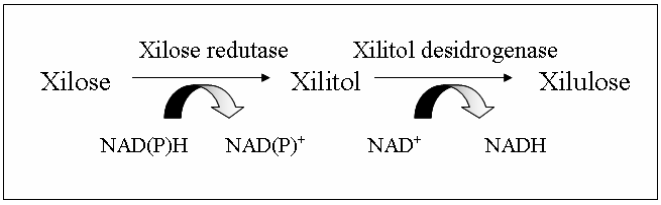


Figura 9. Representação esquemática das primeiras etapas da metabolização da xilose em leveduras.

Assim, sob condições limitadas de oxigênio, não há a completa re-oxidação de NADH a NAD^+, promovendo um desequilíbrio redox. A baixa concentração de NAD^+ diminui a oxidação do xilitol a xilulose, induzindo a liberação de xilitol ao meio de cultura (DU PREEZ et al., 1989).

Em microaerofilia, pode ocorrer a formação não só do xilitol, mas também de etanol, ou de ambos, dependendo, basicamente, dos co-fatores das enzimas envolvidas nas reações de metabolização da xilose, bem como, a regeneração de tais co-fatores (PANAGIOTOU et al., 2006). 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. De uma forma geral, o fornecimento de oxigênio é o parâmetro chave que determina se ocorrerá maior produção de xilitol ou maior crescimento celular (ROBERTO et al., 1999).

2.4. Imobilização celular

A produção de bioetanol de forma eficiente exige uma rápida fermentação, acarretando em uma alta concentração final de etanol. Neste caso, é necessário o uso de leveduras com alta taxa de crescimento em hidrolisados com elevadas atividades osmóticas, concentrações diferentes de inibidores e resistentes às concentrações finais de etanol.

Muitas estratégias vêm sendo exploradas para superar a inibição pelo produto, melhorar a tolerância ao etanol e aos inibidores presentes no meio. Entre eles, um dos mais explorados é a imobilização de leveduras na/em matriz adequada, tais como alginato de cálcio, κ-carragenan gel, poliacrilamida, γ-alumina, apara de madeira, gel de PVA, laranja casca, etc (NICOLÍC, et al., 2009). Entre as diferentes técnicas de imobilização, encontram-se quatro categorias principais com base no mecanismo físico

empregado: ligação a superfícies sólidas, aprisionamento em matrizes porosas, floculação (natural ou artificial) e contenção por membranas (KOURKOUTAS et al., 2004).

Entre as diferentes técnicas de imobilização de células, a realizada com alginato de cálcio tem sido uma das mais amplamente utilizadas para aprisionamento de células, devido à sua simplicidade e caráter não tóxico (YAN et al., 2012). O aprisionamento ocorre após a adição das células de leveduras em uma suspensão em alginato de sódio, e gota a gota ocorre o gotejamento em solução de cloreto de cálcio. Ao final, as células encontram-se imobilizadas em esferas de alginato de cálcio (ROSEVEAR, 1984).

As células imobilizadas apresentam algumas vantagens sobre células livres, tais como a relativa facilidade de separação do produto, a reutilização de biocatalisadores, produtividade volumétrica elevada, melhor controle dos processos e reduzida sensibilidade das células a contaminações (GHORBANI et al., 2011). Como principal desvantagem decorrida desses sistemas, encontram-se as limitações de transferência de massa do substrato e produtos formados.

2.5. Sacarificação e fermentação separada (SHF - *Separate Hydrolysis and Fermentation*)

A hidrólise enzimática (sacarificação) pode ser realizada separadamente da fermentação alcoólica, um processo conhecido como hidrólise e fermentação separada (*Separate Hydrolysis and Fermentation* – SHF). A hidrólise enzimática do processo SHF pode ser feita a temperaturas elevadas como 50 ºC, aproveitando a estabilidade das enzimas, que nesta temperatura aumenta taxas de conversão e minimiza a contaminação

bacteriana. A atividade máxima da celulase na maioria das vezes é observada em aproximadamente 50 ºC com um pH de 4,0 - 5,0; no entanto, as condições ótimas variam de acordo com o tempo de hidrólise e são dependentes do tipo de enzima (OLOFSSON et al., 2008).

As celulases pertencem a dois grupos de enzimas conhecidas como endoglucanases (EG) e celobiohidrolases (CBH), respectivamente. As EG atacam aleatoriamente a cadeia de celulose, criando extremidades livres para CBH clivar dímeros de glicose (celobiose). Um terceiro tipo de enzima, β-glucosidase, hidrolisa celobiose em duas moléculas de glicose (HAHN-HAGERDAL et al., 2006). TAHERZADEH e KARIMI, 2007, relataram que a inibição da atividade de celulases é causada pelo próprio produto da sacarificação, principalmente celobiose e glicose. Quando a concentração de celobiose alcança 6 g L^{-1} a atividade celulolítica das enzimas

pode diminuir até 60 % (TAHERZADEH e KARIMI, 2007). Esta inibição afeta as taxas de reação e rendimentos, sendo uma desvantagem deste sistema.

Porém, como vantagem deste sistema, está a separação fácil dos açúcares da fase líquida da lignina hidrofóbica, que pode ser usada como combustível sólido (queima), gerando energia. Como a fermentação é executada separadamente e após o passo de hidrólise, as células de levedura podem ser recicladas ou utilizadas como ração animal, uma prática usual na indústria brasileira de etanol (SOCCOL et al., 2010).

2.6. Sacarificação e fermentação simultânea (SSF - *Simultaneous Saccharification and Fermentation*)

Dentre os sistemas de produção de etanol a partir da celulose, a sacarificação e fermentação simultânea (SSF - *Simultaneous Saccharification and Fermentation*) é um dos processos mais estudados. Após o pré-tratamento, a celulose restante necessita ser hidrolisada enzimaticamente à glicose, e com a tecnologia SSF isso ocorre

concomitantemente à fermentação. Além disso, a combinação de hidrólise e fermentação diminui o número de vasos necessários e, desse modo, os custos de investimento. A diminuição no investimento de capital quando utilizado o sistema SSF foi estimada como sendo maior do que 20 % (OLOFSSON et al., 2008).

O processo de produção de etanol por SSF é mais rápida que a SHF, pois a glicose formada é fermentada simultaneamente em etanol. Além disso, o risco de contaminação é pequeno, devido à presença de altas concentrações de etanol, uso de condições anaeróbias e a retirada contínua de glicose. Neste contexto, é interessante notar que o etanol que se acumula no meio não afeta significativamente as atividades das enzimas (TAHERZADEH e KARIMI, 2007; SOCCOL et al., 2010).

Apesar da vantagem econômica do SSF frente à hidrólise e fermentação em separado (SHF), o maior problema com o SSF é a diferença de temperatura ótima das celulases e do microrganismo usado na fermentação. Linhagens de *Saccharomyces*, por exemplo, suportam uma temperatura máxima de 35 ºC. Enzimas celulases que são frequentemente aplicadas uma temperatura ideal de 50 ºC (BINOD et al., 2010). Uma possível solução para resolver este problema é usar leveduras termotolerantes que permitem altas temperaturas de processamento (KÁDÁR et al., 2004). Além disso, o uso da temperatura ótima não varia só em função do microrganismo, mas também da matéria-prima a ser hidrolisada e da fonte da enzima. GALBE e ZACHI, 2002, relataram que quando os tempos de residência mais longos foram empregados (maiores que 24 h), uma temperatura de 38 ºC foi ideal. Apesar das novas tecnologias ainda não é possível definir um tratamento ótimo para a hidrólise enzimática, pois as condições podem mudar dependendo principalmente da composição química do material

lignocelulósico a ser hidrolisado. A Figura 10, apresentada por OLOFSSON et al., 2008, é uma representação esquemática da SSF.

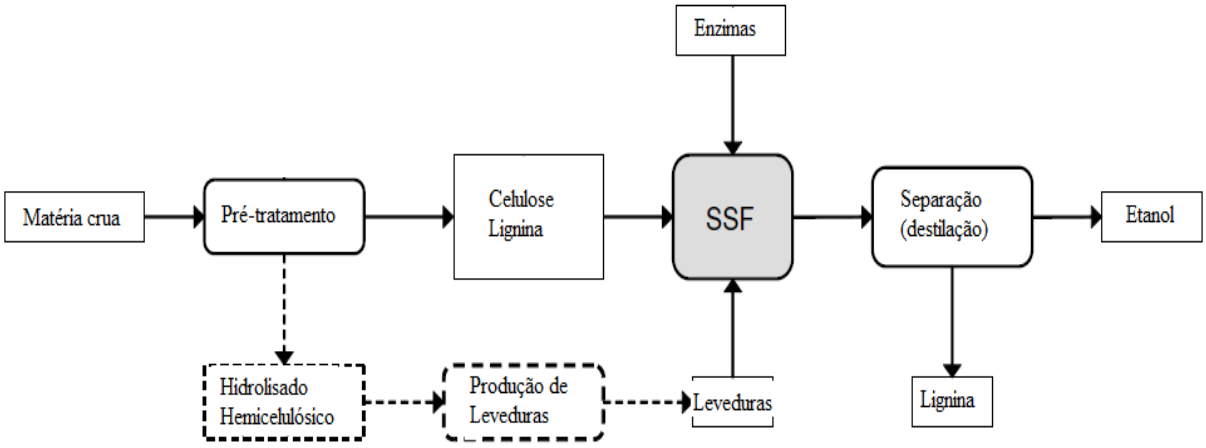


Figura 10. Representação esquemática da sacarificação e fermentação simultânea (Adaptada de OLOFSSON et al., 2008).

Em uma indústria, o processo de SSF deve ter concentrações de enzimas e de células microbianas balanceadas para minimizar o custo com microrganismo e produção de enzima (OLOFSSON et al., 2008).

2.7. Co-fermentação

Para o processo de produção de etanol a partir de materiais lignocelulósicos ser viável economicamente, todas as hexoses (glicose) e as pentoses (xilose, arabinose etc) devem ser convertidas. Não há um microrganismo naturalmente capaz de fermentar todos estes açúcares a etanol (FU et al., 2008). Estes açúcares obtidos através de hidrólise ácida e/ ou enzimática podem ser eficientemente utilizados para a fermentação de etanol utilizando co-culturas. Vários trabalhos relatam um aumento de produção de etanol quando os dois microrganismos são utilizados (DA CUNHA-PEREIRA et al., 2011). No entanto, as melhores condições de crescimento das leveduras geralmente não

são alcançadas, podendo acarretar em menor eficiência e rendimento mais baixo do produto (GUPTA et al., 2009).

A levedura *S. cerevisiae* é a mais utilizada para o processo de produção de etanol em batelada, sendo uma eficiente fermentadora do hidrolisado. Porém esta levedura não fermenta nem utiliza pentoses no seu metabolismo (OHGREN et al., 2006). Um co-cultivo entre dois microrganismos, um fermentador de hexoses e um fermentador de pentoses vem sendo estudado. A maioria desses estudos relata que, enquanto a fermentação de glicose numa mistura de açúcares prossegue de forma eficiente com um microrganismo fermentador tradicional, a fermentação de xilose muitas vezes é lenta e de baixa eficiência devido ao conflito de oxigênio entre as duas linhagens (GROOTJEN et al., 1991). Observa-se também certas dificuldades para encontrar pHs ideais para a co-fermentação, além da dificuldade de encontrar microrganismos resistentes aos inibidores presentes no hidrolisado (HUANG et al., 2009).

Neste contexto, o estudo de culturas co-imobilizadas ganha força. LEE et al., 2012, estudaram a co-imobilização de diferentes microrganismos (co-cultura) no interior de matriz porosa visando diminuir os efeitos pelos diferentes requisitos das cultura, tais como pH, temperatura, nutrientes e carência de oxigênio.

Além da co-fermentação e da co-imobilização, estudos mais recentes relatam a utilização de sacarificação e co-fermentação simultânea (SSCF - simultaneous saccharification and co-fermentation). Em SSCF os microrganismos utilizados na co-fermentação devem ser compatíveis, em termos de pH operacional e de temperatura (SARKAR et al., 2012), tanto um com o outro, quanto com a temperatura em que ocorre a sacarificação. Uma combinação de *C. shehatae* e *S. cerevisiae* foi relatada por DAS

NEVES et al., 2007, como sendo adequada para o processo de SSCF. A fermentação sequencial com dois microrganismos diferentes em diferentes períodos do processo de fermentação para melhor utilização de açúcar também tem sido empregada com um fermentador de hexoses (*S. cerevisiae*) na primeira fase, e *C. shehatae*, por exemplo, na segunda fase para a utilização das pentoses (DAS NEVES et al., 2007).

2.8. Biorrefinaria

Biorrefinaria é um conceito para a coleção de processos utilizados na conversão de biomassa em produtos químicos e energia. O objetivo final de uma biorrefinaria é converter a biomassa (por exemplo, hidratos de carbono, lignina, proteínas, gorduras e em menor grau outros diversos produtos químicos tais como corantes, aromas e vitaminas) em produtos de valor agregado usando várias tecnologias e processos (DAS NEVES et al., 2007). A biomassa lignocelulósica pode ser fracionada em diversos componentes por tratamentos sequenciais que separam produtos que podem ser utilizados para diferentes aplicações, permitindo a maximização dos benefícios de um recurso renovável (AMIDON et al., 2008).

A biorrefinaria pode ser dita de primeira geração quando utiliza açúcares considerados reservas metabólicas da planta, o caso da sacarose e do amido, que são açúcares facilmente acessíveis e que já têm tecnologias firmadas para produção de bioetanol tanto no Brasil quanto nos EUA. Ou ainda, a biorrefinaria pode ser dita de segunda geração, a qual se baseia na produção de bioetanol, entre outros produtos, através da utilização do complexo lignocelulósico (celulose, hemicelulose e lignina). As vantagens na utilização de materiais lignocelulósicos como matéria-prima são a abundância na natureza e o fato de não haver concorrência com produção de alimentos (ZHANG et al., 2008). A vantagem da variedade na composição da biomassa é que as biorrefinarias podem produzir um conjunto maior de classes de produtos do que as

refinarias de petróleo, já que são alimentadas por amplas variedades de matérias-primas, porém um grande número de processos tecnológicos é necessário, sendo muitos destes processos ainda em estágio de desenvolvimento (DALE et al., 2006). Os principais procedimentos para diferentes cenários de uma biorrefinaria – através de processos termoquímicos, bioquímicos e químicos - de materiais lignocelulósicos empregados na transformação da biomassa estão resumidos na Figura 11.

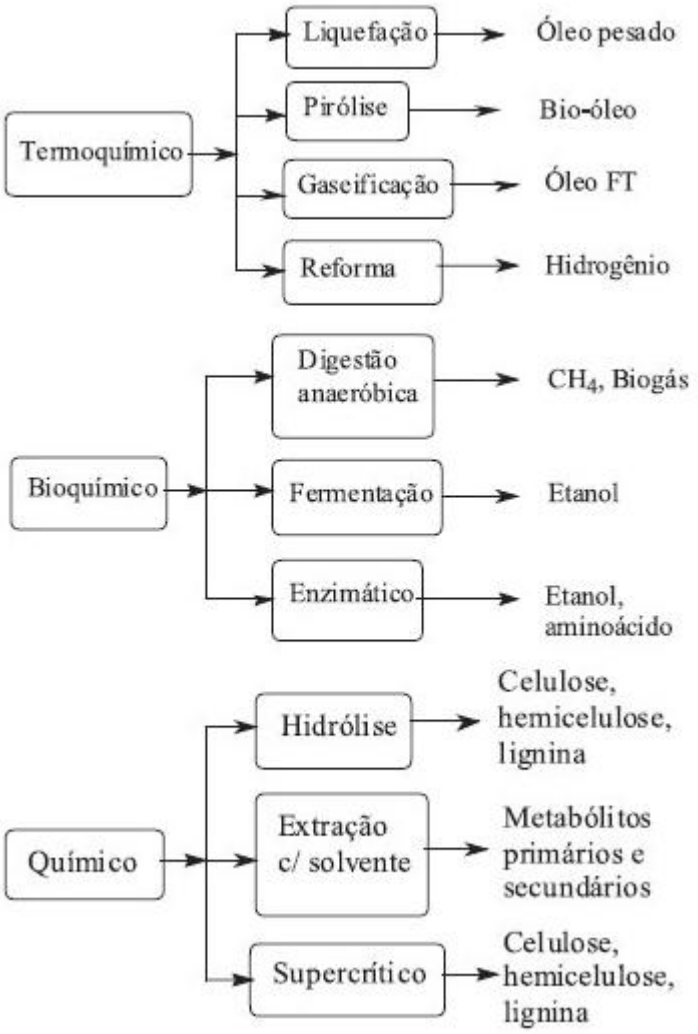


Figura 11. Esquema de biorrefinaria lignocelulósica através de plataformas (Adaptada de RODRIGUES, 2011).

A partir da Figura 12, podemos observar uma estrutura de biorrefinaria através da rota bioquímica: (a) simples utilização (todo açúcar convertido em etanol e lignina como combustível de queima); (b) utilização parcial (todo açúcar convertido em etanol, metade da lignina como combustível de queima e a outra metade transformados em materiais poliméricos e ácido acético como *commodities*); (c) utilização completa (glicose convertida em etanol, xilose e outros açúcares menores em produtos de alto valor agregado, toda lignina usada na produção de materiais poliméricos e ácido acético como *commodities*), respectivamente (DAS NEVES et al., 2007).

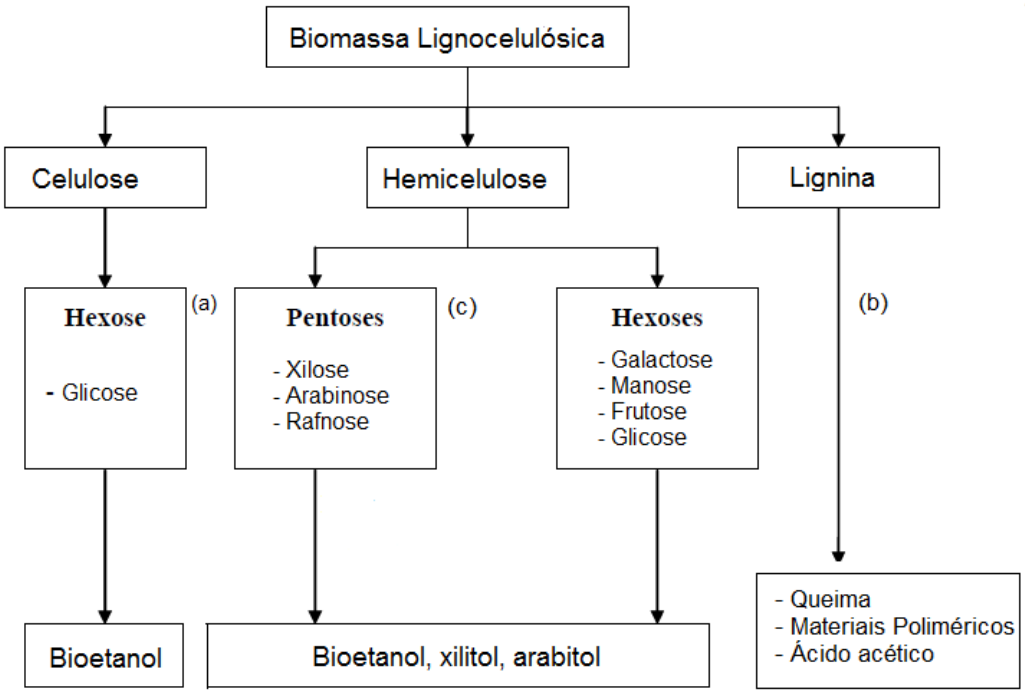


Figura 12. Esquema de biorrefinaria lignocelulósica multi produtos (Adaptada de DAS NEVES et al., 2007).

Segundo ZHANG e colaboradores (2008), as refinarias de petróleo com o objetivo de atingir taxas máximas de eficiência econômica, acabam sempre produzindo múltiplos produtos (diesel, gasolina, óleo lubrificante etc). Isso porque uma planta que produz apenas um produto será um grande risco para os investidores e obterá um fraco desempenho perante as flutuações do mercado econômico. Dessa forma, é preciso

aproveitar as sinergias entre a produção de biocombustíveis e bioprodutos de alto valor agregado, pois mais tarde isso irá aumentar a diversidade econômica da biorrefinaria de biomassa (WYMAN et al., 2003).

Os biocombustíveis tem potencial para substituir uma grande parte dos combustíveis fósseis, no entanto considera-se que a utilização de biomassa lignocelulósica, através de tecnologias de segunda geração, será necessária para que isso seja alcançado de forma economicamente sustentável. A matriz lignocelulósica é complexa e recalcitrante à conversão, mas o estudo em biorrefinarias está avançando rapidamente e instalações comerciais são esperadas no curto prazo (HAYES et al., 2009).

Capítulo 3

Resultados

Os resultados deste trabalho estão apresentados na forma de artigos científicos, sendo três destes aceitos e um 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 é apresentado uma introdução ao assunto abordado, materiais e métodos detalhados, resultados, discussões e referências bibliográficas.

3.1. Resultado I

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 131 (2013) 508–514.

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 physic chemical 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°51'04"S and 51°48'44"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 × 6.25 conversion factor. Ashes were determined by weight difference before and after incineration of the rice hull sample in a muffle furnace at 600 °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°17'14"S and 42°32'01"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 0.0 2%) (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 °C 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×6.25) 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 2 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.

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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 Captions:

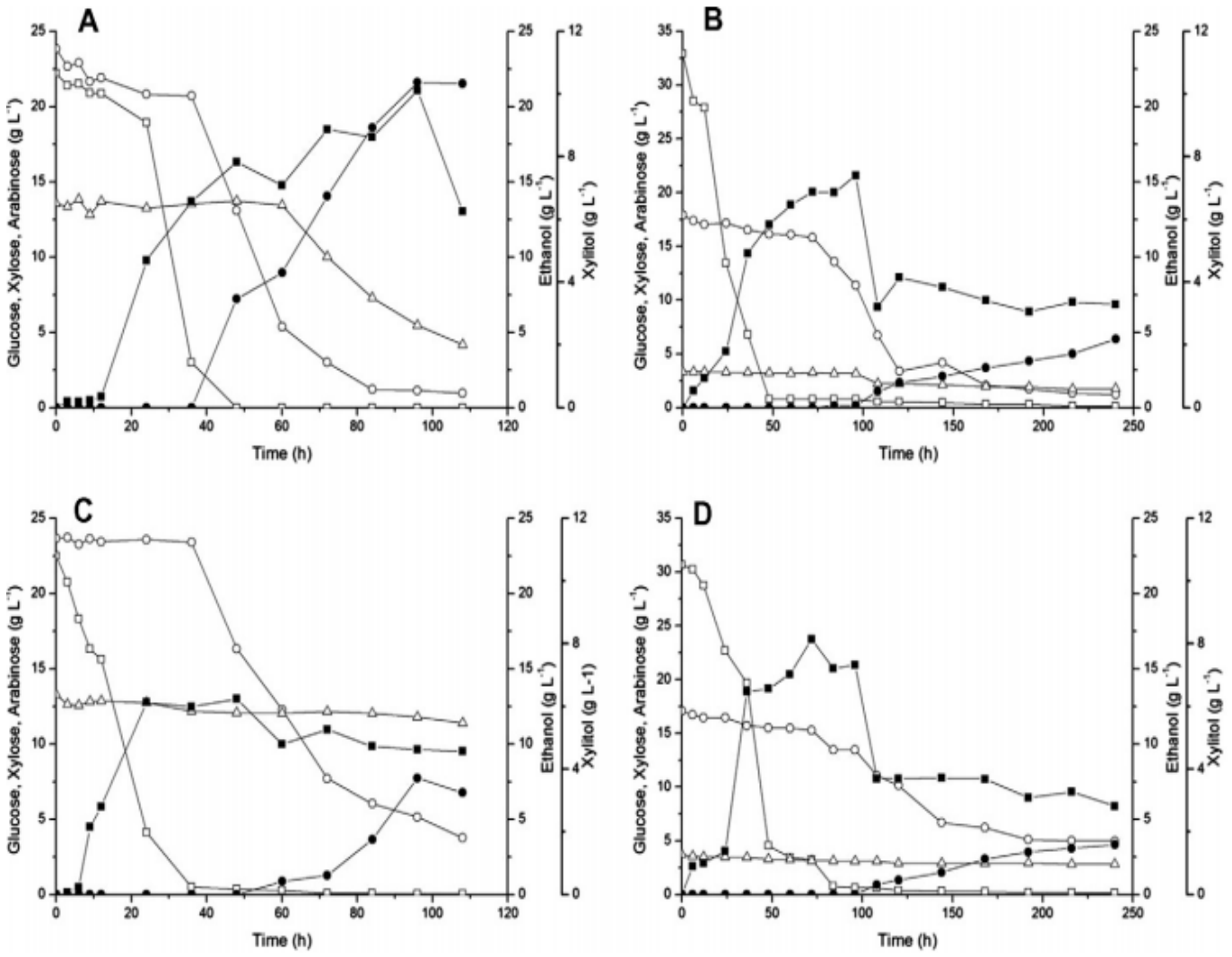

Figure 1. Shake flask kinetics of substrate consumption, ethanol and xylitol production: (A) *C. shehatae* HM 52.2 cultivated in synthetic medium, and (B) in rice hull hydrolysate (RHH); (C) Co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* HM 52.2 cultivated in synthetic medium, and (D) in rice hull hydrolysate (RHH). Glucose (□); xylose (○); arabinose (△); ethanol (▪); and xylitol (●). Results represent the mean of triplicates.

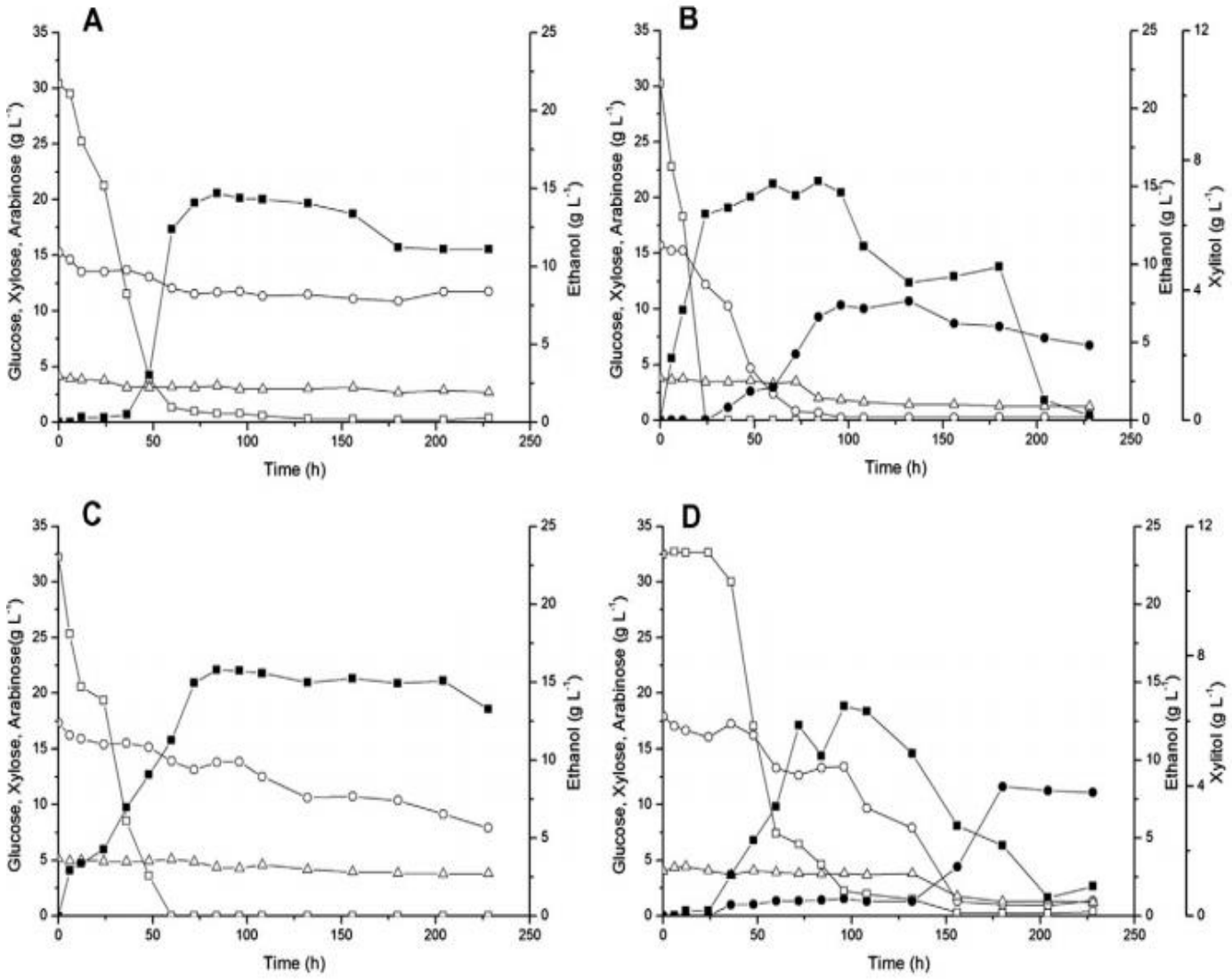

Figure 2. Bioreactor kinetics of substrate consumption, ethanol and xylitol production of co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* HM 52.2 cultivated in (A) synthetic medium under anaerobiosis, and (B) under oxygen limitation; (C) in rice hull hydrolysate (RHH) under anaerobiosis, and (D) under oxygen limitation. Glucose (□); xylose (○); arabinose (△); ethanol (▪); and xylitol (●). Results represent the mean of duplicates.

3.2. Resultado II

'Simultaneous saccharification and co-fermentation of un-detoxified rice hull hydrolysate by *Saccharomyces cerevisiae* ICV D254 and *Spathaspora arborariae* NRRL Y-48658 for the production of ethanol and xylitol.'

Este artigo consiste no estudo da fermentação do hidrolisado de casca de arroz (HCA) por *Saccharomyces cerevisiae;* estudo da co-fermentação deste hidrolisado por um consórcio de *S. cerevisiae* e *Spathaspora arborariae*; e sacarificação e co-fermentação simultânea deste hidrolisado para a produção de etanol e de xilitol através da combinação de ambas as leveduras, em condições de oxigênio limitado em biorreatores. Este artigo foi publicado na revista Bioresource Technology 143, 112–116.

Simultaneous saccharification and co-fermentation of un-detoxified rice hull hydrolysate by *Saccharomyces cerevisiae* ICV D254 and *Spathaspora arborariae* NRRL Y-48658 for the production of ethanol and xylitol

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ABSTRACT

Co-fermentation and simultaneous saccharification of un-detoxified rice hull hydrolysate (RHH) were investigated for the production of ethanol and xylitol by *Saccharomyces cerevisiae*, *Spathaspora arborariae*, or the combination of both yeasts. In bioreactor cultures under oxygen limitation, *S. cerevisiae* was capable of metabolizing glucose present in RHH, which is heavily contaminated with acetic acid, furfural and hydroxymethylfurfural (HMF), while achieving ethanol yields ($Y_{P/S}$) of 0.45. The co-culture of *S. cerevisiae* and *S. arborariae* was able to metabolize pentoses and hexoses present in RHH, with ethanol ($Y_{P/S}$) and xylitol ($Y_{X/X}$) yields of 0.48 and 0.39, respectively. The process of simultaneous saccarification and co-fermentation with both yeasts, produced yields of ethanol and xylitol of 0.50 and 0.39, respectively. Results showed near-theoretical yields of ethanol, pointing out the good prospects for

the use co-cultures with *S. cerevisiae* and *S. arborariae* for the bioconversion of RHH without detoxification, in ethanol and xylitol production.

Keywords: Bioethanol; simultaneous saccharification and co-fermentation; rice hull hydrolysate; *Spathaspora arborariae*; *Saccharomyces cerevisiae*.

1. INTRODUCTION

The interest for ethanol production from renewable resources has been on the increase in the last decade in response to declining oil reserves and environmental concerns. Biomass residues available from agricultural processing constitute a potential source for fermentation bioproducts such as ethanol and xylitol, after their matrix-bound reducing sugars are made soluble, using enzymes or acid-catalysed hydrolyses. Rice hull, which represents 20 % (mass fraction) of the harvested rice, is one of the most abundant lignocellulosic by-products, accounting for more than 120 million metric tons generated per year (YU et al., 2009). Its lignocellulosic composition comprises around 20–25 % lignin, 35–40 % cellulose (glucose), and 15–20 % hemicellulose (mainly as xylose and arabinose) (YU et al., 2009). In order to liberate these fermentable sugars, interesting for bioconversion into bioethanol and other fine chemicals such as xylitol, it is necessary to hydrolyze this material, either by using chemicals (particularly effective over the hemicellulose fraction), enzymes (mainly for cellulose hydrolysis), or a combination of both approaches (SCHIRMER-MICHEL et al., 2008). There are several obstacles in the fermentation of hemicellulosic hydrolysates for ethanol production. Inhibitors, such as weak acids and furans, are often generated during pretreatment with diluted acids (LIN et al., 2012). The detoxification of furans has generally been postulated in order to allow yeasts to convert sugars during fermentation, but it

increases the cost of the process and sugar loss incurs (PURWADI et al., 2004). The enzymatic hydrolysis can be carried out separately from the alcoholic fermentation, a process known as Separate Hydrolysis and Fermentation (SHF), or both processes can run together as Simultaneous Saccharification and Fermentation (SSF). In SHF, hydrolysis and fermentation are carried out in separate vessels under their optimal conditions. However, end-product inhibitors of enzymes activity and contamination problems are associated with this process (TALEBNIA et al., 2010). In the SSF process, the production of ethanol is faster, as the glucose formed is simultaneously fermented to ethanol. Furthermore, the combination of hydrolysis and fermentation decreases the number of vessels needed and thereby investment costs (SOCCOL et al., 2010).

The fermentation process using lignocellulosic biomass would be economically viable only if both hexose and pentoses present in the hydrolysates are converted to ethanol (KUHAD et al., 2011). Traditionally-used microorganisms used for ethanol fermentation, namely *Saccharomyces cerevisiae* and *Zymomonas mobilis*, are incapable to metabolize pentoses (YADAV et al., 2011), unless genetically modified (LIN et al., 2012), while yeasts such as *Scheffersomyces* (*Pichia) stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* have been used for the xylose conversion (FU and PEIRIS, 2008), but with lower productivities. Moreover, yeasts like *S. stipitis* exhibit a low tolerance to inhibitory compounds in un-detoxified lignocellulosic hydrolysates; require a small and well-controlled supply of oxygen for maximal ethanol production; and are sensitive to ethanol, which limit their use for industrial ethanol production (MATSUSHIKA et al., 2009). One possibility to circumvent this problem is the use of co-cultures of different yeasts, capable of both hexoses and pentoses metabolism. Co-fermentations have been described for immobilized *Z. mobilis* and free cells of *Pichia stipitis* (FU et al., 2009); *Pachysolen tannophilus* and *Z. mobilis* (FU and PEIRIS,

2008), and *S. cerevisiae* and *S. stipitis* (YADAV et al., 2011), with mixed results in terms of yields of ethanol and productivities.

In this context, the aims of this research were to investigate the use of rice hull hydrolysate (RHH) as substrate for ethanol and/or xylitol production and the kinetics of glucose, xylose and arabinose consumption by *S. cerevisiae* and its co-culture with *Spathaspora arborariae*, a new strain that has demonstrated the capability to convert pentoses into ethanol (DA CUNHA-PEREIRA et al., 2011). Acid hydrolysis was used to liberate sugars from the biomass matrix, and simultaneous fermentation and saccharification with enzyme was also attempted. Bioreactor fermentations were run with media without the purification of inhibitory compounds formed during acid hydrolysis (acetic acid, furfural, and hydroxymethylfurfural), under oxygen limitation conditions.

2. MATERIALS AND METHODS

2.1. Microorganisms, cell maintenance, and materials

The strains used in this study were *Saccharomyces cerevisiae* ICV D254 (Lalvin, Institut Coopératif du Vin, France), and *Spathaspora arborariae* (NRRL Y-48658). *S. cerevisiae* ICV D254 is a commercial wild-type strain isolated from Syrah grapes from the Rhône Valley region, in France, used for wine fermentation and it has been chosen for this research due to its good ethanol resistance (DA CUNHA-PEREIRA et al., 2011). *S. arborariae* NRRL Y-48658 was recently isolated from rotting wood collected in the Serra do Cipó National Park, State of Minas Gerais, Brazil, and was characterized (CADETE et al., 2009) as an efficient D-xylose fermenting yeast. Yeasts were kept frozen at -20 ºC in stock cultures of 20 % glycerol and 80 % of culture medium,

containing (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, and glucose, 5. Rice hull was obtained from a local rice mill as dried material and processed without any further treatments before hydrolysis (see below). The simultaneous saccharification and fermentation was performed in the culture bioreactor, under the same cultivation conditions, using the complex enzymatic PowerCell (Prozyn, 15 FPU g^{-1}). The enzyme complex PowerCell is an enzyme system developed for the saccharification of lignocellulose biomass. It was kindly provided by Prozyn (São Paulo, Brazil). All chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA), unless otherwise stated.

2.2. Inocula preparation

Inocula for all cultivations were prepared by cultivating the yeasts in synthetic medium (composition described below) in 500 mL Erlenmeyer flask 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, and the pellet formed was washed with sterile distilled water and resuspended directly into the medium to be used in the fermentation to obtain a cell concentration of optical density 1 at 600 nm. Fractions of 10 % (volume fraction) of these cell suspensions were then used as the inocula in all experiments.

2.3. Media composition and cultivation conditions

The microorganisms either isolated or in consortium, were cultivated in rice hull hydrolysate (RHH). 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 sulfuric acid; DA CUNHA-PEREIRA et al., 2011). The liquid

fraction was recovered by filtration and the pH was adjusted to 5 with solid drops of sodium hydroxide for cultivations without enzyme. For the simultaneous fermentation and saccharification, the hydrolyzed biomass was used without filtration, with the addition of 15 FPU g^{-1} dry matter (biomass) of enzyme, resuspended in 100 mL of sodium acetate buffer at pH 4.7. The acid fraction hydrolysate was vacuum-concentrated at 70 ^{o}C in order to increase its final sugar and protein concentrations to (in g L^{-1}): glucose, 27; xylose, 13; arabinose, 5; 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 supplementations were made to the RHH.

2.4. Co-cultures in bioreactor

Experiments were carried out in fully equipped 2 L bioreactors (Biostat B, Braun Biotech International, Germany). For the co-cultures a volume of 75 mL pre-inoculum 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 1 vvm, controlled by a needle valve and with a rotameter. Temperature and agitation speed were maintained at 28 °C and 180 rpm, respectively, in all bioreactor experiments. Samples were collected at stipulated points for determination of biomass (as colony forming units), and for the quantification of sugars, xylitol, and ethanol. All experiments were performed in duplicate.

2.5. Analytical methods

Hydrolyzed samples were analyzed 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 sulfuric acid as eluent, flow rate of 0.6 mL min^{-1} 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. Biomass was estimated as viable cells, using CFU (colony forming units) plated on yeast morphology agar (YMA) medium. The osmotic pressure of RHH was measured using an osmometer (VAPRO 5520), following the manufacturer recommendations.

2.6. Kinetic parameters calculation

The ethanol conversion yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose consumed when *S. cerevisiae* was used as the sole microorganism. When cultivation was with *S. arborariae*, 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. Culture kinetics of *S. cerevisiae* on RHH

Since RHH was used without detoxification and with a very high osmotic pressure (1.539 mOsm kg^{-1}), it was necessary to determine whether yeast cells were going to grow on this hydrolysate. Figure 1 A show the biomass formation of *S. cerevisiae* ICV D254 cultivated in RHH. Exponential growth lasted for about 50 to 60 h of cultivation with at least 2-logs of cell concentration increase. In Figure 1 B kinetics of sugar consumption and products formation are shown. *S. cerevisiae* consumed all available glucose, showing good ethanol production, with conversion yields ($Y_{P/S}$) of 0.44 g g^{-1} showing that this yeast can be cultivated in hydrolysates containing toxic compounds and without supplementation. Furfural and HMF, which are formed by thermal degradation of pentoses and hexoses, can inhibit ethanolic glucose fermentation, causing longer lag phases and lower growth rates (WAHLBOM and HAHN-HAGERDAL, 2002). In this work, results suggest that the presence of toxic compounds did not affect ethanol production and cell mass. As expected, xylose and arabinose were not metabolized. SAHA et al., 2005, cultivated recombinant *Escherichia coli* on rice hull hydrolysate (obtained under the same hydrolysis conditions employed in this work) as substrate, and reported yields of 0.43 g g^{-1} of ethanol considering the conversion of all fermentable sugars present in the medium. CHANDEL et al., 2011 reported the cultivation of *S. cerevisiae*-VS3 in detoxified sugarcane bagasse hydrolysate containing 32.84 g L^{-1} of total reducing sugars, obtaining ethanol yields of 0.20 g g^{-1}. In this work cultivations were run with a limited supply of oxygen, and the ethanol formed was subsequently consumed, suggesting that *S. cerevisiae* ICV D254 was capable of undergoing oxidative metabolism in presence of furans and under high osmotic pressure.

3.2. Kinetics of co-cultures of *S. cerevisiae* and *S. arborariae*

Because *S. cerevisiae* is unable to metabolize xylose, co-cultures with pentose-fermenting yeasts such as *S. arborariae*, could be postulated in order to increase overall sugar conversion. In Figures 2 A and B are shown the biomass formation and kinetics of sugar consumption and products formation of co-cultures of *S. cerevisiae* ICV D254 and *S. arborariae* NRRL Y-48658. Exponential growth was faster than for isolated cultures of *S. cerevisiae*, but final biomass was similar (Figure 2 A). The highest conversion yield of ethanol ($Y_{P/S}$) was 0.48 g g^{-1}, while yield of xylitol ($Y_{X/X}$) reached 0.34 g g^{-1}, with efficiencies of xylose and arabinose utilization in the co-culture around 39 % and 31 %. Although many yeasts are capable of aerobically assimilating L-arabinose, most are unable to ferment it to ethanol (VAN MARIS et al., 2006). Furfural can function as an external electron acceptor, regenerating NAD^+, which is a cofactor of xylitol dehydrogenase that converts xylitol to xylulose that is finally fermented to ethanol (WAHLBOM and HAHN-HAGERDAL, 2002) rerouting the metabolism away from xylitol, therefore, furfural present in RHH might be beneficial for xylose fermentation. The increased ethanol yield in the co-culture is due to the increased rates of glucose and xylose conversion. Initially, the amount of glucose may have inhibited the transport of xylose and prevented the synthesis of the xylose catabolic enzymes, causing the inhibition of xylose metabolism, up to the point where glucose was depleted or below repressive levels (KASTNER et al., 1999). The preferential use of glucose over xylose is not an unusual phenomenon, and was reported by DA CUNHA-PEREIRA et al., 2011, in co-cultures of *S. cerevisiae* ICV D254 and *S. arborariae* NRRL Y-48658. The results obtained in this work show high ethanol and xylitol production using this co-culture when compared to co-cultures using other pentose-

fermenting yeasts, such as *S. stipitis* and *C. shehatae* (CHANDEL et al., 2011; HICKERT et al., 2013). YADAV et al., 2011 used concentrated rice straw hydrolysate medium fermented with a co-culture of *S. cerevisiae* and *S. stipitis,* obtaining an ethanol yield of 0.40 g g $^{-1}$on total sugars. Comparatively, results in this work show that *S. arborariae*, which is new in bioprocesses, is very competitive with other yeasts capable of metabolizing both pentoses and hexoses, converting them into ethanol. For instance, SCHIRMER-MICHEL et al., 2008, using *C. guilliermondii* NRRL Y-2075 grown under oxygen limitation on detoxified soybean hull hydrolysate, obtained ethanol yield of 0.40 g g $^{-1}$, and xylitol of 0.22 g g $^{-1}$.

3.3. Kinetics of simultaneous saccharification and co-fermentation with *S. cerevisiae* and *S. arborariae*

Bioreactor co-cultures of *S. cerevisiae* and *S. arborariae* in RHH were run with the simultaneous saccharification of cellulose using cellulose PowerCell, and results are shown in Figure 3 A and B. Kinetic parameters are presented in Table 1 in comparison with the other cultivations. In a preliminary test, different concentrations of enzyme (10, 15, 20, 25, and 30 FPU g^{-1}) were used, with the most efficient conversion obtained for 15 FPU g^{-1}, with the liberation of 20 g L^{-1} of glucose and 13 g L^{-1} of xylose (data no show). Cell growth with a maximum of 1.73×10^8 CFU mL^{-1} was similar to cultures without added enzyme. Glucose took longer to be completely consumed (168 h) than for co-culture without enzyme (120 h). Xylose and arabinose utilization were 53 % and 30 %. The maximum ethanol production was obtained in 108 h of cultivation with 14.5 g L^{-1} with ethanol yield of 0.50 g g^{-1} and xylitol yield of 0.36 g g^{-1}. SAHA et al., 2005, reported that the acid pretreatment of rice hull (sulfuric acid 1.0 % (volume fraction), 60 min, 121ºC), generated 189 mg fermentable sugars per g, representing 40 % yields of

sugars based on the total carbohydrate content. After enzymatic saccharification of the solid residue using a combination of cellulose and β-glucosidase (Celluclast, Novozyme 188), the authors obtained only a small increase of 5 % in the yields of sugars. The authors then proceeded to fermentation of the hydrolysate, obtaining an ethanol production of 18.7 g L^{-1} ($Y_{P/S}$ = 0.43 g g^{-1}) in SHF, and only 9.1 g L^{-1} of ethanol under SSF with their recombinant strain of *E. coli*. OHGREN et al., 2006, run SSF with *S. cerevisiae* TMB3400, a xylose-fermenting strain, on pretreated corn stovers with 15 FPU g^{-1} Celluclast 1.5 L and Novozyme 188, obtaining 0.33 g g^{-1} yields of ethanol based on the total glucose and xylose available in the raw material. These authors postulated that the use of SSF could be useful, since the low concentrations of glucose in the vessel by the simultaneous release and consumption improved xylose fermentation.

4. CONCLUSION

It was demonstrated the possibility of using RHH as a substrate for ethanol production by co-cultures of *S. cerevisiae* ICV D254 and *S. arborariae* NRRL Y-48658 in bioreactor under oxygen limitation conditions. *S. cerevisiae* proved to be an efficient converter of hexoses to ethanol in the presence of toxic compounds, namely furans and acetic acid, and high osmotic pressure. Ethanol production with yields as high as $Y_{P/S}$ = 0.48 were achivied. The SSF produce ethanol and xylitol yields of $Y_{P/S}$ = 0.50, and $Y_{X/X}$ = 0.39, respectively. Optimization of this process is granted for second-generation bioethanol production.

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Table 1. Kinetic parameters obtained for pure cultures of *S. cerevisiae* ICV 254D, and for their co-cultivations with *S. arborariae* NRRL Y-48658 in rice hull hydrolysate (RHH).

	Consumption (%)				
Microorganism	Glucose	Xylose	Arabinose	$Y_{P/S}$ (g g^{-1})	$Y_{X/X}$ (g g^{-1})
S. cerevisiae	100	-	-	0,44	-
Co-culture	100	39	31	0,48	0,34
Co-culture with enzyme	100	53	36	0,5	0,39

$Y_{P/S}$: ethanol coefficient yield (g ethanol per g total sugar consumed);
$Y_{X/X}$: xylitol coefficient yield (g xylitol per g xylose consumed);

Figures:

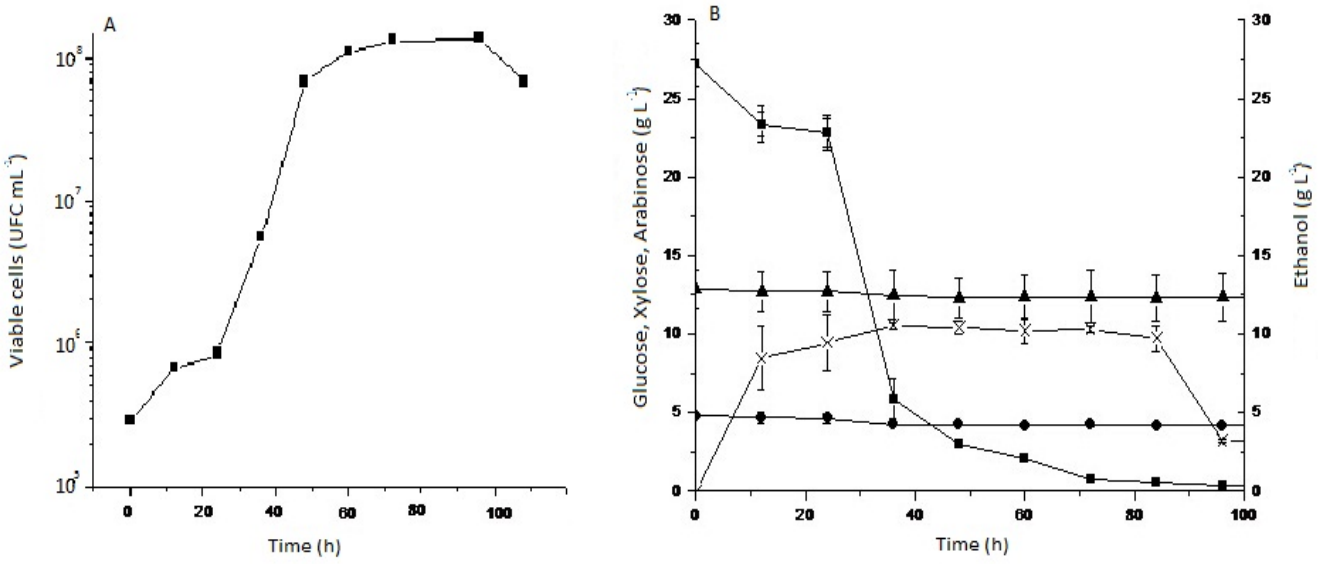

Figure 1. Biomass production of *S. cerevisiae* strain ICV D254 (A); bioreactor kinetics of substrate consumption, ethanol production of this culture cultivated in rice hull hydrolysate (RHH) under oxygen limitation (B). Glucose (■); xylose (▲); arabinose (♦) and ethanol (×). Results represent the mean of duplicates.

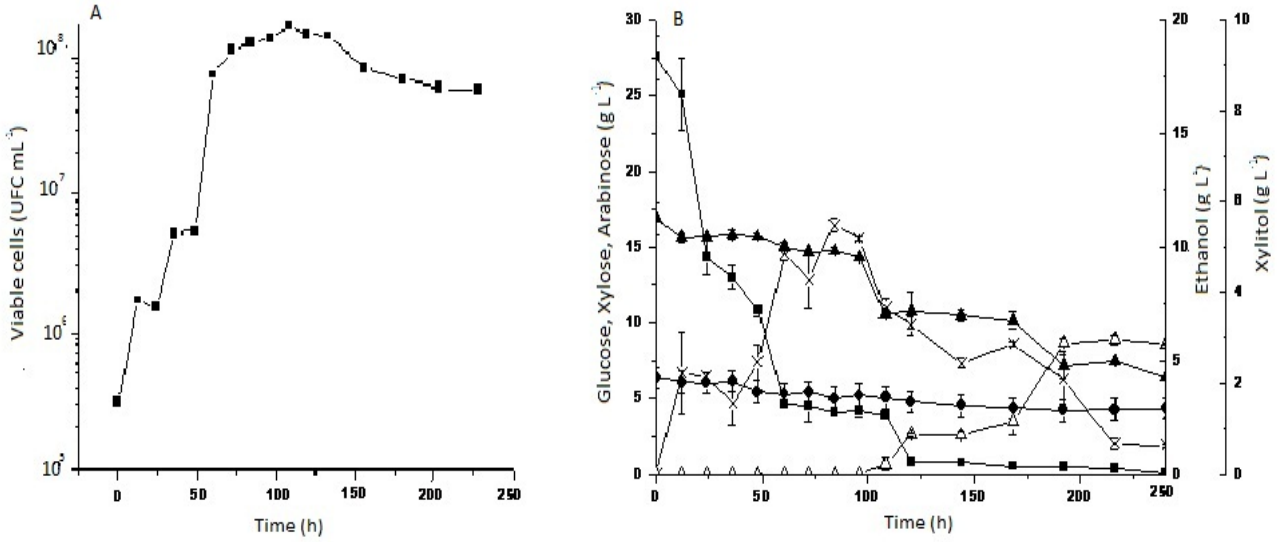

Figure 2. Biomass production of co-cultures of *S. cerevisiae* ICV 254D and *S. arborariae* NRRL Y-48658 (A); bioreactor kinetics of substrate consumption, ethanol production of these cultures cultivated in rice hull hydrolysate (RHH) under oxygen limitation (B). Glucose (■); xylose (▲); arabinose (♦); ethanol (×) and xylitol (●). Results represent the mean of duplicates.

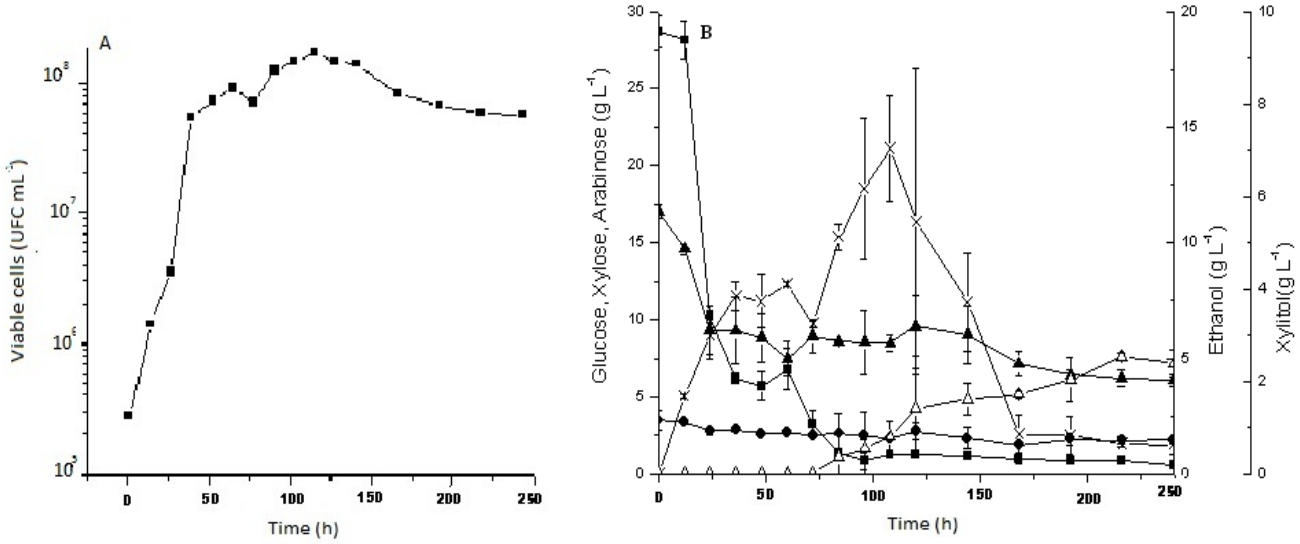

Figure 3. Biomass production of co-cultures of *S. cerevisiae* ICV 254D and *S. arborariae* NRRL Y-48658 with addiction of POWERCELL enzyme (A); bioreactor kinetics of substrate consumption, ethanol production of these cultures cultivated in rice hull hydrolysate (RHH) under oxygen limitation (B). Glucose (■); xylose (▲); arabinose (♦); ethanol (×) and xylitol (●). Results represent the mean of duplicates.

3.3. Resultado III

"Fermentation kinetics of acid-enzymatic soybean hull hydrolysate in immobilized-cell bioreactors of *Saccharomyces cerevisiae, Candida shehatae*, *Spathaspora arborariae*, and their co-cultivations"

Este artigo consiste no estudo da sacarificação da casca de soja pós-hidrólise ácida diluída pelo complexo enzimático de *Penicillium echinulatum* S1M29 e posterior fermentação deste hidrolisado para a produção de etanol por células imobilizadas de *Saccharomyces cerevisiae*, *Spathaspora arborariae* e *Candida shehatae,* e pela co-cultura de *S. arborariae* ou *C. shehatae* com *S. cerevisiae,* em condições de oxigênio limitado. Posteriormente, visando um escalonamento do processo, foram testadas células de *C. shehatae* e *S. cerevisiae* em biorreatores imobilizados. Este artigo foi publicado na revista Biochemical Engineering Journal, 15, 61 – 67.

Fermentation kinetics of acid-enzymatic soybean hull hydrolysate in immobilized-cell bioreactors of *Saccharomyces cerevisiae, Candida shehatae, Spathaspora arborariae,* and their co-cultivations

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ABSTRACT

We investigated the conversion of non-detoxified, high osmotic pressure soybean hull hydrolysate (SHH) into ethanol by immobilized *Saccharomyces cerevisiae, Candida shehatae,* and *Spathaspora arborariae,* or their co-cultivations. Soybean hull was hydrolysed in a two-steps sulphuric acid-enzyme treatment using an enzymatic complex of *Penicillium echinulatum* S1M29, resulting in more than 72 % of saccharification. In shaker cultivations, the bioconversion of SHH into ethanol showed yields ($Y_{P/S}$) of 0.43, 0.47, and 0.38, in cultures of *S. cerevisiae*, *C. shehatae*, and *S. arborariae*, respectively. Co-cultures of *S. cerevisiae* and *C. shehatae* or *S. cerevisiae* and *S. arborariae*, produced $Y_{P/S}$ of 0.48 and 0.40, respectively. *S. cerevisiae* and *C. shehatae* were immobilized in Ca-alginate and cultivated in bioreactors to analyse the possibility of scaling up this process. Immobilized-cell cultures showed yields of 0.45 and 0.38, respectively. Results suggest the viability of acid-enzymatic saccharification of soybean

hulls for second-generation ethanol production by immobilized yeast cells in bioreactors.

Keywords: Soybean hull hydrolysate; enzymatic saccharification; immobilized-cells yeast cultures; ethanol; xylitol; *Penicillium echinulatum*.

1. INTRODUCTION

The interest for ethanol production from renewable resources has increased in the last decade, directly related to environmental and economic concerns over fossil fuels. Bioethanol obtained from the fermentation of a variety of biomasses is widely recognized as one of the most promising technologies for biofuels [1]. Biomass feedstock needs to be decomposed into its monomers in order to release fermentable sugars, which is achieved through technologies using diluted acids and enzymes. The diluted acid hydrolysis approach usually depolymerises the hemicellulose at low temperatures, liberating pentoses (predominantly xylose), whereas the cellulosic fraction of he biomass is scarcely affected by this treatment [2], requiring other physico-chemicals hydrolyses at higher temperatures resulting in sugar decomposition, which may lead to metabolic inhibition during fermentation [3]. Alternatively, enzymatic hydrolysis can be employed to release glucose, generating no toxic compounds for fermentation [4]. In a typical enzymatic hydrolysis of the cellulose fraction, hexoses, (basically glucose) are liberated [5].

The costs involved in biomass fractioning and cellulase enzymes are still the major bottlenecks for the potential applications of lignocelluloses bioconversion into ethanol [6]. The major challenge for second-generation ethanol technology is the availability of efficient and economical enzymatic preparations containing cellobiohydrolases, endoglucanases, and β-glucosidases for biomass hydrolysis [7]. Cellulases (or cellulase

complexes) are commonly produced by fungi, especially of the genera *Trichoderma*, *Penicillum* and *Aspergillus* [3]. Although these enzymatic complexes are usually used as raw preparations, their production costs are still very high [8].

The process of pretreated biomass hydrolysis and fermentation of the liberated sugars can proceed as a separate hydrolysis followed by fermentation (SHF), or as a simultaneous process, in which saccharification and fermentation (SSF) are performed in the same reactor. The advantage of SHF is the ability to carry each step out under their optimal conditions, whereas in SSF operational conditions favour fermentation over hydrolysis, but with fewer unit operations involved [3].

Soybean is one of the most cultivated crops in the world, with a global production of approximately 240 million tons [9], generating about 18-20 million tons of hulls, the major by-product of soy industry [10]. Soybean hull is a lignocellulosic material containing a small proportion of lignin when compared to other agro-residues, thus having a good potential for saccharification because lignin is a major hindrance for the enzymatic hydrolysis of biomass [11]. Despite the large amounts that are generated, soybean hull has received little attention as a low-cost raw material and its use is still limited to animal feeding.

Immobilized-cell systems are intended to enclose the biocatalyst into a defined space in such a way that it retains its bioactivities and can be reused over a long period. Immobilization of yeast cells shows a number of advantages over free-cells systems for industrial fermentation, such as the relative facility on product separation, improved process control, and reduced susceptibility of contaminations. Therefore, immobilization systems are frequently associated to increased products yields and cellular stability, while reducing costs due to the ease of cell recovery and reutilization [12]. Among common supports for cell immobilization, Ca-alginate gel beads are

widely used because they are non-toxic, inexpensive and easy to prepare. The co-immobilization of different microorganisms within the same porous matrix and the combination of two-stage fermentation process in a single-step (i. e., the bioconversion of pentoses and hexoses to ethanol), has been reported to reduce the energy inputs and to solve some problems arising from the use of co-cultures, such as optimal ranges of pH, temperature, nutrients, oxygen demand, among others [13].

In this context, the aims of this research were to investigate the hydrolysis of soybean hulls using a combination of diluted acid and an enzymatic cellulolytic complex produced by Penicillium echinulatum, and the use of this hydrolysate without purification steps as a substrate for fermentation. We evaluated the kinetics of sugars consumption (glucose, xylose, and mannose), and ethanol and xylitol formation by immobilized *Saccharomyces cerevisiae*, *Candida shehatae*, and *Sphataspora arborariae*, and the co-cultures of *C. shehatae* or *S. arborariae* with *S. cerevisiae* in the presence of the inhibitory compounds formed during hydrolysis (acetic acid, furfural, and hydroxymethylfurfural), and high osmotic pressure. The strains showing the best ethanol yields (*C. shehatae* and *S. cerevisiae*) were used to scale up the cultivations using immobilized-cells bioreactors.

2. MATERIALS AND METHODS

2.1. Microorganisms, cell maintenance and materials

The strains used in this study were: (1) *S. cerevisiae* ICV D254, a commercial wild-type strain isolated from Syrah grapes from the Rhône Valley region (France), used for wine fermentation. This strain was chosen for this research because it shows good ethanol resistance [14]. (2) *Candida shehatae* HM 52.2 and (3) *Sphataspora arborariae* NRRL Y-48658, both recently isolated strains from rotting wood used for

rice hull fermentation, showing good yields of conversion [14, 15]. Yeasts were kept frozen at -20 ^{o}C in stock cultures of 20 % glycerol (volume fraction) and 80 % of culture medium, containing (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, and glucose, 5.

The Solae Company (Esteio, RS, Brazil) kindly supplied the soybean hulls. The hulls were milled to a particle size < 1 mm in diameter and stored until further processing or analysis.

The enzymatic complex used in this study was produced and extracted from cultures of *Penicillium echinulatum* strain S1M29 (DSM 18942), in lab scale fermenters, as described and characterized by Dillon et al. [7]. *P. echinulatum* S1M29 cellulase complex has been described as an efficient enzymatic preparation for the hydrolysis of biomasses [7].

2.2. Immobilization technique

The immobilization of cells was carried out as follows: yeasts were grown in 2 L flasks filled with 800 mL of medium, containing (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, 5 and glucose, 10. Cultivations were carried out in an orbital shaker (Marconi MA 830, Brazil) at 180 rpm, 28 ºC for 20 h until cells reached exponential-phase. Cells were then harvested by centrifugation (3 000 *g*, 15 min), washed and resuspended in 10 mL of sterile distilled water at 4 ºC. A sterile solution of 4 % sodium alginate (weight fraction) was mixed with the cell suspension (30 mg dry biomass mL^{-1} alginate solution) and immediately dropped through a 14 G needle (2.1 mm of diameter) using a peristaltic pump into a flask containing 0.1 M $CaCl_2$ sterile solution at 35 ºC, and gently agitated for 30 min to stabilize the system. The formed beads were washed thrice with distilled water at 4 ºC. Average alginate beads of 3.8 mm in diameter were obtained. For co-immobilization, cell suspensions of *S. cerevisiae* and *C. shehatae* or *S.*

arborariae were mixed together and immobilized as described above. Yeasts growth were determined by absorbance at 600 nm and correlated with dry cell weight (g L^{-1}) using a calibration curve.

2.3. Soybean hull hydrolysis, composition, and cultivation conditions in orbital shaker

SHH was obtained by the diluted acid hydrolysis of soybean hulls in autoclave (121° C, 40 min, solid-liquid ratio of 1:10, 1 % sulphuric acid (volume fraction)). This acid hydrolysis was based on a previously published optimized experimental design [16]. The liquid fraction was recovered by filtration. The remaining solid fraction was washed with water until neutral pH, resuspended to a solid-liquid proportion of 1:20 (dry matter in citrate phosphate buffer pH 4.8). This solid suspension was added of the *P. echinulatum* S1M29 enzyme preparation (10, 15, or 20 FPU g^{-1} (filter paper unit per gram of dry solid matter) and incubated in a rotatory shaker at 120 rpm, 50 ºC for 96 h. After the enzymatic hydrolysis, both fractions (enzymatic and acid) were mixed, autoclaved at 0.5 atm, 30 min, and vacuum-concentrated at 70 ^{o}C in order to increase its sugar concentrations to the following final amounts (in g L^{-1}): glucose, 38; xylose, 21; arabinose, 4; mannose, 6; and cellobiose, 7. This sugar concentration was determined by an external laboratory of our university (Chemical Engineering Department), using a Perkin Elmer Series 200 HPLC, fitted with a refractive index (RI) detector and a Phenomenex RHM-Monosaccharides column (Phenomenex, USA). The amount of toxic compounds (inhibitors of microbial growth) in the final SHH, formed during hydrolysis, was determined to be (in g L^{-1}): HMF, 0.58; furfural, 0.08; acetic acid, 2.1. Neither supplementation were applied to the SHH. The pH was adjusted to 5 using solid pastilles of sodium hydroxide. Cultures were carried out in 1 L Erlenmeyer flasks

containing 250 mL of SHH, which were inoculated with 75 mL of Ca-alginate beads, in an orbital shaker at 120 rpm, 28 °C for 120 h. Samples were collected at stipulated points for quantification of sugars, ethanol, xylitol, and acetic acid. All experiments were conducted in duplicates.

2.4. Bioreactor cultivations

Bioreactor experiments were performed in glass column bioreactors (in-house project and manufacture), with a total volume of 370 mL, filled with 75 mL of Ca-alginate beads and 250 mL of fermentation medium [17]. Temperature was kept constant in the water jacket coupled to a thermostat bath. Batch fluidized bed fermentations were carried out for 72 h at 28 ºC with medium recirculation through the column kept by a peristaltic pump promoting the fluidization of the system (upward flow). The yeasts that showed highest ethanol yields during shaker cultivations (above) were tested in the bioreactors. All experiments were made in duplicates.

2.5. Analytical methods

Cultivation samples were analysed by HPLC. Sugars, ethanol, glycerol, xylitol, 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 ^{o}C, using 0.005 M sulphuric acid as eluent, flow rate of 0.6 mL min^{-1} and sample volumes of 20 μL. Under these conditions, xylose and mannose, eluted at the same retention time and were integrated as a single peak and, therefore, the curve depicting xylose in the figures represents the sum of these 2 sugars. Furfural and hydroxymethylfurfural were determined in the same HPLC, using 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. The osmotic pressure of SHH was determined by placing 30 μL samples into the chamber of an osmometer (VAPRO 5520).

2.6. Kinetic parameters calculation

The ethanol yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose and mannose consumed when *S. cerevisiae* was used as the sole microorganism. When *S. arborariae* or *C. shehatae* were used, the yields of ethanol production ($Y_{P/S}$, g g^{-1}) were defined as the ratio between the amount of ethanol produced and total sugars consumed present in the medium up to the moment xylitol started to appear in the medium. The xylitol conversion yield ($Y_{x/x}$ g g^{-1}) was calculated as the ratio of the concentration of xylitol produced and xylose consumed.

3. RESULTS AND DISCUSSION

3.1. Enzymatic saccharification of soybean hulls

The optimal enzyme loading in a hydrolysis process is particularly important to maximize the amount of glucose liberated from the lignocellulosic material, at the same time reducing costs. Figure 1 shows the results of enzyme application on the solid fraction of soybean hulls recovered after acid hydrolysis. A steady increase in sugars was observed until 96 h of reaction, with maximal yields of saccharification (72 % of total sugars of soybean hulls, being composed of approximately 52 % of glucose and 20 % of cellobiose, mass fractions) obtained using either 15 or 20 FPU g^{-1} dry matter. These results show that the cellulase complex preparation of *P. echinulatum* S1M29 is a suitable source for the enzymatic hydrolysis of biomasses.

Commercially available cellulase complexes containing low levels of β-glucosidase lead to an incomplete cellulose hydrolysis, liberating cellobiose into medium, which can not be metabolized by industrial glucose-fermenting yeasts thus affecting conversions to ethanol [18]. Martins et al. [8] reported that β-glucosidase activity is higher in cellulase complexes produced by fungi of the genus *Penicillium* as compared to *Trichoderma*. In special, Camassola and Dillon [19] reported that the β-glucosidase and endoglucanase activities found for *P. echinulatum* were higher when compared with other microorganisms, supporting the good results obtained in this work.

It is important to observe that lignin can bind to cellulosic fibres to form a complex chemical structure that reduces the accessibility of cellulases to the substrate [20]. However, soybean hull has a very small amount of lignin compared with other lignocellulosic materials [16].

3.2. Shake flask cultivations of immobilized yeasts and their co-cultures in SHH

We carried out a test using suspended-free cells of *S. cerevisiae*, *S. arborariae*, and *C. shehatae* in SHH, in which yeasts lost their viability after 24 h of cultivation and only approximately 50 % of glucose present in the medium was consumed, whereas xylose concentration remained intact (data no shown). The final composition of SHH contained toxic compounds, and its osmotic pressure was approximately 2 916 mOsm kg^{-1}. Clearly, these compounds and high osmotic pressure inhibited yeasts growth. Talebnia et al. [21], reported that free cells of *S. cerevisiae* exposed to medium containing 5 g L^{-1} of furfural failed to grow or had its growth arrested, whereas immobilized cells were able to tolerate furfural and take up sugar for growth showing almost no lag phase. The SHH obtained in this work had a lower amount of the inhibitory compounds compared to the that reported by Talebnia et al. [21], therefore,

the differences between free and immobilized cells must be explained by the combination of toxics present in the medium and its very high osmotic pressure. Similar physiological effects were observed for free cells of *C. guilliermondii*, as reported by Schirmer-Michel et al. [22], who grew this yeast in soybean hull hydrolysates with osmotic pressures around 2 950 mOsm kg^{-1} and 2.8 g L^{-1} of combined toxic compounds.

Therefore, it was important to test the immobilization technology for these yeast strains on SHH. Results of cultivations of immobilized cells of *S. cerevisiae* ICV D254, *C. shehatae* HM 52.2, and *S. arborariae* NRRL Y-48658 in SHH are depicted in Figure 2. *S. cerevisiae* entirely consumed glucose, showing high ethanol yields of 0.43 g g^{-1} (Figure 2 A). Comparatively, da Cunha-Pereira et al. [14] cultivated free-cells of *S. cerevisiae* ICV D254 on rice hull hydrolysate with a lower osmotic pressure (1 539 mOsm kg^{-1}) than SHH in this work, reporting yields of 0.53 g g^{-1}. Because *S. cerevisiae* contains an elaborated hexose transport system, the mannose was also metabolized in a diauxic kinetic [23].. In the cultivations of *C. shehatae* (Figure 2 B) and *S. arborariae* (Figure 2 C), however, mannose and xylose were metabolized along with glucose, suggesting that the metabolism of sugars of these two yeasts are not repressed by glucose. Ethanol was produced with yields of 0.47 and 0.38, for *C. shehatae* and *S. arborariae,* respectively. Apparently, the amount of toxic compounds in the medium did not affect cell metabolism or ethanol production. We reported in a previous work an ethanol yield of 0.40 g g^{-1} for suspended-free cells cultures of *C. shehatae* HM 52.2 in rice hull hydrolysate under similar conditions of this research [15]. Efficiencies of xylose utilization present in SHH by *C. shehatae* and *S. arborariae* were 39 % and 33 %, respectively. In general, glucose, mannose, and xylose share the same, unspecific cell transporter system, and the metabolism of the two latter sugars can be repressed by glucose or by high concentrations of substrate, as it has been observed for yeasts such as

S. stipitis and *C. shehatae* [24]. The preferred uptake of glucose over xylose is known to occur for *C. utilis*, *C. shehatae*, and *S. stipitis* because of glucose repression of enzymes involved in the xylose metabolism or by inactivation of enzymes in the high affinity transport system [25]. This fact has not been observed for *C. shehatae* and *S. arborariae* under the conditions of this work, suggesting a significant, but unclear, effect of immobilization on cell metabolism. None of the yeasts tested in this work were able to ferment arabinose (results not shown), as it has also been reported for other yeasts [14, 26]. In this work, cultivations were run with a limited supply of oxygen, therefore, it would be expected that the ethanol formed during glucose metabolism should be subsequently consumed, along with xylose, in a second growth phase, which was clearly shown in the diauxic kinetics (Figure 2). This profile has been demonstrated in experiments with *C. shehatae*, *S. arborariae*, and *C. guilliermondii* [14, 15, 22].

C. shehatae and *S. arborariae* were capable to produce xylitol in SHH medium, reaching up 4.1 g L^{-1} ($Y_{x/x}$ = 0.23 g g^{-1}) and 3.2 g L^{-1} ($Y_{x/x}$ = 0.25 g g^{-1}), respectively. Mussatto et al. [27] reported that when the xylose concentration in the medium is lower than 50 g L^{-1}, yeast metabolism is deviated from xylitol production to cell mass formation in cultures of *C. guilliermondii* in brewers spent grain hemicellulosic hydrolysate. The authors also concluded that the yeast metabolism was strongly affected by the presence of toxic compounds in the hydrolysate. The low productions of xylitol obtained in this work might be explained by the low concentrations of xylose in SHH and the non-ideal medium oxygen concentration during cultivations, which requires tight controls not attainable in shaker flask.

Schirmer-Michel et al. [28] cultivated *C. guilliermondii* under oxygen limitation on soybean hull acid hydrolysate, reporting 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, glycerol was detected at

maximal concentrations of 5.1 g L^{-1}, 4.8 g L^{-1} and 3.7 g L^{-1} for *S. cerevisiae*, *C. shehatae*, and *S. arborariae*, respectively. Glycerol is produced, in part, as cell regulator under osmotic stress [29], which is the case with SHH (2 916 mOsm kg^{-1}). Liu at al. [30] reported that an osmotic pressure of 2 108 mOsm kg $^{-1}$ completely arrested cell growth of several yeasts, including an osmophilic strains of *C. krusei*.

Because *S. cerevisiae* is unable to metabolize pentoses liberated from lignocellulosic matrixes, co-cultures of this yeast with other genera have been proposed as a way to increase ethanol production [2]. In this work we co-immobilized *S. cerevisiae* and *C. shehatae*, or *S. cerevisiae* and *S. arborariae*, within the same porous matrix and the kinetics results of these cultivations in shaker are shown in figures 2 D and 2 E, respectively. In these experiments, a two-stage fermentation process (C5 and C6 fermentations) could be carried out as a one-step bioreaction [13]. Ethanol yields of 0.48 g g^{-1} and 0.40 g g^{-1} were obtained in these co-cultures. The kinetics of co-cultures of free-cells of *S. cerevisiae* and *S. arborariae*, and *S. cerevisiae* and *C. shehatae* using rice hull hydrolysate (RHH), were reported by da Cunha-Pereira et al. [14] and Hickert et al. [15], respectively. The authors reported higher ethanol yields for the co-cultures of *S. cerevisiae*-*S. arborariae*, and for the combination of *S. cerevisiae*-*C. shehatae.* But, it is important to note that their experiments were carried out using free-cells, compared to our immobilized system, where mass transfer problems might be in place. Although SHH and RHH have similar amounts of sugars (63 g L^{-1} and 52 g L^{-1} of total sugars, with 38 g L^{-1} and 35 g L^{-1} of glucose, respectively), their osmotic pressures are significantly diverse, with SHH (2 916 mOsm kg^{-1}) twice as high as RHH (1 539 mOsm kg^{-1}).

In both co-cultures systems xylitol was also produced, reaching up to 4.0 g L^{-1} ($Y_{x/x}$ = 0.25 g g^{-1}) and 3.2 g L^{-1} ($Y_{x/x}$ = 0.27 g g^{-1}) for *S. cerevisiae*-*C. shehatae* and *S.*

cerevisiae-S. arborariae, respectively. The xylitol yields are presented in Table 1. In general terms, the bioconversions obtained in this work were lower than those reported by other authors. However, the results may shed some lights on the conditions to produce xylitol from lignocellulose hydrolysates. The microbial bioconversion of xylose into xylitol depends on a number of culture conditions, such as pH, initial xylose concentration, oxygen supply, and the amounts of furfural, hydroxymethylfurfural, and acetic acid present in the medium [27].

3.3. Scaling up to immobilized-cells bioreactor using *S. cerevisiae* or *C. Shehatae*

The scaling up of fermentations to bioreactors allows identifying problems that were not significantly affected in shaker cultivations, at the same time checking the fermentation parameters to bigger production scales. The use of bioreactors and the specific advantages of immobilized cells are dependent on the type of microorganisms, the reactor configuration and the way the process is conducted [31].

The kinetics of anaerobic batch fluidized-bed bioreactors cultivations of *S. cerevisiae* and of *C. shehatae* in SHH are shown in Figures 3 A and B, respectively. Ethanol was produced to yields of 0.45 for *S. cerevisiae* and 0.38 for *C. shehatae*. Kinetic parameters are presented in Table 1 and compared with the results obtained in the other cultivations. Gabardo et al. [17] tested fluidized and packed-bed bioreactors using cheese whey hydrolysate as substrate for *Kluyveromyces marxianus* and concluded that the better performance of fluidized-bed bioreactor should be reflecting the improved mass transfer mechanisms of this system over the packed-bed configuration, because homogenization of the medium is provided by recirculation. In

the immobilized bioreactor, *S. cerevisiae* showed higher ethanol production than in orbital shaker, amounting 18.6 g L^{-1}.

Pentose-fermenting yeasts usually require oxygen for growth and to produce ethanol, and very few xylose-metabolizing yeast strains have been shown to grow anaerobically on xylose [26]. In our work, in the immobilized bioreactors xylose was not consumed by *C. shehatae*. Possibly for this reason, the yields of ethanol using this yeast in the bioreactor were lower than for the orbital shaker cultivation under limited oxygen conditions (0.38 and 0.47 g g^{-1}), respectively. Accurate dosage of oxygen in large-scale processes is not only economically undesirable, but also virtually impossible, thus underlining the importance of the ability for strict anaerobic growth of some yeasts [23]. For instance, Fu et al. [32] used sugarcane bagasse hydrolysate containing approximately 31 g L^{-1} of glucose and 20 g L^{-1} of xylose in co-cultures of immobilized *Zymomonas mobilis* and free cells of *Scheffersomyces* (*Pichia*) *stipitis* in a modified bioreactor. The authors reported overall ethanol yields of 0.43 g g^{-1} for the total amount of sugars present this hydrolysate.

Talebnia et al. [21] compared cultivations of free and Ca-alginate immobilized cells of *S. cerevisiae* CBS 8066 under anaerobiosis in synthetic medium (glucose as sugar), reporting average ethanol yields of 0.46 g g^{-1} for the immobilized system and 0.42 g g^{-1} for the free-cells system. When these authors cultivated the free-cells in spruce-tree hydrolysate, no growth or sugar consumption and no ethanol was produced in the first 24 h of cultivation, suggesting the toxicity of the hydrolysate resulted in either a very long lag phase, or total metabolic arrest of cells. However, when the immobilized-cells were cultivated in the hydrolysate, ethanol yields of 0.43 g g^{-1} were obtained, whereas less acetic acid and glycerol were formed when compared to the free-cells cultivation.

In the bioreactor cultivations, *S. cerevisae* and *C. shehatae* produced 5.6 g L^{-1} and 5.2 g L^{-1} of glycerol, respectively. Glycerol was formed under anaerobic conditions, as expected, because when the oxidative pathway can not be used by yeast cells for the consumption of reducing equivalents, glycerol is formed for the reoxidation of excess NADH [33]. D`Amore et al. [34] reported that increased glycerol production has also been correlated with high osmotic pressure of medium to counteract cell stress under these conditions. As it was mentioned before, SHH medium, as it was prepared and used in this work, has a high osmotic pressure, one of the highest so far reported in the literature for lignocellulose hydrolysates.

4. CONCLUSION

Immobilized cells of *S. cerevisiae*, *C. shehatae*, and *S. arborariae*, or their combinations, can convert C5-C6 from non-detoxified, acid-enzymatic soybean hull hydrolysate into ethanol. Results of this research point to the possibility of using SHH as a substrate for ethanol production, after its saccharification using cellulases produced by *P. echinulatum* strain S1M29. It was possible to scale up this process with immobilized cells of *S. cerevisiae* and *C. shehatae*, with reasonable ethanol yields. Further studies are granted in order to optimize cultures in the immobilized-cells fluidized bioreactor, using these microorganisms under different oxygen conditions, especially in lignocellulosic hydrolysates and using different feeding strategies.

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Table 1. Ethanol yields obtained in pure cultures of *S. cerevisiae* ICV 254D, *C. shehatae* HM 52.2, or *S. arborariae* NRRL Y-48658, and for the co-cultivations of *S. cerevisiae* and *C. shehatae* or *S. arborariae* on soybean hull hydrolysate.

Yeasts	**Conditions**	**Ethanol** $Y_{P/S}$ **(g g^{-1})**	**Xylitol** $Y_{x/x}$ **(g g^{-1})**
S. cerevisiae	Orbital shaker	0.43	-
C. shehatae		0.47	0.23
S. arborariae		0.38	0.25
S. cerevisiae + *C. shehatae*		0.48	0.25
S. cerevisiae + *S. arborariae*		0.40	0.27
S. cerevisiae	Bioreactor	0.45	-
C. shehatae		0.38	-

Figures:

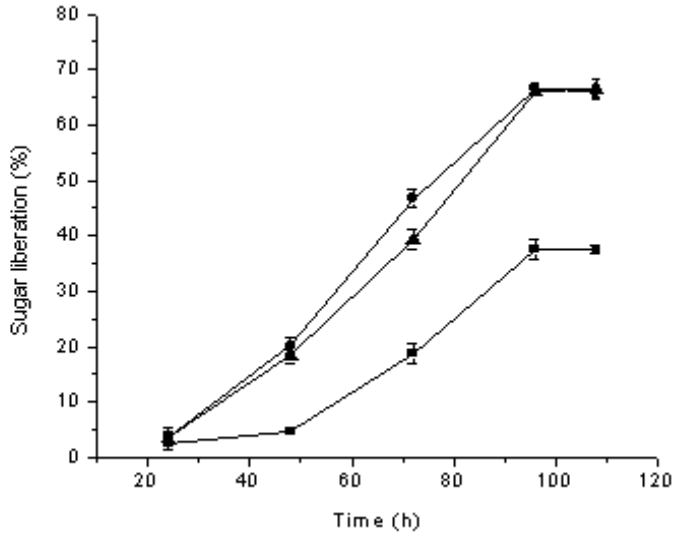

Figure 1. Enzymatic hydrolysis of the solid fraction of soybean hull acid hydrolysate using *P. echinulatum* cellulase complex. Enzyme activities (FPU g^{-1} dry matter): 10 (■), 15 (●), and 20 (▲). Hydrolysis was carried out at 120 rpm, 50 ºC for 110 h. The results are the mean of triplicates.

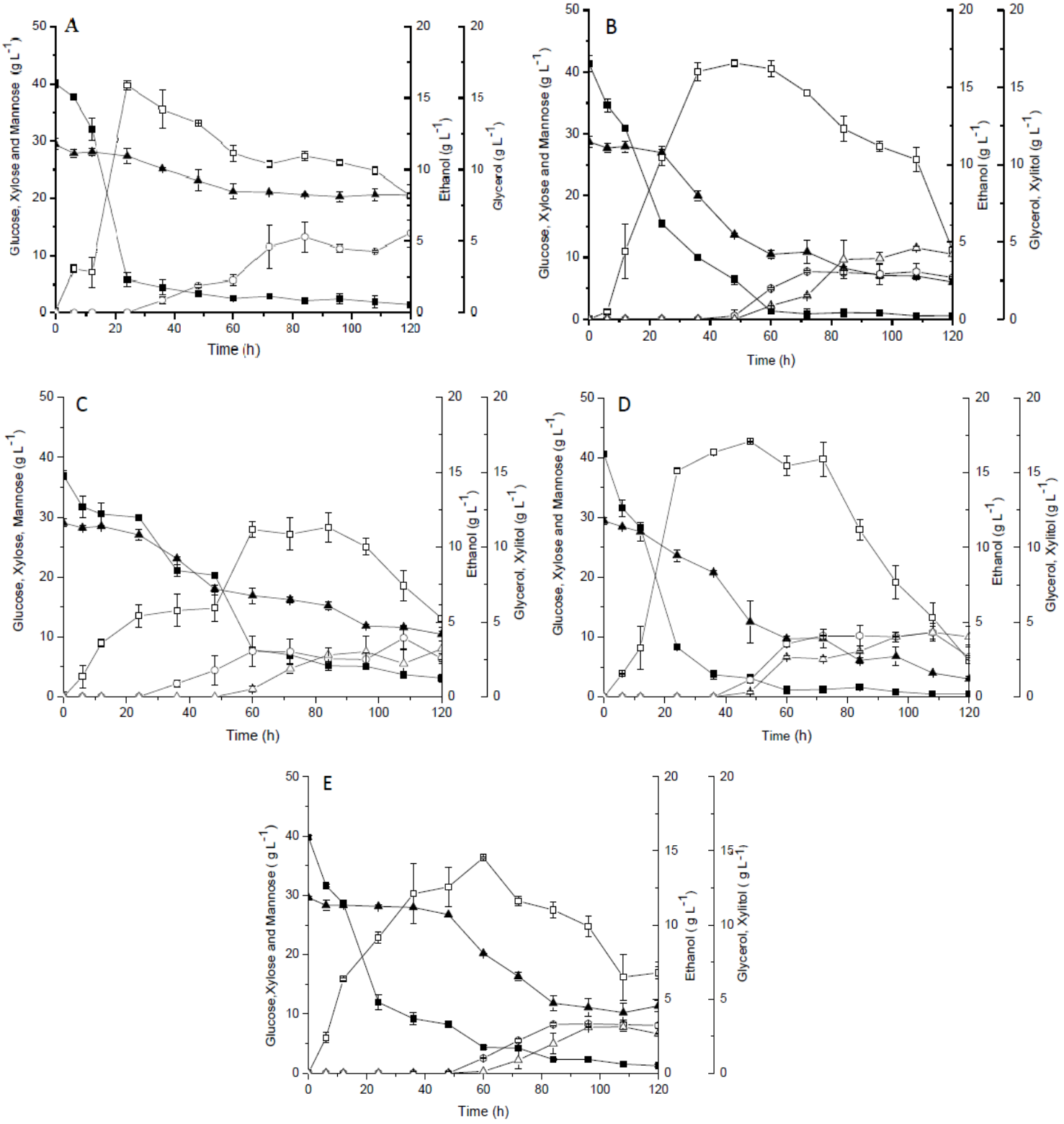

Figure 2. Orbital shaker kinetics of substrate consumption, and ethanol and xylitolproductions on SHH by *S. cerevisiae* (A); *C. shehatae* (B); *S. arborariae* (C); co-cultures of *S. cerevisiae* and *C. shehatae* (D); co-cultures of *S. cerevisiae* and *S. arborariae* (E). Glucose (■); xylose and mannose (▲); ethanol (□); xylitol (△); glycerol (◊). Results are the mean of duplicates.

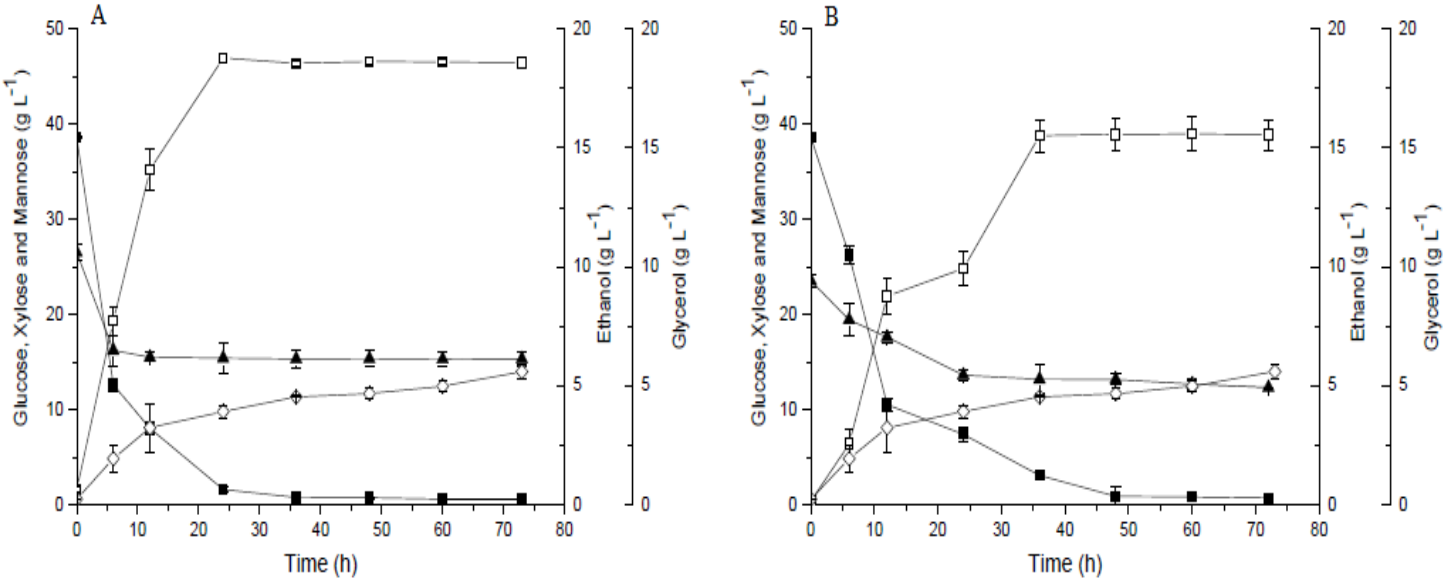

Figure 3. Bioreactor immobilized kinetics of substrate consumption, ethanol and xylitol production by *S. cerevisiae* (A) and *C. shehatae* (B) on soybean hull hydrolysate. Glucose (■); xylose and mannose (▲); ethanol (□); glycerol (◊). Results are the mean of triplicates.

3.4. Resultado IV

“Bioconversion of hexoses and pentoses liberated from soybean hull hydrolysates into ethanol by *Candida guilliermondii* BL 13”

Este artigo consiste no estudo da capacidade de uma linhagem recentemente isolada de *Cândida guilliermondii* BL13 em converter hexoses e pentoses a partir de hidrolisados de casca de soja em etanol. Inicialmente, através da ferramenta estatística Plackett-Burman, tendo como variáveis: peptona, extrato de levedura, milhocina e Tween 80, investigou-se a necessidade de suplementação por nutrientes deste hidrolisado. Após estes experimentos, um planejamento fatorial composto central (CCD), com quatro repetições no ponto central e seis pontos axiais, resultando em um total de dezoito experimentos, foi usado para investigar as condições de fermentação : temperatura, pH, e tamanho do inóculo para a produção de etanol por *C . guilliermondii* BL 13. As melhores condições encontradas foram empregadas em um cultivo com hidrolisado ácido e enzimático – com maior carga de açúcares – por esta levedura. Este artigo foi submetido a revista Process Biochemistry.

Bioconversion of hexoses and pentoses liberated from soybean hull hydrolysates 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 from acid-enzymatic soybean hull hydrolysates into ethanol. Operational conditions and medium formulation were optimized using statistical experimental designs (Plackett-Burman and CCD). Results showed that *C. guilliermondii* BL 13 was capable of growing in non-supplemented, non-detoxified hydrolysate, 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 expected theoretical yields, were observed. These results suggest that *C. guilliermondii* BL13 is potentially useful for applications in second-generation ethanol production from lignocellulosic biomasses.

Keywords: Ethanol production; Soybean hull hydrolysate; Candida guilliermondii; Fermentation optimization.

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 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. 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 ºC for 72 h. 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 [13, 14, 15]. 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:

$$\hat{y} = b_0 + b_1.x_1 + b_2.x_2 + b_{11}.x_1.x_2 + b_{12}.x_1^2 + b_{22}.x_2^2 \quad (1)$$

where ŷ was the predicted response, b0, b1, b2, b11, b12, and b22 were the regression coefficients, and x1 and x2 were the coded levels of the independent 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 oC, 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 % on the basis of their effects and the results for ethanol productivities and yields (measured at the peak production) are shown in Table 3. All coefficients exhibited high p-values for ethanol yield and productivity, 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 by CCD

The effects of temperature, pH, and inoculum size on fermentation of SHH by *C. guilliermondii* BL 13 as determined by the CCD are shown in Table 4. After running the eighteen trials of the CCD, the insignificant effects were excluded to create a reduced model. Using the data shown in Table 4, the experimental model was statistically tested

and the regression coefficients estimated for the variables and their significances, which are shown in Table 5. Regression analysis of experimental data was performed and the quadratic models obtained for each of the response variables can be described by the following reduced equations:

$$Q_P = 0.123 + 0.011x_2 + 0.024x_2^2 + 0.055x_3 \quad (2)$$

$$Y_{P/S} = 0.329 - 0.010x_1 + 0.0105x_2 - 0.0253x_2^2 - 0.0165x_3^2 - 0.0188x_2x_3 \quad (3)$$

where Q_P is ethanol productivity, $Y_{P/S}$ is ethanol yield, and x_1, x_2 and x_3 are the coded levels of pH, temperature, and inoculum size, respectively.

The data provided by the model equations indicates that 91.24 % of the variability ($R^2 = 0.9124$) for the ethanol productivity response, and 90.00 % ($R^2 = 0.9$) for the ethanol yield response can be explained by the models. It was observed a calculated F-value for ethanol productivity (33.84) and yield (6.21) greater than the tabulated F-value (3.18 and 3.09, respectively), denoting that the models are good predictions of the experimental results and the estimated factor effects on the response are real.

The model for ethanol productivity was found to be a second-order (quadratic) model for which the effect of inoculum size can be easily investigated. This model suggests that within the range chosen, the ethanol productivity is independent of the pH and increases proportionally with the inoculum size. Figure 1 shows the response surfaces described by the model Equation (2) to estimate ethanol productivity based on the independent variables pH (x_1) and inoculum size (x_3) (Figure 1 A), and temperature (x_2) and inoculum size (x_3) (Figure 1 B).

Laopaiboon et al., [16], 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 Equation (3). *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 [17, 18], 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., [19], studied the influences 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 ºC, 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 [13] studied the combined effects of initial xylose concentration and inoculum size on xylitol production by *C. guilliermondii* growing in rice straw hydrolysate. They reported the optimum xylose concentration and inoculum level were found to be 82 and 3 g L^{-1}, respectively. The authors did not comment on ethanol production. Powchinda et al., [20], 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 [21, 22].

The best conditions for ethanol productivity by *C. guilliermondii* BL 13 in SHH was found to be: pH 5.0, inoculum size of 1 x 10^9 CFU mL^{-1}, and 28 °C, whereas for ethanol yields the best conditions were: pH 4.6, inoculum size of 7 x 10^6 CFU mL^{-1}, and 28.7 °C. Although bigger inoculum sizes increased ethanol productivity, it negatively affected ethanol yields, probably because of more energy being channeled for cells maintenance. Similar results were reported by Yamada et al., [23], 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} (Table 4, assay 14). 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, 13, 24]. 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. guilliermondi*i 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 1.6 g of glucose and 7.2 g of xylose [25].

3.4. 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}. Using *C. guilliermondii* strain FTI 20037 in supplemented enzymatic hydrolysate of the sugarcane bagasse, which was delignified and pretreated by hydrothermal processing, Silva et al. [26] 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., [24], 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 6, 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 biomasses. Comparing the experiments of this work with those using starch, sweet sorghum, brown rice, and cassava pulp [16, 23, 31], the results were promising, considering that the SH-AEH was not supplemented [16, 23, 31], genetically modified or adapted yeast strains were not used [16, 23, 31] in the fermentation. The comparison of our results against other lignocellulosic biomasses such as corn stalk, corn stover, soybean hull, rice straw, and sugarcane bagasse [25, 27, 28, 29, 30], shows better ethanol volumetric productivity (1.4 g L^{-1} h^{-1}), even when compared to experiments where pretreatment and enzymatic hydrolysis was employed in combination with adapted strains to inhibitors or adapted strains to ethanol, with productivities not higher than 0.89 g L^{-1} h^{-1} [28, 29].

4. 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		Range of levels				
	Symbols	-1.68	-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 (CFU mL^{-1})	x_3	10^5	10^6	10^7	10^8	10^9

Table 3. Plackett-Burman design matrix showing the effects of SHH supplementation with yeast extract, corn steep liquor, peptone, and tween 80 on ethanol productivities and yields.

Assays	Yeast Extract ($g\ L^{-1}$)	Corn Steep Liquor ($g\ L^{-1}$)	Peptone ($g\ L^{-1}$)	Tween 80 ($g\ L^{-1}$)	$Y_{P/S}$ ($g\ g^{-1}$)	Q_P ($g\ L^{-1}\ h^{-1}$)
1	+1 (4)	-1 (0)	-1 (0)	+1 (0.4)	0.357	0.127
2	+1 (4)	+1 (4)	-1 (0)	-1 (0)	0.319	0.131
3	+1 (4)	+1 (4)	+1 (5)	-1 (0)	0.408	0.159
4	-1 (0)	+1 (4)	+1 (5)	+1 (0.4)	0.285	0.113
5	+1 (4)	-1 (0)	+1 (5)	+1 (0.4)	0.468	0.148
6	-1 (0)	+1 (4)	-1 (0)	+1 (0.4)	0.308	0.126
7	-1 (0)	-1 (0)	+1 (5)	-1 (0)	0.319	0.114
8	-1 (0)	-1 (0)	-1 (0)	-1 (0)	0.446	0.154
9	0 (2)	0 (2)	0 (2.5)	0 (0.2)	0.376	0.151
10	0 (2)	0 (2)	0 (2.5)	0 (0.2)	0.388	0.141
11	0 (2)	0 (2)	0 (2.5)	0 (0.2)	0.393	0.146

Table 4. Experimental values of ethanol productivity (Q_P) and yield ($Y_{P/S}$) resulting from the application of the central composite design.

Assay	pH	Temperature (°C)	Inoculum (CFU mL^{-1})	Q_P (g L^{-1} h^{-1})	$Y_{P/S}$ (g g^{-1})
1	-1 (4.5)	-1 (25)	-1 (10^6)	0.10	0.28
2	+1 (5.5)	-1 (25)	-1 (10^6)	0.10	0.26
3	-1 (4.5)	+1 (31)	-1 (10^6)	0.11	0.34
4	+1 (5.5)	+1 (31)	-1 (10^6)	0.11	0.33
5	-1 (4.5)	-1 (25)	+1 (10^8)	0.19	0.29
6	+1 (5.5)	-1 (25)	+1 (10^8)	0.18	0.26
7	-1 (4.5)	+1 (31)	+1 (10^8)	0.20	0.27
8	+1 (5.5)	+1 (31)	+1 (10^8)	0.20	0.26
9	-1.68 (4.16)	0 (28)	0 (10^7)	0.07	0.35
10	+1.68 (5.84)	0 (28)	0 (10^7)	0.11	0.36
11	0 (5.0)	-1.68 (23)	0 (10^7)	0.15	0.33
12	0 (5.0)	+1.68 (33)	0 (10^7)	0.21	0.30
13	0 (5.0)	0 (28)	-1.68 (10^5)	0	0.34
14	0 (5.0)	0 (28)	+1.68 (10^9)	0.24	0.30
15	0 (5.0)	0 (28)	0 (10^7)	0.12	0.25
16	0 (5.0)	0 (28)	0 (10^7)	0.12	0.27
17	0 (5.0)	0 (28)	0 (10^7)	0.12	0.27
18	0 (5.0)	0 (28)	0 (10^7)	0.12	0.30

Table 5. Regression coefficients estimated by means of the ANOVA for ethanol productivity in function of temperature (T), pH and inoculum size (I).

Response variable	Source	Regression coefficient	Standard Error	*t*-value	*p*-value
Q_P	Mean/Interaction	0.1233	0.0079	15.7003	[a] 0.0000
	Quadratic pH	-0.0075	0.0054	-1.3789	0.1912
	Linear T	0.0111	0.0054	2.0643	[a] 0.0595
	Quadratic T	0.0244	0.0054	4.4758	[a] 0.0006
	Linear I	0.0552	0.0054	10.3104	[a] 0.0000
$Y_{P/S}$	Mean/Interaction	0.3294	0.0084	39.284	[a] 0.0000
	Linear pH	-0.0101	0.0057	-1.759	[a] 0.1063
	Linear T	0.0105	0.0057	1.841	[a] 0.0927
	Quadratic T	-0.0253	0.0058	-4.354	[a] 0.0011
	Linear I	-0.0058	0.0057	-1.021	0.3294
	Quadratic I	-0.0165	0.0058	-2.831	[a] 0.0163
	T x I	-0.0188	0.0075	-2.512	[a] 0.0289

a Significant coefficients (p-value ≤ 0.1).

Table 6. 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., [16]
Brown rice	-	*S. cerevisiae*	28.8	0.43	1.2	Yamada et al., [23]
Cassava pulp (5 %)	Hydrothermal reaction and enzymatic hydrolysis	*S. cerevisiae* displaying *Rhizopus oryzae* glucoamylase	18.6	0.50	0.77	Kosugi et al., [31]
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., [29]
Corn stover	Steam exploded and enzymatic hydrolysis	*S. cerevisiae*	43.21	0.47	0.72	Li et al., [28]
Soybean hull	Acid hydrolysis	*C. guilliermondii*	5.78	0.53	0.24	Schirmer-Michel et al., [25]
Rice straw	Acid hydrolysis	*P. stipitis*	18.7	0.37	0.39	Silva et al.,[27]
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., [30]

Figures Captions

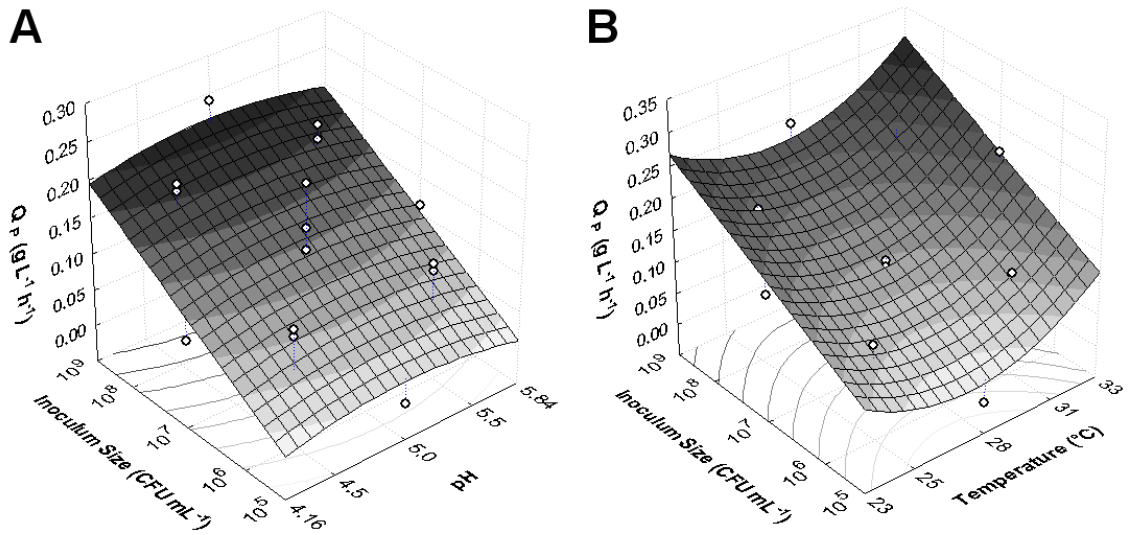

Figure 1. Response surface plots for ethanol productivity (Q_P, g L^{-1} h^{-1}) as function of (A) inoculum size and pH and (B) inoculum size and temperature.

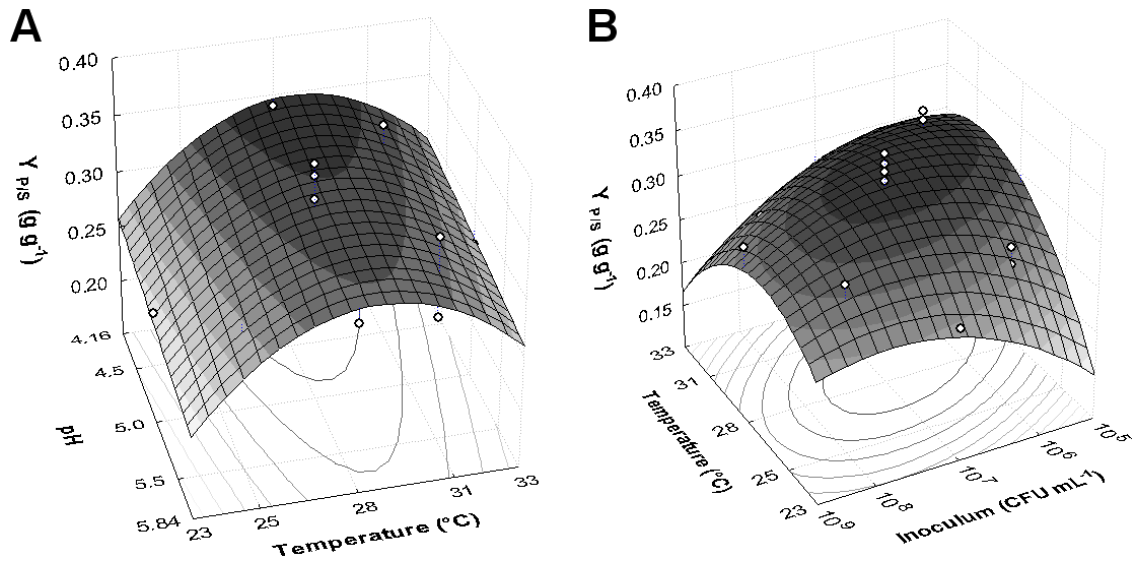

Figure 2. Response surface plots for ethanol yields ($Y_{P/S}$, g g^{-1}) as function of (A) pH and temperature and (B) inoculum size and temperature.

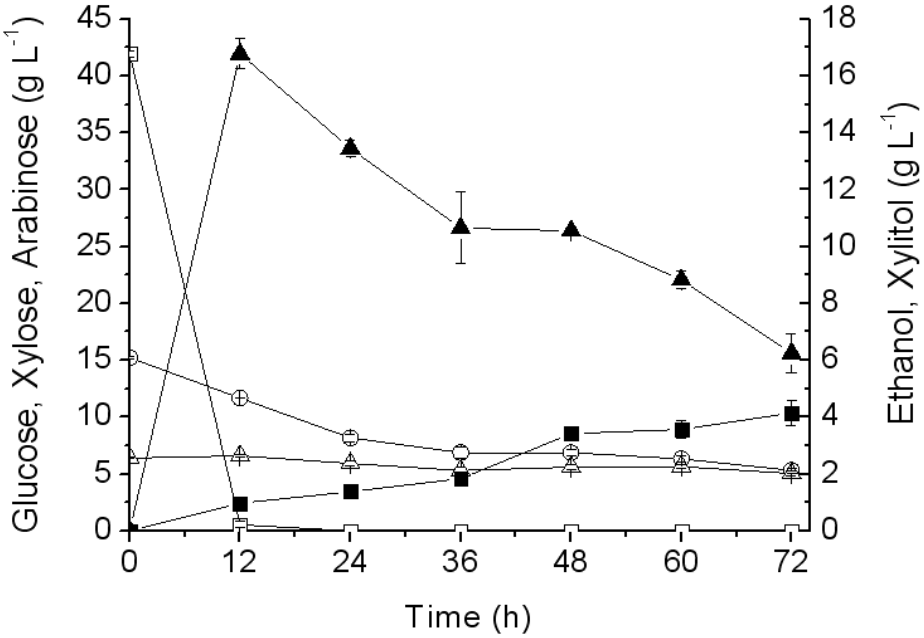

Figure 3. Kinetics of substrate consumption, ethanol and xylitol production by *C. guilliermondii* BL13 cultivated on soybean hull acid-enzymatic hydrolysate. Experiments were carried out in orbital shaker at 28 °C, pH 5.0, 180 rpm. Glucose (□); xylose (¤); arabinose (Δ); ethanol (▲); xylitol (■).Results are the mean of triplicates.

Capítulo 4

Considerações Finais

Estudos relacionados à inserção da produção de etanol de 2ª geração no Brasil são essenciais para a viabilização desta tecnologia e a manutenção do País na posição de grande produtor de etanol no mundo. Não apenas através da produção de bioetanol a partir do bagaço de cana-de-açúcar, mas de todo e qualquer material lignocelulósico, como o caso da casca de arroz e da casca de soja, resíduos hoje subaproveitados. Neste sentido, o Brasil se encontra em uma posição de destaque pela grande variedade de culturas, pela abundância de resíduos e principalmente pela sua biodiversidade.

A viabilidade econômica da produção de etanol de 2ª geração é extremamente interligada à oferta de matéria-prima no local da hidrólise, ao uso de tecnologias enzimáticas eficientes e de baixo custo, e principalmente, de microrganismos capazes de fermentar todos os açúcares presentes no meio dos hidrolisados sem nenhuma detoxificação, aumentando assim a produtividade de etanol e outros subprodutos da fermentação.

No presente trabalho, diferentes tecnologias e microrganismos foram utilizados visando o aumento na produção e/ou produtividade a etanol. Apenas a levedura *S.*

cerevisiae é comercial, os outros microrganismos são todos isolados recentemente, sendo, em grande parte, publicados em bioprocessos utilizando hidrolisados pela primeira vez. Em todos os resultados apresentados, a hidrólise ácida diluída foi utlizada visando à liberação dos açúcares no meio (glicose, xilose, arabinose e manose), principalmente pelo seu fácil manuseio e baixo custo, quando comparado a outros métodos de pré-tratamento. As condições de hidrólise foram otimizadas em trabalhos anteriores do grupo Bioteclab.

No primeiro artigo, a composição da casca de arroz foi estudada, quanto á celulose, hemicelulose, lignina e extrativos presentes. Frente ao indicativo favorável de presença de açúcares, testou-se o uso do hidrolisado ácido de casca de arroz (HCA) com co-culturas de *S. cerevisiae* e *C. shehatae* em meio sintético simulando HCA e no próprio, obtendo altos rendimentos de etanol, tanto em frascos agitados quanto em biorreatores. Em agitador orbital os índices do fator de conversão dos açúcares a etanol foram menores quando comparados aos biorreatores, possivelmente pelo maior controle do processo possibilitado pelos últimos (pH, agitação uniforme e temperatura). Estes resultados demonstraram a possibilidade do uso de HCA para a produção de etanol, tendo como subproduto o xilitol, um produto também visado em bioprocessos pelo alto custo de mercado.

No segundo artigo, relatou-se o uso do HCA em cultivos com a levedura *S. cerevisiae*, em condições de oxigênio limitado em biorreatores. Posteriormente, utilizou-se a levedura *S. arborariae*, um microrganismo recentemente isolado já utilizado pelo grupo para fermentações de C5 (xilose e arabinose), em co-cultivos. Finalizando, testou-se o uso da enzima POWERCELL®, para sacarificação e co-fermentação simultânea (SSCF) do HCA, utilizando ambas as leveduras (*S. cerevisiae* e *S. arborariae*) para a produção de etanol e de xilitol, em biorreatores. Os resultados

encontrados foram satisfatórios, porém os biorreatores onde ocorreram a SSCF foram de difícil manuseio, devido principalmente pela composição do hidrolisado (com sólidos resultantes da hidrólise).

A casca de soja, outro resíduo encontrado em grandes quantidades no RS, foi testada em hidrólise ácida seguida de hidrólise enzimática. O uso de enzimas é um dos grandes gargalos econômicos na viabilidade do etanol de segunda geração. Por isso, no terceiro artigo, o uso da enzima de *Penicillium echinulatum* S1M29, uma enzima não purificada nem comercial, no resíduo sólido de casca de soja pós-hidrólise ácida branda, foi investigado. O hidrolisado de casca de soja (HCS) ácido juntamente com o enzimático, após concentração, possui alta concentração de inibidores do processo fermentativo, além de uma alta pressão osmótica. Por esta razão, não pode-se utilizar células livres de leveduras. Então, as leveduras foram imobilizadas em esferas alginato de cálcio e posteriormente utilizadas na fermentação deste hidrolisado, com rendimentos variados de produção de etanol, em agitador orbital. As melhores produções de etanol foram obtidas com células imobilizadas de *S. cerevisiae* e *C.shehatae*. Visando tornar a produção mais próxima possível do ambiente industrial, estas leveduras foram testadas em biorreatores com células imobilizadas, nos quais mantiveram índices semelhantes encontrados nos agitadores orbitais. Os resultados deste trabalho comprovam as boas perspectivas na utilização da celulase de *P. echinulatum* para hidrólise enzimática da casca de soja, e da combinação com culturas imobilizadas das leveduras *S. cerevisiae* e *C. shehatae*, na produção de etanol.

Pode-se notar que as fermentações utilizando as leveduras de *S. cerevisiae, C.shehatae, S. arborariae* obtiveram fatores de conversão de açúcares a etanol bastante satisfatórios tanto em hidrolisados de casca de arroz quanto em hidrolisados de casca de soja. Neste contexto, no quarto artigo, a levedura *C. guilliermondii* BL13 foi testada em

fermentações do hidrolisado de casca de soja (HCS). Inicialmente, fez-se um planejamento seguindo a metodologia Plackett-Burman, onde a suplementação do meio de HCS foi investigada quando a adição de nutrientes: peptona, extrato de levedura, milhocina e Tween 80. A análise dos resultados não foi significativa para nenhuma suplementação. Desta forma, um planejamento fatorial composto central, com as variáveis temperatura, pH e tamanho do inoculo foi realizado. As condições ideias para cultivo desta levedura, visando uma maior produtividade do processo, foram: 28 °C de temperatura, pH 5.0, e 10^9 CFU mL^{-1} de tamanho de inóculo.

Em conclusão, os estudos realizados neste trabalho, demostraram que tanto o hidrolisado de casca de arroz quanto o de casca de soja possuem grande potencial para a produção de etanol de 2ª geração, assim como outros produtos, como o xilitol. Também pode-se observar que os microrganismos utilizados obtiveram altos rendimentos de conversão dos açúcares presentes nos hidrolisados, mesmo sem suplementação ou destoxificação dos meios. Diferentes estratégias fermentativas são importantes para o aumento da viabilidade econômica do processo. Enfim, estes resultados foram importantes principalmente do ponto ambiental, indicando um destino nobre a resíduos agroindustriais.

PERSPECTIVAS

Ao longo do desenvolvimento deste trabalho verificaram-se algumas oportunidades para aprimoramento e detalhamento das simulações desenvolvidas; algumas destas sugestões de trabalhos futuros são listadas a seguir.

Estudo detalhado do metabolismo de consumo das pentoses pelas leveduras e aperfeiçoamento do mesmo; Estudar o mecanismo de cooperação das leveduras quando utilizados co-cultivos; Utilizar a engenharia metabólica como ferramenta para inserir ou inibir uma determinada rota bioquímica, ampliando a produção dos produtos de interesse;

Avaliar o uso de hidrólise ácida - possibilitando a liberação de pentoses - seguida de hidrólise alcalina – para deslignificação – na casca de arroz, possibilitando maior ataque enzimático à celulose;

Estudo de temperaturas e pHs ideias para sacarificação e fermentação simultânea dos hidrolisados;

Testar diferentes estratégias fermentativas, como cultivos em batelada alimentada e contínuos, tanto com células livre quanto com células imobilizadas;

Analisar outros produtos possíveis de serem obtidos por leveduras utilizando estes hidrolisados, como butanol, 2,3 butanodiol, entre outros;

Avaliar a viabilidade econômica do processo de produção do etanol de 2ª geração a partir destes hidrolisados;

Estudar a etapa de recuperação dos produtos formados.

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