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BÁRBARA RIBEIRO ALVES ALENCAR

AVALIAÇÃO DO POTENCIAL DA PRODUÇÃO DE ETANOL, A PARTIR DE BIOMASSAS LIGNOCELULÓSICA E MELAÇO, COMO ALTERNATIVA ENERGÉTICA PARA O PERÍODO DE ENTRESSAFRA DA CANA-DE-AÇÚCAR NO ESTADO DE PERNAMBUCO

Recife
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de doutora em Ciências Biológicas.

Área de concentração: Biotecnologia.

Orientador: Profº. Dr. Marcos Antonio de Morais Júnior.

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Aprovada em: 23 / 02 / 2022.

BANCA EXAMINADORA

_____

Prof. Dr. Marcos Antonio de Morais Júnior
Universidade Federal de Pernambuco

_____

Profº. Dr. Jorge Luiz Silveira Sonego
Universidade Federal de Pernambuco

_____

Profª. Drª. Sarita Cândida Rabelo
Universidade Estadual Paulista

_____

Prof. Dr. João Ricardo Moreira de Almeida
Embrapa Bioenergia

_____

Profª. Drª. Inês Conceição Roberto
Universidade de São Paulo

Aos meus pais, meus avós, meu irmão e
ao meu companheiro

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“People are gonna tell you who you are your whole life. You just gotta puch back and say: “No, this is who I am”. You want people to look at you differently? Make them. You want to change things? You’re gonna have to go out there and change them yourself”.

“There are always people who want you to give up. Never facilitate their work”.

(Kitsis; Horowitz, 2011)

RESUMO

As limitações geográficas e climáticas de Pernambuco impossibilitam a produção de cana-de-açúcar o ano inteiro e por toda a sua extensão, sem a adição de sistema de irrigação adequado, já que 48% da região Nordeste está inserida no clima árido/semiárido. Dentro deste cenário, avaliou qual a melhor época do ano e período do dia para a colheita da biomassa de palma forrageira, considerando-se o pH ideal para a realização da etapa de hidrólise enzimática. Além disso, determinou a melhor temperatura e o tempo de secagem, desse vegetal, para a etapa de produção de carboidratos. Os resultados sugeriram que o início da manhã e a estação seca são os melhores períodos de colheita, enquanto que a melhor temperatura e tempo de secagem da biomassa são 105°C e 12h, respectivamente. Já o segundo capítulo objetivou a avaliação da fermentação dos hidrolisados ácidos dos bagaços de cana-de-açúcar (HBC) e sorgo (HBS), além da biomassa de palma forrageira (HPF), por *Meyerozyma Caribbica*. Além disso, realizou-se a fermentação de blends com diferentes proporções de melaço e hidrolisado, avaliando-se a influência da concentração de carboidratos na produção de etanol, por esta levedura. *M. Caribbica* fermentou tais substratos, apresentando eficiências superiores a 75%, para a produção de etanol 1G e, >80%, para a 2G. No meio M3, composto pela mistura de 25% de melaço, 25% do HBC, 25% do HBS e 25% do HPF, obteve-se a melhor concentração de etanol (50 g/L) e eficiência fermentativa (90,60%) com *M. caribbica*, na comparação com *S. cerevisiae* (37 g/L, 66,48%). Esses dados sugerem que, em condições de não suplementação nutricional, *M. caribbica* apresenta-se mais adaptada a meios estressantes que *S. cerevisiae*. Assim, a princípio, a condição proposta de utilizar 25% de cada um dos substratos parece ser a mais adequada, sob a ótica de realizar um processo com produção de etanol satisfatória e baixo custo.

Palavras-chave: Biomassa; Hidrólise; Palma forrageira; Sorgo sacarino; Cana-de-açúcar.

ABSTRACT

The geographic and climatic limitations of Pernambuco make it impossible to produce sugarcane throughout the year and throughout its extension, without the addition of an adequate irrigation system, since 48% of the Northeast region is located in an arid/semi-arid climate. Within this scenario, it evaluated the best time of year and time of day for the harvesting of cactos pear biomass, considering the ideal pH for carrying out the enzymatic hydrolysis step. In addition, it determined the best temperature and drying time for this vegetable for the carbohydrate production stage. The results suggested that the early morning and the dry season are the best harvest periods, while the best temperature and biomass drying time are 105°C and 12h, respectively. The second chapter aimed to evaluate the fermentation of acid hydrolyzates from sugarcane bagasse (HBC) and sorghum (HBS), in addition to forage cactus biomass (HPF), by *Meyerozyma Caribbica*. In addition, blends with different proportions of molasses and hydrolyzate were fermented, evaluating the influence of carbohydrate concentration on ethanol production by this yeast. *M. Caribbica* fermented such substrates, showing efficiencies above 75% for the production of 1stG ethanol and >80% for 2ndG ethanol. In the M3 medium, composed of a mixture of 25% molasses, 25% SCH, 25% SSH and 25% CPH, the best concentration of ethanol (50 g/L) and fermentative efficiency (90.60%) with *M. caribbica*, in comparison with S. cerevisiae (37 g/L, 66.48%). These data suggest that, under conditions of no nutritional supplementation, *M. caribbica* is more adapted to stressful environments than *S. cerevisiae*. Thus, at first, the proposed condition of using 25% of each of the substrates seems to be the most adequate, from the point of view of carrying out a process with satisfactory ethanol production and low cost.

Keywords: Biomass; Hydrolysis; Cactus pear; Sweet sorghum; Sugarcane.

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SUMÁRIO

1 INTRODUÇÃO

Soluções para produção de etanol, a partir de biomassas lignocelulósicas, são vastamente estudadas pelo mundo, com base nas características locais de cada região. Entender o comportamento da agricultura local e suas limitações faz-se importante e necessário. Portanto, o Brasil, o qual é um país majoritariamente agrário e de dimensões continentais, apresenta características de clima e culturas completamente diferentes, nas suas mais diversas regiões. Deste modo, avaliações regionais, e até mesmo estaduais, são necessárias.

O Estado de Pernambuco apresenta cerca de 8.000 km^2 de área plantada de cana-de-açúcar, uma região que vai do litoral até a cidade de Vitória de Santo Antão. Porém, a indústria sucroalcooleira do estado sofre com o período de entressafra, no qual se diminui consideravelmente a produção de etanol. Desta forma, faz-se necessário aumentar e diversificar a área produtiva pernambucana, de modo a suprir essa carência. Assim, fontes alternativas de biomassa lignocelulósica, como sorgo sacarino e palma forrageira, juntamente com resíduos da produção de açúcar, como o bagaço da cana e melaço, podem contribuir para reduzir o efeito da entressafra na produção de etanol, gerando álcool, por meio de hidrolisados ácidos, ricos em carboidratos, e melaço. Ademais, utilizar vegetais oriundos de climas áridos e semiáridos pode alterar cenários econômico-sociais no sertão pernambucano.

Utilizar fontes alternativas de carbono implica em usar micro-organismos fermentadores não convencionais, já que serão fornecidos não apenas a glicose, o qual é o monossacarídeo base do processo, mas também pentoses, como a xilose, a qual não pode ser fermentada pela tradicional levedura *Saccharomyces cerevisiae*. Por consequência, processos precisam ser reavaliados, de modo a tornar as etapas da produção de etanol de segunda geração, cada vez mais, viáveis economicamente, principalmente no que se refere a produtos com maior valor agregado, como o xilitol.

As fontes de carbono supracitadas foram e são largamente estudadas pelos grupos de pesquisa “Energia da biomassa” (DEN/UFPE) e “Biologia Molecular e Engenharia Metabólica da UFPE” (DGEN/UFPE), no qual esta tese está inserida. Porém a atual proposta é ampliar estes estudos, tentando entender os modos como biomassas/fontes de carbono não convencionais, além de micro-organismos não

tradicionais, podem contribuir no desenvolvimento e no aumento da produção energética do Estado.

2 REVISÃO DE LITERATURA

2.1 CANA-DE-AÇÚCAR, SORGO SACARINO E PALMA FORRAGEIRA: COMPOSIÇÃO, CULTIVO E PRODUTIVIDADE

2.1.1 Cana-de-açúcar e melaço

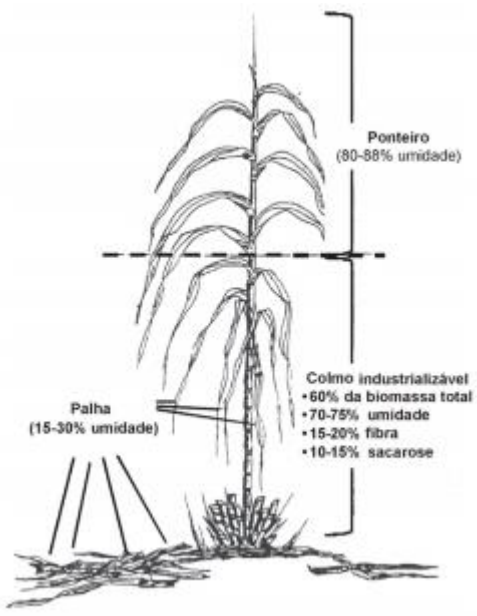
Originária da Nova Guiné, localizado no continente Oceania, a cana-de-açúcar (Figura 1) foi largamente difundida pelo mundo, durante do período das grandes navegações. Esse vegetal apenas chegou ao Brasil no ano de 1532 e, desde então, tem a finalidade de produção de açúcar e álcool, sendo este primariamente utilizado para consumo humano, e, posteriormente, produção de energia (NOVACANA, 2018).

Pertencente à família Poaceae, a cana-de-açúcar apresenta, em sua estrutura, 3 regiões: a raiz, as folhas e o caule, do tipo colmo, cujo, ainda é subdividido em “ponteiro” e o “colmo industrializável” (Figura 2). Esta última porção é a principal fonte energética da planta, por ser a região central de reserva de carboidratos (MATSUOKA et al., 2012), a partir da qual será extraído o caldo da cana, para produção, principalmente, de açúcar e álcool.

Figura 1 - Plantação de cana-de-açúcar e seu caule em destaque.

Fonte: NOVACANA, 2018.

Figura 2 - Estrutura da cana-de-açúcar, ressaltando-se as regiões do colmo e ponteiro.

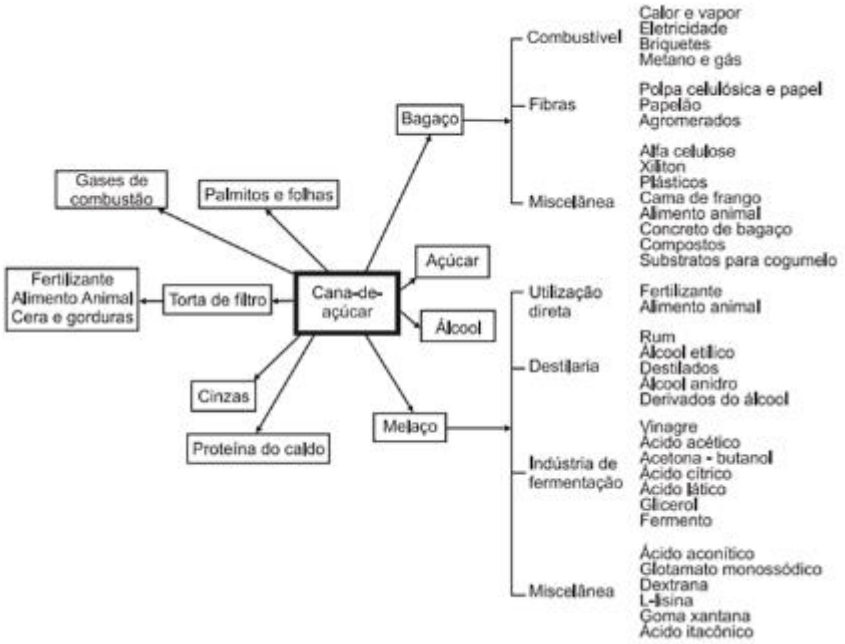


Fonte: MATSUOKA et al., 2012.

O Brasil é o maior produtor de cana-de-açúcar do mundo, tendo sua produção aumentado em mais de 100% nas últimas décadas, com a intenção de atender às demandas energéticas globais (BORDONAL et al., 2018; CONAB, 2019). Na safra de 2019/2020, a área plantada desta biomassa, no Brasil, correspondeu a 8.442 mil ha, com uma produtividade média de 76.133 kg/ha. Já no estado de Pernambuco, os valores foram iguais a 237,3 mil ha de área cultivada, com produtividade de 52.768 kg/ha (CONAB, 2020). No sentido leste-oeste, a área possível de ser utilizada para plantação de cana-de-açúcar segue do litoral até a altura da cidade de Vitória de Santo Antão.

Durante a produção de açúcar e álcool, existe a geração de subprodutos, desde a etapa de colheita, até o término do processo, como o melaço, a torta de filtro, palmitos e folhas, proteínas do caldo e o bagaço da cana-de-açúcar, cujos podem ser utilizados para as mais diversas finalidades (MATSUOKA et al., 2012) (Figura 3). Esta revisão será centralizada apenas no bagaço e no melaço.

Figura 3 - Resíduos da produção de açúcar e álcool, a partir da cana-de-açúcar.



Fonte: MATSUOKA et al., 2012.

O bagaço de cana-de-açúcar é produto da extração do caldo produzido no caule do vegetal. A sua composição química é caracterizada por teores de celulose e hemicelulose relativamente elevados (Tabela 1), porém, neste caso, cerca de 50% da estrutura do homopolímero está na sua conformação cristalina, o que dificulta o acesso à glicose ali existente (GUILHERME et al., 2015; ROCHA et al., 2015). Devido a esta dificuldade, pesquisadores sempre relataram a necessidade do uso de pré-tratamentos, sejam químicos e/ou físicos, para melhorar a obtenção do monossacarídeo em etapas subsequentes do processo (HOANG et al., 2020; LADEIRA-ÁZAR et al., 2019; SAVOU et al., 2019). Porém, o uso de pré-tratamentos acarreta um aumento de 16-30% dos custos de produção do etanol de segunda geração (também denominado de etanol celulósico), sendo este um dos fatores que dificulta a sua viabilidade (HUMBIRD et al., 2011).

Por outro lado, a porção denominada de hemicelulose é amorfa e mais facilmente removível, em relação à celulose, quando o heteropolímero é hidrolisado, principalmente na presença de ácidos fortes, como H_2SO_4 e HCl (KUMAR et al., 2015; LAVARACK; GRIFFIN; RODMAN, 2002). Deste modo, sendo realizada em etapa única, diminuindo assim os custos de produção, a hidrólise ácida da hemicelulose pode fornecer, relativamente, altas concentrações de xilose, a qual pode ser

fermentada a etanol (PHAIBOONSILPA et al., 2020; RECH et al., 2016; TIZAZU; MOHOLKAR, 2018). Assim, essas condições fornecem, ao bagaço da cana-de-açúcar, uma relevante função na produção de etanol de segunda geração, fazendo dele não o ator principal, mas, um importante coadjuvante neste cenário. Isso se deve, principalmente, ao fato do mesmo, a princípio, já estar no local onde será produzido o etanol celulósico, facilitando, portanto, a sua utilização.

O outro subproduto, denominado de melaço, é derivado da produção do açúcar e é o seu último estágio, a partir do qual não é possível mais retirar a sacarose para a produção do alimento, através dos processos tradicionalmente realizados. Porém, ainda é possível encontrar carboidratos em sua composição, os quais podem ser utilizados no processo fermentativo (PALMONARI et al., 2020; RASMEY et al., 2018; WU et al., 2020). A Tabela 1 descreve a composição química e mineral do melaço de cana-de-açúcar, a partir da qual é possível identificar que cerca de 62% da matéria seca, deste resíduo, é composto por açúcar. Destes, quase 50% é sacarose, seguido de frutose (8%), e glicose (5%), não sendo identificado xilose na sua composição. Porém, é identificada uma quantidade relativamente elevada de cátios e ânios, os quais agem inibindo o metabolismo celular, através da pressão osmótica gerada, afetando negativamente o processo fermentativo (D'AMORE et al., 1988; NISHINO; MIYAZAKI; TOHJO, 1985). Apesar disso, trabalhos recentes têm reportado o potencial da produção de etanol, a partir do uso do melaço, como meio de fermentação (JAGTAP et al., 2019; MAYZUHROH; ARINDHANI; CAROENCHAI, 2016; SHARMA; KUMAR; SHUKLA, 2018).

Tabela 1 - Descrição da composição química do melaço de cana-de-açúcar (% de matéria seca, exceto quando indicado o oposto).

Item	Média (%)	Desvio Padrão (%)	Mínimo (%)	Máximo (%)
Matéria seca	76,80	1,00	75,70	79,60
Proteína bruta total	6,65	1,79	2,22	9,31
Carboidratos totais	62,30	4,70	57,00	71,00
Sacarose	48,80	6,40	39,20	67,30
Glicose	5,29	2,69	1,30	12,07
Frutose	8,07	2,83	2,30	14,28
Rafinose	0,03	0,00	0,02	0,03
Galactose	0,04	0,00	0,04	0,04
Arabinose	0,01	0,02	0,00	0,04
Xilose	ND[1]	ND	ND	ND
Amido	0,33	0,25	0,06	1,07
Levanas	0,86	0,26	0,26	1,21
Dextranas	0,79	0,42	0,27	1,63
Arabinanas	0,20	0,05	0,06	0,28
Ácido cítrico	1,42	0,85	0,24	3,78
Ácido láctico	6,10	2,82	1,62	12,75
Ácido málico	0,10	0,05	0,03	0,21
Ácido cítrico	0,13	0,04	0,08	0,22
Ácido pirocarbônico	0,34	0,13	0,18	0,62
Ácido oxálico	0,06	0,02	0,04	0,09
Ácido glicólico	0,00	0,00	0,00	0,00
Ácido acético	0,44	0,28	0,16	1,04
Cinzas	13,10	1,50	10,20	16,30
Ca	1,39	0,55	0,82	3,13
Mg	0,43	0,14	0,19	0,63
Na	0,08	0,10	0,01	0,42
K	1,82	1,91	0,31	7,99
Sulfatos	2,09	0,88	0,81	4,09
Enxofre[2]	0,69	0,29	0,27	1,36
Fosfatos	2,03	0,77	0,70	2,97
Nitratos, mg/kg	464,00	337,00	17,00	999,00
Cloretos, mg/kg	60,00	86,00	1,00	340,00

[1]ND: Não detectado; [2]Valor de enxofre obtido a partir de sulfatos, considerando seus respectivos pesos moleculares.

Fonte: Adaptado de (PALMONARI et al., 2020).

2.1.2 Sorgo sacarino

A revisão de sorgo sacarino será apresentada através da revisão de literatura, publicada pela autora na revista “Cadernos do semiárido – Riquezas e Oportunidades”, no ano de 2020, a qual está intitulada “Potencial de produção de etanol a partir da biomassa de sorgo sacarino”. Os autores deste capítulo de livro foram Rômulo Simões Cezar Menezes, Bárbara Ribeiro Alves Alencar, Emmanuel Damilano Dutra e José Nildo Tabosa.

O crescimento mundial da produção de biocombustíveis é motivado pela diminuição da disponibilidade de combustíveis fosseis, bem como pela ação destrutiva, proveniente da queima desses combustíveis, no meio ambiente (GAURAV et al., 2017). A escolha da matéria-prima para a produção do biocombustível depende das condições ambientais da região. No Brasil, em relação ao etanol, predomina–se o cultivo da cana-de-açúcar. Porém, em algumas regiões do país a limitação hídrica afeta diretamente a viabilidade dessa cultura. A limitação hídrica atual, bem como o cenário de possíveis reduções das precipitações, como consequência de mudanças climáticas, além da redução nas produtividades das culturas energéticas, exige estudos com biomassas de elevada produtividade e menor exigência hídrica. Nesse sentido, apresenta-se como alternativa o sorgo sacarino (ROONEY et al., 2007).

O sorgo sacarino é uma cultura tradicionalmente utilizada como forragem, ração e fibras (MURRAY et al., 2008; REDDY et al., 2005). Como principais características, destacam-se a eficiência no uso de água (1/3 da cana-de-açúcar e 1/2 do milho) e bom desenvolvimento em diferentes condições edafoclimáticas. Em geral, o sorgo sacarino produz 2 t ha^{-1} de grãos rico em amido e 50 t ha^{-1} de colmos rico em açúcares solúveis como sacarose, glicose e frutose (WU et al., 2010) no caldo e carboidratos insolúveis (celulose e hemicelulose) no bagaço gerado após extração do caldo. Devido a essas características, o sorgo sacarino é relatado como uma opção a produção de bioetanol (ALMODARES; HADI, 2009; VASILAKOGLOU et al., 2011).
De uma forma geral, essa produção, a partir do caldo, situa-se em aproximadamente 3.450 L.ha^{-1} (PRASAD et al., 2007), do amido em 800 L.ha^{-1} e da fração celulósica e hemicelulósica do bagaço em 5400 L.ha^{-1} (ZHAO et al., 2009).

Dessa forma, é importante no contexto do semiárido brasileiro reunir informações referentes à utilização da biomassa do sorgo sacarino como matéria-prima para a produção de etanol, a partir das diferentes frações da biomassa. Além disso, discutir as principais dificuldades da implantação desta cultura e as novas perspectivas do em relação às biorrefinarias.

2.1.2.1 Aspectos gerais da biomassa de sorgo sacarino

Na mesorregião semiárida, dados de cultivares sacarina apontam baixas produtividades influenciadas por fatores edafoclimáticos. Mas, quando manejado adequadamente, com adubação química e orgânica, e sob regime de irrigação,

durante o ciclo de cultivo, a cultivar SF 15 pode atingir níveis de produtividade experimental de 194 $t.ha^{-1}$ sob excepcionais condições de fertilidade e de irrigação (TABOSA et al., 2013a).

Apesar da grande variabilidade para a produção de biomassa, o aproveitamento da biomassa de sorgo sacarino para produção de etanol está centrado em três tecnologias principais: 1) fermentação direta dos açúcares presentes no caldo dos colmos; 2) hidrólise enzimática do amido presente nas panículas e fermentação dos hidrolisados e 3) aproveitamento do material lignocelulósico presente no bagaço, após pré-tratamento, hidrólise e fermentação dos açúcares liberados para a produção de etanol.

2.1.2.2 Composição química da biomassa de sorgo sacarino

As partes vegetais de sorgo sacarino, com potencial para a produção de biocombustíveis, são o colmo, cuja é a maior fração, as folhas, o caldo e os grãos. O caldo é rico em glicose, frutose e sacarose, os quais são passiveis de fermentação pela principal levedura da indústria alcooleira: a *S. cerevisiae*. Em algumas cultivares de sorgo sacarino também é relatado pequenas frações de amido (BARCELOS, 2012). A composição dos açúcares pode variar fortemente dependendo do tipo de cultivar avaliada além do local de cultivo e a época de colheita dos colmos (TEETOR et al., 2011).

O teor de carboidratos presente no caldo classifica a cultivar de sorgo para a produção de açúcar ou exclusivamente para a produção de biocombustíveis. Dessa forma, é importante salientar a lacuna atual de informações agroindustriais em relação às cultivares, tais como grau de pureza e fibra, os quais são parâmetros utilizados na indústria sucroalcooleira para determinar a qualidade da matéria-prima (COSTA et al., 2011). Além dos teores de açúcares, o caldo possui macro e micronutrientes que são essenciais para a viabilidade do processo de bioconversão dos açúcares em biocombustíveis. Além do caldo, o grão de sorgo é uma fração importante da planta. Essa porção apresenta composição química semelhante à do grão de milho, com teores de amido entre 60 a 80%. Além do amido, o grão também possui lipídeos e proteínas. O percentual de cinzas é geralmente baixo, com valores entre 1-2%, assim como os teores de fibra bruta (1,4%). Algumas cultivares de sorgo apresentam

elevados teores de taninos, o que dificulta a conversão enzimática e, consequentemente, a produção de etanol.

Além do caldo e do grão de sorgo sacarino, o bagaço e as folhas são materiais lignocelulósicos, com grande variação em sua composição. O percentual de celulose é de 32 a 45%, o de hemicelulose entre 16 a 27% e o de lignina varia de 14 a 20%, sendo os teores de lignina mais elevado nas folhas que no bagaço (KIM et al., 2012).

9.3. Produção de etanol a partir do caldo do sorgo sacarino

A produção de etanol, a partir do caldo do sorgo sacarino, é realizada de forma semelhante à produção a partir do caldo da cana-de-açúcar. Após moagem dos colmos, o caldo é purificado e submetido à fermentação com leveduras industriais. A eficiência da etapa fermentativa apresenta valores entre 85 – 90 % de conversões dos açúcares em etanol para caldos com 120 g.L-1 de açúcares redutores total inicial (LAOPAIBOON et al., 2007). Um importante parâmetro tecnológico de produção é a eficiência de extração de caldo dos colmos, com valores aceitáveis entre 50 a 70% de eficiência de extração (EGGLESTON, COLE; ANDRZEJEWSKI, 2013).

Estima-se que o sorgo sacarino possa produzir cerca de 2500 a 4000 litros de etanol por hectare, a partir do caldo do colmo (ALMODARES; HADI, 2009). Por se tratar de uma cultura de ciclo curto, pode-se ocorrer mais de um ciclo por ano, dependendo das condições ambientais, aumentando, dessa forma, a produção de etanol por unidade de área. O caldo é composto principalmente por açúcares, sacarose, glicose e frutose, prontamente fermentescíveis pelas leveduras industriais (WU et al., 2010). Uma desvantagem em relação à cultura da cana-de-açúcar é a rápida decomposição dos açúcares presentes no caldo do sorgo sacarino. Esta observação é crítica quando se postula a introdução do sorgo sacarino como matéria-prima na região Nordeste do Brasil, uma vez que são observadas altas temperaturas ambientais que podem maximizar a decomposição dos açúcares presentes no caldo. Grande parte dos estudos com a fração do caldo de sorgo sacarino busca novas cultivares com potencial de produção de etanol. Em recente estudo conduzido na região Nordeste do Brasil (DUTRA et al., 2013) avaliaram oito cultivares de sorgo sacarino quanto à concentração de açúcares redutores no caldo para a produção de etanol. Os resultados indicaram diferenças significativas entre as cultivares avaliadas (64 $g.L^{-1}$ a 164,8 $g.L^{-1}$), sendo as mais promissoras para a produção de etanol a BR 506 (59,07 ± 1,3 $g.L^{-1}$), Willey (64,77 ± 4,4 $g.L^{-1}$), Wray (59,07 ± 1,3 $g.L^{-1}$) e IPA 467-

4-2 (52,10 ± 0,7 $g.L^{-1}$). Para o conjunto dos dados experimentais, a eficiência de conversão dos açúcares presentes nos caldos para etanol foi, teoricamente, 88,4 %. Os maiores potenciais de produção de etanol por hectare foram observados nas cultivares BR 506 e SF 15.

Objetivando formas de diminuir a decomposição dos carboidratos no caldo de sorgo sacarino, Laopaiboon et al. (2007) investigaram a produção de etanol a partir do caldo da variedade keller, na Tailândia, em sistemas em batelada e batelada alimentada com a levedura *S. cerevisiae*. No sistema operado em batelada, com concentração inicial celular de 1x108 células mL^{-1} e sólidos solúveis totais (SST) de 24 °Brix, a concentração de etanol (P), rendimento (Yp/s) e produtividade (Qp) foram 100 g L^{-1}, 0,42 g g^{-1} e 1,67 g L^{-1} h^{-1}, respectivamente. No sistema em batelada alimentada, os autores sugeriram uma única alimentação como a melhor estratégia, com o teor de sólidos solúveis inicial de 24 °Brix, com resultados para os parâmetros cinéticos P, Yp/s e Qp de 120 g L^{-1}, 0,48 g g^{-1} e 1,11 g L^{-1} h^{-1}, respectivamente. Alternativas como fermentações com altas concentrações de açúcares iniciais (>270 g.L-1) são avaliadas como modo de diminuir os custos energéticos do processo, já que produzem, por conseguinte, altas concentrações de etanol (LAOPAIBOON et al., 2007). Porém, uma das limitações destas condições são as baixas produtividades volumétricas obtidas, devido ao elevado tempo de processo (40 - 120h), além de afetar a viabilidade celular.

Na abordagem com altas concentrações iniciais de açúcares, esforços são realizados para melhorar a eficiência de fermentação. Um estudo com fermentação de caldo de sorgo sacarino, avaliando as influências de Zn, Mg, Mn e de extrato de levedura como fonte de nitrogênio no processo, indicou que o extrato de levedura é o que mais influencia positivamente, seguido do Mn^{+2}, Zn^{+2} e Mg^{+2} (DEESUTH et al., 2012).

2.1.2.3 Produção de etanol a partir dos grãos do sorgo sacarino

O processo de produção de etanol, a partir dos grãos de sorgo sacarino está baseado na transformação do amido em glicose, através de hidrólise enzimática, com α-amilase e glucoamilase e posterior fermentação dos hidrolisados com leveduras. No entanto, ainda são escassas as publicações sobre o uso dos grãos de sorgo para produção de etanol, principalmente devido ao aproveitamento alimentício dos grãos.

A produção de grãos de sorgo granífero e sacarino situa-se entre 2 a 3 t ha^{-1}. A concentração média de amido nos grãos de sorgo é bastante variável e depende da cultivar e do local de produção. Em média, pode-se produzir aproximadamente 815 L de etanol por hectare (ZHAO et al., 2009).

2.1.2.4 Produção de etanol a partir do bagaço e das folhas do sorgo sacarino

Estudos com a fração lignocelulósica do bagaço do sorgo sacarino são fundamentais para aumentar a produção de etanol por unidade de área plantada e tornar o balanço energético mais positivo. Estima-se que a produção de etanol a partir do bagaço seja de aproximadamente 2.423 litros de etanol por hectare (PRASAD et al., 2007).

Para transformar os polissacarídeos insolúveis constituintes do bagaço e das folhas, geralmente são necessárias etapas de pré-tratamento e hidrólise, enzimática ou ácida. Em relação à produção de etanol, a partir da palhada (colmos e folhas) de sorgo forrageiro, avaliou-se a cultivar BRS 655, desenvolvida pela Embrapa, com três tipos de pré-tratamento (alcalino com NaOH, ácido com H_2SO_4 e H_2SO_4 seguido de NaOH) e dois tempos de reação (15 e 30 min). Observou-se melhoras significativas na etapa de hidrólise enzimática, com eficiências acima de 90% de conversão de celulose em glicose (CARDOSO et al., 2013).

Otimizações na etapa de hidrólise enzimática são fundamentais para viabilizar a tecnologia de conversão pela rota bioquímica. Neste cenário, os estudos com hidrólise enzimática da biomassa de sorgo, que inicialmente eram desenvolvidos para baixas concentrações de biomassa (20 a 100 $g.L^{-1}$), (Shen et al., 2012), já utilizam cargas de sólidos > 250 $g.L^{-1}$, obtendo, dessa forma, maiores concentrações de etanol (WANG et al., 2013).

2.1.2.5 Biomassa de sorgo como matéria-prima para biorrefinarias

O conceito recente de biorrefinaria refere-se a um sistema integrado de instalações e equipamentos desenvolvidos para conversão da biomassa, por rota termoquímica ou bioquímica, com o objetivo de produzir combustíveis, energia e produtos químicos (PATRICK et al., 2010). Neste cenário, o sorgo sacarino é avaliado como matéria-prima para o desenvolvimento de biorrefinarias, em diversas rotas de aproveitamento integral da biomassa, pois apresenta características como elevada

produção de biomassa, com menor consumo de água e insumos (XIN; WANG, 2011). O balanço geral para a produção de etanol a partir de 1 ton de biomassa de sorgo foi de 59 kg de etanol (KIM et al., 2012).

Na região Nordeste do Brasil, ainda não foi implantado estudos que integrem as diferentes fases de produção de biomassa (agronômica) com processamento (engenharias, química), desta biomassa, para a produção de biocombustíveis, energia e produtos de alto valor agregado, o que indica um alto nicho de aproveitamento.

2.1.2.6 Considerações finais

A região litorânea do Nordeste do Brasil é uma área de tradição no cultivo de cana-de-açúcar para a produção de açúcar e etanol. Esta região abrange uma pequena fração da área total do Nordeste e, em algumas partes, o gradiente de precipitação se reduz e inviabiliza o cultivo desta cultura energética. É neste contexto que o cultivo do sorgo sacarino pode ser inserido, atuando como matéria-prima complementar à produção de combustíveis, energia e produtos de alto valor agregado. Como uso potencial, no semiárido brasileiro, apresenta-se as áreas irrigáveis para produção de energia e do subproduto (bagaço) para a alimentação animal.

Para o aproveitamento da biomassa de sorgo sacarino, ainda são necessários vários esforços em diferentes áreas de pesquisa: 1) Melhoramento vegetal de cultivares de sorgo sacarino; 2) Melhora nas eficiências dos processos fermentativos, a partir do caldo; 3) Viabilidade de produção de etanol, a partir dos grãos de cultivares de sorgo, sem interferir, ou interferindo minimamente, na oferta de alimentos; 4) Design de processos de pré-tratamentos, hidrólise enzimática ou ácida, e fermentação de hidrolisados competitivos; 5) Integração das diferentes abordagens na produção de etanol, a partir da biomassa de sorgo sacarino e aproveitamento dos seus resíduos para geração de energia e outros produtos; e 6) Levantamento dos custos de produção de etanol a partir da biomassa de sorgo sacarino.

2.1.3 Palma forrageira

Pertencente à família Cactaceae, a palma forrageira é uma planta do tipo CAM (processo fotossintético conhecido como metabolismo ácido das crassuláceas), a qual é caracterizada por apresentar alta resistência aos longos períodos de estiagem e, sendo assim bem adaptadas à ambientes áridos e semiáridos (PINHEIRO et al.,

2014). Esse mecanismo de adaptação é caracterizado pelo uso eficiente da água devido à absorção de dióxido de carbono à noite e sua conversão em biomassa pela luz solar durante o dia. Essa adaptação, em seu metabolismo, confere à planta a capacidade de crescer naquelas regiões onde a água é um importante fator limitante para o desenvolvimento agrícola. (PINHEIRO et al., 2014; QUEIROZ et al., 2015). Além disso, devido a essa propriedade, são 10 vezes mais eficientes no uso de água do que as plantas C4, que fixam carbono na forma de oxaloacetato, como a cana-de-açúcar. Para vegetais C3 (vegetais que fixam carbono na forma de 3-fosfoglicerato), como a soja, essa capacidade pode chegar a 20 vezes. (SAMPAIO, 2005).

Entre as variedades resistentes à cochonilha, as variedades Orelha de Elephant México e IPA-Sertânia foram as mais eficientes na produção de matéria fresca em condições de sequeiro, com valores em torno de 112,1 e 101,1 kg/ha/mm, respectivamente em relação à taxa de evaporação real das culturas (MOURA et al., 2014). Por outro lado, a cana-de-açúcar, sob o mesmo regime, possui uma eficiência de 59,6 kg/ha/mm (OLIVEIRA et al, 2011).

O Nordeste do Brasil é a maior área de palma forrageira do mundo, com cerca de 600.000 hectares. (JÚNIOR et al. 2013). Nesta região, são encontradas algumas variedades. Dentre elas, as mais abundantemente encontradas são a gigante, a redonda (ambas das espécies *Opuntia ficus-indica*) e a miúda (*Nopalea cochenilifera*) (SILVA; SANTOS, 2006, VASCONCELOS et al., 2009). No Instituto Agronômico de Pernambuco tem desenvolvido cultivares que sejam resistentes à cochonilha do carmim (VASCONCELOS et al., 2009). O cultivo de variedades resistentes à colchonilha é necessário, visto que essa praga dizima plantações da cactácea em questão. Assim, o cultivo dessas variedades garante a plantação dessa biomassa, principalmente pelo fato dela ser usada como reserva forrageira, de grande valor e impacto na pecuária da região (SILVA et al., 2014).

A palma forrageira se credencia como alternativa para a produção de etanol também devido algumas características químicas/bioquímicas. Dentre elas, destacam-se a sua alta conversão de carboidratos fermentescíveis e sua baixa exigência nutricional, o que diminui os custos do seu cultivo (CUSHMAN et al., 2015), Ademais, esse vegetal dispõe de vasta disponibilidade, fácil plantio, ausência de entressafra (pelo fato de ser um vegetal perene), baixa necessidade de tecnologia para cultivo, baixo teor de lignina e alto conteúdo de celulose amorfa (NETO, 2010; YANG et al. 2015), o que diminui a necessidade de pré-tratamentos agressivos.

A produtividade de etanol, a partir da palma pode atingir valores correspondentes a 1490-1875 L/ha.ano^{-1} (Santos et al., 2016), em detrimento da cana-de-açúcar (5300-9400 L/ha.ano^{-1}) e a beterraba sacarina (5000-6000 L/ha.ano^{-1}). No entanto, Alencar et al. (2018) fizeram apontamentos importantes, relacionados ao processamento desta biomassa. Eles destacaram que, devido as variedades IPA-Sertânia e Orelha de elefante mexicana apresentarem valores de lignina <10%, e cristalinidade <8%, a palma não necessita de pré-tratamentos químicos. Isso se faz relevante, visto que a etapa de pré-tratamento corresponde de 15-30% dos custos do etanol de 2G. No entanto, o processo de secagem dessa biomassa requer muita energia, visto que ela armazena grandes volumes de água. Tratamentos biológicos, através do uso de bactérias, para obtenção de carboidratos para a produção de etanol de palma forrageira também foram relatados. Nesse sentido, *Pectobacterium cacticida*, dentre as eubactérias, demonstrou ser a mais promissora (BLAIR; YIM; CUCHMAN, 2021).

2.2 HIDRÓLISE DE BIOMASSAS LIGNOCELULÓSICAS

A hidrólise é uma das principais etapas da produção de etanol de segunda geração. É nela em que ocorre a quebra das cadeias de celulose e hemiceluloses, com o objetivo de se obter os monossacarídeos originários dessas matrizes. Aqui, destacamos a obtenção de glicose, pelo homopolímero, e xilose através do heteropolímero. A glicose e a xilose são os monossacarídeos base para produção de etanol e xilitol, respectivamente, devido ao fato da hexose ser a molécula que iniciará a via glicolítica, a qual, em anaerobiose, irá culminar na produção do álcool. Já em relação à pentose, na presença de oxigênio, essa molécula será convertida a xilitol.

A hidrólise poder ser realizada através do uso de duas classes de moléculas: os ácidos ou as enzimas. Ambos os processos apresentam vantagens e desvantagens. No caso dos ácidos, as vantagens estão relacionadas ao fato de serem mais baratos, as reações serem realizadas com menos tempo, além de poderem agir sobre as matrizes celulósicas e hemicelulósicas indiscriminadamente, devido a ação não específica de soluções ácidas. Como exemplos mais comuns, estão os H_2SO_4 ou HCl. Em contrapartida, existe a necessidade do uso de altas temperaturas (100-150°C), formação de inibidores e correção de pH, para que os hidrolisados possam ser usados na etapa de fermentação (CHEUNG; ANDERSON, 1996; WINGREN; GALBE;

ZACCHI, 2003). Os compostos obtidos, através da hidrólise ácida da biomassa de cana-de-açúcar, são descritos na Tabela 2. A facilidade de obtenção desses compostos, da matriz hemicelulósica, se dá pelo fato do heteropolímero ser amorfo.

Tabela 2 - Composição de hidrolisado hemicelulósico de bagaço de cana-de-açúcar, em reator piloto.

Propriedades físicas	pH	0,97
	ºBrix	4,00
	Condutibilidade (mS)	40,20
Carboidratos (g/L)	D-xilose	19,19
	D-glicose	0,98
	L-arabinose	1,82
Produtos da degradação dos carboidratos (g/L) (compostos furfurais)	Furfural	0,08
	5-hidroxumetilfurfural	0,07
Compostos fenólicos (g/L)	Ácido gálico	0,04
	Ácido Vanílico	nd*
	Ácido siríngico	nd*
	P-Ácido coumarílico	0,15
	Ácido ferulíco	0,12
	Aldeído protocatecuico	0,07
	Vanilina	0,08
	Ácido hidroxibenzóico	nd*
Total de compostos fenólicos (g/L)	Compostos fenólicos (g/L)	1,95
Ácido Acético (g/L)	Ácido acético (g/L)	3,49
Compostos Inorgânicos (mg/L)	Cobre	<0,10
	Ferro	554,40
	Cromo	<0,10
	Cálcio	34,10
	Magnésio	51,10
	Sódio	41,00
	Potássio	103,90
	Manganês	8,20
	Zinco	6,50
	Níquel	27,80
	Enxofre	3433,60

*nd = não detectado.

Fonte: Adaptado de (RODRIGUES et al., 2010).

Tizazu e Moholkar (2018) avaliaram o perfil de formação de carboidratos e compostos de degradação, a partir da hidrólise ácida do bagaço de cana-de-açúcar,

em diferentes temperaturas. Eles observaram que as condições ótimas, para a produção de hidrolisados, ricos em xilose (concentração: 9,00 g/L; $y_{p/s}$ = 0.76 g/g), foi 120°C, durante o período de 30 minutos, usando 2% v/v de H_2SO_4, com carga de sólidos de 1:30 m/v. A partir da mesma biomassa, Sá et al. (2020) relataram a formação de 24,42 g/L de xilose, ao usar a proporção de 1:4 m/v de biomassa, usando o mesmo tempo de hidrólise, porém, com a concentração de 1,6% v/v de H_2SO_4.

Já em relação a hidrólise enzimática, as vantagens estão relacionadas, principalmente, à maior especificidade da reação, não formação de inibidores e maior rendimento de produto. Por outro lado, essas reações são mais demoradas e, os processos que usam enzimas ainda são mais onerosos que àqueles que utilizam compostos inorgânicos (SUBHEDAR; BABU; GOGATE, 2015). Nesse tipo de hidrólise, um coquetel enzimático atuará, principalmente na matriz celulósica. Nesse liquor, haverão enzimas da classe das exo-glucanases, as quais atuarão entre dois monômeros de celobiose. Em contrapartida, o outro grupo, denominado, endoglucanases, agirá na região amorfa da cadeia de celulose. Existe ainda um terceiro grupo, o qual é responsável por quebrar o dissacarídeo celobiose em dua moléculas de glicose. A ele, damos o nome de β-glucosidases (GALBE; ZACCHI, 2002). Na hidrólise enzimática da biomassa de palma forrageira, Alencar et al. (2018), obtiveram, ao aplicar a carga de 30 m/v de sólidos, valores de glicose maiores que 80 g/L. Isso lhes conferiu, no processo fermentativo, concentrações de etanol >35 g/L e eficiências de fermentação >85%.

No entanto, algumas estratégias tem sido utilizadas para para melhorar ainda mais os rendimentos desse tipo de hidrólise. Uma alternativa é uso de proteínas acessórias como as expansinas. Essas moléculas atuam com o objetivo de afrouxar a estrutura rígida da celulose, e promover a hidrólise dessa fração da parede celular (ZHANG et al., 2021). Sendo assim, a partir do uso da expansina BsEXLX1, de *Bacillus subtilis*, num meio contendo endogluganases, a taxa de adsorção desse grupo de enzimas, à matriz celulósica, pode aumentar em quase 5 vezes (ZHANG et al., 2021).

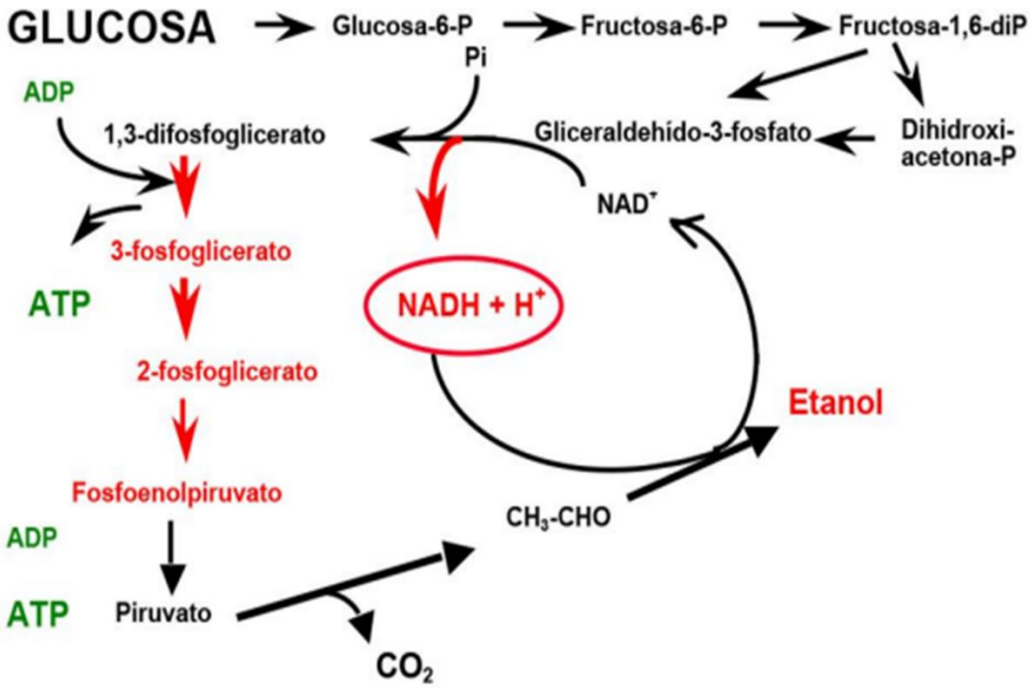
Faz-se importante ressaltar que as enzimas celulolíticas podem ser inibidas pelo seu próprio produto, conforme foi demonstrado por Xiao et al. (2004). Nesse ensaio, eles observaram a inibição de 50% da atividade de β-glucosidase, ao adicionar 100 g/L de glicose, em uma carga de sólidos celulósicos de 10%. No entanto,

alternativas como a fermentação e sacarificação simultâneas (SSF) podem diminuir esse efeito inibitório.

2.3 FERMENTAÇÃO

A produção de etanol é dependente de uma etapa denominada fermentação. Essa etapa inicia na fosforilação da glicose, no citoplasma celular, e culmina na conversão de acetaldeído a etanol. Essa reação ocorre devido a uma necessidade biológica da levedura, em equilibrar as concentrações molares de NAD^+/NADH, disponíveis no citoplasma, com o objetivo de manter a via glicolítica em funcionamento. Na reação de conversão de gliceraldeído-3-fosfato a 1,3-bifosfoglicerato, na glicólise, são reduzidos 2 NAD^+, já que as reações, a partir da conversão de Frutose-1,6-bisfosfato a gliceraldeído-3-fosfato são duplicadas. Como a disponibilidade de oxigênio no meio é limitada, o direcionamento do piruvato para a cadeia respiratória é reduzido, e, por conseguinte, não haveria como restituir a concentração de NAD^+ para manutenção da via glicolítica, havendo, por conseguinte o acúmulo de NADH no citoplasma.

Figura 4. Produção de etanol, *Saccharomyces cerevisiae*, em condições anaeróbias.



Fonte: GURDO (2016).

Sendo assim, como modo de manter essa via, o piruvato, através de uma descarboxilização (↑CO_2), é convertido a acetaldeído, o qual, pela oxidação de NADH, na presença da acetaldeído desidrogenase, é convertido a etanol. Nessa oxidação, o NAD^+ é restituído e pode voltar a ser utilizado na conversão de gliceraldeído-3-fosfato a 1,3-bifosfoglicerato. Por estas razões, a metabolização da glicose produz 2 mols de etanol e 2 mols de CO_2, conferindo, à reação, um rendimento de 0,511 de etanol, para cada mol de glicose.

Os organismos que conseguem fazer isso com maior eficiência são as leveduras. Não obstante, *Saccharomyces cerevisiae* é a levedura mais adaptada ao processo, devido alta eficiência fermentativa, alta capacidade de competição e sobrevivência no ambiente industrial (ANDRIETTA et al., 2007). Sendo assim, relatos dessa levedura atestam sua capacidade de fermentar desde hidrolisados ácidos, quanto enzimáticos, com altas eficiências e diversas biomassas. A variedade de vegetais estudados, para essa finalidade, permeia desde os mais tradicionais, como bagaço de cana-de-açúcar, até mesmo outros menos comuns, como sorgo sacarino, palma forrageira, algodão e batata doce (WANDERLEY et al., 2013, DUTRA et al., 2013, ALENCAR et al., 2018; MALIK et al., 2020; RIZZOLO et al., 2021).

No entanto, *S. cerevisiae* não dispõe da capacidade de metabolizar xilose a produtos como etanol e xilitol. Essa consideração se faz importante pois, hidrolisados provenientes de biomassas lignocelulósicas são compostos por uma riqueza de carboidratos, dentre os mais abundantes são a glicose e a xilose. O não aproveitamento da pentose restringe o potencial energético da biomassa. Sendo assim, pesquisadores identificaram uma levedura denominada *Spathaspora passalidarum*, a qual apresenta a capacidade de co-fermentar carboidratos como glicose, xilose e celobiose (NGUYEN et al., 2006; LONG et al., 2012).

Já em relação à produção de xilitol, leveduras do gênero Meyerozyma, como a *Meyerozyma guilliermondii* e *Meyerozyma caribbica*, tem apresentado a capacidade de produzir esse carboidrato, além de produzir etanol (NAGARAJAM et al., 2021; TADIOTO et al., 2022). No entanto, a produção do açúcar de interesse industrial é dependente da disponibilidade de oxigênio no meio, em contrapartida à fermentação etanólica. Assim, a presença de produtos com alto valor agregado, na rota de produção de etanol de segunda geração, principalmente quando realizado por uma levedura que tenha a capacidade de gerar ambos, é recomendado para auxiliar na viabilidade econômica do processo. Isso ocorre devido aos custos ainda relativamente

elevados para a produção de etanol 2G, exigindo, assim, que outros subprodutos sejam sintetizados.

3 OBJETIVOS

3.1.1 Objetivo geral

Avaliar aspectos da produção de etanol, a partir de blends de hidrolisados ácidos de biomassas lignocelulósicas e melaço, como alternativa para o período de entressafra da cana-de-açúcar no estado de Pernambuco.

3.1.2 Objetivos específicos

a) Determinar o melhor horário, e época do ano, para colheita da biomassa de palma forrageira;

b) Determinar o tempo e a temperatura necessários para a secagem de palma forrageira, para o processo de hidrólise;

c) Produzir etanol a partir de blends de hidrolisados ácidos de bagaço de cana de açúcar, bagaço de sorgo sacarino e palma forrageira, usando *M. caribbica*;

d) Comparar a produção de etanol, nesses substratos, entre *S. cerevisiae* e *M. caribbica*;

e) Avaliar a produção de xilitol por *M. caribbica*.

4 ARTIGO 1 - *MEYEROZYMA CARIBBICA* URM 8365 ISOLATED FROM SUGARCANE PLANTATION SOILS AND ITS POTENTIAL FOR ETHANOL AND XYLITOL PRODUCTION FROM ACID HYDROLYSATES OF LIGNOCELLULOSIC BIOMASSES

INTRODUÇÃO

A área de produção de cana-de-açúcar no Brasil é de cerca de 9.750 mil há, com a produção total de 642.070 toneladas de cana e produtividade média de 76,4 toneladas ha^{-1}. O estado de São Paulo lidera ambos os parâmetros, enquanto que Pernambuco é o segundo estado com maior área plantada e produção no nordeste brasileiro, com 251 mil ha de plantio e 11.500 mil toneladas de cana, e produtividade média de 54,1 toneladas ha-1 (CONAB, 2020). Todos esses valores de produção são reduzidos na entressafra da cana, fazendo com que a produção de etanol diminua nesse intervalo. Alternativas como o uso de melaço e bagaço de cana são sugeridas e já aplicadas, como forma de suprir essa demanda energética (LALUCE et al., 2016; CRUZ et al., 2021). Além disso, há o agravante de que cerca de 80% da área do estado de Pernambuco apresenta déficit hídrico, o qual se torna ainda limitante, na mesorregião do semiárido. Assim, sugere-se a ampliação da área plantada, por meio da utilização de outras biomassas lignocelulósicas, mais adaptadas aos climas áridos e semiáridos, como o sorgo sacarino e a palma forrageira. Essas plantas já foram relatadas como alternativas para esse processo, pois apresentam eficiências de fermentação satisfatórias, sem a necessidade de suplementação nutricional dos respectivos substratos, o que reduz os custos do processo (SANTOS et al, 2016; ALENCAR et al, 2018; DUTRA et al., 2018). A partir dessas fontes de biomassa lignocelulósica, é possível produzir hidrolisados ácidos, ricos em carboidratos. Esses licores açucarados podem, quando misturados ao melaço, contribuir para reduzir o efeito da entressafra na produção de etanol (PALMONARI et al., 2020; RASMEY et al., 2018; WU et al., 2020). Assim, essa demanda poderia ser alcançada, a partir da formulação de substratos mistos, utilizando uma levedura capaz de produzir etanol nestas condições. Nessas condições, leveduras do gênero Meyerozyma tem apresentado resultados associados tanto a produção de etanol, quanto xilitol, a partir de substrato como hidrolisados de biomassas lignocelulólicas e melaço (SANTOS et al., 2020; TADIOTO et al. 2022). A geração de produtos secundários, no conceito de

biorrefinaria, é importante para se tentar viabilizar, economicamente, os custos ainda elevados, inerentes à produção de etanol 2ºG.

Neste contexto, o presente trabalho objetiva avaliar o potencial de M. caribbica, previamente isolada de canaviais pernambucanos, na fermentação de hidrolisados ácidos de biomassas lignocelulósicas e melaço, para a produção de etanol e xilitol.

MATERIAIS E MÉTODOS

Biomassa vegetal e Melaço

A biomassa do bagaço de cana-de-açúcar foi cedida gentilmente por pela usina "Olho d'água", enquanto que o bagaço do sorgo sacarino foi doado pelo Instituto Agronômico de Pernambuco (IPA). Já a palma forrageira foi coletada em uma estação do IPA, na cidade de Arcoverde - PE, e levada para o Laboratório de Energia da Biomassa, em Recife. Ao chegar, os cladódios desta biomassa foram lavados com água destilada, cortado em cubos e transferidos para liquidificador industrial, no qual foram convertidos a uma textura semelhante a um purê. Essa pasta foi disposta em bandejas de alumínio, e mantidas em estufa de circulação de ar por 48h, a 105°C. Em contrapartida, os bagaços foram lavados com água destilada, sendo secos em estufa de circulação de ar, a 65°C, durante 48h. Após o processo de secagem, as biomassas foram trituradas, em moinho de facas, até atingir a granulometria de 20 mesh, e armazenadas em bombonas de 200 L. O melaço foi gentilmente cedido pela Destilaria Agroindustrial de Ipojuca, localizada no município de Ipojuca, Pernambuco, Brasil.

Produção de hidrolisados ácidos e formulação dos blends com melaço

Os hidrolisados de bagaços de cana-de-açúcar, sorgo sacarino e biomassa de palma forrageira foram produzidos aplicando-se uma carga sólida de 10 m/v para os bagaços e 15% m/v para palma forrageira. As hidrolises foram realizadas em erlenmeyers de 500 mL, usando a solução de H_2SO_4, a 1,5 % v/v, com volume reacional de 300 mL (CHEN et al., 2012). A reação ocorreu em autoclave, a 121°C, durante 30 minutos. Posteriormente, os frascos foram resfriados em banho de gelo e a suspensão foi transferida para tubos do tipo Falcon, de 50 mL, para centrifugação. Os parâmetros, para este processo, foram 3600 rpm e 5 minutos. Em seguida, a fração líquida foi armazenada, a -20C, em frascos âmbar de 1L. Este procedimento foi repetido até obter-se o volume de cerca de 2L de cada tipo de hidrolisado.

Já os blends foram formulados conforme descrito na Tabela 2.1, tendo sido preparados em volumes de 2L.

Tabela 2. 1. Blends formulados a partir de hidrolisados ácidos de bagaços de cana-de-açúcar e sorgo sacarino, biomassa de palma forrageira e melaço.

Blends	Composição (%)				Concentração inicial de carboidratos fermentescíveis
	Melaço	SCB	SSB	CPB	
M1	70,00	10,00	10,00	10,00	504,56
M2	50,00	16,67	16,67	16,67	306,97
M3	25,00	25,00	25,00	25,00	108,80
M4	10,00	30,00	30,00	30,00	36,63

Fonte: A autora (2022).

Crescimento microbiológico e fermentação de hidrolisados ácidos e blends

As fermentações, usando como substrato, o melaço, os hidrolisados ácidos e os blends foram realizados conforme descrito por Alencar et al., 2018. Primeiramente, a levedura *M. caribbica* foi crescida em meio YPD, contendo 20 g/L de glicose, 20 g/L de peptona e 10 g/L de extrato de levedura. O volume de 100 mL deste meio de crescimento foi transferido para um erlenmeyer de 250mL, sendo, em seguida, o conjunto, esterilizado, em autoclave, por 20 minutos, a 121°C. Ao frasco, foram transferidas, a partir de colônias previamente crescidas em placas de petri, com células de *M. caribbica*. Este pré-inóculo foi mantido em mesa incubadora rotativa, a 30°C e 250 rpm. Após 24h, esta suspensão microbiológica foi dividida e transferida para 2 erlenmeyers de 2L, previamente esterilizados, os quais continham, cada um, 200 mL meio YPD. A cada 24h, o volume reacional de inóculo era dobrado, a partir da adição do meio de crescimento. Esse procedimento foi repetido por 4 dias. Ao final, as suspensões foram distribuídas em tubos do tipo Falcon, de 50 mL cada. O conjunto foi centrifugado, a 3600 rpm, durante 5 minutos. Esse procedimento foi repetido até cada um dos tubos ter 2g de levedura. Ao final da última centrifugação, foi adicionado 10mL de solução de NaCl, a 0,9% m/v, para a manutenção celular. Os tubos com as células foram mantidos, até o dia seguinte, em geladeira, a 4°C. No dia seguinte, ocorreu nova centrifugação e, após o descarte do sobrenadante, foram transferidos 18 mL de cada um dos substratos para cada um dos tubos, a fim de se realizar as primeiras fermentações. Nestas, avaliou-se a capacidade de *M. caribbica* produzir

etanol, a partir dos hidrolisados ácidos das biomassas lignocelulósicas e do melaço, diluído 6 vezes, previamente. As fermentações ocorreram no período de 72h, a 30°C, em modo estático, em duplicata. Houve coleta de amostras em 0, 6, 24, 48 e 72h.

O segundo conjunto de fermentações ocorreu, utilizando como substrato, os blends apresentados na Tabela 2.1. Porém, nestes experimentos, cujo objetivo era avaliar a possibilidade de produção de etanol, em altas concentrações de carboidratos, utilizou-se, além da *M. caribbica*, *S. cerevisiae* como micro-organismo referência. Os ensaios aconteceram sob as mesmas condições que as fermentações anteriores, ou seja, mesma capacidade do tubo Falcon, relação micro-organismo:volume reacional, tempo, temperatura, modo estático e intervalo de coleta de amostras, às 0, 6, 24, 48 e 72h.

Para o ensaio da hierarquia de consumo, durante a fermentação, por *M. caribbica*, foi produzida uma solução de sacarose, glicose e frutose, contendo 8g/L de cada carboidrato. Desde modo, foi transferido, para o tubo Falcon, contendo a levedura previamente crescida e centrifugada, 45 mL da solução de carboidratos. Amostras foram coletadas a cada 1 hora, durante o período de 12h.

A relação glicose/xilose adequada também foi avaliada, em ensaios utilizando meio sintético, com a proporção de glicose e xilose de 1:5, 1:8, 1:10, 1:15 e 1:25 em duas condições de cultivo: estático (sem agitação) ou com agitação a 70 rpm. Os experimentos foram realizados por 120 horas, a 30°C, com coletas de amostras a cada 24 horas. A biomassa inicial 100 g/L, previamente preparada em meio YPD, conforme descrito acima. Todos os ensaios foram realizados em duplicata biológica.

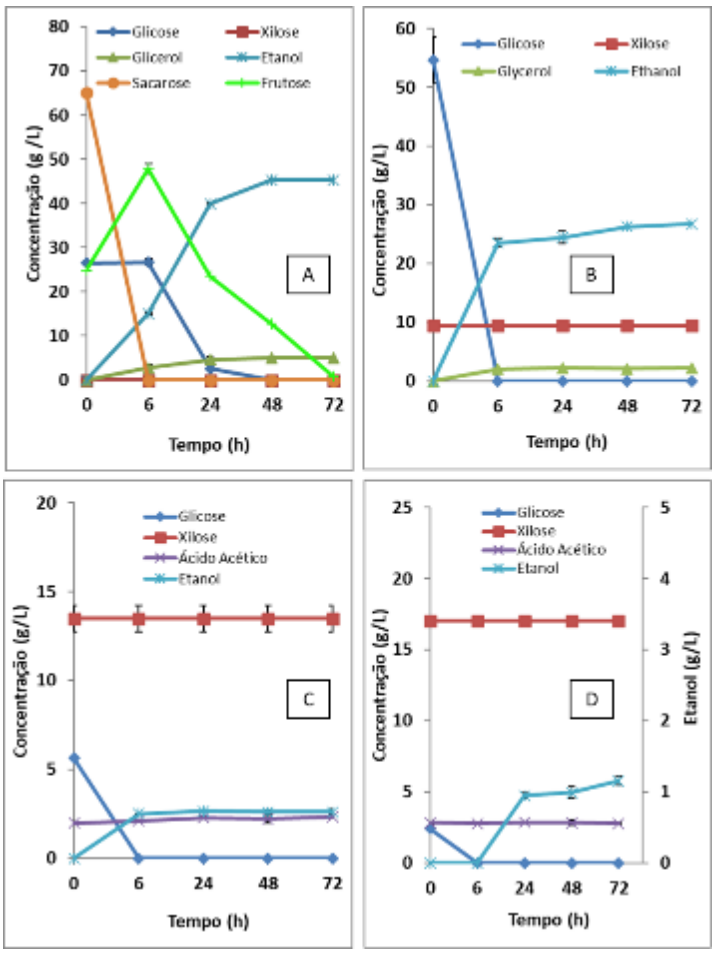
Métodos analíticos

Os metabólitos (glicose, xilose, ácido acético, glicerol, sacarose, frutose e etanol) foram identificados por Cromatografia líquida de alta eficiência (CLAE), no dispositivo Agilent Technologies 1200 Series, usando coluna HPX87H$^+$ (BioRad), a 35°C. Como fase móvel, foi utilizado o ácido sulfúrico, 5 mM, na vazão de 0,6 mL/min.

RESULTADOS E DISCUSSÃO

A capacidade de *M. caribbica* URM8365 fermentar hidrolisados de biomassas lignocelulósicas foi avaliada. A referência foi o melaço de cana-de-açúcar, o qual é composto por uma mistura de sacarose, glicose e frutose (Figura 1a). A sacarose foi completamente consumida, seguida pela glicose. A concentração de frutose aumentou durante o consumo de sacarose, indicando que as células excretam invertase e hidrólise de sacarose no meio. O etanol foi produzido para atingir o rendimento final de 0,39 g/g (Figura 1a). Foram produzidos três hidrolisados vegetais diferentes.

Figura 2. 1. Produção de etanol, por M. caribbica, em melaço e hidrolisados ácidos de biomassa de cana-de-açúcar, sorgo sacarino e palma forrageira.



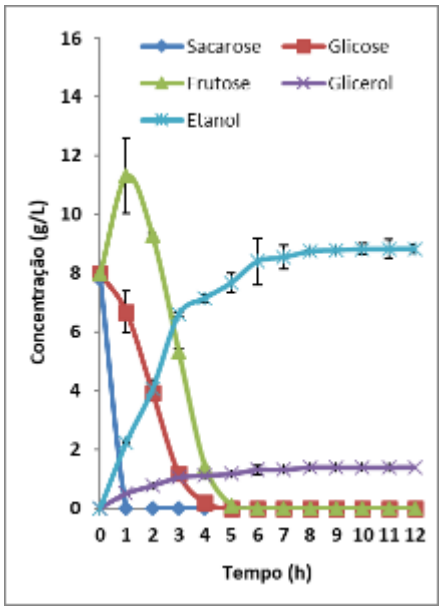
Fonte: A autora (2022).

O bagaço da cana-de-açúcar liberou mais xilose e menos glicose (Figura 1b) do que o bagaço do sorgo sacarino (Figura 1c). Os hidrolisados de palma forrageira apresentaram uma enorme quantidade de glicose devido ao alto índice de celulose amorfa, cerca de 70%, além de baixo teor de lignina (YANG et al, 2015). Em

experimentos estáticos, toda a glicose foi consumida pelas células para produzir etanol nos três substratos, enquanto a xilose permaneceu intocada. Os rendimentos de etanol foram calculados como 0,42 g/g para cana-de-açúcar, 0,45 g/g para sorgo e 0,49 g/g para palma forrageira.

Posteriormente, os testes realizados com os blends de hidrolisados e melaço foram fermentados por *M. caribbica* URM8365 e *S. cerevisiae* JP1. Os resultados obtidos sugerem que *M. caribbica* pode se adaptar a ambientes com altas concentrações de carboidratos iniciais, além de detalhar sua preferência de consumo, relacionada aos carboidratos glicose, sacarose e frutose (Figura 2). Nesses dados, observa-se que a sacarose é metabolizada primeiramente a partir de uma invertase extracelular, independentemente da concentração inicial de carboidratos. Isso é suportado pelo acúmulo de carboidratos glicose e frutose no meio (Figura 3).

Figura 2. 2. Hierarquida de consumo, na fermentação dos carboidratos sacarose, glicose e fructose, por M. caribbica URM8365.



Fonte: A autora (2022).

Nos ensaios M1 (Figuras 3a e 3b), para ambas as leveduras, mais de 73% da sacarose disponível foi metabolizada. Além disso, em *M. caribbica*, houve, concomitantemente, a assimilação de glicose no meio. No entanto, em ambos os testes, a produção de etanol não foi observada. Entretanto, nos ensaios com substrato M2 (Figuras 3c e 3d), o etanol foi produzido exclusivamente por *M. caribbica*, porém, em baixas concentrações (5 g/L) e somente após 48 horas do processo. Isso indica que *M. caribbica* pode se adaptar e produzir etanol em meios cujas concentrações

são superiores a 300 g/L de carboidratos fermentáveis. Deve-se notar também que isso ocorreu, mesmo sem quaisquer suplementos nutricionais, que visam melhorar essas assimilações.

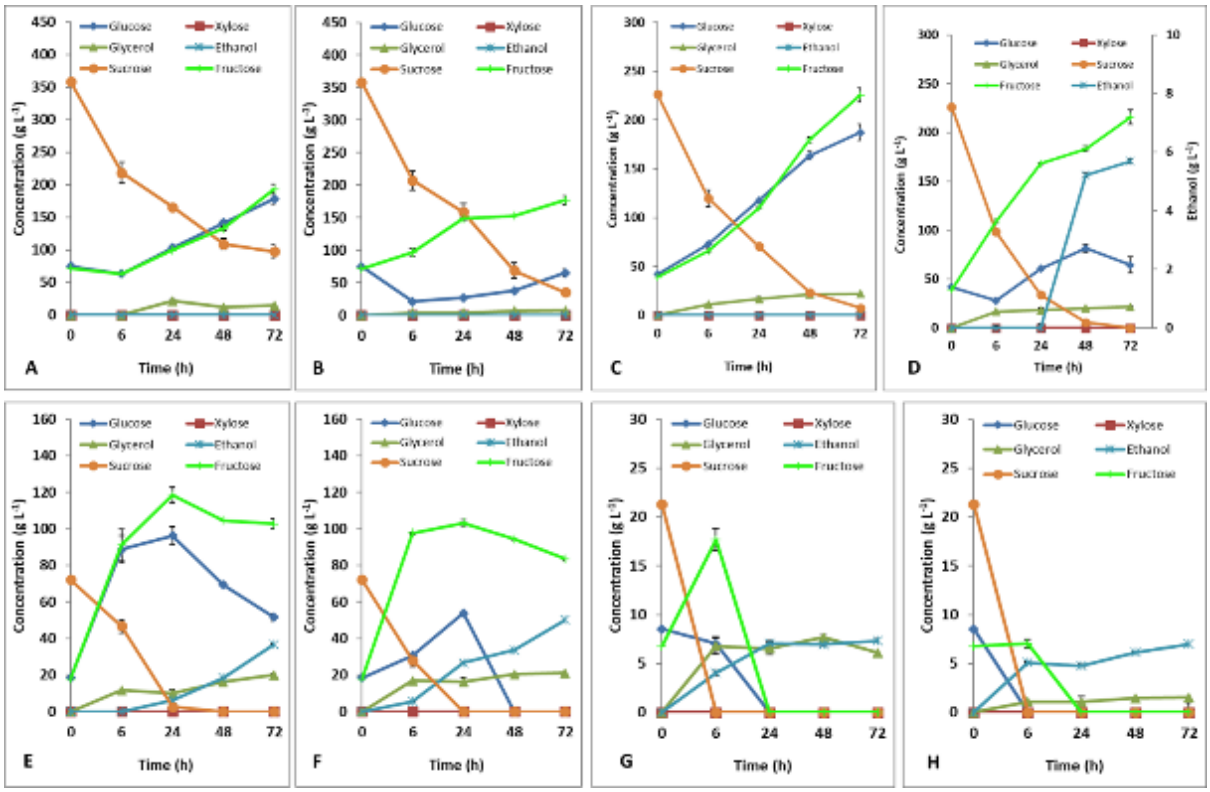
Já no M3 (Figuras 4e e 4f), a resposta parece ser a mais promissora. Ao utilizar 25% de cada um dos substratos testados, a produção de etanol chega a 50 g/L, superando o mínimo exigido, segundo Larsen et al. (2008). Os parâmetros de fermentação para M3 foram Y = 0,46 g/g e Ef = 90,60%. Além disso, toda a sacarose foi convertida em glicose e frutose, em 24h, e toda a glicose foi assimilada em 48h. Ainda há frutose residual, o que pode gerar ainda mais álcool ao longo do tempo. Isso é sugerido pela curva ascendente de produção de etanol, a partir de 48h, e pela curva descendente de frutose, a partir de 24h. Nos ensaios com *S. cerevisiae*, para o mesmo meio, a assimilação dos monossacarídeos foi mais lenta, com concentrações ainda elevadas de glicose e frutose ao final do tempo de 72h, quando comparadas com *M. caribbica*. Esse comportamento indica certa dificuldade de *S. cerevisiae* em converter esses carboidratos em etanol, nas condições de meio propostas.

Na última condição avaliada, M4 (Figuras 4g e 4h), glicose, frutose e sacarose foram completamente consumidos em 48h. A produção de etanol, para ambas as leveduras, foi de aproximadamente 7 g/L. Ou seja, em baixas concentrações de carboidratos, as diferenças nas respostas, no que diz respeito à concentração de etanol produzido, na comparação entre as duas leveduras, não são significativas.

Em testes realizados com uma mistura de 40% v/v de melaço e 60% de hidrolisado de palha de arroz pré-tratado, com suplementação de nitrogênio e fontes minerais, obteve-se uma concentração de etanol de 97 g/L e rendimento de 0,46 g/g (PANDEY et al., 2022). Na mistura de xarope de açúcar celulósico (CCS) e melaço, na proporção 20:80, suplementado com uréia, após 48 horas de fermentação, obteve-se 75 g/L, com 92% de eficiência (NETSOPA et al., 2022). Esses ensaios são semelhantes às condições de substrato propostas em M3, ou seja, com concentrações de carboidratos fermentáveis abaixo de 250 g/L. No entanto, em ambos os estudos, as fermentações foram realizadas por cepas de *S. cerevisiae*. Em comparação com os resultados obtidos no presente trabalho, observa-se que a eficiência fermentativa de *M caribbica*, em M3, é semelhante à obtida por *S. cerevisiae*, com suplementação nutricional, relatada em outros trabalhos. Isso confere uma vantagem econômica ao uso de *M caribbica*, durante o processo, já que não

haveria a necessidade desse tipo de adição nutricional, em detrimento de *S. cerevisiae*.

Figura 2. 3. Fermentação de M. caribbica e S. cerevisiae, em blends de hidrolisados ácidos e melaço.



Fonte: A autora (2022).

Por outro lado, as fermentações de alta gravidade (HGV), caracterizadas por concentrações de carboidratos >250 g/L, são normalmente realizadas com a adição gradual do substrato ao reator, a fim de não afetar substancialmente o metabolismo celular. No entanto, neste trabalho os processos foram realizados em batelada, o que confere uma situação de maior estresse e rearranjo metabólico às leveduras. Isso indica que *M. caribbica* parece se adaptar melhor a essas situações de estresse do que *S. cerevisae* JP1, conseguindo, mesmo assim, produzir etanol.

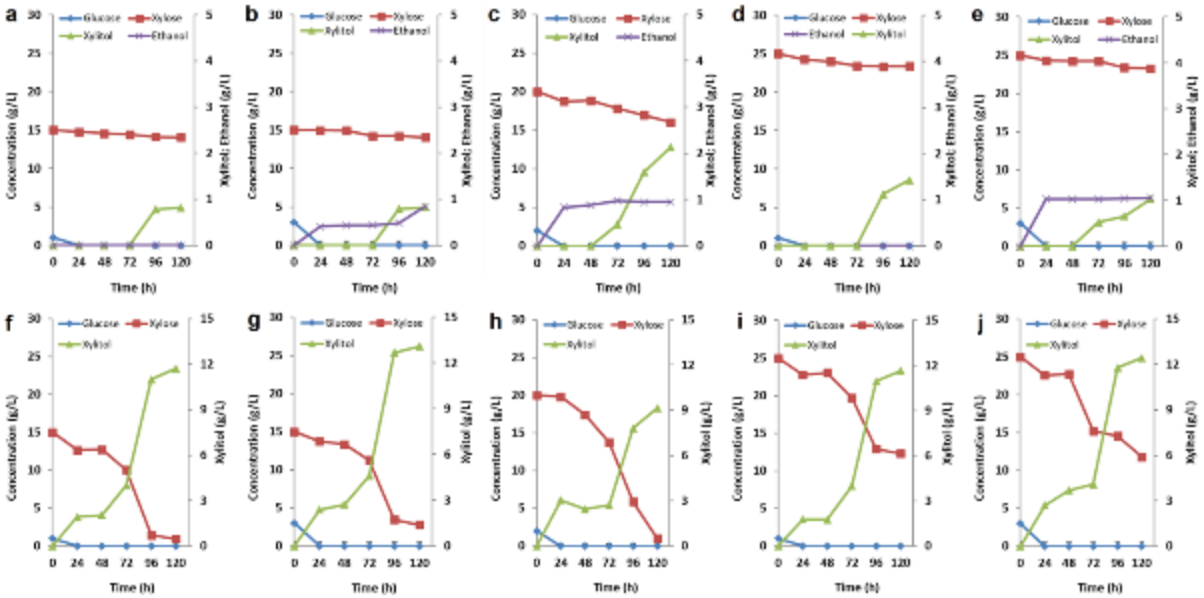
Outro fator que parece interferir substancialmente, para esse tipo de fermentação, é a nutrição e a carga inicial das células. Na fermentação da mistura de caldo de cana, rico em minerais, e melaço, com 15% v/v de carga de *S. cerevisiae*, em sistema contínuo, obteve-se a produção de 135 g/L de etanol, em 30h, (CRUZ et al., 2021). A concentração total de carboidratos foi de 300 g/L, gerando uma eficiência fermentativa de 90%. Nos ensaios com caldo de sorgo sacarino, as concentrações de etanol podem chegar a 160 g/L, a partir de uma concentração de carboidratos de 330

g/L, em meio suplementado com 16 mM de uréia (APPIAH-NKANSAH et al. 2018). Esses dados indicam, portanto, que de fato, fatores como nitrogênio e fontes de nutrientes são essenciais para obter melhores resultados, no que diz respeito às fermentações de alta gravidade. Assim, o atraso e a baixa eficiência na produção de etanol nos testes M2 podem ser justificados.

Além dos ensaios com blends, também foram realizados testes envolvendo a fermentação e xilose por *M. caribbica*. Deste modo, glicose e xilose foram misturadas em diferentes proporções para definir a melhor composição de carboidratos nos substratos (Figura 4). Em experimentos estáticos, no qual o sistema rapidamente se torna anaeróbico, devido à alta densidade celular, a produção de etanol não foi observada quando a quantidade de glicose foi muito baixa (1 g/L), mesmo em excesso de xilose. A pouca xilose consumida foi transformada em xilitol, após 72 h de cultivo (Figuras 4a a 4d). Nesses casos, a eficiência de produção de xilitol se aproximou de 100%, embora quase toda a xilose tenha ficado no meio. Quando a quantidade de glicose foi aumentada (3 g/L), o etanol foi inicialmente produzido até a exaustão da glicose e o rendimento de etanol foi de 0,16 g/g na proporção de glicose para xilose de 1:5 (Figura 5b) e 0,33 g/g a 1:8 (Figura 4e). O xilitol foi produzido após 72h na proporção 1:5 (Figura 4b) e após 48h na proporção 1:8 (Figura 4e).

Na proporção glicose-xilose de 1:10, o maior rendimento de etanol (0,48 g/g) foi observado simultaneamente com o maior rendimento de xilitol (0,46 g/g). Neste caso, observou-se um consumo mais rápido de xilose (Figura 4c). Quando o sistema foi submetido à baixa agitação (70 rpm), o mínimo aporte de oxigênio fez com que a xilose fosse assimilada mais rapidamente e transformada, em xilitol independentemente da presença de glicose (Figuras 5f a 5j). Neste caso, ficou claro que a menor quantidade de xilose (15 g/L) com a maior proporção de glicose para xilose (1:5) resultou na melhor eficiência geral de fermentação, com um rendimento de xilitol de 0,88 g/g e muito pouca xilose residual (Figura 4b).

Figura 2. 4. Experimentos de fermentação utilizando Meyerozyma caribbica URM836,5 em meio contendo diferentes proporções de glicose e xylose, em condições estáticas (a-e) e agitadas a 70 rpm (f-j).



Fonte: A autora (2022).

Quando a quantidade inicial de xilose foi aumentada para 25 g/L, o rendimento de xilitol foi um pouco superior a 0,9 g/g com a penalidade de maior açúcar residual, independentemente da quantidade inicial de glicose (Figuras 4i e 4j). Curiosamente, o menor rendimento de xilitol de 0,46 g/g foi calculado na concentração média de xilose e proporção intermediária de açúcar de 1:10 (Figuras 4h). Esta foi a melhor condição para a produção de xilitol em experimentos estáticos (Figuras 4c). Assim, é necessário adequar as condições de fermentação para produzir simultaneamente etanol e xilitol pelas células da levedura. Este valor de rendimento de xilitol pareceu muito atrativo do ponto de vista industrial quando comparado a relatórios recentes. Tadioto et ai. (2022) obtiveram rendimento de xilitol de 0,32 g/g a partir de meio YNB contendo xilose a 20 g/L, enquanto Nagarajan et al. (2021) relataram rendimento de xilitol de 0,44 g/g em meio sintético de xilose após suplementação com $(NH_4)_2SO_4$, Na_2HPO_4 e extrato de levedura. Embora a suplementação de substrato com sais seja acessível, o uso de suplementos complexos como extrato de levedura e peptona é proibitivo devido aos altos custos que pode impor ao processo industrial.

CONCLUSÃO

Os ensaios demonstraram que, na mistura de melaço e hidrolisados ácidos dos bagaços de sorgo sacarino e cana-de-açúcar e da biomassa de palma forrageira, com as proporções de 25% para cada um dos substratos (condição M3), é possível produzir mais de 50 g/L de etanol, utilizando *M. caribbica* como levedura fermentadora. A proposta do uso deste micro-organismo se faz devido a resposta fermentativa de *S. cerevisiae* JP1 não ter sido promissora, para as condições avaliadas. Nesse sentido, considerando-se apenas as condições testadas no presente trabalho, sugere-se que a composição do meio de fermentação M3 é a ideal para auxiliar a produção de etanol, no período de entressafra da cana-de-açúcar, desde que se utilize, na dorna de fermentação, a levedura *M. caribbica*.

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5 CONSIDERAÇÕES FINAIS

A partir dos dados obtidos, tanto no artigo intitulado “Bioethanol production from cactus cladode biomass: considerations of harvesting time, dry matter concentrations, and enzymatic hydrolysis”, quanto no artigo submetido “*Meyerozyma caribbica* URM 8365 isolated from sugarcane plantation soils and its potential for ethanol and xylitol production from acid hydrolysates of lignocellulosic biomasses” é possível fazer as seguintes considerações: (1) A biomassa de palma forrageira, visando sua hidrólise enzimática, deve ser coletada até as 4:00h da amanhã; (2) Para se atingir a carga de 30% m/v de sólidos, de modo que se tenha o menor gasto energético, deve-se secar a palma forrageira durante 12h, a 105ºC; (3) Que a partir do blend composto por 25% de melaço, 25% de hidrolisado ácido do bagaço de sorgo sacarino, 25% de hidrolisado ácido de cana-de-açúcar e 25% do hidrolisado ácido de palma forrageira (condição M3), é possível produzir mais de 50 g/L de etanol, utilizando *M. caribbica* como levedura fermentadora. Nesse sentido, considerando-se apenas as condições testadas no presente trabalho, sugere-se que a composição do meio de fermentação M3 é a ideal para auxiliar a produção de etanol, no período de entressafra da cana-de-açúcar, desde que se utilize, na dorna de fermentação, a levedura *M. caribbica.*

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APÊNDICE - BIOETHANOL PRODUCTION FROM CACTUS CLADODE BIOMASS: CONSIDERATIONS OF HARVESTING TIME, DRY MATTER CONCENTRATIONS, AND ENZYMATIC HYDROLYSIS

Biomass Conversion and Biorefinery
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ORIGINAL ARTICLE

Bioethanol production from cactus cladode biomass: considerations of harvesting time, dry matter concentrations, and enzymatic hydrolysis

Bárbara Ribeiro Alves Alencar[1] · Nilson Medeiros[2] · Carolaine Larissa Lira da Silva[2] · Aldo Torres[2] · Emmanuel Damilano Dutra[2,3] · Everardo Valadares de Sá Barreto Sampaio[2] · Rômulo Simões Cezar Menezes[2] · Marcos Antônio Morais Júnior[1]

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Abstract
Cactus pear biomass has the potential for bioethanol production in dry regions. However, its low solids concentration and pH variations may hinder the process of alcoholic fermentation. The objectives of this study were as follows: (1) to evaluate the effects of harvest time and season on biomass pH and dry matter concentration of cladodes of two cactus pear species commonly used as forage (*Nopalea cochenillifera* and *Opuntia stricta*); (2) to compare the hydrolysis of fresh and dried biomass (10% w v^{-1} solids); (3) to compare increasing periods of two temperatures (65 °C or 105 °C) to concentrate the biomass to 10% and 30% solids; and (4) to perform enzymatic hydrolysis and fermentation of biomass dried to 30% solids. Biomass pH ranged from 3.0 to 5.6, from early morning to late afternoon, with higher diurnal variation during the dry season, when their solids concentrations were higher than in the rainy season (12–16% × 7–10%). Hydrolyzed fresh and dried biomass had similar glucose, xylose, and galacturonic acid concentrations. Drying at 105 °C for 12 h was the best temperature and period to reach 30% of solids. The enzymatic hydrolysis of the biomass dried to 30% solids yielded 65.3 and 80.0 g of glucose L^{-1}. After fermentations (33 °C; 8 h; *Saccharomyces cerevisiae*), ethanol was produced to 29.4 and 37.5 g L^{-1} from *N. cochenillifera* and *O. stricta* biomasses, respectively. Therefore, early morning during the dry season is the best moment to harvest the cladodes, whose biomass can be partially dried at 105 °C for 12 h to 30% solids load before being hydrolyzed and fermented for bioethanol production. This procedure reduces the time, energy, and inputs needed in the process.

Keywords Cactus forage · *Opuntia* · Bioenergy · Ethanol

1 Introduction

In the last decades, the potential of lignocellulosic biomass as an option for biofuel production has been extensively investigated [1–3]. These studies have as common characteristics the use of chemical and/or physical pretreatment of the biomasses and the use of C3 (3-phosphoglyceric acid fixatives) or C4 (oxaloacetate fixatives) photosynthetic type plants, such as wheat or corn stover and sugarcane or sorghum, as sources of fermentable carbohydrates. These plants require a large amount of water for their cultivation when compared with CAM metabolism (Crassulaceae acid metabolism) species. Currently, most of the biofuels and food produced in the world comes from C3 and C4 plants, which account for about 70% of the world's freshwater use. Future climate scenarios point to a decline in the volume of water reserves by 55% by 2050. For this reason, the use of C3 and C4 biomasses to produce biofuels may not meet with future water availability [4, 5].

✉ Emmanuel Damilano Dutra
emmanuel.dutra@ufpe.br

[1] Research Group in Metabolic Engineering, Department of Genetics, Federal University of Pernambuco, Recife, PE 50670-901, Brazil

[2] Research Group on Biomass Energy, Department of Nuclear Energy, Federal University of Pernambuco, Recife, PE 50740-540, Brazil

[3] Departamento de Energia Nuclear, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, Cidade Universitária, Recife, Pernambuco 50740-540, Brazil

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Moreover, UNESCO [5] also predicts an increase in the land area under semiarid climates in the world. Such environments do not support intensive traditional agriculture, but they are suitable for the growth of Cactaceae species, which have the CAM metabolism type [6]. Some of the cactus species with the highest biomass productions belong to the *Opuntia* and *Nopalea* genera, the cladodes of both genera being largely used as forage sources for ruminants, human food, and other products [7, 8]. In recent years, due to their high biomass productivity, carbohydrate content, and water use efficiency, cactus pear species have been studied as potential biofuel sources [7, 9–11].

However, the production of biofuels from cactus biomass in semiarid regions still faces several technological challenges at all stages of production. Cultivation in the field requires improvements in management practices and planting and harvesting systems, especially with the mechanization of the cropping operations. Further, the conversion of the biomass into biofuels still needs optimizations. One of the main challenges is related to the high-water content of the cactus biomass. On average, the fresh biomass of cactus is around 10% of total solids, which, when subjected to enzymatic or acidic hydrolysis, generates hydrolysates with low sugar contents and, consequently, low concentrations of bioethanol or other bioproducts [7].

An alternative to the use of the high-water content fresh cladode biomass was proposed by Alencar et al.[12], who concluded that the enzymatic hydrolysis of cactus was better when the biomass was at 30% w v^{-1} solids loading. However, that work did not estimate the time spent to dry the biomass.

In general, cactus species have a CAM metabolism that evolved in response to water scarcity in terrestrial environments. These plants open their stomata during the night time, accumulating malic acid, which is decarboxylated during the light period to produce glucose [13]. This physiological pattern results in pH variation of the cladode biomass along the 24-h day period. Finding the most suitable time to harvest the cladodes helps in determining the most suitable buffer solution to control the acidity during the hydrolysis stage, which is best at pH 4 to 5 [12]. Subsequently, it may improve the feasibility of using this biomass for bioethanol production.

Considering the above arguments, the objectives of this study were as follows: (1) to evaluate the effects of harvest time (day-night) and season (dry-rainy) on the pH and dry matter concentration of the cladodes of two cactus pear species (*Nopalea cochenillifera* (L.) Salm-Dyck and *Opuntia stricta* (Haw.) Haw.); (2) to compare the hydrolysis of fresh and dried biomass (10% w v^{-1} solids); (3) to compare increasing periods of two temperatures (65 °C or 105 °C) to concentrate the biomass to 10% and 30% solids; and (4) to perform enzymatic hydrolysis and fermentation of biomass dried to 30% solids.

2 Material and Methods

2.1 Biomass sampling and pH determination

Cladodes of two cactus pear species, *Opuntia stricta* (Haw.) Haw. (variety "Orelha de elefante mexicana") and *Nopalea cochenillifera* (L.) Salm-Dyck (variety "IPA-Sertânia"), were sampled at the experimental station of the Agronomical Institute of the State of Pernambuco, in Caruaru municipality, Pernambuco state, Brazil (08°14'18"S; 38°00'00"W and 537 MSW). Two large fields of each species, which were planted 1 year before, were selected for sampling. In each field, ten cladodes were harvested from randomly selected plants every 2 h in a time course of 24 h in 1 day of the dry season (December) and 1 day of the rainy season (July). The cladodes belonged to different vertical orders in the plant structure (the higher orders corresponding to younger cladodes), excluding the basal, lignified ones and the highest, still developing ones, and any cladode with signs of disease, insect attack, or any abnormality. Immediately after harvest, in a laboratory close to the cultivation field, the cladodes were washed and processed in an industrial blender. After that, the pH of 20 ml of the pulp was measured using a portable pH meter (Model Metter Toledo). Samples (500 g) of the pulps were taken to the laboratory, placed in 5-L flasks, dried at 105 °C for 48 h and their dry matter contents were calculated according to the methodology described by [14] and calculated by

$$SI(\%) = \left[\frac{wbf - wf}{wi}\right] * 100 \quad (1)$$

where *SI* was the solids load, *wbf* was the mass of the dry biomass of the cactus plus the flask weight, *wf* was the weight of the flask, and *wi* was the initial biomass of cactus pear.

Cellulose, hemicellulose, lignin, ashes, and extractive concentrations of the cladode biomasses were also determined according to the methodology described by [14]. These results were already published elsewhere [12].

2.2 Hydrolysis of fresh and previously dried and rehydrated biomass

Based on the pH results, another cladode sampling was conducted in the same fields in the early morning of the dry season. This time, 30 cladodes were collected from each field, treated as described above, and the pulp was submitted to a series of tests. The first test was to compare the effect of hydrolysis using the fresh biomass and the biomass previously dried and then re-hydrated since this is the usual procedure when hydrolysis is performed. The biomass pulp was dried at 105 °C for 48 h, milled, sifted to 20-mesh (2 mm) granulometry, and stored. Part of this biomass was then rehydrated with a 50-mM citrate buffer to 10% solids, a similar

concentration of the fresh biomass. Both this re-hydrated and the fresh biomasses were submitted to hydrolysis.

Portions of 2 g of each biomass treatment were placed in 20-mL flasks, together with 10 FPU g^{-1} of the enzyme Celluclast 1.5 L Novozymes (with an initial activity of 6.39 FPU/mL) and distilled water. The kinetic profile of the formation of carbohydrates and organic acids was evaluated after 0, 6, 12, 24, and 48 h from the start of the hydrolysis, taking samples of the suspension, centrifuging, and analyzing the liquid fraction by high-performance liquid chromatography (HPLC). The HPLC used H_2SO_4 (5 mM) as a mobile phase with a flow of 0.6 mL min^{-1}, the analyte being detected by refractive index on an Aminex R column (HPX-87H, Bio-Rad, USA) at 60 °C. All procedures were carried out in duplicate.

2.3 Drying for increasing periods at different temperatures

Based on the hydrolysis results of the fresh and previously dried and re-hydrated biomasses, it was decided that it was worthwhile to test hydrolysis with only partially dried biomasses. Before conducting this test, a third test was conducted to characterize the curve of solids concentrations obtained with increasing drying periods at two temperatures: 65 and 105 °C. Partial drying can save time, energy, and inputs needed in the process of ethanol production.

Portions of 2 kg of the fresh cladode biomass, described in Section 2.2, were placed in 5-L flasks and oven-dried at 65 °C for 0, 12, 24, 48, 72, 96, 120 h and 105 °C for 0, 6, 12, 24, 36, and 48 h. The solids content (% w v^{-1}) in the biomass was determined as described in Section 2.1.

2.4 Hydrolysis and fermentation of partially dried biomass

Based on the previous tests, it was decided to dry the cladode biomasses of both cactus species to 30% solids, the most usual solids load, and to complete the process of hydrolysis and fermentation with these biomasses. Hydrolysis followed the procedures described in the previous sections. The enzymatic hydrolysates were mixed with *Saccharomyces cerevisiae* JP1 strain previously grown in YPD medium (glucose, 20 $g.L^{-1}$; peptone, 20 g L^{-1}; yeast extract, 10 g L^{-1}) in 1-L Erlenmeyer flasks maintained at 33 °C and 150 rpm, for 24 h.

The volumes of the culture medium were doubled every 24 h for 1 week, aiming to obtain 56 g of yeast biomass. At the end of this step, the cells were centrifuged (10,000 rpm, 5 m), resuspended in NaCl solution (0.08%), and kept in a refrigerator for 24 h. The cells were then centrifuged again (10,000 rpm, 5 min), and 7 g of microbial biomass were resuspended in 63 ml of the enzymatic hydrolysates. The fermentative stage was statically incubated with 10% weight per volume for 8 h at 33 °C and aliquots of 0.5 ml were withdrawn every hour. Samples of the aliquots were filtered on 0.22-μm membranes, and the liquid fractions were analyzed for carbohydrate and ethanol concentrations by high-performance liquid chromatography, as described in Section 2.2. The procedure was performed in triplicate. Equations 2, 3, and 4 correspond to the yield (g g^{-1}), efficiency (%), and volumetric productivity (g L^{-1} h^{-1}) of the alcoholic fermentation:

$$EtOH = \frac{\Delta EtOH\left(gL^{-1}\right)}{\Delta G\left(gL^{-1}\right)} \quad (2)$$

$$Qp = \frac{EtOH\left(gL^{-1}\right)}{t(h)} \quad (3)$$

$$Ef = \frac{Y}{0.511} * 100 \quad (4)$$

Where $EtOH$ is the ethanol concentration (g L^{-1}), G is the glucose concentration (g L^{-1}), Y is the yield of ethanol production, and t is the fermentation time (h).

2.5 Statistical analysis

The pH, dry matter, hydrolysis, and fermentation concentration data were submitted to the Kolmogorov-Smirnov test to check the assumption of normality, and to the Levene test to check for homoscedasticity. The data were then submitted to analysis of variance, and the averages were compared by the paired Student's t test or the Tukey test, both at the 0.05 probability level.

3 Results and Discussion

3.1 Effect of harvest time and season on pH and dry matter concentration

The cladode biomass of *O. stricta* and *N. cochenillifera*, harvested in dry and rainy seasons, had their highest pH values from 2 to 6 pm (Fig. 1). During the night, the pH constantly decreased until it reached the lowest values around 6 to 8 am (Fig. 1). These variations in pH corroborate those reported in Mexico [15], which also found that the higher acid content of the cladodes was observed when they were harvested early in the morning. The pH of both species was lower (3.7) in the dry than in the rainy season. The lower pH values during the dry season probably resulted from low water availability and lowered hydration of the cladode tissues, which decrease photosynthesis and increase the relative H^+ content. Therefore, considering that pH values from 4 to 5 are preferable for enzymatic hydrolysis and fermentation [12], early morning is the best time to harvest the cladodes of both species.

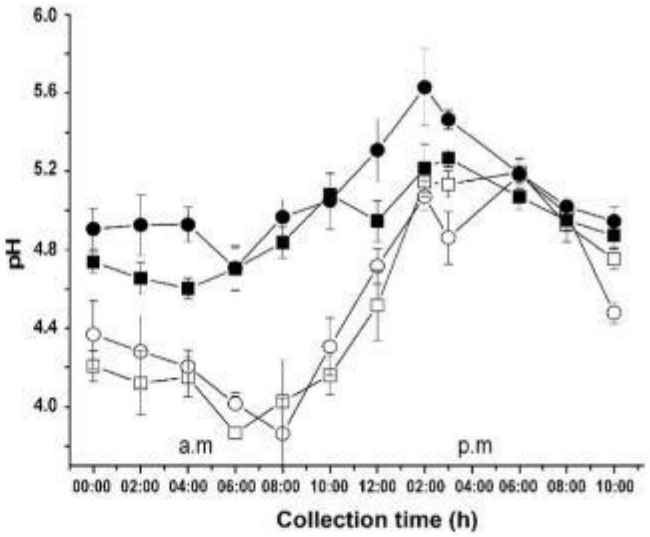

Fig. 1 Cladode biomass pH values of *Nopalea cochenillifera* (L) Salm-Dyck. (circle) and *Opuntia stricta* (Haw.) Haw (square), along 1 day, during the rainy (July) and dry (December) seasons, in the semi-arid region of Pernambuco state, Brazil. Full symbols, rainy season; empty symbols, dry season

The dry matter concentrations, in both species, were higher in the dry than in the rainy season, and they were similar along the day. Therefore, only the averages in each season are shown (Fig. 2). Dubeux et al. [16] also reported that the water content of cactus cladodes is reduced during the dry season, but without adversely affecting their chemical composition. Assuming that a load above 30% w v^{-1} is near to an optimum solid concentration for enzymatic hydrolysis, harvesting during the dry season may save energy and time for biomass dehydration [12]. From a social point of view, this is an important result because this is the season when the rural workers are less occupied with agricultural activities.

Regardless of the season, *N. cochenillifera* had, on average, 23% higher dry matter than *O. stricta*. This trait indicates that *N. cochenillifera* has a higher potential as a feedstock for bioethanol production. However, in rainfed cactus production systems in Brazil [16], *N. cochenillifera* often requires higher

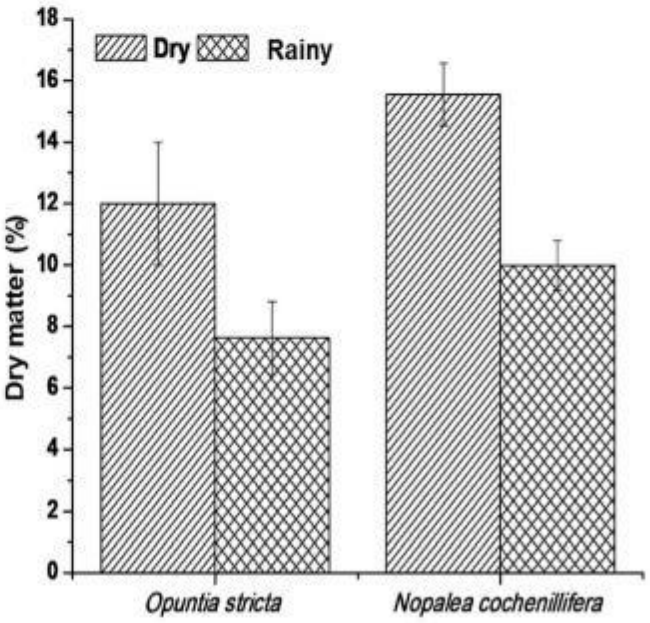

Fig. 2 Dry matter content of *Opuntia stricta* (Haw.) Haw. and *Nopalea cochenillifera* (L.) Salm-Dyck. cladode biomasses harvested in the dry and rainy seasons in the Brazilian semi-arid region

rainfall (> 600 mm year^{-1}) than *O. stricta* (> 400 mm year^{-1}) for maximum biomass production. For this reason, production of *N. cochenillifera* in the Brazilian semiarid region is limited to a smaller land area compared with *O. stricta*. It is noteworthy that even *O. stricta* is not appropriated for cultivation in drier areas, indicating that this ecosystem could be more suited for the production of other cactus species. Therefore, the research efforts for biomass conversion into bioethanol must investigate other Cactaceae species.

3.2 Enzymatic hydrolysis of fresh and dry biomass

The cladode biomass of *N. cochenillifera* had 25.85% cellulose, 9.08% hemicellulose, 5.77% lignin, 30.73% extractives, and 11.65% ashes, while the cladode biomass of *O. stricta* had 28.51% cellulose, 10.59% hemicellulose, 6.40% lignin, 31.03% extractives, and 11.15% ashes. These chemical characterizations were already published elsewhere [12]. Other authors have also characterized these biomasses. Kuloyo et al. [17] stated that the concentration of fibrous carbohydrates (cellulose and hemicellulose) is 42% in the genus *Opuntia*, while Souza-Filho et al. [18] pointed that the cellulose concentration is 31.6% in *N. cochenillifera*. The lignin and ashes concentrations were similar to those previously described in the literature, ranging between 4 and 16% for lignin and 6 and 20% for ashes [17, 19].

The enzymatic hydrolysis of the fresh biomass (10% w v^{-1}) of *N. cochenillifera* released significantly (t = 16.28; p = 0.04) more glucose (26.72 g L^{-1}, 93.1% cellulose conversion) than that of the dry biomass (21.54 g L^{-1}, 75.1% cellulose conversion) after 12 h (Fig. 3). On the other hand, the glucose released from the fresh and dry biomass of *O. stricta* showed no significant differences, reaching 24.28 g L^{-1} and 27.75 g L^{-1}, respectively (Fig. 3). Santos et al. [7] hydrolyzed the fresh biomass (2% w v^{-1}) of *Opuntia ficus-indica*, heat pretreated in an autoclave. In this study, 11.26 g L^{-1} of glucose were obtained, while Kuloyo et al. [17] obtained 45.5 g L^{-1}, from the cladode biomass of *Opuntia ficus-indica* (L.) Mill. pretreated with diluted sulfuric acid (1.5% w w^{-1}) and using a 10% w v^{-1} solids charge. The values observed in our study diverge from those reported by Kuloyo et al. [17], probably because of the different species, cultivation systems, and hydrolysis conditions. It is also important to mention that hydrolysis was performed by enzyme blend of Pectinex Ultra SP-L, Celluclast 1.5 L, and Novozyme 188 (β-glucosidase) in high loads, and this may have caused an increase in the final amount of carbohydrates obtained [17]. However, the use of high enzyme loads increases the cost of the process, which goes against the aim of bioenergy producers.

The glucose released after the biomass hydrolysis of the two species did not significantly differ (Figs. 3 a and b), despite the tendency of more glucose being released from *N. cochenillifera*. The absence of a significant difference indicates that there is no need to distinguish the two species from an industrial point of view. It eliminates the skilling labor of plant identification and allows the simultaneous processing of biomass coming from fields of both species. It is worth noting that both species are resistant to wild cochineal, which is negatively affecting cactus fields in many parts of the world, including Brazil [20]. Despite the advantages of

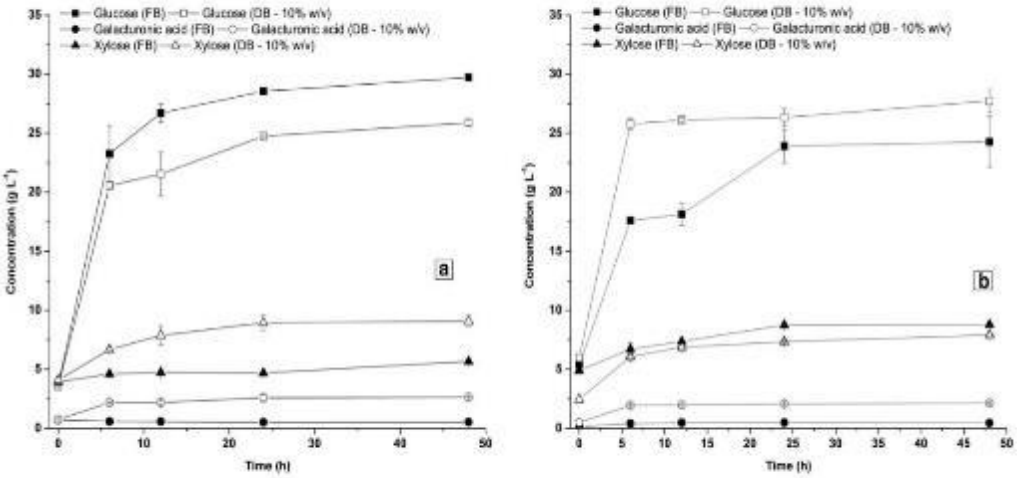

Fig. 3 Glucose, galacturonic acid and xylose concentrations in enzymatic hydrolysates obtained with increasing hydrolyzation times of fresh cladode biomass (FB) and cladode biomass dried to 10% w v^{-1} (DB) of *Nopalea cochenillifera* (L.) Salm-Dyck (**a**) and *Opuntia stricta* (Haw) Haw (**b**)

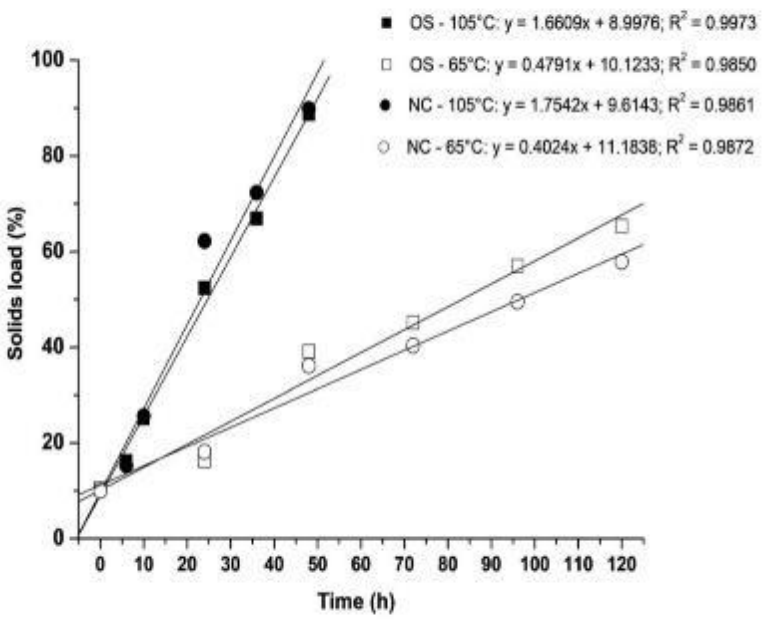

Fig. 4 Solids load of cladode biomass obtained with increasing drying times under 65 °C and 105 °C of *Nopalea cochenillifera* (L.) Salm-Dyck (Nc) and *Opuntia stricta* (Haw) Haw (Os)

using fresh cactus biomass, the glucose concentrations obtained at the end of the enzymatic hydrolysis of fresh biomasses are low. Based on the current bioprocess technologies, these low glucose concentrations do not allow sustainable ethanol production chains. The literature reported similar values of carbohydrates to those of this study, with the use of 10% w v^{-1} of loaded solids in hydrolysis [21]. Thus, it is necessary to concentrate the solids of the cladode biomass to make the process more viable from an economic point of view, and the solid load of 30% has been reported as adequate for hydrolysis [12].

3.3 Solids concentration of fresh biomass

In order to concentrate solids, we tested conditions for water evaporation from fresh cladodes. For both species, biomass reached 30% w v^{-1} load at 105 °C for 12 h, or at 65 °C for

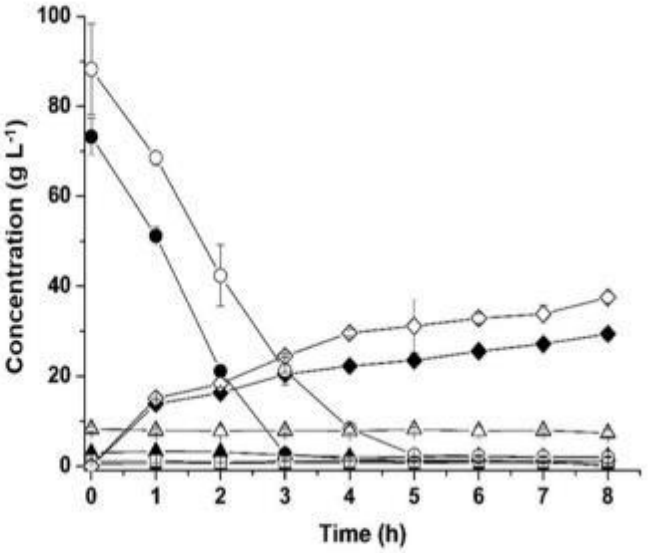

Fig. 5 Glucose (circle), xylose (triangle), galacturonic acid (square), and ethanol (diamond) concentrations in cladode biomasses of *Nopalea cochenillifera* (L.) Salm-Dyck. (complete symbols) and *Opuntia stricta* (Haw.) Haw. (empty symbols) submitted to increasing hydrolysate fermentation times

Table 1 Fermentation parameters of the enzymatic hydrolysates of the concentrated biomass (30% w v^{-1}) of *Nopalea cochenillifera* and *Opuntia stricta* cactus cladodes

Parameters	*Nopalea cochenillifera*	*Opuntia stricta*
Ethanol yield (g g^{-1})	0.40[a1]	0.43[a]
Volumetric productivity (g L^{-1} h^{-1})	3.67[a]	4.69[a]
Efficiency of fermentation (%)	78.49[a]	85.10[a]
Ethanol (g L^{-1})	29.39[a]	37.54[a]
Residual glucose (g L^{-1})	0.00	1.92

[1] Means with the same letter in the line are not significantly different (Tukey, $p < 0.05$)

approximately 47 h for *N. cochenillifera* and 42 h for *O. stricta* (Fig. 4). Kuloyo et al. [17] proposed to sundry the biomass, eliminating the electrical cost of the process. However, this drying alternative would require 10 to 15 days. At 105 °C, 48 h were required to completely dry the cladode biomasses [12]. Partially drying the biomass at 105 °C, to reach a load of 30% w v^{-1}, results in a reduction of energy costs and processing time in relation to complete drying. In addition, a load of 30% w v^{-1} promoted the increase of carbohydrate content in the hydrolysis step, turning it more feasible to produce ethanol [12].

3.4 Enzymatic hydrolysis of the concentrated biomass and fermentation of the enzymatic hydrolysate

The hydrolysis of *N. cochenillifera* biomass concentrated at 30% w v^{-1} resulted in 65.30 g L^{-1} of glucose (75.8% cellulose conversion) and that of *O. stricta* in 79.97 g L^{-1} of glucose (92.9% cellulose conversion). Subsequently, the fermentation of the enzymatic hydrolysates generated 29.39 g L^{-1} of ethanol for *N. cochenillifera* and 37.54 g L^{-1} for *O. stricta* (Fig. 5). The xylose and galacturonic acid of the two species were similar, obtaining 21.98 g L^{-1} and 2.39 g L^{-1} for *N. cochenillifera* and 21.82 g L^{-1} and 2.35 g L^{-1} for *O. stricta*, respectively.

It is important to note that for an ethanol production process to be considered profitable, its concentration at the end of the process must be ≥ 40 g L^{-1} [22].

From the fermentation parameters (Table 1), the potential for ethanol productivity was determined considering yields of 500 ton ha^{-1} $year^{-1}$ of fresh mass [16]. With this productivity and the average dry matter concentration observed in this study, the estimated dry matter productivity would be 85 ton ha^{-1} $year^{-1}$, and that of ethanol would be more than 15 thousand L per ha and year. Recent studies reported values of fermentative parameters greater than those obtained in the present study (Table 1): Sousa Filho et al. [18], Ef = 95.6% and Yp/s = 0.49 g g^{-1}; and Kuloyo et al. 2014 [17], 0.9 g L^{-1} h^{-1} and Yp/s = 0.46 g g^{-1}. However, they supplemented the enzymatic hydrolysate with yeast extract, peptone, and elements such as nitrogen, sulfur, magnesium, and phosphorus. In addition, it has been reported that the supplementation of hydrolysates with magnesium increases ethanol production by 28.7% [17]. Adding such compounds to the cladode biomasses became dispensable under the conditions of the present work since the fermentative parameters were satisfactory. Besides, avoiding the use of such compounds represents huge cost reductions in the process, aiding to the viability of the use of cactus biomass for ethanol production.

4 Conclusions

Early morning during the dry season is the best time to harvest cactus pear cladodes of *Nopalea cochenillifera* and *Opuntia stricta* to be used for enzymatic hydrolysis and bioethanol production. In addition, 12 h and 105 °C are the time and temperature required to dry these biomasses to the 30% w v^{-1} load. These partially dried cactus biomasses can be hydrolyzed and fermented with high ethanol production efficiency, which may lead to reductions in costs, time, energy, and chemical inputs compared with the usual procedure of complete drying and re-hydrating.

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ANEXO A – ARTIGOS PUBLICADOS SEM VÍNCULO COM A TESE

Os artigos adicionados à esta sessão foram produzidos durante o período de doutorado da aluna. Ao longo dos quatro anos de doutoramento, a aluna desenvolveu pesquisas relacionadas à produção de etanol, a partir de biomassas lignocelulósicas. Os estudos abordaram desde problemáticas relacionadas às etapas de pré-tratamento e hidrólise (ácida e/ou enzimática), quanto a fase fermentativa. No primeiro artigo adicionado, a doutoranda foi responsável por toda a parte experimental, bem como escrita, relacionada às sessões de “resultados e discussão” e “materiais e métodos”. No processo de revis No segundo artigo adicionado, “Production and Application of Lignin-Based Chemicals and Materials in the Cellulosic Ethanol Production: An Overview on Lignin Closed-Loop Biorefinery Approaches”, a autora desta tese redigiu toda a sessão relacionada ao uso de lignina na etapa de hidrólise enzimática. No terceiro artigo, “Valorization of Sugar-Ethanol Industry Waste Vinasse for Increased Second-Generation Ethanol Production Using Spathaspora passalidarum Yeast Strains”, a autora da presente tese colaborou na organização dos dados e escrita da sessão de “resultados e discussão”. No último manuscrito, “Chemical pretreatment of sugarcane bagasse with liquid fraction recycling” a doutoranda colaborou no ensinamento da técnica de reciclo de pré-tratamento à primeira autora do manuscrito, bem como escreveu sessões relacionadas a pré-tratamento e hidrólise.

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ORIGINAL PAPER

Concentration of Alkaline Hydrogen Peroxide (AHP) Affects the Recycle of the Liquid Fraction in the Pre-treatment and Enzymatic Hydrolysis of Corn Stover

Bárbara Ribeiro Alves Alencar[1] · Fernanda Leitão Vaz[2] · Adauto Gomes Barbosa Neto[3] · Katia Aparecida Aquino[4] · Everardo Valadares de Sa Barretto Sampaio[2] · Rômulo Simões Cezar Menezes[2] · Emmanuel Damilano Dutra[2,5]

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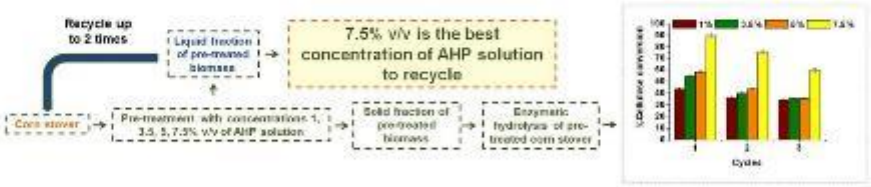
Abstract

Pre-treatment is one of main economic and technological challenges to render feasible the production of biofuels and chemical compounds from lignocellulosic biomass. Alkaline hydrogen peroxide (AHP) is the most used pre-treatment and recycling of its liquid fraction can help reduce production costs. The effects of four AHP concentrations (1, 3.5, 5 and 7.5% v/v) on the recycling performance of the liquid fraction of pre-treated corn stover was evaluated for five consecutive cycles. Delignification rates increased with increasing AHP concentrations in the first cycle: 15, 26, 43 and 76% with 1, 3.5, 5 and 7.5% v/v H_2O_2, respectively. In the following cycles, the rates decreased linearly reaching less than 40% in the last two recycles. These delignification rates and hemicellulose solubilization were corroborated by spectroscopic analyses with Fourier transformation showing reductions in lignin and hemicellulose absorbance and increases in crystallinity indices. Considering the low delignification rates in the last two cycles, the pre-treated biomasses obtained until the third cycle were submitted to enzymatic hydrolysis at 1:10 solid–liquid ratio. The delignification rates affected the efficiency of the enzymatic hydrolysis at all AHP concentrations and all recycles. The highest AHP concentration (7.5% v/v) was required to efficiently remove lignin and solubilize hemicellulose, maintaining cellulose conversion into glucose greater than 50% up to three recycles. Therefore, the technology of recycling the liquid solution of AHP pre-treatment is recommended with high initial concentrations (7.5% v/v).

Graphic Abstract



Keywords Lignocellulosic biomass · Pre-treatment · Recycle liquid fraction · Enzymatic hydrolysis

✉ Emmanuel Damilano Dutra
emmanuel.dutra@ufpe.br

Extended author information available on the last page of the article

Statement of Novelty

This work innovates in testing the possibility of recycling the liquid fraction of the pre-treatment of corn stover with alkaline hydrogen peroxide in order to lower pre-treatment costs.

Introduction

Fossil fuels use has increased in many industrial sectors, especially the energy one [1], and the energy demand will rise from its current 550 EJ to 860 EJ until 2040 [2]. The transportation sector is one of the main consumers, using liquid fuels derived from fossil sources. The chemical industry is another important sector that uses petroleum derivatives. In Brazil, the market for chemical substances has grown annually between 3 and 5%, in the last years [3].

To replace fossil sources, gaseous, liquid and solid biofuels and high value-added chemical compounds derived from lignocellulosic biomass have a great potential [2, 4]. Currently, approximately 2.8 billion tons of lignocellulosic residues are produced in the world [5], corn stover being the main one [6]. However, lignocellulosic biomass conversion into biofuel and chemical compounds is still a technological challenge. To produce liquid biofuels from lignocellulosic biomass three processing steps are usually needed [7]: (1) pre-treatment; (2) acid or enzymatic hydrolysis; and (3) fermentation. Each step still presents serious economic and technological difficulties, but the most challenging step is pre-treatment, that may be performed by physical, chemical, biological and mixed methods [8], and may represent up to 20% of production costs [9].

Baral and Shah et al. [10] made a techno-economic evaluation of four types of lignocellulosic biomass pre-treatments: steam explosion, diluted sulfuric acid, explosion with ammonia and a biological method. Steam explosion and diluted sulfuric acid were more attractive from an economic point of view, but they concluded that all four pre-treatments still needed technological improvements. The efficient use of chemical reagents that work under mild conditions of temperature and pressure is a major objective [11, 12], together with reduction of inputs, like water and chemical reagents, and obtaining some profit from the generated waste [13]. The use of alkaline hydrogen peroxide (AHP) to pretreat lignocellulosic biomass has increased in the last years [14, 15], mainly because it satisfies the conditions of mild reaction, temperatures between 25 and 70 °C and normal atmospheric pression, and because it provides high delignification and hemicellulose rates [16, 17]. However, its large-scale use is still challenged by the cost of reagents (H_2O_2 and NaOH), indicating the need to search ways to reduce these inputs [15]. Input reduction can be achieved by recycling the liquid solutions used in the pre-treatment, which certainly reduces the cost of biofuel production [18].

Several studies have demonstrated the potential of reusing the liquid fraction for different types of lignocellulosic biomass pre-treatment, with significant reductions in the use of chemical inputs and water. Wang et al. [3] evaluated the effect of recycling the liquid fraction of the pre-treatment of sugarcane bagasse with sodium hydroxide to reduce the water consumption and Rocha et al. [19] demonstrated the possibility of reusing the liquid fraction of the same process up to 11 times, with four cycles reducing water consumption by 65%. Yao et al. [20] obtained good results recycling the liquid fraction of the pre-treatment of the wheat straw with phosphoric acid added with hydrogen peroxide.

Previous work by our group [18] demonstrated significant reductions of input consumption recycling the alkaline hydrogen peroxide solution (7.5% v/v) used in the pre-treatment of corn stover. Three recycles of the liquid solution could be made, obtaining cellulose conversions into glucose above 60%, and reducing water, hydrogen peroxide and sodium hydroxide consumption up to 60%. However, this recycling certainly depends on the initial concentration of the alkaline hydrogen peroxide concentration and maybe more than three cycles could be tried [19] or concentrations lower than 7.5% could still allow efficient recycling. Besides, more information on how the H_2O_2 concentration modifies the physical–chemical structure of corn straw biomass is still needed. Therefore, the aims of this work were to evaluate the influence of different alkaline hydrogen peroxide concentrations on successive recycling of the liquid fraction of the pretreatment and enzymatic hydrolysis of corn stover and to determine the effect of these concentrations and recycling on delignification, hemicellulose removal and physical–chemical structure change.

Experimental Section

Raw Material

Corn (*Zea mays* L.) stover was harvested in 2012 and 2013 at *Riacho do Papagaio* Farm, São João municipality (8°52′ S, 36°22′ W), Pernambuco state, Brazil. The stover (here called lignocellulosic biomass) was dried in a forced air circulation oven at 65 °C for 48 h, ground in a Willey mill (MA 680, Marconi, São Paulo) and stored at room temperature. Subsamples, which were homogenized, were sieved through a 10 mesh for chemical characterization and to be submitted to pre-treatment.

Corn Stover Pre-treatment

The pre-treatment of the biomass was performed according to Alencar et al. [18], evaluating the influence of four alkaline hydrogen peroxide (AHP) concentrations: 1%, 3.5%, 5% and 7.5% v/v. The liquid fraction resulting from the pre-treatment of each concentration was cycled five times (four recycles). Stover samples (25 g) were submitted to the AHP pre-treatment, for 1 h, at 25 °C, maintaining the 1:10 proportion of biomass to H_2O_2 (25 g of biomass and 250 mL of H_2O_2 solution) and adjusting the pH to 11.58, using NaOH 5 M). The volume recovered in the first cycle (~200 mL) was subjected to a new cycle, maintaining the 1:10 ratio and this process was repeated to a total of five cycles, with volumes of approximately 140, 110 and 90 mL in the third, fourth and fifth cycle, respectively. The lignocellulosic biomasses in the second to fifth cycle were 20, 14, 11 and 9 g as determined by the volumes recovered in the previous cycle. The biomasses were kept inside propylene bags (0.17 × 0.06 cm) during the whole process.

Subsequently, the bags were dried at 65 °C for 24 h to determine the biomass they contained, according to Eq. 1:

$$Biomass\ recovery(\%) = \frac{Wbb - Wbag}{Wbio} * 100 \quad (1)$$

Where *Wbb* corresponds to the mass of polypropylene bags with the corn stover, *Wbag* corresponds to the mass of the empty polypropylene bags and *Wbio* to the lignocellulosic biomass.

The percentages of delignification or hemicellulose solubilization efficiency for the pre-treated biomass were determined using Eq. 2:

$$E(\%) = 100 - \left[\left(\frac{Pc}{Pin}\right) * 100\right] \quad (2)$$

where *E* corresponds to the percentages of delignification or hemicellulose solubilization efficiency, *Pc* to the percentage of lignin or hemicellulose remaining in the pre-treated sample of the corresponding cycle, and *Pin* to the lignin or hemicellulose content present in the initial dried biomass before pre-treatment.

Enzymatic Hydrolysis of the Pre-treated Corn Stover

Two g of pre-treated corn stover, dried at 65 °C for 24 h, were transferred to Erlenmeyer flask (50 mL), to which were added citrate buffer (50 mM, pH 4.8) and Celluclast 1.5L Novozymes enzyme complex (6.39 FPU/mL; 3.20 FPU/g), totalizing a reaction volume of 20 mL, equivalent to 10% solid load. The flasks were heated (50 °C) and shaked (150 rpm) for 48 h, without addition of β-glucosidase. The enzymatic hydrolysate was then filtered on qualitative paper by gravity and samples of liquid fraction were collected and stored in a freezer at − 20 °C for future analysis. Enzymatic hydrolyses were performed in triplicate. Cellulose conversion of all assays was determined by Eq. 3.

$$\%Cc = \frac{G\left(\frac{g}{L}\right)}{\left[1.11 * \left(\frac{\%C}{100}\right) * Sl\left(\frac{g}{L}\right)\right]} \quad (3)$$

where *%Cc* corresponds to cellulose conversion, *G* to glucose concentration, *%C* to percentage of cellulose in the biomass and *Sl* to solids load in the system.

Analytical Methods

Chemical Analysis

Cellulose, hemicellulose, lignin, and ash were determined in biomass samples, according to fiber analysis method of Van Soest et al. [21]. Carbohydrate and organic acids contents of pre-treatment liquid fractions and enzymatic hydrolysis were determined using High-Performance Liquid Chromatography (HPLC) on an Agilent HP 1100 chromatograph, with a refractive index detector (RID). Samples were diluted in milli-q water, filtered through a 0.22 µm cellulose acetate membrane and analyzed at a flow rate of 0.6 mL min^{-1}, at 60 °C, using 5 mM H_2SO_4 solution as the mobile phase in an Aminex HPX-87-H column (Bio-Rad, Hercules, CA, USA). Sigma-Aldrich® standard solutions (St Louis, MO) were used to construct calibration curves.

Structural Analyses

Untreated and pre-treated corn stover samples were dried at 65 °C for 24 h and submitted to physical analysis to verify changes in the biomass crystallinity index. The dried samples were analyzed by X-ray diffraction using a Bruker Advanced X-ray diffractometer model D8 with scan angle from 10° to 50° (Bragg- angle 2θ), 0.02° increments and count time of 1 s. The crystallinity index was calculated according to Eq. 4:

$$CrI(\%) = \frac{I_{crystalline} - I_{amorphous}}{I_{crystalline}} \quad (4)$$

where *CrI* corresponds to the crystallinity index, $I_{crystalline}$ to the intensity value at angle 21° and $I_{amorphous}$ to the intensity value at angle 18.8°.

Fourier Transform Infrared Spectroscopy (FTIR) analysis were conducted in a Vertex® 70 spectrometer device (Bruker Optics, Germany), with a resolution of 4 cm^{-1}, performing

64 scans per sample at a wave number range from 500 to 4500 cm^{-1}, in absorbance mode, and at total reflectance attenuation mode (TRA) [22]. In this mode, there is no need to prepare KBr pellets and, at each measurement, the software automatically subtracts from the obtained spectrum the spectrum of dioxide carbon previously obtained, which is taken as the background measurement.

Statistical Analysis

All experiments were performed in a completely randomized design in triplicate. Data on efficiency of delignification and cellulose conversion were analyzed by regression as a function of the number of recycles using the Origin® Software.

Results and Discussion

Effect of Hydrogen Peroxide Concentration on the Corn Stover Chemical Composition

Table 1 shows the cellulose, hemicellulose, acid detergent lignin (ADL) and extractives contents for the initial corn stover biomass and after this biomass was submitted to pre-treatment with the four initial H_2O_2 concentrations and after four recycles of the liquid fraction of these pre-treatments. The chemical composition of the "in natura" corn stover was 31.7 ± 0.5% cellulose, 34.9 ± 1.1% hemicellulose, 4.46 ± 0.18% detergent acid lignin and 8.22 ± 0.15% extractives. The highest proportions of cellulose were obtained in the first cycle, for all peroxide concentrations, with significant reductions in further recycles (Table 1). The percentages of cellulose from the first to the last cycles decreased from about 39 to 33% for the 1% and 3.5% v/v AHP, and from 47 to 34% for the two higher concentrations (5% and 7.5% v/v).

The hemicellulose and acid detergent lignin (ADL) contents were highest for stover pre-treated with the lowest hydrogen peroxide concentrations (1% and 3.5% v/v), indicating that the pre-treatment was not effective at these concentrations. Similar results were observed in the pre-treatment of bamboo chips with alkaline hydrogen peroxide at low H_2O_2 concentrations (1.5% v/v), without using the recycle technology [23]. The hemicellulose was lowered at 5% (v/v) concentration, demonstrating its solubilization, which was even more evident at 7.5% (v/v) concentration, when lignin and hemicellulose decreased to 77% and 52% of their initial values, respectively, after the first cycle. In subsequent cycles, lignin decreased consecutively to 56%, 50%, 33% and 12%. These results indicate that the delignification and hemicellulose solubilization capacities are limited at low H_2O_2 concentrations, due to decreased action of hydroxyls

Table 1 Chemical composition (content %) of corn stover "in natura" and pretreated with alkaline hydrogen peroxide at different concentrations, obtained in the five cycles reaction

Compounds	In natura	1 cycle	2 cycle	3 cycle	4 cycle	5 cycle
1%						
Cellulose	31.74 ± 0.45	39.60 ± 1.29	38.66 ± 2.43	36.84 ± 1.92	34.80 ± 0.89	31.88 ± 0.00
Hemicellulose	34.95 ± 1.07	40.53 ± 3.29	38.12 ± 3.96	37.65 ± 3.00	36.02 ± 0.06	35.38 ± 1.11
ADL	4.46 ± 0.18	3.80 ± 0.04	3.99 ± 0.16	4.02 ± 0.42	4.37 ± 0.28	4.48 ± 0.13
Extractives	8.22 ± 0.15	ND	ND	ND	ND	ND
3.5%						
Cellulose	31.74 ± 0.45	38.19 ± 1.80	36.94 ± 1.19	35.87 ± 0.66	34.28 ± 2.54	34.26 ± 1.41
Hemicellulose	34.95 ± 1.07	40.81 ± 3.52	39.32 ± 4.09	38.52 ± 0.27	36.60 ± 1.75	36.52 ± 1.89
ADL	4.46 ± 0.18	3.31 ± 0.61	3.54 ± 0.02	4.00 ± 0.20	4.32 ± 0.38	4.39 ± 0.05
Extractives	8.22 ± 0.15	ND	ND	ND	ND	ND
5%						
Cellulose	31.74 ± 0.45	46.10 ± 0.51	44.07 ± 1.65	38.71 ± 0.59	35.29 ± 0.49	33.54 ± 1.16
Hemicellulose	34.95 ± 1.07	29.03 ± 0.13	30.14 ± 0.91	31.96 ± 2.84	32.67 ± 2.44	34.88 ± 1.97
ADL	4.46 ± 0.18	2.62 ± 0.32	3.32 ± 0.25	3.34 ± 0.30	3.60 ± 0.44	3.71 ± 0.27
Extractives	8.22 ± 0.15	ND	ND	ND	ND	ND
7.5%						
Cellulose	31.74 ± 0.45	47.66 ± 2.96	45.76 ± 2.14	40.93 ± 2.75	35.80 ± 1.94	33.57 ± 1.94
Hemicellulose	34.95 ± 1.07	16.89 ± 1.73	24.97 ± 1.31	28.63 ± 2.14	34.22 ± 0.67	34.83 ± 0.58
ADL	4.46 ± 0.18	1.03 ± 0.23	1.96 ± 0.04	2.23 ± 0.20	2.99 ± 0.26	3.93 ± 0.57
Extractives	8.22 ± 0.15	ND	ND	ND	ND	ND

ADL acid detergent lignin, *ND* not determined

and super oxides of the AHP radicals in removing heteropolymer contents. The differences in percentages from first to fifth cycles can also be caused by the decreasing capacity of the H_2O_2 whose decomposition generates radicals that preferentially act on lignin resulting in the solubilization of hemicellulose [18]. In the present study, the lower H_2O_2 concentrations in the system produced lower concentrations of reactive radicals. Therefore, concentrations lower than 7.5% v/v do not have the capacity to remove considerable proportions of acid detergent lignin and hemicellulose from corn stover, and more H_2O_2 needs to be added to the recycled liquid solution in order to facilitate the enzymatic action during the hydrolysis step.

The delignification efficiencies were calculated knowing the acid detergent lignin concentrations (Fig. 1). Maximum delignification was observed for the 7.5% (v/v) peroxide concentration in the first reaction cycles. Significant negative linear regression model fit the data for all concentrations. Delignification efficiencies higher than 50% until the third cycle were only obtained using the 7.5% v/v concentration. Delignification values vary considerably in the literature, depending on the peroxide concentration, solid load of the reactor, reaction temperature and pre-treatment time length [14]. With a single cycle and similar conditions to our 7.5% treatment (AHP, 7.36% v/v; 4% w/w solid load and one hour) but treating sugarcane bagasse [24], delignification of 86% was obtained. Also in a single cycle but with even lower AHP concentration (2% v/v), delignification reached 74% [25] because corncob was pre-treated with higher temperature (50 °C) and a slightly longer time period (1.5 h).

FT-IR and DRX

FT-IR spectra of the initial and pre-treated corn stover samples are shown in Fig. 2. The assignments of characteristics

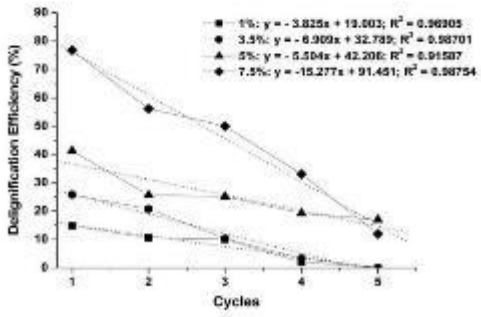


Fig. 1 Linear regression between efficiency of delignification and number of cycles for four concentrations of alkaline hydrogen peroxide solution recycle

bands found in the spectrum of untreated corn stover are shown in Table 2. Significant differences in the characteristic bands of both untreated and pre-treated spectra were not observed using H_2O_2 at 1% v/v concentration (Fig. 2a), indicating that this concentration is not efficient as a corn stover pre-treatment.

On the other hand, the broad band around 3300 cm^{-1}, which represents the stretching vibration of the −OH bonds of cellulose [26] in the untreated corn stover, was shifted to around 3500 cm^{-1}. After pre-treatment, for all cycles (Fig. 2b–d), showing that part of the cellulose molecules was broken. The bands had lower intensities after pre-treatment with H_2O_2 at 7.5% v/v (Fig. 2d), more so after the first cycle, meaning that not only the hydrogen bonds were disrupted but also part of the crystalline cellulose.

Other changes in FT-IR spectra reinforce that H_2O_2 at 7.5% v/v (cycle 1) was the best pre-treatment for corn stover. The change in the band position around 2900 cm^{-1}, which is attributed to C–H stretching within the methylene of cellulose [27], indicate a slight decrease in its relative absorbance after pre-treatment that can be attributed to rupture of methyl and methylene portions of cellulose. The weak band at 1730 cm^{-1} of the untreated corn stove practically disappears after pre-treatment, due to removal of hemicelluloses [28]. The intensity of the band at 1650 cm^{-1}, which is attributed to C=C stretch of lignin, was reduced after pre-treatment, suggesting that some linkages between lignin and carbohydrates were cleaved [29], leading to some lignin fractions with low molecular weight. Reduction in intensity of the band at 1160 cm^{-1} also occurred, being attributed to the cleavage and/or alterations of acetyl groups [30], indicating the removal of hemicellulose by the H_2O_2 (at 7.0% v/v) pre-treatment.

Therefore, the analysis of FTIR spectra showed the removal of hemicellulose and lignin by all pre-treatments with H_2O_2 alkaline solution, with best results in the first cycle at 7.5% v/v. The increases of peak intensities at 3500 cm^{-1} and 1160 cm^{-1} were evidences that pre-treatment efficiencies were reduced in cycles 2 and 3, most likely due the reduction of H_2O_2 in the alkaline solution.

Removal of amorphous components like lignin and hemicelluloses increased the crystallinity index (CI) of the corn stover biomass after pre-treatment in all cycles (Table 3), the highest value being obtained in the first cycle of the 7.5% v/v concentration. This result agrees with the FTIR analysis and it was a consequence of lignin removal (Table 1; Fig. 2d) and possible relocation of lignin due to the oxidation reaction of H_2O_2 [31]. Increases of the crystallinity index from 39.2 to 55% were also observed after pre-treating corn stover with hydrogen alkaline peroxide and ammonia fiber expansion [32].

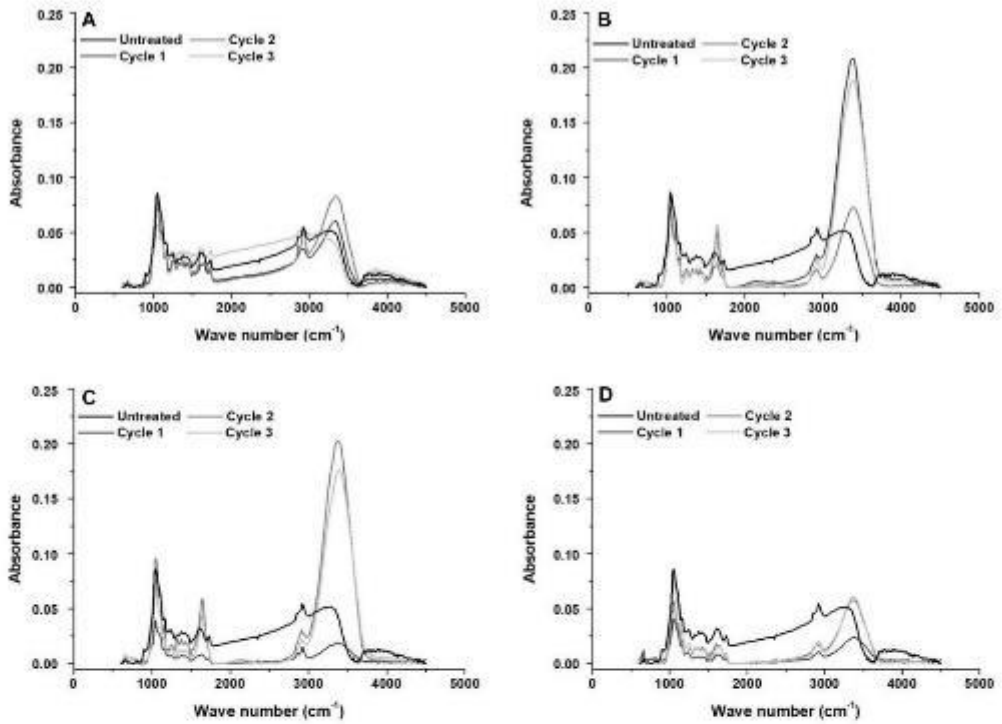


Fig. 2 FTIR spectra of "in natura" and pre-treated corn stover at different H_2O_2 concentrations: a 1% v/v, b 3.5% v/v, c 5% v/v, and d 7.5% v/v

Table 2 FTIR absorption peak location and assignment of corn stover (Nuopponen 2006)

Wave number (cm^{-1})	Absorption peak assignment
3330	O–H stretching
2920	C–H symmetrical and asymmetrical stretching in $-CH_3$ and $-CH_2$ groups
1731	C=O stretch of ester acetyl groups in hemicelluloses
1650–1630	C=C stretching vibration of lignin aromatic ring
1509–1515	C=C skeletal stretch of lignin aromatic ring
1460	C–H deformations (asym. in $-CH_2-$) in lignin and carbonhydrates
1325	C–H vibration in cellulose
1240	C O C stretching of acetyl group
1160	C–O–C vibration in cellulose and hemicellulose
1110–1125	C–O stretch in cellulose and hemicellulose
890	C–H deformation in cellulose and saccharide

Parameters of Pre-treatment Evaluated in the Recycling Technology

Rapid declines in pH over the cycles at 1% and 3.5% v/v concentrations were observed, whereas at 5% and 7.5% v/v the pH remained close to 11.5 until cycle 3 (Fig. 3). At lower hydrogen peroxide concentration, the hydroxyls formed from the degradation of the hydro peroxide radical are consumed by the superoxide radical ($OH^- + O^{3-} + H^+ \rightarrow O_2 + H_2O$), in a shorter time interval [33]. Banerjee et al. [34] also observed this pH decrease when a low peroxide concentration (0.125 g/g) was used

Table 3 Crystallinity index of corn stover biomass obtained by different concentrations of alkaline hydrogen peroxide solution as pretreatment

Cycles	Concentration (% v/v)			
	7.5	5	3.5	1
1	39.52	34.64	35.49	35.47
2	38.59	33.57	33.62	32.97
3	35.74	30.94	30.91	32.03
"In natura"	28.7			

with corn stover so they corrected the pH throughout the pre-treatment. With 5% and 7.5% v/v of H_2O_2, the pH was even increased in relation to the first two cycles. This increase can be explained by the chemical reaction $H_2O_2 + HOO^- \rightarrow OH^- + O^{2-} + H_2O$, where the anion hydroperoxide reacts with another molecule of hydrogen peroxide, forming hydroxylates [33, 35].

The percentage of solids recovered at concentrations of 1%, 3.5% and 5% v/v were similar to each other over the cycles, but were lower than those at 7.5% concentration (Fig. 3), indicating that the AHP content in the medium does not interfere with the hemicellulose solubilization and delignification capacity of system at concentrations equal to or below 5%. This implies in a smaller portion of the biomass being solubilized in the liquid fraction, providing a higher recovery of solids. In the recycling process, the recovered solids content increased due to the decreased capacity of the system to solubilize lignin and hemicellulose in the pre-treatment stage. On the other hand, the recovered volumes with the four AHP concentrations were similar, around 70–85%, implying in reductions of the use of water and hydrogen peroxide in the pre-treatment.

In a non-recycled system, under the conditions of the present work, 0.75 mL of H_2O_2 per g of biomass at 32% w/v of solids loading (considering the biomass used in the five cycles) would be necessary. This contrasts with only 0.24 mL of H_2O_2 per g of biomass reusing the AHP solution and maintaining the same solids loading [18]. Martins et al. [36] used 0.35 mL of H_2O_2 per g of sugar cane bagasse, pretreating with a high solids loading 20% w/w at 90 °C. Therefore, the recycling of the alkaline hydrogen peroxide solution may significantly reduce the costs of the pre-treatment step and may be a more feasible strategy than high solids loading.

Effect of AHP Concentration on the Enzymatic Hydrolysis of Corn Stover

In order to evaluate the efficiency of the pre-treatments with the different AHP concentrations and along the recycles, enzymatic hydrolysis of the pre-treated biomass was carried out using an enzymatic load of Celluclast 1.5L Novozymes complex (6.39 FPU mL^{-1}) at 3.20 FPU g^{-1} of pre-treated biomass (Fig. 4). For all AHP concentrations, linear negative regression models were adjusted for the first three cycles (1% $R^2 = 0.96$; 3.5% $R^2 = 0.94$; 5% $R^2 = 0.98$; 7.5% $R^2 = 0.99$). The maxima saccharification conversions in the first cycle were 89.6% when using the concentration of 7.5% (v/v), 58.3% with 5% (v/v),

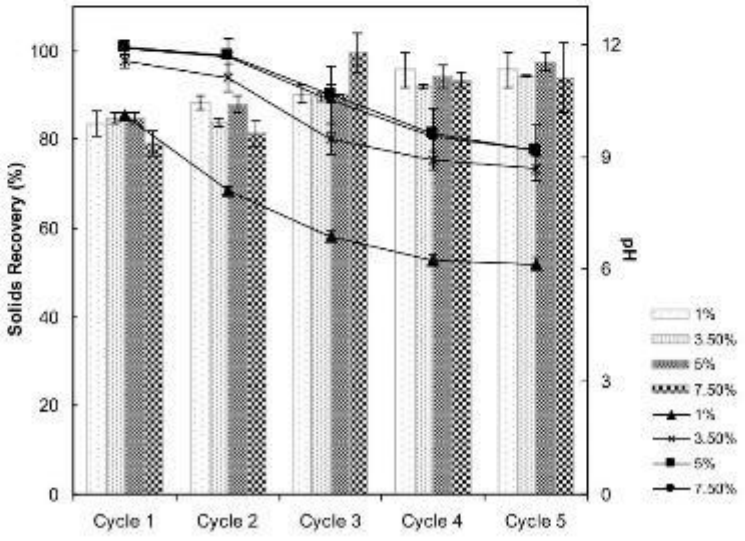


Fig. 3 Relation between recovery volume and pH for four concentrations of alkaline hydrogen peroxide solution recycle

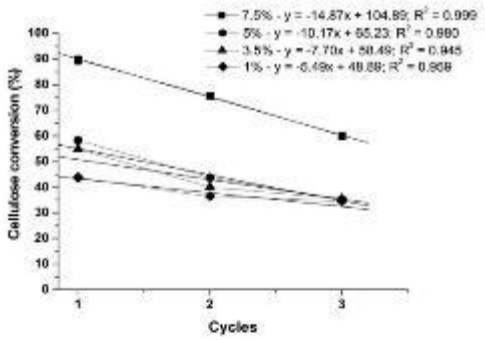


Fig. 4 Cellulose conversion of the corn stover enzymatic hydrolysate pretreated, for 3 cycles, under different concentrations of the AHP solution

54.9% with 3.5% (v/v) H_2O_2, and 43.8% using 1% (v/v) H_2O_2. The enzymatic hydrolysis efficiency was highly dependent on the initial hydrogen peroxide concentration due to its effect on hemicellulose solubilization and delignification [17]. Saha and Cotta [37], pre-treating rice husk with 7.5% H_2O_2 (v/v) AHP (pH 11.5, 35 °C in 24 h) obtained a high yield of saccharification (96%) in only one cycle, but they used a combination of three enzymes (cellulase, β-glucosidase, and xylanase), which probably favored saccharification. Using a lower AHP (5%), to pre-treat bagasse of cashew fruits the efficiencies were lower, 65–85% [16].

The saccharification value was less than 50% in the first cycle of the 1% peroxide concentration and became less than 50%, from the second cycle on for concentrations of 3.5% and 5% v/v. Therefore, they were considered as not feasible for the recycle technology. Therefore, even using commercial enzyme preparations supplemented with b-glucosidases and other hydrolases capable of digesting hemicellulose oligomers, the recycle technology is not feasible at low peroxide concentrations. The concentration of 7.5% v/v is ideal for these recycle systems, since up to the third cycle, the cellulose conversion was greater than 50%. It is important to highlight that the alkaline hydrogen peroxide concentrations were not corrected along the cycles; thus, the effects were based on the residual concentrations. Under these recycling conditions, enzymatic hydrolysis above 50% are only obtained with initial AHP concentrations above 7.5%. Otherwise, the concentration in subsequent cycles has to be corrected with new additions of H_2O_2 at pH 11.5, as was proposed by Rocha et al. [19] when recycling the liquid solution of pre-treatment of sugarcane bagasse with sodium hydroxide.

Conclusions

The alkaline hydrogen peroxide concentration of 7.5% v/v H_2O_2 is more indicated than lower concentrations to be used in the recycling of the liquid solution in the pre-treatment of corn stover. The results of hemicellulose solubilization and delignification, as well as FT-IR and XRD analyzes, indicated that lower H_2O_2 concentrations are insufficient for an efficient pre-treatment of corn stover for 1 h at ambient temperature and pressure. Using the 7.5% v/v concentration, three cycles could be done, based on the residual concentration, with cellulose conversions greater than 50%. The resulting input reduction can be translated into lower costs and higher viability of alcohol production from lignocellulosic biomass.

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Affiliations

Bárbara Ribeiro Alves Alencar[1] · Fernanda Leitão Vaz[2] · Adauto Gomes Barbosa Neto[3] · Katia Aparecida Aquino[4] · Everardo Valadares de Sa Barretto Sampaio[2] · Rômulo Simões Cezar Menezes[2] · Emmanuel Damilano Dutra[2,5]

[1] Interdepartmental Research Group in Metabolic Engineering, Department of Genetics, Federal University of Pernambuco, Recife, PE 50670-901, Brazil

[2] Research Group on Biomass Energy, Department of Nuclear Energy, Federal University of Pernambuco, Recife, PE 50740-540, Brazil

[3] Program of Postgraduation in Genetics, Department of Genetics, Federal University of Pernambuco, Recife, PE 50670-901, Brazil

[4] Laboratory of Polymers and Nanotechnology, Department of Nuclear Energy, Federal University of Pernambuco, Recife, PE 50740-570, Brazil

[5] Departamento de Energia Nuclear, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, Cidade Universitária, Recife, PE 50740-540, Brazil

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RESEARCH ARTICLE

Valorization of Sugar-Ethanol Industry Waste Vinasse for Increased Second-Generation Ethanol Production Using *Spathaspora passalidarum* Yeast Strains

Fernanda Leitão Vaz[1,2] · Raquel de Fátima Rodrigues de Souza[1] · Emmanuel Damilano Dutra[2] · Bárbara Ribeiro Alves Alencar[3] · Esteban Espinosa Vidal[1]

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Abstract In this study, sugarcane bagasse hydrolysate supplemented with different proportions of sugar-ethanol industrial waste (vinasse) was used to optimize fermentative ethanol production using two *Spathaspora passalidarum* yeast strains isolated from decaying wood in Amazonian biome. The bagasse samples were pretreated chemically and enzymatically before using a binary factorial experimental design. The factorial design was carried out in two stages: in the first, a 2^2 factorial design was done to assess the effects of pretreated sugarcane bagasse hydrolysate and vinasse, ammonium sulfate concentration, yeast biomass concentration, and two different strains of yeast, *S. passalidarum*; in the second stage, a 2^3 factorial design was done to provide a more detailed analysis of the effects of vinasse supplementation on the fermentation process—this time, by modifying the ratio of sugarcane bagasse hydrolysate and vinasse and, more significantly, the same factor—biomass concentration. The results showed that the most significant factors for increasing bioethanol production in the 2^4 factorial design proved to be the following: inoculum and hydrolyzed bagasse supplementation with vinasse (the optimal being 75:25), followed by yeast concentration (optimal at 14% w/v cell wet weight) and then the type of strain used (*S. passalidarum* UFMG-HMD-14.1). The use of only hydrolyzed bagasse without vinasse (2^3 factorial design) had no significant influence on the results, nor did varying the concentration of nitrogen. This increase in ethanol production, which allows the same amount of ethanol productivity through a reduction in the carbon source, suggests it can lead to an improved final use for vinasse.

Keywords Factorial design · Lignocellulosic material · Agro-industrial waste · Cellulosic yeast · Alcoholic fermentation

✉ Fernanda Leitão Vaz
nandavaz@gmail.com

[1] White Biotechnology Group, Bioprocess Laboratory, Center for Strategic Technology of the Northeast (CETENE), Recife, PE CEP 50.740-540, Brazil

[2] Research Group on Biomass Energy, Department of Nuclear Energy, Federal University of Pernambuco, Av. Prof. Moraes Rego, 1235, Cidade Universitária, Recife, PE 50740-540, Brazil

[3] Interdepartmental Research Group in Metabolic Engineering, Department of Genetics, Federal University of Pernambuco, Recife, PE 50670 901, Brazil

Introduction

Owing to the need to satisfy the increased global demand for energy and the trend toward more cost-effective and ecologically-friendly energy strategies, renewable fuels are currently emerging as feasible alternatives to fossil fuels and beginning to be incorporated in the energy market (Sørensen 2008). Society is driving initiatives aimed at sustainable biotechnological strategies with a less harmful environmental impact, and thus leading to reduced greenhouse gas emissions and helping maintain a balanced carbon cycle, and these techniques are even proving to have financial benefits (Zhao et al. 2015; Qureshi et al. 2016).

Sugarcane bagasse has been cited as a potential source of cellulose and hemicellulose because of its widespread use in ethanol industrial plants (Soccol et al. 2010; Ballesteros et al. 2004; Wanderley et al. 2013). Assuming a yield of 0.25 Mg of bagasse/Mg of sugarcane, Brazil will have generated 171 million Mg of bagasse, by the end of the 2016–2017 harvest alone (Petrini et al. 2016).

Currently, bagasse is a by-product obtained in bioethanol plants and re-used for steam generation or for electric co-generation, resulting in an energy industry that is self-sufficient (Basso et al. 2008). At the same time, the amount of bagasse is sufficient to be redistributed for second-generation (2G) bioethanol production.

Another by-product from the sugar-ethanol industry is a liquid stream called stillage or distillery vinasse, which is the residue from the distillation of fermented sugarcane juice. Approximately 10–15 l of vinasse is generated for each liter of alcohol produced (Basso et al. 2008). This meant that in the case of the 2016–2017 harvest in Brazil, there was a vinasse production of approx. 412.5 million tons (Petrini et al. 2016). This residue is rich in phosphorus and potassium salts content and B-complex vitamins and is used in soil fertilization (de Oliveira et al. 2013). The problem of disposing of vinasse in this way is that it gives rise to changes in the physicochemical soil properties, and causes groundwater contamination (Salomon and Lora 2009). Because of its potential nutrient content, vinasse has aroused interest among researchers as a yeast nutritional supplement, resulting in a noticeable increase in its use for protein biomass production (Tauk 1982; Silva et al. 2011). Previous studies investigating the use of vinasse have already shown an enhanced biomass production, which could be linked to a significant presence of sodium (Na), potassium (K) and calcium (Ca) (internal report, White Biotechnology Group, Bioprocess Laboratory, CETENE). The reason for investigating this potential vinasse reuse is that it could be integrated into the ethanol production process and, hence, be a way of reducing its detrimental effect on the environment.

Another important factor to consider in 2G ethanol production is the selection of yeast. The yeasts employed in this bioindustrial process must have a series of physiological features required for high-scale fermentation: high fermentation rates; good carbon source assimilation; increased osmotic, acid and alcohol tolerance; high yields and good adaptability. *Saccharomyces cerevisiae* is the principal yeast species used in the first generation (1G) of ethanol production because it efficiently ferments sucrose and glucose (Beato et al. 2016). However, its inability to use compounds that are mainly found in bagasse hydrolysate cellulose, such as xylose, arabinose or the disaccharide cellobiose as carbon sources in 2G ethanol production, results in incomplete carbon source consumption and very low yields (Jojima et al. 2010). With this in mind, research has focused on isolating yeast from lignocellulosic material that is capable of assimilating xylose, arabinose, or cellobiose, and thereby improving the ethanol yield (Tanimura et al. 2012; Cassa-Barbosa et al. 2015; Reis et al. 2016). This ability is made possible by the presence of enzymes such as xylose reductase and xylitol dehydrogenase, which enable xylose to be metabolized so that it can produce ethanol (Hou and Yao 2012). To this end, yeast from the genus *Spathaspora* was recently isolated from rotten wood in the Brazil Amazon Rainforest. This newly isolated yeast has demonstrated a capacity to ferment xylose as the only carbon source and achieved an ethanol yield of 0.34 g/g (Cadete et al. 2009, 2012).

In light of the above, the purpose of this study was to evaluate and optimize the production of second-generation ethanol using sugarcane bagasse and vinasse as a nutritional supplement in two stages of factorial design. At the same time, changes in the concentrations of both nitrogen and yeast cells in the inoculum were evaluated, together with the effect of two promissory *Spathaspora passalidarum* strains, using laboratory-scale methods to emulate industrial 2G ethanol production.

Materials and Methods

Yeast Strains

The yeasts used in this study were two strains of *S. passalidarum* UFMG-HMD-14.1 (S.pa-14.1) and *S. passalidarum* UFMG-HMD-1.3 (S.pa-1.3), isolated from rotting wood collected in the Amazonian rainforest in the state of Roraima, Brazil (Cadete et al. 2012). The strains belong to the Collection of Microorganisms, DNA and Cells of the Federal University of Minas Gerais (CM-UFMG), kindly provided by Dr. Carlos A. Rosa, from the Microbiology Department of the Institute of Biological Sciences (ICB/UFMG, Belo Horizonte, Brazil).

Sugarcane Bagasse and Vinasse

The sugarcane bagasse and vinasse were kindly provided from sugar-ethanol companies from Pernambuco State, Brazil. The bagasse was obtained from sugarcane [fiber] crushed in an industrial press and then dried at room temperature. The bagasse was then oven-dried for 48 h and ground in a knife mill using mesh number 20. The characterization of the bagasse, both before and after the pretreatment for the cellulose, hemicellulose, lignin (acid detergent lignin/ADL), extractives and ash contents, was carried out in accordance with the methodology of Van Soest (1963). Before pretreatment, the bagasse had 39.21 ± 1.10% of cellulose, 37.96 ± 0.10% of hemicellulose, 11.82 ± 1.50% of ADL, 6.30 ± 0.06% of extractives and 2.22 ± 0.14% of ash. After pretreatment, it had 56.13 ± 0.3% of cellulose, 30.20 ± 2.40% of hemicellulose, 6.58 ± 0.70% of ADL, 2.52 ± 0.08% of ash and non-determined extractives.

The vinasse was defrosted from storage at − 20 °C and then clarified by centrifugation at 10,000 rpm for 5 min. The resulting vinasse in suspension was submitted to an elemental composition analysis using flame photometry at the Nuclear Energy Department of the Federal University of Pernambuco (UFPE), Brazil. The analysis showed the presence of sodium (Na) at 44.50 ± 00 mg/l, potassium (K) 945.47 ± 16.07 mg/l and calcium (Ca) 196.80 ± 00 mg/l, in values similar to those described by Prada et al.(1998). When analyzed in HPLC, the concentration of sugars in vinasse was 2.24 g/l of glucose and 0.41 g/l of xylose.

Chemical Delignification and Enzymatic Hydrolysis

The delignification of sugarcane bagasse was conducted by employing the alkaline hydrogen peroxide method (Rabelo et al. 2011; Reis et al. 2016; Alencar et al. 2017). To 20 g of milled dry bagasse were added 160 ml of 7.5% hydrogen peroxide and 40 ml of sodium hydroxide (5 M). A 1-l Erlenmeyer flask was placed in an orbital shaker at 150 rpm, at 25 °C for 1 h. The contents were then washed with 1.5 l of distilled water at approximately 70 °C. The washed, solid material was put in a Petri dish and placed to dry in an oven at 60 °C for 48 h (Rabelo et al. 2011).

The enzymatic hydrolysis was carried out using the commercial enzyme FybreZyme (Dyadic, Florida, EUA) with cellulolytic activity of 9 FPU/ml. Into a 250-ml Erlenmeyer flask were placed 5 g of delignified bagasse, enough enzyme to reach a final of 10 FPU/g of delignified bagasse (using the standard measurement for cellulase (Ghose 1987), and citrate buffer to a concentration of 50 mM (pH 4.8) in a final volume of 50 ml. The Erlenmeyer flasks were placed on the shaking table at 150 rpm at 50 °C for 48 h. After this period, the material was centrifuged at 12,000 rpm for 5 min at 20 °C. The hydrolyzed supernatant was frozen for subsequent sugar quantification and fermentation assays.

Yeast Preparation

The strains were subcultured from 0.1 ml of glycerol stock by inoculation in 10 ml of YPD liquid medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose. The yeasts were grown in this medium for 1 day at 200 rpm. After this, the volume of the medium was doubled every 24 h, to a final volume of approximately 800 ml. After 5 days, the biomasses were determined in grams of wet weight cells per 100 ml of medium (% w/v WCW).

Before yeast inoculation, a measured volume for achieving 6 or 14% w/v WCW of the culture was centrifuged at 4500 rpm for 5 min, the supernatant discarded, and the yeast cells resuspended in the stipulated fermentation medium, which had been prepared according to the factorial design.

Optimized Bioethanol Fermentation Production by Factorial Design

The optimization of the variables affecting the ethanol production was achieved by means of two factorial designs. In the first, a 2^4 factorial design was carried out to assess the effects of 25% of pretreated sugarcane bagasse hydrolysate (SBH) to 75% of vinasse (V), and the reciprocal (SBH:V 75:25 and 25:75), ammonium sulfate concentration [$(NH_4)_2SO_4$], yeast biomass concentration (YBC), and two different strains of yeast, *S. passalidarum* UFMG-HMD-14.1 and *S. passalidarum* UFMG-HMD-11.3 (S.pa) (Table 1).

In this factorial design, 16 experimental runs were carried out, which gave 16 factorial points. All the factors were assayed at two levels, and the ethanol concentration was measured as the response. Fermentations were conducted in 250-ml Erlenmeyer flasks using a volume of 50 ml reactive medium according to the specifications for Factor 1 (Table 1). The assay conditions were as follows: temperature 32 °C, stirring at 120 rpm, pH 4.5 for 72 h. Samples were collected periodically and analyzed for consumption of carbohydrates, ethanol and by-product formation, and other components to determine the yield and efficiency of the process. The yeast biomass was quantified spectrophotometrically by absorbance at 600 nm.

Subsequently, a second 2^3 factorial design was performed to analyze further the effect of vinasse supplementation on the fermentation process, this time, by modifying the ratio of 75% of sugarcane bagasse hydrolysate (SBH): 25% vinasse, (75:25) as the low-level (that showed the best results in a previous experiment), and 100% of SBH without vinasse (SBH:V 100:00) as the high-level variant. The others factors were the yeast biomass concentration (YBC) and yeast strain (S.pa). The experiments were carried out using eight experimental runs, which gave eight factorial points. All the factors were assayed at two levels (Table 2).

The fermentative procedures and conditions were the same as those in the first factorial design. In both experimental designs, the statistical analysis was conducted with the assistance of Statistica 7.0 software from Statsoft Inc. (2325 East 13th Street, Tulsa, OK, 74104, USA).

Sugar and Ethanol Quantification

Concentrations of ethanol, acetic acid, glycerol, xylose, cellobiose, xylitol and glucose were determined by a high-

Table 1 Combination of variables, code levels, real values (in parentheses) and corresponding ethanol production (g/l) for the first 2^4 factorial designs

Run	Variables								Fermentative parameters					
	SBH:V (%)		$(NH_4)_2SO_4$ (g/l)		YBC (%)		S.pa		Ethanol (g/l)	Sugars consumption[a] (%)	Yp/s[b] (g/g)	Qp[c] (g/l h)	H[d] (%)	Grow rate[e] (%)
1	− 1	(25:75)	− 1	(3)	− 1	(6)	− 1	(Spa-1.3)	7.46	96.1	0.39	0.16	76.11	15.57
2	1	(75:25)	− 1	(3)	− 1	(6)	− 1	(Spa-1.3)	6.21	53.04	0.21	0.13	41.74	28.95
3	− 1	(25:75)	1	(7)	− 1	(6)	− 1	(Spa-1.3)	5.05	96.2	0.26	0.11	51.47	33.99
4	1	(75:25)	1	(7)	− 1	(6)	− 1	(Spa-1.3)	6.3	54.33	0.21	0.13	41.34	3.62
5	− 1	(25:75)	− 1	(3)	1	(14)	− 1	(Spa-1.3)	5.77	96.25	0.3	0.12	58.77	8.55
6	1	(75:25)	− 1	(3)	1	(14)	− 1	(Spa-1.3)	16.01	89.29	0.33	0.33	63.92	13.23
7	− 1	(25:75)	1	(7)	1	(14)	− 1	(Spa-1.3)	6.81	96.4	0.35	0.14	69.26	7.87
8	1	(75:25)	1	(7)	1	(14)	− 1	(Spa-1.3)	14.09	90.53	0.28	0.29	55.49	16.09
9	− 1	(25:75)	− 1	(3)	− 1	(6)	1	(Spa-14.1)	6.14	95.95	0.32	0.13	62.74	33.79
10	1	(75:25)	− 1	(3)	− 1	(6)	1	(Spa-14.1)	11.62	70.29	0.3	0.24	58.94	13.8
11	− 1	(25:75)	1	(7)	− 1	(6)	1	(Spa-14.1)	4.8	95.65	0.25	0.1	49.20	39.45
12	1	(75:25)	1	(7)	− 1	(6)	1	(Spa-14.1)	12.05	66.62	0.33	0.25	64.49	32.68
13	− 1	(25:75)	− 1	(3)	1	(14)	1	(Spa-14.1)	4.99	94.55	0.26	0.1	51.74	8.98
14	1	(75:25)	− 1	(3)	1	(14)	1	(Spa-14.1)	20.54	99.42	0.38	0.43	73.65	10.49
15	− 1	(25:75)	1	(7)	1	(14)	1	(Spa-14.1)	4.26	96.4	0.22	0.09	43.32	15.22
16	1	(75:25)	1	(7)	1	(14)	1	(Spa-14.1)	18.67	98.71	0.34	0.39	67.43	12.09

Proportion of mixing pretreated sugarcane bagasse hydrolysate and vinasse (SBH:V), ammonium sulfate concentration $[(NH_4)_2SO_4]$, yeast concentration (YBC) and yeast strain (S.pa)

[a]Sugars consumption (%)—percentage of initial glucose and xylose consumed

[b]Yp/s (g/g)—ethanol yield: correlation between ethanol produced and sugars (glucose and xylose) consumed

[c]Qp (g/l h)—ethanol productivity: ratio of ethanol concentration (g/l) and 48 h of fermentation time

[d]H (%)—fermentation efficiency: percentage of the maximum theoretical ethanol yield (0.51 g ethanol/g sugars)

[e]Grow rate (%)—percentage of cell grow

performance liquid chromatography (HPLC) system comprising an Alliance 2695 Separations Module (Waters, Milford, MA, USA), a 2414 refractive index detector (Waters, Milford, MA, USA), and a size exclusion column RHM-Monosaccharide H^+ (8%) (Rezex™, LC Column 300 × 7.8 mm × 8 µm, PhenomenexInc, Torrance, California, EUA) fitted with a pre-column (SecurityGuard™, Phenomenex Inc, Torrance, California, EUA). The mobile phase (ultra-pure water) was pumped at a flow rate of 0.6 ml/min. The column temperature was regulated at 35 °C, and the sample volume injected was 20 µl. The compounds were identified by their relative retention times and quantified with external standard calibration curves. The linear range of the calibration curve was determined for xylose, cellobiose, xylitol, and glucose, and this was 0.3–10 g/l with a significance at the 0.05 level ($p < 0.05$). The correlation coefficients (R^2) of the standard curves for all the compounds were superior to 0.999, and the relative standard deviation was less than 5%. The quantification and detection limits were approximately 0.2 and 0.3 mg/l, respectively.

Results and Discussion

The factorial design 2^4 showed that the best conditions for ethanol production, using vinasse, were of #14, because it was the one that produced the highest concentration of ethanol and obtained the best yields (Table 1). The significant factors in the Pareto chart were SBH, YBC, S.pa, as well as the interaction between SBH/YBC and SBH/S.pa (Fig. 1a). In fact, the response surface indicated that the increase in ethanol production is dependent on the fact that the SBH:V, YBC and S.pa levels increase in the system (Fig. 1b–d). The data from the first factorial design were employed to design a mathematical model for describing the fermentation of sugarcane bagasse hydrolysate (supplemented with vinasse by *S. passolidarum*) over a 48-h period, with a 95% confidence interval. The variables were

Table 2 Combination of variables, code levels, real values (in parentheses) and corresponding ethanol production (g/l) for the second 2^3 factorial designs

Run	Variables			Fermentative parameters					
	SBH:V (%)	YBC (%)	S.pa (%)	Ethanol (g/l)	Sugars consumption[a] (%)	Yp/s[b] (g/g)	Qp[c] (g/l h)	H[d] (%)	Grow rate[e] (%)
1	− 1 (75:25)	− 1 (6)	− 1 (Spa-1.3)	6.21	53.04	0.21	0.13	41.74	28.95
2	1 (100:00)	− 1 (6)	− 1 (Spa-1.3)	10.34	47.26	0.34	0.24	61.29	19.58
3	− 1 (75:25)	1 (14)	− 1 (Spa-1.3)	16.01	89.29	0.33	0.33	63.93	13.23
4	1 (100:00)	1 (14)	− 1 (Spa-1.3)	21.12	95.17	0.32	0.44	62.16	14.87
5	− 1 (75:25)	− 1 (6)	1 (Spa-14.1)	11.62	70.29	0.3	0.24	58.94	13.8
6	1 (100:00)	− 1 (6)	1 (Spa-14.1)	5.36	37.96	0.2	0.11	39.56	14.86
7	− 1 (75:25)	1 (14)	1 (Spa-14.1)	20.54	99.42	0.38	0.43	73.65	10.49
8	1 100:00	1 (14)	1 (Spa-14.1)	18.34	72.19	0.36	0.38	71.17	25.71

Proportion of mixing pretreated sugarcane bagasse hydrolysate and vinasse (SBH:V), yeast concentration (YBC) and yeast strain (S.pa)

[a]Sugars consumption (%)—percentage of initial glucose and xylose consumed

[b]Yp/s (g/g)—ethanol yield: correlation between ethanol produced and sugars (glucose and xylose) consumed

[c]Qp (g/l h)—ethanol productivity: ratio of ethanol concentration (g/l) and 48 h of fermentation time

[d]H (%)—fermentation efficiency: percentage of the maximum theoretical ethanol yield (0.51 g ethanol/g sugars)

[e]Grow rate (%)—percentage of cell grow

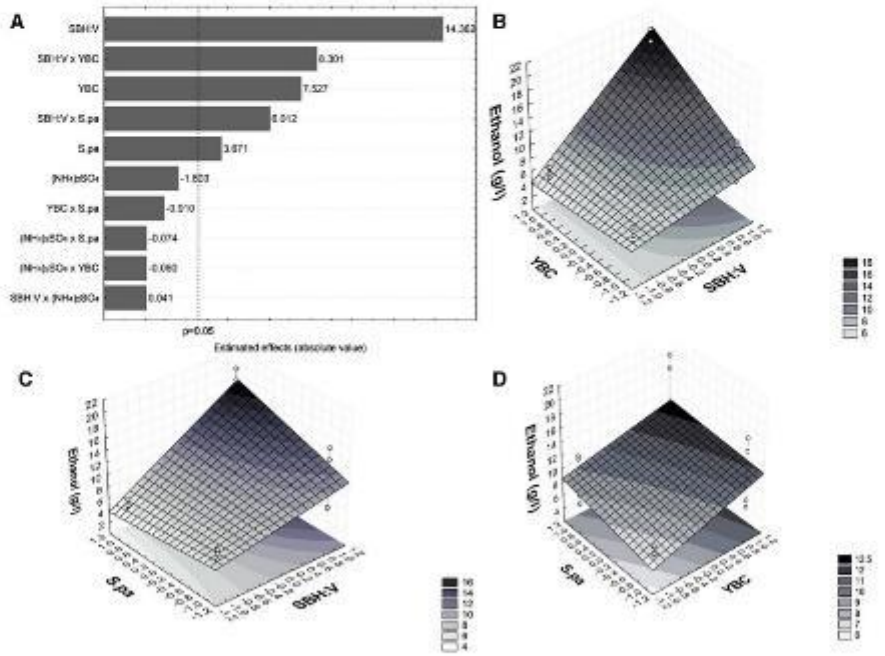

Fig. 1 Pareto's chart and response surfaces displaying the effect on ethanol production at 48 h in 2^3 factorial design; **a** Pareto's chart with all the effects obtained; **b** influence of sugarcane bagasse hydrolysate/vinasse (SBH:V) and yeast concentration (YBC) factors; **c** influence of sugarcane bagasse hydrolysate/vinasse (SBH:V) and yeast strain (S.pa) factors; **d** influence of yeast concentration (YBC) and yeast strain (S.pa)

fitted to a first-order model equation and examined for goodness of fit (Eq. 1).

$$\text{Ethanol production (g/l)} = 9.420 + 3.763X_1 - 0.419X_2 + 1.969X_3 + 0.960X_4 + 0.010X_1X_2 + 2.171X_1X_3 + 1.573X_1X_4 - 0.015X_2X_3 - 0.019X_2X_4 - 0.238X_3X_4 \quad (1)$$

where X_1 is the proportion of sugarcane bagasse hydrolysate/vinasse (SBH:V); X_2 is the ammonium sulfate concentration [$(NH_4)_2SO_4$]; X_3 is yeast concentration (YBC); and X_4 is the yeast strain (S.pa).

Equation 1 can be employed to further maximize the ethanol production, by increasing the number of levels of determinative variables and analyzing how the model responds. On an industrial scale, it is difficult for biomass concentration to surpass 14% (w/v WCW), and thus, it is only practicable to make the carbon source more available, i.e., by raising the proportion of hydrolysate to vinasse to, say, 80:20, 90:10 or even 100:00. Hence, by substituting values into Eq. 1 for coefficient X_1 (1.2, 1.6, and 2), representing increasing proportions of hydrolyzed sugarcane bagasse to vinasse (80:20, 90:10, and 100:00, respectively), the X_2 coefficient was substituted for the level − 1 variant [3 g/l $(NH_4)_2SO_4$]; and X_3 and X_4 for the level + 1 (14% w/v WWC and S.pa-14.1 strain, respectively); the model predicts that the level in ethanol production rises from 21.56 to 27.56 g/l, with the increased proportion of bagasse hydrolysate. This could be a direct consequence of the increase in the carbon source that is available in the hydrolysate. The ANOVA test indicated a good level of reliability between the experimental values and those predicted by the model, from which it can be concluded that the model is indeed predictive. The coefficient of determination (R^2) obtained was close to unity at 0.99, indicating that 99% of the variability in the data can be explained by the empirical equation that was established.

Some authors obtained ethanol production of 24.14 g/l in 72 h, using only bagasse hydrolysate without vinasse and strain S.pa-14.1, which represents a productivity of 0.34 g/l h (de Souza et al. 2018) lower than that presented in this study (0.43 g/l h). In a similar study using S.pa-1.3 and S.pa-14.1 strain in a D-xylose synthetic medium, 17.2 and 16.4 g/l ethanol were obtained, with yields of 0.35 and 0.37 g/g, respectively, for the two strains (Cadete et al. 2012). When taken together, these results suggest an improved fermentation performance by the *S.pa*-14.1 strain, under the conditions optimized for this study, when compared with previous studies related to 2G ethanol production.

The significance of the above mathematical prediction was validated by conducting, a second 2^3 factorial design. This involved modifying the ratio of 75% of sugarcane bagasse hydrolysate (SBH): 25% vinasse, (75:25) as the low-level (that showed the best results in a previous experiment), and 100% of SBH without vinasse (SBH:V 100:00) as the high-level variant (Table 2). However, only an increase in yeast cell biomass was significant for the production of ethanol (Fig. 2). The coefficient of determination (R^2) obtained, from ANOVA, was again close to unity, at $R^2 = 0.99$. Since the organic matter contained in vinasse is not fermentable, it was found that the vinasse supplementation was effectively diluting the carbon source. Thus, we would expect to see a decrease in ethanol production if the supplement were not beneficial to it. However, the parity of the results obtained in the second experiment strongly indicates that vinasse supplementation has a positive effect on ethanol production. Furthermore, this result demonstrates that vinasse supplementation benefits from a higher degree of fermentation efficiency. Vinasse is a residue rich in proteins; minerals such as nitrogen, phosphorus, potassium, calcium, and magnesium; organic matter (mainly sugars) and B-complex vitamins (de Oliveira et al. 2013). Some studies have shown the potential and viable use of vinasse as a supplement for *Candida* sp. biomass production (Tauk 1982; Silva et al. 2011), so it is reasonable to suggest that it has a positive nutritional influence on yeast metabolism, as well as upon the fermentation process.

In this second experiment, the increase in the carbon source to 100% SBH did not produce a significantly different amount of ethanol from the 75:25 ratio (Table 2). As

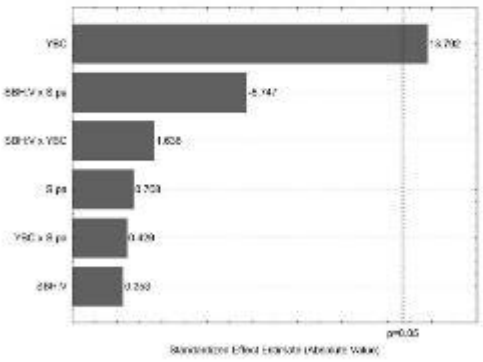

Fig. 2 Pareto's chart displaying the effect on ethanol production at 48 h in 2^3 factorial design [proportion of mixing pretreated sugarcane bagasse hydrolysate and vinasse (SBH:V), yeast concentration (YBC) and yeast strain (S.pa)]

explained above, this strongly suggests that fermentation medium supplementation with vinasse can increase the yield and productivity of ethanol through a positive nutritional influence that can overcome the problem of the dilution of the carbon source. In view of this, it should be possible to determine the exact optimal proportion of the carbon source-vinasse, so that it can maximize ethanol production. We intend to analyze other proportions of sugarcane bagasse hydrolysate/vinasse (e.g., 80:20 and 90:10) in a future factorial design. Another feasible option could be to complement the SBH solution with different amounts of dehydrated vinasse powder, to reduce the volume of water. This would mean that the detrimental effect of the carbon source dilution would be lessened. In this approach, it would be necessary to evaluate any possible adverse effects of the dehydration process on the viability of the vinasse, e.g., by heat damage to thermo-sensitive constituents such as B-complex vitamins. This is unlikely, however, since vinasse is already a by-product of a high-temperature distillation process.

Techno-economic studies are required to assess the feasibility and cost-benefits of introducing vinasse supplementation into the large-scale ethanol production process. Since there already exists a common practice of diluting high-gravity sugarcane juice with water in 1G ethanol production, it could be possible to use appropriate proportions of vinasse to supplement this water, or to supplement the SBH in the 2G ethanol production.

Another significant variable that determined was the cell biomass concentration in the inoculum. However, it is not feasible to increase this in practice, because, as mentioned earlier, it is difficult to reach levels above 14% w/v cell wet weight on an industrial scale (Basso et al. 2008). Finally, the yeast strain *S. passalidarum* UFMG-HMD-14.1 displayed a promising fermentative performance.

Conclusion

The results of this study demonstrate that supplementation during the fermentation process with vinasse benefits the consumption of the carbon source and hence increases the yield and ethanol production.

As expected, the results help to confirm 14% w/v cell wet weight as an optimal inoculum biomass concentration for boosting ethanol production while simultaneously advocating the use of yeast strain *S. passalidarum* UFMG-HMD-14.1.

The novel reuse strategy outlined here for increasing ethanol production by means of vinasse, an agro-industrial residue, could thus be considered to be the means of achieving a similar productivity rate to the traditional process, as well as having the added benefit of encouraging a more correct treatment of industrial waste.

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REVIEW

Production and Application of Lignin-Based Chemicals and Materials in the Cellulosic Ethanol Production: An Overview on Lignin Closed-Loop Biorefinery Approaches

Carlos Eduardo de Araújo Padilha[1,2] · Cleitiane da Costa Nogueira[1] · Bárbara Ribeiro Alves Alencar[3] · Íthalo Barbosa Silva de Abreu[3] · Emmanuel Damilano Dutra[3] · Juan Alberto Chavez Ruiz[4] · Domingos Fabiano de Santana Souza[2] · Everaldo Silvino dos Santos[1]

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Abstract
Lignocellulosic biomass is the most abundant biological resource on the planet and has been extensively researched to produce cellulosic ethanol. However, there is a consensus that the presence of lignin hinders the biomass conversion. Lignin is often considered a villain in cellulosic ethanol production studies due to its adverse effects on cellulases and yeasts. Despite this, recent studies indicate that lignins can be transformed into useful inputs to produce cellulosic ethanol. These approaches aim to establish closed-loop biorefineries to improve economic metrics and reduce the environmental impact due to the substitution of products based on fossil sources. The present review addresses the successful cases in transforming lignin into chemicals and materials to increase cellulosic ethanol titers. A contextualization was first carried out, considering aspects of biomass characteristics and lignin valorization. The impact of lignin-based chemicals and materials in the pretreatment, detoxification, and enzymatic hydrolysis steps was discussed in detail. Economic aspects and future perspectives were also included in this review. These reports open a new point of view on lignin valorization and its integration with the cellulosic ethanol production chain.

✉ Carlos Eduardo de Araújo Padilha
carlospadilha.eq@gmail.com

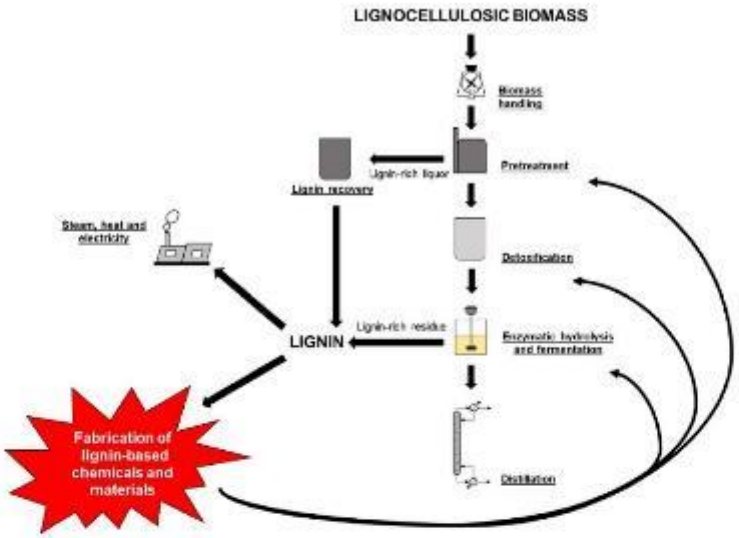
[1] Laboratory of Biochemical Engineering, Chemical Engineering Department, Federal University of Rio Grande Do Norte (UFRN), Natal, RN 59078-970, Brazil

[2] Laboratory of Alternative Energy and Transport Phenomena, Chemical Engineering Department, Federal University of Rio Grande Do Norte (UFRN), Natal, RN 59078-970, Brazil

[3] Research Group On Biomass Energy, Department of Nuclear Energy, Federal University of Pernambuco (UFPE), Recife, PE, Brazil

[4] Laboratory of Sustainability, Senai Institute of Innovation in Renewable Energy, Av. Capitão Mor Gouveia, Natal, RN 59063-400, Brazil

Graphic abstract



Keywords Valorization · Enzymatic hydrolysis · Surfactant · Ionic liquid · Activated carbon

Abbreviations

AC	Activated carbon
A-LMS	Amino-modified lignin microspheres
[*p*-AnisEt$_2$NH][H_2PO_4]	P-anisaldehyde-2-ylmethyl) ethanamine, H_3PO_4 salt
BSG	Brewer's spent grain
CELF	Co-solvent enhanced lignocellulosic fractionation
ChCl	Choline Chloride
CHPTAC	3-Chloro-2-hydroxypropyl trimethylammonium chloride
DES	Deep eutectic solvent
[EMIM][OAc]	1-Ethyl-3-methylimidazolium acetate
EHL	Enzymatic hydrolysis lignin
[FurEt$_2$NH][H_2PO_4]	Furan-2-ylmethyl)ethanamine, H_3PO_4 salt
FPU	Filter paper unit
GCF	Green coconut fiber
GVL	γ-Valerolactone
HBD	Hydrogen bond donor
HHV	Higher heating value
HMF	Hydroxymethylfurfural
HRCA	Herb residues of *Cortex albiziae*
IL	Ionic liquid
KL-PEG	Kraft lignin-polyethylene glycol
LPMO	Lytic polysaccharide monooxygenases
MESP	Minimum ethanol selling price
PB	*p*-Hydroxybenzoic acid
PCA	*p*-Coumaryl alcohol ether
PEG	Polyethylene glycol
PHA	4-Hydroxybenzaldehyde
SEM	Scanning electron microscopy
SScF	Simultaneous saccharification and co-fermentation
[VanEt$_2$NH][H_2PO_4]	Vanillin-2-ylmethyl)ethanamine, H_3PO_4 salt
VOC	Volatile organic compounds
U	Unit of enzyme activity

Statement of Novelty

The replacement of fossil fuels with cellulosic ethanol is in line with the perspective of a more sustainable future, but this technology still suffers from difficulties for its consolidation. Lignin limits cellulosic ethanol titers due to the effects of non-productive adsorption of cellulases and

fermentation inhibition, and, therefore, it is often removed through physicochemical pretreatments. Despite this, recent studies have shown that lignin-based chemicals and materials could improve cellulosic ethanol production. Although these studies propose an unusual closed-loop biorefinery, the use of lignin as input for cellulosic ethanol production offers an opportunity to mitigate commercial chemicals' acquisition and increase the profits from the process.

Introduction

Fossil fuels are still fundamental for maintaining human activities, but there is a search for more sustainable and less aggressive alternatives to the environment [1, 2]. Since the last decade of the twentieth century, extensive studies have been carried out to consolidate the production of plant-based fuels, such as cellulosic ethanol [3]. Cellulosic ethanol, or second-generation bioethanol, is a liquid fuel obtained from the transformation of carbohydrates from the lignocellulosic matrix (cellulose and hemicellulose) into monosaccharides, which are subsequently fermented by microorganisms [2, 4]. In the production of cellulosic ethanol, pretreatment steps are crucial to reducing the biomass recalcitrance and, consequently, increase its enzymatic digestibility [5, 6]. The use of pretreatments focusing on delignification has become popular in cellulosic ethanol schemes since the high lignin content limits accessibility to cellulose fibers and can lead to non-productive adsorption of cellulases [7–11]. After the delignification, the pretreated lignocellulosic biomasses are directed to the enzymatic hydrolysis followed by fermentation, while the lignin from the liquid fraction is considered a cheap source of energy [12]. Solid residues from enzymatic hydrolysis, which are lignin-rich materials, are also directed towards the cogeneration of heat and electricity [13]. However, economic viability remains a bottleneck for the cellulosic ethanol schemes since it is a low value-added product [13, 14].

The improvement of lignin processing has been identified as a critical factor to guarantee the economic viability of biorefineries of lignocellulosic biomasses since it will be possible to convert this biopolymer into value-added products [13, 15, 16]. Lignin-based products can be applied in different fields of science, including the cellulosic ethanol scheme itself. This idea is at first contradictory due to the known adverse effects of lignin on the performance of enzymes and yeasts. The enzymatic digestibility of biomass is generally an inverse function of the lignin content since the concentration of free enzymes is reduced by the phenomenon of non-productive adsorption [17, 18]. Phenolic compounds derived from lignin can inhibit the catalytic activity of cellulases as well as cause cell disruption of ethanol-producing strains [8, 9, 19, 20]. Despite this, recent studies have shown that lignin can be converted into inputs with a fundamental role in producing cellulosic ethanol. They suggest establishing a closed-loop biorefinery concept, in which isolated lignins become raw materials for the preparation of chemicals and materials to be included in the pretreatment, detoxification, or enzymatic hydrolysis steps. The addition of lignin-based inputs aims not only to increase ethanol titers but also to reduce the generation of waste and minimize the acquisition of external products.

Thus, the present study addresses lignin valorization questions and the use of lignin-based chemicals and materials to produce cellulosic ethanol. Section 2 will deal with the structure and recalcitrance of the lignocellulosic biomass and the need for pretreatments. Section 3 will address the conventional and alternative ways to add value to lignin in different industrial sectors. Section 4 will deal with studies that propose cellulosic ethanol production in the presence of lignin-based chemicals and materials. Sub-sections were defined to delimit the success cases and economic aspects of the use of lignin in the pretreatment, detoxification, and enzymatic hydrolysis steps (in order). Also, insights and future perspectives were commented on in Sect. 5.

Lignocellulosic Biomass

Lignocellulosic biomass is formed mainly by cellulose, hemicellulose, and lignin, assembled in a recalcitrant structure with multiple layers [14–20]. Cellulose ($(C_6H_{10}O_5)_c$) is a linear polysaccharide formed by glucose units linked together by β-1,4 glycosidic bonds. Cellulose molecules are associated with others by intermolecular forces (hydrogen bonds and Van der Waals forces) in cellulose fibers, which explains the fact that cellulose is insoluble in water and conventional organic solvents [21] (see Fig. 1). Hemicellulose ($(C_5H_8O_4)_c$) is a branched polysaccharide formed by pentoses (xylose, arabinose), hexoses (glucose, mannose, and rhamnose), and uronic groups (glucuronic acid and methyl-glucuronic acid) [9]. The composition of hemicellulose is random, being strongly dependent on the source of lignocellulosic biomass. Hemicellulose from hardwoods and agro-industrial residues is made up of xylan, while glucomannans make up the most considerable fraction of softwood hemicellulose [22]. Unlike cellulose, hemicellulose is mostly amorphous and generally has a low molecular weight, making this polysaccharide susceptible to acid/enzymatic hydrolysis [3, 21]. According to Isikgor and Becer [23], hemicellulose can also be considered a crosslinking agent between cellulose and lignin. Lignin is an aromatic biopolymer formed by the coupling of monolignol radicals and phenolic radicals [24]. Lignin consists of syringyl (S-unit), guaiacyl (G-unit), and hydroxyphenyl (H-unit) units, which are linked by C–C and C-O bonds, including

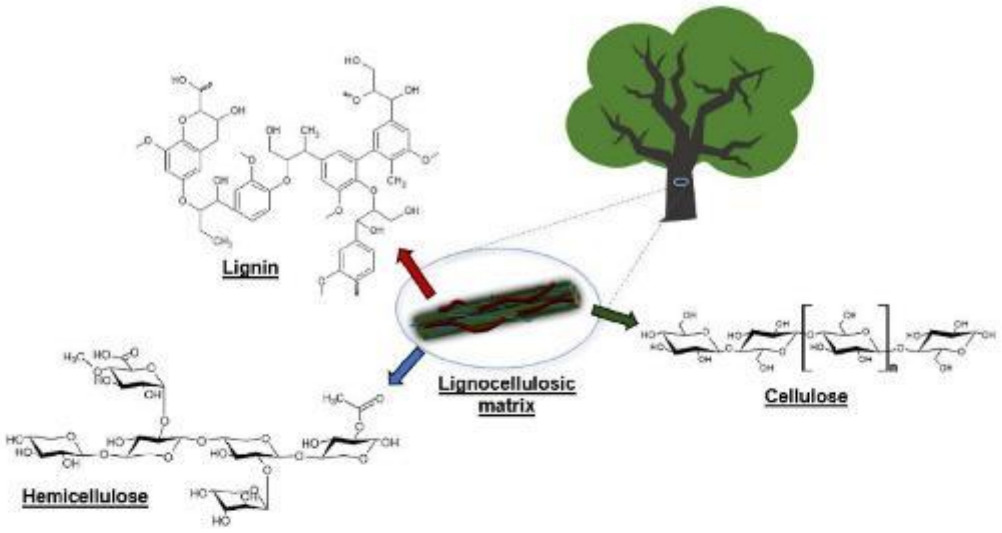


Fig. 1 Simplified description of the lignocellulosic matrix

β-O-4, β-β, β-5, β-1, 5–5 and 4-O-5 bonds [25]. The lignin content is commonly attributed to the biomass recalcitrance since it provides rigidity and protection against biotic and abiotic stresses [25, 26].

Conventional strategies for cellulosic ethanol production aim to depolymerize cellulose into glucose, an easily metabolizable molecule, by enzymatic route or chemical route (using inorganic or acid salts) [27, 28]. It is noteworthy that several engineered strains can effectively convert pentoses into ethanol [29]. Due to less specificity for cellulose, more significant environmental impact, and the occurrence of corrosion in reactors, processes based on the acid route have been replaced by enzyme technology [30]. Cellulases, xylanases, β-glucosidases, and accessory enzymes are some of the enzymes used to digest the lignocellulosic matrix [2, 6]; however, the presence of lignin is an obstacle to the success of the process. According to Börjesson et al. [31], lignin can cause non-productive adsorption of cellulases, mainly due to the hydrophobic interaction between the aromatic lignin structures carbohydrate-binding module (CBM). CBM is responsible for adhering, guiding, and increasing the concentration of cellulases on cellulose, and it is a hydrophobic protein portion of endoglucanases and cellubiohydrolases. Lignin can limit the free movement of cellulases on cellulose fibers during enzymatic hydrolysis of lignocellulosic biomass, as shown in Fig. 2. Xylanases and β-glycosidases are less susceptible to non-productive adsorption onto lignin [32, 33]. Rahikainen et al. [34] and Silva et al. [35] suggest that electrostatic interactions and hydrogen bonds may also contribute to the non-productive adsorption of cellulase onto lignin due to the phenolic hydroxyl groups. This phenomenon limits the concentration of free enzymes within the liquid phase, which leads to a lower amount of sugars after enzymatic hydrolysis. According to Tejirian and Xu [36], phenolic compounds derived from lignin can form complexes with cellulases, inhibiting the enzyme catalytic activity. Therefore, methods to solve lignin problems must be implemented, and one of the most classic options is the pretreatment of lignocellulosic biomass.

Pretreatments consist of processes that disorganize the lignocellulosic matrix through the solubilization of hemicellulose and lignin, cellulose depolymerization, and comminution [38]. Physical (milling, microwave, and sonification), chemical (dilute acid, alkaline, oxidation, and organosolv), physical–chemical (steam explosion, supercritical technologies, ammonia fiber expansion), and biological (fungi and bacterial) pretreatments can be used in cellulosic ethanol production [39]. However, some factors must be taken into account before choosing a pretreatment. The final destination of the lignocellulosic matrix components is decisive for the choice of pretreatment; however, other factors are also important, such as the cost of pretreatment, the formation of fermentation inhibitors, and the environmental impact [39]. For example, fermentation inhibitors accumulate in the pretreatment liquid fraction and can become impregnated with pretreated biomass. Therefore, to improve

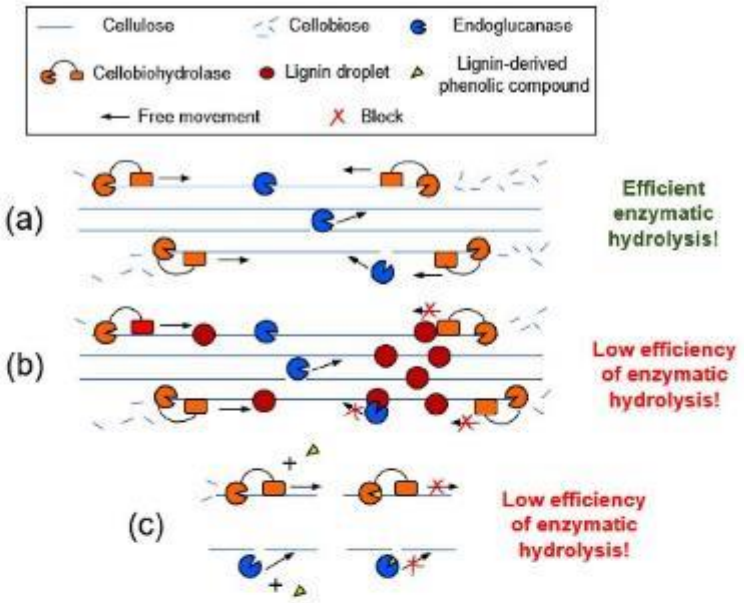


Fig. 2 Impact of lignin on enzymatic hydrolysis of lignocellulosic biomass. **a** Efficient enzymatic hydrolysis in a lignin-free environment; **b** Low efficiency of enzymatic hydrolysis due to the non-productive adsorption of cellulases onto lignin; **c** Low efficiency of enzymatic hydrolysis due to inhibition by phenolic compounds derived from lignin. [37] Adapted from Saini et al.

the fermentation performance, detoxification methods are recommended, such as the addition of adsorbent (or other chemical agents) and the washing of pretreated biomass. Studies of cellulosic ethanol production conventionally seek to enrich the cellulose content in the pretreated lignocellulosic biomass [10, 17]. Hydrothermal pretreatments are used to solubilize hemicellulose from lignocellulosic biomass and can be found in the form of liquid hot water pretreatments, thermolysis, subcritical water treatment, steam explosion, or autohydrolysis. They are commonly operated under high temperatures (> 150 °C) to allow the autoionization of water and, consequently, the release of acetate groups from hemicellulose [40]. The glycosidic bonds of hemicellulose are easily broken in the hydrothermal pretreatment, which leads to the formation of oligomers and monomers (such as xylose and arabinose). As a result, the pretreated biomass shows a proportional increase in cellulose and lignin. Lignins are also fragmented during hydrothermal pretreatment, but they repolymerize and reorganize onto pretreated biomass (see details in Sect. 4.1.2). In addition to changing the chemical composition, hydrothermal pretreatments lead to increased crystallinity (due to the removal of amorphous components), increased porosity, and increased wettability [40]. All of these factors contribute to the increase in enzymatic digestibility, and, therefore, hydrothermal pretreatment should be considered a potential candidate for inclusion in biorefineries of lignocellulosic biomass.

On the other hand, cellulose enrichment can be achieved by using pretreatments focusing on lignin removal. Masarin et al. [41] and Siqueira et al. [42] reported that the generation of fermentable sugars in enzymatic hydrolysis is inversely proportional to the lignin content. Due to their simplicity and effectiveness, alkaline and alkaline oxidative pretreatments are commonly used to delignify the lignocellulosic matrix and increase its enzymatic digestibility [43]. The delignification promoted by the use of sodium hydroxide or other bases occurs due to the cleavage of ester bonds between lignin and polysaccharides and the cleavage of internal lignin ether bonds. The addition of hydrogen peroxide is commonly performed in alkaline pretreatments since reactive oxygen species generated can cleave C–C bonds of the biomass components [44]. In addition to removing amorphous components, oxidative alkaline pretreatments also reduce the degree of cellulose polymerization and improve the water retention capacity [45]. Recent studies have shown great interest in processes involving organic solvents to remove lignin from biomass, which are called organosolv pretreatments. Organosolv pretreatments can use as different types of solvents, such as alcohols, short-chain organic acids, organic peracids, tetrahydrofuran (in the method entitled co-solvent enhanced lignocellulosic fractionation, CELF) and γ-valerolactone (GVL) [15, 46]. Also, delignification methods can involve ionic liquids (IL) and deep eutectic solvents (DES). Acidic ionic liquids can promote biomass fractionation, allowing high lignin yield

in the liquid fraction [47–49]. Moniruzzaman and Ono [50] proposed the use of 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) to maximize the delignification of lignocellulosic biomass using laccases. Kumar et al. [51] and Lim et al. [52] reported the success of acidic and alkaline DES in delignifying agro-industrial residue under mild temperature conditions. Proposals for combining hydrothermal pretreatments and delignificant pretreatments have also shown remarkable results in fractionation and enzymatic hydrolysis. Ruiz et al. [40] highlighted that the sequence of hydrothermal/alkaline or hydrothermal/organosolv pretreatments allows the generation of multiple products, including sugars, bioactive compounds, phenols, and lignin. In Gonçalves et al. [53], biomasses obtained from the sequenced pretreatments sodium chlorite-acetic acid/autohydrolysis showed a higher initial hydrolysis rate (up to 40% higher) and a higher sugar release (up to 6.5% higher) than pretreated biomasses only by sodium chlorite-acetic acid. Matsakas et al. [54] proposed a hybrid pretreatment strategy based on the combination of the steam explosion pretreatment and organosolv pretreatment with ethanol. The authors reported that this pretreatment increased the yield of sugars in enzymatic hydrolysis up to 61% (w/w) in an environment with high solid loadings. More data about delignification and enzyme digestibility can be found in the specialized literature, but this is not the focus of the present study.

It is undeniable that the consolidation of cellulosic ethanol production can bring many advantages to society and the environment in a future low-carbon economy [14]. However, it is essential to highlight that this scheme still faces serious challenges to ensure competitive prices with the first generation technology [55, 56]. The costs for enzyme production/concentration have a considerable impact on economic viability metrics. Despite this, Liu et al. [57] reported that the minimum ethanol selling price (MESP) values remained high even though the enzyme cost was zero. Other strategies (such as energy integration between first and second technology) have also been investigated to improve the economic indicators for cellulosic ethanol schemes [56, 58–60]; however, the results obtained were unsatisfactory or inconclusive. A potential way to get extra income in the cellulosic ethanol scheme comes from lignin processing. Knowledge of lignin's historical use and the effects of pretreatments on the biopolymer structure is necessary to improve the lignin valorization paths. It is noteworthy that the isolation of lignins from the liquid fraction is a simple procedure. Lignins obtained from alkaline pretreatments can be recovered from acidification of the medium to pH 2.0–3.0 due to the protonation of phenolic hydroxyl groups [10]. In the organosolv, IL, and DES pretreatments, the solvents are responsible for the lignin solubilization, being subsequently evaporated from the liquid phase or diluted with water to recover the biopolymer [46].

Lignin Valorization

Two technical lignins stand out in the lignin production scenario, both from the pulp and paper industry: lignosulfonate and Kraft lignin. Lignosulfonate is obtained by the reactions of sulfonation and hydrolysis during the sulfite process. Lignosulfonate has a negative charge density, high water solubility, high molecular weight, and high sulfur content (around 3.5–8.0% w/w) [61]. The lignosulfonate applications include dye dispersant, metal chelating agent, and water reducer in cement [61, 62]. Kraft pulping is the primary method for obtaining high-quality pulp in industries and represents 85% of the total lignin. Kraft pulping consists of treating wood feedstock using sodium hydroxide and sodium sulfide solutions at high temperatures (~ 170 °C), promoting the cleavage of β-O-4 bonds and introducing hydrophilic groups [63]. Like lignosulfonate, Kraft lignin is burned in a recovery boiler to produce energy and recover cooking chemicals [25]. Kraft lignins also have a specific commercial appeal and can be used in fertilizers, binders, and resins [64, 65]. Due to the greater volume generated in comparison with lignosulfonate, the authors also suggest changes in Kraft lignin structure via sulfonation and sulfomethylation reactions to use it as a dispersant [61].

The structural complexity of lignins is even more evident when we deal with lignins from cellulosic ethanol schemes due to the variety of pretreatments and lignin sources, which can be obtained from hardwood, softwood, and agro-industrial residue [25]. Despite this, it is evident that these lignins have characteristics closer to Kraft lignins than lignosulfonates. Lignins isolated from alkaline and alkaline oxidative pretreatments not only have low molecular weight (~ 1–3 kDa) but are also highly condensed (high content of C–C bonds) and have a high content of phenolic hydroxyl groups [66]. Gonçalves et al. [67] studied the isolation of lignins from the liquid fraction of the mature coconut alkaline pretreatment and observed the presence of hydroxycinnamates (such as p-coumarate and ferulate) in the lignin samples. Alkaline oxidative pretreatments can cause additional effects, such as aromatic rings' cleavage to form carboxylate groups [18]. In the literature, it is possible to find several mentions about organosolv pretreatment to obtain high-quality lignins; however, this statement is not necessarily correct. Organosolv pretreatments catalyzed with mineral acids and pretreatments involving a low concentration of organic solvents and high temperatures lead to the generation of highly condensed lignin fractions compared to native lignins [15, 18]. Eventually, organosolv pretreatments under high concentrations of low molecular weight alcohols or high concentrations of organic acids ensure the maintenance of β-O-4 bonds due to alkoxylation and esterification in α-carbon, respectively [25]. This characteristic was observed

by Romaní et al. [68]. They reported that organosolv lignins isolated from pretreatment of *Eucalyptus* wood had lower content of hydroxyl groups than lignins extracted from alkaline pretreatment; this probably occurred due to the stabilization of β-O-4 motifs. The effects caused by pretreatments with ionic liquids are similar to organosolv pretreatments. For example, George et al. [69] observed the lignin depolymerization during pretreatments with ionic liquids and determined that this phenomenon is dependent on the type of anion (sulfates > lactates > acetates > chlorides > phosphates), which acts as a nucleophile. In turn, Kim et al. [70] reported that isolated poplar lignin via pretreatment based on 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) has similar content of β-O-4 bonds to milled wood lignin (lignin without commercial interest, but with a high content of β-O-4 bonds). Pretreatments with DES based on organic acids (acetic acid, levulinic acid, and lactic acid) are effective in isolating lignins but lead to partial or total degradation of β-O-4 bonds, as reported by Alvarez-Vasco et al. [71], Lyu et al. [72], and Chen et al. [73]. It is important to highlight an interest in applying useful biorefinery concepts to convert plant biomass, including multiple outlet systems. Despite this, the existing industrial plants mainly focus on cellulosic ethanol production, while the extracted lignin is underutilized [13]. Like sulfur-containing technical lignins, lignins obtained from cellulosic ethanol schemes are generally used as an energy source for the cogeneration of steam, heat, and electricity [60]. This application is also valid for post-enzymatic hydrolysis residues since it is a lignin-rich product but has polysaccharide contamination. Lignins can have higher heating values (HHV) between 20.0–30.0 MJ/kg, while cellulose and hemicellulose have HHV of only 18.6 MJ/kg [74, 75]. The burning of lignins/enzymatic hydrolysis residues enables the energy self-sufficiency of cellulosic ethanol plants, which is achieved by processing approximately 34–40% of the amount generated of dry lignin [13]. According to Hodásová et al. [76], the commercialization of lignin does not allow a substantial financial return since the price of lignins can reach up to $750.0/T for high-quality organosolv lignins. Enzymatic hydrolysis residues have low-purity lignin and, therefore, the price range of these products varies between $50.0 and $280.0/T. The sale of surplus electricity to the local grid also does not solve biorefineries' economic viability producing cellulosic ethanol. The structural complexity of lignins has always been considered a significant challenge for its wide use, but recent efforts to enhance this biopolymer are notorious.

A series of studies show that lignins are potential raw materials for the manufacture of healthcare products. In vitro studies show that lignins have remarkable antioxidant activity, as reported by Amizanzadeh et al. [77], Gordobil et al. [78], and Nogueira et al. [8]. The availability of phenolic hydroxyl groups in the lignin structure has been linked to free radicals' scavenging. Gordobil et al. [78] reported that Kraft and organosolv lignins obtained from spruce and *Eucalyptus* wood have high antimicrobial activity against pathogenic microorganisms *Escherichia coli*, *Salmonella typhimurium*, and *Aspergillus niger*. The addition of lignins in cosmetics can provide additional protection against ultraviolet radiation, as can be seen in Zhang et al. [79], Padilha et al. [18], and Widsten et al. [80]. The authors indicate that this fact can be attributed to the excellent availability of aromatic groups in lignin; however, they highlight the need to attenuate the color of lignin-based blends by cleaving quinone groups or blocking phenolic hydroxyl groups via acetylation. Due to the low solubility in water, lignins (except lignosulfonate) can be transformed into nanoparticles by diluting lignin solutions or adjusting pH (changing from alkaline to acidic pH). The preparation of nanoparticles occurs due to the self-assembly of lignin molecules due to aromatic moieties [81]. Lignin nanoparticles have high absorption retention, high biodegradability, and low toxicity and, therefore, are considered successful carriers of bioactive substances [82]. Lignin nanoparticles are capable of causing the controlled release of bioactive substances retained inside the nanoparticle or via the Pickering emulsion [81–84]. Dai et al. [85] reported that magnetic resveratrol loaded lignin nanoparticles showed efficient controlled release of the bioactive and reduced tumor size in mice.

The manufacture of materials of industrial importance from lignins has been extensively studied in the literature. Lignin is a thermoplastic material, and it has compatible functional groups (hydroxyl and carboxylate) with other polymers. Changes in the lignin structure are sometimes recommended by polymer grafting, alkylation, phenolation, esterification, among others [86, 87]. The incorporation of lignin in polymeric matrices has resulted in significant mechanical and thermal properties [88]. Gordobil et al. [89] and Lazzari et al. [90] reported that the addition of lignins increased Young's modulus values in polymeric composites based on poly(lactic acid) and polyethylene terephthalate, respectively. The use of lignins as a filler improved the viscoelastic properties of rubbers, as reported by Bahl et al. [91], Barana et al. [92], and Mohamad Aini et al. [93]. In addition to the antioxidant activity and protection from ultraviolet radiation, lignins' addition reduces the flammability of polymeric matrices, as reported by Zhang et al. [94] and Chen et al. [95]. Due to their high carbon content, lignins can be used to prepare carbon-based materials, such as activated carbon and carbon fibers [88].

Lignins are also considered a prominent source of aromatic monomers [25]. The choice of lignin isolation methods is a critical factor in this route of lignin valorization. The occurrence of lignin condensation reactions during severe pretreatments leads to a higher C–C bonds content, which are more resistant to catalysis [96, 97]. According to Feghali

et al. [98], the theoretical yield of monomers is proportional to the square of the content of ether bonds (C–O). In the case of Kraft lignin, the situation is more complicated since the presence of sulfur poisons noble metal catalysts and, consequently, limits the process of lignin depolymerization [99]. There are several ways to depolymerize isolated lignins, including reductive processes, oxidative processes, acid/base-catalyzed processes, and thermal and solvolytic processes. In addition to the different operating conditions used, each method involves different results of oil yield, monomer yield, and selectivity [25]. Particularly, advances have been reported regarding the conversion of isolated lignins to water-soluble monomers using hydrogenolysis [100–104], oxidation [105–109], and supercritical technology [110–113]. The production of lignin monomers can also be carried out through reductive catalytic fractionation (RCF). Unlike conventional lignocellulosic biomass processing approaches, RCF prioritizes lignin valorization over the use of cellulose and hemicellulose, which is commonly called the lignin-first biorefinery approach [114]. RCF is the combination of delignification, stabilization, and depolymerization by active redox catalysts (such as Pd/C), whose final product is a stable oil rich in monomers, dimers, and low molecular weight oligomers. It is preferably operated in batch reactors under high pressures and temperatures in the range of 180–250 °C [114], but researchers have already pointed out that other conditions can also be used. Qiu et al. [115] suggest that the addition of catalysts should be carried out before the reactor temperature reaches 200 °C to avoid lignin repolymerization problems. To simplify the execution of the RCF and mitigate its capital costs, Ren et al. proposed the operation of the RCF under ambient pressure, which was called atmospheric RCF (ARCF) [116]. Despite the proposed approach achieving a 25% lower yield than conventional RCF, ARCF obtained bio-oil rich in propylguaiacol and propylsyringyl (more than 95% of lignin monomers) using ethylene glycol as a solvent and in the absence of external hydrogen source.

Lignin-Based Chemicals and Materials to Increase Cellulosic Ethanol Production

The application field for lignin-based products is vast. The versatility of lignin is such that it can return to the cellulosic ethanol scheme as an input. The number of publications on cellulosic ethanol involving lignin-based materials and chemicals has increased in recent years due to the strengthening of research on lignocellulosic biomasses and a better understanding of the lignin structure. These reports are unusual because lignins are often seen as an obstacle to enzymatic digestibility and sugar fermentation. Interestingly, the approach of inserting lignin-based products into the cellulosic ethanol scheme is somewhat similar to the production of cellulolytic enzymes, which can occur in a plant integrated with the biofuel plant. Thus, this approach contributes to the consolidation of cellulosic ethanol biorefineries by converting lignin into value-added products and improving cellulosic ethanol titers. Advances in the approach will be discussed in more detail in the following topics.

Application of Lignin-Based Chemicals on the Pretreatment of Lignocellulose

Lignin-Based Ionic Liquid and DES

Although delignification has been extensively reported as a favorable event for the enzymatic hydrolysis of lignocellulosic biomasses, the recovered lignin can return to the cellulosic ethanol scheme as chemicals for pretreatments. The monomers that make up this macromolecule can be reused for formulations of bio-ionic liquids (bio-ILs) and DES.

ILs represent a class of organic salts with a melting point below the boiling point of water, which are formed by organic cations and inorganic/organic anions [117]. ILs' properties can be easily modulated from changes in cations and anions, including melting point, viscosity, density, solubility, hydrophobicity, among others. ILs are attractive choices in different applications such as catalysis, biocatalysis, synthetic chemistry, and electrochemistry [118]. In pretreatments, IL based on imidazolium cation has received much attention due to its ability to remove lignin and hemicellulose [119, 120] but its synthesis is still linked to reagents derived from fossil sources [121]. In this scenario, studies have developed varieties of ILs with improved compatibility, which have been called bio-ILs. Bio-ILs are biodegradable substances and composed only of reagents obtained in nature [122]; in our case, using phenolic compounds as cation substitutes (after chemical modification). Figure 3 shows some examples of bio-ILs prepared from phenolic compounds obtained from lignin.

DES are also solvents with modulating properties but have some advantages compared to ILs, i.e., low toxicity, more eco-friendly, and lower preparation cost [123]. DESs are formed by mixing a hydrogen bond acceptor (urea, choline chloride, and betaine) and a hydrogen bond donor (alcohols, acids, amines, carbohydrates, among others) so that the eutectic point of the mixture is lower than the eutectic point of the isolated substances [124]. This fact occurs due to the strong hydrogen bonding and Van der Waals forces established between the acceptor's halogen anion and the acid hydrogens of the hydroxyl groups of the hydrogen donor compound [125]. The properties of DES are dependent on the selection of the acceptor: donor pair and the proportion

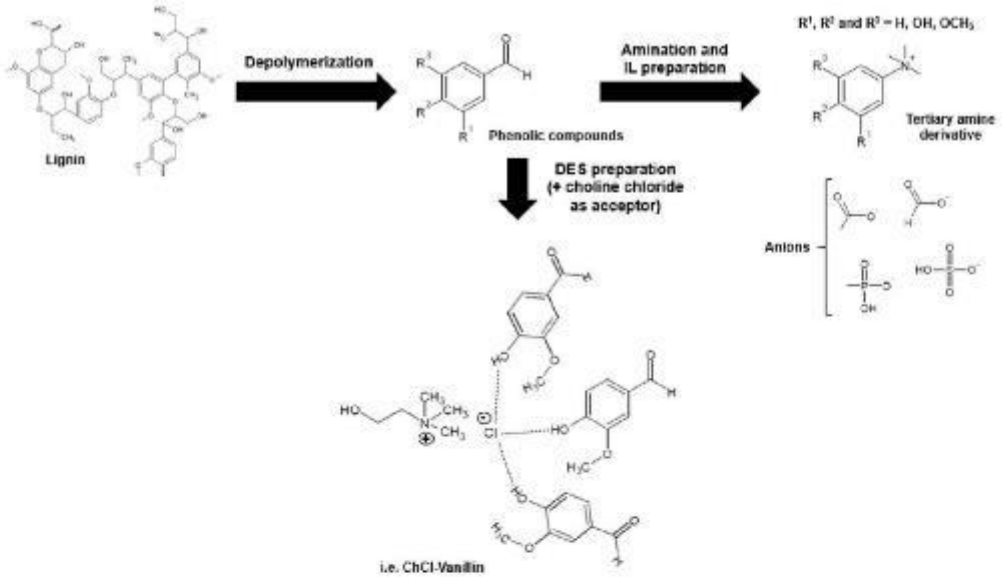


Fig. 3 Synthesis of lignin-based bio-IL and DES

of them, which strongly impact the pretreatment performance. According to Xia et al. [126] and Xu et al. [127], DES can fractionate lignocellulosic biomass from the dissociation of C–C bonds and constituent aryl ether bonds. Figure 3 illustrates the displacement of charges during the preparation of DES from phenolic compounds as a hydrogen donor.

It is essential to point out that phenolic compounds are inhibitory to enzymatic hydrolysis and fermentation processes, so the material must be washed to remove bio-IL or DES. The use of these substances is an alternative to achieve a closed-loop biorefinery. A summary of studies involving pretreatments with bio-ILs and lignin-based DES is shown in Table 1.

Socha et al. [121] proposed an innovative method of preparing ILs from lignin and hemicellulose derivatives for use in the pretreatment of lignocellulosic biomass. The reagents used by the authors include furfural (derived from hemicellulose) and vanillin, and *p*-anisaldehyde (derived from lignin). Through reductive amination, tertiary amine-based ILs were produced ($[FurEt_2NH][H_2PO_4]$, $[VanEt_2NH][H_2PO_4]$, and $[p\text{-}AnisEt_2NH][H_2PO_4]$), which were used in the pretreatment of switchgrass under 160 °C for 3 h. Although cellulose crystallinity was not affected, the use of bio-IL has shown promising results. Pretreatments with $[p\text{-}AnisEt_2NH][H_2PO_4]$ led to the delignification of 51.4% of xylan and 43.0% of switchgrass lignin, being similar to the results of pretreatment with [EMIM][OAc], a commercial IL. The materials pretreated with $[FurEt_2NH][H_2PO_4]$ and $[p\text{-}AnisEt_2NH][H_2PO_4]$ showed good enzymatic digestibility after 24 h of the process, being possible to achieve > 80% glucose recovery and > 60% xylose recovery.

Diez et al. [128] proposed switchgrass pretreatments using quaternary ammonium bio-ILs from phenolic compounds derived from lignin: benzaldehyde, *p*-anisaldehyde, vanillin, and syringaldehyde. The authors used pretreatments with [EMIM][OAc], a commercial IL, to compare bio-IL pretreatments' performance. As reported by Socha et al. [121], this pretreatment promotes a severe delignification, but the dissolution of cellulose is not significant. The cellulose dissolution varied between 3 and 10% (w/w) with the pretreatments involving bio-ILs. The bio-IL prepared from vanillin reached 70% delignification, being comparable with the results obtained from the pretreatment of [EMIM][OAc] (71%). Except bio-IL prepared from syringaldehyde, the other proposed bio-ILs achieved high sugar recovery (70% for glucose and 60% for xylose). Cellulosic conversion value greater than 90% was obtained in enzymatic hydrolysis experiments using vanillin-based IL pretreated switchgrass after 48 h of process.

Lignin-derived aldehydes and phenols have been used as hydrogen bond donors (HBD) in studies of the manufacture of lignin-based DES. Kim et al. [129] proposed different DESs from choline chloride (ChCl) as an acceptor, while

Table 1 Summary of pretreatment studies of bio-ILs and lignin-based DESs

	Chemicals	Lignocellulosic biomass	Operational condition	Pretreatment performance	Reference
Bio-IL	FurEt$_3$NH][H$_2$PO$_4$], [VanEt$_3$NH][H$_2$PO$_4$], and [*p*-AnisEt$_3$NH][H$_2$PO$_4$]	Switchgrass	10% (w/v) solid loading, 160 °C, 3 h	44.8% xylan removal and 52.4% lignin removal (using [EMIM][OAc]; control) 33.9–51.4% xylan removal and 3.9–43.0% lignin removal	[121]
	Quaternary ammonium bio-ILs based on benzaldehyde, *p*-anisaldehyde, vanillin, and syringaldehyde	Switchgrass	10% (w/v) solid loading, 160 °C, 3 h	51.3% xylan removal and 44.5% lignin removal (using [EMIM][OAc]; control) 23.3–51.3% xylan removal and 24.1–57.5% lignin removal	[128]
Lignin-based DES	ChCl:vanillin	*Arabidopsis thaliana*	5% (w/v) solid loading, 80 °C, 3 h	33.6–54.7% lignin removal;	[129]
	ChCl:PCA	herb residue of *Cortex albiziae* (HRCA)	1:1 molar ratio, 10% (w/v) solid loading, 140–200 °C, 1–7 h	48.1% cellulose conversion (using untreated material, control) 20–100% xylan removal and 0–70% lignin removal; 84.6% cellulose conversion	[131]
	ChCl–vanillin–4-hydroxybenzaldehyde	Arabidopsis thaliana seeds (wild type and CAD double mutant type)	2:1–1:2 molar ratio, 5% (w/v) solid loading, 80 °C, 3 h	5–10% lignin removal (using wild substrate) 25–32% (using the mutant type)	[130]
	ChCl:PCA	*Cortex albiziae* residue and *Heterosmilax yunnanensis* residue	1:1–4:1 molar ratio, 5% (w/v) solid loading, 140–180 °C, 0.2–2.5 h	~5% xylose yield (using only water, control) 72.47% xylose yield	[132]
	ChCl:PB, ChCl:PCA, ChCl:PHA	Poplar wood	1:1–1:3 molar ratio, 10% (w/v) solid loading, 100–160 °C, 1–9 h	15.5% cellulose conversion (using untreated material, control) 90% cellulose conversion at 72 h, 15.5–69.6% lignin removal;	[133]

lignin subunits (4-hydroxybenzyl alcohol, catechol, vanillin, and *p*-coumaryl alcohol ether (PCA)) were used with HBD. The authors justify that the availability of the phenolic hydroxyl group is essential for the DES synthesis. Under a temperature of 80 °C for 3 h, the pretreatments of *Arabidopsis thaliana* using ChCl-PCA and ChCl-vanillin reached delignification of 60.8% and 52.5%, respectively. The performance of the pretreatment with ChCl-4-hydroxybenzyl alcohol was disappointing, reaching a delignification of only 0.4%. Although the pretreatment stands out for requiring mild temperature conditions, a severe xylan loss was recorded after pretreatments. Glucose release increased by 34.3% (equivalent to 102 mg/g) after pretreatment assisted by ChCl-vanillin compared to untreated *A. thaliana*. In 2019, the same authors produced another paper involving a biorefinery approach using *A. thaliana* as a substrate and lignin-based DES [130]. Here, pretreatments with ChCl-HBA showed better delignification performances than ChCl-vanillin, either for the wild type (10% vs. 5%) or the mutant type (32% vs. 28%). Using only cellulases in the enzymatic cocktail, the cellulose conversion values from wild *A. thaliana* were 66% and 70% in the biomasses pretreated with ChCl: vanillin and ChCl: HBA, respectively.

Chen et al. [131] investigated the increased enzymatic digestibility of herb residues of *Cortex albiziae* (HRCA) after pretreatment with lignin-based DES. DES was prepared from the mixture between ChCl and PCA at a temperature of 160 °C using molar ratios of 1:1, 2:1, and 4:1. High temperatures (higher than 160 °C) and longer exposure times (higher than 5 h) were not indicated in pretreatments due to cellulose loss. The following operational conditions were defined as the optimal pretreatment condition: temperature of 160 °C, 10% (w/v) solid loadings, DES prepared with a 1:1 molar ratio (ChCl-PCA), and exposure time of 5 h. The pretreated HRCA reached 84.6% digestibility after 72 h of the process, which was almost double the performance of the untreated material. The authors also suggested reducing the viscosity or altering the polarity of the reaction medium as a way to increase enzymatic digestibility. After adding water (50% v/v), the enzymatic digestibility of the pretreated HRCA increased to 99.4%. Scanning electron microscopy (SEM) images showed that the pretreatment increased the roughness of the lignocellulosic biomass, which may have explained the better enzymatic digestibility results. This effect was maximized in the pretreatments with the highest water content in the pretreatment. Finally, the authors proposed the direct reuse of ChCl-PCA to reduce the total costs of the process. They suggested that the procedure be performed without compromising enzymatic digestibility (from 84.6% in the process without recycling to 78.1% in the process with two liquid fraction recycles). To assess the role of water and DES in HRCA polysaccharide depolymerization, the same research group published another paper in 2020. They performed HRCA pretreatments by adding water and ChCl:PCA at different times (one-step and two-step) and testing the substitution of water for ethanol. They observed that hemicellulose hydrolysis is initiated by water, and the generated xylooligosaccharides are finally cleaved to xylose by DES. The xylose yield in DES-water pretreatments was fourfold higher than pretreatment with water-free DES (72.5% vs. 19.1%) [132].

Wang et al. [133] proposed a pretreatment with lignin-based DES, more specifically using *p*-hydroxybenzoic acid (PB), PCA, and 4-hydroxybenzaldehyde (PHA). The use of ChCl-PB in pretreatments was more effective when compared to other DES. The delignification of poplar using ChCl-PB obtained 69%, and the enzymatic digestibility reached 90.8% for glucose and 88.9% for xylose. The lignin obtained from the liquor was recovered, and analyses showed that it has high-purity, low molecular weight, and uniform size.

Some of the studies cited also evaluated how pretreatments with lignin-based DES would affect the chemical structure of the biomass constituent lignins through 2D $^{1}H—^{13}C$ heteronuclear single-quantum coherence (HSQC) analyzes. Kim et al. [129] reported no differences between the signals from the representative lignin units and the signs of the internal lignin bonds in the pretreated switchgrass saccharification residues. These results indicated that the main internal lignin bonds remained intact after pretreatments with DES. In particular, Wang et al. [133] studied the impact of the pretreatment incubation time on the structural characteristics of lignin isolated via HSQC and using enzymatic hydrolysis lignin (EHL) as a standard. The authors noticed the cleavage of β-aryl ether bonds and differences in the amount of bonds between monomers. Using shorter incubation times (3 h), the authors observed that the recovered lignin has high purity. On the other hand, the pretreatment incubation for 9 h proved to be inadequate for the future valorization of the macromolecule due to the occurrence of condensation reactions. In this lignin sample, the content of aliphatic hydroxyl groups was fivefold lower than in EHL, while an increase in the content of substituted C_5 hydroxyl was observed (5.5-fold increase).

The use of lignin as a raw material for bio-IL and DES can lead to advantages over conventional preparation methods. In a preliminary economic analysis, Socha et al. [121] reported that the cost of [VanEt$_2$NH][H$_2$PO$_4$] and [p-AnisEt$_2$NH][H$_2$PO$_4$] is estimated to be between $12.0 to $15.0/kg, while the current price of a traditional ionic liquid is between $17.0 to $25.0/kg. The authors also cited that the reductive amination of phenolic compounds has a substantial impact on the final cost of ILs and that the substitution of reagents such as diethylamine and sodium triacetoxyborohydride with ammonia and hydrogen may allow the final cost of ILs to decrease to up to $4.0/kg. Due to the

simplicity of preparation, the final costs of the DES are only linked to the costs of the precursor compounds. In fact, the depolymerization of lignins to obtain phenolic compounds (which function as HBD of the lignin-based DES) is not yet economically competitive due to the difficulties encountered in separating the products. The use of petroleum as a raw material for the synthesis of phenolic compounds has been improved over decades so that the price of vanillin and 4-hydroxybenzaldehyde has been severely reduced and can be purchased in the range of $15.0–$100.0/kg. For example, the price of vanillin obtained from lignin is estimated to be $100.0–$200.0/kg, and vanillin extracted from vanillin pods is estimated to be between $1200–$4000/kg [134, 135]. Despite this, the production of phenolic compounds from lignin is still attractive, as it gives a destination to solid waste from the cellulose recovery stages, and the products generated have a lower carbon footprint than those based on petroleum [136, 137].

In addition to the costs of raw materials, other characteristics such as the easy recovery and maintenance of the pretreatment performance are fundamental for the commercial use of pretreatments based on IL and DES. It is essential to highlight that the recyclability of IL and DES is also an issue regarding the disposal of the substance, environmental impact, and toxicity [138]. In both pretreatments, the addition of volatile organic compounds (VOC) and their aqueous mixtures are required for successful pretreatment. In IL pretreatments based on imidazolium salts, cellulose and hemicellulose are solubilized in the post-pretreatment liquid phase and must be regenerated from the addition of VOC (such as ethanol and acetone), which act as anti-solvents. VOC and its aqueous mixtures are also used to wash pretreated biomass to remove excess impregnated IL or DES. Therefore, IL and DES can be recovered from the evaporation of VOC and then recycled to a new batch of pretreatment. Unfortunately, to the authors' knowledge, the studies referenced on lignin-based IL did not investigate this issue, and we found data only for lignin-based DES. Kim et al. [129] reported that 95% of ChCl:PCA was recovered from the liquid fraction resulting from the pretreatment after evaporation of ethanol and water in the rotary evaporator. They also recorded a slight decrease in sugar release with the increase in the number of recycles. The use of fresh ChCl:PCA reached a sugar yield of 87%, while the pretreatment with one recycle and two recycles reached a sugar yield of 78% and 70%, respectively. Wang et al. [108] reported that lignin-based DESs were successfully recovered after ethanol addition and evaporation in the wash step. The delignification results reported were 69%, 49%, and 46% in the pretreatment with fresh ChCl:PB, pretreatment with one liquid fraction recycle, and pretreatment with two liquid fraction recycles. HSQC spectra showed disturbances in the ChCl:PB spectra and the authors suggest more in-depth studies on recycling should be performed to minimize DES losses.

Addition of Lignosulfonate and Lignin Derivatives

Lignin derivatives can be useful for tuning hydrothermal and acidic pretreatments. It is widely known that these pretreatments are helpful for removing hemicellulose but have limited performance for delignification. Under acidic conditions, the β-aryl ether bonds of lignin are easily broken, which leads to the formation of carbocations, intermediates highly susceptible to nucleophilic attack [25]. Condensation reactions also occur through the Michael addition, in which quinone methide act as an acceptor and phenolates as nucleophiles [139]. The origin of lignin condensed by the presence of formaldehyde has already been described by Gierer and Lindberg [140]. In this condensation reaction, formaldehyde derived from the lignin depolymerization acts as an acceptor and reacts with two phenolates to form a diarylmethane structure [139]. In practice, lignin fragments generated in the liquid phase are repolymerized and deposited on the surfaces of the pretreated materials (see Fig. 4. This phenomenon was confirmed through SEM images in the studies of Selig et al. [141], Donohoe et al. [142], and Hu et al. [143]. According to Kumar et al. [144], lignin repolymerization causes adverse effects on the substrate, such as reduced enzyme accessibility and increased non-productive adsorption of cellulases, limiting the enzymatic digestibility. It is important to note that this phenomenon is generally reported in softwoods, a material rich in guaiacyl units [145].

Carbocation scavengers can be added at pretreatments to solve this problem. Carbocation scavengers prevent lignin repolymerization via competition for carbocation [146, 147]. In particular, repolymerization problems caused by formaldehyde can be solved using ethanol as a pretreatment solvent [148]. The use of carbocation scavengers is not innovative for treating lignocellulosic biomasses, especially when considering the consolidation of the Kraft and sulfite processes. In cellulosic ethanol production, aromatic compounds with high electron density have been used as carbocation scavengers. Wayman and Lora first proposed the use of 2-naphthol, a potent carbocation scavenger, to increase the delignification of aspen wood after autohydrolysis pretreatment [149]. Li et al. [150] reported the suppression of lignin repolymerization from aspen wood meal after adding 2-naphthol in the steam explosion pretreatment. The authors achieved delignification of up to 91% using 2-naphthol-assisted pretreatment and reported that the pretreated material contained only 4% (w/w) Klason lignin. Pielhop et al. [146] used a loading of 0.02 g of naphthol per g of biomass in the steam explosion pretreatment of spruce wood and reported an increase in enzymatic digestibility of 55% compared to the

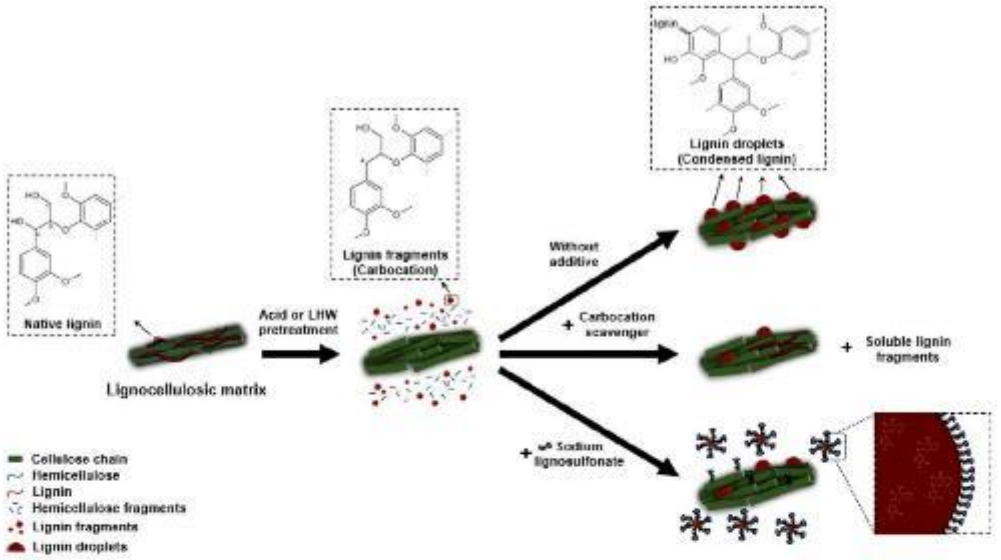


Fig. 4 Strategies to minimize the formation of lignin droplets in hydrothermal and acid pretreatments

control experiments. Using wood sawdust as a substrate, Lai et al. [151] observed that 2-naphthol-7-sulfonate reduced the adsorption of cellulases by 30% and increased the sugar yield from 36.4 to 53.8%.

The use of aromatic compounds from lignocellulosic biomass as carbocation scavengers has only recently been tested. Pielhop et al. [145] proposed the addition of catechol and resorcinol as carbocation scavengers for the autohydrolysis pretreatment of spruce wood, but satisfactory results of enzymatic digestibility were not achieved. In turn, Zhai et al. [152] observed that syringic acid, a phenolic acid derived from lignin, showed high potential as an additive for acid pretreatment of lodgepole pine. Pretreated materials with syringic acid showed a significant increase in cellulosic accessibility and an increase in the cellulosic conversion from 18 to 70%. This fact may represent an advance for replacing 2-naphthol with aromatic compounds derived from lignocellulosic biomass as carbocation scavengers.

The production of carbocarbon scavengers, such as 2-naphthol, is still dependent on the petroleum industry. 2-naphthol is commonly synthesized from the caustic fusion of sodium 1-naphthalenesulfonate with sodium hydroxide or oxidation followed by tetralin aromatization [153]. While the price of oil-derived 2-naphthol is estimated at approximately $210.0/kg (on Sigma-Aldrich platforms), the costs involved in producing naphthalenes from lignin are not yet well established. Advances in the production of naphthalenes from hydrodeoxygenation of lignin have recently been reported in Kim et al. [154], Kong et al. [155], and Zhang et al. [156], but the purification of products is a gap for more consistent techno-economic assessments. In a way, this aspect is also valid for the production of syringic acid (a potential carbocation scavenger) from lignin. In 2015, a patent (CN104693022A) establishes syringic acid production with high purity and yield from syringaldehyde [157]; the latter is commonly obtained from lignin via oxidation methods and its price is estimated at $22.0/kg [158, 159].

Unlike carbocation scavengers, it is possible to solve problems of lignin repolymerization using surfactants. Surfactants are amphipathic molecules with two parts of the opposite nature, one hydrophilic termination, and the other hydrophobic termination. Surfactants are specialized molecules to reduce interfacial tensions, and they are applied in different industrial sectors as emulsifiers, dispersants, foam agents, among other functions [160, 161]. In pretreatments of lignocellulosic biomass, surfactants can stabilize any lignin fragments in the liquid phase to prevent the deposition of lignin in the pretreated material. Also, surfactants can improve reagents' penetration into lignocellulosic biomass during pretreatments, leading to better xylan solubilization and delignification [162]. Several studies have already reported the success of nonionic surfactants for this purpose, with emphasis on polyethylene glycol [162–165], Tween 20 [162, 165, 166], and Tween 80 [162–165, 167].

However, these surfactants can raise the total costs and are usually obtained from petroleum, so researchers have looked for greener alternatives. Lignosulfonates are inexpensive, renewable, biodegradable, and environmentally benign surfactants compared to nonionic surfactants commonly used in pretreatments [168]. According to Hodásová et al. [76], the price of lignosulfonates varies between $0.18 and 0.50/kg, while the price of PEG is around $1.4/kg. There is little data on pretreatments assisted by lignosulfonates, and they were found only in the studies by Xu et al. [168] and Chandra et al. [169].

Xu et al. [168] evaluated lignosulfonate's role in alkaline pretreatments of corn stover in terms of final chemical composition and enzymatic digestibility of pretreated biomass. Pretreatments with only 2% (w/w) lignosulfonate did not lead to better delignification values but increased glucose yields from 80.5 to 83% using corn stover without mechanical refining. According to the authors, more intense effects of lignosulfonate were observed in the presence of anthraquinone due to the surfactant's ability to promote the penetration and distribution of anthraquinone. The values of xylan recovery and delignification increased significantly by 5% ($p < 0.05$) and 6% ($p < 0.01$) compared to the control experiments. They also proposed to prepare lignosulfonate from the lignin removed after pretreatment, obtaining a lignosulfonate *in loco*. The product was used for a new batch, obtaining results similar to the commercial additive.

Chandra et al. [169] proposed a way to improve hemicellulose recovery and enzymatic digestibility using two-step pretreatment of poplar and lignosulfonate as additive. Initially, a pretreatment used acid catalysts to increase the recovery of hemicelluloses, and then a hydrothermal pretreatment was applied under severe conditions (200 °C and 5 min) to obtain a cellulose-rich material for enzymatic hydrolysis. The authors reported that the addition of 6% lignosulfonate increased the hemicellulose solubilization in sulfuric acid pretreatments. The addition of lignosulfonate did not affect the composition of pretreated poplar (either cellulose content or Klason lignin content); however, it improved enzymatic hydrolysis performance. Using cellulases at 10 FPU/g of cellulose, cellulosic conversion increased from 75% (control) to 90% (to 3% lignosulfonate) and 92% (to 6% lignosulfonate), respectively. This behavior was attributed to the adsorption of surfactant onto the substrate, being confirmed in adsorption analyses with Direct Orange dye.

In addition to the improvements in enzymatic hydrolysis already mentioned, the presence of additives (scavengers and surfactants) can benefit lignin valorization. Since the lignin repolymerization in the liquid fraction is avoided, the lignin recovered after pretreatments assisted by additives has a higher content of β-O-4 bonds and less size distribution than condensed lignins, which makes lignin more valuable [146]. This fact occurs mainly in pretreatments with lignin-based carbocation scavengers so that lignin is not only stabilized but also the content of aromatic groups is increased [146].

Detoxification of Hydrolysates via Lignin-Based Activated Carbon

The use of severe conditions in pretreatments guarantees the disruption of the lignocellulosic matrix, which increases the accessibility of enzymes to cellulose fibers in pretreated materials [170]. However, this procedure can lead to the generation of fermentation inhibitors such as hydroxymethylfurfural (HMF), furfural, aliphatic acids (acetic acid, formic acid, levulinic acid), and phenolic compounds derived from lignin [10, 20]. Fermentation inhibitors accumulate in the hydrolyzate, so it is recommended that pretreated materials be washed after the pretreatment step [6]. However, this procedure cannot be performed in the hydrolyzate to avoid sugar dilution, and another process must be included for the treatment of fermentation inhibitors [171, 172].

Detoxification refers to the processes that eliminate or mitigate the effects of fermentation inhibitors. In the context of cellulosic ethanol production, adsorption stands out as a potential detoxification method due to its high effectiveness in removing furan aldehydes and phenolic compounds and its easy implementation [173, 174]. Adsorbents can also be used for long periods of operation after regeneration via heating or eluents [175]. Despite the undeniable performance of polymeric adsorbents in treating hemicellulose hydrolysates, activated carbon has generally been used for this purpose on the laboratory scale.

Activated carbon is a carbonaceous material manufactured from the carbonation or pyrolysis of organic raw materials at high temperatures (500–900 °C) followed by an activation step via the physical route (e.g., 500–900 °C in the presence of air, CO_2, oxidizing agents, and steam) or chemistry (e.g., use of $ZnCl_2$, H_3PO_4, $FeCl_3$, H_2SO_4, and K_2CO_3) [176, 177]. AC has a porous structure with a high surface area (500–5000 m^2/g) and a broad spectrum of functional groups on its surface, which explains its high adsorption capacity [178, 179]. Due to these advantageous characteristics, AC is a useful and consolidated adsorbent for several industrial sectors to treat liquid and gas streams [180]. However, the industrial use of AC in hemicellulose treatment represents additional pressure on cellulosic ethanol's profits. One of the strategies to make this application of AC feasible is to reduce the costs of preparing the adsorbent, being possible through cheaper raw materials to the detriment of hardwood and oil products [180]. Authors have already shown success in the use of agro-industrial residue as precursors of AC, such as coconut fiber [181, 182], palm seed [183], and sugarcane bagasse [184]. Recent studies have proposed isolated lignin and lignin-rich fractions as precursors of AC

for detoxification applications to fully value lignocellulosic biomass.

Mussatto et al. [176] reported the lignin conversion into AC to detoxify hydrolysates obtained from brewer's spent grain (BSG). It is important to note that lignin was also recovered from the liquor after alkaline pretreatment of BSG. The operational conditions for the carbonation/chemical activation of lignin were optimized via an experimental design, which led to obtaining values of surface area up to 692 m^2/g and micropore size up to 0.453 cm^3/g. The authors observed that the use of lower temperatures (300 °C) and an acid: lignin ratio (g/g) equal to 1.0 were more appropriate in terms of yield. In detoxification experiments, AC from BSG lignin reduced the levels of phenolic compounds without compromising the sugar content of the hydrolyzate. These results imply that AC from lignin can be competitive compared to commercial AC.

Similar reports were observed in Yoshioka et al. [185]. They developed an in situ detoxification with AC from lignin for ethanol production from *E. globulus* wood. The lignin used in this process was obtained from the enzymatic hydrolysis of pretreated *E. globulus* wood, and the carbonization was carried out at 250–350 °C for 2 h (see Fig. 5). The lignin AC reached a surface area of 525 m^2/g and significant removal of inhibitors (~ 85%), including furfural, HMF, vanillin, and syringaldehyde. The authors also extolled lignin AC's role in reducing the inhibition of *Zymomonas mobilis* and increasing ethanol production in simultaneous saccharification and co-fermentation (SScF) strategy. The authors observed that the hydrolyzate environment obtained from the microwave pretreatment of *E. globulus* led to complete inactivation of the microorganism (~ 0 g/L ethanol), while experiments with the presence of the adsorbent showed ethanol production equal to 54 g/L.

These studies make it clear that lignin residues are attractive options for the production of AC even because the biopolymer has a high carbon content (around 50–60%) [76] and the polysaccharide fraction is converted into products. The integration of the currents generates a scenario of self-sufficiency, in which the purchase of commercial adsorbents is dispensable, reduces the waste generation, and takes advantage of the energy of the soluble inhibitors (since the saturated activated carbon can be burned) [185]. Comparing prices between commercial AC (~ $0.5-$1.5/kg) and the AC obtained from lignin residues is complicated. The chemical structure and lignin content vary according to lignocellulosic biomass valorization strategies, which strongly impacts AC yield. Hodásová et al. [76] also reported that the AC properties, such as the available surface area, affect the final price.

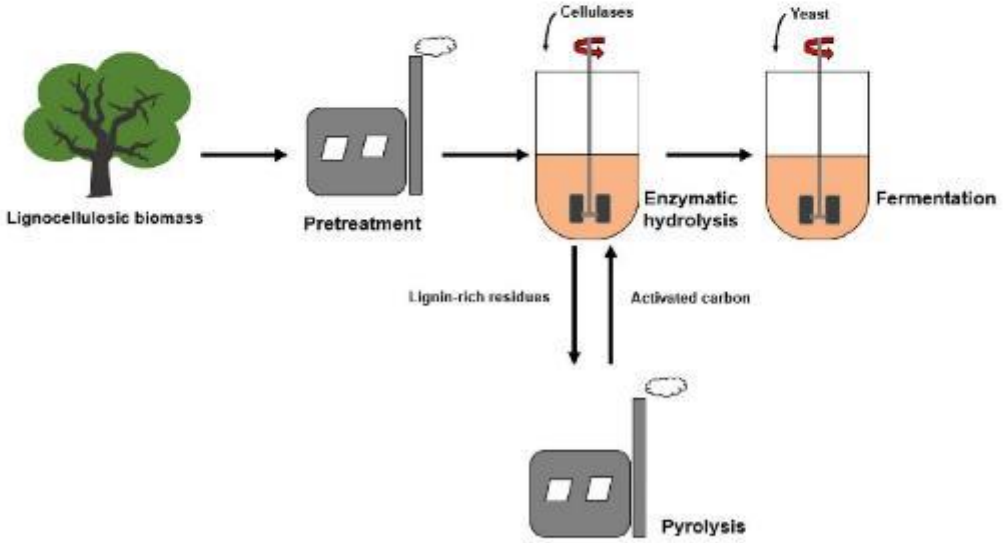


Fig. 5 Activated carbon synthesis from enzymatic hydrolysis residues and its use in the in situ detoxification approach. [184] Adapted from Yoshioka et al.

Use of Lignin-Based Inputs to Improve Enzymatic Digestibility

Enzymatic Hydrolysis Assisted by Lignin-Based Surfactants

The previous sections (especially Sect. 2) were devoted to showing the purpose of delignification, but this is not the only solution to mitigate the effects of non-productive adsorption of enzymes onto lignin. Studies on the use of surfactants to improve enzymatic hydrolysis of lignocellulosic biomass have been carried out for some time. Small dosages of surfactants are added at the start of enzymatic hydrolysis and can interact with the hydrophobic portions of lignin, which blocks the non-productive adsorption of enzymes [186]. Surfactants can also stabilize enzymes' conformation and even increase their catalytic activities [9, 160]. Other possible mechanisms of surfactants include protection from thermal denaturation, protection from denaturation at the air–liquid interface, and minimizing the adverse effects of lignin and hemicellulose inhibitory compounds [160]. Glimpsing the closed-loop biorefinery scenario, some authors have investigated the use of lignin-based surfactants as additives for enzymatic hydrolysis.

The effects of lignosulfonate on enzymatic hydrolysis were first assessed in Liu and Zhu [187]. The authors reported that complexes formed between lignosulfonates and metals could increase the yields of enzymatic hydrolysis of aspen wood after sulfite pretreatment, also called sulfite pretreatment, to overcome recalcitrance of lignocelluloses (SPORL). It is essential to highlight that this pretreatment is similar to the sulfite process of the paper and cellulose industry and that lignosulfonates are also generated in the liquid phase. This behavior was proven after the authors registered that the results of enzymatic hydrolysis of unwashed aspen wood with the addition of 1 mmol/g Mg were equivalent to washed aspen wood performance. In 2013, other studies also reported that cellulolytic conversion of lignocellulosic biomass increased in the presence of lignosulfonates. Zhou et al. [188] discussed the influence of the lignosulfonate chain size on enzymatic hydrolysis. They observed that the saccharification yield is inversely proportional to the molecular weight of the lignosulfonate used. Wang et al. [189] observed that the addition of lignosulfonate in the enzymatic hydrolysis of pretreated lodgepole was beneficial in an environment with pH 5.5. Zheng et al. [190] suggest that lignosulfonates occupy the lignin binding sites of the lignocellulosic biomass and establish electrostatic repulsion forces between cellulases and lignin.

Modified lignins are also used as additives for enzymatic hydrolysis of lignocellulosic biomass. Table 2 presents a compilation of studies on the preparation of lignin-based surfactants and their use in enzymatic hydrolysis of

Table 2 Recent publications on enzymatic hydrolysis of lignocellulosic biomass assisted by lignin-based surfactants

Type of surfactant	Lignocellulosic biomass	Operational condition	Hydrolysis performance	Reference
EHL-PEG	Corn stover	2% (w/v) pretreated corn stover; cellulase loading at 5 FPU/g; 50 °C; 200 rpm; 3–72 h	16.7% (control) 70.1% (2 g/L EHL-PEG)	[191]
pH-responsive lignin carrier	Corncob residue	2% (w/v) substrate; cellulase loading at 10 FPU/g; 50 °C; 150 rpm; 48 h	78.1% (control) 93.0% (3 g/L pH-LC)	[193]
Amphiphilic-lignin derivate	Sweet bagasse sorghum	1% (w/v) sweet sorghum, 0–10% (w/w) PEGylated lignin; cellulase loading at 10 FPU/g; 50 °C; 150 rpm; 72 h	45.57% sugar yield (control) 81.33% sugar yield (10% w/w A-LD)	[196]
KL-PEG	*Eucalyptus* wood	2% (w/v) acid pretreated *E. wood*; 5–10 FPU/g; 50 °C; 200 rpm; 3–72 h	100% (at 2 g/L KL-PEG)	[196]
Lignin amphoteric surfactant	Corncob residue	2–15% (w/v); 5–9 FPU/g; 50 °C; 200 rpm; 72 h	52% (control using 2% solid loading and 5 FPU/g) 70% (1 g/L BS12 at using 2% solid loading and 5 FPU/g) 65% (control at using 15% solid loading and 9 FPU/g) 76% (5 g/L BS12 at using 15% solid loading and 9 FPU/g)	[194]
pH-responsive lignin-based surfactant	*Eucalyptus* wood	10% (w/v) substrate, 5–10 FPU/g, 50 °C; 150 rpm; 96 h	35.8% (control) 89.7% (10 g/L additives)	[195]
PEGylated lignin	*Eucalyptus* wood	2% (w/v) acid pretreated substrate; 10 FPU/g; 50 °C; 200 rpm; 48 h	38.2% glucose yield (control) 84.5% glucose yield (3 g/L L-PEG)	[192]

lignocellulosic biomass. Modifications of the EHL via PEG 1000 graft resulted in EHL-PEG, a polymer with surfactant properties, as reported by Lin et al. [191]. The addition of EHL-PEG led to a 70% increase in corn stover digestibility, while PEG 1000 increased digestibility by only 52.3%. Using dynamic light scattering analyses, it was possible to observe that EHL-PEG reduced the aggregation of cellulases, which may have contributed to the reduction of non-productive adsorption onto lignin. Cai et al. [192] also investigated the beneficial effects of EHL-PEG on enzymatic hydrolysis. In addition to reducing the non-productive adsorption of cellulases, EHL-PEG made it possible to recycle the enzymes by lowering the pH to 3.0, making it possible to reduce the total costs. In other papers in the same group, reducing total costs remained a concern, and pH-responsive surfactants based on lignin were prepared from modification with 3-chloro-2-hydroxypropyl trimethylammonium chloride (CHPTAC) [193–195]. The addition of these surfactants reduced the non-productive adsorption of cellulases and improved the sugar release compared to control experiments (surfactant-free experiments). In Cai et al. [193], the authors observed that the surfactant obtained from the CHPTAC grafting could successfully recycle enzymes from the Cellic Ctec2 cocktail, mainly endoglucanases III, endoglucanases IV, β-glucosidases, and xylanases. Fatriasari et al. [196] reported that the hydrolysis of kraft pulp from sorghum bagasse increased after the addition of PEGylated lignin from *Acacia mangium*. The yield of enzymatic hydrolysis reached up to 76% in the presence of 10% (w/w) PEGylated lignin, while the control experiments reached only 45.57%. Lin et al. [197] reported the success of the addition of polymer kraft lignin-polyethylene glycol (KL-PEG) in the enzymatic hydrolysis of dilute acid pretreated *E.* wood. KL-PEG showed high solubility and good surface activity, which increased the sugar yields by up to 94%. Also, KL-PEG increased enzyme longevity by 44% and its stability, facilitating its recovery and decreasing the amount of enzymes applied to the system. PEG grafting on in-situ lignin can also be an attractive way, as shown in Lai et al. [198].

Although surfactants are used extensively in converting lignocellulosic biomass into ethanol on the laboratory scale, there is no extensive literature on the techno-economic evaluation of these approaches. Tu and Saddler [199] evaluated the economic viability of ethanol production schemes from pretreated lodgepole pine using different concentrations of Tween 80 (0.2–0.005%, w/v). The authors noted that the use of 0.2% (w/v) Tween 80 could reduce the costs associated with enzymes by 66% and the total production costs by 8%. In 2018, Kadhum et al. [200] elaborated a techno-economic evaluation of the conversion of Banagrass to ethanol via a separate saccharification and fermentation strategy using PEG and Tween 80 as additives. They observed that surfactant concentrations greater than 2% (w/w) compromise the ethanol profitability even though it increases the ethanol titers generated. Based on these results, there is a good scenario for using lignosulfonate as an enzymatic hydrolysis additive considering its low cost compared to non-ionic surfactants (see Sect. 4.1.2) that there are no substantial differences in performance. Possibly, this is not the same situation as the surfactants obtained from the chemical lignin modification. The use of expensive reagents for the grafting of chemical groups and the purification of products are required, which raises the prices of these surfactants. Despite this, the ability to recycle cellulases is an asset for lignin-based surfactants and, therefore, more economic data should be evaluated in future studies.

Enzyme Recycling from Liquid and Solid Phase After Saccharification

Other aspects must be discussed considering the phenomenon of non-productive adsorption of cellulases. Nakagame et al. [201] investigated the influence of protease-treated lignin and fractions of cellulolytic enzymatic lignin on the enzymatic hydrolysis of microcrystalline cellulose. It was observed that both isolated lignins increased the sugar release and that this effect is directly proportional to the content of carboxylic acid in the lignins. The authors suggested that carboxylic acids partially alleviate the non-productive binding of cellulases to lignin. Non-productive adsorption onto lignin is an undesirable phenomenon for enzymatic hydrolysis performance, but it is useful for enzyme recycling. The conventional strategy of recycling enzymes from the liquid phase is described in Fig. 3 and involves the contact of the post-enzymatic hydrolysis liquid phase with fresh lignocellulosic biomass, while the sugars are directed to ethanol fermentation. Examples of this enzyme recycling strategy were carried out in the studies of Steele et al. [202], Tu et al. [203], Du et al. [204], Wacnukul et al. [205], and Kim et al. [206]. The recycling of enzymes from the post-enzymatic hydrolysis residue is also possible since it is a lignin-rich material. Different results can be obtained with the variation of the enzyme cocktail, type of biomass, and operational conditions; however, it is often reported that the enzymes retained in the enzymatic hydrolysis residue are not necessarily disabled even if this adsorption is irreversible. Thus, surfactant solutions or pH adjustments can be easily performed to desorb the enzymes retained onto the solid residue (see Fig. 6). Yuan et al. [207] proposed the addition of PEG to desorb 55% and 40% of the enzymes obtained from the acid pretreated corn stover and alkaline pretreated corn stover, respectively. Due to the greater adsorption capacity of acid pretreated lignin compared to alkaline pretreated lignin, a more significant amount of enzymes could be recycled. Shang et al. [208] reported that a drastic change in pH

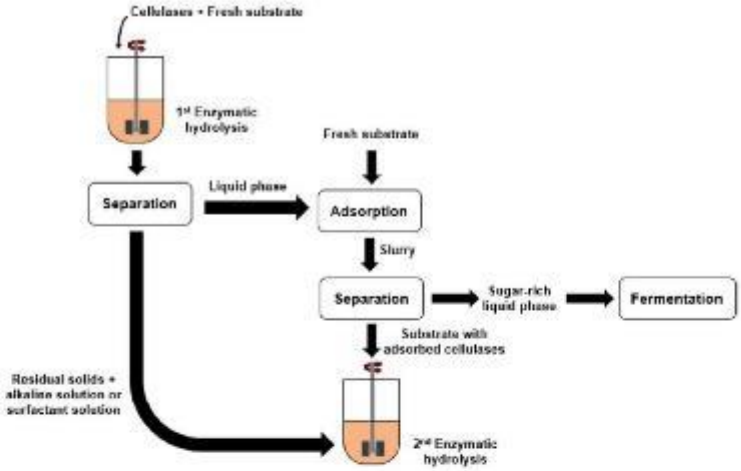


Fig. 6 Cellulase recycling via adsorption strategy using supernatant and residual solids after enzymatic hydrolysis

value from 4.8 to 10.0 could desorb up to 85% of Spezyme CP cellulases and achieve a glucose yield of 70.3%. This result was similar to the performance of the first run. It is essential to highlight that the enzyme recycling using solid phase in a single vessel can lead to mass transfer problems due to solid material accumulation.

Lignins as an Electron Donor for LPMO

Recently, evidence has shown that lignin in enzymatic hydrolysis goes further than cellulose adsorption and may contribute to the activity of lytic polysaccharide monooxygenases (LPMOs). LPMOs are copper-dependent enzymes that catalyze the cellulose chain's breakdown and other polysaccharides (such as xylan, chitin, xyloglucan) and are currently included in commercial enzyme cocktails [209]. Unlike cellulose hydrolases, the mechanism of action of LPMOs involves an oxidation reaction using the presence of molecular oxygen or hydrogen peroxide as a co-substrate and in the presence of a reducing agent [210]. It is noteworthy that they can act in the most recalcitrant strongholds of polysaccharides, the crystalline portions, which leads to the disruption of the structure and greater accessibility of hydrolytic enzymes [211]. Different reducing

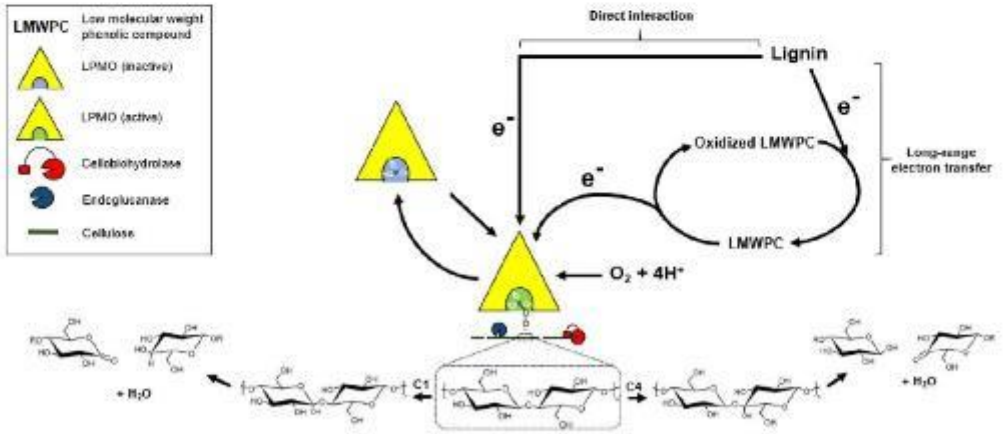


Fig. 7 Lignin as an electron reservoir for the action of LPMOs on cellulose enzymatic hydrolysis

agents can trigger the action of LPMOs, including enzymes [212, 213], photosynthetic pigments [214], and phenolic compounds [215–218]. As previously mentioned, lignin has a high electron density and, therefore, it is considered an excellent reducing agent for LPMOs via direct interaction or long-range electron transfer [219]. Lignin can perform long-range electron transfer by mediating phenolic compounds, which serve as electron transporters for LPMOs. After oxidation, phenolic compounds are reduced again by donating electrons from lignin and can restart the mechanism (see Fig. 7) [209, 219].

Dimarogona et al. [220] reported that the addition of enzymes of thermophilic fungus *Sporotrichum thermophile* increased the sugar release in experiments with lignocellulosic biomasses. The authors suggest a synergistic effect between lignin and the hydrolyzed substrate since enzymatic hydrolysis performance increased linearly with the lignin content. Rodriguez-Zuñiga et al. [216] investigated the role of oxidative enzymes, such as LPMO, in the enzymatic hydrolysis of agro-industrial residues (sugarcane bagasse, wheat straw, and corn stover). When compared to the other pretreatments, it was observed that the hydrothermal pretreatment increased the lignin content in the pretreated material and, therefore, provided higher values of LPMO activity. Ni et al. [221] observed that lignin deposited on cellulose could reduce LPMO for cellulose oxidation and increase the cellulose-based film yield by 26.0%. The authors reported that the results of the in-situ reduction by lignin exceed the performance of ascorbic acid, a recognized LPMO-reducing agent, in terms of oxidation efficiency and lower production of hydrogen peroxide.

Lignins as an Enzyme Immobilization Support

The improvement of the enzymatic hydrolysis of lignocellulosic biomass can also be achieved through the enzyme immobilization onto lignin-based supports. The enzyme immobilization consists of a procedure that restricts the mobility of the enzyme, which allows the reuse of enzymes in new batches and facilitates product recovery [222]. According to Mörschbächer et al. [223], the immobilization of enzymes also guarantees better operational stability and enables the enzymatic operation in a continuous flow regime. It is worth considering that the success of enzyme immobilization is strongly linked to the immobilization method (such as adsorption, covalent bonding, entrapment, and combinations) and the choice of immobilization support.

Natural polymers (such as cellulose, chitosan, collagen, k-carrageenan, among others) are often used as immobilization supports since they are abundant and are eco-friendly [224]. Enzyme immobilization studies involving lignin-based supports emerged after lignin valorization became a hot topic. Lignin offers low cost, low toxicity, and biocompatibility. Also, lignin has a high availability of functional groups susceptible to chemical modifications, being a desirable characteristic for the immobilization of enzymes by covalent bonding [225]. It is worth considering that the phenomenon of cellulases' adsorption is somewhat a method of enzyme immobilization, but it has already been discussed in the previous sections and does not require efforts to make the immobilization support. Here, successful cases of preparing lignin-based supports for enzyme immobilization were discussed.

The first studies of enzyme immobilization with lignin-based supports involved enzymes distinct from the cellulosic ethanol scheme, such as lipases [226], α-amylase [227], β-galactosidase [225], and laccase [225]. Although these studies do not follow the main direction of the paper, they did provide indications of the performance of lignin as an enzyme immobilization support. Zdarta et al. [226] proposed using a chitin-lignin composite to immobilize lipases from *Aspergillus niger*, a hydrolase capable of degrading fats. The authors mentioned that the composite achieved a catalytic activity of 5.72 mU and more excellent stability to temperature and pH stresses than free lipase. The catalytic activity decreased slightly with the number of recycles, and 80% of the cellulolytic activity was maintained after 20 cycles. Gong et al. [227] evaluated the feasibility of α-amylase activation and immobilization using lignin from bamboo shoots. The enzyme immobilization mechanism is based on the adsorption of α-amylase, and it was observed that the catalytic activity increased by two times. Immobilized α-amylase also improved its catalytic efficiency and storage stability. Unexpectedly the immobilized enzyme showed less operational stability than the free enzyme; however, the residual activity remained above 50% after 14 recycles of the immobilized enzyme. Amino-modified lignin microspheres (A-LMS), derived from materials such as kraft lignin, can also be used to support the immobilization of β-galactosidase and laccase as described by Bebić et al. [191]. Different immobilization conditions were tested, including various alginate concentrations (A-LMS_5 and A-LMS_10) and pH values. Electrostatic interaction is predominantly between β-galactosidase and A-LMS_5, while electrostatic and hydrophobic interactions are involved in laccase immobilization. It was also observed that immobilized β-galactosidase demonstrated reasonable specificity for the synthesis of galactooligosaccharides from lactose.

In the context of cellulosic ethanol production, Padilha et al. [33] proposed magnetic immobilization support with lignin coating for β-glucosidase immobilization. β-glucosidase plays a fundamental role in the course of enzymatic digestibility of lignocellulosic biomass. Green coconut fiber (GCF) was used as a lignin source after organosolv pretreatment, which was extracted from sodium hydroxide pretreatment, and was precipitated in the presence of Fe_3O_4

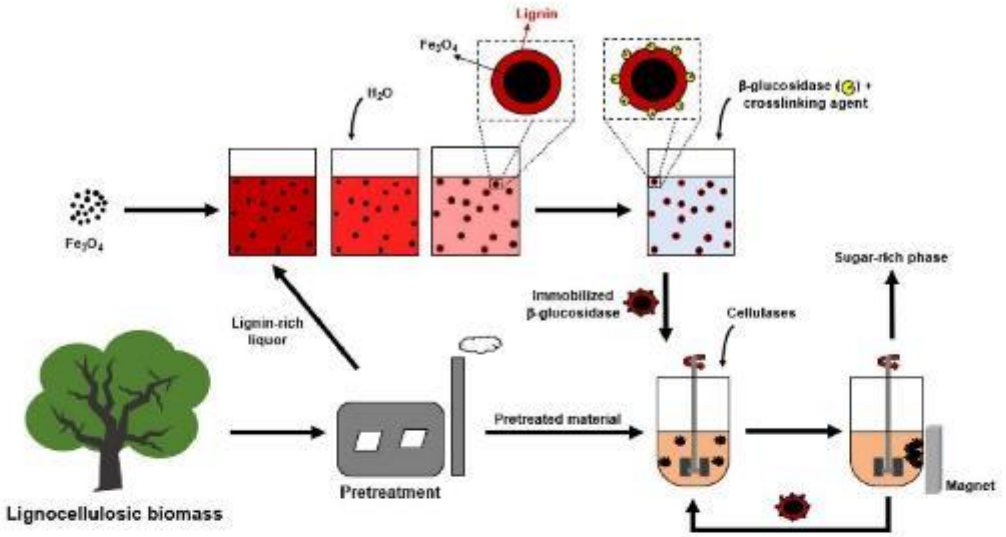


Fig. 8 Enzymatic hydrolysis assisted by β-glucosidase immobilized on a lignin/Fe_3O_4 composite. [33] Adapted from Padilha et al.

nanoparticles to obtain a nanometric composite lignin/Fe_3O_4 (see Fig. 8). The immobilization of β-glucosidase by the addition of epichlorohydrin, a covalent/crosslinking agent, showed better catalytic activity and stability than the enzyme immobilized by adsorption. In comparison with the free enzyme, β-glucosidase immobilized on lignin/Fe_3O_4 nanoparticles obtained a good digestibility performance of crystalline cellulose and organosolv pretreated GCF, presenting approximately 22 and 7 g/L reducing sugars, respectively. Since the immobilization support has a magnetic core, the recycling step was simplified by using a magnet, and the immobilized enzyme was reused three times without strongly altering the results of reducing sugars. Magnetic separation can be an attractive technology to simplify the recovery of immobilized enzymes, but some difficulties should be considered for larger scales, as noted by Jørgensen and Pinelo [227]. The need for high ethanol titers in the industrial scale forces the operation with high solid loadings; this fact can be a complicating factor since there is a decline in separation efficiency due to increased viscosity and mass loss by penetration into the biomass (in the case of nanometric enzyme immobilization supports).

Insights and Future Perspectives

The integration of lignin recovery with cellulosic ethanol technology gives hope not only for solving economic problems but also can promote environmental benefits. Looking at the consolidation of biorefinery concepts, the approaches shown in this paper should partially solve the controversial use of chemicals obtained from petroleum in the pretreatment and enzymatic hydrolysis. The approaches represent progress in reducing waste generation and reducing the carbon footprint of the whole process. The economic and environmental aspects raised throughout the papers must be investigated more deeply in future studies. We point out that most of the studies with cellulosic ethanol production assisted by lignin-based inputs have been carried out in the last five years, and there was a greater concern in extolling the originality brought by the strategy.

For example, lignin-based bio-ILs and DES are prepared from phenolic compounds, such as vanillin, p-coumaryl alcohol, syringic acid, or 4-hydroxybenzaldehyde, which are considered to be high-priced products. According to Hodásová et al. [76], the prices of phenolic compounds are estimated at $10.0–$ 110.0/kg and exceed the prices of carbon fibers ($18.0–$26.0/kg) and primal substances such as benzene ($1.0–$3.0/kg), toluene ($1.0–$2.7/kg) and xylene ($0.5–$2.2/kg). In this sense, the use of bio-ILs and lignin-based DES in pre-treatments may be problematic since the scheme aims to obtain cellulosic ethanol, whose price is

estimated at only $0.95/kg. The sale of most of the production of phenolic compounds derived from lignin is probably a more assertive decision, considering that a small fraction is destined for the preparation of bio-ILs and DES. Investigations on the recycling yield of lignin-based bio-ILs and DES and their effects are also recommended.

Among the proposals shown, we believe that there is a greater chance of success with the approaches using lignin-based activated carbon, lignin-based surfactants, and lignins as enzyme immobilization support. The addition of lignin-based activated carbon can eliminate the presence of furans and hydrolyzed phenolic compounds and can trigger the scheme to finally reach attractive ethanol titers (see Yoshioka et al. [185]). The preparation of lignin-based surfactants based and enzymes immobilized on lignin supports involves simple reactions so that steps of lignin depolymerization and separation of monomers are discarded; also, these approaches lead to reduced enzyme consumption, as mentioned in Sects. 4.3.1 and 4.3.4. Considering surfactants and enzyme immobilization supports, we still see gaps to be explored. Lignin-based surfactants showed good results in enzymatic hydrolysis, but there are no data about their effects on ethanol fermentation performance. Except Lou et al. [194], there is no study of the cytotoxicity of lignin-based surfactants on fermentation-producing yeasts or even investigations on the SScF performance. With the discovery that lignins can contribute to the cellulose breakdown by LPMO action (see Sect. 4.2.3), there is also the doubt whether lignin-based surfactants' benefits can be attributed only to the minimization of non-productive adsorption of cellulases or if there is any contribution from lignin-LPMO interaction. In Padilha et al. [33], lignin was used as a cheap matrix to immobilize β-glucosidases, a key enzyme for enzymatic synergism, but no attempt was made to immobilize multiple enzymes. Like β-glucosidases, xylanases have difficulties being recycled after enzymatic hydrolysis or SScF, and, therefore, it would be interesting to check how effective the immobilization of β-glucosidases and xylanases in lignin composites would be. Also, it is interesting to investigate whether this enzyme immobilization support limits the availability of cellulases by non-productive adsorption.

Another insight obtained from the aforementioned studies comes from the fact that the synthesis of lignin-based chemicals and materials was carried out mainly with lignins acquired on the market, among them Kraft lignins, lignosulfonates, and EHL. Understandably, the authors have chosen these technical lignins since there is a better knowledge of their chemical structures and physicochemical properties. Studies involving Kraft lignins, lignosulfonates, and EHLs provide valuable information and validate the idea of integrating lignin recovery and cellulosic ethanol production, but we cannot necessarily consider them as closed-cycle biorefineries. Kraft lignins and lignosulfonates are produced in the paper and cellulose industries and, therefore, must be purchased for the cellulosic ethanol scheme. There is also no coincidence between the sources of biomass (substrate for ethanol) and the lignin source obtained from enzymatic hydrolysis (raw material for lignin-based additives); the latter usually comes from corncobs. As reported in Sect. 3, lignin is a heterogeneous polymer whose chemical composition is strongly dependent on the biopolymer source. This fact raises the question of whether the lignins from the cellulosic ethanol scheme will have reactivity comparable to the technical lignins mentioned. This situation is more complicated for the preparation of lignin-based bio-ILs, and DES since the yields of lignin depolymerization and monomer separation are difficult to predict.

Conclusions

The establishment of sustainable schemes with lignocellulosic biomass invariably involves the enhancement of lignin. Lignin-based products can increase gains in cellulosic ethanol production and reduce dependence on petroleum products in a closed-loop biorefinery approach. Recent studies indicate that lignin derivatives can act as pretreatment agents that offer lower cost, less toxicity, and biodegradability. Residual enzymatic hydrolysis lignins can be easily converted to activated carbon to detoxify hydrolysates from lignocellulosic biomass. In addition to lignosulfonate, lignin-rich fractions can be transformed into surfactants in the presence of PEG to minimize non-productive adsorption of cellulases. The improvement in the catalytic activity of LPMO and the possibility of anchoring auxiliary enzymes should also be extolled as positive effects of lignin. Techno-economic assessment and life cycle analysis studies should be carried out in future studies to strengthen the proposed approaches. The results of approaches assisted by lignin inputs, which were observed on a laboratory scale, should also be validated on larger scales. In summary, old conceptions about lignin should be left out in the face of new evidence that appears in the literature, whether it is the fact that lignin cannot generate money or that it is just a villain in the production of cellulosic ethanol.

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Chemical pretreatment of sugarcane bagasse with liquid fraction recycling

Fernanda Leitão Vaz [a], Jennyfer da Rocha Lins [b], Bárbara Ribeiro Alves Alencar [c], Ithalo Barbosa Silva de Abreu [a], Esteban Espinosa Vidal [d], Ester Ribeiro [c], Everardo Valadares de Sá Barretto Sampaio [a], Rômulo Simões Cezar Menezes [a], Emmanuel Damilano Dutra [a,*]

[a] Research Group on Biomass Energy, Department of Nuclear Energy, Federal University of Pernambuco, 50740-540, Recife, PE, Brazil
[b] Technical School Professor Agamenon Magalhães (ETEPAM), 52050-385, Recife, PE, Brazil
[c] Interdepartmental Research Group in Metabolic Engineering, Department of Genetics, Federal University of Pernambuco, 50670-901, Recife, PE, Brazil
[d] White Biotechnology Group, Bioprocess Laboratory, Center for Strategic Technology of the Northeast (CETENE), 50.740-540, Recife, PE, Brazil
[e] Antibiotics Department (DANTI), Federal University of Pernambuco (UFPE), 50740-535, Recife, PE, Brazil

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ABSTRACT

Pre-treating lignocellulosic biomass is a major technological challenge. Recycling the liquid fraction may reduce chemical inputs and water use but may have lower efficiency. Seven chemical pre-treatments of sugarcane bagasse were compared: four acids (sulfuric, citric, phosphoric, and oxalic), two alkaline (sodium and calcium hydroxides) and an oxidative (alkaline hydrogen peroxide). The liquid fractions resulting from these pre-treatments were reused up to five times. After each pre-treatment cycle, the solid fraction was hydrolyzed with Celluclast 1.5L enzyme in a 1:10 solid-liquid ratio. Sulfuric and oxalic acids solubilized 80% of the hemicellulose over the cycles. Sodium and calcium hydroxides and hydrogen peroxide removed lignin until the fourth cycle. About 70% of lignin were removed with alkaline hydrogen peroxide. This pre-treatment lead to the highest glucose release after the enzymatic hydrolysis, above 50% in the three cycles analyzed. The best efficiency in enzymatic hydrolysis followed the order H_2O_2-alkaline > NaOH > oxalic acid > phosphoric acid > H_2SO_4 > citric acid > $Ca(OH)_2$. With the recycling, more than 62% in reagents used in the pre-treatment of sugarcane bagasse were saved, enabling an economy in the costs at this stage of the process.

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1. Introduction

The search for more sustainable technological solutions has driven an energy revolution to replace fossil fuels and their derivatives. This replacement is necessary in view of the environmental impacts on the hydrosphere, lithosphere, and atmosphere associated with the use of fossil fuels [1]. Biomass plays a fundamental role in this revolution. It is able to offer large quantities of liquid, gaseous, and solid biofuels, in addition to high value-added chemicals, and can supply up to 30% of the energy demand of the humanity by 2050 [2].

The worldwide lignocellulosic biomass production, excluding wood, is estimated to reach 2.8 billion tons [3], being 520 million tons in Brazil [4]. Sugarcane bagasse stands out for its expressive production associated with the cultivation of sugarcane to produce sugar and ethanol. The 2019/2020 Brazilian sugarcane harvest is estimated at 622 million tons, generating approximately 180 million tons of bagasse [5].

Currently, biomass is used mainly by direct burning to produce thermal and electric energy [6], with less production of consolidated biofuels, whether liquid or gaseous, such as ethanol, biodiesel, and biogas [2]. It is estimated that the annual production of these biofuels reaches 130 billion liters of ethanol and biodiesel [7] and 25 billion m^3 of biogas [2]. In addition to thermal, electrical energy and consolidated biofuels, biomass has been used to produce advanced fuels and chemical compounds with high added value through the biochemical biomass processing pathway.

* Corresponding author. Departamento de Energia Nuclear, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50740-540, Recife, Pernambuco, Brazil.
E-mail address: emmanuel.dutra@ufpe.br (E.D. Dutra).

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Examples are the production of the ABE mixture (acetone, butanol, and ethanol) [8], sugarcane diesel, 1,3-propanediol, polyhydroalkonates, and aviation biofuel [9,10].

The use of this lignocellulosic biomass to produce second-generation biofuels by the biochemical pathway generally requires several process steps: (1) pre-treatment, (2) hydrolysis, (3) fermentation, and (4) product recovery. Each stage presents technical and economic challenges, the greatest of which is pre-treatment. Pre-treatment methods aim to solubilize and separate one or more components of the plant cell wall and release them for subsequent steps of enzymatic or acid hydrolysis and fermentation or biodigestion [11]. Pre-treatment is essential due to the crystalline nature of cellulose, the physical barrier formed by lignin and the presence of complex interactions between hemicellulose and cellulose. Pre-treatment must achieve four basic objectives: (1) separate the matrix from lignin; (2) reduce cellulose crystallinity; (3) increase the amorphous fraction of cellulose; and (4) solubilize hemicellulose. These objectives aim to make cellulose more accessible to chemical and biological hydrolysis [12].

Pre-treating can be physical, chemical, biological, or combined methods [13]. Each method has advantages and disadvantages, such as the high energy consumption of the physical methods [14], the high input consumption of the chemical methods [15], and the long time required by the biological methods [16].

Chemical pre-treatments have characteristics that allow their widespread use in the pre-treatment of different types of lignocellulosic biomass [17], such as solubilization of hemicellulose in acid pre-treatments, removal of lignin in alkaline/oxidative pre-treatments, and separation of polysaccharides by ionic liquid pre-treatments [18]. Examples are the use of diluted acid [19], sodium hydroxide [20], peroxide alkaline hydrogen [21], ozone [22], and ionic liquids [23], for the pre-treatment of sugarcane bagasse.

After pre-treatment, the liquid and the solid fractions are separated, the solid fraction being the pre-treated biomass which is used in the continuation of the process and the liquid fraction may be discarded or re-used. Re-use of the liquid fraction can take advantage of the chemical reagents still available but their efficiency tend to decrease after each re-use cycle [24]. Several studies have already highlighted the importance of recycling the liquid fraction during the chemical pre-treatment of different types of lignocellulosic biomass, and have addressed how this technology can contribute to the reduction of inputs and energy in the processes [25].

Thus, the objective of this work is to compare seven types of sugarcane bagasse pre-treatments and to evaluate the use of successive recycles of the liquid fraction after each chemical pre-treatment cycle without adding or correcting the reagents, and to measure the efficiency of the enzymatic hydrolysis after each cycle.

2. Material and methods

2.1. Biomass source and processing

Sugarcane bagasse was obtained from the "Olho d'Água" sugar and alcohol plant in Pernambuco, Brazil. It was washed under running water, dried in an oven at 65 °C for 48 h, crushed with a knife mill, sieved to a 20-mesh particle size, and stored at −20 °C for later analysis of chemical composition and use.

2.2. Pre-treatments of sugarcane bagasse

2.2.1. Acids

Solutions of sulfuric (1.5%) [26], citric (5.0%), oxalic (5.0%) [27], and phosphoric acids (1.0%) [28] were used separately in the pre-treatment of sugarcane bagasse at a ratio of 10% w/v, in autoclave, at 121 °C and 1 atm for 30 min, with a reaction volume of 250 mL.

2.2.2. Alkaline

A 3% (w/v) sodium hydroxide solution was added to the sugarcane bagasse (10% w/v) and autoclaved at 121 °C and 1 atm for 30 min [24]. Calcium hydroxide was used at a ratio of 0.55 g of Ca $(OH)_2$ g^{-1} of dry biomass for a final volume of 250 mL of solution on a shaking table at 150 rpm and 60 °C for 24 h [25].

2.2.3. Oxidative

Hydrogen peroxide solution 7.5% v/v, with acidity adjusted to pH 11.5 with 5M NaOH solution [29], was added at the same ratio to bagasse biomass as already described (10% w/v) and the mixture was transferred to Erlenmeyer flasks placed on a shaking table at 150 rpm and 25 °C for 1 h.

2.3. Recycling liquid fractions

All reactions, except for that with hydrogen peroxide, were carried out in 100% semipermeable polypropylene bags, which are inert [30], to recover biomass in the most efficient way possible. As the reaction with hydrogen peroxide and lignocellulosic biomass releases a great volume of oxygen, the polypropylene bag would prevented its expansion, making this pre-treatment inefficient. Thus, the reaction was carried out in 2-L Erlenmeyer flasks, and then filtered through polypropylene fabric. The processes were carried out in triplicate.

At the end of the reaction, the mixtures were removed from the autoclave or from the stirring table, cooled and squeezed by hand (manual pressure) to recover the largest possible volume of solution, separating the liquid and the solid fractions. The solid fractions were washed until reaching pH 7 and placed in an oven at 65 °C to dry. After drying, the solid fractions were weighed to determine the proportion of recovery. The liquid fractions were used for a new pre-treatment cycle. The recovered volumes were measured to calculate the amount of new bagasse biomass to be mixed to maintain the 1:10 ratio of biomass/volume already used in the first pre-treatment cycle.

In the new cycle, the liquid fractions obtained from the previous cycle were used to react with the new amounts of bagasse without any addition of chemical reagents and following all procedures already described above. Since the volumes of the liquid fractions decrease after each cycle, after some cycles the remaining solution volumes would be too small to be worthwhile to run a new cycle. Based on a previous work [25], five cycles (four repetitions of the pre-treatment) were expected to be run. However, in the cases of the citric and phosphoric acids and the calcium hydroxide, only four cycles were performed because the volume of liquid fraction resulting from the previous cycle was too small. Since the number of possible cycles is an inherent characteristic of the pre-treatment solutions, they were compared considering their total cycles. To analyze the technical feasibility of using the applied pre-treatments, three recycling parameters were measured along the cycles: pH, volume of solution recovered, and biomass recovered, as established by Alencar et al. [30].

To calculate biomass recovery, bags containing the biomass were dried at 65 °C for 24 h to determine the recovered biomass content, according to Equation (1):

$$\text{Biomass recovery } (\%) = \frac{Wbb - Wbag}{Wbio} * 100 \qquad \text{Eq. 1}$$

Where *Wbb* is the mass of polypropylene bags containing the sugarcane bagasse, *Wbag* is the mass of the empty polypropylene bags, and *Wbio* is the biomass mass.

2.4. Characterization of biomass

The raw bagasse and the pre-treated sugarcane bagasse were used to determine the levels of extracts, polysaccharides (cellulose and hemicellulose), lignin, and ash using the method proposed by Van Soest [31]. Neutral and acid detergents were used to quantify the parameters of NDF (neutral detergent fiber) and ADF (acid detergent fiber), and digestion with sulfuric acid 72% v/v was used to quantify the ADL (acid detergent lignin).

The percentages of delignification or hemicellulose solubilization efficiency for pre-treated biomass were determined using Equation (2):

$$E\,(\%) - 100 - \left[\left(\frac{Pc}{Pin}\right) * 100\right] \qquad \text{Eq. 2}$$

Where E is the percentages of delignification or hemicellulose solubilization efficiency, Pc is the percentage of lignin or hemicellulose remaining in the pre-treated sample of the corresponding cycle, and Pin is the lignin or hemicellulose content present in the fresh biomass.

2.5. Calculation of input reduction

The reduction in the use of chemical reactors was estimated by recycling the liquid fraction for each type of pre-treatment. This reduction was calculated based on the conditions of the initial pre-treatment reaction and is described in Equation (3):

$$Rr(\%) = \left(1 - \frac{Nr}{Nwr}\right) * 100 \qquad \text{Eq. 3}$$

Where Rr is the percentage of reagents reduction, N is the H_2O or reagent volume or total NaOH mass used in the tests with recycling, and Nw is the H_2O or reagent volume or mass used only in first cycle tests. The reagent volume used in the pre-treatment was also determined in relation to the pre-treated biomass mass, according to Equation (4):

$$V = \frac{Vhp\ (mL)}{m\ (g)} \qquad \text{Eq. 4}$$

Where Vhp is the reagent volume used for tests with or without solution recycling, and m is the pre-treated biomass in this solution.

2.6. Enzymatic hydrolysis

The pre-treated biomass was subjected to enzymatic hydrolysis in 125-mL Erlenmeyer flasks containing 2 g of pre-treated biomass, with an enzymatic load of 10 FPU/g of bagasse of the enzyme Celluclast 1.5L Novozymes, and citrate buffer 50 mmol/L pH 4.8, for a reaction volume of 20 mL. The Erlenmeyer flasks were incubated on a rotating shaker at 150 rpm and 50 °C for 48 h. After this period, the material was centrifuged at 12,000 rpm for 5 min at 20 °C. The supernatant was frozen for subsequent quantification of carbohydrates. The conversion of cellulose and hemicellulose was determined by Equation (5):

$$C = \left(\frac{C}{Sl * \left(\frac{P}{100}\right) * Cf}\right) * 100 \qquad \text{Eq. 5}$$

Where C is the percentages of efficiency conversion of cellulose or hemicellulose into glucose or xylose, Sl is the solid load of biomass (g/L), P is the percentage of cellulose or hemicellulose in the *in natura* bagasse biomass, and Cf is the conversion factor of cellulose or hemicellulose into glucose (1,11) or xylose (0,88).

2.7. Analytical methods

The contents of carbohydrates and ethanol were determined by high performance liquid chromatography (HPLC; Shimadzu Corporation – Kyoto, Japan) in an AMINEX-IRON HPX-87H$^+$ exclusion column (Bio-Rad, Hercules, CA, USA), and detector by index of refraction (RID; Shimadzu, RID 10^a). The mobile phase was H_2SO_4 (5 mM) at a flow of 0.6 mL min^{-1} and kept in an oven at 60 °C.

3. Results and discussion

3.1. Pre-treatment liquid fraction recycling variables

In general, there was a tendency to increase the pH in pre-treatments with sulfuric, citric, oxalic, and phosphoric acids (Fig. 1A). The greatest variation over the cycles occurred with sulfuric acid ($\Delta_{pH} = 1.0$). Sulfuric and oxalic acids acted within the pH range of 0.4–1.42 while the organic acids (citric and oxalic) acted within a broader range (2.0–2.8).

The alkaline (sodium and calcium hydroxides) and the oxidative solutions (hydrogen peroxide corrected with NaOH) had different behaviors in relation to pH (Fig. 1B). Those with NaOH varied by up to four points on the logarithmic scale (pH 14.5–10.5) and that of $Ca(OH)_2$ varied only 0.5, a variation similar to that of the organic acids. The two solutions with NaOH (H_2O_2 with NaOH and NaOH alone), at the end of cycle 5, had almost the same pH value (about 10.7). The pH reduction during the pre-treatment occurs due to the consumption of hydroxyl radicals resulting from the decomposition of the hydroperoxide anion [32] in the pre-treatment with hydrogen peroxide and in the consumption of hydroxyls that react with lignin in the alkaline pre-treatment [29].

The volumes of pre-treatment solutions recovered over the cycles gradually decreased, especially those of the citric and phosphoric acids, with which only four cycles were run, since at the end of the fourth cycle only 40 mL remained, a volume too low to justify a fifth cycle (Fig. 1C). This reduction is due to the property of organic materials to absorb aqueous solutions [33]. The recovered volume is an important parameter, since it limits the amount of biomass that can be pre-treated by recycling, since the biomass/volume ratio must be constant to maintain the load of solids in the pre-treatment [30].

The patterns of bagasse biomass recovery varied both among pre-treatments and along the cycles (Fig. 1D). Even pre-treatments with the same group of solutions, acidic or alkaline, differed. The biomass recoveries with oxalic acid were around 70% in the first four cycles and those with NaOH varied from 60 to 82%, being greater in the intermediate cycles. Therefore, the cation of the solution interferes with the recovery patterns of the pre-treatments. This is the most important parameter, as it indicates whether the pre-treatment was effective or not in removing the fractions of interest, which are hemicellulose for acids and lignin for alkali and oxidative solutions.

Bagasse *in natura* (without pre-treatment) had 41% cellulose, 30% hemicellulose, and 15% lignin (Table 1), values similar as those found in the literature: Rocha et al. [34] found 37–46% for cellulose, 26–30% for hemicellulose, and 19–26% for lignin. The lower value of lignin in the present work may have been due to the difference between the characterization methods. This work followed the methodology of Van Soest [37] and Rocha et al. [40] developed their own methodology.

Sulfuric and oxalic acids removed more hemicellulose than the other solutions. Both removed more than 75% over the cycles,

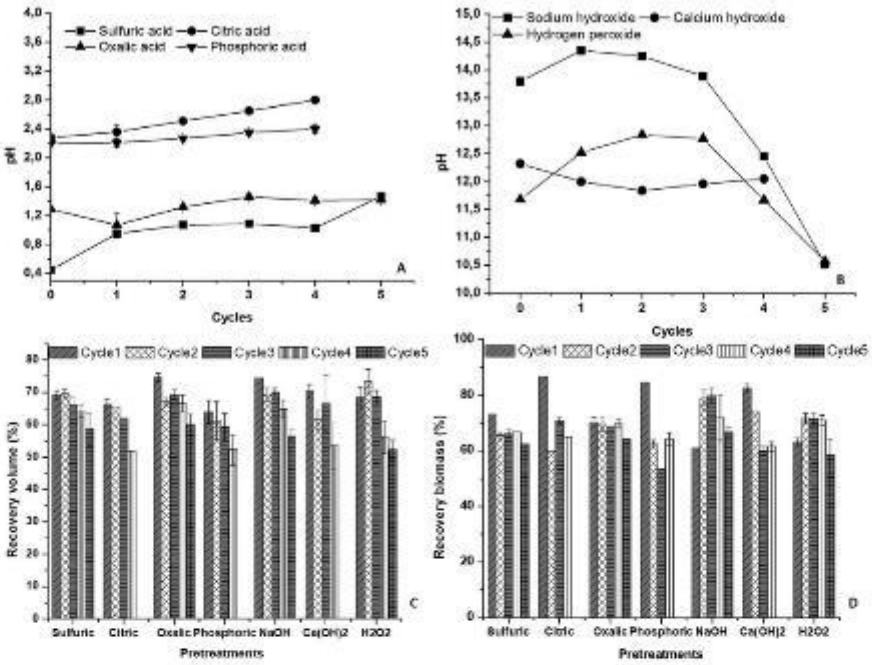

Fig. 1. Parameters of pre-treatments of sugarcane bagasse re-using the liquid fraction of the previous cycle. A) pH of the liquid fraction of acid solutions throughout the cycles; B) pH of the liquid fractions of basic/oxidative solutions throughout the cycles; C) Volume of recovered liquid fractions after each cycle; D) Bagasse biomass recovered after each cycle.

Table 1
Sugarcane biomass characterization after chemical pre-treatments of an initial bagasse portion and of new portions pre-treated re-using the liquid fraction of the previous cycle.

Pre-treatments	Contents (%)	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
Sulfuric acid	Cellulose	55.51 ± 1.87	56.68 ± 2.82	57.52 ± 1.90	55.86 ± 1.36	56.80 ± 1.37
	Hemicellulose	5.57 ± 1.12	4.30 ± 0.54	5.31 ± 0.44	4.85 ± 0.62	3.75 ± 0.33
	Lignin ADL	26.48 ± 2.66	26.45 ± 3.25	24.31 ± 2.50	25.08 ± 2.53	25.33 ± 1.65
	Ashes	4.88 ± 0.69	4.47 ± 1.00	3.00 ± 1.02	3.65 ± 1.03	3.35 ± 0.47
Citric acid	Cellulose	48.51 ± 1.49	48.53 ± 1.95	47.58 ± 1.08	48.29 ± 1.06	
	Hemicellulose	22.07 ± 1.02	21.97 ± 0.71	23.63 ± 0.70	23.60 ± 0.63	
	Lignin ADL	15.93 ± 3.28	17.45 ± 2.35	15.47 ± 1.89	15.59 ± 1.03	
	Ashes	3.16 ± 1.05	3.47 ± 0.91	2.82 ± 0.68	3.06 ± 0.17	
Oxalic acid	Cellulose	54.29 ± 2.90	55.71 ± 0.20	55.27 ± 2.20	56.19 ± 2.92	52.83 ± 1.03
	Hemicellulose	8.47 ± 0.80	7.11 ± 0.32	6.55 ± 1.44	7.27 ± 0.34	6.55 ± 0.23
	Lignin ADL	23.59 ± 3.48	22.39 ± 1.24	23.33 ± 4.27	21.25 ± 2.86	24.22 ± 1.26
	Ashes	3.81 ± 1.43	3.35 ± 0.06	4.13 ± 1.54	3.56 ± 0.68	4.07 ± 0.54
Phosphoric acid	Cellulose	50.25 ± 0.60	48.26 ± 1.31	46.46 ± 3.87	48.14 ± 0.36	
	Hemicellulose	23.60 ± 6.94	19.57 ± 5.29	26.05 ± 3.21	21.61 ± 0.39	
	Lignin ADL	17.70 ± 1.07	18.29 ± 1.88	17.51 ± 3.26	18.48 ± 2.46	
	Ashes	3,88 ± 0.44	3,38 ± 0.91	3.45 ± 1.52	3.78 ± 0.96	
Sodium hydroxide	Cellulose	67.36 ± 1.87	66.80 ± 1.31	59.01 ± 5.53	54.27 ± 2.46	50.33 ± 1.28
	Hemicellulose	11.17 ± 0.28	13.12 ± 1.65	17.78 ± 2.38	19.39 ± 1.51	20.38 ± 0.85
	Lignin ADL	11.42 ± 1.70	10.75 ± 1.44	11.37 ± 2.59	14.84 ± 1.48	16.12 ± 0.68
	Ashes	4,57 ± 0.62	3,41 ± 0.78	2.93 ± 0.68	3.89 ± 0.35	3.16 ± 0.17
Calcium hydroxide	Cellulose	50.65 ± 1.70	44.15 ± 0.73	45.97 ± 1.22	44.91 ± 0.89	
	Hemicellulose	17.71 ± 2.79	25.93 ± 2.19	27.70 ± 1.73	29.86 ± 0.58	
	Lignin ADL	10.58 ± 1.80	12.87 ± 0.86	12.03 ± 0.57	15.85 ± 0.97	
	Ashes	2.62 ± 0.62	2.38 ± 0.30	1.67 ± 0.34	2.38 ± 0.45	
Hydrogen peroxide	Cellulose	70.70 ± 1.32	64.03 ± 2.39	57.81 ± 1.20	49.98 ± 3.75	46.08 ± 0.61
	Hemicellulose	21.00 ± 2.64	18.27 ± 2.52	25.11 ± 1.80	30.02 ± 4.76	32.62 ± 1.00
	Lignin ADL	4.61 ± 1.04	11.08 ± 0.83	11.09 ± 1.17	15.27 ± 1.58	13.85 ± 1.44
	Ashes	2.82 ± 0.91	3.50 ± 0.13	2.19 ± 0.61	2.45 ± 0.48	2.58 ± 0.69

reaching more than 80% in the first cycle (Fig. 2). Phosphoric acid removed less than 30%. Therefore, regarding hemicellulose removal the first two are indicated for the pre-treatment of sugarcane bagasse biomass. The similarity of removal with the inorganic (sulfuric) or organic (oxalic) acids shows that the nature of the acid does not directly affect pre-treatment efficiency. Rocha et al. [35] obtained a removal of more than 90% in a single cycle pre-treating bagasse with 1% w/v sulfuric acid and 1% v/v acetic acid and applying a 10% w/v solid load. Although the combined action of both acids led to a greater efficiency in removing hemicellulose than that of the present study, the increase was relatively small and the process costs were higher, especially without recycling the solutions. Chandel et al. [36] also obtained the removal of more than 90% of bagasse hemicellulose pre-treating it with 3.5% w/v oxalic acid at 160 °C, for 20 min and 10% solids load. The higher concentrations of the acid and the higher temperature may justify the greater removal compared to that of the present study. However, Awci et al. [33] obtained a greater removal of corn straw hemicellulose with phosphoric acid (68%) using 0.78% v/v of acid at 161.81 °C, for 9.78 min. This removal contrasts with that of 30% obtained by Amnuaycheewa et al. [32] from rice straw using 12.21% v/v acid at 126.19 °C for 1 h. This comparison and the work of Chandel et al. [42] emphasize the importance of high temperatures in increasing hemicellulose removal efficiency.

The removal of lignin by the oxidative-alkaline pre-treatment (H_2O_2, corrected with NaOH) was more efficient than that with the alkaline pre-treatment, mainly in the first cycle (70%), decreasing to around 35% in the second cycle (Fig. 3). This decrease indicates that the radical products of the decomposition of H_2O_2, in the presence of NaOH, are more effective in removing lignin especially in the first cycle than the radicals resulting from the ionization of bases. However, the oxidative-alkaline solution was the most unstable over the five cycles. The removals with NaOH and Ca $(OH)_2$ were similar (30%) and both lower than that with the oxidative pre-treatment since the first cycle. Delignification between 50 and 90% has been reported [37] and the variation can be attributed to the type of pre-treated biomass and the conditions of pre-treatment, i.e., different temperatures, peroxide concentrations and applied solids loads [38].

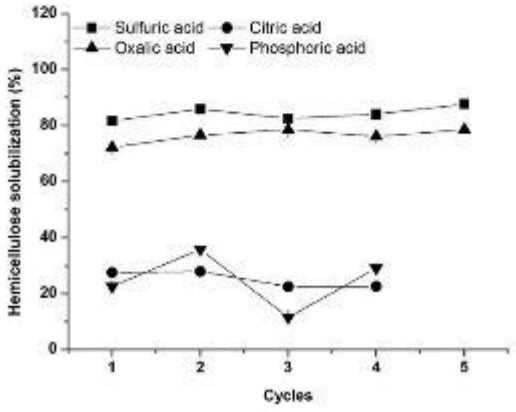

Fig. 2. Hemicellulose solubilization with acid pre-treatments of an initial sugarcane bagasse portion and of new portions pre-treated re-using the liquid fraction of the previous cycle.

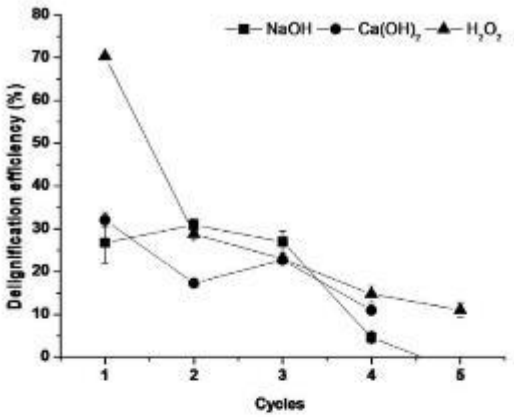

Fig. 3. Delignification efficiency with basic/oxidative pre-treatments of an initial sugarcane bagasse portion and of new portions pre-treated re-using the liquid fraction of the previous cycle.

3.2. Saving inputs during recycling

The quantities of reagents used in the pre-treatments during the five cycles were relatively small, reaching 1.37 g per gram of biomass with hydrogen peroxide (Table 2). Recycling resulted in reductions of more than 50% of reagents and water in all pre-treatments, corroborating previous works, which obtained reductions of 60% of reagents and water by recycling the alkaline hydrogen peroxide solution in the pre-treatment of corn straw [30]. In contrast, Cheng et al. [28] reported reductions of 26% in water and 40% in reagents after three pre-treatment cycles of sugarcane bagasse with alkaline solution (NaOH) supplemented at low concentrations (0.6%) of H_2O_2. These lower savings can be attributed to the low concentration of sodium hydroxide used (0.092 g/g of biomass). By comparing with pre-treatments without recycling (Table 2), there is greater reagent savings that can be obtained with recycling, which proves to be a promising alternative to reduce expenses with the pre-treatment stage.

3.3. Quantification of carbohydrates in the net fraction of pre-treatments

At the end of each pre-treatment cycle, glucose and xylose were detected only in the liquid fractions of pre-treatments with sulfuric and oxalic acid (Fig. 4). The concentrations increased throughout the cycles due to the indirect removal of amorphous cellulose during the process and its accumulation in the liquid fraction with each new cycle. This occurred because new portions of fresh biomass were treated with each new cycle. At the end of the cycles, approximately 10 g L^{-1} of glucose accumulated in the pre-treatments with oxalic acid and 8 g L^{-1} in those with sulfuric acid.

The accumulations of xylose after the fifth cycle were 68 and 61 g L^{-1}, respectively (Fig. 5). Aguilar et al. [49] obtained 10.7 g L^{-1} of xylose in experiments at the same temperature as the present study, but using 2% v/v sulfuric acid and 20 min of pre-treatment, and 20.4 g L^{-1} when the reaction time was increased to 40 min. Comparing only the first cycle, the lower xylose concentration in the present study (12.3 g L^{-1}) than that obtained by Aguilar et al. [39] probably resulted from the intermediate pre-treatment time (30 min) and the lower acid concentration (1.5% v/v).

Table 2
Comparison of the reagents used in the pre-treatment of sugarcane bagasse and the reduction of inputs, when recycling the pre-treatment liquid fraction.

Pre-treatments		Inputs reduction (%)	Present work (g/g)[a]	Researches without using recycle (g/g)[b]
H_2SO_4		62.68	0.11	0.29[a]
Oxalic acid		64.03	0.18	0.50[b]
Phosphoric acid		54.55	0.05	0.10[c]
Citric acid		57.63	0.21	0.50[b]
$Ca(OH)_2$		57.63	0.23	0.55[d]
NaOH		64.79	0.11	0.20[e]
Alkaline hydrogen peroxide solution (AHP)	H_2O_2	62.12	1.31	2.32[f]
	NaOH	62.12	0.15	0.27[f]

[a] Chen et al., 2012.
[b] Amnuaycheewa et al., 2016.
[c] Avci et al., 2013.
[d] Rabelo et al., 2011.
[e] Sindhu et al., 2014.
[f] Rabelo et al., 2014.
[a] Amount of reagents used for pretreatment of sugarcane bagasse, in the present work, with the recycling of the pre-treatment liquid fraction, in the ratio of reagent/g of biomass.
[b] Amount of reagents used for pretreating sugarcane bagasse, reported by other authors, in the ratio of reagent/g of biomass.

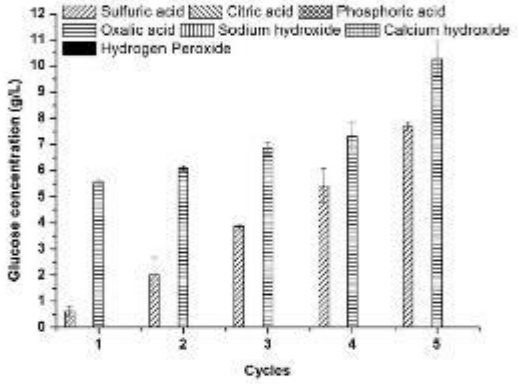

Fig. 4. Glucose concentration in the liquid fraction of pre-treatments of an initial sugarcane bagasse portion and of new portions pre-treated re-using the liquid fraction of the previous cycle (no glucose was found in the fractions obtained with citric and phosphoric acids, sodium and calcium hydroxides and hydrogen peroxide).

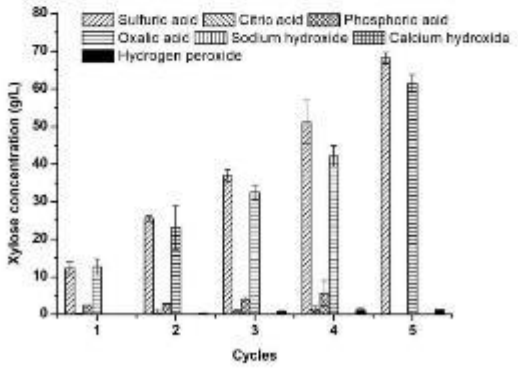

Fig. 5. Xylose concentration in the liquid fractions of pre-treatments of an initial sugarcane bagasse portion and of new portions pre-treated re-using the liquid fraction of the previous cycle (no xylose was found in the fractions obtained with citric acid, sodium and calcium hydroxides).

The accumulations of glucose and xylose throughout the cycles are important to increase the productivity of ethanol in the fermentation [40] or in other industrial processes that use these sugars, mainly xylose for the production of xylitol [41], succinic acid [42], gluconic and xylonic acids [43].

3.4. Efficiency of enzymatic hydrolysis

The greatest efficiency of enzymatic hydrolysis occurred after the pre-treatment with hydrogen peroxide, resulting in a cellulose conversion of 97% in the first cycle, and greater than 50% in the following two cycles (Fig. 6). Alencar et al. [30] reported the same behavior using conditions similar to those in this study but treating corn straw. The pre-treatment with sodium hydroxide reached 68% cellulose conversion in the first cycle and above 50% in the two subsequent cycles, as obtained by Rabelo et al. [25], using the same conditions as the present study. In contrast, the pre-treatment with calcium hydroxide proved to be inefficient as it resulted in less than 10% conversion. In the literature, better efficiencies with this pre-treatment have been obtained in experiments carried out at higher temperatures [44].

Among the acid pre-treatments, the greatest conversion of cellulose occurred with phosphoric acid, reaching 71% in the first

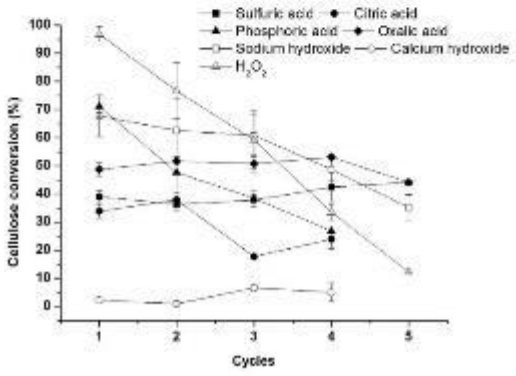

Fig. 6. Cellulose to glucose conversion of pre-treatments of an initial sugarcane bagasse portion and of new portions pre-treated re-using the liquid fraction of the previous cycle.

cycle, but with conversions below 50% in the subsequent cycles. Conversions with sulfuric and oxalic acids were around 40% and 50%. The result of the first cycle with phosphoric acid contrasts with the greater removal of hemicellulose obtained with sulfuric and oxalic acids. This indicates that chemical characterization alone is not sufficient to predict the efficiency of a pre-treatment, and that it is necessary to confirm the effectiveness of the process with hydrolysis. Despite the lower efficiency in the first cycle, pre-treatments with sulfuric and oxalic acids deserve attention because they maintained constant conversions in all five cycles. This shows a great potential of these pre-treatments with the reuse technique, which can result in savings on reagents. Amnuaycheewa et al. [32] compared several acid pre-treatments of rice straw and obtained better results using oxalic and sulfuric acids. However, they did not tested phosphoric acid.

The best hemicellulose into xylose conversions were obtained by pre-treatments with sodium hydroxide and hydrogen peroxide, reaching more than 90% conversion in the first cycle and remaining above 50% in the first three cycles (Fig. 7).

The best conversions with the acid pre-treatments were obtained using sulfuric (63%) and oxalic acids (56%), and it must be highlighted that this conversion was maintained throughout the five cycles analyzed. In the review by Dutra et al. [38], hemicellulose conversions (54–81%) were lower than those of the present study. However, they were carried out with other biomasses, such as rice husks [45] and microalgae [46].

4. Conclusions

The recycling of the liquid fractions in chemical pre-treatments was efficient with all pre-treatments, except that with calcium hydroxide. The greatest efficiency in the first cycle was obtained with hydrogen peroxide added with NaOH, followed by NaOH alone. Among the acid pre-treatments, the greatest efficiency was obtained in the pre-treatment with phosphoric acid. Sulfuric and oxalic acids also resulted in adequate efficiencies because they maintained constant conversions throughout the five cycles, allowing the reuse of the same solution for more cycles. The order of pre-treatments according to their efficiencies of enzymatic hydrolysis were H_2O_2-alkaline > NaOH > oxalic acid > phosphoric acid > H_2SO_4 > citric acid > $Ca(OH)_2$. With the recycling, more than 62% in reagents and water used in the pre-treatment of sugarcane bagasse were saved, enabling an economy in the costs at this stage of the process.

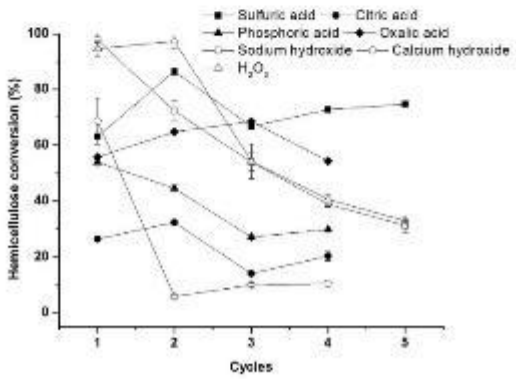

Fig. 7. Hemicellulose to xylose conversion of pre-treatments of an initial sugarcane bagasse portion and of new portions pre-treated re-using the liquid fraction of the previous cycle.

CRediT authorship contribution statement

Fernanda Leitão Vaz: Conceptualization, Methodology, Writing – review & editing. **Jennyfer da Rocha Lins:** Data curation, Methodology. **Bárbara Ribeiro Alves Alencar:** Conceptualization, Methodology, Writing – review & editing. **Ithalo Barbosa Silva de Abreu:** Conceptualization, Methodology, Formal analysis. **Esteban Espinosa Vidal:** Visualization, Supervision. **Ester Ribeiro:** Writing – review & editing, Investigation. **Everardo Valadares de Sá Barretto Sampaio:** Writing – review & editing, Funding acquisition. **Rômulo Simões Cezar Menezes:** Writing – review & editing, Supervision, Funding acquisition. **Emmanuel Damilano Dutra:** Conceptualization, Validation, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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