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PRODUÇÃO DE ETANOL DE SEGUNDA GERAÇÃO E DE ÁCIDO LÁCTICO A PARTIR DA CASCA DE ARROZ E DO CAPIM-ELEFANTE UTILIZANDO PRÉ-TRATAMENTO A VAPOR E HIDRÓLISE ENZIMÁTICA

SHEILA MONTIPÓ

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção de grau de Doutor em Biotecnologia.
Orientadora: Profª. Drª. Marli Camassola
Coorientador: Prof. Dr. Ayrton F. Martins
Coorientadora: Drª. Mercedes Ballesteros P.

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TESE APROVADA EM 25 DE AGOSTO DE 2017.

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2G etanol – etanol de segunda geração
AA – ácido acético
AFEX – explosão de fibras com amônia (*ammonia fibre expansion*)
AL – ácido láctico
BAL – bactérias ácido-lácticas
CA – casca de arroz
CE – capim-elefante
CGEE – Centro de Gestão e Estudos Estratégicos
CONAB – Companhia Nacional de Abastecimento
DCCR – delineamento composto central rotacional
FAO – Organização das Nações Unidas para Alimentação e Agricultura (*Food and Agriculture Organization of the United Nations*)
GAIN Report – Relatório da Rede de Informação Agrícola Global (Global Agricultural Information Network Report)
G_{WIS} – glicose contida no WIS
HPLC – cromatografia líquida de alta eficiência (*high performance liquid chromatography*)
INPI – Instituto Nacional da Propriedade Industrial
LC-MS/TOF – cromatografia líquida acoplada à espectrometria de massas por tempo-de-vôo (*liquid chromatography with time-of-flight mass spectrometry*)
MEV – microscopia eletrônica de varredura
MRS – Mann, Rogosa & Sharpe
P&D – Pesquisa & Desenvolvimento
PLA – poli (ácido láctico) (*poly (lactic acid)*)
SHF – hidrólise e fermentação separadas (*separate hydrolysis and fermentation*)
SSF – sacarificação e fermentação simultâneas (*simultaneous saccharification and fermentation*)
UFC – unidade de formação de colônias
USDA – Departamento de Agrigultura dos Estados Unidos (*United States Department of Agriculture*)
WIS – sólidos insolúveis em água (*water insoluble solids*)
X_{LIQ} – xilose contida no licor
YPD – extrato de levedura-peprona-dextrose (*yeast extract-peptone-dextrose*)

RESUMO

A casca de arroz (CA) e o capim-elefante (CE) são matérias-primas lignocelulósicas abundantes no sul do Brasil, sendo fontes disponíveis para a conversão a inúmeros insumos químicos com valor agregado e biocombustíveis. Neste estudo, ambas as biomassas foram pré-tratadas por explosão a vapor não-catalisada, seguido de hidrólise, com o intuito de obter teores elevados de hexoses nos sólidos insolúveis em água (*water insoluble solids* – WIS) e pentoses no licor, para posterior fermentação a etanol e ácido láctico (AL), respectivamente. Assim, o efeito das variáveis temperatura (181 a 229 °C para a CA e 178 a 212 °C para o CE) e tempo (1,6 a 11,4 min para a CA e 5,9 a 10,1 min para o CE), utilizadas nos pré-tratamentos, foram avaliadas através de um delineamento experimental. De modo geral, dentre os distintos pré-tratamentos executados, aqueles envolvendo o uso da CA favoreceram a recuperação da hemicelulose contida no pré-hidrolisado, enquanto que aqueles envolvendo o uso do CE foram mais susceptíveis à recuperação da celulose contida no WIS. Adicionalmente, foi possível verificar que condições intermediárias de temperatura e períodos prolongados de tempo conduziram à obtenção de teores balanceados de glicose no material pré-tratado e de xilose no filtrado, em um único experimento. Pré-tratamentos posteriores com o CE, conduzidos com agente catalisador ácido, evidenciaram uma melhora no rendimento dos açúcares de interesse em cada uma das frações-alvo. Para as hidrólises e fermentações seguintes, a condição otimizada de 205 °C, por 11,5 min, foi usada para o pré-tratamento da CA; por sua vez, 190 °C, por 5 min, foi empregada para o pré-tratamento do CE, usando-se 2% (*m/m*) de ácido sulfúrico. Para ambos os materiais pré-tratados, ensaios de hidrólise enzimática indicaram as vantagens em empregarem-se cargas de sólidos elevadas, resultando solução mais concentrada em glicose, favorecendo a subsequente produção de etanol. Assim, os WIS foram submetidos à hidrólise usando elevada carga combinada com diferentes doses de enzimas. Ensaios empregando carga de 20% (*m/v*) de sólidos, aliados a uma dose de enzima de 20 FPU g^{-1} substrato, beneficiaram a conversão de celulose em glicose, liberando 33,68 g L^{-1} (CA) e 95,57 g L^{-1} (CE) de glicose. Estratégias distintas de fermentação foram empregadas visando-se à produção de etanol: pré-hidrólise, hidrólise e fermentação separadas, e, sacarificação e fermentação simultâneas, sendo que esta última incrementou globalmente a produtividade do processo. A produção de etanol por *Saccharomyces cerevisiae* CAT-1 foi de 19,17 g L^{-1} (CA) e 42,25 g L^{-1} (CE) após 72 h de cultivo, empregando-se WIS como substrato. Ainda, os filtrados foram destoxificados e empregados como substrato na produção de AL por *Lactobacillus buchneri* NRRL B-30929. No caso específico da CA explodida, o licor foi previamente submetido à hidrólise com um complexo enzimático produzido pelo fungo *Penicillium echinulatum* S1M29. Já o pré-tratamento catalisado para o CE atuou também como uma hidrólise ácida. Logo, os açúcares totais de cada um dos licores foram consumidos pela bactéria ácido-láctica, produzindo-se uma mistura racêmica de DL-AL, totalizando 12,69 g L^{-1} (CA) e 13,35 g L^{-1} (CE) após 30 h de cultivo. Os dados resultantes indicam que tanto a CA quanto o CE são potenciais recursos renováveis, que podem gerar bioprodutos da plataforma de *building blocks* com base em pentoses e, ao mesmo tempo, produzir etanol de segunda geração a partir de hexoses. Ademais, a possível inserção destas matérias-primas, de baixo custo de mercado, pode fomentar a geração de processos com inovação tecnológica, contribuindo, além disso, para a redução de problemas de caráter ambiental.

Palavras-chave: casca de arroz; capim-elefante; explosão a vapor; etanol; ácido láctico.

ABSTRACT

Rice husk (RH) and elephant grass (EG) are abundant lignocellulosic raw materials in the South of Brazil and a feasible resource that can be converted into a range of value-added chemicals and biofuels. In this study, both biomasses were investigated by uncatalyzed steam explosion followed by hydrolysis, in order to obtain a satisfactory content of C6-sugar in the water insoluble solids (WIS) and C5-sugar in the liquor to subsequent fermentation aiming ethanol and lactic acid (LA), respectively. Thus, the effect of the variables temperature (181 to 229 °C for RH and 178 to 212 °C for EG) and residence time (1.6 to 11.4 min for RH and 5.9 to 10.1 min for EG) employed in the pretreatments was evaluated by means of experimental design. Altogether, among the different pretreatments performed, those involving the use of RH favoured the recovery of hemicellulose contained in the liquor, whereas those involving the use of EG were more susceptible to the recovery of cellulose contained in the WIS. In addition, it was possible to verify that intermediate temperature conditions and prolonged periods of time led to the obtaining of balanced contents of glucose in the pretreated material and xylose in the filtrate, in a sole experiment. Further pretreatments with EG, conducted with the aid of an acid catalyst, have shown an improvement in the yield of the sugars of interest in each of the target fractions. For the following hydrolysis and fermentations, the optimized condition of 205 °C for 11.5 min was employed for RH pretreatment; in turn, the condition of 190 °C for 5 min was employed for EG pretreatment, which was impregnated with 2% (*w/w*) of sulfuric acid. For both pretreated feedstocks, enzymatic hydrolysis assays indicated the advantages in the use of high solids loading, resulting in a more concentrated glucose and promoting the subsequent ethanol production. Thus, the WIS was subjected to hydrolysis using high solids consistency arranged with distinct doses of enzymes. Assays employing 20% (*w/v*) solids loading and an enzyme dosage of 20 FPU g^{-1} substrate augmented the conversion of cellulose into glucose, releasing 33.68 g L^{-1} (RH) and 95.57 g L^{-1} (EG). Different fermentation strategies were employed aiming ethanol production: prehydrolysis, separate hydrolysis and fermentations, and simultaneous saccharification and fermentation; the latter of which increased the productivity of the overall process. Ethanol production by *Saccharomyces cerevisiae* CAT-1 reached 19.17 g L^{-1} (RH) and 42.25 g L^{-1} (EG), after 72 h of cultivation, using WIS as substrate. Filtrates were detoxified and used as a substrate for the production of LA by *Lactobacillus buchneri* NRRL B-30929. In the specific case of steam-exploded RH, the liquor was previously subjected to hydrolysis with an enzymatic complex produced by *Penicillium echinulatum* S1M29 strain. Already the pretreatment catalyzed for the EG also acted as an acid hydrolysis. Therefore, the total sugars belonging to each of the liquors were consumed by LA bacteria, and a racemic blend of DL-LA was produced, totaling 12.69 g L^{-1} (RH) and 13.35 g L^{-1} (EG), after 30 h of cultivation. The findings of this study suggest that both RH and EG are potential renewable resources which can generate bioproducts from C5-sugars, while producing second generation ethanol from C6-sugars. Likewise, the possible insertion of these raw materials, with low market cost, can foment the generation of processes linked with technological innovation, also contributing to the reduction of environmental problems.

Keywords: rice husk; elephant grass; steam explosion; ethanol; lactic acid.

1. INTRODUÇÃO

A preocupação com a minimização dos impactos ambientais diretos e indiretos, decorrentes do uso intensivo de combustíveis fósseis, aliada ao agravamento da crise energética e ao aumento do preço do petróleo, tem conduzido à investigação de novas fontes de energia e de matérias-primas para a indústria. A biomassa, em especial aquela de composição lignocelulósica, disponível e renovável, é a substituta natural para o petróleo, e, se completamente aproveitada, pode ser usada na geração de insumos químicos, potência e energia, além de mitigar a crescente poluição atmosférica.

De acordo com o Centro de Gestão e Estudos Estratégicos (CGEE, 2010), a utilização de biocombustíveis é estimada como crescente a taxas de 10% até 2030, mesmo que ainda persista algum debate sobre o tipo e a forma de produção dos mesmos. Igualmente, os demais produtos que integram a chamada *white biotechnology* também apresentam projeções de crescimento expressivo. Dentre os combustíveis renováveis da bioenergia moderna estão o etanol, o biodiesel e o biogás. O etanol e o biodiesel podem ser usados como combustíveis para transporte, sendo o etanol um importante insumo na indústria química (Yuan *et al.*, 2008).

A produção biotecnológica de etanol empregando biomassas lignocelulósicas é um empreendimento promissor, figurando, no âmbito da química e da engenharia verde, como tecnologia sustentável. De modo geral, as publicações envolvendo biomassas apontam um aproveitamento regional de produtos, constatando-se publicações científicas sobre o uso de subprodutos advindos do processamento do milho, nos EUA, e da cana-de açúcar, no Brasil. Porém, além da cultura da cana-de-açúcar, concentrada na região sudeste do País, há as culturas de arroz e de capim-elefante, largamente cultivadas no Estado do Rio Grande do Sul (RS). A casca de arroz, advinda do processo de beneficiamento do grão, resulta em um resíduo agroindustrial sem aplicação direta na indústria, enquanto que o capim-elefante é

considerado uma herbácea de elevada produtividade (Gallego *et al.*, 2015; Montipó *et al.*, 2016) – sendo que, ambas as biomassas, possuem potencial para conversão a etanol.

Contudo, os componentes lignocelulósicos, que compõem a casca de arroz e o capim-elefante necessitam ser previamente separados para que as porções de celulose e hemicelulose sejam despolimerizadas, de modo a facilitar a etapa de hidrólise enzimática dos polissacarídeos em açúcares fermentescíveis. Dentre os distintos pré-tratamentos desenvolvidos, o processo de explosão a vapor, conduzido à pressão e temperatura de vapor saturado, destaca-se como promissora alternativa que cumpre este requisito (Jönsson & Martín, 2016). Cabe ressaltar, que os resultados finais de conversão dos açúcares nos produtos de interesse são muito dependentes dos pré-tratamentos utilizados.

Após a fermentação alcoólica por *Saccharomyces cerevisiae*, levedura usualmente empregada na produção de etanol, as pentoses remanescentes podem ser fermentadas por micro-organismos hábeis em metabolizar tais açúcares, como é o caso de distintas bactérias ácido-lácticas. O ácido láctico é um importante insumo industrial, com vasto mercado em expansão devido à sua versatilidade. Recentemente, a demanda deste ácido orgânico aumentou consideravelmente, tendo uma projeção estimada de 600.000 t para o ano de 2020, principalmente pelo uso como monômero na produção de poli (ácido láctico), um polímero biodegradável (Dusselier *et al.*, 2013; Zhang & Vadlani, 2015).

A biorrefinaria é uma instalação de processamento que integra múltiplas vias de reação química para converter biomassa em produtos de valor agregado juntamente com calor e energia (Fernando *et al.*, 2006; Ng *et al.*, 2015). Deste modo, o conceito de biorrefinaria adapta-se muito bem ao processo que enfatiza a conversão eficiente de todos os carboidratos presentes no material lignocelulósico em diversos bioprodutos, o que favorece a maximização da rentabilidade do processo. A utilização destas matérias-primas renováveis para a produção

de etanol e demais bioprodutos é uma vantajosa alternativa, pois, além de constituir uma via mais limpa, o custo de produção pode ser efetivamente reduzido. Sendo assim, com a implementação deste estudo pretende-se impulsionar as ações integradoras visando o desenvolvimento de processos de produção de etanol e ácido láctico, a partir de açúcares fermentescíveis, liberados após hidrólise da casca de arroz e do capim-elefante, pré-tratados.

1.1 Objetivo geral

Avaliar processos de pré-tratamentos a vapor e hidrólise enzimática da casca de arroz e do capim-elefante visando à obtenção de hexoses e de pentoses, com subsequente conversão a etanol e ácido láctico, respectivamente.

1.2 Objetivos específicos

- otimizar as condições de pré-tratamento por explosão a vapor das biomassas lignocelulósicas, em termos de temperatura e tempo, de modo a elevar os teores de glicano na fração sólida e, ao mesmo tempo, de xilanas na fração líquida;
- caracterizar as biomassas lignocelulósicas *in natura* e pré-tratadas quanto à composição química;
- avaliar diferentes condições de hidrólise enzimática das frações pré-tratadas;
- avaliar a produção de etanol por *Saccharomyces cerevisiae*, a partir das hexoses advindas do material pré-tratado, e, concomitantemente, avaliar a produção de ácido láctico por bactérias ácido-lácticas, a partir das pentoses advindas do licor;
- selecionar bactérias ácido-lácticas com habilidade na metabolização de pentoses;
- analisar distintas estratégias de sacarificação e fermentação.

2. REVISÃO BIBLIOGRÁFICA

2.1 Biorrefinarias

As tendências mundiais em Pesquisa e Desenvolvimento (P&D) para a utilização diversificada de matérias-primas renováveis, em substituição às fontes fósseis, têm consolidado a ideia de biorrefinaria. Estas, ao produzirem biocombustíveis, bioprodutos e bioenergia, contribuirão para a viabilização econômica da exploração da biomassa, em geral, melhorando o desempenho do ponto de vista ambiental. O uso destas matérias-primas, portanto, não deve ser visto apenas como uma reação aos altos preços do petróleo dos últimos anos, mas como um processo mais consistente de desenvolvimento de novos conhecimentos neste setor (CGEE, 2010).

A crescente importância atribuída à ideia de futuras biorrefinarias sugere que a exploração de biomassas precisa integrar uma visão multiproduto, explorando diversas correntes e processos, à semelhança das refinarias de petróleo (CGEE, 2010). O desenvolvimento de biorrefinarias possui duas vertentes estratégicas: *i)* o incentivo em favor da utilização das biomassas locais, e *ii)* a criação de uma indústria robusta de base biológica (Bozell & Petersen, 2010).

Os recursos da biotecnologia moderna, aliados aos da indústria química, constituem via essencial na consolidação da ideia de biorrefinaria, na prática. A produção de insumos químicos a partir da indústria biotecnológica adere aos princípios da química e da engenharia verde, tal como redução da demanda de energia e da geração de resíduos, ampliando-se o uso de catálise seletiva e a produção de compostos biodegradáveis. Adicionalmente, a condução dos procedimentos em uma única etapa facilita a otimização da produção envolvendo menores gastos energéticos, emprego de menores quantidades de reagentes e, ainda, no caso,

permitindo a elaboração de produtos de difícil síntese por via química (Sijbesma & Schepers, 2003).

Conforme Octave & Thomas (2009), dependendo do composto majoritário da estrutura química das biomassas, as biorrefinarias podem ser classificadas como: biorrefinaria base carboidrato, biorrefinaria base lignocelulósica e biorrefinaria base lipídica. A primeira propõe a utilização dos açúcares, que são as reservas de energia nos vegetais (sacarose e amido). São açúcares mais acessíveis às necessidades energéticas dos vegetais e objeto de uso pleno na indústria alimentícia. A segunda se baseia na utilização dos constituintes estruturais do vegetal. São frações de maior complexidade estrutural e, por isso, de mais difícil acesso. Já a terceira se baseia em grupos específicos de vegetais capazes de produzir e armazenar triglicerídeos, que também atuam como reserva energética para o vegetal, tendo aplicabilidade em diversos setores industriais.

2.1.1 Biorrefinaria de base lignocelulósica

As biomassas lignocelulósicas, incluindo resíduos de processamento agrícola, florestal e de alimentos, além de culturas energéticas, compõem as fontes de carboidratos mais abundantes na natureza. Aproximadamente 220 bilhões t (massa seca) de materiais lignocelulósicos são produzidos anualmente no mundo. Na maioria das vezes não são integralmente utilizados pelas indústrias e, ultimamente, vêm se destacando como potenciais matérias-primas, passíveis de conversão em *commodities* e energia através de processos bioquímicos (Chandra *et al.*, 2012; Nissilä *et al.*, 2014). No Brasil, diversos tipos de biomassas residuais estão disponíveis, variando de região para região.

Por tratar-se de uma biomassa extremamente disponível e com elevada capacidade de processamento, ou seja, renovável, os substratos lignocelulósicos contendo elevado conteúdo

sacarídico e de lignina se tornam mais atraentes à produção de biocombustíveis, bioprodutos e energia (Damartzis & Zabaniotou, 2011). Uma gama de subprodutos da agroindústria tem sido empregada para o crescimento de micro-organismos pela alta disponibilidade e baixo custo. No entanto, a conversão sustentável de biomassa em produtos de valor agregado carece ainda de melhor estruturação do mercado e da economia.

Os principais constituintes das biomassas lignocelulósicas são celulose (~40-50%), hemicelulose (~25-30%) e lignina (~15-20%), além de extraíveis e distintos materiais inorgânicos (Knauf & Moniruzzaman, 2004). Devido à heterogeneidade destes materiais, tal composição varia conforme as propriedades particulares de cada vegetal. A lignina, juntamente com a hemicelulose, forma uma matriz que envolve a celulose, fazendo com que estes três componentes majoritários encontrem-se estritamente associados em uma estrutura cristalina e complexa (Figura 1) (Keshwani & Cheng, 2009; Zhang, 2008).

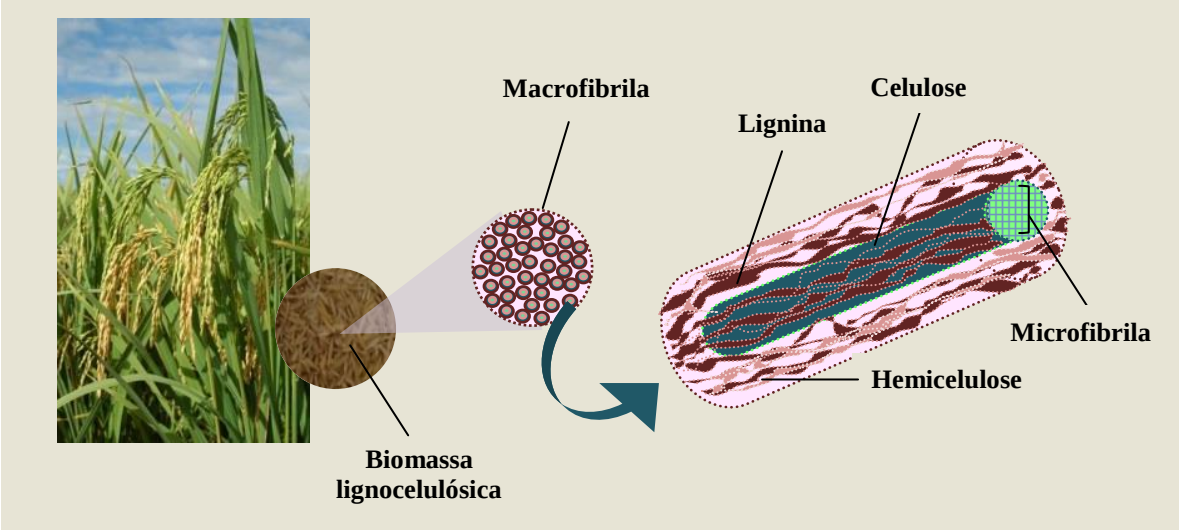

Figura 1. Estrutura de uma biomassa lignocelulósica. Fonte: adaptado de Rubin, 2008.

Tal como evidenciado na Figura 1, a celulose é empacotada em microfibrilas, que são estabilizadas por ligações de hidrogênio. Estas microfibrilas são interligadas umas às outras

pela hemicelulose, por polímeros amorfos de diferentes açúcares, bem como por polímeros de pectina, sendo, finalmente, envolvidos pela lignina. As microfibrilas celulósicas, que estão presentes na matriz hemicelulose-lignina, estão associadas na forma de macrofibrilas. As moléculas individuais de microfibrilas estão empacotadas tão firmemente que, não somente enzimas, mas pequenas moléculas como a água, não conseguem adentrar no complexo (Arantes & Saddler, 2010). As regiões cristalinas das microfibrilas são mais resistentes à biodegradação em relação às partes amorfas e, por isso, são ditas pouco reativas (Hallac & Ragauskas, 2011). Cabe salientar que a separação efetiva de cada um destes constituintes durante o processamento tem papel essencial na composição de fatores de viabilidade econômica dos múltiplos produtos gerados (Menon & Rao, 2012).

A hidrólise total da celulose gera apenas glicose, considerada o principal substrato para processos biotecnológicos, podendo ser convertida a uma série de compostos químicos (Bevilaqua *et al.*, 2015; Camassola & Dillon, 2007; Lian *et al.*, 2010; Menegol *et al.*, 2016; Montipó *et al.*, 2016; Pedroso *et al.*, 2017; Scholl *et al.*, 2015a,b). O produto da hidrólise da fração hemicelulósica é uma mistura de açúcares, predominando a xilose, que pode ser convertida a xilitol (Baudel *et al.*, 2005; Rambo *et al.*, 2013) ou demais bioprodutos, quando se empregam agentes químicos ou micro-organismos capazes de converter tal açúcar. Quando não é queimada para gerar energia, a maioria da lignina remanescente do processo hidrolítico oferece perspectivas de utilização para a obtenção de produtos com elevado valor agregado. Adicionalmente, pesquisas têm demonstrado que a lignina possui um potencial que vai muito além do que lhe foi atribuído até o momento – geração de energia através de sua queima. Alguns exemplos destas aplicações são: na produção de dispersante para a indústria química, aditivo/substituinte de resinas fenólicas para produção de painéis, na produção de fibra de

carbono, como veículo de fertilizantes e pesticidas, fonte de distintos compostos químicos, etc. (Arkell *et al.*, 2014; Doherty *et al.*, 2011; Zhang, 2008).

Conforme mostrado na Figura 2, a planta de uma biorrefinaria lignocelulósica típica deve basear-se no princípio de que: *i)* todos os açúcares isolados são utilizados na produção de bioprodutos, biopolímeros e biocombustíveis; e *ii)* todos os resíduos do processo (incluindo lignina, hemicelulose e celulose) são utilizados na geração de energia. Ainda, segundo Menon & Rao (2012), na biorrefinaria lignocelulósica, a conversão da biomassa geralmente envolve cinco etapas: *i)* escolha da biomassa adequada; *ii)* pré-tratamento eficaz; *iii)* hidrólises ácida e/ou enzimática; *iv)* fermentação a partir das hexoses (glicose, galactose, manose e frutose) e pentoses (xilose e arabinose); e *v)* recuperação e purificação dos produtos obtidos. Todavia, os principais impedimentos no que diz respeito ao desenvolvimento de uma tecnologia economicamente viável para a biodegradação da celulose são: a intrínseca associação com a hemicelulose e com a lignina, a cristalinidade da estrutura, o grau de polimerização e a área superficial.

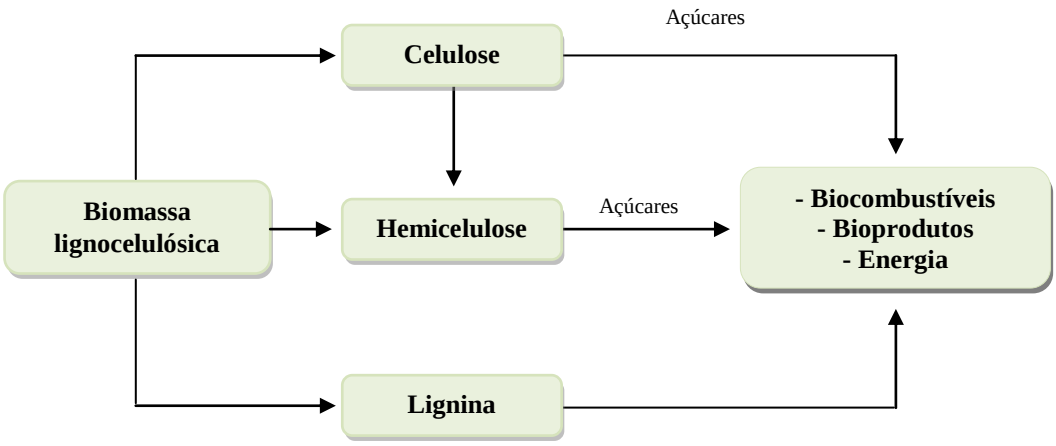

Figura 2. Estrutura simplificada para biorrefinaria com base em biomassa de composição lignocelulósica. Fonte: adaptado de CGEE, 2010.

2.1.1.1 Casca de arroz

O arroz (*Oryza sativa*) está entre os cereais mais consumidos do mundo. Conforme a Organização das Nações Unidas para Alimentação e Agricultura (FAO, 2017), o Brasil é o 11º maior produtor mundial e colheu 12,13 milhões t na safra 2016/2017. O cultivo de arroz irrigado, praticado na região Sul do Brasil, contribui, em média, com 81,41% da produção nacional, sendo o RS o maior produtor brasileiro, com 71,0% (CONAB, 2017).

Após o processo de beneficiamento, a casca de arroz (CA) surge como um rejeito agrícola, caracterizado pela natureza abrasiva, baixa densidade, elevado conteúdo de cinza/sílica, grande resistência à degradação e difícil digestibilidade (Saha & Cotta, 2008), sendo que estas particularidades fazem da CA um subproduto com pouco valor nutritivo. Adicionalmente, a CA é comumente eliminada através da queima em campos abertos, desencadeando problemas ambientais e de saúde, bem como o desperdício de energia (Lim *et al.*, 2012).

Devido ao teor energético da CA, a pirólise tem sido proposta como processo de valorização deste resíduo (Lim *et al.*, 2012). A cinza resultante da queima é rica em sílica (SiO_2), constituindo matéria-prima econômica para a produção de distintos materiais, em diferentes ramos industriais da construção civil (Carlos & Khang, 2008). A CA é um dos resíduos lignocelulósicos que tem atraído a atenção dos pesquisadores devido ao seu conteúdo de celulose e hemicelulose e ao seu potencial para a produção de bioprodutos e biocombustíveis (Ang *et al.*, 2012; Montipó *et al.*, 2016). A sua composição inclui, em média, 32,4% de celulose, 16,3% de hemicelulose e 27,9% de lignina, além de elevado teor de cinzas (17,2%) e demais componentes (Pedroso *et al.*, 2017).

Assim, tais fatos incentivam o estudo do aproveitamento desta biomassa, largamente disponível, renovável e, praticamente, sem valor comercial (Economou *et al.*, 2011; Suxia *et*

al., 2012). A utilização adequada deste resíduo poderá minimizar os problemas ambientais vinculados ao seu acúmulo e diminuir o consumo de combustíveis fósseis, além de resultar em melhora no quadro atual do aproveitamento desta matéria-prima.

2.1.1.2 Capim-elefante

As culturas energéticas de gramíneas, a exemplo do switchgrass (*Panicum virgatum* L.), são vistas como potenciais matérias-primas a serem utilizadas como substrato para a produção de etanol em longo prazo (Keshwani & Cheng, 2009). Tais plantas diferem quanto à produtividade, às propriedades físico-químicas, às demandas ambientais, às necessidades de manejo, e flexibilidade de uso (Keshwani & Cheng, 2009; Lemus & Parrish, 2009).

Dentre estas culturas, o capim-elefante (CE) (*Pennisetum purpureum*), é uma cultura herbácea que tem sido utilizada como forragem animal por muitos anos, amplamente reconhecida por sua elevada produtividade devido aos seus ciclos de colheita curtos. Uma cultura de CE pode produzir até 29,0 t de matéria seca/ha/ano (teor de umidade de 15%), enquanto que uma cultura de cana-de-açúcar baseada em bagaço produz 20,8 t de matéria seca/ha/ano (50% de teor de umidade) (Fontoura *et al.*, 2015; Gallego *et al.*, 2015; Pérez-Boada *et al.*, 2014).

O CE, espécie nativa das pastagens tropicais da África e introduzida na maioria dos países tropicais e subtropicais, é uma gramínea robusta, com caules perenes, atingindo mais de 3 m de altura (Pérez-Boada *et al.*, 2014). Esta gramínea requer pouco nutrientes suplementares para o crescimento e, além disso, pode ser colhida até quatro vezes por ano (Pérez-Boada *et al.*, 2014; Strezov *et al.*, 2008; Xie *et al.*, 2011).

As aplicações energéticas atuais desta gramínea são a combustão direta, a gasificação e o carvoejamento. No entanto, o CE é considerado uma excelente matéria-prima alternativa

para fornecer recursos abundantes e sustentáveis de biomassa lignocelulósica para a produção de biocombustíveis (del Río *et al.*, 2012; Somerville *et al.*, 2010), uma vez que a sua composição inclui, aproximadamente, 36,0% de celulose, 20,5% de hemicelulose e 20,8% de lignina, além de cinzas, extraíveis e demais componentes (Menegol *et al.*, 2014), o que também torna esta planta uma potencial 'precursora' à síntese de insumos químicos. Estudos laboratoriais e em escala piloto têm constatado as potencialidades desta biomassa para a produção de etanol de segunda geração (2G etanol) e, também, como substrato alternativo para a produção de enzimas, que podem ser empregadas na sacarificação de biomassas lignocelulósicas (Menegol *et al.*, 2016; Scholl *et al.*, 2015a, 2015b).

2.2 Pré-tratamento das biomassas lignocelulósicas

A conversão da biomassa lignocelulósica em biocombustível e bioproduto envolve três etapas majoritárias: pré-tratamento, hidrólise enzimática e fermentação, sendo a primeira considerada a mais crítica dentre as mesmas (Ruíz *et al.*, 2012). Neste contexto, o conceito de biorrefinaria pressupõe a separação seletiva de frações constituintes da biomassa de acordo com suas características químicas e com o perfil dos produtos a serem obtidos. Tendo em vista que o pré-tratamento interfere nas etapas posteriores, a seleção por um pré-tratamento efetivo é imprescindível, uma vez que a intenção é desorganizar e provocar o rompimento do complexo lignocelulósico, aumentando a digestibilidade da celulose e tornando-a mais acessível à etapa hidrolítica (Perez-Cantu *et al.*, 2013).

Conforme Taherzadeh & Karimi (2008) e Zhang (2008), um pré-tratamento efetivo e econômico deve englobar os seguintes requisitos: *i)* tornar as fibras celulósicas acessíveis às hidrólises ácida/enzimática sem que ocorra degradação da celulose; *ii)* trabalhar em condições menos severas de temperatura para evitar a degradação dos açúcares e a formação de

possíveis inibidores de enzimas hidrolíticas e de micro-organismos fermentadores; *iii)* consumir o mínimo possível de reagentes químicos; *iv)* reduzir custos relacionados à construção dos equipamentos; *v)* minimizar a demanda energética; *vi)* gerar o mínimo de resíduos; e *vii)* diminuir o número de etapas envolvidas no processo global.

Conceitualmente, os pré-tratamentos podem ser divididos em quatro tipos: físicos, físico-químicos, químicos e biológicos, ou, ainda, uma combinação destes, que dependerá da natureza do material a ser tratado, do grau de separação requerido e do fim a que se destina o processo (Keshwani & Cheng, 2009). A Figura 3 ilustra o processo de conversão da celulose e da hemicelulose em monossacarídeos fermentescíveis e, da mesma maneira, a formação de compostos provenientes da ruptura da lignina durante o processo de pré-tratamento físico-químico.

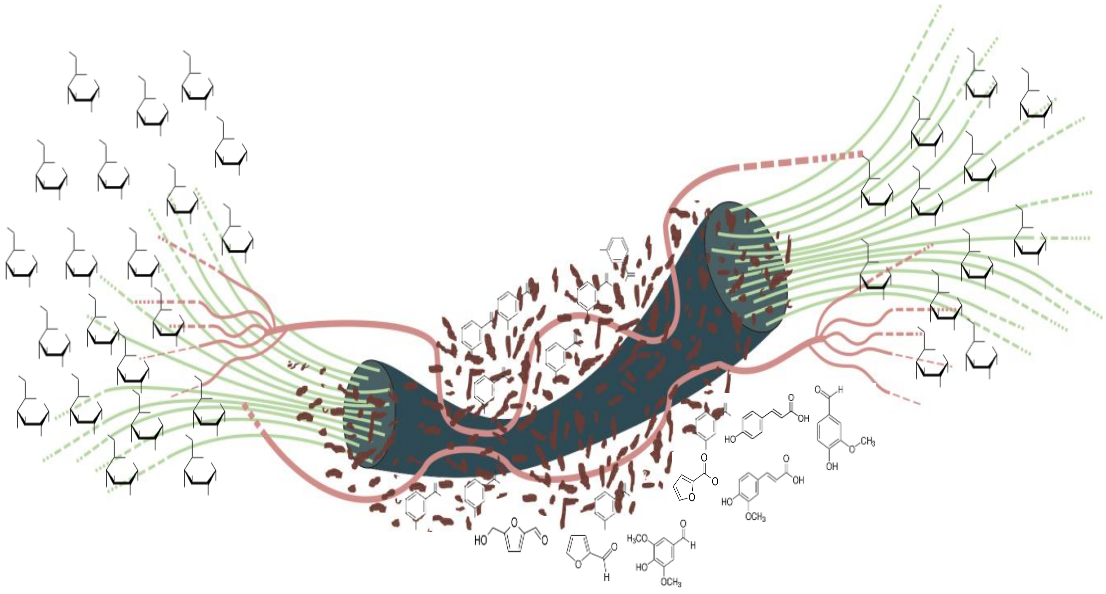

Figura 3. Estrutura lignocelulósica pré-tratada. A parte central em vermelho representa a lignina; o tubo em azul e as linhas verdes indicam as fibrilas de celulose e as microfibrilas, respectivamente; as linhas em rosa representam a hemicelulose. Fonte: adaptado de Pedersen & Meyer, 2010.

Os pré-tratamentos físicos, normalmente, envolvem moagem e extrusão. O objetivo principal é aperfeiçoar o processo hidrolítico, reduzindo a cristalinidade das fibras celulósicas e eliminando as limitações de transferências de massa e calor. Os mais importantes pré-tratamentos físico-químicos incluem o hidrotérmico, a explosão a vapor (catalisada ou não por ácidos inorgânicos, CO_2, SO_2, etc.), a explosão de fibras com amônia (*ammonia fibre expansion* – AFEX) e a oxidação úmida, além de tratamentos com micro-ondas e ultrassom. Dentre as categorias de pré-tratamentos existentes, a classe dos químicos tem sido estudada extensivamente e inclui tratamentos ácidos e alcalinos, principalmente; ademais, o emprego de líquidos iônicos, organossolventes e ozonólise (Alvira *et al.*, 2010; Menon & Rao, 2012; Taherzadeh & Karimi, 2008).

Conforme comentado por Taherzadeh & Karimi (2008), a hidrólise ácida diluída é, provavelmente, a técnica mais usual entre os pré-tratamentos químicos devido à relação custo-benefício. Em que pese o fato de ser uma tecnologia amplamente estudada, alguns "gargalos" tecnológicos podem ser identificados nesse processamento: *i)* formação de compostos tóxicos, oriundos da degradação dos açúcares e da degradação da lignina, que podem acarretar problemas de inibição ao processo de conversão biológica; *ii)* problemas relacionados à corrosão de equipamentos quando se trabalha com meios ácidos com quecimento; e *iii)* remoção da lignina e dos compostos resultantes da sua degradação parcial (Almeida *et al.*, 2007; CGEE, 2010; Mussatto & Roberto, 2004). Além disso, há ainda os problemas de ordem ambiental vinculados ao uso destes agentes químicos.

A eficiência dos processos de pré-tratamento é resultante do sinergismo entre as condições do processo, envolvendo pH do meio, tempo de exposição da matriz e temperatura, uma vez que intencionam desconstruir o complexo lignocelulósico. O conceito que pode relacionar tais fatores é denominado grau de severidade, sendo empregado como parâmetro

por muitos pesquisadores. Basicamente, há um grau de severidade ótimo, acima do qual será menor a eficiência de hidrólise em razão da degradação dos açúcares e da formação de outros inibidores (Mc Millan, 1994; Perez-Cantu *et al.*, 2013). Vale menciomar a grande importância de o substrato conter baixos níveis de compostos tóxicos, permitindo a fermentação com o mínimo de pré-tratamento e, paralelamente, com índices superiores de produtividade e rendimento, havendo reduzida ou nenhuma formação de subprodutos.

Os fungos ligninolíticos e suas enzimas apareceram como uma nova alternativa para os processos de pré-tratamento. Esta tecnologia tem sido útil tanto na etapa de deslignificação quanto na etapa de destoxificação (Plácido & Capareda, 2015). O pré-tratamento biológico, envolvendo o uso de micro-organismos, é uma técnica sustentável e promissora, que apresenta a vantagem de não exigir o uso de reagentes químicos, operar em condições amenas de temperatura e necessitar baixa demanda energética (Kurakake *et al.*, 2007; Salvachúa *et al.*, 2011). Todavia, os desafios relacionados com este tipo de pré-tratamento incluem o desempenho do micro-organismo durante o processo, exigindo longos períodos de tempo; bem como a tendência dos mesmos na degradação dos açúcares, acarretando na diminuição do rendimento global (Jönsson & Martín, 2016). Por isso, faz-se necessário a busca por fungos basidiomicetos com capacidade de deslignificação rápida e eficiente (Alvira *et al.*, 2010).

2.2.1 Pré-tratamento por explosão a vapor

O pré-tratamento por explosão a vapor é uma das tendências tecnológicas mais adotadas para aumentar a acessibilidade das enzimas às moléculas de celulose. Este processo atua na transformação dos materiais lignocelulósicos e envolve o tratamento da biomassa com vapor saturado a temperaturas entre 160 e 240 °C durante um tempo de reação que varia de 2

a 30 min. Após o tempo de reação, ocorre a descompressão explosiva e a suspensão pré-tratada, também referida como *slurry*, é coletada em um tanque de expansão. Durante o processo, parte das hemiceluloses é hidrolisada a açúcares solúveis e a lignina é parcialmente modificada, tornando a biomassa mais susceptível à solvatação e, por conseguinte, aos processos de hidrólise enzimática (Moreno *et al.*, 2013; Romaní *et al.*, 2013; Singh *et al.*, 2015; Sun & Cheng, 2002).

Quando não há a adição de reagentes químicos, diz-se que o processo é catalisado por autohidrólise, e este método propicia uma rota mais sustentável para o tratamento da biomassa, sendo mundialmente empregado em unidades-piloto responsáveis pela conversão de materiais lignocelulósicos a etanol, por exemplo (Jönsson & Martín, 2016; Kont *et al.*, 2013). A autohidrólise ocorre quando as altas temperaturas promovem a formação de ácido acético a partir de grupos acetilo; além disso, a água também pode atuar como um ácido a temperaturas elevadas (Alvira *et al.*, 2010).

Não obstante, a explosão a vapor pode ser assistida por impregnação com catalisadores, incluindo ácidos inorgânicos, CO_2 e SO_2, que visam o aumento da eficiência do processo de explosão a vapor. Nesse sentido, várias pesquisas têm sido conduzidas envolvendo o uso de agentes químicos – ácido sulfúrico, principalmente, com concentrações variando entre 0,1 e 5% *v/v* (Ballesteros *et al.*, 2006; CGEE, 2010; Ferreira-Leitão *et al.*, 2010; Jönsson & Martín, 2016).

2.3 Enzimas

2.3.1 Produção de enzimas

Nos últimos anos, o interesse em celulases e hemicelulases aumentou devido às inúmeras aplicações potenciais para essas enzimas. No contexto da produção de etanol e

bioprodutos a partir de matérias-primas lignocelulósicas, micro-organismos (hemi)celulolíticos são empregados na geração das enzimas necessárias à degradação da celulose e da hemicelulose (Camassola & Dillon, 2007; Steffien *et al.*, 2014).

Embora um grande número de micro-organismos seja capaz de degradar a celulose, apenas alguns destes produzem quantidades significativas de enzimas capazes de hidrolisar completamente a celulose cristalina (Wang *et al.*, 2012). A maioria das preparações comerciais de celulases e hemicelulases é produzida por fungos filamentosos dos gêneros *Aspergillus* e *Trichoderma* (Bischof *et al.*, 2016; Shin *et al.*, 2011). Recentemente, as variantes do fungo *Penicillium* foram descritas como ótimas produtoras de celulases e hemicelulases, e consideradas promissoras para a indústria de bioetanol (Camassola & Dillon, 2007; Dillon *et al.*, 2011; Dillon *et al.*, 2006; dos Reis *et al.*, 2013; Jørgensen *et al.*, 2005; Schneider *et al.*, 2016).

A seleção de uma fonte de carbono apropriada é uma questão importante na produção de celulases e hemicelulases. Além da celulose e das xilanas comerciais, os resíduos agroindustriais na forma de bagaço, farelo, palha e serragem têm sido empregados como substratos na produção destas enzimas (Sajith *et al.*, 2016). Estas matérias-primas são bons substratos para a produção de xilanases, com destaque para farelo de trigo, palha de trigo e bagaço de cana-de-açúcar; as quais resultam em altos rendimentos de xilanase, permitindo custos inferiores de produção (Knob *et al.*, 2014).

O desenvolvimento comercial de hemicelulases para a hidrólise de materiais lignocelulósicos não está tão avançado quanto o de celulases, pois as preparações celulásicas comerciais correntes têm sido desenvolvidas principalmente para a hidrólise de biomassa pré-tratada com ácido diluído, onde a hemicelulose é removida antes da sacarificação de celulose. No entanto, com o desenvolvimento de pré-tratamentos não ácidos, nos quais a fração

hemicelulósica permanece intacta, as hemicelulases serão compulsoriamente requeridas (CGEE, 2010).

Os processos de produção de enzimas podem envolver a fermentação submersa ou a fermentação em estado sólido, sendo esta última uma alternativa promissora para a produção eficiente de enzimas (hemi)celulolíticas (Farinas, 2015). Este sistema apresenta muitas vantagens em relação à fermentação submersa, incluindo alta produtividade, concentração relativamente maior dos produtos, menor geração de efluentes, exigência de equipamentos de fermentação simples, etc. (Pandey *et al.*, 1999).

2.3.2. Hidrólise enzimática

A hidrólise enzimática da lignocelulose é um processo complexo, afetado por muitos fatores, considerando que diferentes métodos de pré-tratamento resultam em diferentes composições e estruturas de biomassa lignocelulósica. Por sua vez, tais alterações influenciam não somente a etapa de hidrólise enzimática, mas também a posterior fermentação. Além disso, a absorção e dessorção da celulase demonstram estar relacionadas ao teor de lignina da biomassa, comprometendo a hidrólise enzimática (Liu *et al.*, 2010; Wang *et al.*, 2012). O processo de hidrólise enzimática é conduzido em condições amenas de pH e de temperatura (faixa de pH de 4,8 e temperatura de 45-50 °C), com reduzida formação de compostos tóxicos, não apresentando problemas de corrosão, como se costuma observar nas hidrólises com ácidos ou bases (Duff & Murray, 1996; Malburg *et al.*, 1992; Szengyel, 2000).

A degradação das matérias-primas lignocelulósicas requer diversas enzimas. As celulases, que coletivamente apresentam especificidade para as ligações glicosídicas ß-1,4, são todas necessárias para a solubilização completa da celulose, mesmo daquela das regiões cristalinas, existindo sinergismo na sua forma de atuar. As enzimas do complexo celulolítico

são classificadas em três grupos: *i)* endoglicanases, que clivam ligações internas da fibra celulósica; *ii)* exoglicanases, que atuam na região externa da celulose; e *iii)* ß-glicosidases, que hidrolisam oligossacarídeos solúveis a glicose (Malburg *et al.*, 1992; Yang *et al.*, 2009). A Figura 4 a seguir esquematiza a atuação dessas enzimas sobre a celulose.

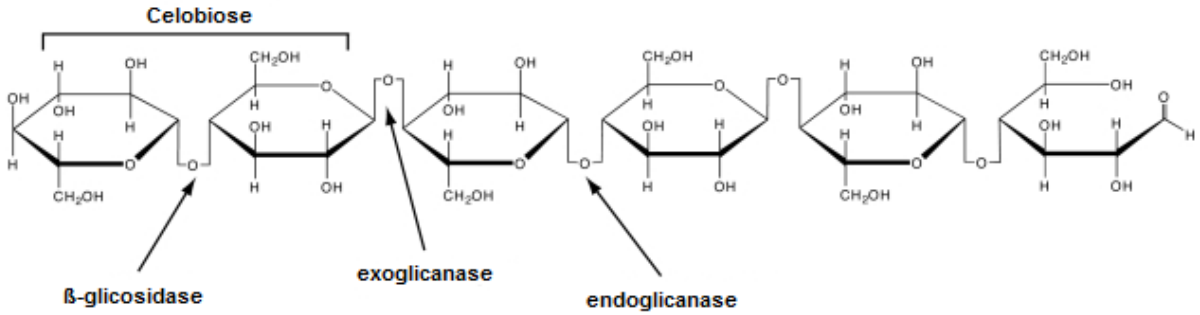

Figura 4. Enzimas envolvidas na hidrólise da celulose. Fonte: adaptado de Yang *et al.*, 2009.

A biodegradação das hemicelulases requer a ação de diversas enzimas: *i)* endoxilanases, que hidrolisam aleatoriamente a cadeia principal das xilanas, produzindo uma mistura de xilo-oligosacarídeos; *ii)* xilosidases, que liberam xilose de oligossacarídeos curtos; *iii)* α-L-arabinofuranosidases, que removem L-arabinofuranose das cadeias laterais; *iv)* α-D-glicuronidases, que hidrolisam os resíduos de metil glicuronato; *v)* acetil-xilana-esterases, que hidrolisam grupos acetato da cadeia principal; *vi)* feruloil/cumaril esterases, que hidrolisam os respectivos ácidos aromáticos ligados aos resíduos de arabinofuranósido (Chávez *et al.*, 2006). A Figura 5 esquematiza a atuação dessas enzimas sobre as hemiceluloses.

2.4 Etanol

O etanol (C_2H_6O) é um dos combustíveis renováveis mais importantes na atualidade, sendo que nas últimas décadas a produção e o consumo global destes expandiram-se significativamente devido as políticas de apoio que visam, sobretudo, reduzir a emissão dos

gases do efeito estufa e diversificar as fontes energéticas (Jonker *et al.*, 2015; Limayem & Ricke, 2012). A partir de 1975, durante o Proálcool, o Governo Brasileiro passou a estimular o setor através de subsídios e garantia de fornecimento de matéria-prima; porém, os incentivos à indústria alcoolquímica tiveram curta duração (CGEE, 2010).

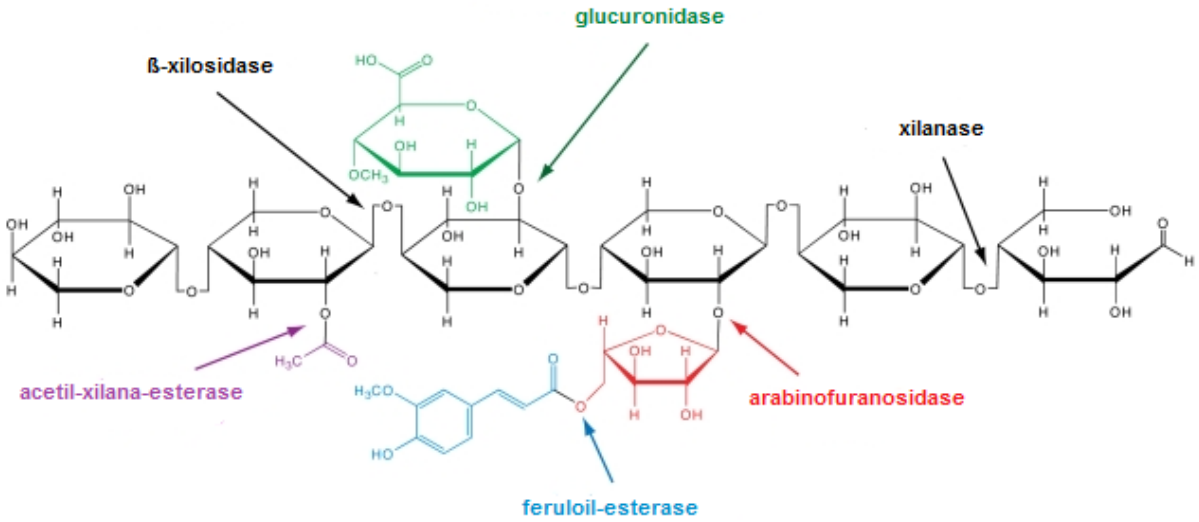

Figura 5. Enzimas envolvidas na hidrólise das hemiceluloses. Fonte: adaptado de Yang *et al.*, 2009.

Atualmente, o Brasil tem um papel central no mercado global de açúcar a etanol, produzindo-o a partir da cana-de-açúcar, e, juntamente com os Estados Unidos, que empregam o milho, responderam por 86% da produção total em 2010 (Mayer *et al.*, 2015; Rezende & Richardson, 2015). Conforme o Relatório da Rede de Informação Agrícola Global, fornecido pelo Departamento de Agricultura dos Estados Unidos (GAIN Report, USDA) a produção de etanol no Brasil foi de 30,37 bilhões de litros em 2016, e há uma previsão de 30,15 bilhões de litros para 2017 (USDA, 2016).

Tendo em vista que o Brasil apresenta potencial para ampliar a produção de etanol, faz-se necessário expandir a área de cultivo, melhorar o rendimento agrícola ou industrial e introduzir novas vias de processamento industrial. Novas rotas de processo requerem

melhorias na produção de etanol de primeira geração, com base na fermentação de açúcares; ou de segunda geração, onde há a conversão de biomassa lignocelulósica. A inserção da tecnologia de 2G etanol é capaz de incrementar a produção de etanol por hectare, utilizando os subprodutos lignocelulósicos do processamento de cana-de-açúcar, por exemplo. Além disso, o uso da tecnologia de segunda geração permitirá a produção de etanol durante todo o ano, uma vez que as culturas lignocelulósicas são menos sazonais em relação àquelas da cana-de-açúcar (Jonker *et al.*, 2015).

Ao lado dos subprodutos da cana-de-açúcar (Pereira *et al.*, 2015; Rudolf *et al.*, 2008), diferentes fontes de biomassa lignocelulósica foram propostas para a produção de 2G etanol devido ao seu rendimento, composição e tolerância ao clima e/ou características do solo nas áreas de cultivo, incluindo: bagaço de agave, capim-elefante, casca de arroz, espiga de milho, madeiras de eucalipto e de oliveira, palha de trigo, dentre outras (Ballesteros *et al.*, 2006; Cara *et al.*, 2006; Jonker *et al.*, 2015; Menegol *et al.*, 2016; Mohagheghi *et al.*, 1992; Pérez-Pimienta *et al.*, 2017; Romaní *et al.*, 2013; Saha & Cotta, 2008; Zheng *et al.*, 2014).

2.5 Ácido láctico

O ácido láctico (AL) ($C_3H_6O_3$) é um ácido orgânico de ocorrência natural, com valor agregado e amplamente versátil, considerando-se inúmeras aplicações na indústria alimentícia, farmacêutica, química, têxtil e do couro (Ghaffar *et al.*, 2014; John *et al.*, 2009). A demanda mundial por AL é estimada em aproximadamente 130.000-150.000 t anuais (Randhawa *et al.*, 2012). No entanto, espera-se que o consumo global de AL aumente rapidamente no futuro próximo, atingindo 600.000 t no ano de 2020, em função da aplicabilidade na indústria de polímeros para a produção de poli (ácido láctico) (PLA) (Dusselier *et al.*, 2013; Tirpanalan *et al.*, 2015; Wang *et al.*, 2015).

A produção do AL pode ocorrer via química ou fermentativa. A síntese química fundamenta-se na hidrólise da lactonitrila com ácidos fortes, resultando em uma mistura racêmica de DL-AL. Já a rota fermentativa é capaz de convergir à produção de isômeros L-(+)- ou D-(–)- opticamente puros, uma vez que a pureza óptica do AL é importante para a síntese do PLA altamente cristalino. Sob o ponto de vista nutricional, somente o isômero L-(+)- é assimilado pelo organismo humano (Datta *et al.*,1995; Karp *et al.*, 2011; Randhawa *et al.*, 2012; Wang *et al.*, 2015).

Com o desenvolvimento da bioconversão industrial, a fermentação com micro-organismos apropriados tornou-se o método predominante de produção de AL, correspondendo à aproximadamente 90% da produção de tal insumo. As preocupações de ordem ambiental, as condições de processo envolvendo temperaturas mais brandas e exigência energética menor, bem como a possibilidade de elevar-se a pureza do produto contribuíram para a substituição da via sintética pela biotecnológica (Wang *et al.*, 2015).

As biomassas lignocelulósicas representam substrato atraente para produção de AL devido a sua abundância e custo inferior destas matérias-primas, servindo como fonte de carboidratos e nitrogênio para os micro-organismos (Abdel-Rahman *et al.*, 2011; Wang *et al.*, 2010). Considerando-se a grande demanda do AL, o uso de tais biomassas é fundamental para assegurar a viabilidade da produção biotecnológica do AL (Bustos *et al.*, 2007; John *et al.*, 2007; Wee *et al.*, 2006).

2.6 Produção de etanol e ácido láctico pela rota fermentativa

A conversão das matérias-primas lignocelulósicas para a produção de etanol e AL pode ocorrer de maneira convencional, com hidrólise e fermentação separadas (*separate hydrolysis and fermentation* – SHF) ou envolver estratégias de sacarificação e fermentação

simultâneas (*simultaneous saccharification and fermentation* – SSF). A principal vantagem da SSF frente à SHF é que a primeira é capaz de incrementar a produtividade do sistema uma vez que ambas as etapas são executadas conjuntamente, reduzindo o tempo global do processo e, com isso, os custos de produção. Além disso, a SSF pode resolver o problema da inibição quando concentrações elevadas de açúcares constituem o meio de cultivo, considerando que os carboidratos são hidrolisados a açúcares fermentescíveis ao mesmo tempo em que há a degradação destes mesmos, produzindo, então, os compostos desejados (John *et al.*, 2009). Entretanto, o sinergismo entre tal integração apenas pode ser atingido quando ambos os processos de hidrólise e fermentação são compatíveis quanto às condições de processo (Kim *et al.*, 2010).

A levedura *Saccharomyces cerevisiae* é um dos micro-organismos mais utilizados na produção de etanol devido à sua eficiência na fermentação de hexoses, sendo tolerante ao baixo pH, ao próprio etanol produzido, bem como aos compostos inibitórios presentes no hidrolisado obtido após o pré-tratamento (Klinke *et al.*, 2004). Todavia, devido à falta de uma via metabólica da xilose em xilulose, *S. cerevisiae* não pode utilizar xilose, apenas se as cepas forem reconstruídas por engenharia genética. Para tanto, pesquisas objetivam o desenvolvimento de linhagens modificadas de tal levedura capazes catabolizar as pentoses presentes nas biomassas lignocelulósicas pré-tratadas e hidrolisadas (Matsushika *et al.*, 2009).

Os micro-organismos produtores de AL, em geral anaeróbios, convertem o ácido pirúvico resultante da rota de Embden-Meyerhof a AL, sob a ação da enzima lactato-desidrogenase (Figura 6). Contudo, a estereoespecificidade do AL é dependente do micro-organismo escolhido.

As bactérias ácido-lácticas (BAL) gram-positivas são capazes de produzir o AL como metabólito principal, com alto rendimento e produtividade; e os gêneros *Lactobacillus* (*L.*),

Streptococcus, *Leuconostoc* e *Enterococcus* são os mais empregados (Reddy *et al.*, 2008). As BAL classificam-se em *i)* homofermentativas, produzindo somente AL, e *ii)* heterofermentativas, que juntamente com AL, produzem etanol, ácido acético, CO_2, etc. (John *et al.*, 2007; Litchfield, 1996; Reddy *et al.*, 2008). As BAL são cultivadas em pH ótimo entre 5,5 e 6,5, sendo tolerantes às condições ácidas. Estes micro-organismos possuem um requisito nutricional complexo devido a sua limitação em sintetizar aminoácidos e vitaminas (Reddy *et al.*, 2008).

Grande parte das BAL consegue converter somente glicose em AL. Em contrapartida, algumas são capazes de fermentar tanto hexoses quanto pentoses pela via das pentoses-fosfato (Figura 6), como é o caso de *L. pentosus* ATCC 8041 e *L. bifermentans* DSM 20003 (Givry *et al.*, 2008; Zhu *et al.*, 2007). A bactéria *L. casei* subsp. *rhamnosus* ATCC 10863 também foi reportada como fermentadora de glicose e xilose, sendo capaz de metabolizar outros açúcares presentes em menores quantidades na hemicelulose (Cui *et al.*, 2011; Iyer *et al.*, 2000). A cepa de *L. buchneri* NRRL B-30929 foi isolada por Liu *et al.* (2008), que demonstraram a capacidade da mesma em utilizar xilose como única fonte de carbono. Ademais, as cofermentações empregando micro-organismos produtores de AL objetivando a utilização tanto de hexoses quanto de pentoses podem elevar a eficiência de conversão do substrato (Cui *et al.*, 2011).

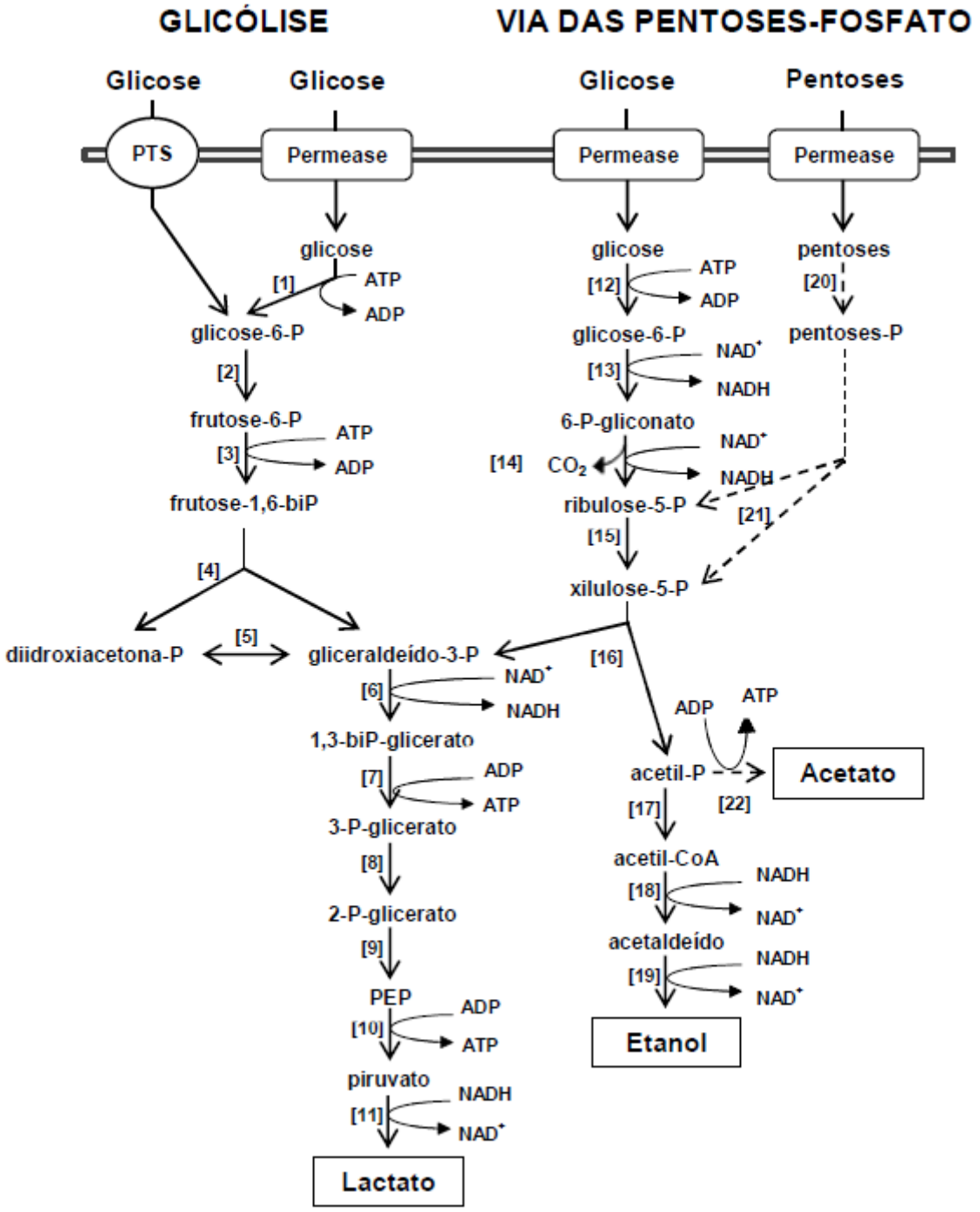

Figura 6. Glicólise e via das pentoses-fosfato nas bactérias ácido-lácticas. (1) hexo-cinase, (2) fosfo-hexose-isomerase, (3) fosfofrutocinase-1, (4) aldolase, (5) triose-fosfato-isomerase, (6) gliceraldeído-3-fosfato-desidrogenase, (7) fosfoglicerato-cinase, (8) fosfoglicerato-mutase, (9) enolase, (10) piruvato-cinase, (11) lactato-desidrogenase, (12) hexocinase, (13) glicose-6-fosfato-desidrogenase, (14) 6-fosfogliconato-desidrogenase, (15) ribose-5-fosfato-epimerase, (16) transcetolase, (17) fosfotransacetilase, (18) acetaldeído-desidrogenase, (19) álcool-desidrogenase; (20) pentose-cinase, (21) pentose-fosfato-epimerase ou -isomerase, (22) acetato-cinase. Fonte: adaptado de Fuente-Hernández *et al.*, 2013.

3. MATERIAL E MÉTODOS

Um fluxograma experimental deste trabalho é apresentado na Figura 7 e na Figura 8, de modo a simplificar a complementar a descrição das etapas desenvolvidas, apresentadas no decorrer dos capítulos a seguir. Ambas as figuras enfatizam a rota bioquímica de uma biorrefinaria de etanol e de AL através do aproveitamento da CA e do CE *in natura* por pré-tratamentos físico-químicos de explosão a vapor, seguido de hidrólise enzimática. Os pré-tratamentos com elevados teores de glicose nos sólidos insolúveis em água (*water insoluble solids* – WIS) (G_{WIS}) e, igualmente, de xilose no licor (X_{LIQ}), serão selecionados para subsequentes fermentações alcoólica e láctica.

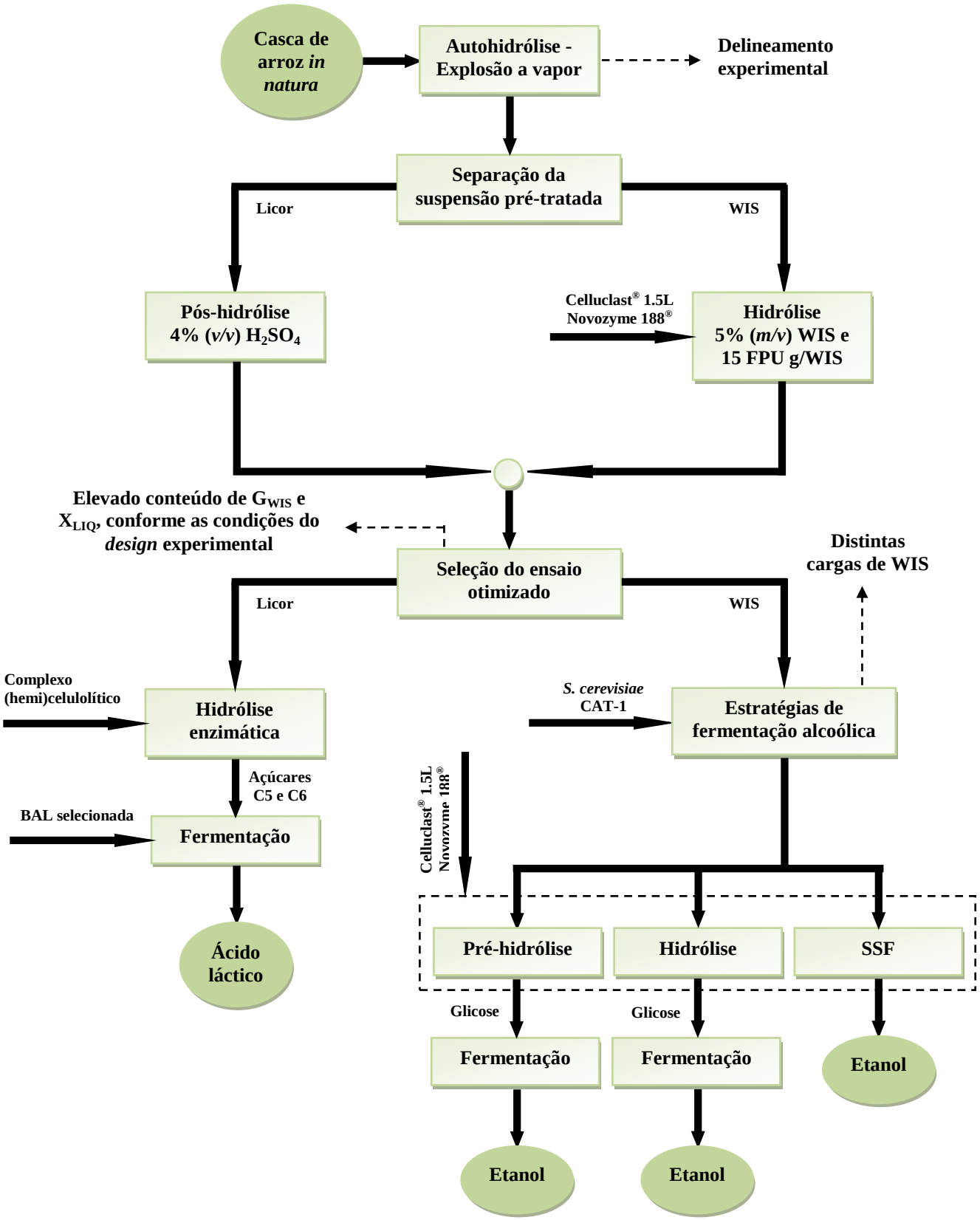

Figura 7. Fluxograma experimental da produção de etanol e ácido láctico a partir da casca de arroz.

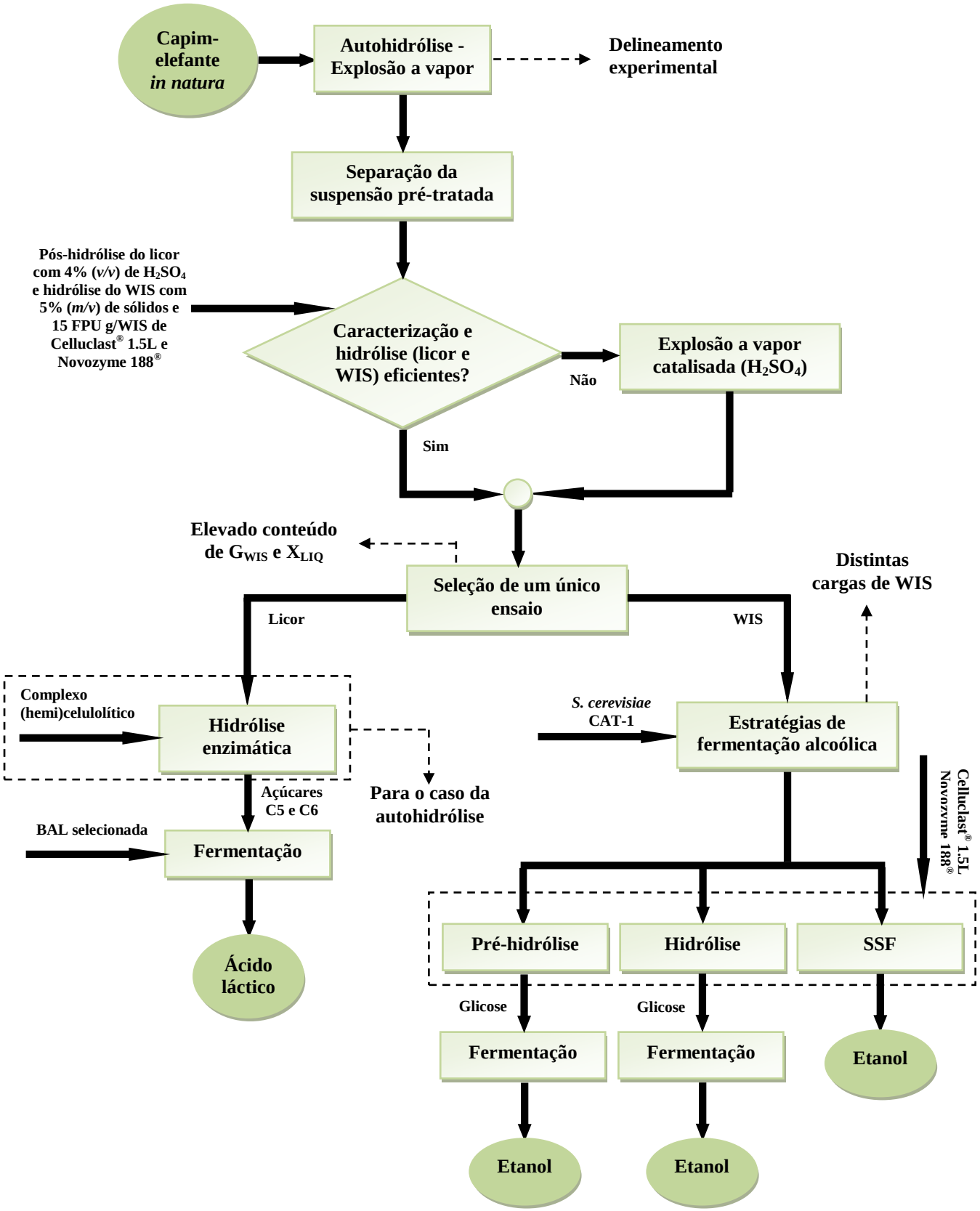

Figura 8. Fluxograma experimental da produção de etanol e ácido láctico a partir do capim-elefante.

4. RESULTADOS E DISCUSSÃO

Os resultados e a discussão estão apresentados em parte no formato de artigos científicos, divididos em três subcapítulos, e outra parte em formato de proposta de patente. No primeiro subcapítulo, buscou-se otimizar o pré-tratamento da CA e do CE, via explosão a vapor, empregando-se delineamento experimental e considerando-se como variáveis independentes a temperatura (°C) e o tempo (min), tendo como intuito produzir altos teores de glicanos e de xilanas nas frações sólida e líquida, respectivamente. O segundo subcapítulo evidencia as produções de etanol e de AL empregando a CA como fonte de carboidratos, a partir do pré-tratamento inicialmente otimizado. Este subcapítulo também conta com um *screening* inicial de cinco cepas de BAL, com vistas à conversão de xilose e, ainda, com a produção de complexo enzimático a partir do fungo *P. echinulatum* S1M29, que atuará na hidrólise exclusiva do licor. O terceiro subcapítulo trata da produção de etanol e de AL a partir do CE explodido na presença de catalisador ácido e da influência deste agente nos resultados quanto à liberação dos açúcares fermentescíveis. Por fim, o quarto subcapítulo, excepcionalmente, trata de um pedido de patente depositado no Instituto Nacional da Propriedade Industrial (INPI), apresentando resultados gerais relacionados à conversão da CA e do CE em etanol e AL.

4.1 Artigo 1

Optimisation of uncatalysed steam explosion of lignocellulosic biomasses to obtain both C6 and C5-sugars

Artigo submetido a periódico internacional.

Optimisation of uncatalysed steam explosion of lignocellulosic biomasses to obtain both C6- and C5-sugars

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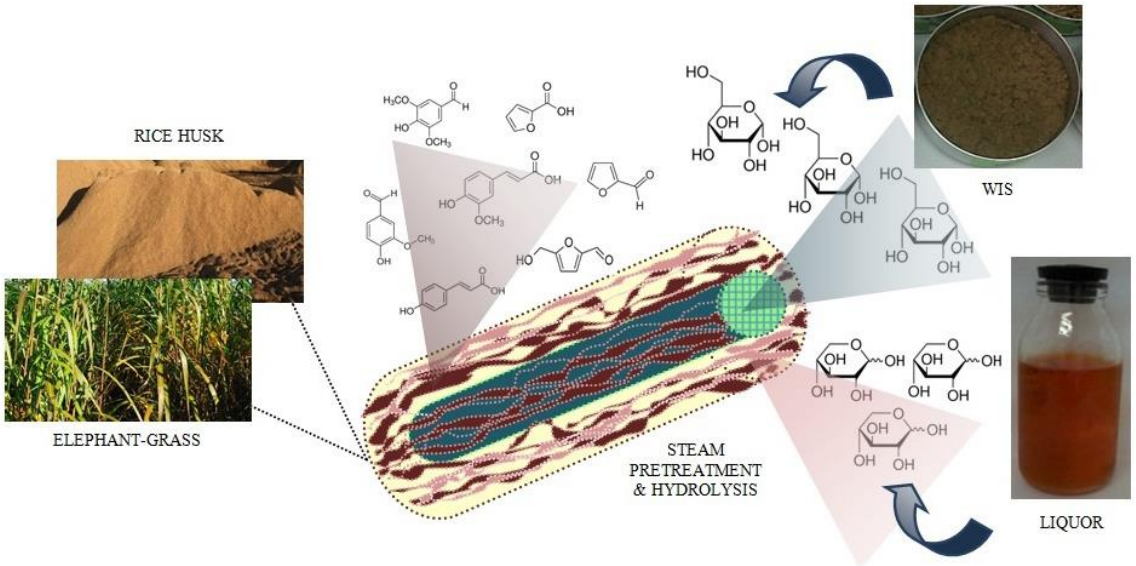
TOC/Abstract graphic



Abstract

The conversion of rice husk and elephant grass into sugars was investigated by uncatalysed steam explosion. The effect of temperature and time was evaluated for the pretreatment steps in order to obtain an optimal content of hexoses in the solid fraction and pentoses in the liquid fraction, according to the conditions of the experimental design. After enzymatic hydrolysis using 5% (*w/v*) of solids and 15 FPU g^{-1} substrate, steam pretreatment at 205 °C for 11.5 min led to 37.38 g glucose/100 g glucose, while the acid hydrolysis of the liquor resulted in 47.53 g xylose/100 g xylose in the untreated rice husk. For elephant grass, a temperature of 207 °C for 9.5 min generated 56.63 g glucose/100 g glucose and 29.20 g xylose/100 g xylose in the untreated elephant grass. The findings of this research introduce a multivariate approach which may be useful in the design of subsequent biochemical processes.

Keywords: rice husk; elephant grass; steam pretreatment; hydrolysis; multivariate design.

INTRODUCTION

An overview of the publications involving biomass-based energy indicates that local use of renewable raw materials is able to minimise capital investments and support the social aspects of sustainability, at the same time as relieving greenhouse gas emissions. In contrast to the sugarcane bagasse and straw used primarily in the South-Central and Northeastern region of Brazil, lignocellulosic biomasses such as rice husk (RH) and elephant grass (EG) are readily available and plentiful in the south, making them appropriate carbohydrate sources for conversion into value-added products and biofuels in this region of the country.[1-4]

The major producer of rice (*Oryza sativa*) in Brazil is the state of Rio Grande do Sul, accounting for 70.9% of the production with an estimated harvest of 8.2 million tons in 2016/2017.[5] During processing, husk represents at least 20% of the dry weight[6] (1.6 million

tons), considered a waste material[7] which is not properly exploited and if accumulated can cause environmental pollution. Pyrolysis has been proposed as a process of appreciation of this by-product, resulting in an ash that is rich in silica.[8] On the other hand, EG (*Pennisetum purpureum*) is a herbaceous crop that has been used as animal fodder for many years and is widely recognised for its high productivity due to its brief harvest cycles.[9,10] An EG crop can produce up to 29.0 tons of dry matter ha^{-1} $year^{-1}$ with a 15% moisture content, while a sugarcane crop, based on bagasse, produces 20.8 tons of dry matter ha^{-1} $year^{-1}$ with 50% moisture content.[11] Thus, there are some initiatives in Brazil to use EG in co-generation processes, such as thermal energy production.[12]

The efficient conversion of lignocellulosic biomass requires pretreatment to disorganise and rupture the lignocellulosic complex. In this process cellulose are prepared for enzymatic digestion,[13] since hemicellulose is degraded to soluble sugars and lignin is modified,[14] making the biomass more susceptible to solvation and, therefore, more easily hydrolysed. Pretreatment by steam explosion (SE) is one of the most widely adopted technologies used to increase the accessibility of enzymes to cellulose molecules,[14,15] and autohydrolysis is an environmentally friendly process that can replace the use of chemical reagents.[16] SE has proved to be the most effective pretreatment for agricultural residues and herbaceous biomass, and involves heating lignocellulose with superheated steam followed by a sudden decompression.[17,18]

In this work, an experimental design approach was employed to investigate the effects of critical parameters of steam pretreatment for RH and EG, and their subsequent hydrolysis to obtain C6-sugars from the solid fraction and C5-sugars from the liquor. The aim was also to promote the use of regional feedstock resources and find suitable uses for RH and EG as renewable and low-cost raw materials.

MATERIALS AND METHODS

Steam explosion (SE) pretreatment

RH was provided by a local supplier in Santa Maria and EG was collected in Nova Petrópolis, Brazil. Pretreatments were undertaken in an SE pilot unit operated in batches, with a reaction vessel volume of 2 L. Due to the different densities of each raw material, batches of 250.0 g (RH) or 200.0 g (EG) of dry matter were transferred to the reactor, which was directly heated to the desired temperature for a specific period of time with saturated steam. Different strategies were evaluated in an experimental design in order to achieve the highest release of sugars from the water insoluble solids (WIS) and the liquor. The following parameters were studied: temperature (181 to 229 °C for RH and 178 to 212 °C for EG) and holding time (1.6 to 11.4 min for RH and 5.9 to 10.1 min for EG).[2,3] A 2^2 Central Composite Rotational Design (CCRD) was planned for these independent variables with three central point repetitions. The overall yields of sugars were selected as the response variables, as well as the concentrations of the main interfering compounds released during the pretreatments. Outcomes were evaluated by using of Statistica 8.0 software. After the process, the slurry was recovered in a cyclone, cooled and filtered to recover the liquid and solid phases. Pretreated material was washed with distilled water before analysis of its sugar production and characterisation of its composition, whereas the liquor was analysed for the presence of sugars and their degradation products. A severity factor $log(R_0)$, based on the temperature (T, °C) and reaction time (t, min) was used to compare the pretreatments through the following equation, where T_{Ref} is the reference temperature of 100 °C, and the arbitrary constant ω was fitted at 14.75.[19]

$$log(R_0) = \int_0^t exp\left(\frac{T - T_{Ref}}{\omega}\right) dt \quad (1)$$

Enzymatic hydrolysis and posthydrolysis

The pretreated insoluble fraction was enzymatically hydrolysed by a cellulolytic complex of Celluclast® 1.5L, containing a cellulase enzyme loading of 15 FPU g^{-1} substrate, and supplemented with Novozyme 188®, containing a ß-glucosidase enzyme loading of 15 IU g^{-1} substrate (Novozymes, Bagsvaerd, Denmark). Hydrolysis was carried out in 50 mL flasks, each containing 20 mL of solution with 5% (*w/v*) of substrate concentration in 50 mmol L^{-1} sodium citrate buffer, pH 4.8. The reaction was incubated in a rotatory shaker at 50 °C and 150 rpm for 72 h.[20] Since most of the sugars present in the liquor were oligomers, a mild acid hydrolysis with 4% (*v/v*) H_2SO_4 at 121 °C for 30 min was required to determine the monomer concentrations.[15] The results were evaluated using GraphPad Prism 5.01 software.

Analytical methods

The chemical composition of the raw and pretreated materials was determined based on the Laboratory Analytical Procedures (LAP) technique established by National Renewable Energy Laboratory (NREL, Golden, USA) for the standardisation of analytical methods for biomass (NREL/TP-510-42618, NREL/TP-510-42619, NREL/TP-510-42620 and NREL/TP-510-42622) (NREL, 2008). Total protein analysis was performed using the method from Kjeldahl, AOAC Official Method 2001.11 (2012).[21]

Sugar content was determined by means of High Performance Liquid Chromatography (HPLC) using a Waters e2695 instrument (Waters, Milford, USA) and the procedure was assisted by a Waters 2414 Refractive Index Detector (RID). The column was a Transgenomic CARBOSep CHO-782 (Transgenomic, San Jose, EUA), the oven temperature was 75 °C, and

ultrapure water was used as the aqueous phase with a low rate of 0.5 mL min^{-1}. Empower software was used for data analysis.

Acetic and formic acid concentrations were determined by means of a Waters HPLC coupled to RID and equipped with a 515 quaternary pump, a 2707 automatic sampler, and a 7971 column oven. Helium gas was used as a degasser. A Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, USA) was used at an oven temperature of 65 °C. The aqueous phase was ultrapure water containing 5 mmol L^{-1} H_2SO_4, and the flow rate was 0.6 mL min^{-1} flow.

Furfural, 5-hydroxymethylfurfural (5-HMF), vanillin, p-coumaric acid and ferulic acid were determined and quantified through an Agilent Technologies HPLC (Waldbronn, Germany) linked to a G1315D diode array detector (DAD) and equipped with a G1311A quaternary pump, a G1322A degasser, a G1313A auto sampler, and a 7971 column oven, using ChemStation software. A Transgenomic Coregel 87H3 column, at an oven temperature of 65 °C, was used in an isocratic mode with 89% of the aqueous phase (ultrapure water containing 5 mmol L^{-1} H_2SO_4) and 11% of acetonitrile, a flow rate of 0.7 mL min^{-1} and detection in the range 190-400 nm. Elution was in isocratic mode and the injection volume was 20 µL for all HPLC analyses.[15]

Phenolic compounds in the liquor were quantified using an adaptation of the vanillin method.[22]

RESULTS AND DISCUSSION

Characterisation of raw materials

The macromolecular compositions of *in natura* raw materials are summarised in Table 1 as a percentage of the dry weight, the moisture content of RH being determined as 6.52%

and that of EG as 7.10%. It is well known that herbaceous feedstocks can contain significant amounts of protein in the stalks and leaves, which can interfere with lignin measurements in compositional analyses, also contributing to the sum of the components. The amount of proteins present was subsequently investigated, and a greater amount was found in EG (8.06±0.11%) compared with RH (2.01±0.02%).

Table 1 Chemical composition of rice husk and elephant grass (% of dry weight)

Components	Raw materials (%)	
	Rice husk	Elephant grass
Extractives	3.73 ± 0.37	16.43 ± 0.99
Water	2.98 ± 0.33	14.51 ± 1.01
Ethanol	0.76 ± 0.08	1.92 ± 0.04
Cellulose	33.79 ± 0.13	33.60 ± 0.11
Hemicellulose	18.07 ± 0.13	20.62 ± 0.02
Xylan	14.55 ± 0.04	17.28 ± 0.08
Galactan	1.50 ± 0.04	1.18 ± 0.02
Arabinan	1.84 ± 0.09	1.90 ± 0.06
Mannan	0.18 ± 0.04	0.25 ± 0.02
Insoluble lignin	24.74 ± 0.10	16.65 ± 0.15
Soluble lignin	0.75 ± 0.04	1.77 ± 0.07
Ashes	17.27 ± 0.07	12.25 ± 0.11
Acetyl groups	1.41 ± 0.03	0.99 ± 0.03
Starch	2.26 ± 0.15	nd
Total	102.02	102.31

These data are mean values of three assessments.
nd: not determined.

Comparing the composition of the untreated RH with data from the literature, besides variability and complexity of this lignocellulosic structure, there was reasonable similarity overall, although the amounts of lignin and the non-structural components were quite variable. Dagnino *et al.*[23] found that total lignin represented 19.0% of the dry mass, a rather lower percentage than the 27.93% found by Pedroso *et al.*[24] and the 25.49% found in the present study. The amount of extractives was intermediate between the content found by Dagnino *et al.*[23] (8.2%) and Pedroso *et al.*[24] (1.0%). The untreated EG used here also contained different percentages of each component compared with previous research,[3,25] especially extractives, with slightly more ash and fewer acetyl groups. The extractives content

was intermediate between the amounts found by Scholl *et al.*[3] (8.84%) and Toscan *et al.*[25] (21.9%).

The high percentage of extractives in EG compared with RH was one of the major differences between these lignocellulosic biomasses. In addition, RH exhibited high lignin and ash content, a high level of ash being a characteristic feature of this raw material.[6] The elevated content of extractives and ash increases the buffering capacity, which decreases the efficiency of the pretreatment process. Li *et al.*[26] speculated that extractives-free corn stover presented higher cellulose digestibility than ordinary corn stover after liquid hot water pretreatment, since it did not inhibit xylan removal. Equally, some extractives could condense on the surface of the corn stover after pretreatment, hindering enzymatic hydrolysis.

Steam explosion (SE) pretreatments: chemical composition of water insoluble solids (WIS) and liquor

Carbohydrates

A mass balance was accomplished to analyse the influence of each independent variable on the pretreatment, and to predict its efficiency. The RH and EG biomass compositions after SE pretreatment, with the coded and the real values for each assay, are listed in Table 2 and Table 3, as a dry weight percentage.

Table 2 Rice husk biomass composition (% of dry weight) after steam explosion pretreatment

Assay	Steam explosion conditions				pH	Solids recovery (%)	WIS[a] composition (%)			
	Temperature (°C)	Time (min)	Pressure (MPa)	Severity *log(R_0)*			Cellulose	Hemicellulose	Lignin[b]	Ashes
1	205.0 (0)	6.5 (0)	1.72	3.9	3.81	74.85	40.05 ± 0.78	5.08 ± 0.26	30.33 ± 0.15	20.52 ± 0.05
2	188.0 (-1)	3.0 (-1)	1.20	3.1	4.57	91.46	36.31 ± 0.18	16.58 ± 0.10	26.65 ± 0.46	16.47 ± 0.07
3	222.0 (+1)	10.0 (+1)	2.41	4.6	3.27	67.91	43.67 ± 0.48	0.62 ± 0.04	33.22 ± 0.35	21.61 ± 0.02
4	205.0 (0)	6.5 (0)	1.72	3.9	3.81	75.92	41.51 ± 0.01	5.08 ± 0.11	33.64 ± 0.06	20.50 ± 0.03
5	181.0 (-1.41)	6.5 (0)	1.03	3.2	4.45	93.98	34.17 ± 0.35	15.87 ± 0.43	25.87 ± 0.79	16.76 ± 0.05
6	188.0 (-1)	10.0 (+1)	1.20	3.6	4.15	82.23	39.98 ± 0.15	12.05 ± 0.15	27.04 ± 0.40	18.17 ± 0.19
7	205.0 (0)	6.5 (0)	1.72	3.9	3.81	72.53	41.76 ± 0.05	5.45 ± 0.15	29.53 ± 0.38	20.94 ± 0.34
8	229.0 (+1.41)	6.5 (0)	2.75	4.6	3.27	65.65	43.35 ± 0.08	0.58 ± 0.00	34.67 ± 0.12	21.73 ± 0.02
9	205.0 (0)	1.6 (-1.41)	1.72	3.3	4.21	80.65	37.78 ± 0.11	11.93 ± 0.26	26.63 ± 0.42	18.36 ± 0.24
10	222.0 (+1)	3.0 (-1)	2.41	4.1	3.38	71.19	41.34 ± 0.61	1.38 ± 0.03	35.89 ± 0.70	21.25 ± 0.34
11	205.0 (0)	11.5 (+1.41)	1.72	4.2	3.47	68.42	43.54 ± 0.15	2.36 ± 0.10	32.19 ± 0.32	21.23 ± 0.04

These data are mean values of three assessments.
[a] water insoluble solids.
[b] acid-insoluble lignin.

Table 3 Elephant grass biomass composition (% of dry weight) after steam explosion pretreatment

Assay	Steam explosion conditions				pH	Solids recovery (%)	WIS[a] composition (%)			
	Temperature (°C)	Time (min)	Pressure (MPa)	Severity *log(R_0)*			Cellulose	Hemicellulose	Lignin[b]	Ashes
1	195.0 (0)	10.1 (+1.41)	1.40	3.8	5.22	63.93	50.41 ± 0.39	17.34 ± 0.12	24.22 ± 0.17	9.19 ± 0.49
2	207.0 (+1)	9.5 (+1)	1.80	4.1	4.42	60.29	53.13 ± 0.17	7.44 ± 0.02	30.95 ± 0.29	9.91 ± 0.21
3	183.0 (-1)	9.5 (+1)	1.07	3.4	5.98	67.69	47.55 ± 1.94	25.29 ± 0.77	23.95 ± 0.81	8.44 ± 1.03
4	195.0 (0)	8.0 (0)	1.40	3.7	5.35	66.61	45.67 ± 0.49	17.69 ± 0.21	24.66 ± 0.24	8.75 ± 0.22
5	183.0 (-1)	6.5 (-1)	1.07	3.3	6.28	75.79	42.90 ± 0.15	24.37 ± 0.04	22.97 ± 0.65	9.55 ± 0.05
6	207.0 (+1)	6.5 (-1)	1.80	4.0	4.84	63.85	47.15 ± 1.33	10.41 ± 0.23	25.58 ± 0.31	10.60 ± 0.02
7	212.0 (+1.41)	8.0 (0)	1.98	4.2	4.41	60.54	50.68 ± 0.10	5.86 ± 0.04	31.20 ± 0.32	11.00 ± 0.25
8	195.0 (0)	8.0 (0)	1.40	3.7	5.5	66.66	44.67 ± 0.62	17.22 ± 0.31	23.93 ± 0.10	9.14 ± 0.35
9	178.0 (-1.41)	8.0 (0)	0.96	3.2	6.42	77.62	38.91 ± 0.04	22.85 ± 0.04	24.51 ± 0.70	9.65 ± 0.29
10	195.0 (0)	5.9 (-1.41)	1.40	3.6	5.45	75.99	42.57 ± 0.03	20.61 ± 0.09	24.70 ± 0.29	10.78 ± 0.11
11	195.0 (0)	8.0 (0)	1.40	3.7	5.61	68.59	43.81 ± 0.34	17.57 ± 0.03	24.96 ± 0.01	10.74 ± 0.14

These data are mean values of three assessments.
[a] water insoluble solids.
[b] acid-insoluble lignin.

As expected, cellulose was the most plentiful sugar, followed by the hemicellulose-derived sugars. For RH, the cellulose and hemicellulose contents varied from 34.17 to 43.67% and 0.58 to 16.58%, respectively, as a result of pretreatment (Table 2). Pretreatment at 222 °C for 10 minutes (assay 3), or at the positive axial points, represented by 229 °C (assay 8) or 11.5 min (assays 11), show how elevated temperatures and/or long residence times favour increased cellulose extraction, while contributing to the removal of extractives and the partial solubilisation of the hemicellulose portion. The EG resulted in higher cellulose and hemicellulose contents compared with RH, as milder conditions were used (Table 3). The cellulose content ranged from 38.91 to 53.13% and hemicellulose content comprised 5.86 to 25.29%. Higher values for cellulose resulted from pretreatments for 10.1 min (assay 1), at 207 °C for 9.5 min (assay 2) or at 212 °C (assay 7).

Overall, the pretreated biomasses present a greater percentage cellulose content compared with the *in natura* materials. The percentage lignin content also increased under all pretreatment, especially in the more severe conditions. However, hemicellulose was more susceptible to SE pretreatment and its content was much lower in the WIS than in the untreated raw materials. This enrichment has been attributed to the extraction of carbohydrates in the pretreated biomass. Losses of WIS are associated with changes in the lignocellulosic structure and, in agreement with Bondesson *et al.*,[27] a gradual loss of solids was observed as the values of the independent variables increased. The recovery of EG solids was lower than for RH, as there was higher recovery of water soluble solids from the EG liquor (data not shown).

The liquor resulting after filtration was rich in oligomers of sugars and Table 4 and Table 5 show their contents as g/100 g raw material after acid hydrolysis treatment. Xylose was present at the highest percentage for both raw materials. For RH, it content ranged from

1.23 to 8.27 g/100 g RH, followed by glucose. Central point assays (205 °C, 6.5 min) showed the highest values for xylose production, along with assay 11 (205 °C, 11.5 min) (Table 4). Using EG, xylose varied from 1.63 to 7.33 g/100 g EG, where a temperature of 207 °C and a holding time of 6.5 min (assay 6) resulted in the highest levels of this sugar (Table 5).

Table 4 Composition of rice husk liquor (g/100 g rice husk) obtained from steam explosion followed by posthydrolysis

Assay	Monosaccharides (g/100 g rice husk)				
	Glucose	Xylose	Galactose	Arabinose	Mannose
1	4.78 ± 0.21	7.73 ± 0.34	0.86 ± 0.04	0.62 ± 0.03	0.15 ± 0.01
2	3.92 ± 0.19	1.23 ± 0.06	0.35 ± 0.02	0.52 ± 0.03	0.00 ± 0.00
3	2.92 ± 0.10	2.44 ± 0.08	0.34 ± 0.01	0.13 ± 0.00	0.11 ± 0.00
4	5.05 ± 0.17	7.87 ± 0.26	0.86 ± 0.03	0.68 ± 0.02	0.18 ± 0.01
5	3.48 ± 0.20	1.38 ± 0.08	0.37 ± 0.02	0.56 ± 0.03	0.00 ± 0.00
6	3.84 ± 0.22	4.73 ± 0.27	0.70 ± 0.04	0.77 ± 0.04	0.05 ± 0.00
7	5.21 ± 0.30	8.27 ± 0.47	0.98 ± 0.06	0.91 ± 0.05	0.07 ± 0.00
8	3.01 ± 0.06	1.98 ± 0.04	0.38 ± 0.01	0.15 ± 0.00	0.09 ± 0.00
9	4.66 ± 0.19	4.93 ± 0.20	0.78 ± 0.03	0.85 ± 0.03	0.00 ± 0.00
10	3.00 ± 0.10	5.42 ± 0.17	0.71 ± 0.02	0.40 ± 0.01	0.00 ± 0.00
11	3.97 ± 0.14	7.82 ± 0.27	0.94 ± 0.03	0.59 ± 0.02	0.00 ± 0.00

	Lignocellulose-derived by-products (g/100 g rice husk)				
	Furfural	5-HMF	Acetic acid	Formic acid	Phenols
1	0.31 ± 0.01	0.06 ± 0.00	0.71 ± 0.03	0.31 ± 0.01	1.58 ± 0.01
2	0.01 ± 0.00	0.00 ± 0.00	0.17 ± 0.01	0.08 ± 0.00	0.75 ± 0.00
3	1.03 ± 0.03	0.48 ± 0.02	1.64 ± 0.05	0.85 ± 0.03	2.29 ± 0.01
4	0.30 ± 0.01	0.08 ± 0.00	0.70 ± 0.02	0.31 ± 0.01	1.84 ± 0.01
5	0.01 ± 0.00	0.01 ± 0.00	0.18 ± 0.01	0.08 ± 0.00	0.59 ± 0.01
6	0.07 ± 0.00	0.02 ± 0.00	0.34 ± 0.02	0.17 ± 0.01	1.28 ± 0.01
7	0.28 ± 0.02	0.06 ± 0.00	0.69 ± 0.04	0.34 ± 0.02	1.57 ± 0.01
8	1.07 ± 0.02	0.64 ± 0.01	1.76 ± 0.03	0.86 ± 0.02	3.20 ± 0.01
9	0.04 ± 0.00	0.02 ± 0.00	0.29 ± 0.01	0.16 ± 0.01	1.10 ± 0.02
10	0.66 ± 0.02	0.17 ± 0.01	1.32 ± 0.04	0.75 ± 0.02	2.14 ± 0.01
11	0.75 ± 0.03	0.15 ± 0.01	1.10 ± 0.04	0.45 ± 0.02	1.25 ± 0.01

These data are mean values of two assessments.

Some assays were able to achieve a reasonable balance between the amount of glucan in the WIS and the amount of xylose released in the liquor. In the case of RH, the assay at the central point and assay 11 generated similar yields of glucan (up to 28.85% higher than for untreated RH) and xylose. For EG, a temperature of 195 °C held for 10.1 min (assay 1) and

conditions of 207 °C for 9.5 min (assay 2) generated an improvement of up to 58.15% of glucan compared with untreated EG.

Table 5 Composition of elephant grass liquor (g/100 g elephant grass) obtained from steam explosion followed by posthydrolysis

Assay	Monosaccharides (g/100 g elephant grass)				
	Glucose	Xylose	Galactose	Arabinose	Mannose
1	1.28 ± 0.04	5.46 ± 0.19	0.90 ± 0.03	1.16 ± 0.04	0.15 ± 0.00
2	1.40 ± 0.04	5.70 ± 0.15	0.77 ± 0.02	0.54 ± 0.01	0.09 ± 0.00
3	1.47 ± 0.09	2.81 ± 0.17	1.06 ± 0.06	1.09 ± 0.07	0.22 ± 0.01
4	1.24 ± 0.05	4.62 ± 0.20	0.87 ± 0.04	1.19 ± 0.05	0.00 ± 0.00
5	1.64 ± 0.07	1.63 ± 0.07	1.05 ± 0.04	0.82 ± 0.03	0.29 ± 0.01
6	1.74 ± 0.03	7.33 ± 0.14	1.03 ± 0.02	0.90 ± 0.02	0.00 ± 0.00
7	1.39 ± 0.04	3.22 ± 0.09	0.60 ± 0.02	0.27 ± 0.01	0.23 ± 0.01
8	1.64 ± 0.07	4.40 ± 0.18	1.04 ± 0.04	1.08 ± 0.04	0.06 ± 0.00
9	1.30 ± 0.07	3.06 ± 0.17	0.87 ± 0.05	0.89 ± 0.05	0.21 ± 0.01
10	1.38 ± 0.05	3.32 ± 0.11	0.92 ± 0.03	0.95 ± 0.03	0.21 ± 0.01
11	1.51 ± 0.05	4.50 ± 0.14	1.06 ± 0.03	1.15 ± 0.04	0.30 ± 0.01

	Lignocellulose-derived by-products (g/100 g elephant grass)				
	Furfural	5-HMF	Acetic acid	Formic acid	Phenols
1	0.03 ± 0.00	0.00 ± 0.00	1.38 ± 0.05	0.68 ± 0.02	2.48 ± 0.01
2	0.17 ± 0.00	0.02 ± 0.00	1.88 ± 0.05	1.81 ± 0.05	3.75 ± 0.01
3	0.00 ± 0.00	0.00 ± 0.00	1.40 ± 0.09	0.33 ± 0.02	1.82 ± 0.02
4	0.02 ± 0.00	0.00 ± 0.00	1.21 ± 0.05	0.52 ± 0.02	2.18 ± 0.01
5	0.00 ± 0.00	0.00 ± 0.00	1.32 ± 0.05	0.27 ± 0.01	1.71 ± 0.02
6	0.07 ± 0.00	0.01 ± 0.00	1.44 ± 0.03	0.91 ± 0.02	2.98 ± 0.02
7	0.17 ± 0.00	0.03 ± 0.00	1.42 ± 0.04	1.62 ± 0.04	3.67 ± 0.02
8	0.01 ± 0.00	0.00 ± 0.00	1.28 ± 0.05	0.49 ± 0.02	2.76 ± 0.01
9	0.00 ± 0.00	0.00 ± 0.00	1.26 ± 0.07	0.22 ± 0.01	1.49 ± 0.02
10	0.01 ± 0.00	0.00 ± 0.00	1.19 ± 0.04	0.47 ± 0.02	1.64 ± 0.02
11	0.01 ± 0.00	0.00 ± 0.00	1.32 ± 0.04	0.53 ± 0.02	2.41 ± 0.01

These data are mean values of two assessments.

Aliphatic carboxylic acids, furans and phenolic compounds

The severity factor $log(R_0)$ was used to take into account the influence of temperature and time on each sample (Table 2 and Table 3). Assays around the central point and using high temperatures or long time periods contributed to increase $log(R_0)$, and, under these conditions, hemicellulose was degraded while cellulose in the solids was subject to significant hydrolysis.[28]

Several degradation end-products were found in the pretreated raw materials (Table 4 and Table 5). According to Table 4, assays 3 (222°C, 10 min) and 8 (229 °C, 6.5 min) contained the highest percentages of interfering compounds in the liquor when using RH. In the case of EG, assays 2 (207 °C, 9.5 min) and 7 (212 °C, 8 min) generated the greatest amounts of these compounds (Table 5).

For RH, the central point assay and assay 11 resulted in the release of intermediate amounts of by-products, and in the central point assay their production was lower, with the exception of phenolic compounds (Table 4). Compared with assays 1 and 2 using EG, assay 1 released smaller amounts of all inhibitors (Table 5). For both biomass types, the products of interest attained satisfactory yields at a severity factor close to 4.0 (Table 2 and Table 3). The equations below show the influence of the independent variables on the release of the main by-products during RH pretreatment. The units are g L^{-1}. Only the significant coefficients ($p<0.05$) were considered, where *T* is temperature and *t* is time.

$$Furfural_{(RH)} = 0.84 + 0.83T \qquad R^2 = 90.0\%;\ F = 81.8 \qquad (2)$$

$$5\text{-}HMF_{(RH)} = 0.19 + 0.38T + 0.20T^2 + 0.12t + 0.14T * t \qquad R^2 = 99.3\%; F = 205.8 \qquad (3)$$

$$Acetic\ acid_{(RH)} = 2.00 + 1.15T \qquad R^2 = 83.1\%; F = 44.4 \qquad (4)$$

$$Formic\ acid_{(RH)} = 0.91 + 0.63T \qquad R^2 = 79{,}6\%; F = 35.0 \qquad (5)$$

$$Phenols_{(RH)} = 4.76 + 1.17T \qquad R^2 = 86.4\%; F = 57.2 \qquad (6)$$

Similarly, the following equations are related to the conditions employed during EG pretreatment.

$$Furfural_{(EG)} = 0.09T + 0.06T^2 + 0.02t + 0.03T * t \qquad R^2 = 97.6\%; F = 96{,}4 \qquad (7)$$

$$5\text{-}HMF_{(EG)} = 0.01T + 0.01T^2 \qquad R^2 = 92.4\%; F = 109.4 \qquad (8)$$

$$Acetic\ acid_{(EG)} = 2.68 - 0.48T \qquad R^2 = 87.4\%; F = 62.3 \qquad (9)$$

$$Formic\ acid_{(EG)} = 1.08 + 0.70T + 0.32T^2 \qquad R^2 = 92.1\%; F = 46.7 \qquad (10)$$

$$Phenols_{(EG)} = 5.16 + 0.50T \qquad R^2 = 78.9\%; F = 33.6 \qquad (11)$$

For the studied range, the above equations show that temperature is the only important variable in achieving high concentrations of by-products from both raw materials. Table S1 and Table S2 (Supporting Information) show the experimental values of the by-products concentration and the predicted values by the coded models, which were validated by the analysis of variance (ANOVA).

Comparing SE pretreatments of RH and EG in terms of the production of inhibitors, it was notable that EG generated higher levels of phenolic compounds, as well as acetic acid and formic acid, and lower amounts of furfural and 5-HMF. Even though the assays were milder than RH, EG seems to be more vulnerable to steam pretreatment due to its particular composition. Therefore, acetyl groups contained in hemicellulose were more easily hydrolysed into acetic acid and, possibly, furfural and 5-HMF from sugar degradation was immediately converted into formic acid during the pretreatment, accounting for their lower amounts. Although pretreated EG exhibited high levels of acetic acid and formic acid, these results were not entirely consistent with the detected pH values, which were less acidic than in the pretreated RH samples (Tables 2-5). As EG had a significant content of extractives and even ashes that may have buffering capacity, it is likely that this feature contributed to the almost neutral pH values that were detected. This study preferred to use only the severity factor, and not the combined severity factor, which involves the final pH of the liquor. In this sense, extractives seem to be strongly connected to buffering capacity regarding ashes — keeping in mind that RH contains much more ash and significant buffering was not evident.

Sugar recovery

Sugar recovery graphs are shown in Figure 1, with recovery represented as a percentage of the initial component in each raw material. Both glucan recovery from the solid

fraction (GR_{WIS}) and xylan recovery from the liquid fraction (XR_{LIQ}) are shown. In general, glucan recovery showed similar behaviour between the two raw materials studied and the rates of glucan loss were lower than those for xylan.

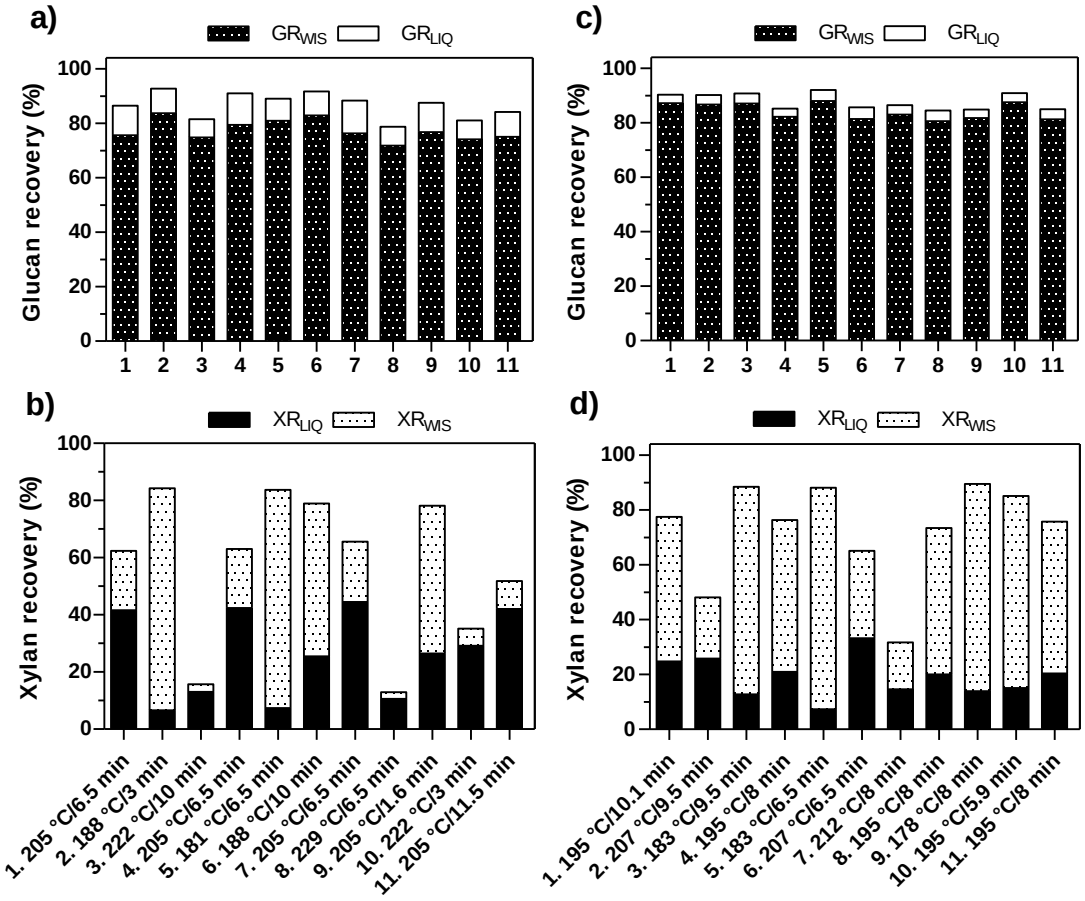


Figure 1 Sugar recovery yields obtained using different conditions of steam explosion expressed as the percentage of glucan (a, c) and xylan (b, d) recovered from the pretreated material or liquor, respectively, in relation to the original sugar content in the rice husk (left columns) or elephant grass (right columns) (GR_{WIS}: glucan recovery in the solid fraction; GR_{LIQ}: glucan recovery in the liquid fraction; XR_{LIQ}: xylan recovery in the liquid fraction; XR_{WIS}: xylan recovery in the solid fraction)

In relation to pretreated RH (Figure 1a, b), most of the glucan was recovered in WIS, with yields greater than 71.82% when temperatures of 188 °C or lower that favoured its recovery were employed. XR_{LIQ} ranged from 6.64 to 44.51% and pretreatments at 205 °C for

6.5 min (assays 1, 4 and 7), or 11.5 min (assay 11), elevated its recovery. Central point assays resulted in a little more sugars than assay 11. Unfortunately, assays with sufficient hemicellulose solubilisation do not always present an increase in XR_{LIQ} owing to sugar degradation,[29] and this phenomenon was rather pronounced in assays with RH. In total, the maximum losses were 21.27% for glucan and 87.08% for xylan at 229 °C, and xylan recovery yields dropped off at temperatures over 222 °C.

In experiments with pretreated EG (Figure 1c, d), glucan recovery from both phases was practically constant in all conditions, with GR_{WIS} more than 80.55%. XR_{LIQ} was between 7.35 and 33.19%, and the pretreatment at 207 °C for 6.5 min (assay 6) gave the highest value. Liquor from pretreated EG exhibited greater sugar losses compared with RH, which might be attributed to the nature of the starting material. The greatest losses were 15.45% for glucan at 195 °C for 8 min (assay 8), and 68.23% for xylan at 212 °C. Assay 2 recovered slightly more sugars of interest than assay 1.

Enzymatic hydrolysis

SE induced improvements in the yield of glucose were analysed after subsequent enzymatic hydrolysis with a 5% (*w/v*) solids loading and an enzyme dosage of 15 FPU g^{-1} substrate. The released glucose was expressed as a percentage of the potential glucose in the pretreated material (Figure 2). Successful enzymatic hydrolysis of pretreated RH and EG was influenced by the operating conditions of the SE, especially high temperatures. It is known that RH contains high amounts of lignin and ashes[30] — even higher than those found in EG — which constrains its use for the release of fermentable sugars. However, glucose yields were higher in RH relative to those in EG. Removal of xylan from the cellulose matrix increases cellulose accessibility and subsequently improves enzymatic hydrolysis,[31] and this

is evidenced in Table 4 and Table 5, where the presence of xylose in the liquor was more pronounced in the case of RH.

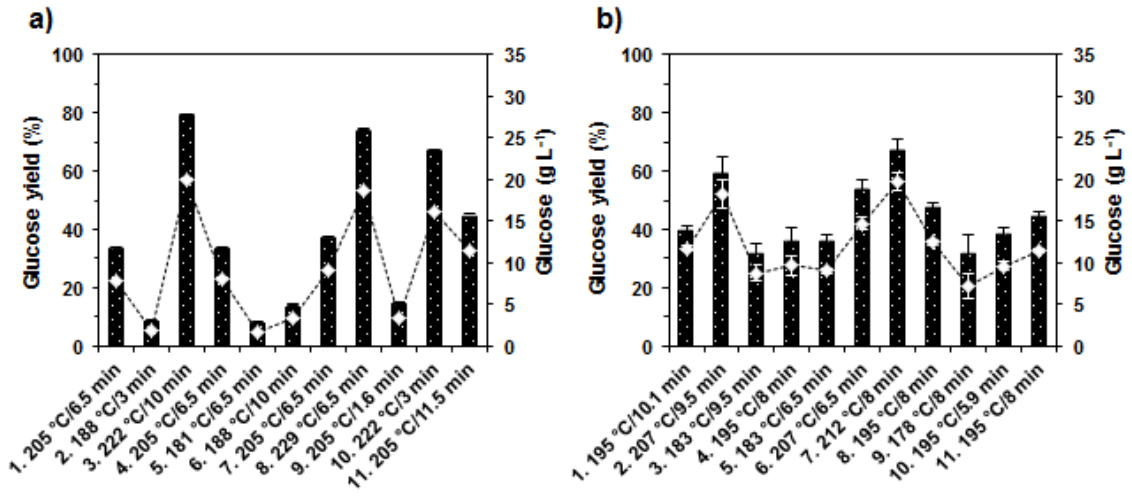


Figure 2 Glucose concentrations and yields obtained after 72 h of enzymatic hydrolysis using steam-exploded rice husk (a) and elephant grass (b) under different conditions (vertical bars: yield; dashed lines: concentration)

It is notable that low temperatures were more suitable for the enzymatic hydrolysis of pretreated EG compared with RH, inasmuch as its yields were demonstrated to be more constant under different conditions. Of the initially selected pretreatments, assay 11 seems to be better than the central point for RH, with a hydrolysis yield of 45.23% — a glucose yield approx. 28.0% higher than at the central point (Figure 2a). For EG, the assay 2 hydrolysis yield was 59.39%, while assay 1 achieved only 40.23% (Figure 2b). Ewanick and Bura[31] pretreated switchgrass and sugarcane bagasse by SE followed by saccharification with 5% (*w/v*) solids loading and 10 FPU g^{-1} cellulose of cellulose. When the dry bagasse was steam-exploded at 205 °C for 10 min, the cellulose conversion of one of the analysed varieties was slightly higher than the results found here for the EG. Moreover, the authors achieved similar

conversion when the dry switchgrass, a herbaceous biomass analogous to EG, was pretreated at 195 °C for 7.5 min. Despite the increased conversion of cellulose when SO_2 was employed by these authors, it is possible to evaluate the potential of RH and EG in relation to sugarcane bagasse — a well-known source for the production of second generation ethanol.

Overall yield of sugars

The conversion of lignocellulosic biomass into sugars is laborious and costly.[32] To allow assessment of the efficiency of the whole process, the overall yields of glucose and xylose, including the soluble sugars obtained after hydrolysis, are presented in Figure 3. Values for the released sugars in each fraction are presented as a percentage of the values of the sugars in the raw materials. In the case of RH, overall glucose yields reached 72.68% and higher temperatures of 222 and 229 °C resulted in higher yields. Overall xylose yields achieved 61.12% and assays at 205 °C for 6.5 min (central point) or 11.5 min (assay 11), contributed to this (Figure 3a, b). For EG, temperatures of 207 and 212 °C enhanced overall glucose yields, reaching 65.19%. Overall xylose yields reached 59.08% with a pretreatment of 207 °C for 6.5 min (assay 6) (Figure 3c, d).

In an experiment with olive tree wood using SE and subsequent delignification, Cara *et al.*[29] reported that higher temperatures resulted in a lower yield of the overall process. However, it must be borne in mind that at high temperatures, the glucose solution obtained after enzymatic hydrolysis is more concentrated, which in turn makes the ethanol conversion process more efficient. Altogether, temperatures around the central point or tending to positive axial points, allied with long processing times, contributed to the efficiency of the whole process, given that the steam pretreatment results must be interpreted in terms of glucose released from WIS (G_{WIS}) and xylose released from the liquor (X_{LIQ}). Satisfactory

values were found when RH was steam treated at 205 °C for 11.5 min (assay 11), releasing 22.64 g sugars/100 g RH (14.82 g of G_{WIS} and 7.82 g X_{LIQ}, for each 100 g of RH, equivalent to 37.38 g glucose per 100 g glucose present in the untreated RH and 47.53 g xylose per 100 g xylose in the untreated RH, respectively). For EG, a temperature of 207 °C for 9.5 min (assay 2) led to 26.63 g sugars/100 g EG (20.93 g of G_{WIS} and 5.70 g X_{LIQ}, for each 100 g of EG, equivalent to 56.63 g glucose per 100 g glucose present in the untreated EG and 29.20 g xylose per 100 g xylose in the untreated EG, respectively).

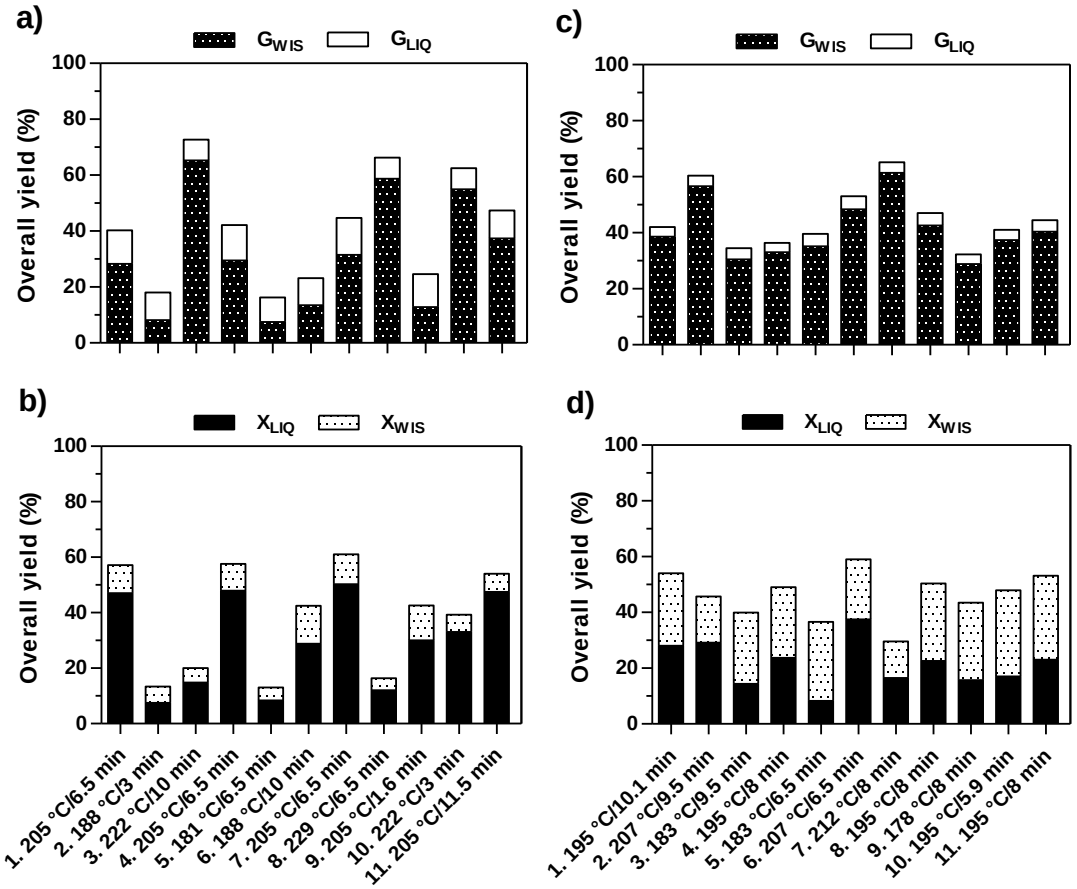


Figure 3 Overall sugar yields after hydrolysis in each fraction from steam explosion pretreatment of rice husk (a) and elephant grass (b) under different conditions, expressed as a percentage of the original sugar content in the raw materials (G_{WIS}: glucose in the solid fraction; G_{LIQ}: glucose in the liquid fraction; X_{LIQ}: xylose in the liquid fraction; X_{WIS}: xylose in the solid fraction)

SE improvement of the overall sugar yield (OY G_{WIS} and OY X_{LIQ}) was also analysed after subsequent hydrolysis, using response surface methodology. The analysis is shown in Figure 4 for RH (left column) and EG (right column). The equations below represent the optimisation pretreatments of OY G_{WIS} and OY X_{LIQ} in RH, as well as the equation for G_{WIS} in EG. Only significant coefficients ($p<0.05$) are included, as functions of the independent variables, where *T* is temperature and *t* is time.

$$OY\ G_{WIS(RH)} = 29.71 + 21.38T + 6.31t \qquad R^2 = 94.9\%; F = 74.6 \qquad (12)$$

$$OY\ X_{LIQ(RH)} = 48.35 - 20.00T^2 - 5.63t^2 - 9.88T * t \qquad R^2 = 96.7\%; F = 67.6 \qquad (13)$$

$$OY\ G_{WIS(EG)} = 38.69 + 10.67T \qquad R^2 = 94.7\%; F = 159.9 \qquad (14)$$

In accordance with ANOVA, these regression coefficients were properly explained by the models. Pareto diagrams shown in Figure S1 and Figure S2 (Supporting Information) corroborate the above equations and show the *t* test for each independent variable. For both types of biomass, there was an increase of OY G_{WIS} when the variables migrate from -1.41 to +1.41, and the surface responses shown in Figure 4a and Figure 4c illustrate these behaviours, where temperature was the main effect variable (Eq. 12 and Eq. 14). For RH, there was a decline in OY X_{LIQ} when harsh conditions were employed (Figure 4b), with temperature (quadratic) being the most significant variable, followed by time (quadratic), and their interactions (Eq. 13). The plot of the experimental responses for the OY G_{WIS} and OY X_{LIQ} versus the polynomial predicted values by the coded models is given in Supporting Information (Figure S3 and Figure S4). Analysis of independent variables that optimise OY G_{WIS} and OY X_{LIQ} yields equally (in the same assay) was also performed using the desirability function (Figure S5 and Figure S6). The results obtained were not consistent with those expected or could not be reproduced, and assays 11(RH) and 2 (EG) were considered the optimised assays.

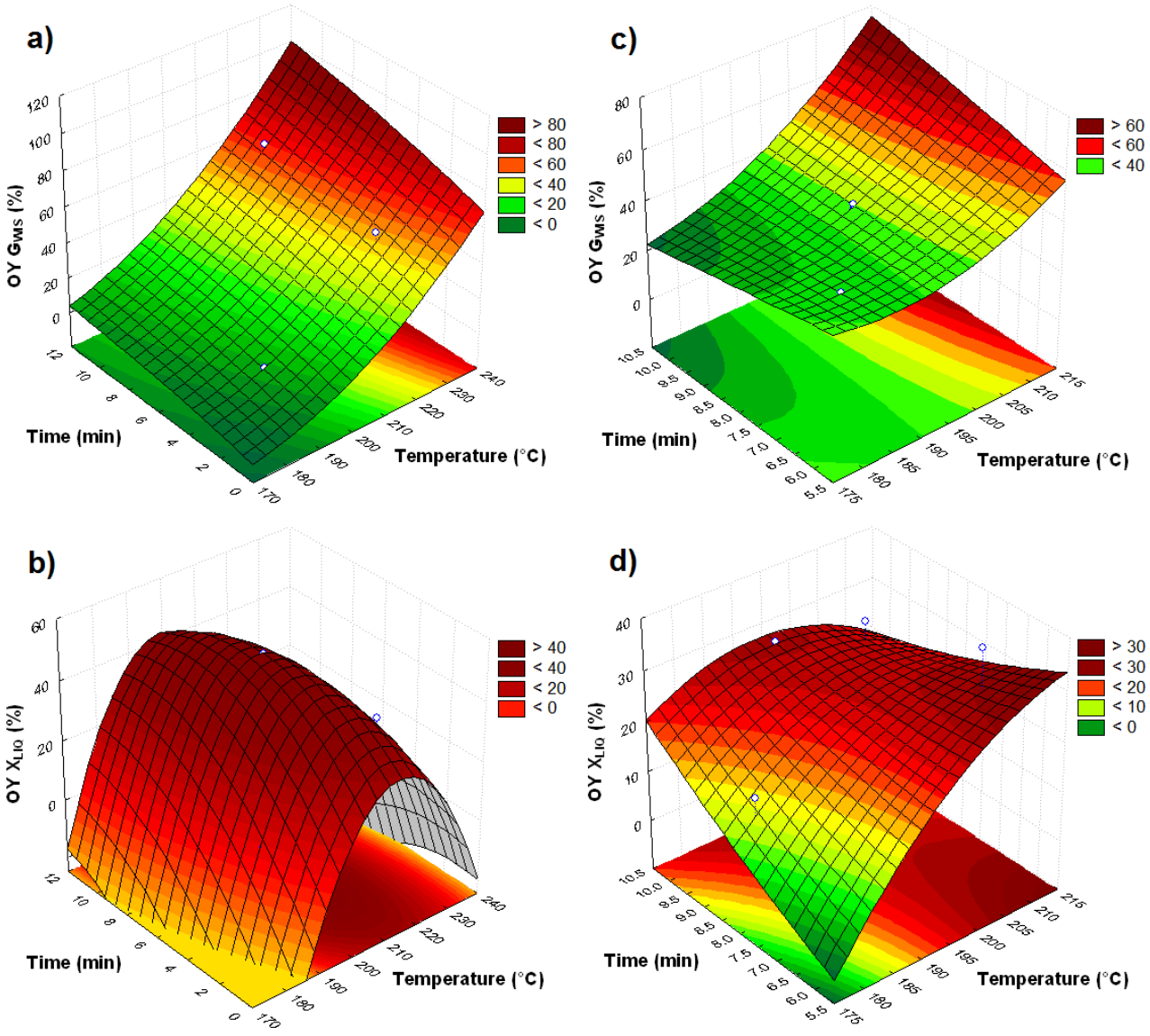


Figure 4 Rice husk (left column) and elephant grass (right column) response surfaces comprising the interactions between temperature (°C) and time (min) for overall yields of glucose (a, c) released in the solid fraction (OY G_{WIS}), and xylose (b, d) released in the liquid fraction (OY X_{LIQ}) after hydrolysis, expressed as the percentage of each sugar generated related to the original sugar content in the raw materials

CONCLUSIONS

This research focused on improving yields of G_{WIS} and X_{LIQ} equally, employing SE as an effective pretreatment to increase the digestibility of underutilised RH and EG, even in

samples with high extractives and ash contents. Apart from the high yields recovered from G_{WIS}, several assays conditions were also able to preserve good yields in X_{LIQ}. In the range of the experimental runs, temperatures around the central point or just to its right, with long treatment times, provided suitable conditions to increase yields of both G_{WIS} and X_{LIQ} for each raw material. To improve the overall process for further experiments, removal of unwanted by-products from the liquor must be performed. Additionally, further studies will focus on superior solid loading in an attempt to produce better yields of value-added products from G_{WIS} and X_{LIQ}.

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The authors declare that they have no conflicts of interest.

Description of Supporting Information

Supporting Information associated with this article can be found, in the online version, at … (to be accomplished).

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Supporting Information

Table S1 Experimental and predicted values of by-products levels after pretreatment using rice husk

Assay	Furfural (g L^{-1})		5-HMF (g L^{-1})		Acetic acid (g L^{-1})		Formic acid (g L^{-1})		Phenols (g L^{-1})	
	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	0.90	0.84	0.19	0.19	2.08	2.00	0.92	0.91	4.61	4.76
2	0.05	0.00	0.02	0.03	0.91	0.74	0.40	0.32	3.76	3.35
3	2.36	2.23	1.09	1.04	3.77	3.39	1.94	1.62	5.27	4.74
4	0.87	0.84	0.22	0.19	2.02	2.00	0.89	0.91	5.28	4.76
5	0.07	0.00	0.03	0.05	0.84	0.60	0.37	0.25	2.49	2.84
6	0.15	0.42	0.04	0.00	0.77	1.11	0.37	0.51	2.88	2.52
7	0.77	0.84	0.17	0.19	1.93	2.00	0.94	0.91	4.39	4.76
8	1.86	2.26	1.11	1.13	3.05	3.84	1.50	2.02	5.56	6.16
9	0.16	0.54	0.07	0.04	1.04	1.68	0.56	0.92	3.91	4.42
10	1.98	1.48	0.52	0.53	3.95	3.06	2.25	1.72	6.42	5.84
11	1.55	1.40	0.31	0.37	2.26	2.18	0.94	0.98	2.58	3.01

Table S2 Experimental and predicted values of by-products levels after pretreatment using elephant grass

Assay	Furfural (g L^{-1})		5-HMF (g L^{-1})		Acetic acid (g L^{-1})		Formic acid (g L^{-1})		Phenols (g L^{-1})	
	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	0.05	0.06	0.01	0.01	2.46	2.67	1.21	1.36	4.44	4.27
2	0.23	0.24	0.03	0.04	2.65	2.60	2.55	2.59	5.10	5.47
3	0.01	-0.01	0.00	0.00	3.34	3.12	0.79	0.59	4.33	4.23
4	0.03	0.03	0.00	0.00	2.75	2.68	1.19	1.09	4.93	5.16
5	0.01	0.01	0.00	0.00	3.64	3.77	0.74	0.88	4.71	4.47
6	0.10	0.13	0.01	0.02	2.06	2.37	1.30	1.68	5.38	5.60
7	0.30	0.28	0.05	0.05	2.61	2.44	2.98	2.72	6.74	6.35
8	0.03	0.03	0.01	0.00	2.69	2.68	1.03	1.09	5.79	5.16
9	0.00	0.02	0.00	0.01	3.71	3.79	0.66	0.73	4.40	4.67
10	0.02	0.00	0.00	0.00	3.27	2.97	1.28	0.94	4.49	4.53
11	0.02	0.03	0.00	0.00	2.60	2.68	1.04	1.09	4.75	5.16

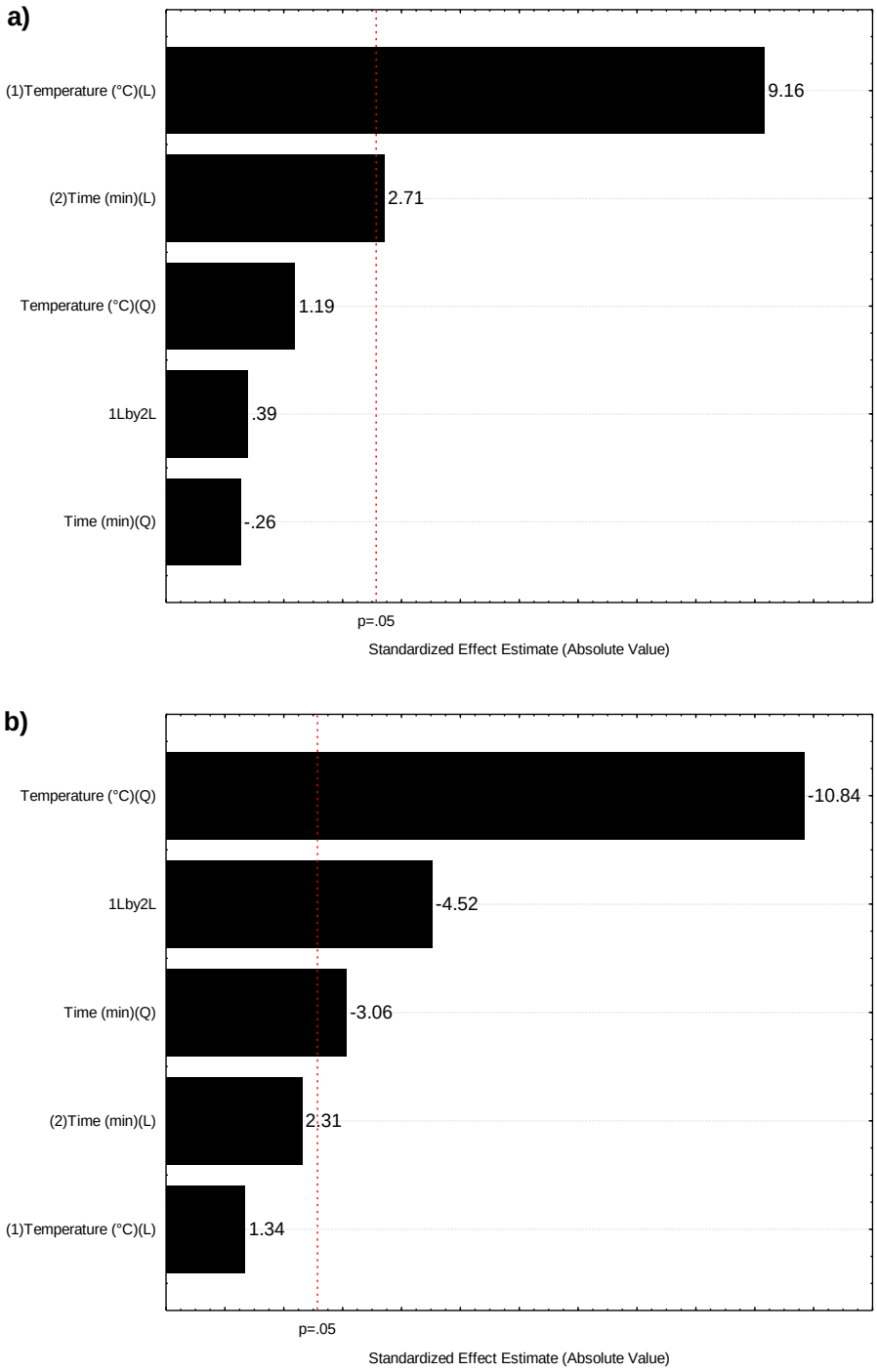

Figure S1. Pareto chart of standardised effects (absolute values) for rice husk in terms of overall yield responses of (a) glucose released in the WIS and (b) xylose released in the liquor. The vertical dashed line indicates a level of significance of 95% (p=0.05); (L) stands for Linear variable and (Q) stands for Quadratic variable

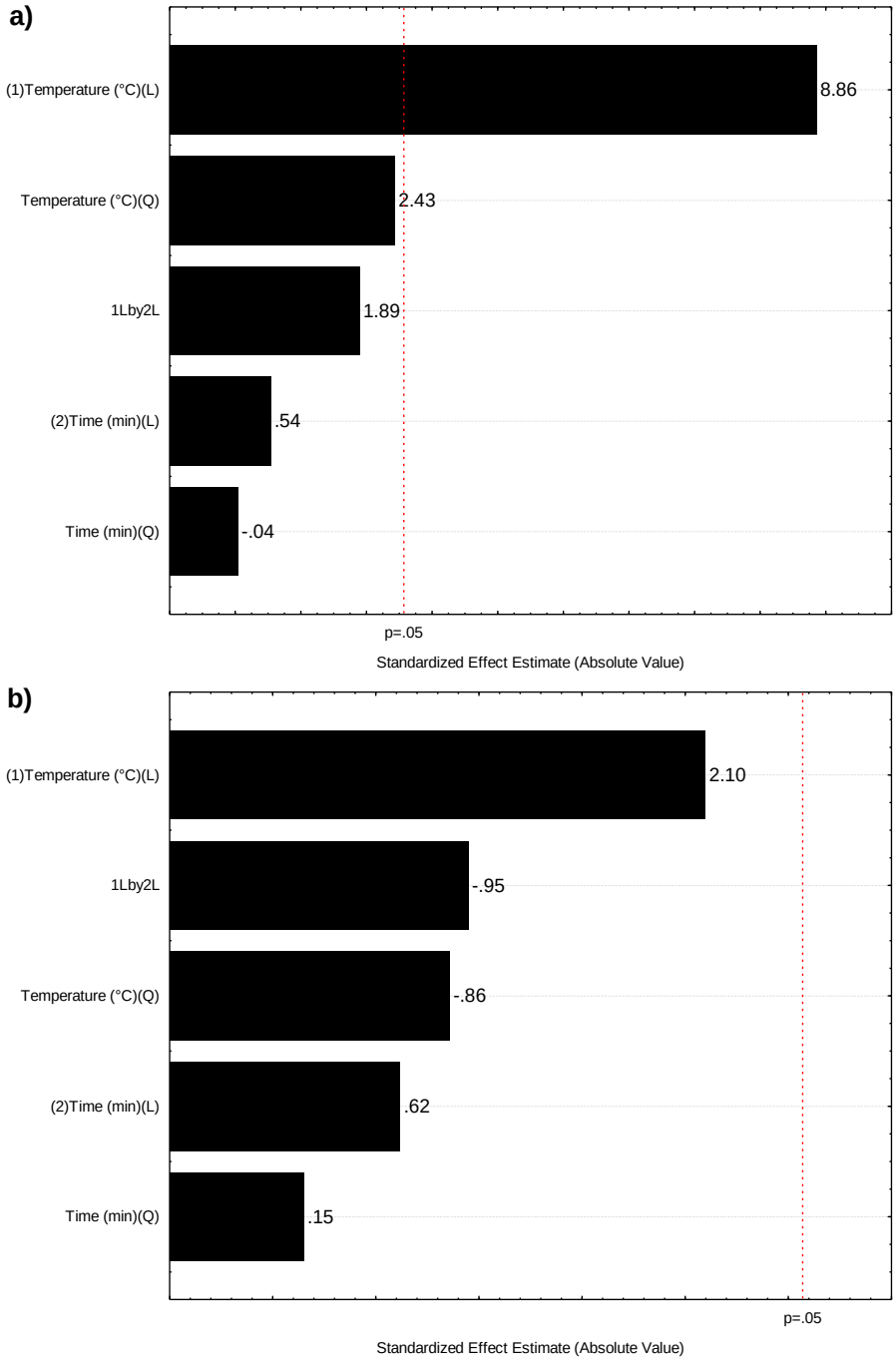

Figure S2. Pareto chart of standardised effects (absolute values) for elephant grass in terms of overall yield responses of (a) glucose released in the WIS and (b) xylose released in the liquor. The vertical dashed line indicates a level of significance of 95% (p=0.05); (L) stands for Linear variable and (Q) stands for Quadratic variable

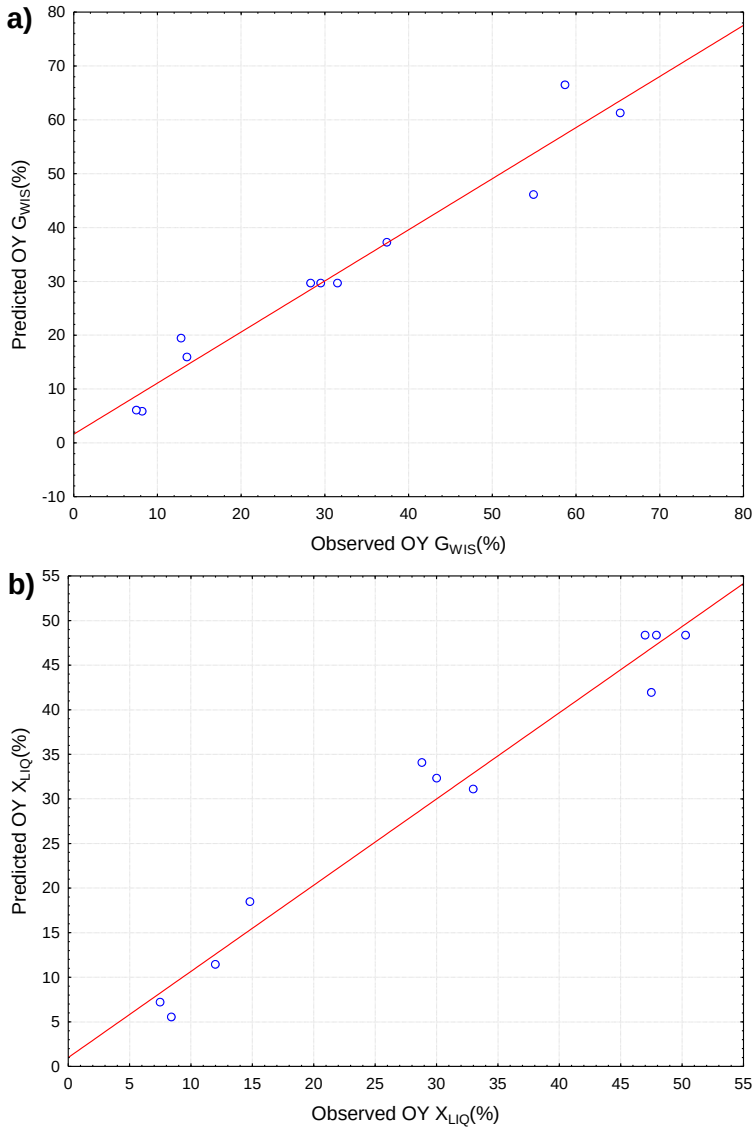

Figure S3 Experimental values versus expected values in terms of overall yield responses of (a) glucose released in the WIS and (b) xylose released in the liquor using pretreated rice husk followed by hydrolysis

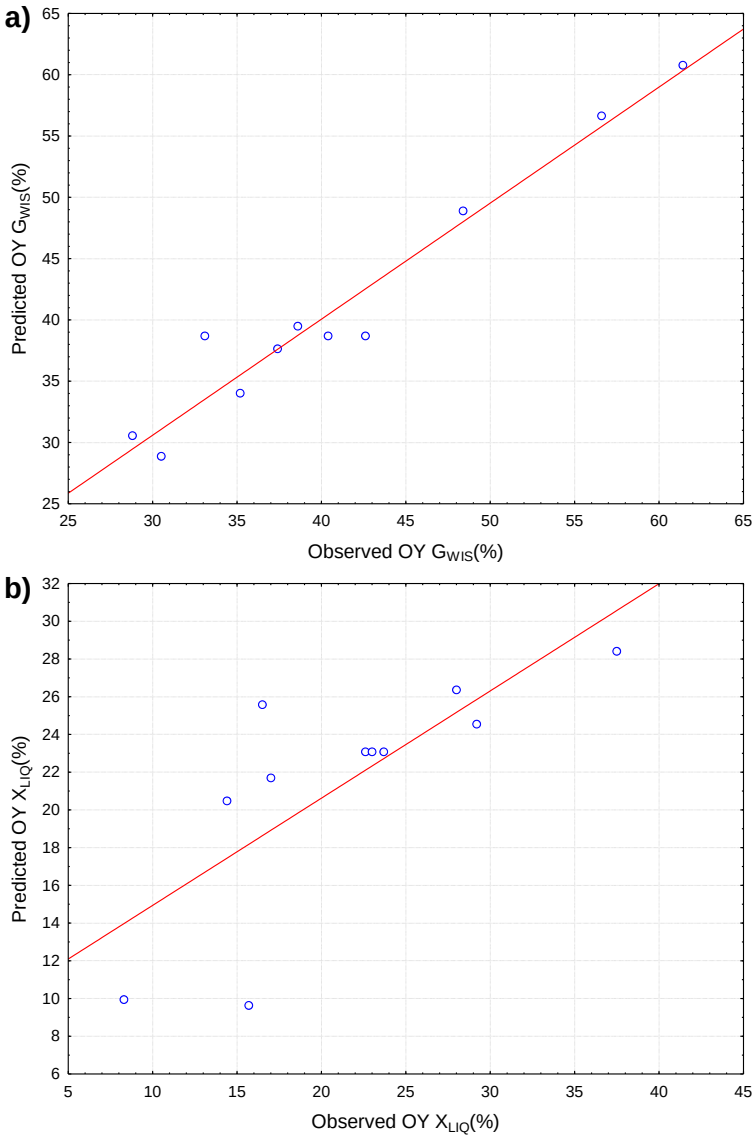

Figure S4 Experimental values versus expected values in terms of overall yield responses of (a) glucose released in the WIS and (b) xylose released in the liquor using pretreated elephant grass followed by hydrolysis

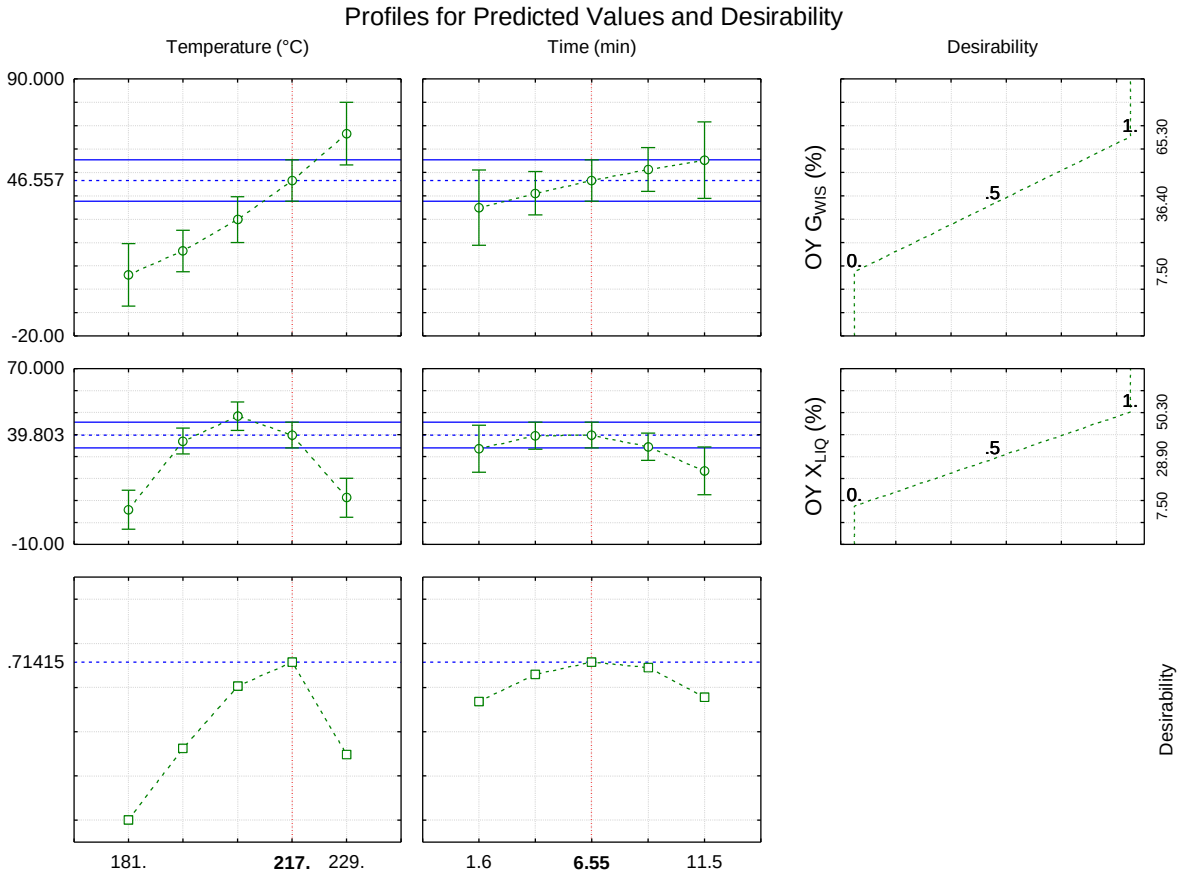

Figure S5. Desirability profiles for rice husk in relation to the overall yields of sugars

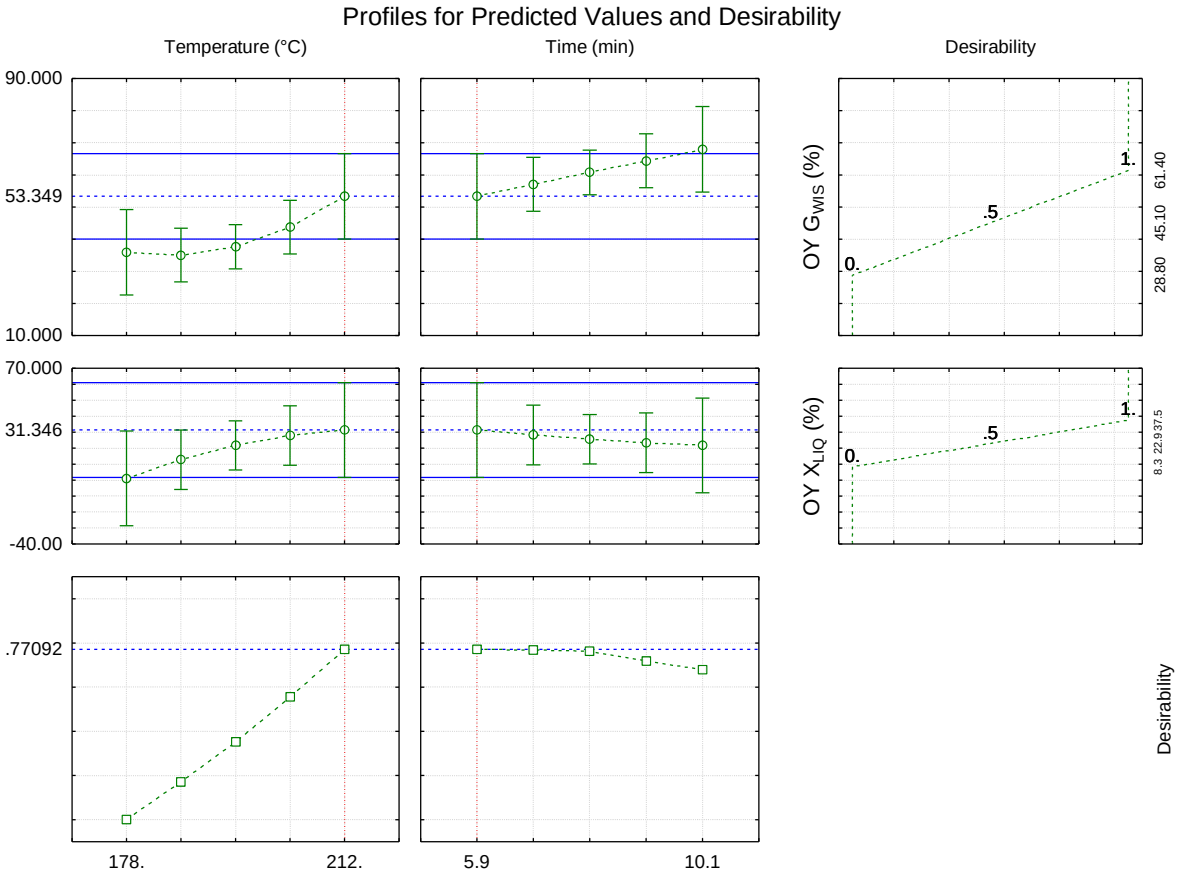

Figure S6. Desirability profiles for elephant grass in relation to the overall yields of sugars

4.2 Artigo 2

Bioprocessing of rice husk into monosaccharides and the fermentative production of bioethanol and lactate

Artigo submetido a periódico internacional.

Bioprocessing of rice husk into monosaccharides and the fermentative production of bioethanol and lactate

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Highlights

- A biorefinery of steamed-rice husk via biochemical routes was proposed.
- Hydrolysis of the liquor with enzymatic cocktail resulted in high sugars yields.
- Lactic acid was produced by *Lactobacillus buchneri* from mixed sugars in the liquor.
- Ethanol was produced by yeast via SSF fermentation from WIS.
- Both products were developed in accordance with the principles of green technology.

Abstract

Rice husk, a residue from agro-industrial processing, is one of the most abundant lignocellulosic feedstocks in the South of Brazil. In this study, the uncatalyzed steam-

exploded rice husk slurry was separated in liquid and solid fractions. The liquor was hydrolyzed by using an enzymatic complex produced from *Penicillium echinulatum* S1M29 strain, then followed by lactic acid production by using *Lactobacillus buchneri* NRRL B-30929 which can efficiently utilize a blend of xylose and glucose presented in the liquor. The water insoluble solids were subjected to simultaneous saccharification and fermentation process using high solid loading combined with distinct doses of commercial enzymes, producing ethanol via *Saccharomyces cerevisiae* CAT-1. In total, 12.69 g L^{-1} of lactic acid and 19.17 g L^{-1} of ethanol were produced, demonstrating that this abundant low valued renewable feedstock can be explored through a cleaner and greener environmentally friendlier production.

Keywords: rice husk; autohydrolysis steam explosion; enzymatic hydrolysis; biorefineries; lactic acid; ethanol

1. Introduction

The multiple alternatives in lignocellulosic raw materials, conversion technologies, and variety of end products compose the current scenario of the biomass-based industry as substitute for fossil fuels. Among of the technologies, the fermentation route of useful substrates from renewable resources has become more attractive (Abdel-Rahman *et al.*, 2011a) in comparison to those obtained by traditional chemical synthesis, integrating a trend focused on environmental sustainability. However, the cost of converting lignocellulosic materials into suitable substrates for the fermentation process has been historically high to attract industrial interest, making it necessary to develop processes with high productivity (Gallego *et al.*, 2015). To achieve cost-effectiveness, most of the processes elaborated toward

industrial scale involve addition of enzymes for cellulose and hemicellulose hydrolysis and use of specific strains to utilize C5- and C6-sugars (Svetlitchnyi *et al.*, 2013).

Alongside the intense production of second generation (2G) ethanol, the high demand for biodegradable polymers has stimulated the use of lignocellulosic feedstocks for lactic acid (2-hydroxypropionic acid) (LA) production. LA is a natural organic acid with unique physicochemical properties, presenting numerous applications in the industry (Tang *et al.*, 2013). The lactic acid bacteria (LAB) are known to possess exclusive fermentative pathways to convert various carbohydrates into LA, and some of them are able to metabolize also C5-sugars. The heterofermentative *L. buchneri* NRRL B-30929 can utilize xylose as a sole carbon source and produce lactate from a blend of sugars (Liu *et al.*, 2008). Likewise, the homofermentative bacterium *L. casei* subsp. *rhamnosus* ATCC 10863 can ferment the mixed sugars present in softwood hydrolysates to produce LA (Iyer *et al.*, 2000).

In Brazil, the state of Rio Grande do Sul is the major South American producer of rice (*Oryza sativa*) (Nunes *et al.*, 2016). According to the National Supply Company (CONAB, 2017), this state accounts for 71.0% of the rice produced and projects a harvest of 8.6 million ton in 2016/2017. After the processing of this cereal, about 20% (*w/w*) of the dry weight consists of rice husk (RH) (Wei *et al.*, 2009). RH has been regarded as a recalcitrant lignocellulosic feedstock mainly due to the amount of silica contained in the cell wall. Nevertheless, recent publications have shown that RH has been used to produce different fine chemicals from its cellulose and hemicellulose fractions, including organic acids, xylitol and ethanol (Bevilaqua *et al.*, 2015, 2013, Montipó *et al.*, 2016, Pedroso *et al.*, 2017, Rambo *et al.*, 2013, Saha *et al.*, 2005). Thus, this residual feedstock might be a good option for the production of LA and 2G ethanol aforementioned.

Autohydrolysis steam explosion (SE) is a suitable environmentally friendly pretreatment process that not implies chemical reagents (Carvalheiro *et al.*, 2004), combining mechanical forces and chemical effects due to the hydrolysis of acetyl groups contained in hemicellulose (Alvira *et al.*, 2010). After pretreatment, depending on the conditions employed, hemicellulose portion is solubilized and recovery in the liquor as xylooligomers, while the water insoluble solids (WIS) contain the more accessible portion of cellulose. Both components can be hydrolyzed into fermentable sugars. It has been proposed that enzymatic hydrolysis performed at elevated WIS concentration will be necessary to render the lignocellulosic conversion process more economically feasible (Modenbach and Nokes, 2013). Furthermore, simultaneous saccharification and fermentation (SSF) has been proven superior to separate hydrolysis and fermentation (SHF) at high solid loading (Öhgren *et al.*, 2006).

In the present study, steam-exploded RH followed by enzymatic hydrolysis of the liquor was carried out to release C5- and C6-sugars, which were employed to LA production. The solid fraction containing basically C6-sugars was used for 2G ethanol production via SSF using high solid loading. It is proposed that both products will be integrated in the same platform, which could reduce the costs due to the total use of the released sugars, thus establishing an efficient biorefinery with multi end products via biochemical routes.

2. Materials and methods

2.1 Raw material and pretreatment

SE was accomplished at the Research Centre for Energy, Environment and Technology – CIEMAT, Madrid, Spain, in a reactor vessel of 2 L filled with 250.0 g of dry RH provided by a local supplier in Santa Maria, RS, Brazil. The pretreatment was held at 205

°C for 11.5 min, according to a previous optimization result (data not shown), and six batches of this assay were carried out. After filtering the slurry, the WIS was washed with distilled water and the resulting WIS was analyzed along with the liquor portion.

2.2 Enzymes production by solid-state cultivation

Enzymes production was performed by the genetic variant S1M29 (Dillon *et al.*, 2011), isolated by mutagenesis from strain 9A02S1 of *Penicillium echinulatum*. S1M29 strain belongs to the microorganism collection from the Laboratory of Enzymes and Biomass, Biotechnology Institute, University of Caxias do Sul, Brazil. *P. echinulatum* was grown as described by Dillon *et al.* (2006) and incubated at 28 °C until the conidia formation.

Solid-state cultivations were conducted based on Menegol *et al.* (2014). Approximately 400 g of dry wheat bran, soaked with a 10× concentrated salt medium (Mandels and Reese, 1957), were packed in plastic bags and autoclaved at 121 °C for 1 h. Each bag was inoculated with a conidial suspension of 1.0×10^6 per g of dry mass and arranged in 32×24 cm trays covered with a gauze layer stuffed with cotton. These trays were incubated at 28 °C under humidity of 90%, for 120 h. Enzymatic extraction was done as described (Camassola and Dillon, 2007) and the supernatant was centrifuged and concentrated by lyophilisation. The enzymatic activity was analyzed according to Camassola and Dillon (2007).

2.3 Enzymatic hydrolysis assays

Liquor was hydrolyzed by enzyme complex produced by *P. echinulatum* S1M29. Hydrolysis were fulfilled in 15 mL flasks containing 5 mL of liquor and 30 FPU g^{-1} substrate of enzyme in 50 mmol L^{-1} sodium citrate buffer (pH 4.8). The experiments were done in

duplicates and maintained at 50 °C shaking at 150 rpm for 32 h. WIS was hydrolyzed by a cellulolytic complex of Celluclast® 1.5L, with an activity of 59.0 filter paper units (FPU) mL^{-1}, supplemented with Novozyme 188® with an activity of 530.0 international units (IU) mL^{-1}, both enzymes were kindly provided by Novozymes (Bagsvaerd, Denmark). Hydrolysis was taken place in 50 mL flasks (working volume of 40 mL) containing 10, 15 and 20% (*w/v*) of solids loading along with 10, 20 and 30 FPU g^{-1} WIS of enzyme, in 50 mmol L^{-1} sodium citrate buffer, pH 4.8. The experiments were done in duplicates and maintained at 50 °C on a rotatory hydrolysis reactor. Samples were collected every 24 h, until 96 h. The performance of enzymatic hydrolysis was valued using the parameters glucose (Y_G) or xylose (Y_X) yields, which were determined as the ratio of grams of sugar released by enzymatic hydrolysis per 100 g of potential sugar in the corresponding fraction.

2.4 Lactate fermentation

2.4.1 LAB screening

Enterococcus mundtii NRRL B-51316, *Lactobacillus brevis* NRRL B-1834, *Lactobacillus buchneri* NRRL B-30929, *Lactococcus lactis* subspecies *lactis* NRRL B-4449, and *Lactobacillus rhamnosus* NRRL B-445 were cordially provided by U. S. Department of Agriculture, Agricultural Research Service – USDA, ARS, Peoria, IL, USA. The strains were maintained in modified MRS medium (de Man, Rogosa and Sharpe, 1960), pH 6.0 with 20 g L^{-1} of xylose as a sole carbon source, without sodium acetate. The cultures were grown at 37 °C and were stored at 4 °C until their use. For the screening phase in submerged culture, growth and production were analyzed through synthetic broth containing 20 g L^{-1} glucose; 20 g L^{-1} xylose; or 10 g L^{-1} glucose and 10 g L^{-1} xylose. The cultures were carried out in 50 mL

flasks. The volume was set at 25 mL at 37 °C under 150 rpm. The experiments were done in duplicates and samples were collected until 48 h.

2.4.2 LA production from liquor via SHF

3 L of liquor was detoxificated by incubating with 2.5% (*w/v*) activated carbon at 50 °C for 1 h, under 200 rpm (Rambo *et al.*, 2013). Afterwards, the solution was vacuum filtered through a grade 1 Whatman qualitative filter paper. The fermentation was carried out in 2 L flasks, with a reaction volume of 1.0 L, the pH was adjusted to 6.0 with 6.0 mol L^{-1} NaOH and the medium was supplemented with the following nutrients (g L^{-1}): proteose peptone (10.0), yeast extract (5.0), ammonium citrate (2.0) and dipotassium phosphate (2.0) when 6 g L^{-1} of *L. buchneri* NRRL B-30929 was used as inoculum. The cultures were incubated at 37 °C and stirred magnetically at 150 rpm. The maximum specific rates of bacterium growth (μX_m), substrate uptake (μS_m), and product formation (μP_m) were determined. The yields were described as g LA per g sugar present in the liquor ($Y_{LA/S}$) and as percentage of the maximum theoretical yield of 0.76 g LA per g sugar (Y_{LA}). This stoichiometric conversion factor (f_{LA}) was calculated according to the percentage of total sugars released in the liquor in relation to the maximum theoretical yield in LA for each sugar:

$$f_{LA} = [Sugars\ C6\ (\%) * 1.0\ g\ g^{-1}] + [Sugars\ C5\ (\%) * 0.6\ g\ g^{-1}] \quad (1)$$

Samples were collected periodically for determination of biomass, quantification of products, and pH evaluation. All experiments were conducted in triplicates and results were evaluated through Origin 8 software.

2.5 Ethanol production from WIS via SSF

S. cerevisiae CAT-1 was kindly provided by Luiz de Queiroz College of Agriculture – ESALQ, University of São Paulo, Piracicaba, Brazil. The CAT-1 strain was reactivated, maintained and replicated in YPD (Yeast Extract-Peptone-Dextrose) (Costa *et al.*, 2014) medium, pH 5.0, grown at 37 °C and stored at 4 °C. SSF experiments for ethanol production were conducted in 1 L flasks containing 20% (*w/v*) of WIS, 20 FPU enzyme per g substrate, and 50 mmol L^{-1} sodium citrate buffer, pH 4.8, supplemented with the following nutrients (g L^{-1}): yeast extract (5.0) and $(NH_4)_2SO_4$ (1.0). Approximately 2.0 g L^{-1} of *S. cerevisiae* CAT-1 was used as inoculum. The total volume was 600 mL and final pH was adjusted to 5.0 with 1.0 mol L^{-1} NaOH. The cultures were kept at 37 °C under orbital agitation of 180 rpm. SSF results were presented as g ethanol per g potential glucose in the WIS ($Y_{E/G}$) and as percentage of the maximum theoretical yield of 0.51 g ethanol per g glucose (Y_E), assuming that all the potential glucose was available for fermentation. The other conditions evaluated were identical to those of LA fermentation.

2.6 Analytical methods

2.6.1 Composition of RH

Chemical composition of the WIS was analyzed according to the Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (NREL, Golden, USA) for the standardization of analytical methods for biomass (NREL/TP-510-42622, NREL/TP-510-42619, NREL/TP-510-42618 and NREL/TP-510-48087).

2.6.2 Scanning electron microscopy (SEM)

Micrographs of selected specimens were generated using a scanning electron microscope (SEM) Sigma 300 VP (Carl Zeiss, England) with field emission gun (FEG) of the

Schotky type (tungsten filament covered with zirconium oxide), equipped with a Gemini column. The images were obtained with the use of secondary electron detector (SE) in high vacuum (HV) mode. The samples were coated with carbon using a metallizer (Q 150R-ES Medium Quorum Technologies, England) and a carbon thread (Nisshin EM Co. Ltd., Japan) and carried out under HV.

2.6.3 Quantification of the products

Sugars contents and by-products concentrations of WIS and liquor, as well as hydrolysis products were determined as described by Romaní *et al.* (2013). Fermentation products were analyzed via High Performance Liquid Chromatography (HPLC) by using a Shimadzu system (Kyoto, Japan) coupled to an ultraviolet detector UV/VIS SPD-20A or a refractive index detector RID-10A, and equipped with quaternary pump LC-20AD, DGU-$20A_3$ degasser, CTO-20A column oven, CBM-20A communicator module and LC Solution *software*. For the quantification of sugars, ethanol and lactic and acetic acids in RID mode, an Aminex® HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA), at an oven temperature of 60 °C, was used beyond an aqueous phase ultrapure water containing 5 mmol L^{-1} H_2SO_4, 0.6 mL min^{-1} flow. The Astec® CLC-D chiral column (Supelco, Sigma-Aldrich, St. Louis, MO, USA), operated at 28 °C, was employed to separate the LA isomers in the UV mode using 5 mmol L^{-1} $CuSO_4$ at a flow rate of 1.0 mL min^{-1}, with detection at 254 nm. Furfural and 5-HMF were also quantified in the UV mode using a Discovery® C18 column (Sigma-Aldrich) working at 30 °C, using water (0.1% formic acid):methanol (1% formic acid), 90:10 (*v/v*) at a 0.5 mL min^{-1} flow, with detection at 284 nm. Elution was in isocratic mode and injection volume of 20 µL for all analysis. The identification of sugars, ethanol,

LA, acetic acid, furfural and 5-HMF was obtained based on the retention time and quantification was determined using a standard curve with known concentrations.

2.6.4 Cell concentration

Biomass was estimated as viable cells using CFU (colony forming units) by the standard plate counting method and related to a standard curve of CAT-1 (0 to 1.0 g L^{-1}) or *L. buchneri* (0 to 4.5 g L^{-1}), previously constructed and determined at 600 nm and 620 nm, respectively, in a spectrophotometer.

3. Results and discussion

3.1 RH

In natura RH comprised of 3.73% ± 0.37 extractive, 33.79% ± 0.13cellulose, 18.07% ± 0.13 hemicellulose, 25.49% ± 0.14 lignin, 17.27% ± 0.07 ashes, 1.41% ± 0.03 acetyl groups and 2.26% ± 0.15 starch (n=3). Scanning electron microscope (SEM) analysis of *in natura* and steamed-exploded RH samples were illustrated in Supplementary data, Fig. S1. The images of pretreated rice husk demonstrated significant breakdown of the RH fiber structures (Fig. S1b). The averages values of the main components present in the WIS, as well as sugars and by-products in the liquor after SE are presented in Table S1 and Table S2, respectively (Supplementary data). The overall sugars yield obtained after post-hydrolysis of the liquor portion was calculated as 7.02 g xylose from 100 g of RH, and 13.40 g glucose of the WIS portion after enzymatic hydrolysis with 5% (*w/v*) of solids loading using 15 FPU g^{-1} WIS from 100 g of RH.

3.2 LA production

3.2.1 Enzymatic hydrolysis using enzymes produced by P. echinulatum S1M29

Post-hydrolysis of the hemicellulosic hydrolysate obtained from autohydrolysis SE pretreatment is a common practice to enable the conversion of xylooligosaccharides (XOs) into fermentable monosaccharides. The acid post-hydrolysis of liquor from different biomasses has been widely reported in the literature (Nakasu *et al.*, 2016). In order to preserve the principle of green chemistry and engineering, the depolymerization of XOs through enzymatic hydrolysis of the liquor was consistently chosen for this study. According to Schneider *et al.* (2016), the expression of cellobiohydrolases, endoglucanases and xylanases indicate a high propensity of the mutant S1M29 for biomass hydrolysis. Thus, the enzymes produced by this mutant strain were used to hydrolyze the liquor which has a high content of XOs. The enzymatic activities of the extract were measured after lyophilisation, and the following specific activities were obtained: FPases (7.0 U mL^{-1}), endoglucanases (42.31 U mL^{-1}), ß-glucosidases (53.28 U mL^{-1}) and xylanases (213.32 U mL^{-1}). The total amount of proteins reached 37.40 mg mL^{-1}. These results are in agreement with those reported by dos Reis *et al.* (2013), when the same mutant strain was utilized to produce enzymes obtained in submerged cultivation batch and fed-batch procedures using cellulose. The higher activity of ß-glucosidases found here is likely related to the solid cultivation process, which favors enzymatic stability (Menegol *et al.*, 2014), and this high content is also a characteristic of *P. echinulatum* when compared to different fungi species (Camassola and Dillon, 2007). In the study of using steam-exploded elephant grass as carbon source for enzyme production in submerged cultivation, Scholl *et al.* (2015) also demonstrated the potential of *P. echinulatum* S1M29 to produce cellulases and xylanases simultaneously.

Time course analyses of the enzymatic hydrolysis of the liquor resulted in a maximum glucose and xylose concentration of 7.25 g L^{-1}, with a Y_G of 88.68%; and 12.60 g L^{1}, with a

Y_X of 78.18%, respectively, demonstrating the competence of the cellulolytic and hemicellulolytic enzymatic complex to hydrolyze the liquor into monomeric xylose (Fig. 1). Thus, the procedure set forth herein provided the beneficial and eco-friendly xylose production. This is the first work reporting the hydrolysis of the XOs contained in the liquor fraction after autohydrolysis SE to xylose by enzymes produced by *P. echinulatum* S1M29. Teng *et al.* (2010) also employed SE pretreatment followed by enzymatic hydrolysis of the liquor using a thermostable xylanase from *Paecilomyces themophila* J18, but focused on the production of XOs from corncobs. A maximum XOs yield of 28.6 g/100 g xylan in corncobs was achieved. Wang *et al.* (2017) introduced 50 U g^{-1} XOs of a commercial xylanase aiming the post-hydrolysis of prehydrolysate originating from eucalyptus pulping. The xylose yield reached 26.67 g L^{-1} from 23.48 g L^{-1} XOs with a total xylose concentration of 54.10 g L^{-1}.

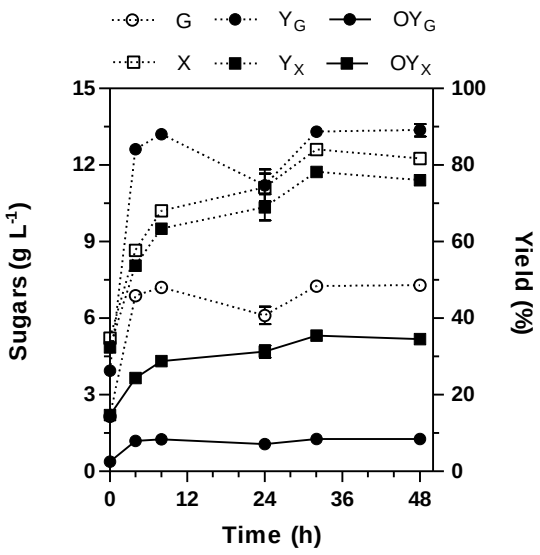

Fig. 1 Sugar concentrations and yields obtained after 48 h of enzymatic hydrolysis with the mutant S1M29 employing the liquor from steam-exploded rice husk. G, X: glucose or xylose. Y_G, Y_X: glucose or xylose yield related to g of each sugar released by enzymatic hydrolysis per 100 g potential glucose or xylose present in the liquor. OY_G, OY_X: overall glucose or xylose yield related to g of each sugar released by enzymatic hydrolysis per 100 g glucose or xylose present in the rice husk.

3.2.2 By-products detoxification

The effects of the inhibitor compounds on the fermentative processes depend on the nature of the microorganism as well as the pH and temperature conditions of the fermentation broth (Oliva *et al.*, 2003). Previous experiments of LA fermentation using the liquor after the enzymatic hydrolysis indicated the need to reduce the concentration of by-products since the consumption of the free sugars by LAB was inefficient, even with the addition of nutrients (data not shown). In order to increase the fermentability of the liquor obtained after SE followed by enzymatic hydrolysis, activated carbon was employed to minimize the content of any toxic derivatives by adsorption. As a result, there were 66% decreases of furfural and 39% decreases of 5-HMF, with slight gains in monomeric sugars contents caused by the action of temperature. This activated carbon adsorption method reduced the toxic effect of the liquor without the necessity of expensive laborious techniques such as solid phase extraction and separation processes with membranes. In a study involving the use of *Aspergillus terreus* for the production of itaconic acid, Pedroso *et al.* (2017) eliminated efficiently the by-products of the RH hydrolysate by the addition of CaO, leading to the formation of insoluble products of the inhibitors including acetic and formic acids, which precipitated and were then separated. Bevilaqua *et al.* (2015) tested different detoxification processes on RH hydrolysates, such as, addition of CaO followed by flocculation with polymeric quaternary ammonium tannate, adsorption in activated carbon, and combination of changes of pH followed by activated carbon. The last method brought an improvement to the growth of *Actinobacillus succinogenes* through the removal of unwanted components in the medium, without any significant loss of sugars, resulting in improved succinic acid fermentation.

3.2.3 LAB screening

One of the main objectives of this research was to find a LAB capable of metabolizing xylose faster and, consequently, producing LA with higher productivities and higher yields. For this purpose, five strains participated in the initial screening. The growth performance of each strain in modified MRS medium can be visualized in Fig. 2. Furthermore, the kinetics of sugar consumption and LA production of all strains can be visualized in Fig. 3.

Among the strains analyzed, *L. buchneri* NRRL B-30929 was the most efficient when the medium contains only xylose as substrate, reaching the exponential phase after 18 h of cultivation (Fig. 2c). In addition, when compared to other LAB, this strain was the only one capable of utilizing xylose almost completely within 24 h, even in a mixture of sugars (Fig. 3h and Fig. 3i). It is noticed that this strain also produced acetic acid (AA) (Fig. 3g, Fig. 3h and Fig 3i). *E. mundtii* NRRL B-51316, *L. brevis* NRRL B-1834 and *L. rhamnosus* NRRL B-445 also were effective in producing LA, despite the longer lag phase in the same medium, which reached up to 36 h of adaptation in the case of the first strain (Fig. 2a, Fig. 2b and Fig. 2e). Regardless, all three strains proved to be a wild-type LAB capable of consuming xylose and turn it into LA (Fig. 3b, Fig. 3e and Fig. 3n). Abdel-Rahman *et al.* (2011b) reported the efficient fermentation of xylose to L-(+)-lactate with minimal by-products by an isolated identified as *E. mundtii* QU 25. Iyer *et al.* (2000) also observed that *L. casei* subsp. *rhamnosus* (ATCC 10863) can ferment xylose and all other minor sugars in hemicellulose, except arabinose, with an initial lag phase lasting 24-72 h before xylose consumption takes place. Analysis of several *L. brevis* isolates indicated that co-utilization of xylose and glucose is a common feature for this species (Kim *et al.*, 2009). Indeed, when glucose and xylose were present in the medium, *L. brevis* NRRL B-1834 appeared more efficient in the consumption of xylose than *E. mundtii* NRRL B-51316 and *L. rhamnosus* NRRL B-445 (Fig. 3c, Fig. 3f and Fig. 3o); however, it is still much slower when compared to *L. buchneri* NRRL B-30929

(Fig. 3i). The data suggested that *L. lactis* NRRL B-4449 was the only strain that, within 48 h of culture, was not able to growth on xylose (Fig. 2d).

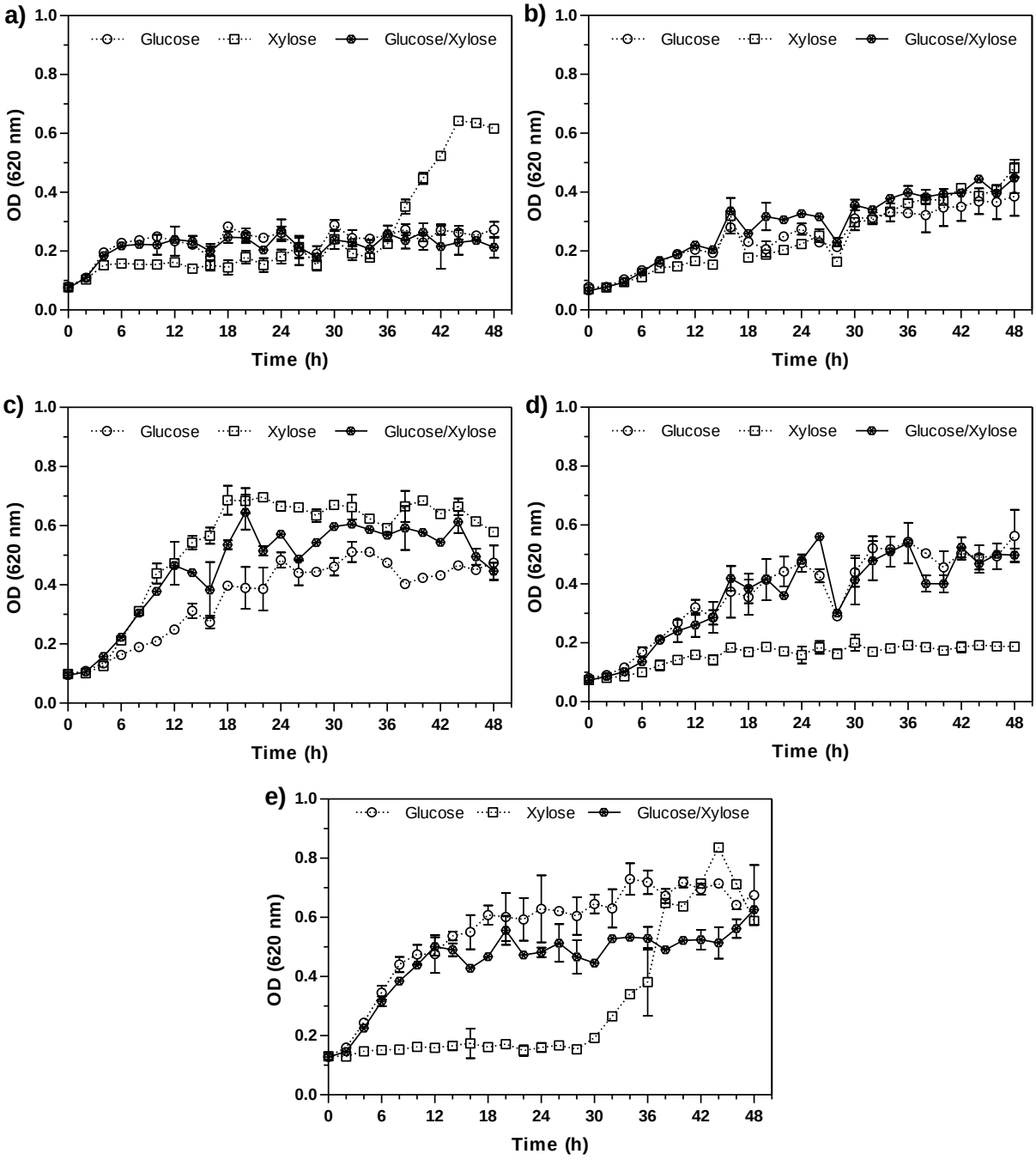

Fig. 2 Lactic acid bacteria growth during cultivation at 37 °C using modified MRS medium containing glucose, xylose, or glucose and xylose involving *Enterococcus mundtii* NRRL B-51316 (a); *Lactobacillus brevis* NRRL B-1834 (b); *Lactobacillus buchneri* NRRL B-30929 (c); *Lactococcus lactis* NRRL B-4449 (d); and *Lactobacillus rhamnosus* NRRL B-445 (e)

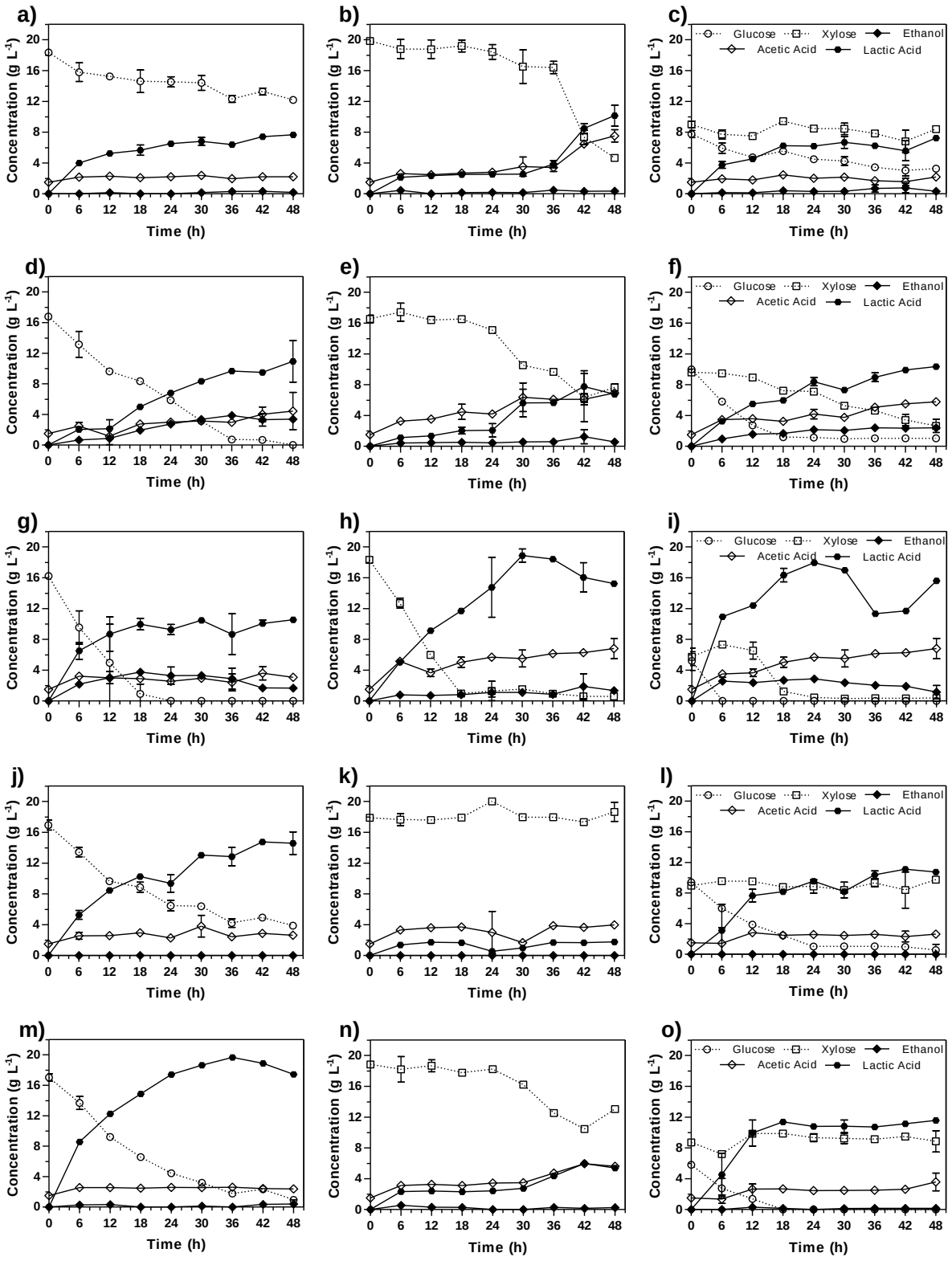

Fig. 3 Lactic acid bacteria performance during cultivation at 37 °C using modified MRS medium containing glucose (left columns), xylose (center columns), or glucose and xylose (right columns) with *Enterococcus mundtii* NRRL B-51316 (a-c); *Lactobacillus brevis* NRRL B-1834 (d-f); *Lactobacillus buchneri* NRRL B-30929 (g-i); *Lactococcus lactis* NRRL B-4449 (j-l); and *Lactobacillus rhamnosus* NRRL B-445 (m-o)

In many LAB cultivated on a mixture of carbohydrates, the sugar consumption is sequential, since glucose consumption represses the utilization of alternative C5- and C6-sugars owing to carbon catabolite repression (Abdel-Rahman *et al.*, 2011a, b). Hofvendahl and Hahn-Hägerdal (2000) compared different carbon sources and it has been demonstrated that glucose resulted in higher LA concentration and yields in relation to other sugars. In the present study, it was evident how these five strains exhibited classical diauxic growth in which showed greater preference for glucose consumption when the medium contained both glucose and xylose. These findings diverge with the results obtained by Kim *et al.* (2010), when using *L. brevis* the authors indicated the tendency of the strains for C5-sugars (xylose and arabinose) over glucose. Liu *et al.* (2008) also showed that, in fermentations with high concentrations of glucose and xylose, *L. buchneri* NRRL B-30929 strain utilized both sugars simultaneously, suggesting that the microorganism maintains an active xylose utilization pathway, with xylose not being repressed by glucose. Knowing that RH liquor is made up of a blend of sugars, it is important that all of them are metabolized completely by the LAB. It has demonstrated that *L. buchneri* NRRL B-30929 and *L. rhamnosus* NRRL B-445 appear to be more efficient in producing LA in medium containing a mixture of xylose and glucose (Fig. 3i and Fig. 3o). Also, the homofermentative *L. rhamnosus* NRRL B-445 strain is characterized by rapid conversion of glucose into L-(+)-LA (Fig. 3m and Fig. 3o) (Montipó *et al.*, 2016), a preferred isomer. Thereby, these two strains were selected for the lactic fermentation of the liquor from RH pretreatment.

3.2.4 LA production from liquor

In view of the complex nutritional requirements of the LAB, it is essential to supplement the hydrolyzed medium (Montipó *et al.*, 2016). Prior experiments on hydrolyzed

liquor obtained after the autohydrolysis SE of RH biomass indicated the necessity to add the aforementioned nutrients in section 2.4.2 to the growing medium containing *L. buchneri* NRRL B-30929 or *L. rhamnosus* NRRL B-445. Detoxified liquor medium supplemented with nutrients was used to carry out fermentations by using these LAB. Initial tests were conducted and both delay in initial glucose uptake and no xylose consumption were observed in the hydrolyzed medium fermented with *L. rhamnosus* NRRL B-445, besides the low production of LA (Supplementary data, Fig. S2). Thus, it was decided to only use *L. buchneri* NRRL B-30929 strain for further fermentations since the toxic level of the detoxified liquor still seemed to affect the microorganism. Furthermore, the inhibiting effect of lactate on growth of LAB is frequently described because of its uncoupling and intracellular anion accumulation or its chelating properties (Yáñez *et al.*, 2008). The performance of *L. buchneri* regarding the utilization of sugars and formation of bioproducts is shown in Fig. 4.

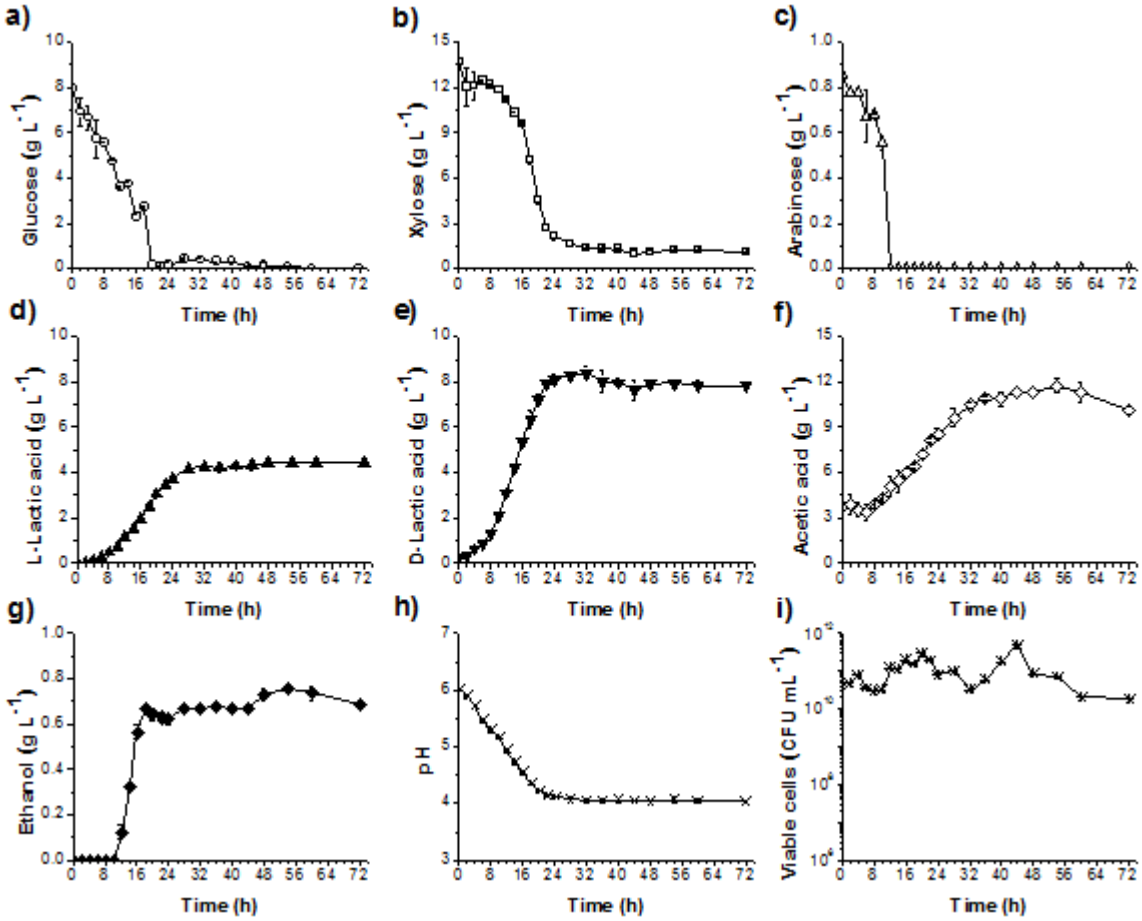

Fig. 4 Kinetics of *Lactobacillus buchneri* NRRL B-30929 fermentation at 37 °C using the liquor of the pretreated rice husk, added with nutrients, as substrate

The sugars blend contained in the RH liquor was rapidly metabolized by *L. buchneri* NRRL B-30929, and after 32 h of cultivation the remaining sugars were ~1.50 g L^{-1} (Fig. 4a, Fig. 4b and Fig. 4c). Arabinose took just 12 h to be consumed, while glucose needed a little more time. Most xylose was consumed within 24 h of fermentation. Approx. 1.10 g L^{-1} of galactose was also consumed during the process (data not shown). The total sugars were converted into a racemic mixture of DL-LA, composed of 4.28 g L^{-1} of L-isomer and 8.41 g L^{-1} of D-isomer, in 32 h of fermentation, a time point when a plateau was achieved (Fig. 4d and Fig. 4e). By this time, the pH was dropped to 4.0 (Fig. 4h). Cell concentration started at 4.3×10^{10} CFU mL^{-1}, corresponding to 6.08 g biomass L^{-1} when confronted with the standard

curve of *L. buchneri* NRRL B-30929. This concentration reached 3.0×10^{11} CFU mL^{-1}, that is, 12.55 g L^{-1} in 20 h of cultivation (Fig. 4i). It should be noted that the RH liquor medium was not sterilized before fermentation, as done by some authors (Saha *et al.*, 2005), without causing damage to the experiment. One of the attributes of this heterofermentative, facultative anaerobe bacterium is the concomitant generation of by-products. Thus, as expected, AA was the secondary product produced by *L. buchneri* NRRL B-30929, reaching approximately 8.0 g L^{-1} in the final fermentation times (discounted value of initial concentrations of AA in liquor and inoculum) (Fig. 4f). AA is an important chemical, with large applications in food, plastic and textile industries, as well as pharmaceuticals (Tammali *et al.*, 2003), which could also enhance this study after appropriate separation and purification. In accordance with Elferink *et al.* (2001), *L. buchneri* is indeed able to degrade LA into AA with the concomitant production of 1,2-propanediol, as well as traces of ethanol, under anoxic conditions without requiring an external electron acceptor. The authors found that the occurrence of LA degradation and its rate depend on culture fermentation temperatures and pH.

Table 1 reveals that specific *L. buchneri* NRRL B-30929 culture parameters favoring lactic fermentation. Conversions of sugars contained in the RH liquor into products ($Y_{LA/S}$) was 0.53 g g^{-1} (32 h) and into cells ($Y_{X/S}$) was 0.27 g g^{-1} (20 h). After 32 h fermentation, Y_{LA} was valued as 70.13% in relation to the theoretical; while productivity reached 0.44 g L^{-1} h^{-1} at 28 h. Montipó *et al.* (2016) investigated the conversion of RH hydrolysates into LA using *L. rhamnosus* ATCC 10863. After successive pressure pretreatment with acid catalysts, a productivity of 0.31 g L^{-1} h^{-1} was achieved when HCl-fermentation was considered, corresponding to the half of the H_2SO_4-fermentation (0.62 g L^{-1} h^{-1}). This latter high productivity is associated with the fact that the pretreatment has released glucose as the main sugar – unlike the pretreatment employed here – which is easily assimilated by this strain,

converging to the exclusive production of LA. The maximum specific rates are also listed, and the delay to reach μX_m can be explained through Fig. 4, given that after the degradation of arabinose and glucose, the xylose continues being consumed.

Table 1 Lactic acid yield employing *Lactobacillus buchneri* NRRL B-30929 after 32 h fermentation

Lactic acid ($g\ L^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	$Y_{LA/S}$ ($g\ g^{-1}$)	Y_{LA} (%)	P_V ($g\ L^{-1}\ h^{-1}$)	OY_{LA} (%)	μX h^{-1}	μS $g\ g^{-1}\ h^{-1}$	μP $g\ g^{-1}\ h^{-1}$
12.69	0.27	0.53	70.13	0.44	5.83	0.07	0.14	0.09

$Y_{X/S}$: ratio yield of cells per substrate referred as g biomass per g sugar in the liquor (calculated at 20 h).
$Y_{LA/S}$: ratio yield of product per substrate referred as g lactic acid per g sugar present in the liquor.
Y_{LA}: lactic acid yield expressed as percentage of theoretical lactic acid yield (0.76 g lactic acid per g sugar).
P_V: volumetric productivity (calculated at 28 h).
OY_{LA}: overall process yield defined as g lactic acid per 100 g untreated rice husk.
μX_m: maximum specific rate of bacterium growth (20 h).
μS_m: maximum specific rate of substrate degradation (20 h).
μP_m: maximum specific rate of product formation (20 h).

3.3 Ethanol production from WIS

3.3.1 Enzymatic hydrolysis tests using high solid loading

In this study, higher solid loadings were processed aiming to achieve higher ethanol concentrations. The implementation of this saccharification process was motivated by the potential to realize significant economic advantages over a conventional low solids process. Previous studies suggested that higher solids resulted in a reduced reaction volume and a more concentrated medium (Hodge *et al.*, 2009, Mohagheghi *et al.*, 1992). Thus, the WIS was utilized as substrate for hydrolysis tests at10, 15 and 20% (*w/v*) solids loading under different enzymatic doses (10, 20 and 30 FPU g^{-1} WIS) to estimate the impact. The average results from these assays after 72 h, in terms of concentrations and yields, are presented in Table 2.

Table 2 Glucose concentration and yields obtained after 72 h of enzymatic hydrolysis at different solids loading (%, *w/v*) and enzyme dosages using as substrate steam-exploded rice husk

Solids loading (%)	Enzyme dosage (FPU g^{-1} WIS)	Glucose concentration (g L^{-1})	Y_G (%)	OY_G (%)
10	10	17.15	31.79	26.92
	20	20.05	37.17	31.47
	30	21.17	39.24	33.23
15	10	24.28	30	25.41
	20	26.74	33.05	27.99
	30	28.50	35.23	29.83
20	10	28.50	26.42	22.37
	20	33.68	31.22	26.44
	30	35.84	33.21	28.13

WIS: water insoluble solids.
Y_G: glucose yield related to g of glucose released by enzymatic hydrolysis per 100 g glucose present in the WIS.
OY_G: overall glucose yield related to g of glucose released by enzymatic hydrolysis per 100 g glucose present in the raw material.

Regarding glucose released, the effect of increasing the solids loading excelled compared to the increase of the enzyme doses (Table 2). Augmentations up to 41.6% were found when the solids load increased from 10 to 15% (*w/v*). However, these values fell down when there was an increment from 15 to 20% (*w/v*). Considering the passage from 10 to 20% of WIS, similar increases were observed for all the enzymatic doses employed, reaching 69.3% when 30 FPU g^{-1} WIS was used. The variation in the enzymatic dose was highlighted when 20% (*w/v*) solids loading was utilized, reaching 25.7% in the transition from 10 to 30 FPU g^{-1} WIS. As expected, in spite of the potential benefits from high solid loading, Y_G are not linear with increasing solids content. Indeed, the decrease in yields at high solids concentrations was denominated by Kristensen *et al.* (2009) as solids effect. Assays utilizing 20% (*w/v*) solids loading plus 20 FPU g^{-1} WIS appeared to result in adequate concentration and yield for the follow-up of this study (Table 2). A concentration of 33.68 g glucose L^{-1} was released at the end of the process, corresponding to a Y_G in the order of 31.22%.

In general, the results obtained were lower when compared to other studies employing distinct lignocellulosic feedstock, such as sugarcane bagasse, corn stover and wheat straw

(Amores *et al.*, 2013, Liu and Chen, 2016, Qiu *et al.*, 2017). In a research involving RH, Yáñez *et al.* (2006) achieved a glucose concentration slightly higher than that obtained in this study. The authors subjected RH to hydrothermal processing in aqueous medium and also treated with hydrogen peroxide in alkaline media before enzymatic hydrolysis. When this treatment was considered, a liquid to solid ratio of 15 g g^{-1} and an enzyme to substrate ratio of 25 FPU g^{-1} releasing up to 43.5 g L^{-1} of glucose after 48 h. The choice of a more recalcitrant biomass, RH, when compared to sugarcane bagasse, corn stover and wheat straw; the conditions of the pretreatment (uncatalyzed); the absence of a delignification step; besides the saccharification system using flasks in orbital agitation, probably interfered in the yields achieved in this study. For the last case, one important impediment to developing a high solids saccharification process may have been the rheological challenges manifested as limitations in the ability to maintain effective mass and heat transfer (Hodge *et al.*, 2009). It is worth mentioning that one of the major objectives of the present investigation was to obtain the products of interest through a cleaner and greener environmentally friendlier production, without the use of catalysts, with a fewer processing steps.

3.3.2 SSF

SSF technique was fulfilled with 20% (*w/v*) solids loading and an enzyme dose of 20 FPU g^{-1} WIS for the hydrolysis and, simultaneously, the yeast *S. cerevisiae* CAT-1 was used for ethanol production. This strain has been used in the Brazilian industrial sector (Costa *et al.*, 2014). The kinetic of CAT-1 fermentation data including average concentrations of substrates and products, viable cell counts and final pH values during 72 h are shown in Fig. 5.

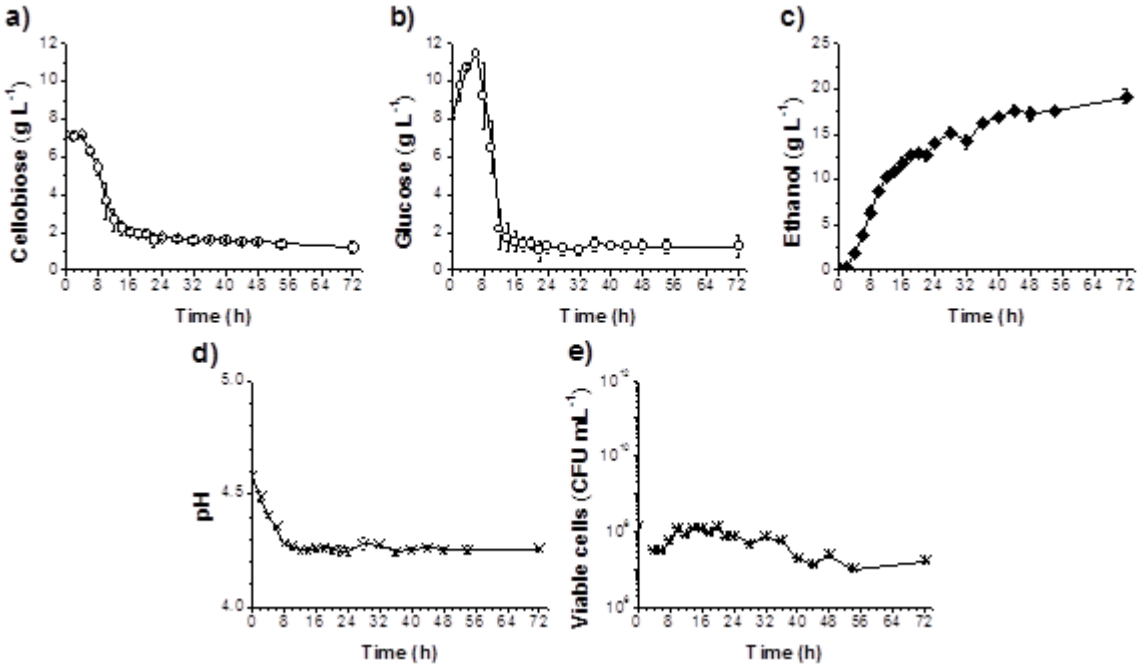

Fig. 5 *Saccharomyces cerevisiae* CAT-1 performance during simultaneous saccharification and fermentation at 37 °C employing 20% (*w/v*) pretreated rice husk, accompanied by nutrients, and an enzyme dose of 20 FPU g^{-1} WIS

Ethanol began to be produced as soon as the SSF starts, reaching 17.71 g L^{-1} in 44 h. The maximum concentration was reached at the ending of cultivation (Fig. 5c). The graphs show that both cellobiose and glucose were not fully consumed (Fig. 5a and Fig.5b). In relation to viable cells, at the onset of cultivation there was about 1.5×10^{8} CFU mL^{-1}, corresponding to 1.86 g biomass L^{-1} when confronted with the standard curve constructed for CAT-1, at the end, reached 1.7×10^{7} CFU mL^{-1}. In general, biomass production remained constant throughout the process, as well as pH (Fig. 5d and Fig. 5e). Table 3 reveals kinetic parameters involved during CAT-1 SSF. When calculating the SSF yield within 72 h fermentation period as Y_E, a value of 34.84% was obtained comparing to the theoretical, while $Y_{E/G}$ was 0.18 g ethanol g^{-1} potential glucose in the WIS. Productivity reached 0.44 g L^{-1} h^{-1} at 36 h of cultivation. Although initial tests of ethanol fermentation using RH were

carried out from separate hydrolysis and fermentation, as well as prehydrolysis (data not shown), the SSF process used here did show increased productivity.

Table 3 Ethanol yield attained by simultaneous saccharification and fermentation using *Saccharomyces cerevisiae* CAT-1 after 72 h fermentation

Solids loading (*w/v*)	Enzyme dosage (FPU g^{-1} WIS)	Ethanol ($g\ L^{-1}$)	$Y_{E/G}$ ($g\ g^{-1}$)	Y_E (%)	P_V ($g\ L^{-1}\ h^{-1}$)	OY_E (%)
20%	20	19.17	0.18	34.84	0.44	6.02

WIS: water insoluble solids.
$Y_{E/G}$: ratio yield referred as g ethanol per g potential glucose in the WIS.
Y_E: ethanol yield expressed as percentage of the maximum theoretical ethanol yield (0.51 g ethanol per g glucose).
P_V: volumetric productivity (calculated at 36 h).
OY_E: overall process yield defined as g ethanol per 100 g untreated rice husk.

Saha and Cotta (2008) produced ethanol by SSF of lime-pretreated whole RH slurry adding a cocktail of three commercial enzymes preparations (cellulase, ß-glucosidase and hemicellulase) and using a recombinant *Escherichia coli* strain FBR5. The ethanol concentration achieved was only 11.0 g L^{-1} in 53 h of culture, proving that the lime pretreatment is not an option for converting RH to sugars. In any case, this result was superior when compared to the SHF process carried out by these authors in the same study. Amores *et al.* (2013) studied steam-exploded sugarcane bagasse for conversion into ethanol. In SSF experiments using solids consistency and enzymatic dosages similar to the present study, the maximum ethanol levels of 56.3 g L^{-1} was achieved by the authors using *S. cerevisiae (EthanolRed)*, with a Y_E of 64.3% and a $Y_{E/G}$ of 0.33 g ethanol g^{-1} potential glucose, demonstrating the feasibility of this typical raw material.

In the present study, conversion of each product of interest was developed in distinct systems, in order to prevent possible inhibition as well as selective fermentation. Simultaneous production of ethanol and LA by co-fermentation could be investigated if the LA producing strain consumed only xylose. Another strategy would be produce ethanol and, sequentially, LA. Thus, the remaining xylose from the alcoholic fermentation would be

immediately converted into LA, making this technology profitable. However, the synergism between such integrations can only be achieved when both processes are compatible, *e.g.* the use of a microorganism resistant to inhibition by formed product. Further experiments for the recovery and purification of the products obtained here need to be accomplished in order to complement this investigation. Assuming that the ethanol produced by fermentation can be directly used for esterification with LA, as suggested by Tang *et al.* (2013), the esterification process followed by hydrolysis will become economically viable to complement this study.

4. Conclusions

RH demonstrates to be a potential resource which can integrate the platform of building blocks from C5-sugars and, concurrently, produce 2G ethanol from C6-sugars. In this research, both products of interest were developed trying to obey the principles of green chemistry and engineering using a local biomass, with the least possible treatments, as well as avoiding the addition of chemicals – thus contributing to a cleaner production. These yields obtained may seem low, but it becomes valuable when it is mentioned that the RH is a feedstock resulting from rice processing, with no commercial value and no direct application in industry. New strategies for the pretreatment process as well as enzymatic hydrolysis of the WIS should be investigated to obtain an improvement in the yield of fermentable sugars into ethanol.

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The authors have declared no conflicts of interest.

Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at … (to be accomplished).

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Supplementary Data

Table S1 Rice husk biomass composition (% of dry weight) after steam explosion pretreatment

Stem explosion		Solids recovery (%)	WIS composition (%)			
Temperature (ºC)	Time (min)		Cellulose	Hemicellulose	Lignin[a]	Ashes
205	11.5	62.25	49.09±0.15	3.28±0.07	31.28±0.28	20.92±0.03

These data are mean values of three assessments.
[a] acid-insoluble lignin.

Table S2 Chemical composition of rice husk liquor after steam explosion

Sugars before post-hydrolysis (g L^{-1})					Sugars after post-hydrolysis (g L^{-1})				
Glucose	Xylose	Galactose	Arabinose	Mannose	Glucose	Xylose	Galactose	Arabinose	Mannose
2.30	4.91	1.10	1.61	0	9.59	15.45	1.93	1.21	0.34

pH	Lignocellulose-derived products (g L^{-1})								
	Furfural	5-HMF	Acetic acid	Formic acid	Coumaric acid	Ferulic acid	Vanillin	Syringaldehyde	4-HBA
3.31	1.91	0.36	2.99	1.61	0.06	nd	0.06	0.003	nd

nd: not detected.

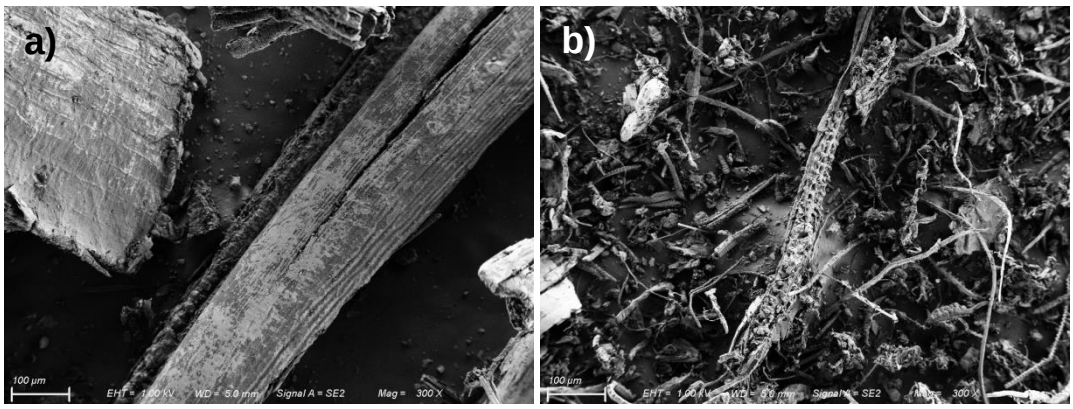

Fig S1 Scanning Electron Microscopy images of a) *in natura* rice husk and b) rice husk pretreated by steam explosion

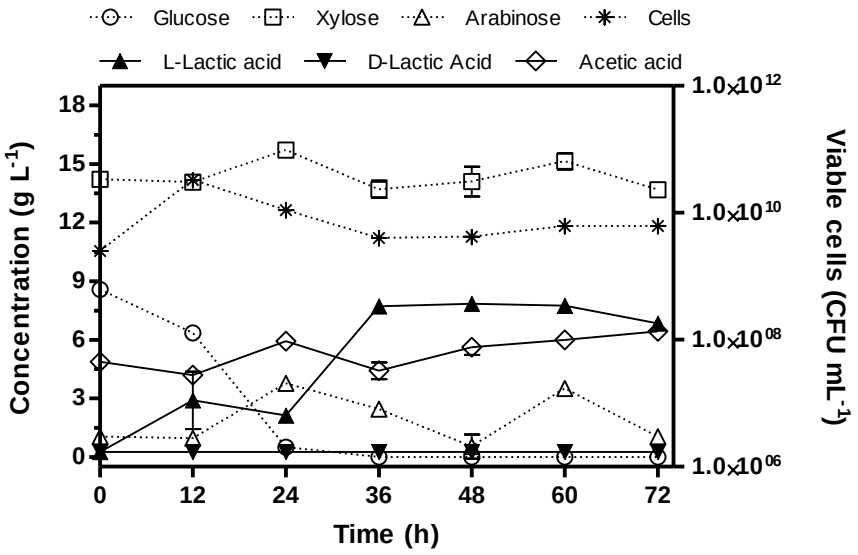

Fig S2 Kinetics of *Lactobacillus rhamnosus* NRRL B-445 at 37 °C using the liquor of the pretreated rice husk

4.3 Artigo 3

Integrated production of second generation ethanol and lactic acid from steam-exploded elephant grass

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Integrated production of second generation ethanol and lactic acid from steam-exploded elephant grass

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Highlights

- A biorefinery platform was designed to use elephant grass for fuels and chemicals.
- Pretreatment and hydrolysis with high solid load resulted in high sugar contents.
- Simultaneous saccharification and fermentation led to high ethanol yield.
- Lactic acid was produced in parallel from pentoses conversion in the liquor.

Abstract

Elephant grass was subjected to steam explosion to enhance cellulose accessibility and convert it into ethanol. After catalyzed pretreatment at 190 °C for 5 min, enzymatic hydrolysis was carried out using high rate of solid loading combined with different enzyme dosages.

Assays employing 20% (*w/v*) solids loading and an enzyme dosage of 20 FPU g^{-1} substrate led to a yield of 86.02 g glucose released per 100 g potential glucose in the water insoluble solids. This condition was selected to carry out the simultaneous saccharification and fermentation procedure through *S. cerevisiae* CAT-1, producing 42.25 g L^{-1} ethanol with a yield of 74.57% regard to the maximum theoretical. The liquor containing C5 and C6-sugars was successfully converted into lactic acid using *L. buchneri* NRRL B-30929, resulting in 13.35 g L^{-1} with a yield of 68.21% in relation to the maximum theoretical.

Keywords: elephant grass; steam explosion; simultaneous saccharification and fermentation; ethanol; lactic acid.

1. Introduction

Research efforts have mounted to validate the use of lignocellulosic feedstocks in order to promote the second generation of ethanol through the stages of pretreatment, enzymatic hydrolysis, sugar fermentation and process design (Svetlitchnyi *et al.*, 2013). Considering that only about 20% of native biomass is hydrolyzed without disrupting the recalcitrant carbohydrate-lignin structure, these previous stages are fundamental to facilitate the hydrolysis of the polysaccharides into monomeric sugars, which can be further fermented (Himmel *et al.*, 2007; Rocha *et al.*, 2015).

Several pretreatment methods have been developed to make biomass more amenable to enzymatic hydrolysis (Shill *et al.*, 2012). Among the pretreatments in vogue, steam explosion (SE) is a successful option carried out at a high pressure and temperature, typically 180 to 240 °C, capable of modifying the cell wall structure, yielding a slurry, which upon filtration renders a filtrate with hemicellulose sugars and a cellulose-rich filter cake containing also lignin and residual hemicellulose (Jönsson and Martín, 2016; nee' Nigam *et al.*, 2009).

Acid impregnation has also been used in combination with SE to enhance hemicellulose hydrolysis efficiency (Egüés *et al.*, 2012). It is envisaged that the filtrate must comprise a low level of by-products, ensuring an economic overall process, with the minimum of treatments as possible and, in parallel, with higher yield and productivity rates.

The products from degradation of lignocellulosic biomass, composed in part of hemicellulose, include large amounts of pentoses sugars (Rubin, 2008), which cannot be used by wild-type *Saccharomyces cerevisiae*. To establish better economic panorama towards a lignocelluloses biorefinery, it is plausible to develop an integrated process for the conversion of the feedstock to achieve full utilization of all co-products (Tippkötter *et al.*, 2014). In this scenario, alongside the production of ethanol from lignocellulosic feedstocks, organic acids, *e.g.*, lactic acid (LA), can advantageously be obtained from microorganisms adjusted to consume xylose, since the remaining filtrate after SE is rich in this sugar.

LA is a natural organic acid with exclusive physicochemical properties (Tang *et al.*, 2013), characterized by its versatility in industrial applications, including its function as a monomer of poly-lactic acid (Tirpanalan *et al.*, 2015). Lactic acid bacteria (LAB) are an important group for the production of LA as main metabolite, besides *Bacillus* genus and fungi. A small parcel of wild types is capable of ferment both hexoses and pentoses sugars, and mutagenesis and metabolic engineering techniques also contribute to this. Heterofermentative LAB, such as *Lactobacillus pentosus* ATCC 8041 and *L. bifermentans* DSM 20003^{T}, were suitable for the production of LA for fermenting hexoses and pentoses from corn stover and wheat bran syrup, respectively (Givry *et al.*, 2008; Zhu *et al.*, 2007). Already, *L. buchneri* NRRL B-30929 can utilize xylose as a sole carbon source, being able to produce lactate as major component from a mixture of glucose and xylose sugars (Liu *et al.*,

2008). Another strain reported was the homofermentative bacterium *L. casei* subsp. *rhamnosus* ATCC 10863, used in softwood hydrolysates to produce LA (Iyer *et al.*, 2000).

The exploitation of elephant grass (EG) (*Pennisetum purpureum*) as a substrate for both ethanol and lactic acid production, separately, has not been reported yet in the literature, but its features such as harvesting times and productivity are very attractive for this purpose. EG is a traditional forage species with high photosynthetic efficiency and an average productivity of 30.0 ton of dry matter ha^{-1} $year^{-1}$ (Fontoura *et al.*, 2015; Jonker *et al.*, 2015), according to seasonality (Anindo and Potter, 1994). This grass requires very little supplementary nutrients for growth and can be harvested up to four times a year, besides presenting a solid centre of the stems, which makes this plant one of the highest potential crops for use as precursors for fine chemicals and bioenergy (Pérez-Boada *et al.*, 2014; Strezov *et al.*, 2008; Xie *et al.*, 2011).

The present investigation was focused on the development of a process for ethanol and lactic acid production through the consumption of C6- and C5-sugars, respectively, aiming for the use of a lignocellulosic feedstock, here represented by the steam-exploded EG. Both processes were conducted separately: the conversion of pretreated EG to ethanol involved a simultaneous saccharification and fermentation (SSF) strategy in order to not only reduce production expenses, but also raise productivity from the proper release of sugars, whereas LA fermentation took place directly from the filtrate of the slurry. This study seeks to contribute to technological innovation and to the solution of environmental problems from the biorefinery.

2. Materials and methods

2.1 Steam pretreatment of elephant grass (EG)

EG was collected in Nova Petrópolis, RS, Brazil. SE was completed at the Research Centre for Energy, Environment and Technology – CIEMAT, Madrid, Spain. The reactor with a capacity of 2 L was filled with 200.0 g of dry matter with or without acid, and heated at 190 °C for 5 min with saturated steam. Three different concentrations of 0, 1 and 2% (*w/w*) of sulfuric acid were tested. Prior to pretreatment, EG was soaked for ~20 h in water or diluted sulfuric acid solution (1:16, solid: liquid ratio). The soaked material was vacuum filtered and then steam-exploded. At the end of the process, the slurry was filtered and the water insoluble solids (WIS) were washed with distilled water. The composition of the WIS and the analysis of the liquor were determined as described below. The combined severity factor $log(R_0')$ (Pedersen and Meyer, 2010) based on temperature (T, °C) and reaction time (t, min), along with final pH, was considered to analyze the severity of each assay:

$$log(R_0') = \int_0^t exp\left(\frac{T - 100\,°C}{14.75}\right) dt - pH \quad (1)$$

2.2 Enzymatic hydrolysis of water insoluble solids (WIS)

WIS was hydrolysed by a cellulolytic complex of Celluclast® 1.5L, containing a cellulase enzyme loading of 15 FPU g^{-1} substrate, and supplemented with Novozyme 188®, containing a ß-glucosidase enzyme loading of 15 IU g^{-1} substrate. Both enzymes were kindly provided by Novozymes (Bagsvaerd, Denmark). Initially, hydrolysis was conducted in 50 mL flasks, each containing 20 mL of solution with 5% (*w/v*) of substrate concentration, and 50 mmol L^{-1} sodium citrate buffer. The hydrolysis was carried out at pH 4.8, and 50 °C on a rotatory shaker at 150 rpm for 72 h. The pretreatment condition releasing higher amounts of glucose was chosen to carry out duplicate experiments using 10, 15 and 20% (*w/v*) of solids loading along with 10, 20 and 30 FPU g^{-1} WIS of enzyme loading in citrate buffer to a total

40 mL. Samples were withdrawn after 24, 48, 72 and 96 h. The assessment of enzymatic hydrolysis was based on glucose yield (Y_G), which was calculated as the ratio of grams of glucose released by enzymatic hydrolysis per 100 g glucose in the WIS. Overall glucose yield (OY_G) was determined as grams of glucose released per 100 g glucose present in the raw material. The optimal combination was selected for further ethanol fermentation.

2.3 Microorganisms maintenance, inoculum preparation and fermentations

S. cerevisiae CAT-1 was kindly provided by Luiz de Queiroz College of Agriculture – ESALQ, University of São Paulo, Piracicaba, Brazil. CAT-1 strain was reactivated according to the pre-established protocol, maintained and replicated in Yeast Extract-Peptone-Dextrose (YPD) medium (Costa *et al.*, 2014), pH 5.0. The culture was grown at 37 °C and stored at 4 °C. SSF for ethanol production were conducted in 1 L flasks containing 20% (*w/v*) of solids loading and 20 FPU g^{-1} substrate of enzyme loading, with the following components (g L^{-1}): yeast extract (5.0) and $(NH_4)_2SO_4$ (1.0), and 1.5 g L^{-1} of CAT-1 as inoculum. Total volume was of 600 mL and the pH was adjusted to 5.0 with NaOH. The cultures were grown at 37 °C at 180 rpm. SSF results were reported as g ethanol per g potential glucose in the WIS ($Y_{E/G}$) and as percentage of the maximum theoretical yield of 0.51 g ethanol per g glucose (Y_E), assuming that all the potential glucose was available for fermentation.

L. buchneri NRRL B-30929 was provided by U. S. Department of Agriculture, Agricultural Research Service – USDA, ARS, Peoria, IL, USA. The strain was maintained in modified MRS medium (de Man, Rogosa and Sharpe) (de Man *et al.*, 1960), pH 6.0, using 20 g L^{-1} of xylose as the merely source of carbon and without sodium acetate. The culture was also grown at 37 °C and stored at 4 °C until its use. The resulting liquor from the pretreatment was used for lactic fermentation. Firstly, it was treated to reduce the inhibitory compounds by

the addition of 2.5% (*w/v*) activated carbon (Bevilaqua *et al.*, 2015), and incubated at 50 °C for 1 h with shaking at 200 rpm. Thereafter, the solution was vacuum filtered through a grade 1 Whatman qualitative filter paper. The fermentation occurred in 2 L flasks, with a reaction volume of 900 mL, pH was adjusted to 6.0 with NaOH and complemented with nutrients (g L^{-1}): proteose peptone (10.0), yeast extract (5.0), ammonium citrate (2.0) and dipotassium phosphate (2.0); using 5 g L^{-1} of *L. buchneri* NRRL B-30929 as inoculum. The cultures were kept at 37 °C and stirred magnetically at 150 rpm. The maximum specific rates of microorganism growth (μX_m), substrate consumption (μS_m), and product formation (μP_m) were determined using the Origin 8 software. The yields of LA were described as g LA per g sugar present in the liquor ($Y_{LA/S}$) and as percentage of the maximum theoretical yield of 0.73 g LA per g sugar (Y_{LA}). This stoichiometric conversion factor (f_{LA}) was calculated empirically from the percentage of total sugars released in the liquor in relation to the maximum theoretical yield in LA for each sugar (1.0 g g^{-1} C6-sugars and 0.6 g g^{-1} C5-sugars):

$$f_{LA} = (\%\ C6\ sugars * 1.0) + (\%\ C5\ sugars * 0.6) \quad (2)$$

Samples were collected periodically for determination of biomass and quantification of sugars, ethanol and lactic and acetic acids, as well pH evaluation. All experiments were conducted in triplicate. Results were evaluated through GraphPad Prism 5.01 software.

2.4 Analytical methods

2.4.1 Chemical composition of pretreated elephant grass (EG)

Chemical composition of the WIS was analyzed according to the Laboratory Analytical Procedures (LAP) technique established by National Renewable Energy Laboratory (NREL, Golden, USA) for the standardization of analytical methods for biomass

(NREL/TP-510-42622, NREL/TP-510-42619, NREL/TP-510-42618 and NREL/TP-510-48087). The results were mean values of three determinations.

2.4.2 Scanning electron microscopy (SEM) analysis

Micrographs of selected specimens were generated using a scanning electron microscope (SEM) Sigma 300 VP (Carl Zeiss, England) with field emission gun (FEG) of the Schotky type (tungsten filament covered with zirconium oxide), equipped with a Gemini column. The images were obtained with the use of secondary electron detector in variable pressure (VPSE) mode.

2.4.3 Determination of sugars, metabolites of interest and by-products

The resulting liquor and WIS referring to sugar contents and by-products concentrations, as well as the sugars released in the enzymatic hydrolysis, were determined as described by Romaní *et al.* (2013). Fermentation products were analyzed via High Performance Liquid Chromatography (HPLC) through a Shimadzu system (Kyoto, Japan) coupled to an ultraviolet detector UV/VIS SPD-20A or a refractive index detector RID-10A, and equipped with quaternary pump LC-20AD, DGU-20A_3 degasser, CTO-20A column oven, CBM-20A communicator module and LC Solution *software*. For the quantification of sugars, ethanol and acetic acid in RID mode, an Aminex® HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA), at an oven temperature of 60 °C, was used beyond an aqueous phase ultrapure water containing 5 mmol L^{-1} H_2SO_4, 0.6 mL min^{-1} flow. The Astec® CLC-D chiral column (Supelco, Sigma-Aldrich, St. Louis, MO, USA), operated at 28 °C, was employed to separate the LA isomers in the UV mode using 5 mM $CuSO_4$ at a flow rate of 1.0 mL min^{-1}, with detection at 254 nm. Furfural and 5-hydroxymethylfurfural (5-HMF) were also

quantified in the UV mode using a Discovery® C18 column (Sigma-Aldrich) working at 30 °C, using water (0.1% formic acid):methanol (1% formic acid), 90:10 (*v/v*) at a 0.5 mL min^{-1} flow, with detection at 284 nm. Elution was in isocratic mode and injection volume of 20 µL for all analysis.

2.4.4 Analysis of liquor by Time-of-Flight Mass Spectrometer (LC/TOF-MS)

The distinct compounds of the liquor were also investigated by HPLC, coupled to a Time-of-Flight Mass Spectrometer (LC/TOF-MS) (Waters, Xevo G2 QT of, Milford, MA, USA). The operational system consisted of an electrospray ionization source (positive and negative modes) that met the following conditions: 3 kV capillary tension; 40 kV sampling cone tension; 4 kV extraction cone tension; 120 °C source temperature; 350 ºC desolvation temperature; 50 L h^{-1} cone gas flow, and 800 L h^{-1}desolvation gas flow. The direct infusion of samples (10 µL min^{-1}) was employed.

2.4.5 Cell concentration

Biomass was estimated as viable cells using CFU (colony forming units) by the standard plate counting method and related to a standard curve of CAT-1 (0 to 1.0 g L^{-1}) or *L. buchneri* (0 to 4.5 g L^{-1}), previously constructed and determined at 600 nm and 620 nm, respectively, in a spectrophotometer.

3. Results and discussion

3.1 Elephant grass pretreatment by steam explosion (SE)

Previous macromolecular characterization of untreated EG, in percentage of dry weight, resulted in extractives (16.43±0.99%), cellulose (33.60±0.11%), hemicellulose

(20.62±0.02%), total lignin (18.42±0.11%), ashes (12.25±0.11%) and acetyl groups (0.99±0.03%). Taking into account the high content of extractives found in this herbaceous lignocellulose related to the prevalence of buffer capacity (Li *et al.*, 2016), which may compromise the overall process yield, in this study it was opted to carry out SE pretreatment associated with sulfuric acid. Furthermore, impregnation with an acidic agent was conducted to improve cellulose conversion of the residue through enzymatic hydrolysis and, concurrently, release high contents of C5-sugars in the filtrate.

3.1.1 Analysis of the resulting solid fraction

The values of percentage (dry weight) of main components in WIS fraction, as well as solid recovery (SR) after SE under 190 °C for 5 min are presented in Table 1. Regarding WIS composition, cellulose was the main component after the pretreatments. It was concentrated in relation to untreated raw material under all conditions assessed. Pretreatment using 2% (*w/w*) H_2SO_4 resulted in the WIS fraction including the highest value of cellulose content, 56.17%, followed by pretreatment using 1% (*w/w*) H_2SO_4, with 53.21% cellulose. Nevertheless, when aqueous pretreatment was considered, cellulose content of the resulting WIS fraction dropped to 42.32% since less hemicellulose was dissolved. Provided that hemicellulose is the most affected portion by physical-chemical pretreatments, the inverse effect was now observed, assuming that a considerable part has been prehydrolyzed. The uncatalyzed mixture accounted for 21.11% of hemicellulose in the WIS, it is approx. 93% higher than the more acidic pretreatment. As noted by Singh *et al.* (2015), the addition of a catalyst in the SE not only decreases temperature and time to improve enzymatic hydrolysis, but also leads to higher hemicelluloses removal in comparison with water-impregnated biomass (Ballesteros *et al.*, 2006). The lignin content was considerably increased in relation to the initial EG, and this

occurred due to the solubilization of other portions of biomass by the chemical agent. It was evidenced as this independent variable also influenced the SR, which is strictly related with variations occurred in the lignocellulosic matrix, recovering 54.69% of WIS in the most extreme condition compared with 69.50% of the uncatalyzed assay.

Table 1 Elephant grass biomass composition (% of dry weight) after steam explosion pretreatment

Stem explosion conditions			Solids recovery (%)	WIS[a] composition (%)			
Temperature (ºC)	Time (min)	H_2SO_4 (*w/w*)		Cellulose	Hemicellulose	Lignin[b]	Ashes
190	5	0%	69.50	42.32±0.58	21.11±0.30	21.65±0.28	7.67±0.04
		1%	59.24	53.21±0.93	4.00±0.22	29.97±0.64	10.90±0.57
		2%	54.69	56.17±0.75	1.44±0.06	31.98±0.17	10.21±0.24

These data are mean values of three assessments.
[a] water insoluble solids.
[b] acid-insoluble lignin.

SEM of *in natura* and steam-exploded EG samples were taken to prove structural changes caused by the extreme condition of 2% (*w/w*) H_2SO_4 (Fig. 1). Evident contrasts appear between both images, since in the untreated sample the recalcitrant structure remains unchanged (Fig. 1a), while in the pretreated sample the disaggregation was noticeable (Fig. 1b), which may provide better access to the hydrolyzing enzymes. Related SE effects have been reported for different raw materials including rice straw (Boonterm *et al.*, 2016), sugarcane bagasse (Rocha *et al.*, 2015), EG (Scholl *et al.*, 2015) and *Eucalyptus globulus* (Romaní *et al.*, 2013).

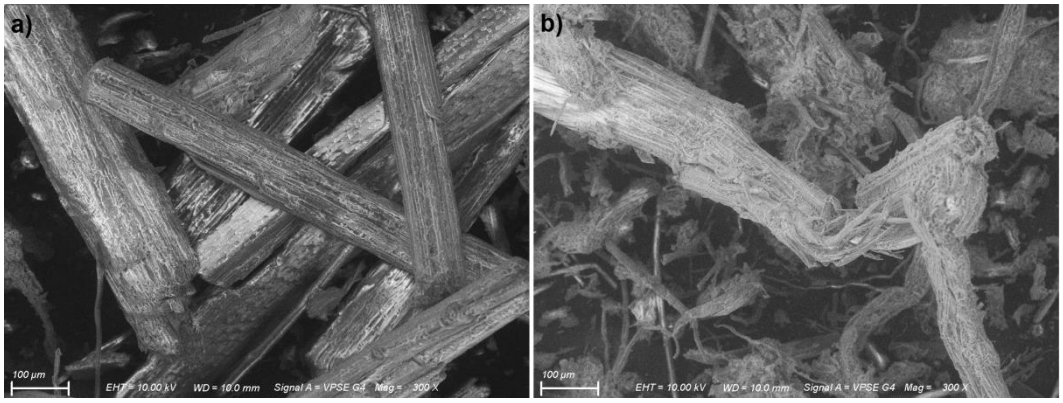

Fig. 1 Scanning Electron Microscopy images of a) *in natura* elephant grass and b) elephant grass pretreated by steam explosion impregnated with 2% (*w/w*) H_2SO_4

3.1.2 Analysis of the resulting liquor

Sugar contents formed during the pretreatment and released in the liquor are described in Table 2. As expected, xylose was the sugar found in greater amounts in this fraction, reaching 16.82 g L^{-1} when 2% (*w/w*) H_2SO_4 pretreatment was used. In the conditions used for this study, 9.62 g xylose per 100 g EG, corresponding to 49.27 g per 100 g xylose in the raw material, was released. Pretreatment without impregnation of sulfuric acid did not generate monomeric xylose, and even after a mild acid hydrolysis step with 4% (*v/v*) H_2SO_4, 121 °C, 30 min, only 2.99 g L^{-1} xylose was produced, that is, 1.86 g xylose per 100 g EG corresponding to 9.54 g per 100 g xylose in the untreated EG.

Table 2 Chemical composition and severity of elephant grass liquor after steam explosion

H_2SO_4 (*w/w*)	Sugars before post-hydrolysis (g L^{-1})					Sugars after post-hydrolysis (g L^{-1})				
	Glucose	Xylose	Galactose	Arabinose	Mannose	Glucose	Xylose	Galactose	Arabinose	Mannose
0%	0.10	0.06	0.07	0.30	nd	1.58	2.99	1.07	1.26	0.18
1%	1.32	10.08	1.40	2.46	nd	3.91	19.64	2.35	2.66	0.55
2%	3.36	16.82	1.91	2.38	0.44	5.02	19.75	2.51	2.92	0.65

H_2SO_4 (*w/w*)	pH	Severity *log (R_0')*	Lignocellulose-derived products (g L^{-1})								
			Furfural	5-HMF[a]	Acetic acid	Formic acid	Coumaric acid	Ferulic acid	Vanillin	Syringaldehyde	4-HBA[b]
0%	5.47	-2.1	0.010	0.002	0.931	0.274	0.168	0.026	0.014	0.005	0.017
1%	2.66	0.7	1.392	0.154	0.784	0.209	0.118	0.039	0.016	0.005	nd
2%	1.85	1.5	1.937	0.300	1.352	0.194	0.119	0.042	0.019	0.011	nd

nd: not detected.
[a] 5-hydroxymethylfurfural.
[b] 4-hydroxybenzaldehyde.

Under typical acidic conditions in pretreatment processes, hexoses are dehydrated into 5- HMF, while pentoses and uronic acids resulting from hydrolysis of the hemicelluloses undergo dehydration to produce furfural (Jönsson and Martín, 2016). Both products are known to inhibit glycolytic enzymes (Pedersen and Meyer, 2010). Formic acid is the component resulting from breakdown of these by-products, and 5-HMF can be degraded into levulinic acid. Meanwhile, acetic acid can be generated as a result of the hydrolysis of the acetyl groups of hemicelluloses (Jönsson and Martín, 2016). Consequently, liquor fraction was also investigated in terms of lignocellulose-derived products and $log(R'_0)$ was calculated to analyze the leverage of sulfuric acid among pretreatments. The major degradation end-products found in the liquor is given in Table 2, in g L^{-1}, and their presence was independent of the impregnation method. Du *et al.* (2010) already observed that the types and concentrations of degradation products were more dependent upon the raw material utilized than on the style of pretreatment employed.

In accordance with the data, the milder assay ($log(R'_0)$ = -2.1) released lower levels of inhibitory compounds in the liquor, generated a liquid fraction with a pH around 5.47. In general, the formation and accumulation of select compounds were dependent upon both raw material and pretreatment chemistry (Du *et al.*, 2010). Thus, in opposition, the harshest assay released higher levels of inhibitory compounds, reaching values of 1.937 g L^{-1} furfural and 0.3 g L^{-1} 5-HMF, as well as 1.352 g L^{-1} acetic acid. In these circumstances, it generated a liquid fraction with a pH around 1.85 ($log(R'_0)$ = 1.5); indicating that even in this severe condition, just a small portion of inhibitors are generated from the corresponding polysaccharides, as observed by Toscan *et al.*(2017). However, it is worth noting that even though they were present in low concentrations, they are detrimental to the progress of subsequent processes, considering that they can inhibit microbial fermentations.

Therefore, activated carbon was employed to minimize the content of toxic derivatives by adsorption. As a result, there were 69% reductions of furfural, 31% of 5-HMF and 40% of acetic acid, with slight gains in glucose and xylose contents caused by the action of temperature. Yang *et al.* (2013) explained that detoxification is necessary before fermentation on the lignocellulosic acid hydrolysates. The authors hydrolyzed EG with sulfuric acid and, prior to being used for conversion, they detoxified the medium by overliming and adsorption with activated carbon. Jung *et al.* (2013) submitted the oil palm empty fruit bunches, previously impregnated with sulfuric acid, in a microwave digester aiming to utilize the whole slurry in SSF. Before ethanol fermentation, activated carbon was added to the liquid fraction entailing the loss of furfural and acetic acid, with a slight drop in glucose content.

Liquor generated from 2% (*w/w*) H_2SO_4 pretreatment was subjected to a screening analysis by LC/TOF-MS aiming to identify the probable components of interest (Table S1 and Fig. S1 in Supplementary material). Among other compounds, the following possible fragments are detected: formic acid, acetic acid, furfural, 2-furoic acid, levulinic acid, 4-hydroxybenzaldehyde (4-HBA), 5-HMF, xylose, vanillin, coumaric acid, glucose, syringaldehyde, ferulic acid and syringic acid. These samples were analyzed prior to the activated carbon treatment.

3.1.3 Sugars recovery and overall process yields

Sugars recovery was referred as percentage of the initial component in EG (Fig. S2a). Generally speaking, glucan recovery was predominant in the WIS while xylan recovery was related to the conditions employed. Satisfactory recovery results was achieved with previous acid impregnation, the catalytic assays were responsible for recovering up to 85.3% of glucan in the WIS (GR_{WIS}) and up to 53.1% of xylan in the liquor (XR_{LIQ}), right after the acid

hydrolysis of the liquor. Overall sugars yield, based on free sugars obtained after enzymatic hydrolysis with 5% (*w/v*) of solids loading using 15 FPU g^{-1} WIS of enzyme dosage, and after post-hydrolysis of the liquor, was given as percentage of sugars released in WIS or in liquor referred to sugar contents in raw material, where G_{WIS} refers to glucose in the WIS and X_{LIQ} to xylose in the liquor (Fig. S2b). It is possible to verify the significant impact of the acid on total glucose and xylose yields during the pretreatments, since the variables temperature and time were fixed in these experiments. In these specific assays, the most extreme condition can generate 39.29 g sugars per 100 g EG (27.99 g of G_{WIS} and 11.30 g X_{LIQ} for each 100 g of raw material, equivalent to 75.73 g glucose per 100 g glucose present in the untreated EG and 57.9 g xylose per 100 g xylose present in the untreated EG). Jung *et al.* (2013) emphasize that the total amount of sugar released can be attributed to the efficiency of the acid pretreatment.

Initial experiments were unsuccessfully conducted aiming to reduce extractive compounds and improve the overall process yield using a prior autoclaving strategy (121 °C, 30 min) followed by a moderate SE pretreatment (195 °C, 8 min) or employing harsh SE condition (220 °C, 5 min) (Fig. S3). The overall yield of X_{LIQ} in the pretreatment at 220 °C for 5 min was four times lower relative to that using 2% (*w/w*) H_2SO_4, even though the G_{WIS} yield was slightly higher in the 220 °C, 5 min SE condition. The buffering effect of extractive compounds was observed, when comparing the amount of 1% (*v/v*) H_2SO_4 required to reach the same pH between water and EG (Fig. S4).

3.1.4 Representative assay

Based on the promising results obtained with the use of 2% (*w/w*) H_2SO_4 as impregnating agent and maintaining the initial focus of this study in finding a balanced consistency between G_{WIS} and X_{LIQ} in a single pretreatment, this assay seems to be the most

appropriate among those studied. Thus, six additional batches of this assay were completed and applied in the subsequent hydrolysis and fermentation experiments. The data about biomass and liquor composition are shown in Table S1 and Table S2.

3.2 Enzymatic hydrolysis with high solid loading

The SE pretreatment chosen was used as substrate for enzymatic hydrolysis assays at high solid loading using distinct dosages of enzymes. The average results of these assays, in terms of concentrations and yields, are given in Fig. 2. It is noticeable how the progressive increase in solid loading positively influences glucose production, much more than the increase related to enzyme dosage. The augmentation in glucose concentrations based on the WIS contents was expressive for all dosages of enzymes (Fig. 2a, Fig. 2b, Fig. 2c). In general, at 96 h, there were increments situated between 47.1-49.2% when the solids load increased from 10 to 15% (*w/v*). These percentages fall considerably when their contents migrate from 15 to 20% (*w/v*), with values of 18.4% (10 FPU g^{-1} WIS), 26.2% (20 FPU g^{-1} WIS) and 30.4% (30 FPU g^{-1} WIS). Probably there may be a maximum solid load above which the glucose release remains practically constant. Considering the transition from a solids load of 10 to 20%, there was an increase of 76.7% when a dose of 10 FPU g^{-1} WIS was used. This percentage rose to 85.7% at an enzymatic dose of 20 FPU g^{-1} WIS, and, finally, to 94.5% using 30 FPU g^{-1} WIS. Therefore, with a load of 20% (*w/v*) solids it was possible to achieve up to 102.83 g glucose L^{-1} employing 30 FPU g^{-1} WIS. Overall a time of 24 h was sufficient to reach high glucose concentration.

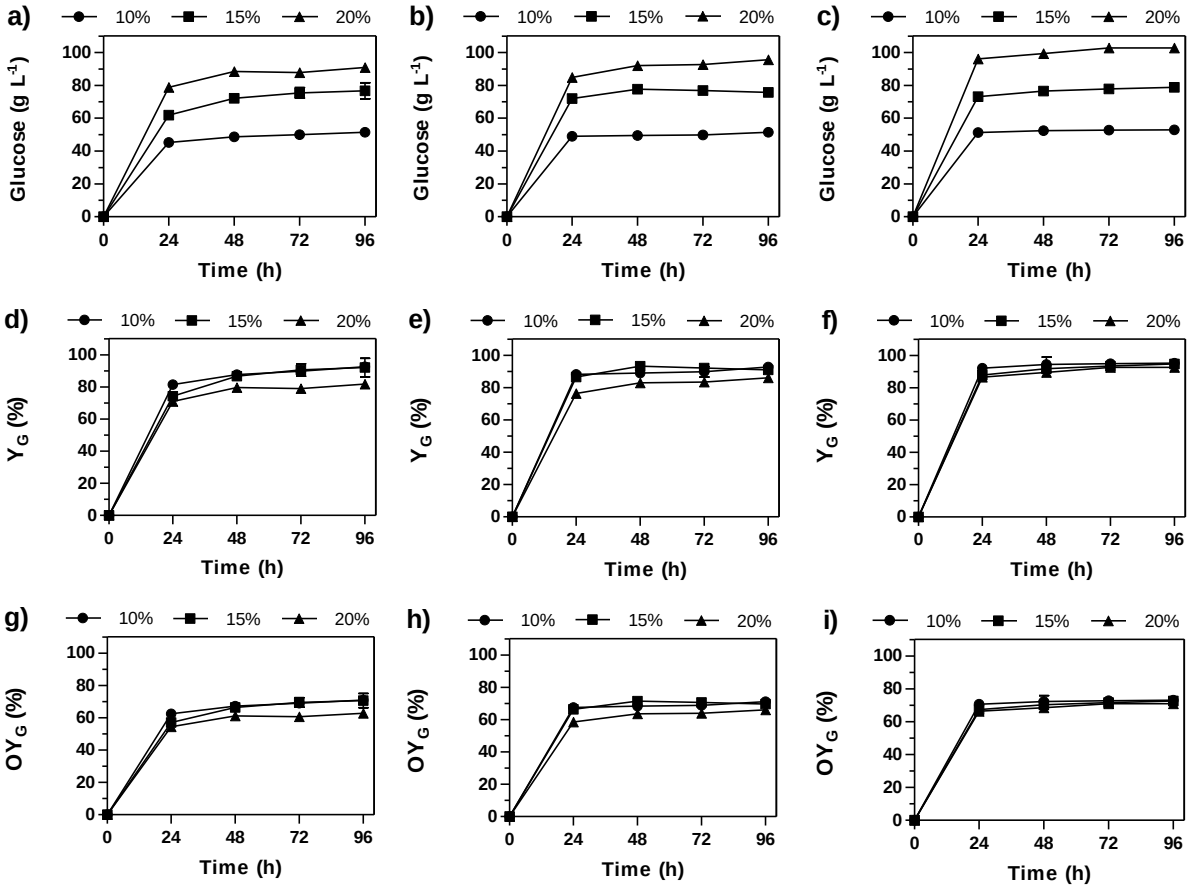

Fig. 2 Time course of the glucose concentration and yields obtained after 96 h of enzymatic hydrolysis at different solids loading (%, *w/v*) and enzyme dosages (10 FPU g^{-1} water insoluble solids (WIS): left column (a), (d) and (g); 20 FPU g^{-1} WIS: central column (b), (e) and (h); 30 FPU g^{-1} WIS: right column (c), (f) and (i)) using as substrate steam-exploded elephant grass under selected condition. Y_G: glucose yield related to g of glucose released by enzymatic hydrolysis per 100 g glucose present in the WIS. OY_G: overall glucose yield related to g of glucose released by enzymatic hydrolysis per 100 g glucose present in the raw material.

As expected, Y_G decreased as the solid load increased. These changes appear to be more pronounced in the first 24 h, becoming stable in the following hours, as shown in the Fig. 2d, Fig. 2e and Fig. 2f. Moreover, by varying the enzyme dose from 10 to 20 and to 30 FPU g^{-1} WIS, an increase in yields was observed for the same solid load, at 24 h. In the specific case of a solids loading of 20% (*w/v*), this characteristic has extended to the other times. Both situations can be evidenced also in Fig. 2g, Fig. 2h and Fig. 2i. For comparative

purposes, the assay previously carried out using 5% (*w/v*) solids loading and 15 FPU g^{-1} WIS reached a superior OY_G, 75.73%.

Assays employing 20% (*w/v*) solids loading added to 20 FPU g^{-1} WIS appeared to result in adequate concentration and yield for the continuity of this study (Fig. 2). A concentration of 95.57 g glucose L^{-1} was released at the end of the hydrolysis, corresponding to yields in the order of 86.02% (Y_G) and 65.98% (OY_G). Ramos *et al.* (2015) investigated the hydrolysis of phosphoric acid-impregnated steam-treated sugarcane bagasse. After experimental design, the highest amount of fermentable sugars was also obtained with 20% (wt%) solids loading in addition to 4.5 FPU g^{-1} substrate of Cellic CTec2®, producing 76.80 g L^{-1} glucose. Taking account the results of these authors with those obtained here, it is possible to perceive the valuable potential of EG against sugarcane bagasse – feedstock widely used in the production of ethanol. Lu *et al.* (2010) used steam-exploded corn stover to investigate the influence of solid concentration ranging from 10-30% (*w/w*) on the enzymatic hydrolysis using commercial acidic cellulose of 20 FPU g^{-1} dry stover. For the washed material at 30% (*w/w*), enzymatic hydrolysis reached 103.3 g glucose L^{-1} with a cellulose conversion of 72.5%. Here, this glucose concentration was found using 20% (*w/v*) solids loading with 30 FPU g^{-1} WIS.

3.3 Simultaneous saccharification and fermentation to ethanol production

High solids operation is a significant mode to elevate the final ethanol concentration (Lu *et al.*, 2010). Therefore, SSF procedure was carried out with a loading of 20% (*w/v*) solids and an enzyme dosage of 20 FPU g^{-1} WIS for the saccharification; concomitantly, the yeast *S. cerevisiae* CAT-1 was used for the fermentation, evaluated for 72 h. CAT-1 is one of the strains most used by Brazilian industries because it has remarkable capacity to compete with

native strains and to survive and dominate during the industrial fermentation process (Costa *et al.*, 2014). The profile of CAT-1 fermentation including average results of concentrations and viable cells pH is shown in Fig. 3.

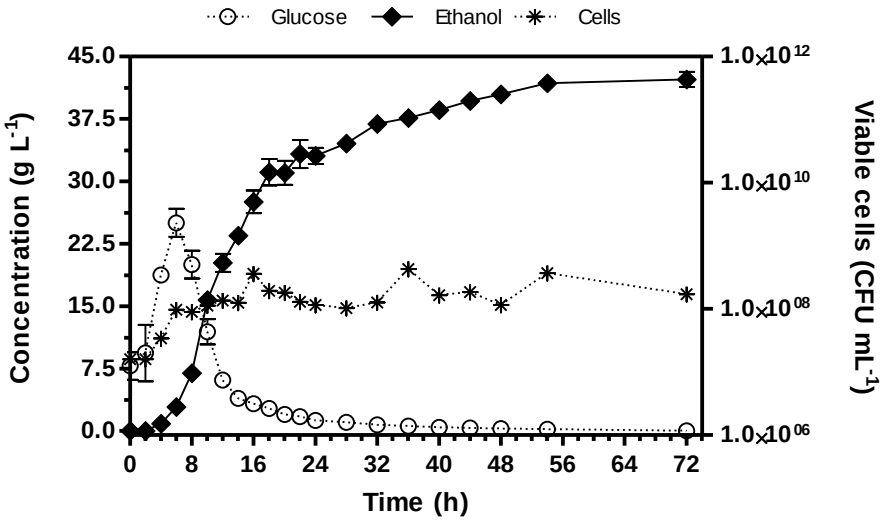

Fig. 3 *Saccharomyces cerevisiae* CAT-1 was used during simultaneous saccharification and fermentation at 37 °C using 20% (*w/v*) pretreated elephant grass and an enzyme dose of 20 FPU g^{-1} water insoluble solids

The experiment initiated with a glucose concentration of approx. 7.80 g L^{-1}, and a maximum of 25.01 g L^{-1} was reached after 6 h of SSF. From this time point, its content started to decrease considerably until 28 h, seeing that it was immediately converted by the microorganism after being released. Fig. 3 also indicates that it has been totally consumed in the remaining hours. Ethanol began to be accumulated after 4 h of SSF, reaching 40.48 g L^{-1} in 48 h. When the glucose released was at its peak, only 2.89 g L^{-1} of ethanol was detected. The upper value of 42.25 g L^{-1} was produced by CAT-1 at the end of fermentation. Knowing that the toxicity of ethanol to the microorganism may be a limiting factor, fermentative medium flowed properly here, without inhibition by the substrate or product formed. The total

numbers of viable cells at the onset of cultivation were 1.6×10^{7} CFU mL^{-1}, corresponding to 1.54 g biomass L^{-1}, and progressively increased until 16 h, reaching 3.6×10^{8} CFU mL^{-1}, that is, 2.41 g L^{-1} of biomass produced. From this point, there were some falls, followed by slight growth of the microorganism, according to the availability of sugars. Cultivation finished at 1.7×10^{8} CFU mL^{-1}. In general, biomass production remained constant throughout the fermentation, as well as pH, reaching 3.94 in the last collected sample. Table 3 summarizes the SSF results.

Table 3 Ethanol yield attained by simultaneous saccharification and fermentation using *Saccharomyces cerevisiae* CAT-1 after 72 h fermentation

Solids loading (*w/v*)	Enzyme dosage (FPU g^{-1} WIS)	Ethanol (g L^{-1})	$Y_{E/G}$ (g g^{-1})	Y_E (%)	P_V (g L^{-1} h^{-1})	OY_E (%)
20%	20	42.25	0.38	74.57	0.84	14.58

$Y_{E/G}$: ratio yield referred as g ethanol per g potential glucose in the WIS.
Y_E: ethanol yield expressed as percentage of the maximum theoretical ethanol yield (0.51 g ethanol per g glucose).
P_V: volumetric productivity (calculated at 48 h).
OY_E: overall process yield defined as g ethanol per 100 g untreated elephant grass.

When calculating the SSF yield within 72 h fermentation period as Y_E, a value of 74.57% was obtained comparing to the maximum theoretical, while $Y_{E/G}$ was 0.38 g ethanol g^{-1} potential glucose in the WIS. Productivity reached 0.84 g L^{-1} h^{-1} at 48 h of cultivation. An alkaline pretreatment of EG followed by SSF using *S. cerevisiae* Ethanol Red, with an enzyme dose of 30 FPU g^{-1} biomass of Accellerase 1500 and a solid to liquid ratio of 10:90 (*w/w*) were performed by Cardona *et al.* (2014). They obtained an OY_E similar to that found in this study, 14.93 g ethanol 100 g^{-1} EG. However, the highest ethanol concentration was 26.0 g L^{-1}, corresponding to 95.1% of the maximum theoretical yield. Narra *et al.* (2015) carried out a SSF with *Kluyveromyces* sp. employing lignocellulosic materials chemically pretreated with 10% (*w/v*) solids loading and 9 FPU g^{-1} substrate, consisting of commercial cellulases. Maximum ethanol attained from rice straw, wheat straw and sugarcane bagasse

was 24.63 g L^{-1}, 19.83 g L^{-1} and 19.05 g L^{-1}, corresponding to 54.38%, 52.44% and 48.07%, respectively, as maximum theoretical yield. When a commercial ß–glucosidase was added to the reaction, these yields rose to 84.56%, 72.47% and 70.55%. Again, this data demonstrate the efficiency of EG *versus* the typical raw materials for the production of ethanol. Previous experiments of ethanol fermentation from EG were also carried out using separate hydrolysis and fermentation (SHF) and prehydrolysis strategies (data not shown), leading to similar results. Considering the time spent with these initial processes, it was decided to apply the SSF strategy in this study to increase productivity.

3.4 Lactic acid (LA) production

LA fermentation was performed from the detoxified liquor using *L. buchneri* NRRL B-30929. The activity of this bacterium in the utilization of sugars and formation of bioproducts is demonstrated in Fig. 4. The first sugar to be metabolized by *L. buchneri* was glucose, and it was instantly consumed and quenched after 16 h of cultivation. The other sugars required double the time since arabinose was slowly converted over 32 h, while xylose demanded approx. 12 h to initiate the conversion process, remaining 0.75 g L^{-1} after 32 h of cultivation. The amount of 3.50 g L^{-1} of galactose and mannose were also metabolized (data not shown). LA was efficiently produced by *L. buchneri*, and a racemic mixture of DL-LA was generated throughout the process. After separation of the peaks by a chiral column, a concentration of 4.0 g L^{-1} was obtained for the L-isomer, compared to 9.0 g L^{-1} for the D-isomer, in 24 h of fermentation, a period in which a plateau was reached. Since the EG liquor was detoxified, no inhibition of cell growth was observed in the experiments. Acetic acid is the main by-product produced by this *L. buchneri* strain (Liu *et al.*, 2008), reaching 9.0 g L^{-1} in only 24 h of cultivation. Due to the high generation of this organic acid in the reaction

medium, preparation of the *L. buchneri* inoculum excluded sodium acetate (5.0 g L^{-1}) from the growth broth without damaging the fermentation. Even though the inoculum having been washed and resuspended in water prior to inoculation, at the onset of the cultivation almost 2.0 g L^{-1} of acetic acid was quantified. Ethanol production by *L. buchneri* was not verified in this experiment. Cell concentration started at 1.7×10^{10} CFU mL^{-1}, corresponding to 5.43 g biomass L^{-1}. This concentration reached 2.2×10^{11} CFU mL^{-1}, that is, 10.50 g L^{-1} in 22 h of cultivation. After a drop and a successive increase, the final viable cells were 5.9×10^{7} CFU mL^{-1}. pH dropped almost halfway through fermentation, ending at 3.3. Table 4 details the results of *L. buchneri* culture parameters to lactic fermentation.

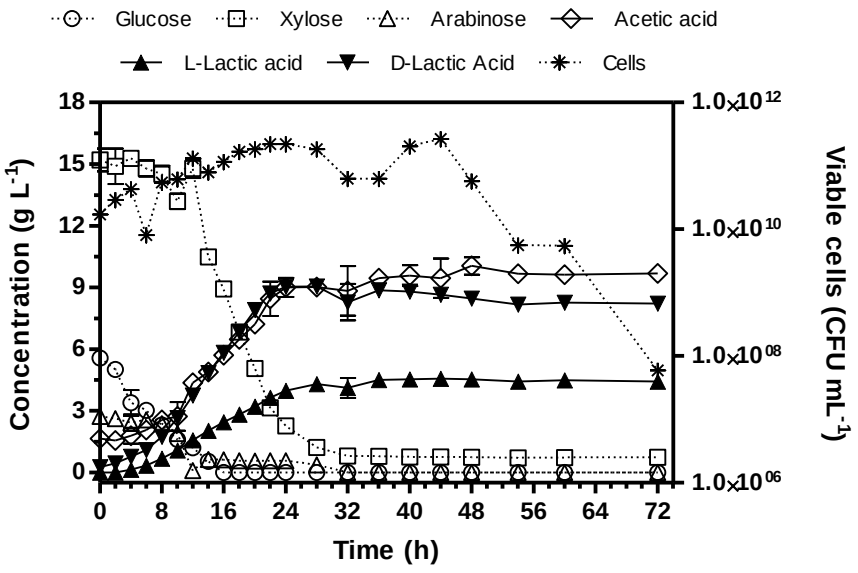

Fig. 4 The fermentation profile of *Lactobacillus buchneri* NRRL B-30929 carried out at 37 °C using the liquor of the pretreated elephant grass

Conversions of sugars contained in the liquor into product ($Y_{LA/S}$) was 0.50 g g^{-1} (28 h) and into cells ($Y_{X/S}$) was 0.19 g g^{-1} (22 h). After 28 h fermentation, Y_{LA} was measured as 68.21% in relation to the maximum theoretical. At that time, productivity reached 0.48 g L^{-1}

h^{-1}. Complementing the kinetic data, the maximum specific rates were established. μX_m corresponding to 0.047 h^{-1} was reached only after 18 h. This delay to reach μX_m becomes evident when analyzing Fig. 4. Firstly, the microorganism converted the glucose and then started to convert the xylose. A μP_m of 0.123 g g^{-1} h^{-1} was achieved almost at 13 h and a μS_m of 0.178 g g^{-1} h^{-1} in 9 h (Fig. S5). After pressurized hydrolysis of lignocellulosic materials catalyzed with sulfuric acid, Montipó *et al.* (2016) employed *L. rhamnosus* ATCC 10863 to convert them into L(+)-LA. In approx. 24 h of fermentation, it was achieved 14.5 g LA L^{-1}, with a yield of 8.71 g LA 100 g^{-1} rice husk; and 11.3 g L^{-1}, with a yield of 6.79 g LA 100 g^{-1} agave bagasse, similar to the results found here. Mixed cultures of *L. rhamnosus* and *L. brevis* were investigated by Cui *et al.* (2011) for improving utilization of both C6- and C5-sugars from corn stover aiming at LA production. LA yield of 0.70 g g^{-1} and productivity of 0.58 g L^{-1} h^{-1} were obtained from NaOH-pretreated corn stover.

Table 4 Lactic acid yield employing *Lactobacillus buchneri* NRRL B-30929 after 28 h fermentation

Lactic acid (g L^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{LA/S}$ (g g^{-1})	Y_{LA} (%)	P_V (g L^{-1} h^{-1})	OY_{LA} (%)
13.35	0.19	0.50	68.21	0.48	7.06

$Y_{X/S}$: ratio yield of cells per substrate referred as g biomass per g sugar in the liquor (calculated at 22 h).
$Y_{LA/S}$: ratio yield of product per substrate referred as g lactic acid per g sugar present in the liquor.
Y_{LA}: lactic acid yield expressed as percentage of theoretical lactic acid yield (0.73 g lactic acid per g sugar).
P_V: volumetric productivity.
OY_{LA}: overall process yield defined as g lactic acid per 100 g untreated elephant grass.

The present study demonstrated what could be an efficient platform for the processing of ethanol and a value-added chemical in order to apply to the concepts of biorefinery. The initial intention was to employ EG not just to use its potential for energy production, unlike most current works, but for the production of an organic acid. To the best of our knowledge, there is no publication involving the use of EG in the generation of both ethanol and LA from

C6- and C5-sugars, respectively. The conversion of each product of interest was developed in separate systems, avoiding problems of inhibition and also selective fermentation processes. Further experiments for the recovery and purification of the products obtained here need to be accomplished.

4. Conclusions

Fermentable sugars contained in the EG can be used for the production of fuel and value-added products. After SE pretreatment employing dilute sulfuric acid, a configuration of SSF at 20% (*w/v*) solids loading targeted the conversion of ethanol with *S. cerevisiae* CAT-1, reaching a yield of 0.38 g g^{-1} potential glucose in the WIS. The filtrate was used in parallel for lactic acid production with *L. buchneri* NRRL B-30929 at a conversion of 0.50 g g^{-1} sugar. This study emphasizes the total use of both G_{WIS} and X_{LIQ} in an integrated system for fuel ethanol and bioproduct LA production.

Acknowledgments

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The authors have declared no conflicts of interest.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at … (to be accomplished).

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Supplementary Material

Table S1 Results of positive and negative electrospray ionization using LC/TOF-MS of selected model compounds from liquor of elephant grass pretreated by steam explosion with 2.0% (*w/w*) H_2SO_4

Model compound	Molecular weight	$M \pm H^+$ / Adducts	
		TOF MS ES+	TOF MS ES-
Formic acid	46.02	118.08 $[M+3Na+3H]^+$	113.02 $[M+3Na-2H]^+$ 163.04 $[M+3K]^+$
Acetic acid	60.05	109.03 $[M+2Na+3H]^+$ 177.05 $[M+3K]^+$ 180.1 $[M+3K+3H]^+$	128.04 $[M+3Na-H]^+$ 134.92 $[M+2K-3H]^+$
Furfural	96.09	120.08 $[M+Na+H]^+$ 136.06 $[M+K+H]^+$ 144.11 $[M+2Na+2H]^+$ 168.07 $[M+3Na+3H]^+$ 176.07 $[M+2K+2H]^+$ 177.05 $[M+2K+3H]^+$	131.03 $[M+K-4H]^+$ 134.92 $[M+K]^+$ 163.04 $[M+3Na-2H]^+$
2-Furoic acid	112.08	136.06 $[M+Na+H]^+$ 152.07 $[M+K+H]^+$ 154.05 $[M+K+3H]^+$ 230.08 $[M+3K+H]^+$	131.03 $[M+Na-4H]^+$ 134.92 $[M+Na]^+$ 180.91 $[M+3Na]^+$ 229.0 $[M+3K]^+$
Levulinic acid	116.11	118.08 $[M+2H]^+$ 120.08 $[M+4H]^+$ 196.1 $[M+2K+2H]^+$	113.02 $[M-3H]^+$ 134.92 $[M+Na-4H]^+$ 180.91 $[M+3Na-4H]^+$ 193.05 $[M+2K-H]^+$ 229.0 $[M+3K-4H]^+$ 232.88 $[M+3K]^+$
4-Hydroxybenzaldehyde	122.12	168.07 $[M+2Na]^+$	
5-Hydroxymethylfurfural	126.11	152.07 $[M+Na+3H]^+$ 168.07 $[M+K+3H]^+$ 176.07 $[M+2Na+4H]^+$ 196.1 $[M+3Na+H]^+$	163.04 $[M+K-2H]^+$ 193.05 $[M+3Na-2H]^+$ 194.93 $[M+3Na]^+$
Xylose	150.13	152.07 $[M+2H]^+$ 154.05 $[M+4H]^+$ 176.07 $[M+Na+3H]^+$ 177.05 $[M+Na+4H]^+$ 196.1 $[M+2Na]^+$	193.05 $[M+2Na-3H]^+$ 194.93 $[M+2Na-H]^+$
Vanillin	152.15	152.07 [M] 154.05 $[M+2H]^+$ 176.07 $[M+Na+H]^+$ 177.05 $[M+Na+2H]^+$ 230.08 $[M+2K]^+$	194.93 $[M+2Na-3H]^+$ 196.93 $[M+2Na-H]^+$ 229.0 $[M+2K-H]^+$ 265.07 $[M+3K-4H]^+$
Coumaric acid	164.16	168.07 $[M+4H]^+$	163.04 $[M-H]^+$ 229.0 $[M+3Na-4H]^+$ 232.88 $[M+3Na]^+$
Glucose	180.16	180.1 [M] 230.08 $[M+2Na+4H]^+$	
Syringaldehyde	182.17	230.08 $[M+2Na+2H]^+$	180.91 $[M-2H]^+$ 259.01 $[M+2K-H]^+$
Ferulic acid	194.18	196.1 $[M+2H]^+$	193.05 $[M-H]^+$ 229.0 $[M+K-4H]^+$ 232.88 $[M+K]^+$ 259.01 $[M+3Na-4H]^+$ 270.84 $[M+2K-H]^+$ 307.07 $[M+3K-4H]^+$
Syringic acid	198.17		194.93 $[M-3H]^+$ 196.93 $[M-H]^+$ 232.88 $[M+K-4H]^+$ 265.07 $[M+3Na-2H]^+$

Table S2 Elephant grass biomass composition (% of dry weight) after steam explosion pretreatment

Stem explosion			Solids recovery (%)	WIS composition (%)			
Temperature (ºC)	Time (min)	H_2SO_4 (*w/w*)		Cellulose	Hemicellulose	Lignin[a]	Ashes
190	5	2%	51.03	50.50±0.38	0.82±0.04	35.11±0.37	11.86±0.13

These data are mean values of three assessments.
[a] acid-insoluble lignin.

Table S3 Chemical composition of elephant grass liquor after steam explosion

H_2SO_4 (*w/w*)	Sugars before post-hydrolysis (g L^{-1})					Sugars after post-hydrolysis (g L^{-1})				
	Glucose	Xylose	Galactose	Arabinose	Mannose	Glucose	Xylose	Galactose	Arabinose	Mannose
2%	5.91	16.32	2.23	2.87	0.64	6.64	16.92	2.34	2.95	0.74

H_2SO_4 (*w/w*)	pH	Severity *log (R_0')*	Lignocellulose-derived products (g L^{-1})								
			Furfural	5-HMF	Acetic acid	Formic acid	Coumaric acid	Ferulic acid	Vanillin	Syringaldehyde	4-HBA
2%	1.69	1.7	2.376	0.556	1.884	0.233	0.073	0.048	0.016	0.010	nd

nd: not detected.

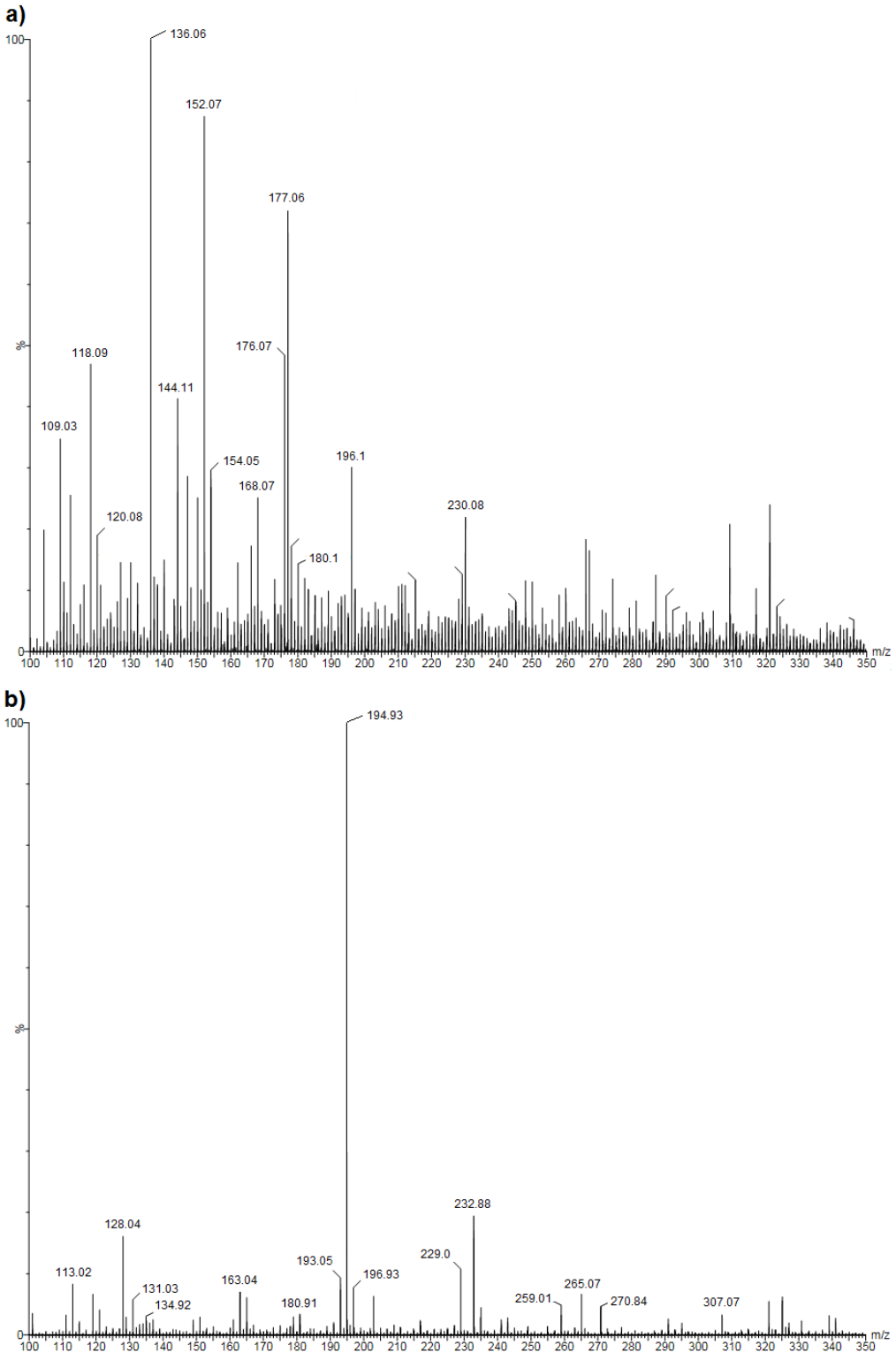

Fig S1 Total ion chromatogram acquired via LC-MS/TOF (a) ES+ and (b) ES- by direct infusion of hydrolyzed sample by steam explosion with 2.0% (*w/w*) H_2SO_4

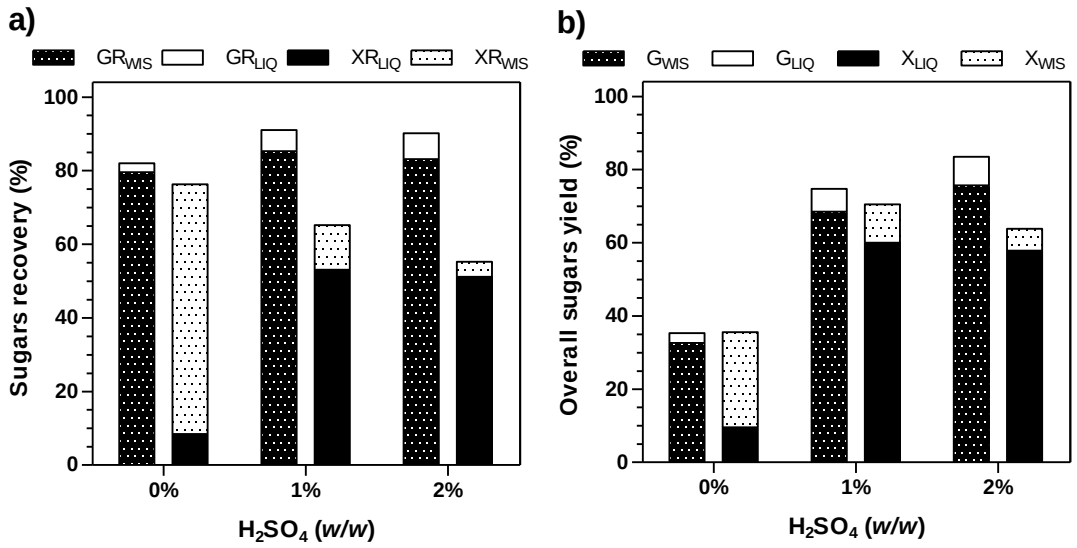

Fig S2 Sugars recovery yields (a) and overall sugars yields (b) obtained from different conditions of steam explosion expressed as percentage of glucose or xylose remained in WIS or in liquor regarding to sugars content on elephant grass, and expressed as percentage of sugars released in each fraction referred to sugars content in the raw material, respectively.

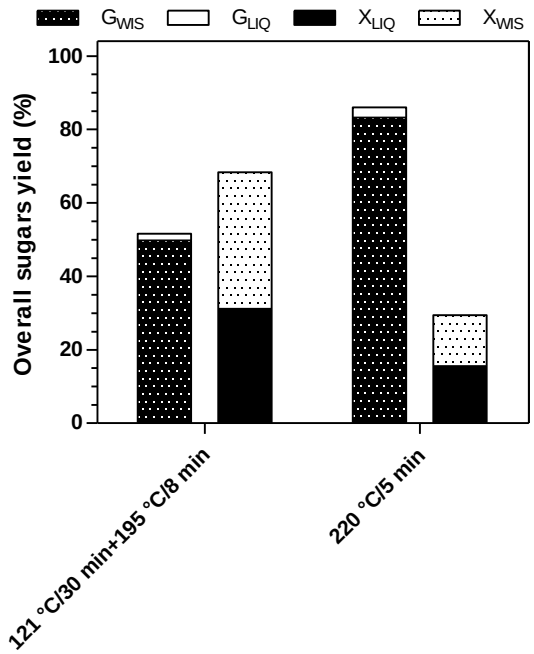

Fig S3 Overall sugars yields in previous steam explosion pretreatment of elephant grass at different conditions expressed as percentage of sugars released in each fraction referred to sugars content in the raw material

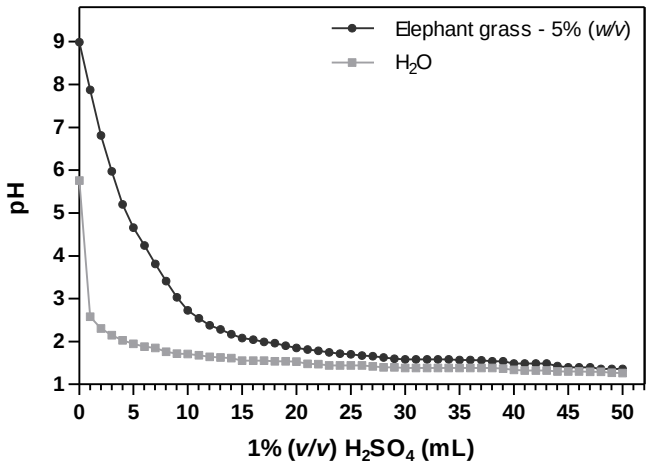

Fig S4 Amount of 1% (*v/v*) H_2SO_4 required to reach the same pH among water and elephant grass solution

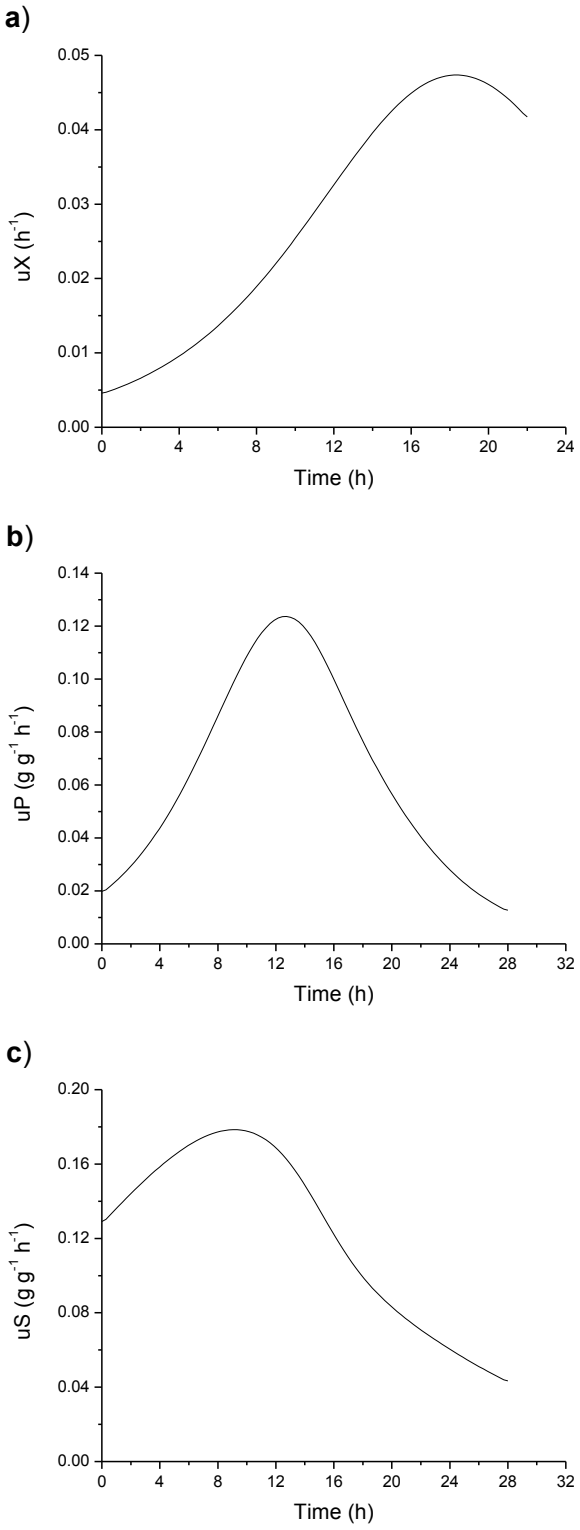

Fig S5 Specific rates of *Lactobacillus buchneri* cultivation: microorganism growth (μX), substrate consumption (μS), and product formation (μP)

4.4 Solicitação de patente

Processo de obtenção de bioproduto e biocombustível

a partir de biomassas lignocelulósicas

Pedido de patente depositado no INPI.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 018206 1

Dados do Depositante (71)

Depositante 1 de 1

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Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): PROCESSO DE OBTENÇÃO DE BIOPRODUTO E BIOCOMBUSTÍVEL A PARTIR DE BIOMASSAS LIGNOCELULÓSICAS

Resumo: A presente invenção visa demonstrar o aproveitamento da casca de arroz e do capim-elefante, propondo a transformação de ambas em etanol e ácido láctico a partir das frações sólida e líquida destas biomassas, respectivamente, pré-tratadas por explosão a vapor e hidrolisadas. Tudo isso aliado à otimização das condições operacionais, desenvolvendo processo de produção de etanol através do consumo das hexoses presentes no material pré-tratado e de ácido láctico através do consumo das pentoses presentes no licor. Ademais, busca contribuir para a mitigação do crescente problema ambiental da casca de arroz e valer-se da elevada produtividade do capim-elefante no estado do Rio Grande do Sul/Brasil. Tal processo situa-se nas áreas da química e da engenharia verdes.

Título: PROCESSO DE OBTENÇÃO DE BIOPRODUTO E BIOCOMBUSTÍVEL A PARTIR DE BIOMASSAS LIGNOCELULÓSICAS

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Relatório Descritivo de Patente de Invenção

PROCESSO DE OBTENÇÃO DE BIOPRODUTO E BIOCOMBUSTÍVEL

A PARTIR DE BIOMASSAS LIGNOCELULÓSICAS

Campo da Invenção

[1] A presente invenção trata do processo de produção de etanol e ácido láctico empregando como substrato a casca de arroz e o capim-elefante previamente pré-tratados em sistema de explosão a vapor e posteriormente hidrolisados, visando à obtenção de glicose e xilose, as quais converter-se-ão nos produtos de interesse – etanol e ácido láctico, respectivamente. Tal processo situa-se nas áreas da química e da engenharia verde.

Antecedentes da Invenção

[2] O etanol é um dos combustíveis renováveis mais importantes, sendo que nas últimas décadas a produção e o consumo global de biocombustíveis expandiram-se significativamente devido às políticas de apoio que visam, sobretudo, reduzir a emissão dos gases do efeito estufa e diversificar as fontes energéticas. A levedura *S. cerevisiae* é um dos micro-organismos mais utilizados na produção de etanol devido à sua eficiência na fermentação de hexoses, sendo tolerante ao baixo pH, ao próprio etanol produzido, bem como aos compostos inibitórios presentes no hidrolisado. No entanto, devido à falta de uma via metabólica da xilose em xilulose, *S. cerevisiae* não consegue utilizar xilose, apenas se as cepas forem reconstruídas por engenharia genética.

[3] O ácido láctico é um ácido orgânico de ocorrência natural, com valor agregado e versátil, considerando-se inúmeras aplicações industriais e biotecnológicas. O ácido láctico pode ser produzido por rota química ou enzimática. A síntese química fundamenta-se na hidrólise da lactonitrila, resultando em uma mistura racêmica de DL-ácido láctico. Já a rota

fermentativa converge à produção de isômeros L-(+)- ou D-(–)- opticamente puros. Atualmente, a produção de ácido láctico ocorre quase que totalmente através da rota fermentativa (~90%) com o consumo de sacarídeos e demais substratos sintéticos altamente nutritivos, os quais elevam o custo do processo. As bactérias produtoras de ácido láctico são capazes de produzí-lo como metabólito principal, com alto rendimento e alta produtividade, sendo os gêneros *Lactobacillus*, *Streptococcus*, *Leuconostoc* e *Enterococcus* os mais empregados. Grande parte destas bactérias consegue converter somente glicose em ácido láctico.

[4] Dentre as patentes pesquisadas nas bases de dados, vinculadas a presente invenção, percebe-se que as iniciativas do emprego de matérias-primas alternativas como substrato à produção de bioprodutos pela rota fermentativa, fazem uso de uma série de biomassas de origem sacarínea, amilácea ou lignocelulósica. Muitas das patentes comentam apenas a possibilidade de biotransformação das matérias-primas, desde os inúmeros pré-tratamentos existentes, passando pela hidrólise enzimática até a fermentação aos produtos desejados. A respeito da casca de arroz e do capim-elefante, não foram encontradas abordagens de processamento semelhantes às propostas na presente invenção.

[5] Durante o processamento das biomassas, os açúcares gerados são passíveis de produzirem uma gama de produtos de interesse, além de compostos intermediários e/ou subprodutos. Não obstante, a biomassa pré-tratada pode originar uma mistura de produtos de difícil separação, tal como a glicose e a xilose, em decorrência das suas semelhanças químicas e físicas. Uma mistura destes açúcares poderá ser seletivamente fermentada para deixar um ou mais açúcares na mistura, juntamente com um produto. O açúcar subutilizado poderá ser fermentado com um sistema diferente e produzir um segundo produto. Diante disso, a patente US 20150368684 A1 propôs a utilização de distintos micro-organismos para produzir etanol a

partir da glicose (*S. cerevisiae*) e ácido butanoico a partir da xilose (*C. tyrobutyricum*), por exemplo, empregando espiga de milho.

[6] De modo geral, ainda no que diz respeito à produção de distintos produtos, a patente WO 2011002824 A1 fez uso de matérias-primas vegetais envolvendo pré-tratamentos ácidos ou básicos à produção de álcoois e demais produtos por micro-organismos do gênero *Clostridium*. Já a patente WO 2014160402 A1 enfatiza a co-conversão de diferentes carboidratos a produtos fermentativos, incluindo ácidos láctico e acético e etanol.

[7] A possibilidade da utilização de biomassas residuais, como a espiga de milho, empregando pré-tratamento para a obtenção de glicose e posterior fermentação a ácidos hidroxicarboxílicos, tal como o ácido láctico, por micro-organismos do gênero *Lactobacillus*, por exemplo, foi proposto na patente US 20160076062 A1. Uma série de patentes citou o emprego de micro-organismos especializados na degradação de pentoses para a produção de ácido láctico: WO 2004063382 A2, US 7098009 B2, EP 1437415 A1, WO 2009025547 A1 e US 5798237 A. O processo de obtenção de ácido láctico a partir de um substrato convencional contendo glicose, xilose, arabinose ou ribose foi o processo empregado na primeira patente, WO 2004063382 A2, em que *Bacillus* spp., capazes de consumir especialmente a xilose, foram utilizados. A utilização de *Bacillus* isolado da natureza, o qual produz elevado rendimento em L-(+)-ácido láctico a partir de açúcares de hexose e pentose de biomassas, foi referido na patente US 7098009 B2. Por sua vez, a patente EP 1437415 A1 relata a produção de ácido láctico a partir de substratos contendo pentoses, em que espécies de *Bacillus* moderadamente termófilas são capazes de fermentar especificamente xilose a ácido láctico enantiomericamente puro, por via homofermentativa. Palha de trigo pré-tratada com agente alcalino foi convertida em ácido láctico por *B. coagulans* ao longo de um processo de sacarificação e fermentação simultânea na patente WO 2009025547 A1, sendo que hexoses e

pentoses derivadas da matéria-prima foram utilizadas concomitantemente. Já a patente US 5798237 A fez uso de *Lactobacillus* MONT4 recombinante, modificado com genes de xilose isomerase e xiluloquinase de *L. pentosus* para conferir ao *Lactobacillus* MONT4 a capacidade de fermentar biomassa lignocelulósica contendo xilose em ácido láctico.

[8] Deste modo, não foram encontrados documentos antecipando ou sugerindo os ensinamentos da presente invenção, uma vez que tanto as condições de pré-tratamento quanto as hidrólises em si, além das estratégias de fermentação foram diferenciadas.

Sumário da Invenção

[9] Tal invento está vinculado à resolução de questões, dentre elas: utilização de substratos alternativos para a produção de açúcares fermentescíveis, aqui representados pela casca de arroz e pelo capim-elefante, desencadeando custo de preparo inferior nos produtos de interesse; emprego de subproduto residual da agroindústria – a casca de arroz não possui aplicação direta na indústria e, tampouco, valor comercial, estando sujeita ao agravamento dos problemas ambientais devido a sua acumulação em grandes áreas; processo de pré-tratamento por explosão a vapor visando à produção ótima de glicanos na fração sólida e de xilanos na fração líquida; hidrólise da fração sólida com enzimas do complexo celulolítico e ß-glicosidases, e hidrólise da fração líquida, no caso da casca de arroz, com enzimas produzidas por *P. echinulatum* S1M29, sendo esta última um procedimento inédito; aproveitamento total das frações sólida e líquida para a produção de etanol e ácido láctico, respectivamente, de modo a degradar as hexoses com CAT-1 e as pentoses com *L. buchneri* NRRL B-30929; a produção de cada produto de interesse foi desenvolvida em sistemas separados, evitando problemas de inibição e, ainda, processos de fermentação seletiva; novo processo de produção de etanol e de um insumo químico de elevado valor agregado, empregando casca de arroz e capim-elefante, de maneira a aplicar os conceitos da biorrefinaria; emprego do capim-elefante

também para a produção de ácido orgânico, e não valer-se do seu potencial apenas para a produção de biocombustíveis.

[10] A presente invenção pretende ampliar os conhecimentos sobre pré-tratamentos à pressão e hidrólise enzimática da casca de arroz e do capim-elefante visando à obtenção de hexoses e pentoses e a conversão destas a etanol e ácido láctico, respectivamente.

[11] Ao mesmo tempo, a invenção visa:

- otimizar as condições operacionais, tal como o pré-tratamento dos materiais lignocelulósicos por explosão a vapor, de modo a maximizar rendimento e produtividade;

- desenvolver processo inovador de produção de etanol (CAT-1) a partir das hexoses da fração sólida e ácido láctico (*L. buchneri* NRRL B-30929) a partir das pentoses da fração líquida, em separado;

- monitorar as produções de etanol, de ácido láctico, dos açúcares e dos subprodutos por cromatografia;

- investigar um processo para a produção de etanol e ácido láctico com o intuito de encontrar um uso adequado e/ou alternativo para a casca de arroz e para o capim-elefante como matérias-primas de baixo custo, e, ao mesmo tempo, ultrapassar um grave problema ambiental regional relacionado à casca de arroz.

[12] Em um objeto, a presente invenção apresenta um processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas compreendendo as etapas de:

a. pré-tratamento da biomassa em sistema de explosão a vapor;

b. separação das porções líquidas e sólidas;

c. hidrólise da fração sólida com enzimas do complexo celulolítico e ß-glicosidases;

d. conversão das hexoses presentes na fração sólida a etanol; e

e. conversão das pentoses presentes na fração líquida a ácido láctico.

[13] Deste modo, a presente invenção apresenta um processo que pode utilizar diferentes biomassas, permitindo a obtenção de ácido lático a partir da fração líquida, e etanol e partir da fração sólida da biomassa.

[14] Estes e outros objetos da invenção serão imediatamente valorizados pelos versados na arte e pelas empresas com interesses no segmento, e serão descritos em detalhes suficientes para sua reprodução na descrição a seguir.

Breve Descrição das Figuras

[15] A invenção poderá ser mais bem compreendida através da seguinte descrição detalhada, conforme as figuras em anexo, onde:

[16] A Figura 1 evidencia os teores de glicose liberados a partir de pré-tratamento e hidrólise da (A) casca de arroz e do (B) capim-elefante contendo 20% (*m/v*) de biomassa pré-tratada e distintas dosagens de enzimas do complexo celulolítico e ß-glicosidases.

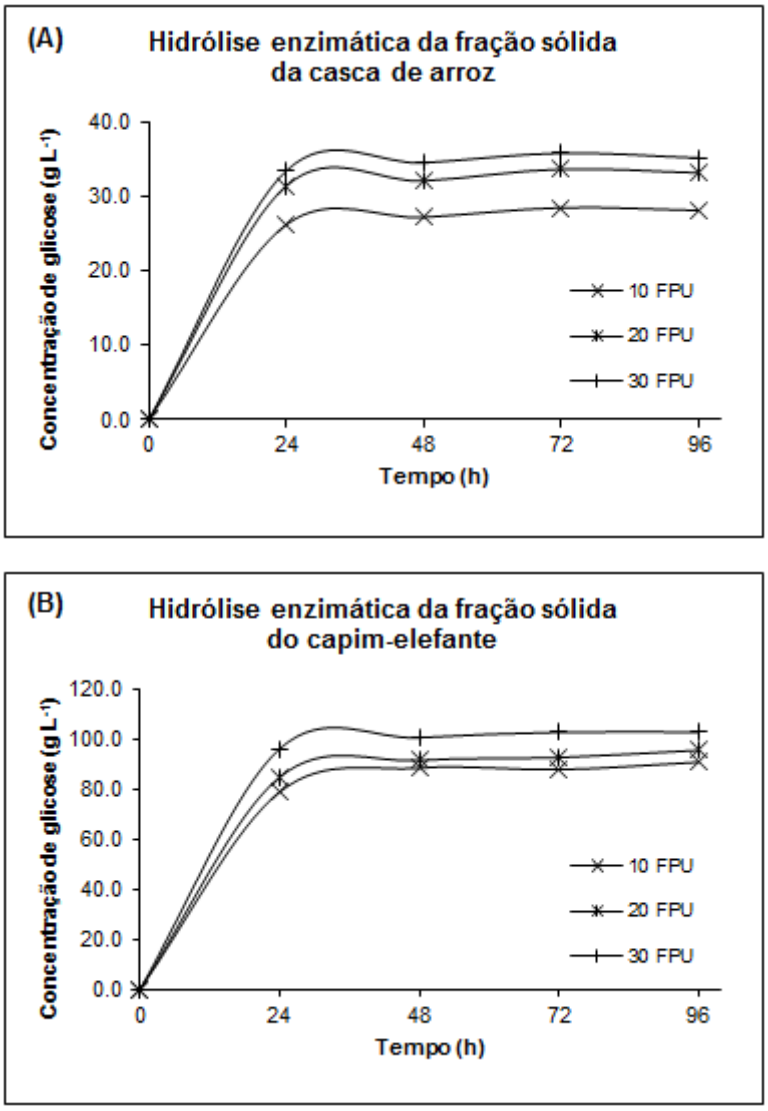


Figura 1

[17] A Figura 2 revela os teores de açúcares liberados a partir da fração líquida da casca de arroz pré-tratada durante hidrólise (30 FPU) com enzimas produzidas por *P. echinulatum* S1M29.

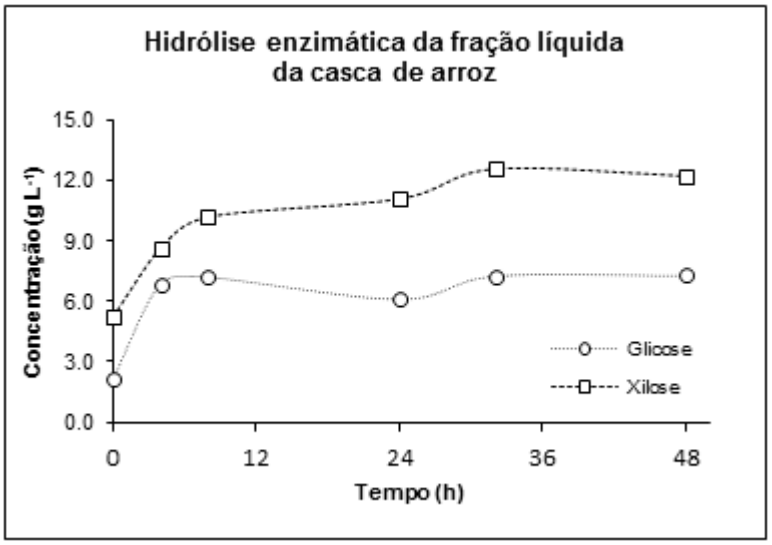


Figura 2

[18] A Figura 3 revela a cinética da produção de etanol e o consumo de açúcares durante a fermentação da fração sólida da casca de arroz com CAT-1, suplementada com nutrientes, a 37 ºC, sob 180 rpm. As estratégias utilizadas foram: (A) pré-hidrólise e (B) hidrólise e fermentação separadas (SHF).

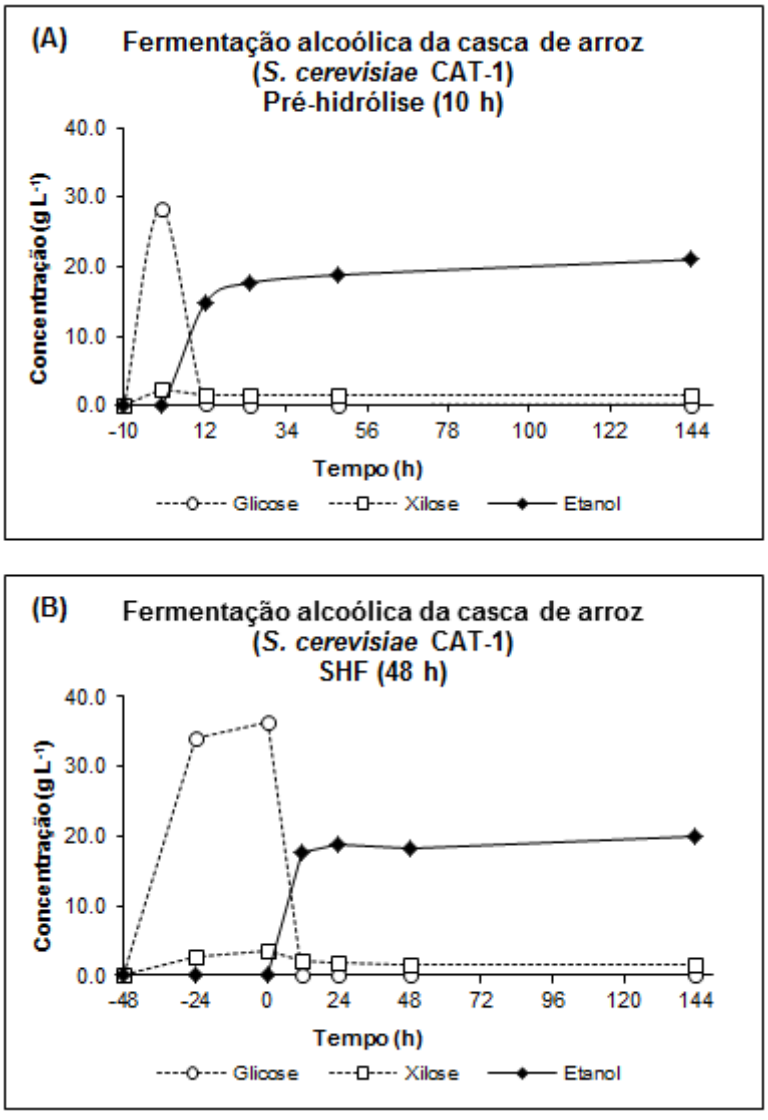


Figura 3

[19] A Figura 4 revela a cinética da produção de etanol e o consumo de açúcares durante a fermentação da fração sólida do capim-elefante com CAT-1, suplementada com nutrientes, a 37 ºC, sob 180 rpm. As estratégias utilizadas foram: (A) pré-hidrólise e (B) hidrólise e fermentação separadas (SHF).

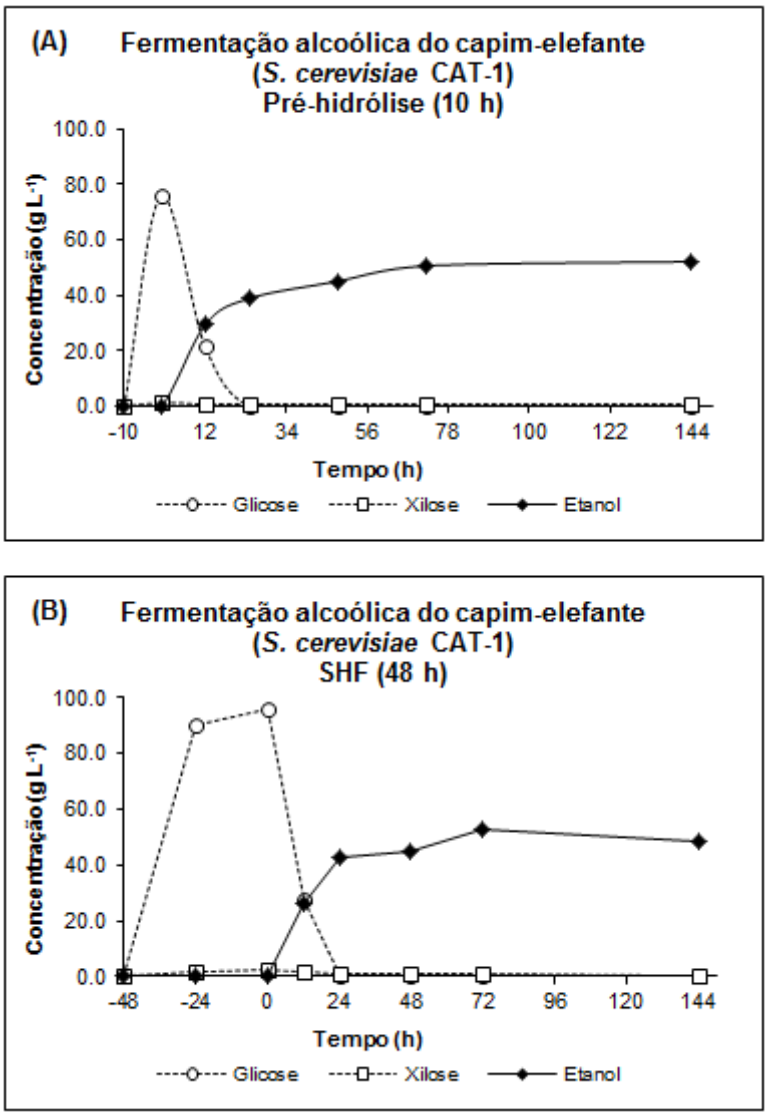


Figura 4

[20] A Figura 5 revela a cinética da produção do ácido láctico e o consumo de açúcares durante a fermentação da fração líquida da (A) casca de arroz e do (B) capim-elefante com *L. buchneri* NRRL B-30929, suplementado com nutrientes, a 37 ºC sob 150 rpm.

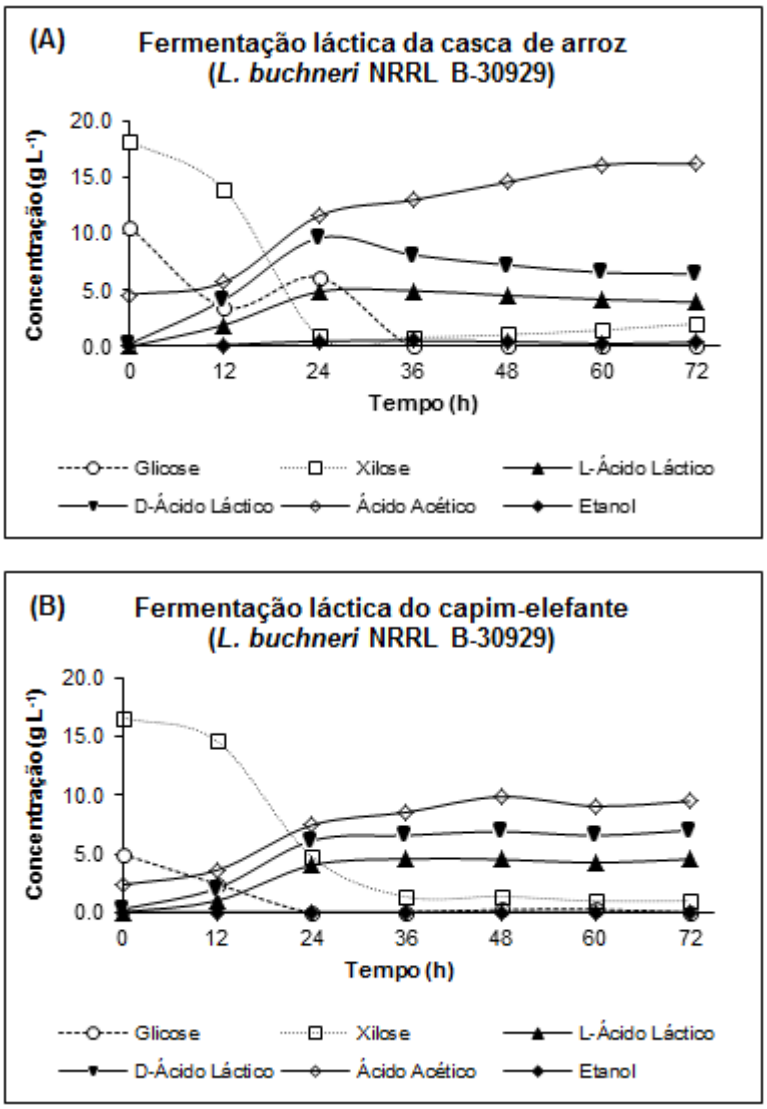


Figura 5

Descrição Detalhada da Invenção

[21] As descrições que seguem farão compreender de forma mais clara o objetivo do presente pedido de patente.

[22] Em um objeto, a presente invenção apresenta um processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas compreendendo as etapas de:

a. pré-tratamento da biomassa em sistema de explosão a vapor;

b. separação das porções líquidas e sólidas;

c. hidrólise da fração sólida com enzimas do complexo celulolítico e ß-glicosidases;

d. conversão das hexoses presentes na fração sólida a etanol; e

e. conversão das pentoses presentes na fração líquida a ácido láctico.

[23] Em uma concretização, a enzima comercial contendo o complexo celulolítico é Celluclast® 1.5L, e a enzima comercial contendo ß-glicosidases é Novozyme 188®.

[24] Em uma concretização, as biomassas lignocelulósicas compreendem casca de arroz e capim-elefante (*Pennisetum purpureum*).

[25] Em uma concretização, a etapa de pré-tratamento do capim-elefante compreende o emprego de um catalisador ácido, sendo o pré-tratamento da biomassa lignocelulósica realizado simultaneamente com a etapa de hidrólise ácida.

[26] Em uma concretização, o catalisador ácido ser 2% (*p/p*) H_2SO_4.

[27] Em uma concretização, o processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas compreende uma etapa adicional de hidrólise da fração líquida da casca de arroz com enzimas produzidas por *P. echinulatum* S1M29 antes da etapa de fermentação ao bioproduto.

[28] Em uma concretização, a etapa de conversão das hexoses presentes na fração sólida a etanol emprega *Saccharomyces cerevisiae* CAT-1 no processo fermentativo.

[29] Em uma concretização, a conversão das hexoses presentes na fração sólida a etanol compreende uma etapa de adição de nutrientes às frações sólidas, tais como extrato de levedura e sulfato de amônio, com vista ao aumento da produtividade da fermentação.

[30] Em uma concretização, a etapa de conversão das pentoses presentes na fração líquida a ácido láctico emprega *Lactobacillus buchneri* NRRL B-30929 no processo fermentativo.

[31] Em uma concretização, a conversão das pentoses presentes na fração líquida a ácido láctico compreende uma etapa de adição de nutrientes às frações líquidas, sendo que tais nutrientes podem ser selecionados do grupo que compreende: peptona, extrato de levedura, citrato de amônio e fostato dipotássico.

[32] As vantagens da presente invenção incluem o aproveitamento de um resíduo agroindustrial para a obtenção de produtos.

Exemplos – Concretizações

[33] Os exemplos aqui mostrados têm o intuito somente de exemplificar uma das inúmeras maneiras de se realizar a invenção, de modo a proporcionar uma melhor compreensão de forma mais clara o objetiva do presente pedido de patente; contudo sem limitar, o escopo da mesma.

Exemplo 1. Pré-tratamento por explosão a vapor

[34] O pré-tratamento por explosão a vapor da casca de arroz ou do capim-elefante ocorreu em vaso de reação com capacidade de 2 L. As biomassas foram adicionadas no reator, sendo este aquecido diretamente com o vapor saturado. Após a explosão, o material foi recuperado em um ciclone, resfriado e filtrado. A composição das frações sólida e líquida foi

analisada. Foram avaliadas estratégias de delineamento experimental visando maior liberação de açúcares fermentescíveis, baseado em valores ótimos de distintas variáveis independentes do processo. Para tanto, no caso da casca de arroz, optou-se por um planejamento completo – delineamento composto central rotacional (DCCR) – para as variáveis temperatura (181-229 °C) e tempo de residência (1,6-11,5 min), com 4 combinações possíveis para os 2 níveis estudados (2^2), acrescidas dos 4 ensaios axiais e de 3 repetições no ponto central. Para o capim-elefante, empregou-se um catalisador no processo de explosão a vapor. Para tanto, fixou-se a temperatura em 190 °C e o tempo em 5 min e testou-se ácido sulfúrico nas concentrações de 1 e 2% (*p/p*). Os pré-tratamentos os quais liberaram quantidades expressivas de glicanos na fração sólida, bem como de xilanos na fração líquida, foram selecionados. Assim, a produção ótima destes açúcares a partir de pré-tratamento da casca de arroz ocorreu a 205 °C por 11,5 min. O ensaio selecionado no caso do capim-elefante foi aquele contendo 2% (*p/p*) de H_2SO_4.

Exemplo 2. Hidrólise enzimática

[35] As hidrólises enzimáticas das frações sólidas da casca de arroz e do capim-elefante foram feitas em frascos de 50 mL, no qual foram adicionados 20% (*m/v*) de biomassa pré-tratada; 10, 20 e 30 FPU g^{-1} de substrato de Celluclast® 1.5L (59 FPU mL^{-1}) e Novozym® 188 (530 UI mL^{-1}) (Novozymes A/S, Dinamarca); e tampão citrato de sódio (pH 4,8, 50 mM) para um volume de 40 mL. Esta mistura foi incubada a 50 ºC, sob 150 rpm. Amostras foram coletadas após 24, 48, 72 e 96 h. A hidrólise da fração líquida da casca de arroz, ocorreu de maneira enzimática, em que 30 FPU g^{-1} de enzima produzida por *P. echinulatum* S1M29 L^{-1} (7 FPU mL^{-1}) foi adicionada em 50 mL de hidrolisado de CA com incubação a 50 ºC, sob 150 rpm, coletando-se alíquotas em 0, 4, 8, 24, 32 e 48 h para análise de açúcares.

Exemplo 3. Fermentação das frações sólidas a etanol

[36] A cultura de *Saccharomyces cerevisiae* CAT-1 foi gentilmente adquirida através da Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo – ESALQ, USP. A mesma foi mantida e repicada em meio YPD modificado, pH 5,0 e incubadas a 37 °C por 24 h. Fermentações visando à produção de etanol foram conduzidas em distintos frascos de 100 mL, a 37 °C sob 180 rpm. Tanto para a casca de arroz quanto para o capim-elefante utilizou-se 20% (*m/v*) de substrato pré-tratado, 20 FPU g^{-1} de substrato de Celluclast® 1.5L e Novozym® 188, além de nutrientes (g L^{-1}): extrato de levedura (5,0); e $(NH_4)_2SO_4$ (1,0). O volume foi de 50 mL, e o pH ~5,0. Fizeram-se duas estratégias de fermentações etanólicas: *i)* pré-hidrólise de 10 h seguido de fermentação em agitador orbital; e *ii)* hidrólise e fermentação separadas (*separate hydrolysis and fermentation* – SHF), com uma hidrólise enzimática inicial de 48 h e posterior fermentação em agitador orbital. Amostras foram coletadas em 0, 12, 24, 48, 72 e 144 h. A concentração celular inicial foi determinada pelo método de contagem padrão em placas, resultando em ~$1,7x10^8$ UFC mL^{-1}, sendo que 2,0 g L^{-1} de inóculo foram adicionados nos meios de cultivo.

Exemplo 4. Fermentação das frações líquidas a ácido láctico

[37] *Lactobacillus buchneri* NRRL B-30929, bactéria anaeróbia facultativa, heterofermentativa, produtora de DL-ácido láctico, proveio do Departamento de Agricultura dos Estados Unidos, Serviço de Pesquisa Agrícola – USDA, ARS. A cultura liofilizada foi reativada, mantida e repicada em meio comercial MRS modificado, pH 6,0 e incubada a 37 °C por 24 h. A produção de ácido láctico ocorreu em frascos de 100 mL, a 37 °C sob 150 rpm, empregando *L. buchneri* NRRL B-30929. As frações líquidas da casca de arroz e do capim-elefante foram tratadas com carvão ativo e, após, hidrólisadas e/ou fermentadas. Para a fermentação, os seguintes nutrientes foram adicionados (g L^{-1}): peptona (10,0); extrato de

levedura (5,0); citrato de amônio (2,0); e fostato dipotássico (2,0). O volume foi de 50 mL, e o pH ajustado a 6,0 com NaOH. Amostras foram coletadas em 0, 12, 24, 36, 48, 60 e 72 h. A concentração celular inicial foi determinada pelo método de contagem padrão em placas, resultando em ~3,9x10^9 UFC mL^{-1}. O volume de inóculo foi de 10% do volume total de cultivo em todos os frascos utilizados nos experimentos.

Exemplo 5. Métodos Analíticos

[38] A concentração de açúcares foi quantificada por cromatografia líquida de alta eficiência (HPLC – *high performance liquid chromatography*) através de sistema cromatográfico Waters e2695 (Waters, Milford, EUA), acoplado a um detector de índice de refração Waters 2414 e *software* Empower. A coluna Transgenomic CARBOSep CHO-782 (Transgenomic, San Jose, EUA), operada a 75 °C, foi empregada para a separação dos analitos, usando água ultrapura como fase móvel (0,5 mL min^{-1}). Ácidos fórmico e acético foram analisados por HPLC (Waters) composto por bomba quaternária 515, He como desgaseificador, amostrador automático 2707, forno para coluna 7971, detector de índice de refração 2414 e *software* Empower. A coluna Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, EUA), operada a 65 °C, foi empregada para a separação dos componentes da amostra, usando 5 mM H_2SO_4 a um fluxo de 0,6 mL min^{-1}, com detecção na faixa de 190-400 nm. O etanol foi analisado por cromatografia gasosa 7890A GC System (Agilent Technologies, Waldbronn, Alemanha) equipado com injetor Agilent 7683B, detector de ionização de chamas e coluna Carbowax 20M (Agilent), mantida a 85 °C. A temperatura do injetor e do detector foi conservada a 175 °C. A quantificação do ácido láctico ocorreu via HPLC com detector ultra-violeta. Para tanto, foi empregado um sistema (Shimadzu, Quioto, Japão) constituído por uma bomba quaternária LC-20AD, desgaseificador DGU-$20A_3$, forno para coluna CTO-20A, detector UV/VIS SPD-20A, módulo comunicador CBM-20A e

software LC Solution. A coluna quiral Astec CLC-D (Supelco/Sigma-Aldrich, St. Louis, EUA), operada a 28 °C, foi empregada para a separação dos isômeros do ácido láctico, usando 5 mM $CuSO_4$ a um fluxo de 1,0 mL min^{-1}, com detecção a 254 nm. Em todos os casos, as amostras foram diluídas em água ultrapura e, após, passadas em filtro para seringa com membrana de nylon (0,22 µm). A eluição foi em modo isocrático, com volume de injeção de 20 µL.

Conclusões

[39] Uma visão geral das patentes depositadas nas bases de dados e das publicações científicas envolvendo a geração de energia e bioprodutos baseados em biomassas aponta que o uso das matérias-primas renováveis locais – especialmente aquelas de composição lignocelulósica – é capaz de minimizar os investimentos de capital e apoiar os aspectos sociais da sustentabilidade, ao mesmo tempo em que contribui para diminuir as emissões dos gases do efeito estufa. Assim, além de contribuir para a mitigação da questão ambiental provocado pelo acúmulo da casca de arroz no ambiente e, ao mesmo tempo, tirar proveito da alta produtividade do capim-elefante, a presente invenção buscou o desenvolvimento de uma alternativa econômica-industrial para o Estado do RS, de maneira inovadora, inserida no conceito de biorrefinaria.

[40] O uso da casca de arroz para a geração de etanol e ácido láctico enfatiza uma abordagem estritamente vinculada aos princípios da química e engenharia verde, desde a escolha da matéria-prima passando pelo pré-tratamento não catalisado, hidrólises enzimáticas das frações sólida e líquida e, finalmente, a conversão aos produtos desejados, com o máximo possível de aproveitamento dos açúcares. Ao contrário de inúmeras publicações as quais fazem uso da hidrólise ácida da fração líquida (*posthydrolysis)*, o licor resultante do pré-tratamento de tal biomassa, contendo uma mistura de oligossacarídeos, foi submetido à

hidrólise a partir de enzimas provenientes de *P. echinulatum* S1M29, não havendo na literatura processos similares de hidrólise de licores a partir de tal complexo enzimático. Após 24 h de fermentação, foram produzidos 18,93 g L^{-1} de etanol via SHF e 14,49 g L^{-1} de ácido láctico. O rendimento global de etanol foi de 5,94 g/100 g de casca de arroz, enquanto que o rendimento global de ácido láctico foi de 6,65 g/100 g de casca de arroz.

[41] A impregnação com ácido sulfúrico favoreceu a digestibilidade da celulose no caso do pré-tratamento do capim-elefante. Hidrólises enzimáticas foram desenvolvidas empregando cargas de sólidos e cargas de enzimas superiores com a finalidade de promover a liberação de glicanos provenientes da fração sólida. Ao aumentar-se o percentual de sólidos há um decréscimo em nível de rendimento global do processo; todavia, a glicose obtida após a hidrólise enzimática torna-se mais concentrada, o que deve ser levado em conta para um processo eficiente de conversão a etanol. Para o capim-elefante, os resultados mensurados na concentração de glicose e, consequentemente, na concentração de etanol, são comparáveis àqueles empregando biomassas usuais e extremamente reconhecidas por sua competência na produção de etanol de segunda geração, como é o caso do bagaço de cana-de-açúcar. Em síntese, o uso do capim-elefante conduziu a uma produção de etanol de 50,65 g L^{-1} após 72 h de fermentação precedida por uma pré-hidrólise, com um rendimento global de 17,48 g/100 g de capim-elefante. Igualmente, foram produzidos 11,13 g L^{-1} de ácido láctico após 36 h, com um rendimento global de 6,08 g/100 g de capim-elefante.

[42] A presente invenção procurou contribuir com o uso de biomassas renováveis locais, mantendo o foco no desenvolvimento de uma biorrefinaria de base bioquímica direcionada ao aproveitamento da glicose para a produção de etanol de segunda geração e, igualmente, da xilose para a produção de um insumo químico de valor agregado e com elevada demanda no mercado atual. Os dados apresentados evidenciam, de fato, que ambas as

biomassas possuem capacidade notável para serem empregadas como matérias-primas alternativas na produção de etanol e ácido láctico.

[43] Os versados na arte valorizarão os conhecimentos aqui apresentados e poderão reproduzir a invenção nas modalidades apresentadas e em outras variantes, abrangidas no escopo das reivindicações anexas.

Reivindicações

1. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas **caracterizado** por compreender as etapas de:

a. pré-tratamento da biomassa em sistema de explosão a vapor;

b. separação das porções líquidas e sólidas;

c. hidrólise da fração sólida com enzimas do complexo celulolítico e ß-glicosidases;

d. conversão das hexoses presentes na fração sólida a etanol; e

e. conversão das pentoses presentes na fração líquida a ácido láctico.

2. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com reivindicação 1, **caracterizado** pelas biomassas lignocelulósicas compreenderem casca de arroz e capim-elefante (*Pennisetum purpureum*).

3. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com reivindicação 1, **caracterizado** pela etapa de pré-tratamento do capim elefante compreender adicionalmente o emprego de catalisador ácido, sendo o pré-tratamento da biomassa lignocelulósica realizado simultaneamente com a etapa de hidrólise ácida.

4. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com reivindicação 3, **caracterizado** pelo catalisador ácido ser 2% (*p/p*) H_2SO_4.

5. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com a reivindicação 1, **caracterizado** por compreender uma etapa adicional de hidrólise da fração líquida da casca de arroz com enzimas produzidas por *P. echinulatum* S1M29 antes da etapa de fermentação.

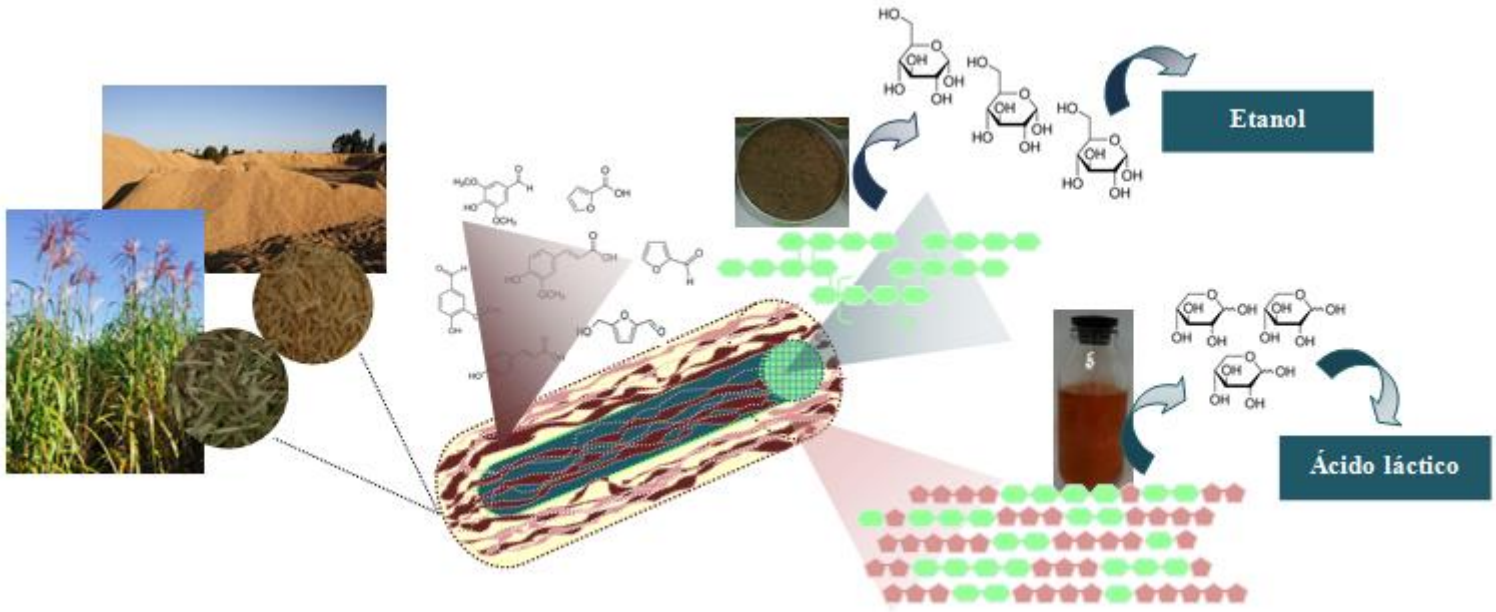
6. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com qualquer uma das reivindicações 1 a 4, **caracterizado** pela etapa de conversão das hexoses presentes na fração sólida a etanol ser através do emprego de *Saccharomyces cerevisiae* CAT-1 no processo fermentativo.

7. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com reivindicação 6, **caracterizado** por compreender a etapa de adição de nutrientes às frações sólidas com vista ao aumento da produtividade da fermentação, sendo que tais nutrientes podem ser extrato de levedura como fonte de nitrogênio, e sulfato de amônio.

8. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com qualquer uma das reivindicações 1 a 5, **caracterizado** pela etapa de conversão das pentoses presentes na fração líquida a ácido láctico por meio de *Lactobacillus buchneri* NRRL B-30929 no processo fermentativo.

9. Processo de obtenção de bioproduto e biocombustível a partir de biomassas lignocelulósicas, de acordo com reivindicação 8, **caracterizado** por compreender a etapa de adição de nutrientes às frações líquidas, sendo que tais nutrientes podem ser selecionados do grupo que compreende: peptona, extrato de levedura, citrato de amônio e fostato dipotássico.

Figura Representativa



5. DISCUSSÃO GERAL

Uma visão geral das patentes depositadas nas bases de dados e das publicações científicas envolvendo a geração de energia e bioprodutos baseados em biomassas aponta que o uso das matérias-primas renováveis locais – especialmente aquelas de composição lignocelulósica – é capaz de minimizar os investimentos de capital e apoiar os aspectos sociais da sustentabilidade, ao mesmo tempo em que traz contribuição na redução de emissões dos gases do efeito estufa (Ruíz *et al.*, 2016, Suzuki *et al.*, 2017, Zheng *et al.*, 2014). Assim, além de contribuir para a mitigação da questão ambiental provocado pelo acúmulo da CA no ambiente (Montipó *et al.*, 2016) e, ao mesmo tempo, tirar proveito da alta produtividade do CE (Gallego *et al.*, 2015), o presente trabalho buscou o desenvolvimento de uma alternativa econômico-industrial para o RS, de maneira inovadora, inserida no conceito de biorrefinaria.

A proposta inicial deste trabalho foi a produção de etanol a partir das hexoses provenientes da porção celulósica e, igualmente, a produção de AL a partir das pentoses provenientes da porção hemicelulósica de ambas as biomassas, com vista ao total aproveitamento dos carboidratos liberados através do pré-tratamento, seguido de hidrólise enzimática. Em meio a isso, o subcapítulo 4.1 retrata as estratégias de um delineamento experimental desenvolvido para o pré-tratamento por explosão a vapor, explorando todo o espaço amostral e visando analisar o sinergismo entre as variáveis independentes do processo e suas influências na recuperação de celulose proveniente da fração sólida e de hemicelulose proveniente da fração líquida.

Ao término do pré-tratamento, valores ótimos dos índices dos açúcares de interesse, G_{WIS} e X_{LIQ}, foram alcançados para cada uma das biomassas lignocelulósicas estudadas e, indiferentemente da matéria-prima, houve uma similaridade entre as variáveis independentes das condições otimizadas (Figura 9). Considerando que tanto a CA quanto o CE *in natura*

contêm teores similares de celulose e hemicelulose, ao confrontarem-se ambas as biomassas no quesito pela busca de valores ótimos de G_{WIS} e X_{LIQ} pertencentes ao mesmo tratamento, CE explodido apresentou maior susceptibilidade à recuperação da celulose contida no material pré-tratado, enquanto que CA explodida demonstrou maior tendência à recuperação da hemicelulose contida no pré-hidrolisado. Tais resultados também são refletidos na hidrólise enzimática da fração sólida (5% *m/v*) e na hidrólise ácida da fração líquida. Provavelmente, esta situação está relacionada com a natureza da matéria-prima considerada.

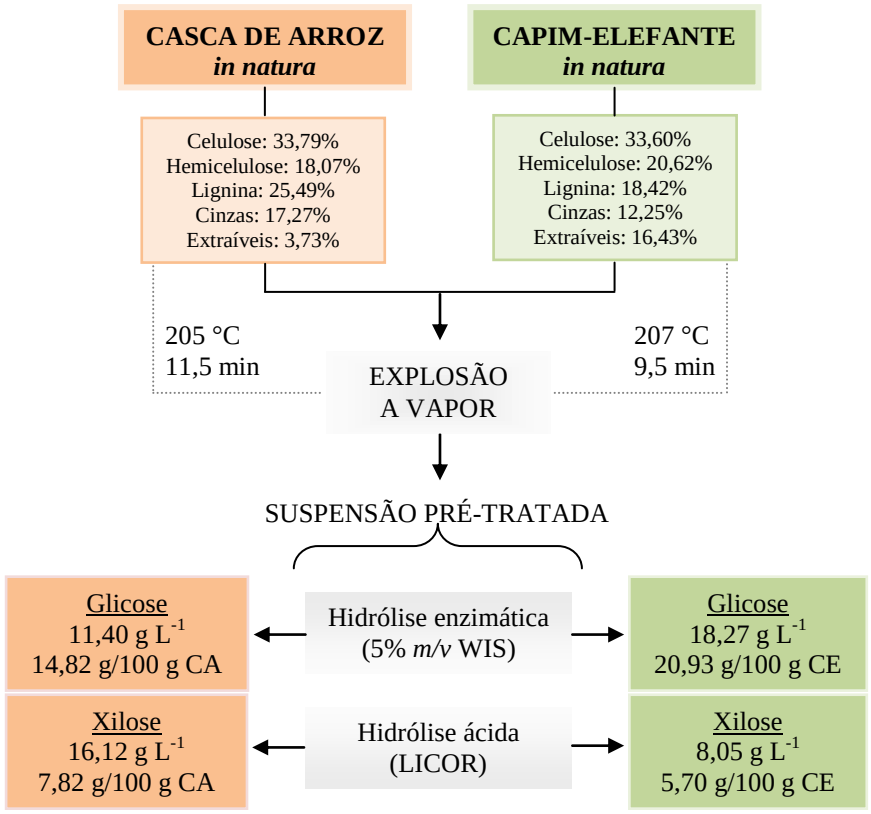


Figura 9. Rendimento global dos açúcares após pré-tratamento otimizado das biomassas lignocelulósicas por explosão a vapor, seguido de hidrólises enzimática e ácida.

O elevado conteúdo de lignina presente na CA não-tratada e, do mesmo modo, o elevado conteúdo de extraíveis presente no CE não-tratado podem estar conectados com tais resultados. A quantidade superior destes componentes não-estruturais encontrada nesta herbácea, aliada à sua capacidade tamponante (Li *et al.*, 2016), tornou o resultado do

delineamento experimental pouco promissor em relação à X_{LIQ}. Cabe ressaltar que a alta incidência de cinzas, característica da própria CA (Lim *et al.*, 2012), também está vinculada ao efeito tamponante da fração líquida e, mesmo assim, tal fato não foi evidenciado quando este resíduo agroindustrial foi considerado.

Hidrólises enzimáticas posteriores foram desenvolvidas empregando-se cargas de sólidos e cargas de enzimas superiores com a finalidade de promover a liberação de G_{WIS} (subcapítulo 4.2 e subcapítulo 4.3). Em ambos os trabalhos, foi constatado que uma concentração de substrato de 20% (*m/v*), aliada a uma dosagem enzimática de 20 FPU foram eficazes no cumprimento de tal proposta. Entretanto, ao aumentar-se o percentual de sólidos há um decréscimo em nível de rendimento global do processo (Kristensen *et al.*, 2009). Todavia, a G_{WIS} obtida após a hidrólise enzimática torna-se mais concentrada, o que deve ser levado em conta para um processo eficiente de conversão a etanol (Cara *et al.*, 2006). Ademais, três estratégias distintas de fermentação alcoólica com o uso de *S. cerevisiae* CAT-1 foram empregadas: pré-hidrólise, SHF e SSF, sendo que a terceira acarretou no aumento da produtividade sem interferir na liberação de glicose. Neste estudo, a celulose presente na fração sólida da CA e do CE foi hidrolisada por complexos celulolíticos comerciais, largamente empregados, produzidos por *Trichoderma reesei* e *Aspergillus niger*.

Para o caso da CA, o pré-tratamento selecionado e considerado otimizado (205 °C, por 11,5 min) foi utilizado na produção de etanol e AL do subcapítulo 4.2 (Figura 10). Hidrólise enzimática contendo 20% (*m/v*) de WIS acarretou em um aumento de 300% da G_{WIS} quando comparada com o mesmo procedimento contendo apenas 5% (*m/v*) de WIS. Diferentemente de inúmeras publicações, as quais fazem uso da hidrólise ácida (Nakasu *et al.*, 2016), o licor resultante do pré-tratamento, incluindo uma mistura de pentoses e hexoses, foi submetido à hidrólise com enzimas previamente produzidas por *P. echinulatum* S1M29. Este mutante se destaca por expressar distintas enzimas, dentre as quais celobioidrolases, endoglicanases e

xilanases (Schneider *et al.*, 2016), indicando o seu elevado potencial na hidrólise de biomassas lignocelulósicas compostas por uma mistura de carboidratos, como é o caso do licor advindo da CA pré-tratada por explosão a vapor. Adicionalmente, sabendo que hidrólises enzimáticas envolvendo o uso de licores são escassas na literatura, optou-se em conduzir este trabalho de modo a demonstrar o potencial de tais hidrólises frente às hidrólises ácidas e, do mesmo modo, promovendo a eficiência de tal complexo (hemi)celulolítico na hidrólise enzimática.

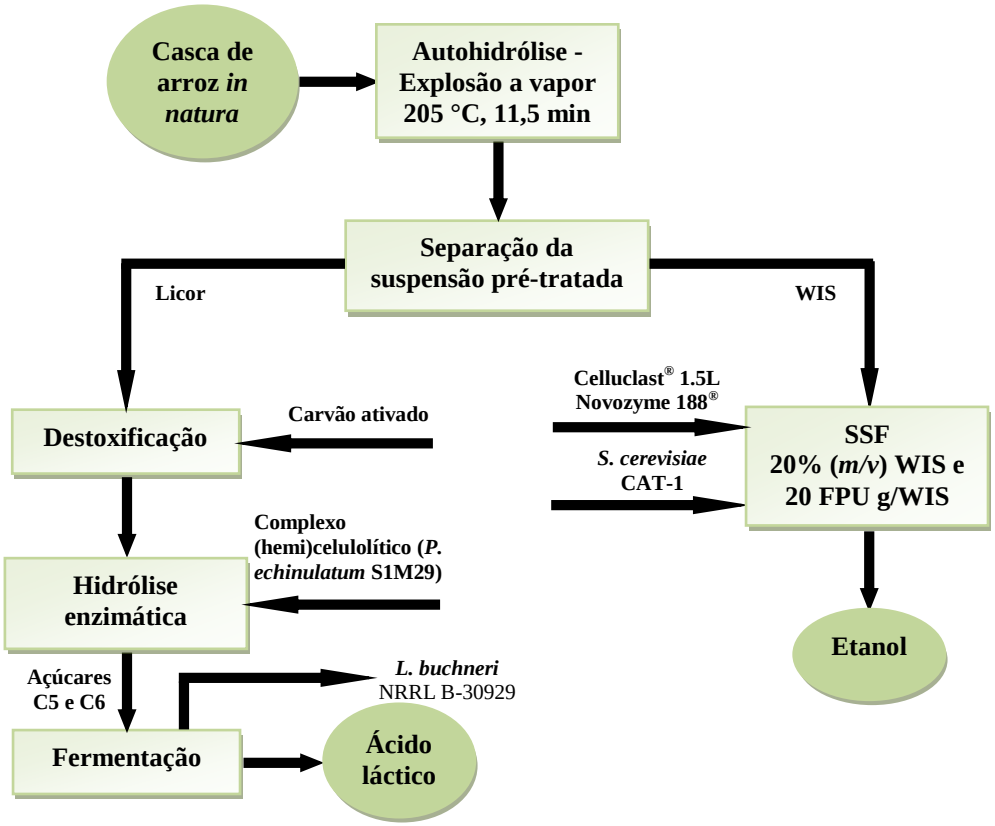


Figura 10. Etanol e ácido láctico a partir do pré-tratamento selecionado para a casca de arroz.

A escolha por este liofilizado enzimático foi fundamental para a sequência do trabalho, influenciando na fermentação láctica, e *L. buchneri* NRRL B-30929 demonstrou ser uma LAB viável no que diz respeito à degradação concomitante de pentoses e hexoses. De fato, experimentos prévios indicaram que, mesmo sendo verificada a superioridade da

hidrólise ácida (~46.0%) frente à hidrólise enzimática no que se refere à liberação de xilose, a produção de AL se mostrou ineficiente no primeiro caso. Deste modo, o subcapítulo 4.2 enfatiza uma abordagem estritamente vinculada aos princípios da química e engenharia verde, desde as escolhas da matéria-prima passando pelo pré-tratamento não catalisado – o qual atua como uma autohidrólise, ambientalmente amigável (Egüés *et al.*, 2012) –, hidrólises enzimáticas das frações sólida e líquida e, finalmente, a conversão nos produtos desejados, com o máximo possível de aproveitamento dos açúcares.

Em síntese, CA permitiu produção de etanol de 19,17 g L^{-1} e de AL de 12,69 g L^{-1} – sendo 4,28 g L^{-1} do isômero L-(+)- e 8,41 g L^{-1} do isômero D-(–)- –, representando 34,84% e 70,13% em relação ao percentual máximo teórico, respectivamente. Decerto, o baixo rendimento referente à geração de etanol foi especialmente influenciado pelo modo de condução da SSF, posto às dificuldades de transferência de massa e calor em sistemas contendo elevada carga de sólidos (Hodge *et al.*, 2009). Entretanto, as condições do planejamento experimental para este resíduo recalcitrante também merecem ser avaliadas diante dos dados aqui expostos.

Com o intuito de incrementar a geração de G_{WIS} e de X_{LIQ} concomitantemente em experimentos abrangendo o uso do CE, o subcapítulo 4.3 evidenciou os benefícios da incorporação de um catalisador no processo de explosão a vapor: elevação dos rendimentos da G_{WIS} na etapa da hidrólise enzimática a uma carga inicial de WIS de 5% (*m/v*), juntamente com o aumento da concentração de X_{LIQ}, sendo este último superior a 100% quando confrontado com o pré-tratamento dito otimizado para o CE (207 °C, por 9,5 min).

A opção por condições mais amenas, 190 °C por 5 min, associada à impregnação com ácido sulfúrico (Figura 11) favoreceram a digestibilidade da celulose. Pode-se inferir que, ao longo do pré-tratamento, houve uma redução dos componentes não-estruturais, desencadeando na remoção das xilanas (Li *et al.*, 2016). Ainda, no que se refere à utilização

do WIS, os resultados mensurados na concentração de glicose e, consequentemente, na concentração de etanol, são comparáveis àqueles empregando biomassas usuais e extremamente reconhecidas por sua competência na produção de 2G etanol, como é o caso do bagaço de cana-de-açúcar (Ramos *et al.*, 2015). Em resumo, o uso do CE permitiu uma produção de etanol de 42,25 g L^{-1} e uma produção de AL de 13,35 g L^{-1} – sendo 4,30 g L^{-1} do isômero L-(+)- e 9,01 g L^{-1} do isômero D-(–)- –, representando 74,57% e 68,21% em relação à percentagem máxima teórica, respectivamente.

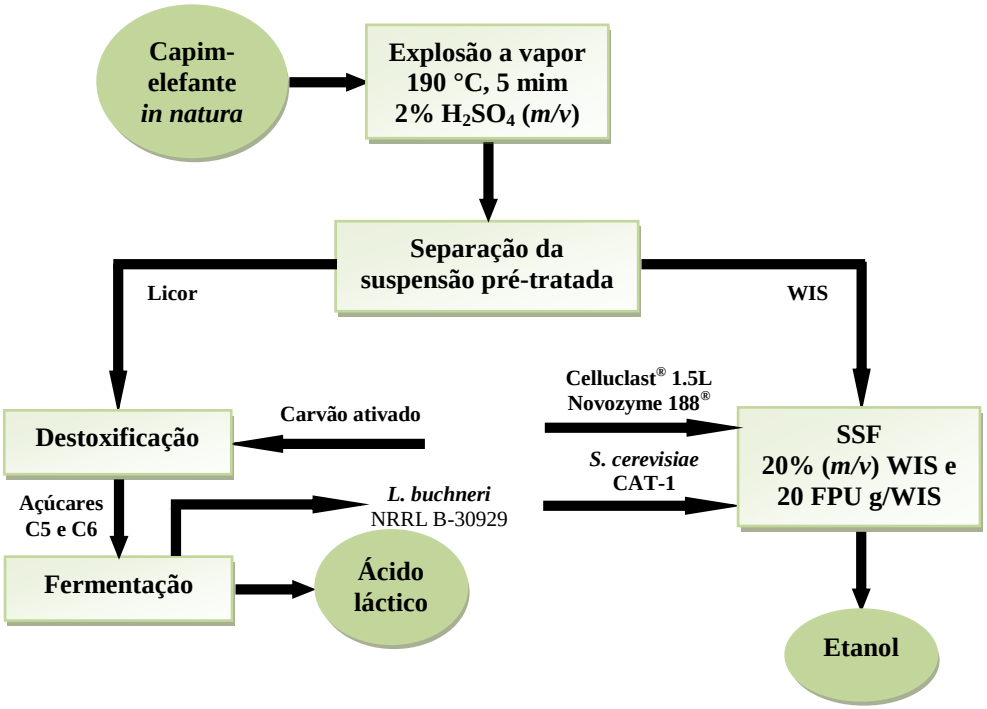


Figura 11. Etanol e ácido láctico a partir do pré-tratamento selecionado para o capim-elefante.

Não obstante, há duas questões importantes referentes à fermentação empregando o licor que merecem ser abordadas: *i)* a interferência dos subprodutos presentes no meio fermentativo, e *ii)* os requisitos nutricionais das BAL. O entrosamento entre a biomassa lignocelulósica e o seu posterior pré-tratamento é uma questão altamente complexa e ainda pouco compreensível. Além dos sacarídeos fermentáveis, uma variedade de outros produtos de degradação, potencialmente inibidores, é produzida nessa etapa, sendo que muitos destes

compostos podem ser cruciais aos processos bioquímicos subsequentes. A presença de tais subprodutos no meio fermentativo afetou desfavoravelmente as BAL, por exemplo, fazendo-se necessário uma etapa adicional de destoxificação do mesmo. Igualmente, critérios envolvendo a suplementação do substrato formulado a partir do pré-hidrolisado foram essenciais, uma vez que os quesitos nutricionais das BAL são extremamente complexos. Neste trabalho, testes iniciais de fermentações lácticas com *L. buchneri* NRRL B-30929 e *L. rhamnosus* NRRL B-445 indicaram a necessidade da suplementação dos licores com nutrientes específicos e, inclusive, demonstraram que tal procedimento é até mesmo mais importante do que a quantidade de monossacarídeos presentes no meio.

Neste trabalho, a produção de cada produto de interesse foi desenvolvida em sistemas independentes, evitando-se problemas relacionados com inibição e, ainda, a degradação seletiva dos monossacarídeos. A utilização total da suspensão pré-tratada visando à produção simultânea de etanol e AL (cofermentação) poderia ser considerada caso o micro-organismo produtor de AL estivesse direcionado exclusivamente ao consumo de pentoses, destacando-se, sobretudo, que tal processo reduziria os tempos mortos ou não-produtivos. Adicionalmente, técnicas de fermentação etanólica por *S. cerevisiae* CAT-1, seguidas do consumo das pentoses remanescentes por meio da fermentação láctica por *L. buchneri* NRRL B-30929 seria outra possibilidade, porém, não resolveria a questão da produtividade.

Dada a natureza de cada uma das biomassas lignocelulósicas estudadas, bem como as distintas condições de pré-tratamento envolvidas, não cabe aqui uma comparação entre a CA e o CE no que concerne à produção dos compostos de interesse. No entanto, vale ressaltar que o emprego da CA, resíduo agroindustrial sem valor comercial e sem aplicação direta na indústria, convergiu a um rendimento global de etanol de 6,02 g/100 g CA e a um rendimento de AL de 5,83 g/100 g CA. Já o uso do CE, herbácea caracterizada pela sua elevada

produtividade e substituta ideal ao bagaço de cana-de-açúcar no RS, levou a um rendimento global de etanol de 14,58 g/100g CE e a um rendimento de AL de 7,06 g/100 g CE.

Com a finalidade de se estabelecer um balanço aproximado da produção de etanol e de AL a partir destas biomassas lignocelulósicas, calcularam-se os rendimentos brutos de tais processos, sem grande preocupação com a aderência à realidade industrial – variável e dependente de inúmeros fatores. Os resultados estão inseridos na Tabela 1 e na Tabela 2, a seguir.

Tabela 1. Avaliação da produção de etanol e de ácido láctico a partir da casca de arroz.

	Input	*Output*	
Saldo Total	**Casca de arroz**	**Etanol**	**Ácido láctico**
	1 t	60,2 kg	58,3 kg

Tabela 2. Avaliação da produção de etanol e de ácido láctico a partir do capim-elefante.

	Input	*Output*	
Saldo Total	**Capim-elefante**	**Etanol**	**Ácido láctico**
	1 t	145,8 kg	72,9 kg

Verifica-se que a produção de etanol e de AL proveniente da CA e do CE demonstra viabilidade tecnológica, ainda que os cálculos tenham sido elaborados com os dados provindos dos ensaios aqui executados. No caso específico da CA, após o beneficiamento do grão, cerca de 1,7 milhões t da casca foram produzidas no Estado do RS em meados da safra 2016/17 (CONAB, 2017; Lim *et al.*, 2012; Wei *et al.*, 2009), sendo que a utilização deste subproduto agroindustrial a etanol e a AL poderá contribuir para a sua valorização. Do mesmo modo, a produção de AL tem a capacidade de tornar o CE uma matéria-prima de apreciação, servindo não apenas à produção de 2G etanol, o qual é comumente abordado (Menegol *et al.*, 2016; Scholl *et al.*, 2015b). Adicionalmente, uma série de outros produtos e subprodutos advindos das etapas de pré-tratamento e de fermentação também foram concomitantemente

produzidos, tal como o ácido acético (AA), integrando, por sua vez, a biorrefinaria do etanol, do ácido láctico e do ácido acético (Figura 12).

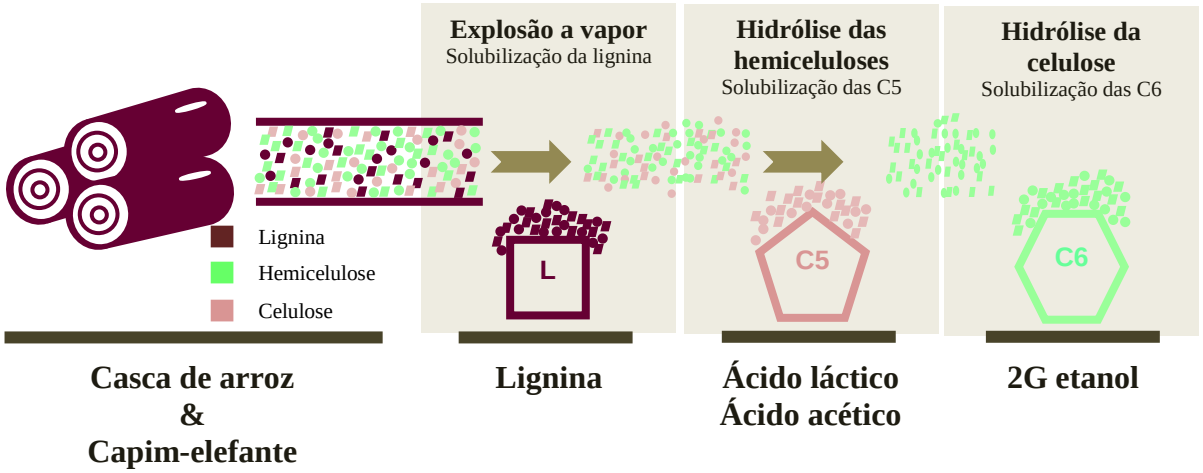


Figura 12. Biorrefinaria do etanol e dos ácidos láctico e acético a partir de biomassas lignocelulósicas. Fonte: adaptado de ARBIOM, 2017.

Assim, visto que as áreas de bioprocessos e biotecnologia oferecem distintas possibilidades para a produção de biocombustíveis e insumos químicos através das chamadas *green technologies*, os resultados apresentados evidenciam, de fato, que a CA e o CE possuem capacidade notável para serem empregadas como matérias-primas alternativas na produção de etanol e AL.

6. CONCLUSÕES

Este estudo procurou contribuir com o uso de biomassas renováveis locais, mantendo-se o foco no desenvolvimento de uma biorrefinaria de base bioquímica direcionada ao aproveitamento da G_{WIS} para a produção de 2G etanol e, igualmente, da X_{LIQ} para a produção de um insumo químico de valor agregado e com elevada demanda no mercado atual.

Os dados obtidos neste estudo permitem concluir que:

- tanto CA quanto CE apresentaram potencial para serem convertidas em açúcares fermentescíveis empregando-se pré-tratamento por explosão a vapor;

- em virtude das características particulares das matérias-primas, a otimização multivariada dos pré-tratamentos via explosão a vapor não-catalisada indicou a tendência da CA à recuperação das hemiceluloses contidas no licor, e do CE à recuperação da celulose contida no WIS;

- através do delineamento experimental foi possível verificar que condições moderadas de temperatura por períodos alongados contribuíram para a obtenção de melhores valores de G_{WIS} e X_{LIQ}, em um único experimento; para o caso da CA, condições de 205 °C, por 11,5 min; enquanto que para o caso do CE, condições de 207 °C, por 9,5 min;

- um *screening* prévio de cinco cepas pertencentes às BAL, consideradas aptas em metabolizar xilose, resultou na seleção de *L. buchneri* NRRL B-30929;

- hidrólises enzimáticas do licor referente a CA foram conduzidas por meio de enzimas produzidas por *P. echinulatum* S1M29, produzindo-se concentrações maiores de pentoses e hexoses, sem a necessidade de pré-concentrar o hidrolisado;

- pré-tratamentos adicionais do CE foram efetuados, sendo que o experimento a 190 °C, por 5 min e catalisado com 2% (*m/m*) de ácido sulfúrico foi o mais eficiente,

possibilitando a utilização imediata do licor uma vez que o mesmo funcionou como uma hidrólise ácida;

- uma etapa prévia de destoxificação foi necessária para permitir a atuação das BAL a partir da conversão total dos açúcares advindos do licor; adicionalmente, houve a necessidade de suplementação do meio com nutrientes específicos;

- hidrólises enzimáticas envolvendo o WIS a partir de concentrações superiores a 10% (*m/v*) promoveram a liberação da glicose, sendo que concentrações de 20% (*m/v*) em conjunto com dosagens enzimáticas de 20 FPU foram selecionadas para a condução dos ensaios subsequentes de fermentação alcoólica;

- dentre as distintas estratégias de fermentação analisadas, a SSF revelou ser a prática mais viável para a produção de etanol;

- a CA e o CE comprovaram ser biomassas lignocelulósicas potenciais na conversão a etanol e AL através da metabolização das hexoses (G_{WIS}) por *S. cerevisiae* CAT-1 e através da metabolização de pentoses (X_{LIQ}) por *L. buchneri* NRRL B-30929, respectivamente.

7. PERSPECTIVAS

Para a continuidade deste trabalho, abrem-se perspectivas:

- investigação de alternativas de pré-tratamento catalisado envolvendo a CA;

- aumento de escala das fermentações, possibilitando o controle adequado do pH, da temperatura e da agitação dos meios reacionais em biorreator, possibilitando também o escalonamento do processo em níveis de planta piloto e industrial;

- condução da SSF em reatores específicos para tratamentos empregando elevada carga de sólidos;

- utilização integral da suspensão pré-tratada visando à cofermentação a etanol e AL a partir de micro-organismos apropriados, reduzindo o tempo de processo; ou ainda utilizar a suspensão pré-tratada com vista à degradação inicial de hexoses por *S. cerevisiae* CAT-1, empregando o caldo remanescente como meio de cultivo à degradação de pentoses por BAL;

- recuperação e purificação tanto do etanol quanto do AL produzidos, e;

- utilização dos demais produtos e subprodutos provenientes do pré-tratamento, tal como a lignina remanescente no WIS.

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