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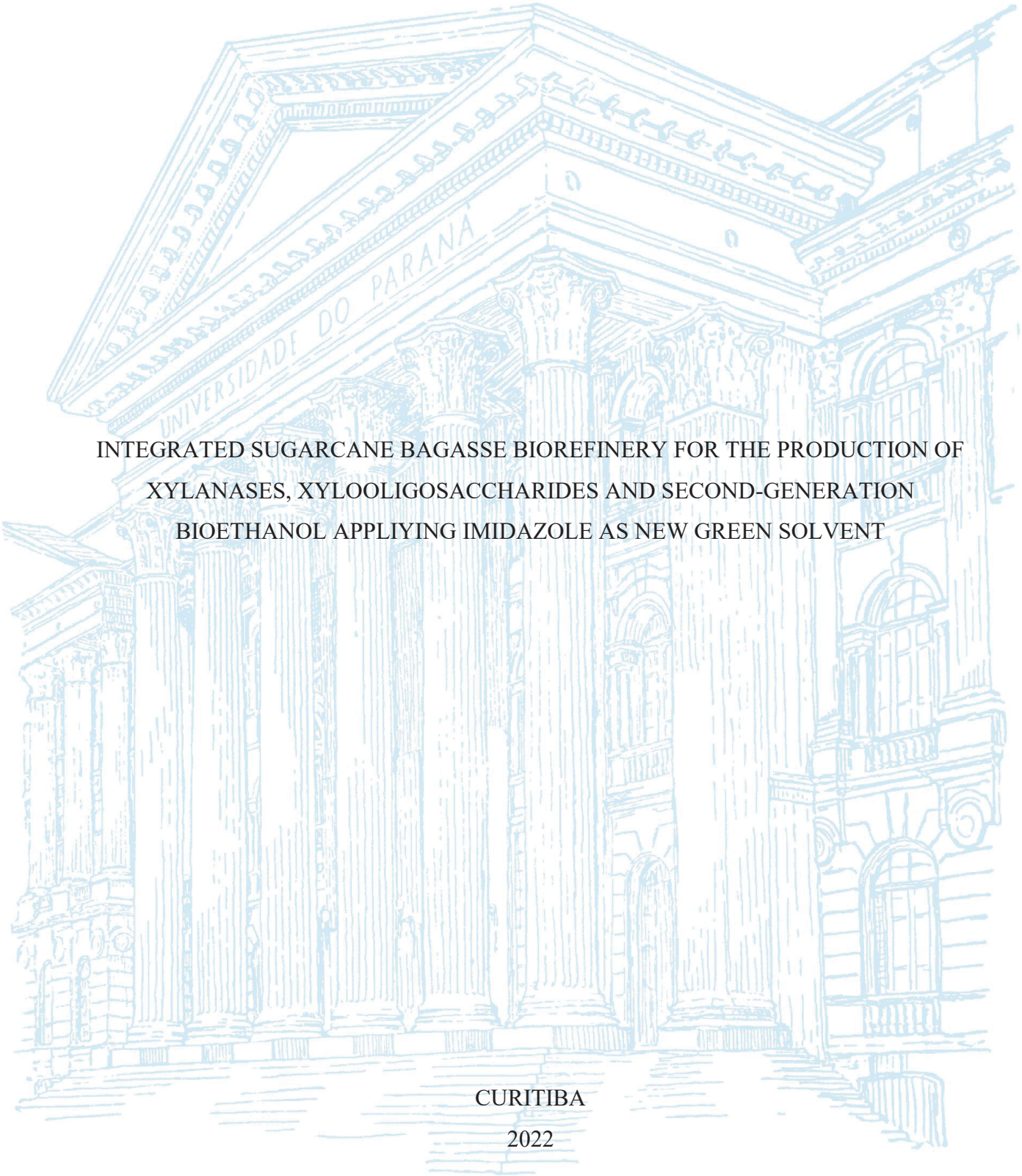
KIM KLEY VALLADARES DIESTRA



INTEGRATED SUGARCANE BAGASSE BIOREFINERY FOR THE PRODUCTION OF XYLANASES, XYLOOLIGOSACCHARIDES AND SECOND-GENERATION BIOETHANOL APPLIYING IMIDAZOLE AS NEW GREEN SOLVENT

CURITIBA

2022

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Tese apresentada como requisito parcial para a obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientadora: Profa. Dra. Luciana Vandenberghe

Co-orientador: Prof. Dr. Carlos Ricardo Soccol

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **KIM KLEY VALLADARES DIESTRA** intitulada: **INTEGRATED SUGARCANE BAGASSE BIOREFINERY FOR THE PRODUCTION OF XYLANASES, XYLOOLIGOSACCHARIDES AND SECOND-GENERATION BIOETHANOL APPLIYING IMIDAZOLE AS NEW GREEN SOLVENT**, sob orientação da Profa. Dra. LUCIANA PORTO DE SOUZA VANDENBERGHE, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
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DEDICATÓRIA

En memoria de mi amada tía Maria Milagritos Diestra Portella y mi querido abuelo Remigio Diestra Flores que siempre quisieron verme como Doctor, para ustedes hasta el cielo.

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RESUMO

Nos últimos anos o mundo vem buscando alternativas limpas e renováveis para fornecer novos materiais e energia, reduzindo a dependência dos combustíveis fósseis derivados do petróleo. No Brasil, as políticas governamentais criaram o cenário ideal para consolidar as usinas de cana-de-açúcar com produção de biocombustíveis, açúcar e bioeletricidade. No entanto, o crescimento populacional gera a necessidade de aumentar a produtividade, evitando o aumento dos impactos ambientais, o que torna necessária a busca por novas estratégias de produção. Por isso, as biorrefinarias lignocelulósicas integradas são uma alternativa para produzir diferentes biomoléculas de valor agregado gerando processos econômicos competitivos, sustentáveis e ecologicamente corretos. Neste trabalho, uma nova estratégia é desenvolvida para a implantação de biorrefinarias lignocelulósicas integradas, aplicando o uso do imidazol como novo solvente verde no pré-tratamento do bagaço de cana-de-açúcar. Essa estratégia busca-se aumentar a produtividade total do bioetanol, evitando-se assim o aumento das áreas de cultivo com a valorização dos resíduos agroindustriais. Além disso, aplicando o conceito de biorrefinaria, busca-se o aproveitamento máximo do bagaço da cana-de-açúcar para a produção de outras biomoléculas de valor agregado, como enzimas e prebióticos. Os resultados mostraram um bom desempenho na produção de xilanases utilizando bagaço de cana (74%) e farelo de soja (26%), no tratados, como substrato, obtendo-se uma produção de 53,1 $U.mL^{-1}$ de xilanases com produtividade de 0,44 $U.mL^{-1}.h^{-1}$. Por outro lado, observou-se um ótimo desempenho do imidazol no pré-tratamento do bagaço de cana-de-açúcar realizado a 160°C por 1h. O pré-tratamento permitiu a recuperação de uma fração rica em celulose com 75% de deslignificação, o que levou a uma conversão de 100% de glucano em glicose após hidrólise enzimática. A bioconversão de hidrolisados por fermentação com *Saccharomyces cerevisiae* proporcionou um alto rendimento produtivo (83,7%), o que representa uma produção de 218 L de etanol por tonelada de bagaço de cana e uma produção integrada de bioetanol (primeira e segunda geração) poderia aumentar a produção total superior a 37%, com aproximadamente 110L por tonelada de cana. Além disso, foi possível a recuperação de uma fração rica em hemiceluloses com alto teor de xilana (28,9%) e 91,2% de deslignificação. A fração rica em hemicelulose foi hidrolisada com o complexo xilanolítico produzido permitindo a produção de 6,06 $g.L^{-1}$ de xilooligossacarídeos onde a xilotriose representou mais de 70%. Os resultados obtidos mostraram o grande potencial do imidazol como nova estratégia de pré-tratamento do bagaço de cana para gerar produtos de valor agregado, levando a um processo eficiente e econômico na valorização de resíduos, redução de impactos ambientais para a implantação de biorrefinarias lignocelulósicas integradas.

Palavras chave: Bagaço de cana, Imidazol, Xilanase, Xilooligossacarídeos, Biorrefinaria

ABSTRACT

In the last years the world has been searching for clean and renewable alternatives to provide new materials and energy, reducing the dependence on petroleum fossil fuels. In Brazil the government policies have created the optimal scenario to consolidate the sugarcane mills with biofuels, sugar and bioelectricity production. However, population growth generates the need for increase the productivity, avoiding the increase in environmental impacts, which makes it necessary to search for new production strategies. For this reason, integrated lignocellulosic biorefineries are an alternative to produce different value-added biomolecules generating economical competitive, sustainable, and environmentally friendly processes. In this work, a new strategy is developed for the implementation of integrated lignocellulosic biorefineries, applying the use of imidazole as a new green solvent in the pre-treatment of sugarcane bagasse. This strategy seeks to increase the total productivity of bioethanol, avoiding the increase in cultivation areas with the valorisation of agro-industrial residues. In addition, applying biorefinery approaches, the maximum use of sugarcane bagasse is sought for the production of other added-value biomolecules such as enzymes and prebiotics. The results showed a good performance in the xylanases production using sugarcane bagasse (74%) and soybean meal (26%) as substrate, obtained a production of 53.1 $U.mL^{-1}$ of xylanases with a productivity of 0.44 $U.mL^{1}.h^{-1}$. On the other hand, a great performance of the imidazole in sugarcane bagasse after pre-treatment at 160 °C for 1h was observed. The pre-treatment allowed the recovery of a cellulose-rich fraction with 75% of delignification, which led to a 100% conversion of glucan to glucose after enzymatic hydrolysis. The bioconversion of hydrolysates by fermentation with *Saccharomyces cerevisiae* gave a high production yield (83.7%), which represents a production of 218 L of ethanol per ton of sugarcane bagasse and an integrated production of bioethanol (first- and second- generation) could increase the total production of more than 37%, with approximately 110 L per ton of sugarcane. Besides, was possible the recovery of a hemicellulose-rich fraction with high content of xylan (28.9%) and 91.2% of delignification. The hemicellulose-rich fraction was hydrolyzed whit the xylanolytic complex allowing the production of 6.06 $g.L^{-1}$ of xylooligosaccharides where xylotriose represented more than 70%. The results obtained showed the great potential of imidazole as new strategy for sugarcane bagasse pre-treatment to generate value-added products, leading to an efficient and economic process with waste valorization, reduction of environmental impacts for the implementation of integrated lignocellulosic biorefineries.

Keywords: Sugarcane bagasse, Imidazole, Xylanases, Xylooligosaccharides, Biorefinery

SUMÁRIO

1. INTRODUÇÃO

O aumento da população humana foi projetado em um total de 9,7 bilhões de pessoas para 2050, o que gera um crescimento potencial na demanda de bens e serviços. Se prevê que a demanda de consumo de energia aumentará em 28% para 2040, sendo assim necessários o aumento de produção, produtividade e implementação de novas tecnologias para poder satisfazer toda estas novas demanda Energy (2019). Por outro lado, novas alternativas límpidas e renováveis vêm sendo presquisadas para a produção de novos materiais e energia, reduzindo a dependência do uso de combustíveis fósseis devido a seu alto impacto negativo frente ao meio ambiente. Nesse sentido, surge a alternativa do uso de biomassa lignocelulósica como matéria prima para a produção de biomoléculas de alto valor agregado como também de biocombustíveis Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021).

A biomassa lignocelulósica é bastante abundante e está distribuída em nível mundial, sendo um material de baixo custo e que não compete com a produção de alimentos. A principal fonte de biomassa lignocelulósica em grande escala são os resíduos agroindustriais, a valorização destes resíduos agroindústrias surgem com um grande interesse devido a seu potencial de reciclagem, reuso e aplicação através de processos biotecnológicos industriais nos setores da alimentação e da bioenergia. As características apresentadas por estes resíduos demonstram suas potencialidades para uso como matéria prima, tal como sua rica composição em polissacarídeos e proteínas. Além, da sua capacidade de renovação em curtos períodos de tempo, o que favorece sua aplicação em grande escala, diminuindo substancialmente o aumento das áreas de cultivo e os grandes impactos negativos para o ambiente Valladares-Diestra; Porto de Souza Vandenberghe; Ricardo Soccol (2021).

Um dos principais resíduos agroindustriais lignocelulósicos é o bagaço de cana, subproduto sólido da indústria sucroalccoleira que processa a cana de açúcar como matéria

prima. O Brasil é o principal produtor de cana de açúcar com uma produção total de 757,1 milhões de toneladas e em aproximadamente de 10 milhões de hectares em 2020. O bagaço de cana representa entre 25-30% da cana de açúcar e o Brasil reporta uma produção de quase 200 milhões de toneladas ao ano Vandenberghe et al. (2022). As características químicas de composição do bagaço de cana são muito diversas sendo principalmente afetadas pelo tipo da variedade de cana, condições de cultivo e região geográfica. Porém, a composição principal do bagaço de cana é de polissacarídeos, constituídos por mais do 50% de celulose e hemicelulose, além de apresentar compostos como a lignina, polímeros extraíveis e metais. Devido a esta combinação de diferentes polímeros (principalmente por polímeros amorfos como hemicelulose e lignina) a biomassa lignocelulósica presenta uma característica recalcitrante, que serve como defesa frente aos ataques microbianos. Nesse sentido para a liberação de açúcares fermentáveis a partir do bagaço de cana é necessária a aplicação de pré-tratamentos físico-químicos.

As estratégias e métodos aplicados para a liberação de açúcares fermentáveis por meio de pré-tratamentos a biomassa lignocelulósica são diversas, sendo utilizados catalisadores químicos com condições severas de tratamento, tais como a aplicação de métodos biológicos. Devido a seu rápido resultado os métodos físico-químicos são os mais aplicados. No caso do bagaço de cana têm sido utilizados métodos como explosão à vapor com ácido diluído, tratamentos alcalinos, e tratamentos sequenciais de ácido diluído e alcalino Bartos et al. (2020); Hemansi et al. (2020) e Ramos et al. (2015). No entanto, o uso convencional de produtos químicos alcalinos ou ácidos em pré-tratamentos de biomassa lignocelulósica pode gerar subprodutos prejudiciais para saúde humana e ao meio ambiente.

Por estas razões novas estratégias e catalisadores estão sendo desenvolvidas para evitar a geração de compostos tóxicos assim como catalisadores que sejam recicláveis e reutilizáveis. Os “solventes verdes” tais como os líquidos iônicos e solventes eutéticos são

uma alternativa ao uso de solventes tradicionais nos processos de pré-tratamento de biomassa lignocelulósica. Porém, seu alto custo inviabiliza sua aplicação em escala industrial. O imidazol surge como uma opção de catalisador de mais baixo custo para pré-tratamento, o qual é um químico de baixa toxicidade, baixa pressão de vapor, alto ponto de ebulição e facilidade de manuseio como solvente no pré-tratamento de biomassa em comparação com outros solventes. Além disso, tem-se relatado que o imidazol tem a capacidade de desestruturar a biomassa lignocelulósica e permitindo a recuperação de suas três frações principais (celulose, hemicelulose e lignina). Ainda, pode ser reciclável e reutilizável nos processos de pré-tratamento Morais et al. (2016) e Toscan et al. (2019). Estas características tornam o imidazol ser considerado um potencial catalisador para o uso no pré-tratamento de bagaço de cana com o objetivo de obter açucares fermentáveis na produção de bioetanol de segunda geração.

A aplicação do imidazol no pré-tratamento de bagaço de cana permite a recuperação da celulose e hemicelulose, possibilitando o uso da celulose para a produção de bioetanol de segunda geração enquanto a hemicelulose pode ser utilizada na produção de xilooligossacarídeos (XOs). Os XOs são oligômeros formados de subunidades de xilose, e devido a seu alto valor nutricional como suplemento alimentar, são considerados prebióticos de alto valor, sendo estimuladores de probióticos e apresentam propriedades antioxidantes e antialérgicas. O mercado que envolve este prebiótico é bastante amplo com um volume de transações financeiras de $93 milhões em 2017 e com um mercado em crescimento para 2023 de $130 milhões Lan et al. (2021). Porém, a produção deste prebiótico ainda é incipiente, devido a seus custos de processo, principalmente na hidrólise enzimática uma das etapas de maior custo em um processo por causa dos altos preços das enzimas comerciais.

As xilanases são as enzimas, mais utilizadas no processo de produção de XOs, sendo as famílias GH10 e GH11 as enzimas de maior uso para este objetivo. A produção dessas

enzimas é bastante diversa, sendo que é possível a utilização de resíduos ricos em hemicelulose como matéria-prima na sua produção Morgan et al. (2017). O bagaço de cana devido a seu alto conteúdo de hemicelulose é um candidato ideal para a produção de xilanases a baixo custo. Porém, é necessária a suplementação do meio de cultivo com fontes de nitrogênio e sais minerais para o crescimento adequado e produção de enzimas por parte do microrganismo. O farelo de soja, outro subproduto da agroindústria brasileira é uma fonte rica em proteínas e de baixo custo, podendo ser utilizada como suplemento para a produção de biomoléculas, tais como as enzimas, em processos fermentativos Maciel et al. (2008).

Nesse sentido o desenvolvimento de biorefinarias lignoceliulósicas é de grande importância nos países agroindustriais como o Brasil. Ainda, as biorrefinarias liginocelulósicas integradas têm como objetivo a utilização de 100% dos resíduos agroindustriais, com a produção de uma maior diversidade de bioprodutos, gerando uma alta flexibilidade diante um mercado bastante volátil e de alto índice de crescimento. Além dos benefícios econômicos, as biorrefinarias lignocelulósicas integradas apresentam processos ecologicamente sustentáveis, o que permite a redução dos gases de efeito estufa chegando-se a mitigar a liberação de CO_2, um dos principais objetivos da 21ª Conferência de Paris (COP21) organizada pela Convenção das Nações Unidas sobre Mudança do Clima (UNFCC) Vandenberghe et al. (2022). Finalmente, as políticas governamentais do Brasil buscam também a diminuição das emissões de gases de efeito estufa por meio de créditos de carbono, o que cria um ambiente propício que impulsiona a implementação de novas biorrefinarias integradase com o mínimo ou nulo impacto ao meio ambiente.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo principal deste trabalho é o desenvolvimento de estratégias para o pré-tratamento de bagaço de cana utilizando o imidazol como um novo solvente verde para a implementação de uma biorrefinaria lignocelulósica integrada para a produção de xilanases, xilooligossacarídeos e bioetanol de segunda geração.

2.2 Objetivos Específicos

a) Implementar um bioprocesso de produção de xilanases de baixo custo com o uso de resíduos lignocelulósicos.
b) Avaliar e determinar as melhores condições de pré-tratamento do bagaço de cana usando imidazol como solvente verde.
c) Produzir bioetanol de segunda geração a partir de bagaço de cana pretratado com imidazol.
d) Implementar um novo bioprocesso de produção integrada de bioetanol de primeira e segunda geração com o uso de imidazol como solvente do pré-tratamento.
e) Obter frações da hemicelulose ricas em em xilana a partir de bagaço de cana pretratado com imidazol.
f) Produzir de xilooligossacarídeos usando o complexo enzimático xilanolítico aplicado à xilana extraída do bagaço de cana com imidazol.

3. Beyond sugar and ethanol: the future of sugarcane biorefineries in brazil

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ABSTRACT

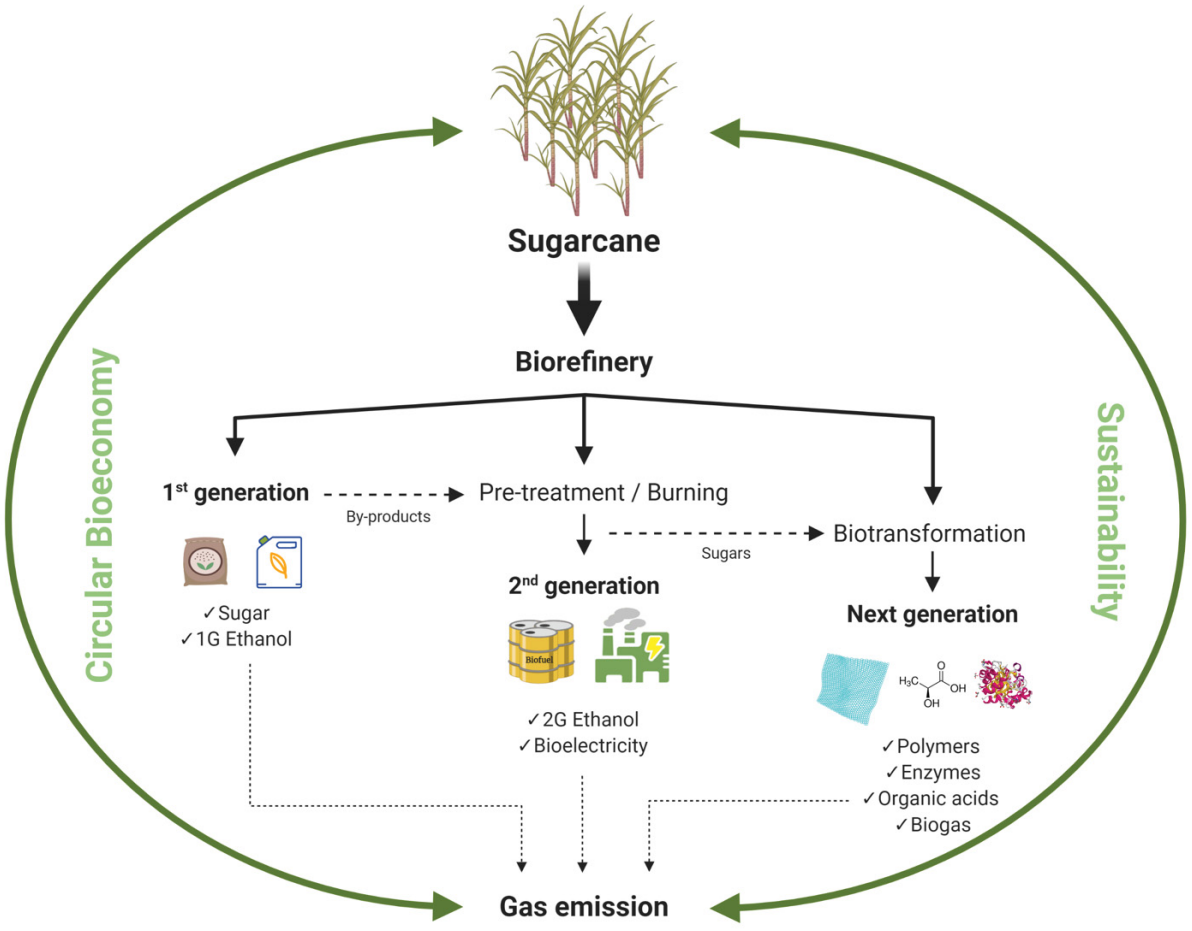
The world has been searching for clean and renewable alternatives to provide new materials and energy, reducing the dependence on petroleum fossil fuels. Sugarcane has become an important raw material as a solution for this demand since it is grown in more than 100 countries and can potentially reduce greenhouse gas (GHG) emissions. In this context, the production of biomaterials and biofuels and their effective use are promising ways to reach these goals. Brazil government policies have created the optimal scenario to consolidate the as the first sugarcane 1G biorefinery with biofuels, sugar and bioelectricity production, trying to reach sustainable and low-cost solutions. After the consolidation of 1G technology, the 2G and 3G technologies are being developed, bringing new possibilities for value-added biochemicals production, in integrated systems with minimized emissions with a non-waste vison and circular bioeconomy. This review presents the current status and future perspectives of sugarcane as a potential biofactory. The present and future of Brazilian sugarcane biorefinery are discussed in terms of the exploitation of sugarcane, research and innovation with the integrated production of sugar, biofuels, bioelectricity, biopolymers, organic acids, enzymes and other biomolecules, as well as the use of process-generated liquid and solid by-products and/or wastes.

Keywords: sugarcane; sugarcane bagasse; biofactory; biofuels; bioenergy; biochemicals

Highlights

- Sugarcane Brazilian biofactories' future and perspectives.
- Biorefinery concept as a sustainable tool for sugarcane circular bioeconomy.
- Brazilian governmental incentives help sugarcane biofuels competition with fossil fuels.
- Re-use of liquid and solid sugarcane wastes for high value bioproducts production.
- Raising number of innovative and development of new sugarcane chain processes.

Graphical abstract



List of abbreviations

1G	First Generation
2G	Second Generation
3G	Third generation
ABE	Acetone-butanol-ethanol
CBios	Decarbonisation Credits
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
COP21	21st Conference of Parties
DES	Deep Eutectic Solvents
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
Emim OAc	1-ethyl-3-methylimidazolium acetate
FAO	United Nations Food and Agriculture Organization
GHG	Greenhouse Gas
IBE	Isopropanol-butanol-ethanol
ILs	Ionic Liquids
IPC	International Patent Classification
IU	International Unity
MTCC	Microbial Type Culture Collection
N	Normal volume
NRRL	Northern Regional Research Lab
PAISS	Plan to Support Innovation in the Sugar and Energy Sectors
PHA	Polyhydroxyalkonoate
RNA	Ribonucleic acid
SB	Sugarcane bagasse
SO_x	Sulphur Oxides
SOFC	Solid Oxide Fuel Cells
SS	Sugarcane Straw
SSF	Solid-State Fermentation
TEA	Triethylammonium Hydrogen Sulphate
UNFCC	United Nations Framework Convention on Climate Change
USD	United State Dollar
XOs	Xylooligosaccharides

1. Introduction

Climate change due to greenhouse gas (GHG) emissions has significant impacts on humans and biodiversity Gómez-Merino et al. (2014). However, the world energy matrix still largely depends on fossil fuels Soccol et al. (2010). A possible solution has been presented at the 21st Conference of Parties (COP21) organised by the United Nations Framework Convention on Climate Change (UNFCC), the so-called "Paris Agreement", as a measure to limit the effects of global warming Klein, Bruno Colling et al. (2019). For this purpose, each country established some targets for CO_2 mitigation and the search for lower emissions. In this context, it is important to point out that fossil fuels' production has its own subsidies and remove them could help to mitigate climate change by discouraging inefficient energy consumption and levelling the playing field for renewable energy Jewell et al. (2018). In line with the worlds' commitments, the Brazilian national policy on biofuels, RenovaBio, established a target for gasoline substitution with bioethanol that will demand an increase of almost 100% in production in the next ten years UNICA (2021a). Although the effects of the Covid-19 pandemic, which has impacted the biofuel market, led the federal government to propose the revision of the targets, the goal for 2030 is of almost 91 million decarbonisation credits (CBios), representing 90% of the original target Bossle (2020). In this sense, the use of sugarcane to produce sugar, alcohol and electricity as major products is a probable scenario in Brazil, at least for the next decades. With these incentives, new possibilities for the implementation of integrated sugarcane biorefineries in a Brazilian perspective will come to light Klein, Bruno Colling et al. (2019).

2. The sugarcane history, production and market evolution

Sugarcane belongs to the grass family (Poaceae) and has been created about a century ago from the combination of polyploid *Saccharum* species. It contains 57% of water, along with straw, bagasse and sugars, and is the main source of sucrose worldwide. It is a semi-perennial plant, originated from tropical regions of Asia, especially India, and worldwide known for its high productivity and as a high-quality raw material for sugar production, mainly sucrose, glucose and fructose Santos; Rabelo; et al. (2020). As a C4 carbohydrate metabolism plant with a perennial life cycle, sugarcane is one of the most productive cultivated plants. Nevertheless, it has one of the most complex genomes, which has stimulated several research groups in the genetics development of the species to support crop improvement programs, turning it in an ideal biofactory as it efficiently converts sunlight and water into biomolecules such as sugar, fibres and waxes, making it the most productive field crop among cultivated plants Gómez-Merino et al. (2014) and Nunes et al. (2020). Sugarcane is considered not only an agricultural crop, but it is also efficiently employed in ethanol production from sugars, making it an important energy crop.

Today, sugarcane, together with cotton, soy, corn, wheat, and rice, dominate culture crops. According to the United Nations Food and Agriculture Organization (FAO), the annual sugarcane production of 1,89 billion tons in an area of 27,000,000 hectares, is distributed in more than 100 countries. In 2019, the global sugarcane production (Figure 1) was led by Brazil (768 Mt), India (348 Mt), China (123 Mt) and Thailand (87 Mt) NationMaster (2019). The 20 largest world producers reached 1,75 billion tons per year in an area of approximately 24,250,000 hectares, with a productivity of 72.91 tons/ha. Some South American, Asian and African countries historically dominate the sugarcane market, of which Brazil has been considered the pioneer of ethanol production since 1970 Klein, Bruno Colling et al. (2019) and Soccol et al. (2010). It is important to mention that sugarcane production areas are located

mainly in the South of the Amazon Rainforest, not competing with native forest areas. South-Central Brazil is the heart of the Brazilian sugarcane production and industry, and the total area of sugarcane cultivation corresponds to less than 1% of the national territory UNICA (2020).

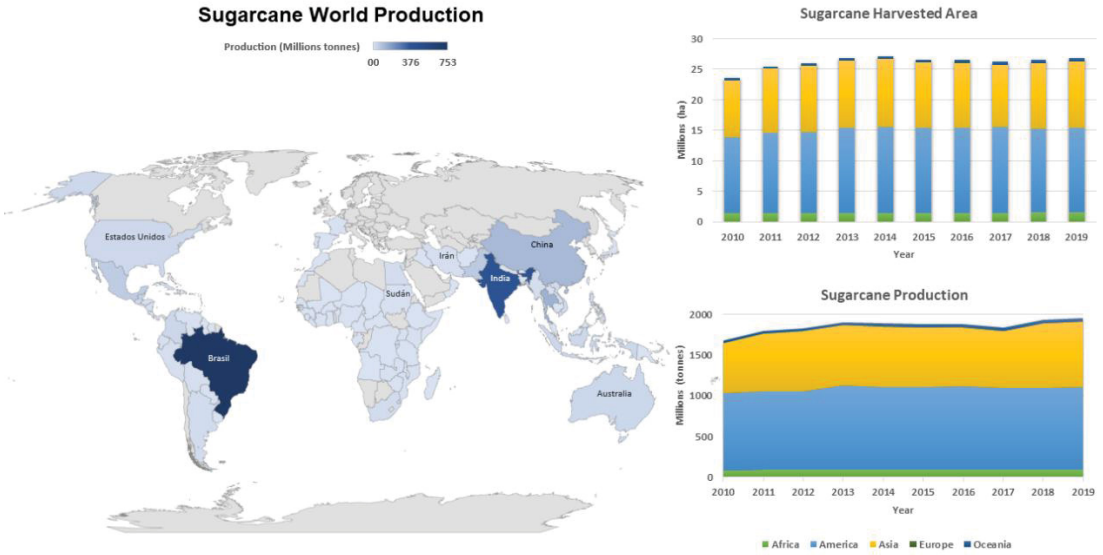


Figure 1. The main sugarcane-producing countries and sugarcane production from 2010 to 2019 U.S Department of Energy (2020)

The exploitation of sugarcane comprises one of the most successful case of biorefinery, due to three major marketable products, specifically sugar, ethanol and bioelectricity, as well and its great diversity of derived by-products. The cogeneration of by-products, such as bagasse burning, edible sugar and 1G ethanol production from sugarcane juice (Fig. 2), are energy self-sufficiency, allowing the producing unities to commercialize the surplus of the bioelectricity production. The sector represents 6.73% of the power granted by the Brazilian Electric Energy Agency (ANEEL), being the fourth most important energy source in the country at 2019 UNICA (2019). Other bioproducts, such as CO_2, molasses, vinasse, biogas and others could amplify the product range, environmental and economic sustainability of traditional sugarcane mills by including value-added products from sugarcane liquid and solid wastes. In the last decades, the sugarcane 2G ethanol has gained increasing importance

Oliveira et al. (2018). In 2017, 67 biorefineries were identified in the world, producing 2G ethanol from sugarcane bagasse (SB) Nguyen, Que et al. (2017). Among them, five are located in South America.

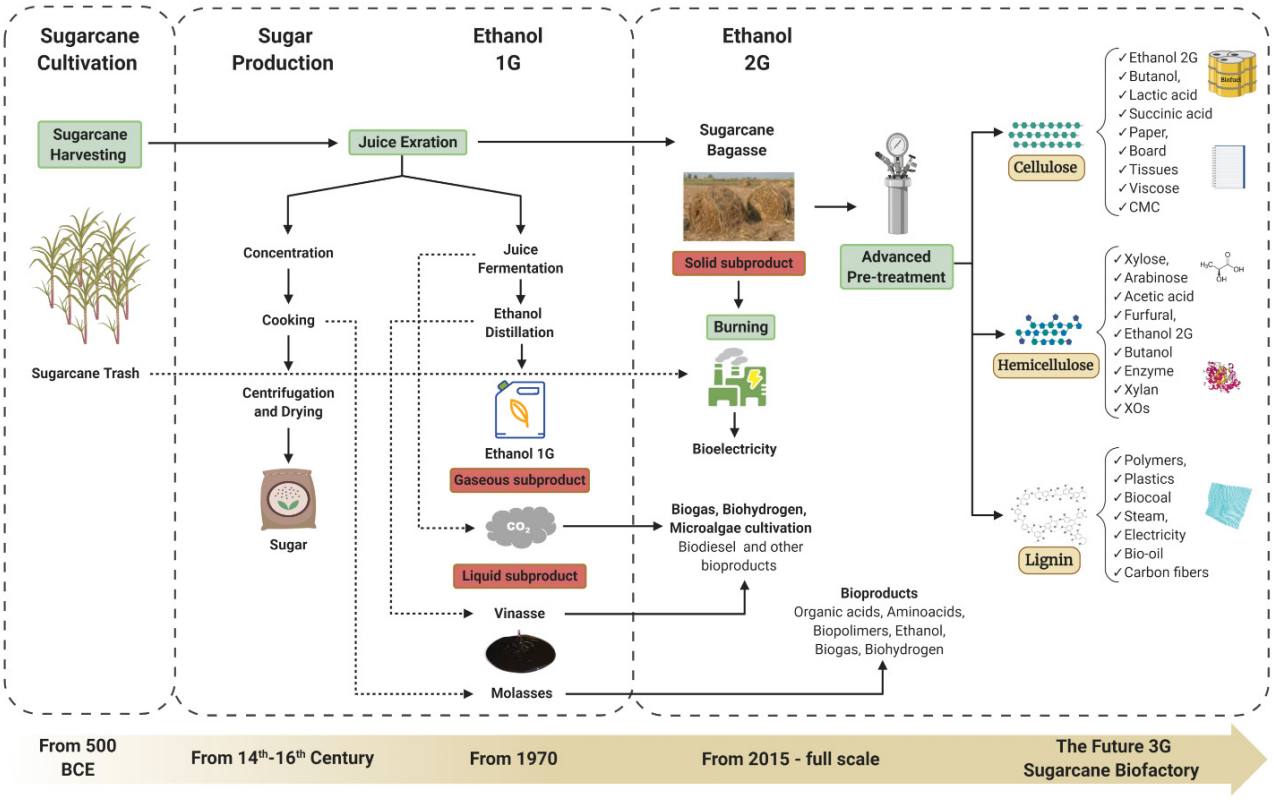


Figure 2. Timeline of sugarcane biofactory evolution.

But, why to invest in biofuels from sugarcane, when the electric vehicles' fleet is continuously growing worldwide? Ternel et al. (2021) and Pan et al. (2021). This discussion is occurring in countries where electricity costs are too high and investments on hybrid *plug in* systems implantation is too expensive. In addition, bioethanol demand increases continuously due to the policy adopted by Brazil and many other countries, to increase the mix of anhydrous ethanol in gasoline, together with flex-fuel vehicles market. The substitution of combustion engines by electric motors in Brazil still has barriers to be overcome, from the availability of charging stations in a country of continental dimensions to the security of electric energy supply. Although the Brazilian electric energy matrix is more than 80% renewable and thus could be a sustainable energy option for the car fleet, the country still

faces problems of logistics and distribution, and the energy supply is in great part dependent on hydric resources that, in turn, are affected by the rainfall regime. Experts from the Center of Cane Technology (CTC, Brazil) reported that Brazil has the sixth world fleet with more than 40 million vehicles (the majority is flex fuel) and is the fourth biofuels' consumer Carpio; Simone de Souza (2017) and Cruz (2021). Following this high demand, the sugarcane biorefineries will work hard to sustain their economic and environmental viability with the inclusion of 2G and 3G technologies. Sugarcane producing countries, such as India, China and others, may also walk in the same direction Cruz (2021).

3. The present and future of sugarcane biorefinery

A biorefinery is an integrated system of hybrid technologies from different areas such as agriculture, chemistry, microbiology, and biotechnology, enabling the efficient use and separation of plant biomass to produce different biomolecules. In addition, the re-use and/or recycling of solid, gaseous and liquid effluents from each processing stream, reduces environmental impacts, process costs and GHG emissions, as well as diversifies products' portfolio, and improves energy self-efficiency Jin et al. (2018); Parsons et al. (2020); and Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). Traditional Brazilian sugarcane mills produce ethanol and sugar from sugarcane juice in a synergic system, generating different by-products/wastes, such as SB, straw, leaves and grasses, molasses, vinasse, and CO_2, each with different levels of integrated process exploration Dias; Junqueira; Jesus; et al. (2012); Ferreira et al. (2018) and De Souza Dias et al. (2015).

3.1 The 1G sugarcane biorefinery – the past and the actual scenario

The synergy between sugar and bioethanol production was responsible for the consolidation of the sugar-alcohol industry. The co-generation of bioelectricity and heat, which are obtained from SB and/or straw burning, allows the energetic auto sufficiency and subsistence of the 1G sugarcane mills, providing its demand integrally with benefits of the

surplus Cervi et al. (2020) and De Freitas et al. (2020). Another point is that 10-15 L of sugarcane vinasse are generated per litre of produced ethanol Da Silva et al. (2021). In this way, vinasse could also be used for energy generation, through anaerobic digestion, and fully supply the energy demand of a sugarcane plant processing, making the allocation of lignocellulosic biomass for medium to high-value biomolecules economically viable Rodriguez et al. (2019). In the case of Brazil, the sale of electricity by sugarcane mills was prohibited. However, starting in 1999, a reform was carried out in the electricity sector, which authorized sugarcane mills to sell electricity, allowing them to access the transmission and distribution network belonging to the Brazilian electricity system. This action caused a boost and growth in the production of bioelectricity from the 1G biorefineries. These factors have sparked a discussion about the most suitable proportion of by-products used in the generation of bioenergy or in the production of other products. This dilemma goes hand in hand with fluctuations in electricity and ethanol prices Carpio; Simone de Souza (2017). There are currently 422 sugar and ethanol sugarcane mills in operation in Brazil, of which 54% are located in the Southeast Region, where the state of Sao Paulo has the largest number of plants (173) NOVACANA (2022).

3.2 The 2G and 3G sugarcane biorefinery – the present and future scenario

Several research groups have intensively studied the re-use of lignocellulosic material, with the efficient exploitation of its different fractions (cellulose, hemicellulose and lignin) through the employment of traditional and advanced pre-treatment strategies Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). The 2G biofuels began to be produced at full commercial scale in 2015 Hassan et al. (2019a) and Nguyen, Q et al. (2017). As shown in Figure 3, the by-products of sugar mills can be separated into two large groups, namely solid and liquid by-products. Solid by-products consist of sugarcane straw, SB and filter cake from sugarcane juice, which are rich in residual sugars and lignocellulosic

material Abdeshahian et al. (2020); Candido; Gonçalves (2019) and Shi et al. (2021). They generally require the application of physicochemical pretreatments to facilitate the sugars release and generate new products Nicodème et al. (2018). Liquid by-products include vinasse and molasses; the first is the liquid by-product obtained after alcohol distillation, and the second is the residual liquid by-product of sugar refining and composed of high concentrations of residual sugar, which can be reused for ethanol production Wu et al. (2020).

The development of new technologies for 1G and 2G ethanol production, in an integrated sugarcane-processing chain with re-use/recycling of by-products/wastes, has great economic and environmental advantages. However, some conscious analyses must be done before implementation. Cellulose and hemicellulose fractions from SB, can be transformed into different products with commercial applications such as the production of biofuels, enzymes, organic acids, xylooligosaccharides (XOS), xylitol, and films Aruna et al. (2021). Molasses and vinasse, from 1G bioethanol and sugar production, which are mainly composed of residual saccharose, can be re-used for the 3G biochemicals production from microalgae, such as biofuels, biopolymers, biopigments and other biochemicals Klein, B. C. et al. (2019). These technologies are being intensively studied for the establishment of large-scale production, with concomitant reduction of effluents' impact, reaching the so called zero-waste condition.

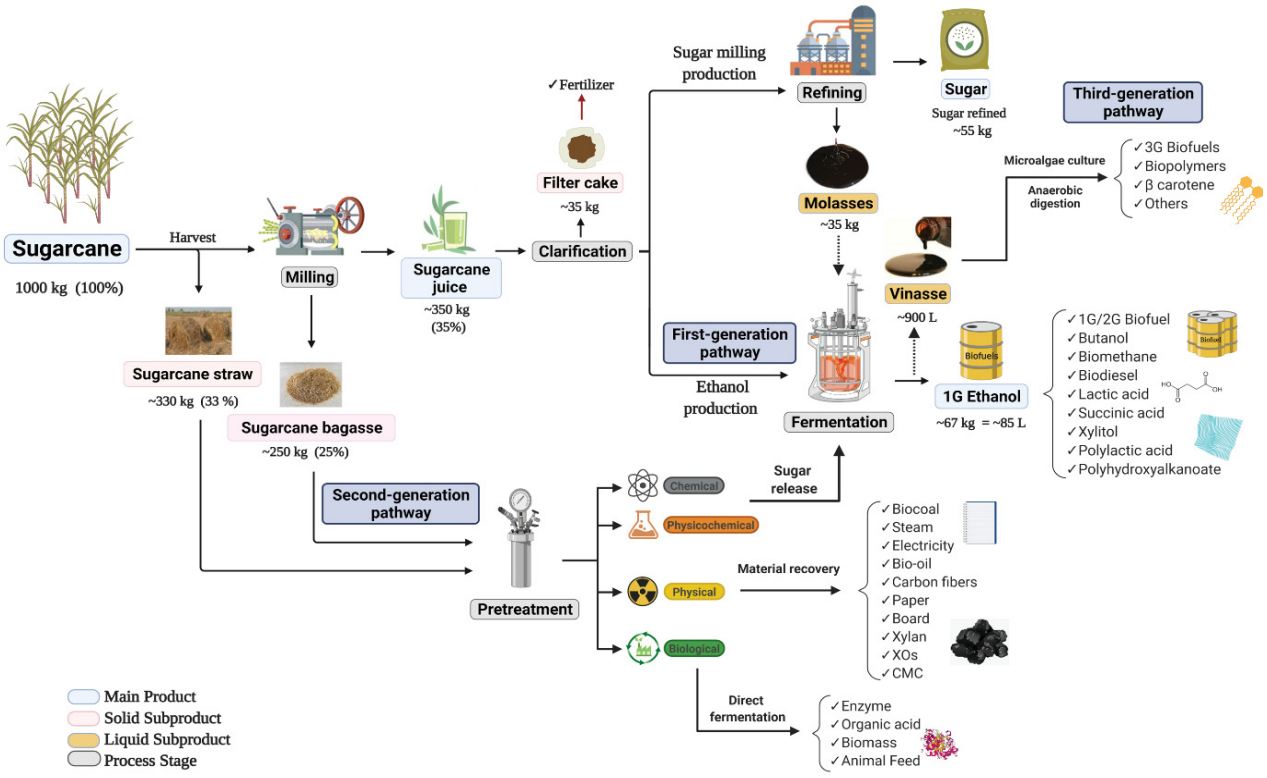


Figure 3. From 1G to the 2G and 3G sugarcane biorefinery.

The global productivity and viability of 1G and 2G sugarcane biorefineries is being continuously discussed due to advantages in sharing the 1G technology equipment with the 2G ethanol production, which leads to the increase of bioethanol production without the need of supplementary sugarcane cultivation areas Dias; Junqueira; Cavalett; et al. (2012). In the Brazilian context, 2G biorefineries are undergoing an implementation process with potential growth within the industry, mainly due to the high benefits and profits that can be obtained from the production of biomolecules or chemicals with high added value. One of the aspects that most drives 2G biorefineries in Brazil is the high supply of electricity due to its great energy diversity, based mainly on renewable sources such as hydroelectric, solar and wind energy, which represent more than 70% of its energy matrix, causing a constant fluctuation in electricity prices. For these reasons, the sugarcane mills are looking for new alternatives to the production of bioelectricity, such as the production of 2G ethanol or biomolecules to produce bioplastics. In this sense, the company Raízen, the largest producer of bioethanol and

derivatives from sugarcane, has implemented a 2G production plant, the first in Brazil and a pioneer worldwide. The use of SB provided an increase of up to 50% of bioethanol production without increasing the planted area, presenting an index of 30 and 97% less greenhouse gas emissions compared to 1G bioethanol and gasoline, respectively Raízen (2022). In this context, complex techno-economic studies of the possible biorefinery improvements, new biomolecules' production, market analysis, raw material costs, as well as the costs of each involved operation time for capital payback and future earnings must be evaluated Mendes et al. (2017) and Özüdoğru et al. (2019). The sustainability of a sugarcane biorefinery may also be analysed through life cycle evaluation show the projection of the net balances (positive, negative or neutral) of environmental impacts Formann et al. (2020) and Pereira et al. (2019).

According to these approaches, different studies have been carried out to evaluate sugarcane biorefineries. De Oliveira et al. (2020) proposed a model to increase ethanol production through the integrated production of 1G and 2G ethanol. The authors showed that by using 100% of SB, the total ethanol production was increased by 29%. The use of by-products obtained from 2G technology (lignin burning and C5 fermentation) could increase energy cogeneration by 10% compared to what was expected in the total burning of bagasse. Another scenario showed that using only 50% of SB, an increase of 14% was reached, while the gradual burning of sugarcane straw (SS) up to 60% would increase the energy cogeneration by 64% compared to total bagasse burning. Rodriguez et al. (2019) studied the integrated production of 1G and 2G ethanol with biogas, as an energy source, which was produced through the anaerobic fermentation of vinasse. The inclusion of the anaerobic fermentation process generated enough energy, which allowed 100% of SB use for 2G ethanol production, doubling its productivity.

A techno-economic analysis of a 1G and 2G integrated sugarcane biorefinery has been reported Vasconcelos et al. (2020). The authors claimed that better economic returns not only

depend on the achievement of high product yields, but also on some industrial factors and process conditions including the duration of the enzymatic hydrolysis step (C6 hydrolysis) and the use of C5 generated by acid pretreatment. There is still great potential for exploitation, mainly in several stages in which the productive efficiency has not been fully taken advantage, and where different generated by-products could be employed in new biomolecules production Candido; Gonçalves (2019); Cherubini (2010) and Garlapati et al. (2020).

Mandegari et al. (2018) evaluated eight different scenarios of a sugarcane lignocellulosic biorefinery, analysing the economic, environmental and energy performance. The multi-co-production of ethanol, lactic acid or methanol from SB was employed, where lactic acid showed a higher probability of economic success, with the higher net present value (U$ 476–1,278). In this scenario, ethanol production would only be viable with a government subsidy of 39% on its price, while methanol production would not be economically viable. Another favourable scenario was the co-production of ethanol and lactic acid. In all scenarios, investment and viability risks improved with the use of coal as an energy source. Despite the use of a fossil fuel, the results of environmental analyses showed positive benefits for the sustainable development of this type of biorefinery.

However, not all techno-economic analyses provided positive results. For example, Fonseca et al. Fonseca et al. (2020), who studied the economic viability of 1G ethanol production expansion for the implementation of a 2G ethanol and bioelectricity production, used bagasse and straw for 2G ethanol production and vinasse for biogas generation. In all evaluated scenarios, the most suitable approach was the complete hydrolysis of bagasse for 2G ethanol production with the burning of cane straw, lignin and biogas, supporting distillation and bioelectricity production. Total investment costs were estimated at U$ 535.2 million, with the highest costs for cogeneration, U$ 289 million; U$ 143.5 million were assessed for 2G technology implementation and U$ 21 million for anaerobic digestion.

However, the techno-economic analysis showed the infeasibility of the project because of the risk assessment (0.62), which, even for the most optimistic scenarios, was below 0, considering only investments with risk assessment greater than 1 as viable.

The use of microalgae biomass as an alternative feedstock to petrochemical and plant-based resources is been studied due to its high biomass productivity, and the eco-friendly ability to absorb GHGs in its autotrophic complex Elrayies (2018). After microalgae cultivation in sugarcane vinasse, Santana et al. (2017) showed that proteins and carbohydrates comprised the major fractions of algal biomass highlighting the potential of using residues derived from ethanol plants for the production of energy and bioproducts.

Some prospects for large-scale integrated sugarcane-microalgae biorefineries were carried out in terms of their techno-economic and environmental feasibility. Process integration was based on fermentation-derived CO_2, CO_2 in biogas, and vinasse as carbon sources for microalgae growth. However, an increase in both fixed and operational expenses was observed, but anhydrous ethanol production costs remained in the range of US$ 0.36–0.42/L, below the market price of US$ 0.51/L. Depending on the scenario, a reasonably-sized integrated biorefinery would be able to mitigate around 500 thousand t CO_2eq/year. The integrated sugarcane-microalgae biorefineries showed an economic feasibility at carbon prices lower than US$ 10/t CO_2eq under the RenovaBio support Klein, B. C. et al. (2019).

4. Circular bioeconomy of sugarcane biorefineries

In recent years, bioeconomy has emerged as a sustainable development strategy, defined as the production of renewable resources, mostly from biological sources, applied in the production of products with high added value Hassan et al. (2019b). Within this process, the management of by-products that are employed as new raw materials to produce other products, avoiding waste generation, increases and drives business volumes, generating financial gains. On the other hand, bioeconomy also plays an important role in the global

transition from fossil carbon sources to new renewable and sustainable sources under the perspective of a circular economy Formann et al. (2020).

That is the case of the Brazilian sugarcane biorefinery, which is still pushed by the high market demand for bioethanol, sugar and bioelectricity. Sugarcane juice´s composition and production volume make it the preferable substrate for 1G bioethanol production. It can be employed directly in bioethanol production, with no need of pretreatment without extra operation costs. The use of solid and liquid wastes, which require additional pretreatment, is then turned to medium to high-value 2G and 3G biomolecules' production. Therefore, an improvement of sugarcane biorefineries depends on the implementation of circular economy approaches through the cascading of waste and residues to promote bioeconomy concepts Hysa et al. (2020). The RenovaBio is the public policies that has most stimulated the biofuel market in Brazil, with decarbonisation credits (CBIO) being the main factor in promoting and growing the regional bioeconomy. CBIO acts as a "green currency" where each ton of CO_2 that is not emitted from the sale of biofuels generates a carbon credit, allowing biofuel producers to obtain dividends from the sale of these assets. In this way, the CBIOs promote three important effects within the Brazilian bioeconomy, which are currency exchange, carbon sequestration and the reduction of biofuel prices. In this sense, it is estimated that by the year 2030, companies will be able to issue around 590 million CBIOs, which would mean an annual profit of US$ 14.4 billion for the sector. However, bioeconomy not only focuses on a scientific or engineering idea, but also becomes an important basis for the socio-economic development of countries and regions. In addition, it also stimulates local enterprises, generating microgrids within the production process and, thus, connecting the population with the industry, aiming for a socio-economically and environmentally sustainable development.

Figure 4 shows a condensed version of the application of bioeconomy within a sugarcane biorefinery, presenting a closed production cycle, which is obtained because of the different approaches applied throughout the process.

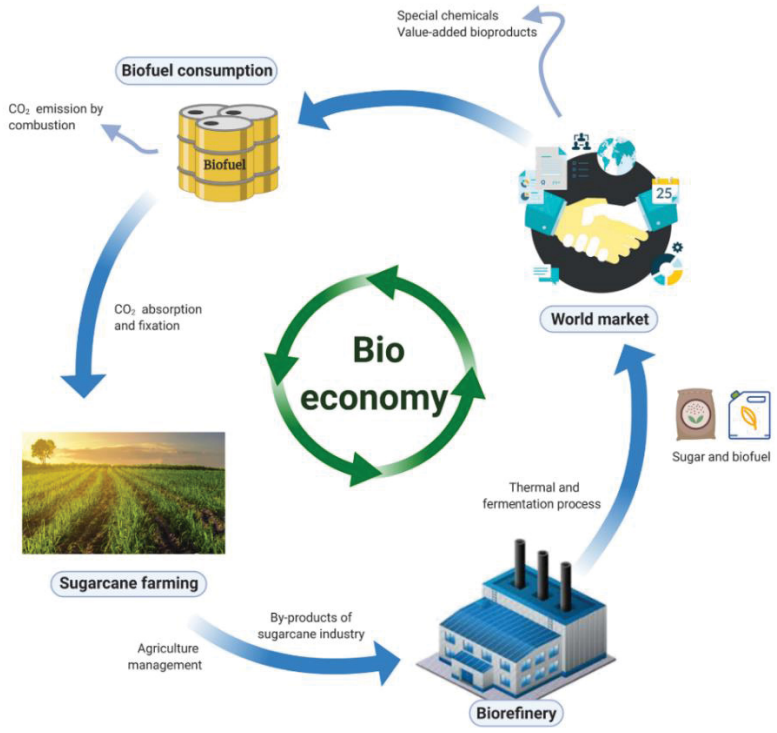


Figure 4. The sugarcane biorefinery and the circular bioeconomy.

5. From sugarcane liquid and solid wastes to value-added bioproducts

The improved biorefinery approach, with the vision of circular bioeconomy, could amplify the product range, environmental and economic sustainability of traditional sugarcane mills by including value-added products from sugarcane liquid and solid wastes. However, the full-scale application is still under development. In the case of Brazil, due to its advances in the sugarcane industry, there are industrial-scale production plants in which high value-added molecules are being developed and produced, aiming at the use of 100% of the sugarcane. Companies like Braskem use sugarcane as raw material to produce renewable plastics, ethanol, and high-value chemicals. Renewable polyethylene is one of its main

products that is used as a thermoplastic resin to produce different packaging. Besides, produce polyethylene waxes that are used in cosmetics and dyes Braskem (2022). Another example is Raizen company, with its multiple bioenergy parks, which is capable of producing 1G and 2G bioethanol at industrial scale. In addition, the company uses residues such as vinasse and filter cake to produce biogas, being able to obtain biomethane and bioelectricity as final products Raízen (2022). Other large-scale investments are being done by Earth renewable technologies company to produce biodegradable bioplastic from sugarcane and its by-products (molasses and SB) ABICOM (2021). Although these large companies represent a step forward in the implementation of biorefineries, the production of other molecules and materials with high added value are being developed and investigated by the scientific community. Table 1 summarizes some worldwide bioproducts' production from sugarcane residues, as well as its market value.

Table 1. Productivities and market sizes of industrial products from sugarcane residues.

By-product	Raw material	Yield (g/g)	Productivity (g/L. h)	Market size	References
Ethanol 2G	Bagasse	0.23-0.47	0.27-1.80	US$632 million (2020)	Ayodele et al. (2020); Research and Markets (2021a)
Lactic acid	Bagasse	0.73-0.87	1.14-1.81	US$2.7 billion (2020)	Oliveira et al. (2019), Van Der Pol et al. (2016), Research and Markets (2021b)
Bio-succinic acid	Bagasse	0.43-0.79	0.61-1.01	US$728 million (2020)	Pateraki et al. (2016), Research and Markets (2021c)
	Molasses	0.76-0.96	0.77-1.15		Sydney, E.B. et al. (2021) Magalhães Júnior et al. (2021)
PHB	Vinasse, molasses and bagasse	8.5-20.9[a] and 56-69[b]	0.16-0.36[c]	US$81.8 million (2020)	Dalsasso et al. (2019), Sakthiselvan; Madhumathi (2018), Research and Markets (2021d)
Xylitol	Bagasse	0.61-0.86	0.38-2.81	US$921 million (2020)	Morais Junior et al. (2019), Research and Markets (2021a), De Godoi et al. (2019)

a: dry cell weight (g/L); b: PHA content (%); c: maximum specific growth rate (μmax, h^{-1})

5.1 Bioproducts from sugarcane liquid wastes

5.1.1. Bioproducts from vinasse

Vinasse is obtained from the distillation columns where the bioethanol is recovered from the yeast-free fermented broth. It is an effluent with a low carbohydrate content. Each litre of produced bioethanol generates 10–15 litres of vinasse. Sugarcane vinasse is mainly composed of cellular residues and organic acids Paz-Pino et al. (2014) and volatile suspended solids, varying from 1,160 to 10,240 mg/L according to the harvesting season De Godoi et al. (2019). Its high conductivity (14,500±2,000 μS cm^{-1}) is due to its high salinity, combined with a low pH of 4.72 ± 0.67 De Godoi et al. (2019). On the other hand, it has high levels of potassium, magnesium and sulphate and relatively low levels of nitrogen and phosphorous, which justifies its traditional use in the fertigation of sugarcane areas. During sugarcane harvesting and bioethanol production, the vinasse production rate reaches 8,400 m^3/day in an average-size industry (560 m^3 ethanol/day). The vinasse produced in the distillation tower, still hot, is transported through underground channels to an equalisation and storage tanks, where it flows through a network of channels distributed through the cane plantation to be fertigated. The vinasse from *annexed plants* (using a mixture of sugarcane juice and molasses) and *autonomous plants* (using only sugarcane juice) presents an average COD of 37,500 and 21,000 mg/L, respectively Bernal et al. (2017) (**Fig. 5**). Because of (i) numerous environmental issues related to the direct use of vinasse as fertiliser, such as the contamination of subterranean water, soil desertification Fuess et al. (2017), methane emissions Oliveira et al. (2017), (ii) the high rate of production resulting in excess vinasse that is underused and (iii) its biodegradable character De Godoi et al. (2019), many technologies for a more rational use of vinasse have been proposed in the last years. Among them, anaerobic digestion is the only one that has reached an industrial production level.

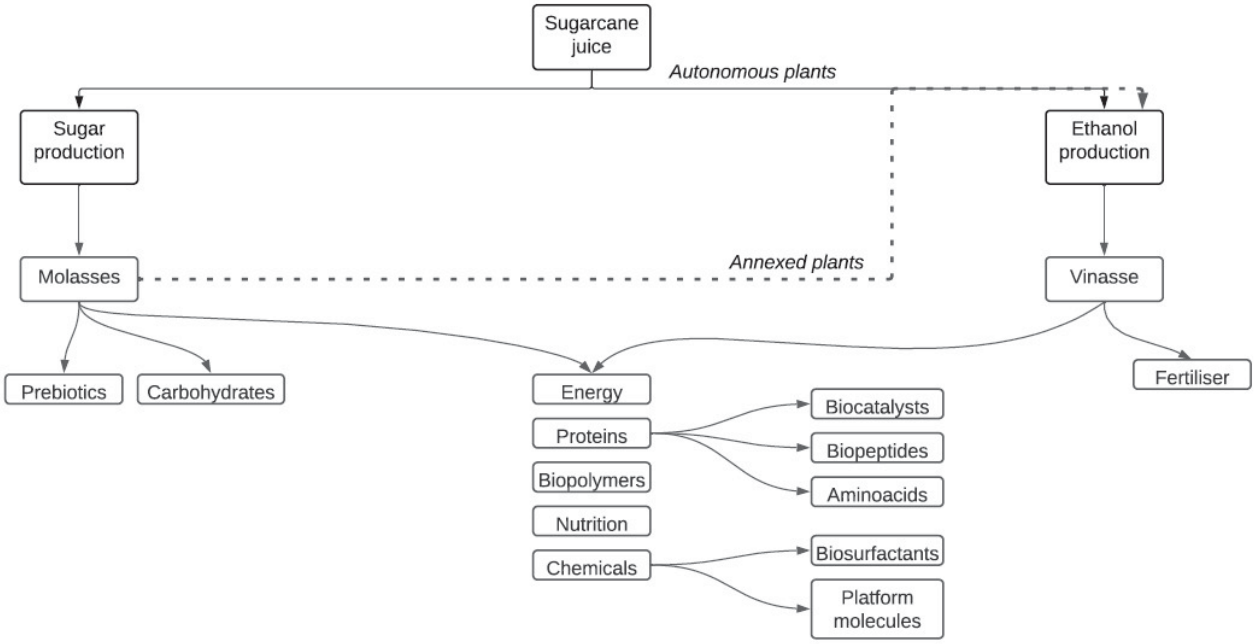


Figure 5. Bioproducts derived from the liquid wastes of sugar and bioethanol production.

The anaerobic digestion of vinasse to methane can convert its remaining organic matter into a source of energy, maintaining its fertiliser capacity with the resulting liquid fraction, which is rich in nitrogen, potassium and phosphorus Silva; Abud (2016). The methane-rich biogas can be used in the production of thermal and electrical energies, which are applied in large amounts within the industry or purified to be used as biofuel in transportation. Anaerobic digestion has also the potential to reduce methane emissions in vinasse storage tanks and distribution channels, which can reach more than 1.36 $kgCO_{2\text{-}eq}/m^3$ of vinasse Oliveira et al. (2017). However, vinasse must be combined with other substrates in co-digestion, such as molasses, sugarcane juice and sugarcane filter cake to balance the C/N ratio and micronutrient contents. Some of the co-substrates that have been studied are molasses, sugarcane juice and sugarcane filter cake; they have been proposed to balance the C/N ratio and micronutrient

contents. Also, the high content of sulphur requires the use of technologies to remove SO_x and H_2S Rodrigues Reis; Hu (2017).

Besides methane, vinasse has also been studied for the production of biohydrogen through dark fermentation, both in mono- and co-digestion, with yields close to the theoretical maximum Fuess et al. (2019) and Sydney et al. (2014). Dark fermentation is an anaerobic digestion process where the conditions are controlled to achieve methanogen inhibition, thus producing biohydrogen and short-chain fatty acids (C2–C6 compounds). The first can be used within the sugarcane-processing plants for the production of thermal and electrical energies or in fuel cells Sudheer et al. (2020), while the second can be used as platform molecules for the chemical industry Kim et al. (2018).

Similar to higher plants, vinasse has a positive effect on the production of microalgae biomass, which is a source of an infinitude of bioproducts such as pharmaceuticals, bioenergy, food, feed, pigments, fertilisers, bioactive compounds with application in cosmetics, phytohormones and others Caporgno; Mathys (2018), Levasseur et al. (2020) and Nethravathy et al. (2019). Producing microalgae in such a dark and turbid wastewater requires vinasse dilution to allow the passage of light and to obtain higher growth rates. Moreover, to support high biomass production, it is recommended to adjust the nitrogen and phosphorus contents to approximate the Redfield ratio (C:N:P 106:16:1), which is not satisfied by vinasse. Most studies have achieved high biomass concentrations and growth rates at maximum vinasse concentrations of up to 50% Ramirez et al. (2014), Santana et al. (2017) and Sydney et al. (2019). The alkaline treatment of sugarcane vinasse with NaOH prior to microalgae cultivation has been presented as a promising method to reduce turbidity without significant variations in nutritional composition, allowing its use without dilution Sydney et al. (2019). Biomass

concentrations higher than 2 g/L were observed for *Arthrospira maxima* and *Botryococcus braunii*.

Besides the bioconversion to methane and hydrogen by bacteria and the production of microalgae biomass, vinasse has also been studied a substrate for the production of fungi-derived biomass and bioproducts. However, the scientific literature is extremely limited in its application in aerobic processes, probably because of the low content of easily fermentable carbohydrates. On the other side, its acidic pH is close to that preferred by fungi. Biomass production of edible fungi for feed application have been studied Sartori et al. (2015). For the production of single-cell protein, the yeast *Candida utilis* was cultivated in a diluted vinasse medium (50% in distilled water) enriched with 50 g/L molasses, achieving 11.78 g/L of biomass and a protein content of 40% Cajo et al. (2011). *Rhizopus microsporus* (var. *oligosporus*) was cultivated in 100% cane- and molasses-vinasse supplemented with nitrogen and phosphorus. Levels of 39.48 and 45.55% of crude protein were obtained, respectively, with significantly high amino acid, arginine and threonine concentrations Nitayavardhana; Khanal (2010).

The production of biodiesel from the single-cell-oil from *Mucor circinelloides*, which achieved approximately 23% in a medium composed of sugarcane vinasse supplemented with corn steep liquor (2% vol) Rodrigues Reis et al. (2019). Expanding the industry portfolio of products, the production of polyhydroxyalkonoate (PHA) in a vinasse-based medium was carried out by *Haloarcula marismortui*. In this case a medium composed of 100% active carbon-pretreated vinasse resulted in a 23% PHA accumulation with a productivity of 0.020 g/L.h Pramanik et al. (2012). In another recent report, the production of 2.7 g/L of biosurfactant (rhamnolipid) from *Pseudomonas aeruginosa* was described in a 50%-diluted vinasse medium supplemented with glycerol and nitrogen source, followed by anaerobic digestion of the residual fermentation medium Naspolini et al. (2017). New technological developments for

vinasse valorisation should also be considered but keeping in mind its use as fertiliser may not be lost.

5.1.2 Bioproducts from molasses

Molasses is the by-product of the manufacture of sugar. It is a dark brown, viscous liquid with a strong odour Vidra et al. (2017), which contains approximately 50% (w/w) sugar, other nutrients such as organic acids, vitamins and minerals, as well as a nitrogen level of about 0.5–0.9% (w/v) Desouky, S E et al. (2017). For each ton of crushed cane, about 0.041 ton of molasses is generated. Sugar production in Brazil in the 2019/2020 harvest was 29,606 thousand tons, and in the 2020/2021 harvest it was 41,503 thousand tons UNICA (2021b).

The composition of molasses varies significantly due to differences in sugarcane varieties, growing conditions and production processes. It is generally characterized by a high sucrose content, ranging from 30–40% (dry weight), and high levels of reducing sugars (mainly glucose and fructose) in the range of 15–20% (dry weight) Qi et al. (2017). Because it contains niacin, pantothenic acid, minerals and some microelements, it is used as a supplement in animal feed, also acting to increase palatability and as a binder for pelleting. Its use in animal feed is limited due to toxicity in ruminants and laxative effects in monogastric animals. Therefore, it is commercially included in the feed at levels of 15% for cattle and swine, 8% for calves and sheep, and 5% for birds Singh et al. (2020). Molasses is generally sent to autonomous distilleries as a substrate for fermentation in the production of bioethanol Meghana; Shastri (2020) and for the production of biogas Detman et al. (2017).

The potential of molasses in biorefineries can be harnessed through its bioconversion into value-added products Sharma et al. (2016). This is an environmentally and economically advantageous alternative, as in addition to providing the destination for the residual fraction of

the sugar industry process, it generates new products. Due to its high sugar content, molasses is an efficient option as a carbon source in microbial production, to obtain biochemicals and bioproducts, Sen; Baidurah (2021). In the production of lactic acid, the use of sugarcane molasses represented 37.7% of the total medium cost, compared to glucose Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). Production of erythritol from sugarcane molasses can significantly reduce process costs Hijosa-Valsero et al. (2021). For the production of polyhydroxybutyrate (PHB) the high cost is a limiting factor, therefore it is necessary to search for more accessible sources. Sugarcane molasses is a viable alternative because it is an abundant and low cost raw material Dalsasso et al. (2019) and Sen et al. (2019). The use of molasses as a culture medium for the fungal strain of *Mucor circinelloides* URM4182 resulted in biomass with 25% by weight of lipids, with potential for producing biodiesel to be coupled with bioethanol plants Reis et al. (2020). In a study of biohydrogen production by thermophilic dark fermentation of sugarcane juice and molasses, molasses showed better results, due to its balance in nutrient content, surpassing bioenergy generation by about 600%, compared to vinasse Fuess et al. (2020). Another study indicated that the energy potential of molasses exceeded that of vinasse by about 25%, justifying that the high availability of organic matter in molasses requires a low organic loading rate for efficient energy recovery Vilela et al. (2021).

Among the various applications of molasses are studies of its antioxidant capacity, production of citric acid Ozdal; Basaran (2019), hydrogen and volatile fatty acids Baima Ferreira Freitas et al. (2020), propionic acid Pereyra et al. (2020) butyric acid Guo, X. et al. (2020), poly (malic) acid Wei et al. (2017); hyaluronic acid Pan et al. (2017), and succinic acid Cao, Weifeng et al. (2018). *Rhodosporidium* sp can be cultivated in molasses for the production of oils accumulated intracellularly, with potential as a renewable raw material for the oleochemical industry Boviatsi et al. (2020). In Asia, molasses is widely used in

fermentation for the production of monosodium glutamate, organic acids and amino acids (lysine) Lopez-arenas (2017) and Santos; Eichler; et al. (2020). A study evaluating growth and pigment production by *Monascus ruber* from molasses indicated that, associating rice flour as a carbon source, and molasses as a rich source of nutrients, there was an increase in pigment production Da Silva et al. (2021). In co-fermentation of glycerol and molasses as carbon sources were employed for the production of hydrogen and metabolites, De Souza Queiroz et al. (2021), aromatic components recovery by fermentation with *Pichia fermentans* Rossi et al. (2017), isomaltulose production by *Yarrowia lipolytica* Wang, Z. P. et al. (2019), microbial oil production by *Mucor circinelloides* Bento et al. (2020) and *Rhodosporidium* sp.

Although it is a product with diverse applications, some factors, such as storage, conservation and costs, must be considered. The treatment and heating operations in the manufacture of sugar generate toxic substances, such as an excess of metal ions and 5-hydroxymethylfurfural, which cause low yields and potentially inhibit the growth of some microorganisms Sun et al. (2019). Due to the high salinity and osmolarity, the implantation of new industrial strains using molasses as raw material is difficult Bento et al. (2020). Some pretreatment processes, such as hot acid clarification, barium sulphate or calcium oxide precipitation, EDTA chelation and activated carbon adsorption, are used alone or in combination to remove heavy metals, pigments and other toxic substances from molasses.

Regarding transportation and storage, molasses has a higher freight cost when compared to sugar because of its consistency, making storage difficult and presenting a high risk of contamination. In sugar and alcohol mills, the ethanol distillery is close to the sugar production sector, reducing transport costs and bringing the value of the product to a minimum Magalhães et al. (2020). In a study with the addition of maltodextrin, the rheological properties of molasses were investigated in order to improve transport issues and future prospects in the manufacture of dry molasses. The flow behavior of pure molasses can be significantly affected by the

increase in temperature, resulting in an increase in the glass transition of the solid mixture Wang et al. (2018). In natura molasses, on the other hand, is more difficult to transport, requiring care and storage in special tanks, and is more prone to fermentation.

Value-added bioproducts obtained from molasses and vinasse includes different types of industries, with an interesting perspective from a sustainable and economic point of view (Table 2). Considering the complex and variable composition of these liquid wastes, reflected in yield and cost advantages, some production processes have a more promising application on an industrial scale than others. Vinasse reuse is more challenging than molasses due to its high content of organics, salts and heavy metals and low pH, which confers toxicity to many microorganisms. Although a large part of the molasses is destined for bioethanol and animal feed, these applications depend directly on the variation in the availability and price of molasses Zhang et al. (2021). Therefore, valuing this by-product in obtaining new products for different markets is economically significant and viable, since the literature identifies its potential to generate products of interest for applications in the pharmaceutical, cosmetics, food and other sectors. The bioethanol production market is established with the use of other sources, and due to the large amounts of molasses generated, the application of this by-product to obtain other products would expand the economy derived from the sugar-alcohol sector. However, considering industrial-scale productions for potential bioproducts from molasses, analyzes are still needed on the difficulties that this by-product presents, as mentioned in this study. By reducing these limiting factors, especially related to compounds that can inhibit microbial activity, it will be possible to increase the yield of bioproducts and improve the use of sugarcane molasses.

Table 2. Bioproducts derived from the microbial bioconversion of sugarcane vinasse and molasses.

Substrate	Product Category	Product	Microrganism(s)	Reference(s)
Vinasse	Energy	Biodiesel	Microalgae, cyanobacteria, *Mucor circinelloides*	Rodrigues Reis et al. (2019) and Sydney et al. (2016)
		Biogas	Microbial community	Parsaee et al. (2019)
		Biomethane		Albuquerque et al. (2019)
		Biohydrogen		Fuess et al. (2019) and Sydney et al. (2014, 2018)
	Fertiliser	Anaerobic digestate		Silva; Abud (2016)
	Nutrition	Feed	*Aspergillus oryzae, Neurospora intermedia, Rizhopusoryzae, Monascuspurpureus, Fusarium venenatum, Pleurotussajor-caju*, *Pleurotusostreatus*, *Pleurotusalbidus* and *Pleurotusflabellatus*	Karimi et al. (2019) and Sartori et al. (2015)
		Single cell protein	*Candidautilis, Rhizopusmicrosporus* (var. *oligosporus)*	Cajo et al. (2011)
	Biocatalysts	Ligninolytic enzymes	*P. sajorcaju* and *P. ostreatus*	Aguiar et al. (2010)
		Cellulolytic Enzymes	*P. sajorcaju* and *P. ostreatus*	Aguiar et al. (2010)
		Manganese-peroxidase	*T. reesei, Pleurotussajorcaju, Pleurotusostreatus, Trametes versicolor*	Aguiar et al. (2010) and Pinheiro et al. (2020),
		Laccase	*Pycnoporus* sp.and *Trametes* sp.	Ahmed et al. (2018)
	Platform molecules	Short Chain Fatty Acids (C2 – C6)	Consortia	Sydney et al. (2014, 2018)
	Biopolymer	Polyhydroxyalkanoates (PHA)	*Haloarcula marismortui*	Pramanik et al. (2012)
	Biosurfactant	Rhamnolipid	*Pseudomonas aeruginosa*	Naspolini et al. (2017)

	Biopeptides	Three peptide fractions	*Arthrospira maxima*	Montalvo et al. (2019)
Molasses	Energy	Ethanol	Microbial community	Meghana; Shastri (2020)
		Biogas		Detman et al. (2017)
		Biohydrogen		Baima Ferreira Freitas et al. (2020)
		Microbial oil	*Rhodosporidium toruloides*NRRL Y-27012 *and R.kratochvilovae*Y-43	Boviatsi et al. (2020)
	Value added chemicals	Organic acids	*Clostridium tyrobutyricum*	Guo, X. et al. (2020)
			Aureobasidium pullulans	Wei et al. (2017)
			Actinobacillus succinogenes 130Z	Cao, Weifeng et al. (2018)
			Aspergillus niger	Ozdal; Basaran (2019)
	Biopolymer	Polyhydroxyalkanoates	*Bacillus flexus*Azu-A 2	Desouky, Said El-sayed et al. (2017)
		Polyhydroxybutyrates	*Cupriavidus necator DSM 545*	Dalsasso et al. (2019)
			Cupriavidus necator	Sen et al. (2019)
	Prebiotics	Oligosaccharides	*Leuconostoc mesenteroides*MTCC 10508	Sharma et al. (2016)
	Carbohydrates	Mannitol	*Lactobacillus brevis NM101-1*	Gomaa; Rushdy (2014)
		Erythritol	*Moniliella pollinis*	Hijosa-Valsero et al. (2021)
		Isomaltulose	*Yarrowia lipolytica*	Wang, Z. P. et al. (2019)
		Xylitol	*Candida tropicalis*	De Souza Queiroz et al. (2021)
	Aminoacids	Lysine	*Corynebacterium glutamicum*	Lopez-arenas (2017)
	Enzymes	Lipase	*Burkholderia sp*	Zhu et al. (2018)
		Phytase	*Bacillus subtilis K46b*	Rocky-Salimi et al. (2017)
	Other	β-carotene	*Rhodotorula rubra*	Banzatto et al. (2013)
		Aroma compounds	*Pichia fermentans*	Rossi et al. (2017)
		Pigment	*Monascus ruber*	Da Silva et al. (2021)

5.2 Bioproducts from sugarcane solid wastes

5.2.1 Pre-treatment – traditional and new methods

As already stated, the solid residues represent good sources of sugars that can be biotechnologically transformed into biofuels or other high-value-added biochemical products Sritrakul et al. (2017). For such a purpose, the release of fermentable sugars from the cell wall polysaccharides requires the use of enzymes. However, the efficiency of the enzymatic attack is extremely limited due to the lignocellulosic biomass recalcitrance, which is a result of the association of glucan with hemicellulose and lignin. To overcome this limitation, lignocellulosic residues require pretreatment to change and/or remove hemicellulose and/or lignin, increasing the accessibility of enzymes to their substrates Santos et al. (2018).

Acid, alkaline and hydrothermal methods are the most popular pre-treatments employed for the improvement of enzymatic saccharification of lignocellulosic biomass Miyamoto et al. (2018). Nevertheless, the use of corrosive reagents, the large volume of residues and the necessity of special equipment are the major drawbacks.

The different pre-treatment methods currently available represent different alternatives for the valorisation of sugarcane residues such as bagasse and straw. The possibility of fractionating the lignocellulosic biomass into its three main components (cellulose, hemicellulose and lignin) allows the production of different high-value-added products (Table 3).

Table 3. Bioproducts from sugarcane solid wastes

Substrate	Pretreatment	Microorganism	Product	Reference
Pretreated bagasse	Alkaline	*Candida shehatae*	Bioethanol	Prajapati et al. (2020)
Pretreated bagasse and corn stover	Alkaline/Hot water	*Saccharomyces cerevisiae*	Bioethanol	Wang, Z. et al. (2019a)
Pretreated bagasse	Imidazole pretreatment	*S cerevisiae*	Bioethanol	Valladares-Diestra et al. (2021)
Pretreated bagasse	Steam-assisted salt/alkaline	*S cerevisiae*	Bioethanol	Jugwanth et al. (2020)
Pretreated bagasse and hydrolysate	Acid	*S. cerevisiae and Candida tropicalis*	Bioethanol/ Xylitol	Unrean; Ketsub (2018)
Pretreated bagasse and hydrolysate	Aqueous ammonia	*C. tropicalis*	Bioethanol/ Xylitol	Raj; Krishnan (2020)
Pretreated bagasse and hydrolysate	Acid	*Kluyveromyces marxianus*	Bioethanol/ Xylitol	Dasgupta et al. (2017)
Pretreated bagasse and hydrolysate	Acid	*C. tropicalis*	Bioethanol/ Xylitol	Antunes et al. (2021)
Bagasse hydrolysates	Steam explosion/acid	*C. tropicalis*	Xylitol	Junior et al. (2019)
Bagasse hydrolysates	Glycerol organossolv	*Candida glycerinogenes*	Bioethanol/ 1,2,4-butantriol	Zhao et al. (2019)
Bagasse hydrolysates	Acid or hydrothermal	Mixed culture	Biohydrogen	Sá et al. (2020)
Bagasse hydrolysates	Autohydrolysis	Mixed culture	Biohydrogen	Baêta et al. (2016)
Bagasse hydrolysates	Steam explosion	Mixed culture	Biohydrogen	Thungklin et al. (2018)
Pretreated bagasse and hydrolysates	Acid	*Clostridium acetobutylicum*	Biobutanol	Gomes et al. (2019)
Glucose and bagasse	Alkaline	*Clostridium beijerinckii*	Biobutanol	Vieira, C. F. dos S. et al. (2020)
Molasses, pretreated bagasse and hydrolysates	Acid	*C. beijerinckii*	Biobutanol	Vieira et al. (2021)
Molasses and bagasse hydrolysates	Acid	*Clostridium saccharoperbutylacetonicum*	Biobutanol	Chacón et al. (2020, 2021)
Bagasse hydrolysates	Acid	*Trichosporon sp.*	Biodiesel	Brar et al. (2017)
Bagasse hydrolysates	Acid	*Enterobacter aerogenes EMY-22*	2,3-butanediol	Kim et al. (2020)
Pretreated bagasse	Alkaline	*Bacillus coagulans*	Lactic acid	Nalawade et al. (2020)
Pretreated bagasse	Acid	*Lactobacillus pentosus*	Lactic acid	Unrean (2018)
Pretreated bagasse	Acid/ EtOH/ hot water	*Actinobacillus succinogenes*	Succinic acid	Chen et al. (2020)

Pretreated bagasse	Alkaline	*Yarrowia lipolytica*	Succinic acid	Ong et al. (2019)
Pretreated bagasse	Acetic acid	*Gluconobacter oxydans*	Gluconic acid and XOS	Zhou; Xu (2019)
Pretreated bagasse	Hot water	Only chemical reactions	Levulinic acid	Schmidt et al. (2017)
Pretreated bagasse	Acid	*Corynebacterium glutamicum*	Xylonic acid	Tenhaef et al. (2018)
Raw bagasse	-	*Cladosporium cladosporioides*	Cellulases	Srivastava et al. (2020)
Pretreated bagasse	Acid or Alkaline	*S. cerevisiae and C. tropicalis*	Cellulases and xylanases	Qadir et al. (2018)
Pretreated bagasse	Hot water	*Trichoderma reesei*	Cellulases	Darabzadeh et al. (2019)
Pretreated bagasse	Steam explosion	*Aspergillus niger*	Endoglucanase	Squinca et al. (2018)
Pretreated bagasse and hydrolysates	Hydrothermal	Only chemical reactions	Cellulose nanofibrils/XOS	Marcondes et al. (2020)
Pretreated bagasse	Microwave/ Ultrasonication	Only chemical reactions	Cellulose nanofibrils	Harini; Chandra Mohan (2020)
Pretreated bagasse	Na_2CO_3/ Ionic Liquid	Only chemical reactions	Cellulose nanofibrils	Sankhla et al. (2021)
Raw bagasse	-	Only chemical reactions	Activated carbon	Vieira et al. (2019) Dantas et al. (2020)
Raw bagasse	-	*Aspergillus niger*	Xylanases	Valladares-Diestra et al (2021)
Sugarcane straw	Straw recovery and burning	-	Bioelectricity	Cervi et al. (2020)
Sugarcane straw	Organosolv pretreatment and acid hydrolysis	Only chemical reactions	Lignocellulose nanocrystals	Bilatto et al. (2020)
Sugarcane straw	Consecutive pretreatment of dilute H_2SO_4 and NaOH	*Lasiodiplodia theobromae*	β-glucan biopolymer	Abdeshahian et al. (2020)
Sugarcane straw	Sequential ionic liquid pretreatment and pyrolysis	Only chemical reactions	Furfural and levoglucosenone	Halder et al. (2020)
Sugarcane straw	Mild deacetylation, followed by hydrothermal pretreatment.	Only chemical reactions	Xylo-oligosaccharides	Halder et al. (2020)
Filter cake	-	*Rhodotorula glutinis*	Biolipids	Shi et al. (2021)
Filter cake	-		Fertiliser	De Lima Vasconcelos et al. (2020)
Filter cake	Incineration		Self-compacting concrete	Sua-Iam; Makul (2017)

5.2.2 Products from sugarcane bagasse

Each ton of processed sugarcane generates 250 kg of SB Soccol et al. (2010) that are currently used for the energy demand of sugarcane extraction plants, which can be provided integrally by the heat and bioelectricity generated from its own produced bagasse Santos et al. (2016) and Sydney, Eduardo Bittencourt et al. (2021). The surplus energy produced plays an important role in industries economics if it is commercialised to the consumer market. The high volatility market prices of electricity and ethanol, along with the competition for their common feedstock, imposes sugarcane industries to invest in flexible biorefineries, with alternation between them Cavalcanti et al. (2020). Carpio and Souza Carpio; Simone de Souza (2017) evaluated different scenarios of market prices and bagasse allocation for the maximization of the financial return from the commercialization of both products. The analysis showed that the bagasse allocation to 2G bioethanol increases with the reduction of its production costs. With a 2G bioethanol production cost of 0.30 US$/L, the optimised bagasse allocation is 84% to ethanol and 16% to electricity. Furthermore, solid residues from 2G bioethanol production can be used for bioelectricity generation. Carvalho et al. Carvalho, D. J. et al. (2020) reported that the cogeneration of lignin residue from bagasse hydrolysis and 36% straw, which is available in the field, can guarantee electrical and thermal self-sufficiency of integrated 1G and 2G bioethanol production, with 63 kWh of surplus electricity per cane ton.

The dominant technologies of bioelectricity and 2G bioethanol will probably maintain their position in the near future due to the high demand for energy in sugarcane production and biofuels on the global market Hassan et al. (2019a) and Sydney, Eduardo Bittencourt et al. (2021). However, SB has great potential as feedstock of many other relevant bioproducts; xylitol, biodiesel, biobutanol, 2, 3-butanediol, biohydrogen, biopolymers, enzymes, organic acids, modified catalysts and other biomolecules were

reviewed in the relevant work of Sindhu et al. Sindhu; Gnansounou; et al. (2016). In recent years, many research groups have developed the biorefinery of bagasse bioproducts.

To reach the desired reduction of 2G bioethanol production costs, many researches evaluated developments of traditional methods in different aspects. The use of enzymatic cocktails for hydrolysis is one of the most critical steps in terms of process costs Kumar; Verma (2020). In addition, the traditionally used *Saccharomyces* yeasts only ferment C6 sugars Azhar et al. (2017). In this context, Prajapati et al. Prajapati et al. (2020) evaluated novel cellulase and hemicellulase cocktail produced by *Aspergillus tubingensis*, reaching 74.9% hydrolysis efficiency. Thereafter, 15.5 g/L bioethanol was produced by xylose-fermenting *Candida shehatae* with a 77.9% yield. Wang et al. Wang, Z. et al. (2019b) evaluated a blend of C5/C6-fermenting *Saccharomyces* yeasts, obtaining 32.9 g/L bioethanol, with 98.0% glucose and 96.1% xylose consumed.

Due to economic difficulties in using yeasts to convert the pentose fraction of bagasse into bioethanol, one alternative is its exploration to co-produce bioethanol with other high-value bioproducts, such as xylitol Raj; Krishnan (2020). Unrean and Ketsub Unrean; Ketsub (2018) co-produced 2G bioethanol and xylitol by *S. cerevisiae* and *Candida tropicalis*, respectively. Acid pretreatment was applied to separate liquid hydrolysate to produce 24.0 g/L xylitol, and pretreated solids to produce 56.1 g/L ethanol.

Biohydrogen, is a promising future energy source due to its high energy content and zero CO_2 emission, from lignocellulosic biomass. Raheem et al. Raheem et al. (2019) evaluated different conditions of a conventional gasification method (700-900 ºC, 10-30 min) to convert bagasse to biohydrogen, reaching 413 NmL/g bagasse (normalized milliliter at normal conditions of temperature and pressure). Cao, Wen et al. (2018) investigated a supercritical water gasification with 20 wt% of Na_2CO_3 as catalyst at

650ºC, reaching a higher yield of 797 NmL/g bagasse. These results shows that bagasse gasification has great potential to add value into sugar and bioethanol plants. Biohydrogen can also be generated from hemicellulosic pretreated fractions of bagasse, promoting a complementary exploration of biomass, which can be further converted to other bioproducts from its cellulosic fraction.

In comparison with bioethanol, biobutanol is more convenient for storage and use because of its higher energy density, lower vapor pressure and lower auto-ignition temperature Li, Y. et al. (2019). Gomes et al. Gomes et al. (2019) reported an acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* from acid pre-treated bagasse, with 0.4 g/g yield, 0.5 g/L.h productivity and 9.1 g/L butanol. The isopropanol-butanol-ethanol (IBE) mixture fuel is an alternative route to produce acetone-free butanol, which otherwise could flood the market Vieira et al. (2019). Vieira et al. Vieira, S. et al. (2020) evaluated different loadings and delignification extents of bagasse as immobilization agent on *Clostridium beijerinckii*. The IBE productivity varied between 0.22 and 0.28 g/L.h and the butanol titre between 6.7 and 8.6 g/L. Techno-economic analysis showed that an IBE mixture fuel production can be competitive with cellulosic ethanol, with minimum selling prices (15 USD/GJ) Dantas et al. (2020). Biodiesel is an important biofuel which can be used in existing diesel engines blended or not with petrodiesel, with a similar energy density. SB acid hydrolysate was used for lipid accumulation and biodiesel production by *Trichosporon* sp., reaching 10.25 g/L lipids and 40.5% yield after 120 h of fermentation Brar et al. (2017).

Organic acids are an important biobuilding blocks and platform chemical biomolecules that offer several industrial applications, such as monomers of biodegradable plastics Ahmad et al. (2020) and Mancini et al. (2020). Thus, Nalawade et al. (2020) evaluated two strategies for lactic acid production from bagasse through alkali

pre-treatment, high solid- enzymatic hydrolysis and a *Bacillus coagulans* fermentation. The results varied between 50.4 and 51.24 g/L lactic acid and 1.75 and 2.4 g/L.h productivity. Chen et al. (2020) evaluated different pre-treatments for succinic acid production by *Actinobacillus succinogenes* from bagasse. A production of 41 g/L succinic acid and a 0.3 g/L.h productivity were obtained.

Cellulases were produced by *Cladosporium cladosporioides* NS2 in solid-state fermentation (SSF) using different types of carbon sources, with raw SB reaching the highest activity results of 16.9 IU/g to exoglucanase, 150 IU/g to endoglucanase, and 200 IU/g to β-glucosidase Srivastava et al. (2020). Qadir et al. (2018) related endoglucanase, β-glucosidase and xylanase production from acid or alkaline pretreated bagasse. Under optimised conditions of 35ºC, 94 h, and 0.5 mL/g yeast inoculation co-culture in alkali-treated bagasse, endoglucanase production reached 9.81 IU/mL.

Other materials such as nano- and microcrystalline cellulose are high-value-added biomaterials that can be obtained in a sugarcane biorefinery Oprea; Voicu (2020). Marcondes et al. (2020) applied a two-stage hydrothermal pre-treatment to co-produce XOs and cellulose nanofibrils. Harini; Chandra Mohan (2020) isolated micro- and nanocrystalline cellulose fibres from different biomass sources, with bagasse presenting higher yields. Activated carbon with different characteristics was also produced in different works. Sarkar et al. produced porous carbon to be used as an electrode material for supercapacitors, applying chemical agents such as K_2CO_3, KOH and NaOH with 650ºC. Similarly, Guo, Y. et al. (2020) produced porous carbon with a high specific surface area and a large pore volume with NaOH treatment for CO_2 adsorption.

5.2.3 Products from sugarcane straw

Sugarcane straw (SS) represents approximately one third of the total mass of cultivated sugarcane, with 10 to 20 tons of dry matter per cultivated hectare Cardoso et al. (2015), although these values depend largely on the type of sugarcane variety and the geographic and climatic conditions. Most of these by-products are wasted by burning or left to decompose in the fields after harvest. The main agronomic benefits of SS left in the cropland are protection against soil erosion, increase in organic carbon, inhibition of weed growth, reduction of water loss from soils and contribution to nutrients recycling Leal et al. (2013), Menandro et al. (2017) and Vasconcelos et al. (2018). For this reason, the balance between positive impacts and the economic/energy value of SS must be considered according to the harvest location and the processing conditions.

Due to its high calorific value, SS has attracted considerable interest in the field of bioelectricity production. However, unlike SB, SS often requires additional operations that generate a greater investment of resources and time, mainly for its transport to energy production plants Cervi et al. (2020) and Watanabe et al. (2020). Many sugarcane mills are therefore coupled with specific boilers for SS burning, generating bioelectricity that can be used within the sugarcane mill (providing energy autonomy) or exporting the energy within the electrical grid Cervi et al. (2020). Although SS is mainly used in the production of bioelectricity, its composition, rich in polymers, similarly to that of SB, presents 42–38% of cellulose, 28–17% of hemicellulose and 22–17% of lignin, making it a potential raw material for the production of different biomolecules through the use of physicochemical pre-treatments Abdeshahian et al. (2020) and Halder et al. (2020).

For example, Bilatto et al. (2020) used SS for cellulose nanocrystals production. After sulfuric acid treatment of the pre-treated solid, cellulose nanocrystals were recovered. High performance values were obtained with thermal stability of the

nanocrystals, showing good perspectives for this process. High-value-added food products, such as prebiotics, can also be obtained from SS. XOs are prebiotics that are composed of xylose chains and can be obtained from lignocellulose material rich in hemicellulose. Brenelli, Lívia B et al. (2020) applied a deacetylation treatment followed by a hydrothermal pre-treatment that facilitated the release of XOs. The process was successfully carried out at pilot scale, showing a high efficiency in hemicellulose solubilisation to obtain XOs (9.1% w/w) with different degrees of polymerisation, providing an encouraging approach for the use of SS to obtain food additives and biofuels. Abdeshahian et al. (2020) developed an innovative process employing SS hydrolysates obtained from sequential dilute acid/alkaline pretreatment. The hydrolysates were fermented using *L. theobromae* to produce extracellular β-glucans.

Recently, new more environmentally friendly pretreatment methods, such as the use of ionic liquids (ILs), have been applied on SS. Halder et al. (2020) compared the efficiency of ILs based on choline cations with imidazolium cations. Higher delignification and crystallinity were reached by ILs based on choline cations, which improved the production of furfural and levoglucosenone after pyrolysis. This indicates a high possibility of using green technologies in the implementation of biorefineries.

5.2.4 Products from other residues

It has been calculated that an average of 3% of filter cake is generated per ton of crushed sugarcane, a by-product obtained after the clarification of sugarcane juice Gupta et al. (2011). Its composition is highly variable, mainly due to variations in clarification methods. However, it is rich in organic substances such as sucrose and polysaccharides, as well as several minerals such as phosphorus and calcium Sua-Iam; Makul (2017) and Vasconcelos et al. (2017). These characteristics suggest that filter cake can be used as raw

material for the fertilisation of crop fields or as a source of substrate for different biomolecules.

Shi et al. (2021) reported that filter cake is composed of 6.52% insoluble solids, 15% sucrose and 2% polysaccharides, showing potential for sucrose recovery and biolipids conversion. On the other hand, Vasconcelos et al. (2017) used filter cake as an additive associated with phosphate fertilization of sugarcane crops, increasing the nutritional status and biometrics of sugarcane, mainly regarding stem yield, increasing its quality and productivity. Filter cake can also be used in the civil construction area. Sua-Iam; Makul (2017) used incinerated filter cake in different proportions to study its effects on self-compacting concrete production. The basis of their study is that calcium oxide, which is commonly used in clarification process, can be applied as building lime.

6. The next generation of sugarcane biorefineries

In the Brazilian scenario, an interesting substitute of the combustion engines could be the solid oxide fuel cells (SOFC), a recent technology for "bio-electric cars" developed as an alternative to conventional electric cars. Biofuels such as ethanol can be used to fuel these cells once they are coupled to a reformer. They use ceramic materials made of oxide solids to separate the catode and the anode and are built of low-cost, eco-friendly materials, as compared to conventional batteries and to the proton exchange membrane cells. In a prototype bio-electric vehicle tested for two years in Brazil, the average performance was 22 km/L ethanol Pereira (2020).

Besides the use as a biofuel, ethanol can be converted to a variety of products including (poly)ethylene, ethylene oxide, ethylacetate, diethyl ether, acetic acid, butanol, butadiene and Guerbet alcohols Stichnothe et al. (2016). In the materials segment, ethanol is already being employed as raw material for green plastics by the Brazilian company Braskem. Sugarcane ethanol is dehydrated and converted to ethene, which is then

polymerised to low-density polyethylene Braskem (2020). Projects for the development, production and commercialisation of new industrial technologies for the processing of sugarcane biomass are supported by the PAISS Program (BNDES - Finep Plan to Support Innovation in the Sugar and Energy Sectors) in three research lines: second-generation bioethanol, new products from sugarcane and gasification technologies, equipment, processes and catalysers FINEP (2021).

On the other hand, the competition with fossil-based sources is still a challenge, and sugarcane-processing technologies have not seen significant disruptive innovations in decades. For more than 20 years, the price paid to ethanol producers has been remaining practically unaltered, while no new technologies were incorporated to compensate for the rising costs of inputs, land and workforce Finguerut (2019). In the current sugarcane harvest, the production of sugar increased by 50% in the Central-southern region of Brazil, motivated by the international demand and the sharp valorisation of the US dollar Calcidoni (2020). As pointed out by other authors, the lack of stable regulations and technology development are two factors that are "holding biofuels back" globally Salgado; Boshell (2019). Two indicators highlighted by the authors were the total number of filed patents on biofuels, which decreased from over 6,000 in 2011 to around 2,500 in 2017, and the global investments, which were reduced from $ 27 billion in 2007 to $ 2 billion in 2017. In the rank of biofuel patent filings, China, Europe and the USA were the leaders, and the dominant technologies were directed to grain-based bioethanol, cellulosic ethanol, biodiesel, bio-pyrolysis and other biofuels. The stabilisation followed by the drop of patent applications, observed in all cases except bio-pyrolysis, indicates that the technology has reached a stage of maturity and saturation. Usually, at this time, a new technology emerges based on the previous one, but in the biofuel scenario, this was not observed, which is also reflected by the reduced investments. In this sense, it is necessary

to search for new alternatives to biofuels with innovations to obtain more competitive products in the market based on sugarcane. Although bioplastics are positioning themselves as a great commodity in the world market, there are other paths such as obtaining new polymers and additives that can be used in the pharmaceutical and food industries.

The patent scope on sugarcane technologies, on the other side, shows an increase in patent filings from 2011 (1,588 documents) to 2015 (2,448 documents), followed by stabilisation, considering the secrecy period of 18 months before publication (data obtained from the Derwent Innovations Index Database on January 5, 2021). In the last 10 years, the dominant knowledge areas related to sugarcane technologies were chemistry (77.5%), agriculture (46.9%), as well as biotechnology and applied microbiology (38.3%); only 3.82% of the documents were related to energy fuels. Other identified subject areas were instruments and instrumentation (36.92%), food science technology (28.54%), polymer science (25.31%), engineering (20.06%), pharmacology and pharmacy (16.42%), and materials science (5.58%).

In the area of chemistry, the dominant technologies represented by the International Patent Classification (IPC) codes were related to fertilizers and recombinant DNA technology. In the area of agriculture, harvesting technologies and fertilizers were dominant. As for the area of biotechnology and applied microbiology, recombinant DNA technology and fertilizers were the most representative subjects. Based on these data, we conclude that most technologies are related to sugarcane cultivation.

The dominant technologies related to energy fuels among the patent documents were “solid fuels essentially based on vegetable substances” (IPC C10L-005/44) and “ethanol, i.e. non-beverage produced as by-product or from waste or cellulosic material substrate containing cellulosic material” (IPC C12P-007/10). These data show the

predominance of second-generation biofuels, and we know that such technologies still have technoeconomic bottlenecks to be vastly applied at commercial scale.

In order to search for alternative pathways for the sugarcane industry based on available technologies, special attention was directed to the areas of food science technology, polymer science, pharmacology and pharmacy and materials science. In the area of food science technology, dominant subjects were related to animal feeding-stuffs; polymer science technologies were mostly related to fertilizers; pharmacology and pharmacy technologies were majorly represented by medicinal preparations of undetermined constitution containing material from plants; and materials science technologies comprised especially pulp from non-woody plants or crops, e.g., straw or bagasse.

Although the patent search was performed for the last ten years, it is expected that traditional technologies are dominant in patent documents, and a more orientated analysis is necessary to identify emerging technologies. For this purpose, terms related to the top bio-based chemicals to be obtained from sugar, as listed in the 2020 IEA Report on Bio-Based Chemicals De Jong et al. (2020), were searched within the patent documents associated to sugarcane. The most innovative fermentation products to be obtained from sugar were itaconic acid, adipic acid, 3-hydroxypropionic acid/aldehyde, isoprene/farnesene, glutamic acid, aspartic acid, and 1,4-butanediol (1,4 BDO). Among the most promising chemical derivatives to be synthesized from sugar, there were levulinic acid, 2,5-furan dicarboxylic acid, mono-ethylene glycol, and methyl vinyl glycolate. Sorbitol, xylitol and furfural are already obtained industrially through this route De Jong et al. (2020). Fig. 6 presents the results of the patent search for fermentation products and chemical derivatives associated to sugarcane. Among the 350 patent documents containing the terms “sugarcane” and “itaconic acid, adipic acid, 3-

hydroxypropionic acid, 3-hydroxypropionic aldehyde, isoprene, farnesene, glutamic acid, aspartic acid, or 1,4-butanediol" (considering their synonyms and orthographical variations), 32 were related to the obtaining of such products through fermentation processes. Among these, Braskem appeared nine times as an assignee, and most of the technologies were related to the preparation of acyclic hydrocarbons (IPC C12P-005/02).

Documents containing the terms "sugarcane" and "levulinic acid, 2,5-furan dicarboxylic acid, mono-ethylene glycol or methyl vinyl glycolate" (considering their synonyms and orthographical variations) were 44 in total, and of these, 17 were related to the obtaining of such compounds through the chemical conversion of sugars. Two of these documents were filed by Coca Cola Co. and were related to the manufacture of bio-based polyethylene terephthalate (PET), from mono-ethylene glycol, two were filed by the Battelle Memorial Institute and were related to the manufacture of alkyl lactate, lactic acid, alkyl levulinate and levulinic acid, and two were filed by Eaux Minerales Evian SA and were also related to the manufacture of bio-PET.

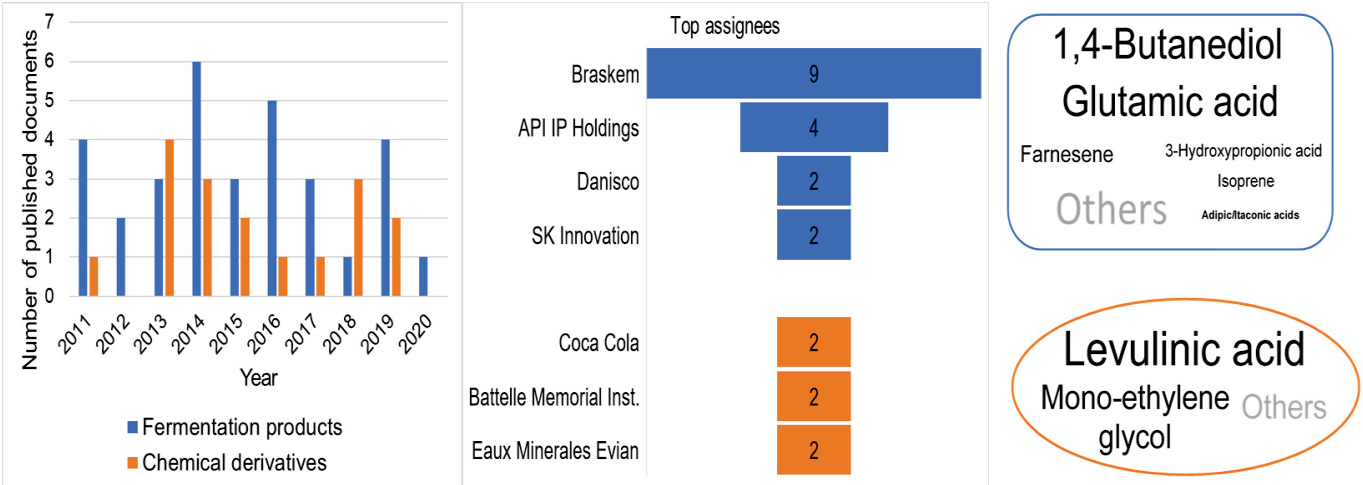


Figure 6. Patent overview on fermentation products (blue) and chemicals (orange) derived from sugarcane: number of published patent documents per year, top assignees, and most frequent subjects (fermentation products – blue rectangle; chemicals – orange ellipse).

Although it is an indicator of technological development, the patent scope does not limit the possible routes for the sugarcane industry. Many innovative solutions may not be protected by patents or may be under development, as pointed out in the previous sections. Also, many promising technologies are still to be developed. In this sense, many possibilities are prospected for value addition in the sugarcane processing chain, but this depends on investments in scientific research, technological development, and technology transfer.

7. Conclusions

Sugarcane is one of the main world crops, with a history of success in its exploitation and research developments. Its processing chain has, however, some bottlenecks and high environmental impacts, which have stimulated researchers on the search of new methodologies, economic and more sustainable solutions. Brazil government policies have created the unique scenario for the sugarcane biorefinery strategy represents a possible way to compete with fossil fuels through the production of biofuels, bioenergy and other bioproducts. The future of sugarcane biorefineries holds numerous questions, and the answers are complex in terms of sustainability and depend on several external factors. Even so, in Brazil, the traditional products obtained from sugarcane – sugar, bioethanol and bioelectricity – remain as strategic goods produced by mature technologies with significant expansion potential for at least the next decades to attend the high internal demand of flex vehicles. However, the 2G and 3G technologies are being installed to amplify the portfolio of bioproducts and guarantee a more sustainable sugarcane biofactory of the future.

4. A biorefinery approach for enzymatic complex production for the synthesis of xylooligosaccharides from sugarcane bagasse

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Abstract

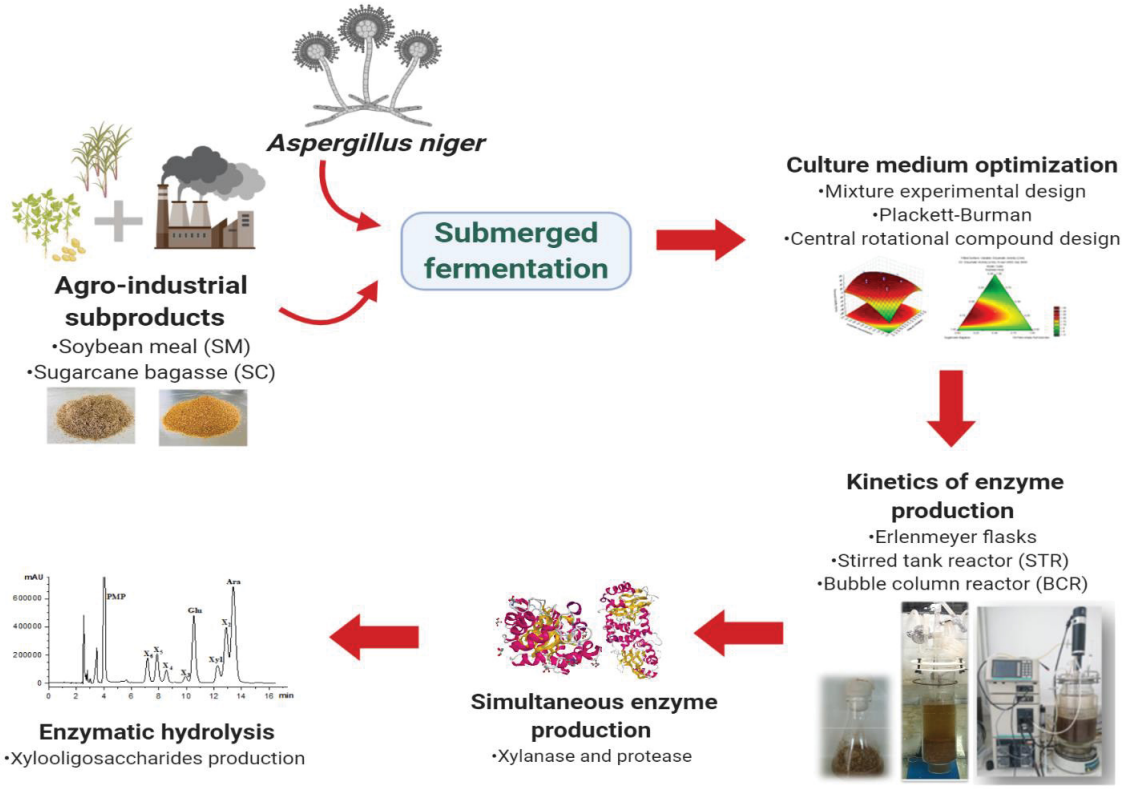
The use of low-cost feedstock for enzyme production is an environmental and economic solution. Sugarcane bagasse and soybean meal are employed in this study for optimised xylanase production with the concomitant synthesis of proteases. The enzymatic complex is produced by submerged fermentation by *Aspergillus niger*. Optimisation steps lead to a 2.16-fold increase in enzymatic activity. The fermentation kinetics are studied in Erlenmeyer flasks, a stirred tank reactor and a bubble column reactor, with the xylanase activities reaching 52.9; 33.7 and 60.5 $U.mL^{-1}$, respectively. The protease production profile is also better in the bubble column reactor, exceeding 7 $U.mL^{-1}$. The enzyme complex is then evaluated for the synthesis of xylooligosaccharides from sugarcane extracted xylan with a production of 3.1 $g.L^{-1}$ where xylotriose is the main product. Excellent perspectives are observed for the developed process with potential applications in the animal feed, prebiotics and paper industries.

Keywords: Xylanase; Protease; sugarcane bagasse; submerged fermentation; xylooligosaccharides

Highlights

- Sugarcane bagasse and soybean meal as substrate for low-cost enzyme production.
- Highly efficient xylanase production through submerged fermentation in BCR.
- Concomitant xylanases and proteases production.
- Application of produced xylanases in XOS production.
- Xylotriose appeared as the main produced XOS.

Graphical abstract



1. Introduction

The awareness of environmental pollution and increased scientific development have allowed for the recovery of an extensive range of valuable and usable by-products, which have previously been considered as waste Wei; Shannon (2020). In recent years, different agroindustrial subproducts have been recycled, reused and introduced into biotechnological processes, thus creating new possibilities for the massive use of these residues in the development of value-added products.

The agroindustry plays a significant role in the Brazilian economy, with Brazil being the largest producer of sugarcane with an annual production of 746.83 Mt. Brazil is also the second largest producer of soybeans with an annual production of 117.89 Mt and one of the growing oil palm fruit producers with an annual production of 1.57 Mt FAO, (2018) and Karuga, (2017). Sugarcane bagasse (SB), soybean meal (SM) and oil palm empty fruit bunches (OPEFB) are the residual wastes generated at ton-scale by the sugar, bioethanol and biodiesel industries. These agroindustrial residues are mainly composed of lignocellulosic compounds Medina et al., (2015), Esa, (2014), and Heuzé et al., (2017). Fungi can degrade these residues through a set of synergistically acting enzymes.

The main enzyme systems produced by fungi from lignocellulosic residues are cellulases Xue et al. (2020) and hemicellulases Alberton et al. (2009) and Dilokpimol et al. (2020). Xylanases (EC 3.2.1.8) are hemicellulases that hydrolyse the β-1,4 linked chains of xyloses to produce small xylooligosaccharides (XOS). The heterogeneity and complexity of xylan has resulted in a wide range of xylanases with different specificities. Different isoforms apparently cooperate to enhance the hydrolysis of xylan. Within the classification system that is based on amino acid sequence similarity, xylanases are usually grouped into glycoside hydrolase (GH), families GH10 and GH11 Morgan et al.

(2017). These enzymes have a variety of industrial applications due their structure-function relationships and are a subject of intense research, including bakery products, animal feed, textiles, cellulose pulp and paper, pharmaceuticals and chemicals Chadha et al. (2019), Orozco Colonia et al. (2019) and Polizeli et al. (2005).

XOS are xylose oligomers, which are mainly derived from hemicellulose that are found in plant biomass. These oligosaccharides can be produced by physicochemical hydrolysis, using different solvents Brenelli, Lívia B. et al. (2020) and Liu et al. (2019) and by enzymatic hydrolysis using xylanases Lian et al. (2020) and Rahmani et al. (2019). Several studies have shown that XOS, which are non-digestible for humans, can selectively stimulate the growth of probiotic bifidogenic and lactic acid bacteria Cho et al. (2020), Gullón et al. (2011) and Lian et al. (2020). Thus, there has been a significant increase in the interest in XOS production with different degrees of polymerisation (DP) that allow their uptake by probiotic strains.

The main purpose of the present work is to produce xylanases by submerged fermentation with solids in suspension using different agro-industrial sub-products, such as SB, SM and OPEFB, obtained from the Brazilian agroindustry. This study also applies the biorefinery concept in the synergistic production of xylanases and other enzymes (such as proteases), and the application of enzyme complexes in XOS production through enzymatic hydrolysis of xylan from SB. As far as we know, this is the first report on the use of SB and SM in xylanase production through submerged fermentation with solids in suspension, without the need for a previous chemical or enzymatic pre-treatment.

2. Material and methods

2.1. Microorganisms

Thirteen strains of the genera Aspergillus sp. and Rhyzopus sp. from the culture bank of the Bioprocess Engineering and Biotechnology Laboratory at UFPR were tested. Strains were transferred to potato dextrose agar slants that were incubated at 30 ºC for 144 hours with periodic renovation.

Xylanase production was performed in Erlenmeyer flasks (250 mL) with 5 g of SB as a substrate and a mineral salt solution composed of ($g.L^{-1}$): K_2HPO_4, 1.5; $MgSO_4.7H_2O$, 0.3; $CuSO_4.5H_2O$, $CaCl_2.2H_2O$, 0.005; $NaNO_3$, 3; $FeSO_4.7H_2O$, 0.009; $CoSO_4.7H_2O$, 0.02 $ZnSO_4.7H_2O$, 0.002 and $MnSO_4.H_2O$, 0.012 Maciel et al. (2009). After sterilisation at 121 °C during 15 min), the medium was inoculated at 10^6 $spores.mL^{-1}$. Enzyme production was carried out at 30 °C and 120 rpm for 96 hours. All experiments were conducted in triplicate.

2.2. Optimisation of xylanase production

Three steps for the optimisation of xylanase production were carried out using fractional and complete factorial designs. Experiments were carried out with the previously selected strain *Aspergillus niger* LPB BC.

An initial optimisation of a substrate mixture ratio for xylanase production is carried out using three alternative lignocellulosic substrates (SB, SM and OPEFB) at different ratio mixtures (Table 1). The influence of medium composition and fermentation factors on xylanase production was then determined using Plackett–Burman (PB) designs with seven factors and eight runs for the screening of significant factors. The studied factors were grouped into two factorial designs: PB1, composed of sodium nitrate, urea, ammonium sulfate, potassium hydrogen phosphate, magnesium sulfate, copper sulfate

and trace elements; and PB2, composed of glucose, saccharose, inoculum ratio, medium pH, SB and SM particle size (Table 2). Finally, a rotational central composite design (RCCD) was employed and analysed by the response surface methodology. The experimental design was composed of five factors: substrate concentration, inoculum rate, medium pH, K_2HPO_4 concentration and $CuSO_4.5H_2O$ concentration; 54 essays and four central points per block, in three blocks.

Table 1. Mixture design for substrate composition optimization for xylanase production

Assays	Factor			Enzymatic activity ($U.mL^{-1}$)
	SB (% $w.v^{-1}$)	OPEFB (% $w.v^{-1}$)	SM (% $w.v^{-1}$)	
1	5.000	0.000	0.000	0.84 ± 0.00
2	0.000	5.000	0.000	0.26 ± 0.00
3	0.000	0.000	5.000	6.76 ± 2.48
4	1.667	3.333	0.000	0.31 ± 0.03
5	1.667	0.000	3.333	2.23 ± 0.03
6	0.000	1.667	3.333	1.88 ± 1.02
7	3.333	1.667	0.000	0.14 ± 0.18
8	3.333	0.000	1.667	31.14 ± 2.88
9	0.000	3.333	1.667	8.71 ± 1.03
10	1.667	1.667	1.667	18.70 ± 1.39

Table 2. Optimization of nutrient solution and medium composition for xylanase production in submerged fermentation

Group	Assay	Factor							Enzymatic activity ($U.mL^{-1}$)
		$NaNO_3$ (g.L-1)	CH_4N_2O (g.L-1)	$(NH_4)_2SO_4$ (g.L-1)	K_2HPO_4 ($g.L^{-1}$)	$MgSO_4$ ($g.L^{-1}$)	$CuSO_4.5H_2O$ ($g.L^{-1}$)	Trace element	
PB1	**1**	0	0	0	2	0.3	0.4	0	24.46 ± 1.80
	2	3	0	0	0	0	0.4	1	13.31 ± 0.56
	3	0	1.5	0	0	0.3	0	1	1.36 ± 0.40
	4	3	1.5	0	2	0	0	0	14.75 ± 0.82
	5	0	0	1.5	2	0	0	1	13.34 ± 0.50
	6	3	0	1.5	0	0.3	0	0	11.21 ± 0.74
	7	0	1.5	1.5	0	0	0.4	0	13.47 ± 1.22
	8	3	1.5	1.5	2	0.3	0.4	1	15.86 ± 0.41

	Assay	Substrate concentration (% $w.v^{-1}$)	Glucose ($g.L^{-1}$)	Saccharose ($g.L^{-1}$)	Inoculum rate ($spores.mL^{-1}$)	pH	SB particle size (mm)	SM particle size (mm)	Enzymatic activity ($U.mL^{-1}$)
PB2	**1**	2.5	0	0	10^6	6.5	0.85-1.18	0.17-0.35	35.79 ± 1.57
	2	5.0	0	0	10^5	5	0.85-1.18	0.35-0.85	30.44 ± 3.50
	3	2.5	10	0	10^5	6.5	0.35-0.85	0.35-0.85	22.87 ± 4.78
	4	5.0	10	0	10^6	5	0.35-0.85	0.17-0.35	19.13 ± 3.55
	5	2.5	0	5	10^6	5	0.35-0.85	0.35-0.85	36.96 ± 1.09
	6	5.0	0	5	10^5	6.5	0.35-0.85	0.17-0.35	24.39 ± 1.90
	7	2.5	10	5	10^5	5	0.85-1.18	0.17-0.35	7.75 ± 2.79
	8	5.0	10	5	10^6	6.5	0.85-1.18	0.35-0.85	34.08 ± 3.42

Trace element ($g.L^{-1}$): $FeSO_4*7H_2O$, 0.009; $CoSO_4*7H_2O$, 0.02 $ZnSO_4*7H_2O$, 0.002; and $MnSO_4*H_2O$, 0.012

2.3. Kinetic study of xylanase production in different bioreactors

Kinetics of xylanase production was conducted in three different bioreactors: Erlenmeyer flasks (250 mL), a stirred tank reactor (STR) and a bubble column reactor (BCR). Flasks were incubated at 30 °C and 120 rpm for 144 h. A 10.5 L BioFlo 110 fermenter (New Brunswick, Edison, NJ, USA) was employed for xylanase production with a work volume of 5 L using previously optimised conditions. The process was conducted at 30 °C, 200 rpm and 1 vvm. A 1.5 L BCR with a working volume of 1 L and a length-to-diameter ratio of 4.4 was employed. Fermentation was carried out at 30 °C with an aeration rate of 2 vvm, resulting in a superficial gas velocity of 0.377 cm/s for 192 h. Silicone oil at 5 $mL.L^{-1}$ (Dow Chemical Inc., USA) was added to control foaming. Samples were withdrawn each 24 h.

2.4. Determination of enzyme activities

Xylanase and cellulase activities were assayed using 1% beechwood xylan (Megazyme, Denmark) and 2% carboxymethilcellulose (Sigma, USA), respectively, in a 50 mM citrate-phosphate buffer (pH 5.8) Bailey et al. (1992). Released reducing sugars were determined according to Miller (1959). A unit of enzymatic activity (U) was defined according to the international system of units (SI) as the enzyme amount that produces 1 μmol of xylose and glucose per minute.

Protease activity was determined according to Siala et al. (2012) using haemoglobin (Sigma, USA). A volume of 100 μL of appropriately diluted enzyme solution was added to a solution composed of 100 μL of haemoglobin 1% ($w.v^{-1}$) and 100 mM glycine-HCl (pH 3). The reaction was carried out at 60 °C for 10 min and interrupted with the addition of 200 μL of trichloroacetic acid 8% ($w.v^{-1}$). Samples were centrifuged at 10,000×g for 10 min at 10 °C, then the absorbance was determined at 285 nm. A standard curve was

generated using tyrosine solutions. The necessary amount of enzymes for the release 1 mol of tyrosine per minute was determined as a unit of enzyme activity (U). Experiments were carried out in triplicate and enzymatic blanks were measured for each essay.

2.5. Xylooligosaccharide production by enzymatic hydrolysis of crude xylan extracted from SB

SB was soaked with 40% ($w.w^{-1}$) of NaOH for 2 h at 60 °C according to Sporck et al. (2017). The liquid fraction was concentrated and then the hemicellulose was precipitated with two volumes of 95% ($v.v^{-1}$) ethanol. The recovered sediment was washed twice with 70% ($v.v^{-1}$) ethanol, dried at 60 °C and used as crude xylan. Crude and commercial xylan (beechwood xylan) samples were hydrolysed using the produced enzymatic complex, with 1.6 g of xylan diluted in 100 mL of a citrate-phosphate buffer solution (50 mM, pH 5.8), the xylanase complex added at 5 $U.mL^{-1}$. Enzymatic hydrolysis was performed in shake flasks at 50 °C and 100 rpm for 1 h. For enzymatic hydrolysis interruption, samples were boiled for 10 min. Then, they wer, centrifuged at 10,000×g for 5 min, filtered and stored at 4 °C for further HPLC analysis.

2.6. Analytical procedures

1-Phenyl-3-methyl-5-pyrazolone (PMP) was used for the derivatisation of XOS, as described by Li et al. (2013). Briefly, 250 µL of hydrolysates, individual or mixed analytical standards of XOS and monosaccharides, were placed in a 2 mL centrifuge tube, followed by the addition of 250 µL of 0.3 M NaOH and 250 µL of 0.5 M PMP in methanol. Samples were maintained at 70 °C for 30 min and then 500 µL of chloroform were added to remove the PMP in excess. After vigorous shaking and centrifugation (6000×g), the organic phase was carefully discarded to remove the excess of reagents. The extraction procedure was repeated three times. The aqueous phase was diluted with

water and filtered through a 0.22 μm membrane. The XOS with DP 2–6 was determined using a HPLC Agilent 1200 series with a C_{18} column and a diode-array detector at 245 nm. Elution was carried out with a solution composed of a ratio of sodium phosphate buffer (40 mM, pH 8.0)/acetonitrile (81:19, $v.v^{-1}$) at 0.5 $mL.min^{-1}$. Standards XOS with DP 2–6 (X2-X6)) (Megazyme, Campinas, Brazil) were used.

Reducing sugars' concentration, including glucose, xylose and arabinose, and the organic acids (citric, acetic and propionic acids) were quantified by HPLC. Samples were filtered through a 0.22 μm membrane (Millipore Corp., Billerica, MA, USA). An Aminex HPX 87H column (300 by 7.8 mm; Bio-Rad, Richmond, CA, USA) was used at 60 ºC with 5 mM H_2SO_4 mobile phase and a flow rate of 0.6 $mL.min^{-1}$. A refractive index detector (HPG1362A; Hewlett-Packard Company, São Paulo, Brazil) was employed.

2.7. Determination of metal ion composition of fermentation medium

ICP-OES (Varian, Model ES 720, Palo Alto, CA, USA) was used for metal ion determination with a solid-state detector using an axial arrangement simultaneously. Mn^{2+} was used as standard solution (5.0 $mg.L^{-1}$) to align the torch horizontally and vertically . A stabilisation time of 15 s was used for sample analysis with a sample washing time of 3 s and sample delay of 30 s. The power and the pressure of the nebulizer were 1.10 kW and 180 kPa, respectively. While the plasma gas flow and the auxiliary gas flow were both kept at $1.5L.min^{-1}$. The triplicate reading time was 3s.

2.8. Statistical analysis

The statistical analysis was carried out by Statistica 8 and Design expert 10 software.

3. Results and discussion

The strain *A. niger* LPB BC was first tested for xylanase production with a concentration of 27 $U.mL^{-1}$ using SB as the substrate suspended in a nutritive solution. The strain was then used in the optimisation steps.

3.1. Optimisation of xylanase production

According to Figure 1, the xylanase activity increased with increased concentrations of SB and SM in the fermented medium. The best xylanase production (31.14 $U.mL^{-1}$) was reached with 66.67% of SB and 33.33% of SM (Table 1). Other authors reported lower xylanase production using only SB as the substrate: 24.31 $U.mL^{-1}$ Alberton et al. (2009), 8.4 $U.mL^{-1}$ Bocchini et al. (2005) and 8.99 $U.mL^{-1}$ Di Marco et al. (2017). This fact shows the importance of substrate composition. In this case, SM with a higher protein concentration probably promoted a better growth of the fungus, leading to higher enzyme production.

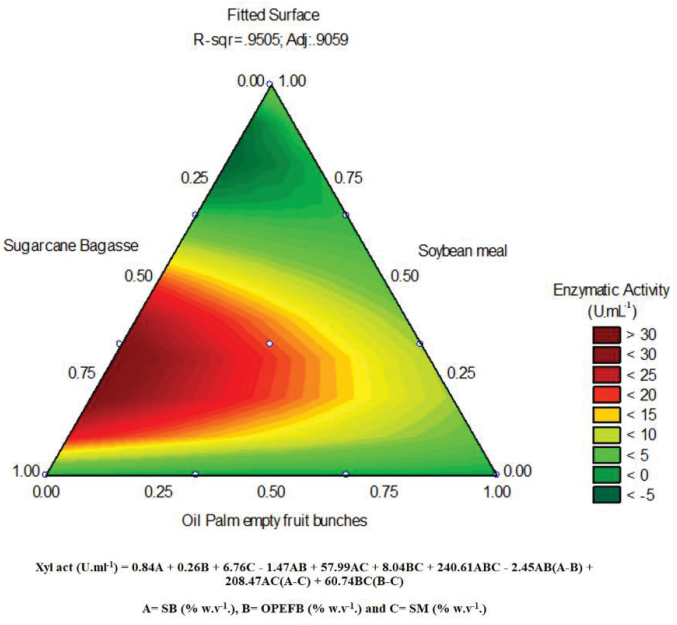


Figure 1. Contour plot of the Mixture experimental design for xylanase production optimization – Study of media composition

Equation shown in Figure 1 gives the correlation, which represents xylanase activity according to the influence of different substrate ratios ($R^2 = 0.95$). The highest estimated production of xylanases, according to the model, was 29.54 $U.mL^{-1}$, which corresponds to a composition of 73.77% of SB, 26.23% of SM and 0% of OPEFB. The experimental activity of xylanases with optimal conditions was 28.89 $U.mL^{-1}$, which represents 97.83% of the prediction.

The production of xylanases is probably induced by the high presence of hemicellulose in SB (27–32%) Esa (2014). In this sense, it could be considered that the high protein content of SM (44–50%) Heuzé et al. (2017) probably stimulated the fungal growth. In the absence of simple and easily metabolised carbon sources, hemicellulose from SB certainly induced xylanase production. In contrast, the use of OPEFB did not have a significant effect on xylanase production due to their low hemicellulose composition (19%) and higher contents of cellulose (30.5%) and lignin (33%) (Medina et al., 2015), which hinders the degradation of hemicelluloses.

Different studies have showed the importance of salts, sugar and physicochemical factors in xylanase production Ghanem et al. (2000) and Pérez-Rodríguez et al. (2014). The compositions of salts in the culture medium act as important osmosis controllers for cells, and several salts, also known as micronutrients, are redox active and can act as active enzyme cofactors Hänsch; Mendel (2009). It was possible to observe the strong effect of potassium hydrogen phosphate and copper sulfate on xylanase production, as previously reported Ghanem et al. (2000) and Park et al. (2002). Nonetheless, magnesium sulfate and trace element solutions did not have a significant effect on xylanase production (Table 2).

In contrast, the positive influence of inoculum rate, SM particle size and pH on xylanase production was observed. The increase in inoculum rate plays an important role in fermentation processes due to the reduction of the latency time observed during the initial phase of enzyme production Rousk et al. (2010), A slightly acidic pH can improve the initial growth of the fungus Niu et al. (2016) but acidic environments can negatively influence the production of xylanases. Finally, small SM particle sizes improved the nutrient bioavailability for the filamentous fungi. Alternatively, glucose and saccharose, as carbon sources, were not positive inducers, possibly because these components stimulate other metabolic pathways and biomolecule production rather than enzyme synthesis (Table 2).

The analysis of the micronutrient composition of the SC and SM medium showed high contents of magnesium, iron, calcium, aluminium, sodium and other micronutrients (Table 3). This could explain why medium supplementation was not necessary for xylanase production. In addition, the use of SM eliminates the need for other nitrogen sources due to its high protein content that helps fungal growth and the synthesis of enzymes.

Table 3: Ions composition of sugarcane and soybean meal

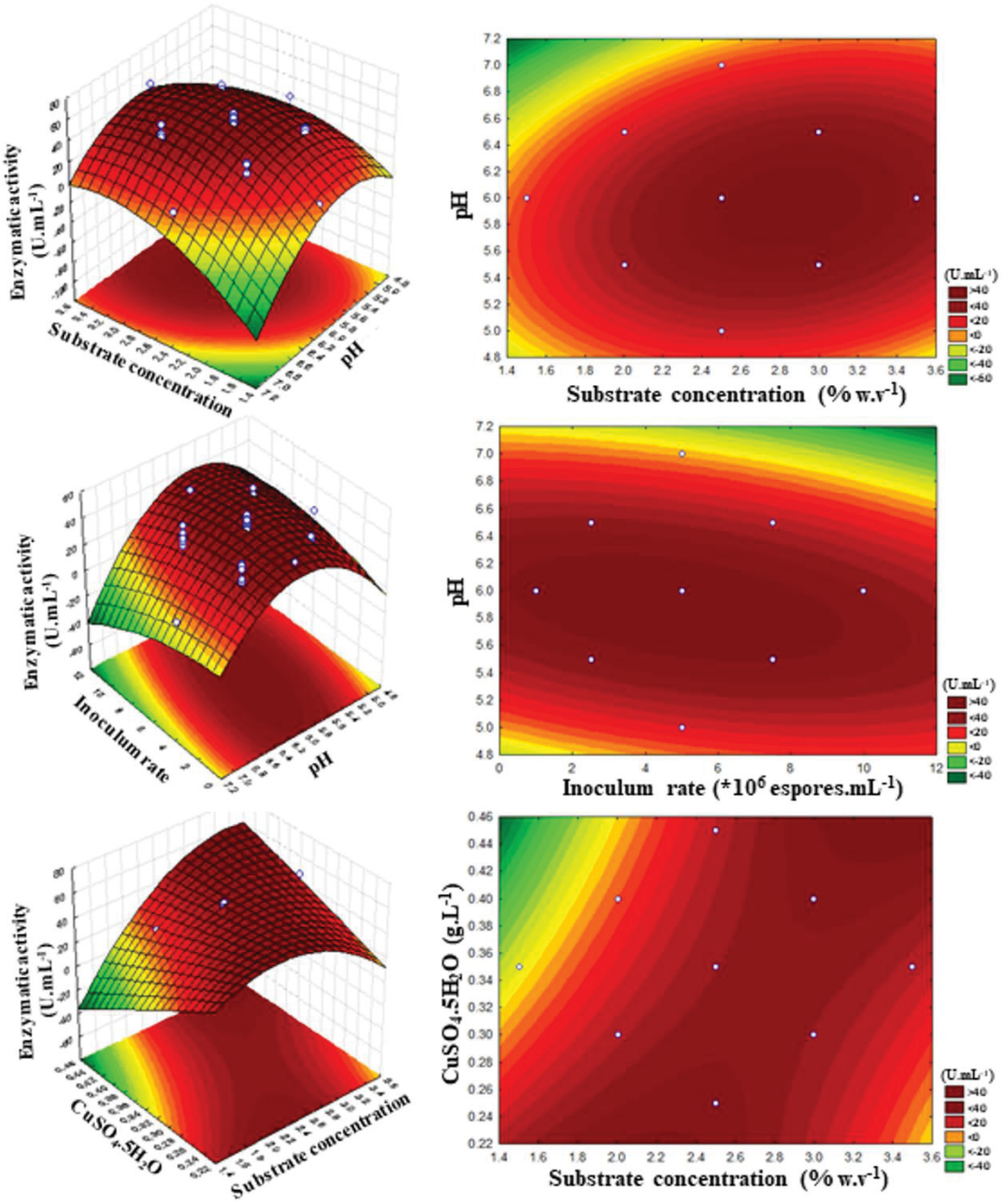
	Substrate	
	Sugarcane Bagasse	Soybean meal
Al	519.11 ± 79.82	554.505 ± 57.03
Ba	2.305 ± 0.77	2.915 ± 0.21
Ba	ND	ND
Cd	ND	ND
Ca	747.03 ± 50.3	2720.79 ± 41.24
Co	ND	ND
Cu	5.435 ± 0.26	17.38 ± 0.68
Fe	978.06 ± 93.95	182.58 ± 25.96
P	213.01 ± 7.39	10242.45 ± 76.16
Li	32.075 ± 1.48	25.3 ± 5.01
Mg	427.39 ± 10.88	3216.93 ± 51.04
Mn	ND	ND
Mo	ND	4.08 ± 0.23
Ni	ND	ND
K	3763.855 ± 49.22	76225.15 ± 12.09
Se	ND	ND
Na	717.535 ± 42.62	873.9 ± 12.28
V	2.28 ± 0.31	ND
Zn	16.195 ± 0.06	75.605 ± 5.013

The third step of optimisation employed a RCCD design with five factors: inoculum ratio, medium pH, copper sulfate, potassium hydrogen phosphate and substrate concentration. Xylanase activities varied from 0.642 to 55.283 $U.mL^{-1}$. The best adjusted model to explain the variation of xylanase activity as a function of the studied factors was the quadratic model ($R^2 = 0.85$, p-value < 0.05 and F-value = 9.37, Table 4). Substrate concentration (A), initial pH (C), copper sulfate (E) and their respective interactions (AC, AE, BC, A^2 and C^2) showed significant influences ($p < 0.05$) on xylanase production (Table 4). The model that represents xylanase activity is given by equation into the Figure 2. Finally, the best conditions for maximum xylanase production (52.87 $U.mL^{-1}$ or 1965.43 $U.g^{-1}$ of substrate) were determined as: substrate concentration (2.69% w/v),

inoculum rate ($4.33*10^6$ spores.mL^{-1}), pH (5.96), potassium hydrogen phosphate (3.186 $g.L^{-1}$) and copper sulfate (0.327 $g.L^{-1}$).

Table 4. ANOVA for Response Surface Quadratic model DCCR of Xylanase production

Source	Sum of Squares	df	Mean Square	F-value	p-value
Block	643.93	2	321.97		
Model	10181.46	20	509.07	9.37	< 0.0001
A-Substrate Concentration	2989	1	2989	55.04	< 0.0001
B-Inoculum rate	30.85	1	30.85	0.57	0.4567
C-pH	724.42	1	724.42	13.34	0.0009
D- K_2HPO_4	175.22	1	175.22	3.23	0.0822
E- $CuSO_4.5H_2O$	425.75	1	425.75	7.84	0.0087
AB	1.45	1	1.45	0.027	0.8713
AC	294.13	1	294.13	5.42	0.0267
AD	34.62	1	34.62	0.64	0.4307
AE	1160.34	1	1160.34	21.37	< 0.0001
BC	252.46	1	252.46	4.65	0.0389
BD	58.74	1	58.74	1.08	0.3064
BE	63.37	1	63.37	1.17	0.2883
CD	1.63	1	1.63	0.03	0.8634
CE	135.82	1	135.82	2.5	0.1239
DE	7.11	1	7.11	0.13	0.72
A^2	1110.33	1	1110.33	20.45	< 0.0001
B^2	54.89	1	54.89	1.01	0.3225
C^2	2202.26	1	2202.26	40.56	< 0.0001
D^2	43.86	1	43.86	0.81	0.3757
E^2	1.04	1	1.04	0.019	0.8906
Residual	1683.37	31	54.3		
Lack of Fit	1485.79	22	67.54	3.08	0.0425
Pure Error	197.58	9	21.95		
Cor Total	12508.76	53			



Xyl act (U.ml^{-1}) = -1151.3 - 11.1A + 8.7B + 388.6C + 37.9D - 123.2E - 0.2AB + 12.1AC - 4.2AD + 240.9AE - 2.3BC + 1.1BD + 11.3BE + 0.9CD - 82.4CE - 18.9DE - 23.1A^2 - 0.2B^2 - 32.5C^2 - 4.6D^2 - 70.8E^2

A=Substrate concentration (% w/v), B=Inoculum rate (espores.mL^{-1}), C=pH, D= K_2HPO_4 (g.L^{-1}) and E= $CuSO_4.5H_2O$ (g.L^{-1})

Figure 2. Three-dimensional response surface plot of the RCCD experiment and contour plot of the calculated response for the studied factors and their influence on xylanase production

3.2. Kinetics of xylanase production in Erlenmeyer flasks

The strong correlation between the experimental and statistical results confirms the validity of the response model and the existence of an optimal point. The xylanase activity production profile by *A. niger* LPB BC in Erlenmeyer flasks is shown in Figure 3A. Maximum xylanase production (52.87 $U.mL^{-1}$) was obtained after 4 d of fermentation with high productivity (13.22 $U.mL^{-1}.d^{-1}$ or 491 $U.g^{-1}d^{-1}$). The cellulase (CMCases) activity was also detected, reaching 5.92 $U.mL^{-1}$ on the third day. Pectinase and mannanase production was not significantly detected.

From Figure 3B, it is possible to observe a low concentration of glucose in the initial phase of the process, which could be due to the hydrolysis of SB during the sterilisation process remaining constant until the end of the fermentation. This may indicate that the microorganisms did not consume this sugar and there was no enzymatic action on cellulose. In contrast, the concentration of xylose increased concomitantly with xylanase production, until reaching its maximum at 48 h. Therefore, it is possible to state that enzyme production is associated with sugar liberation and consumption for microbial growth Mali et al. (2019).

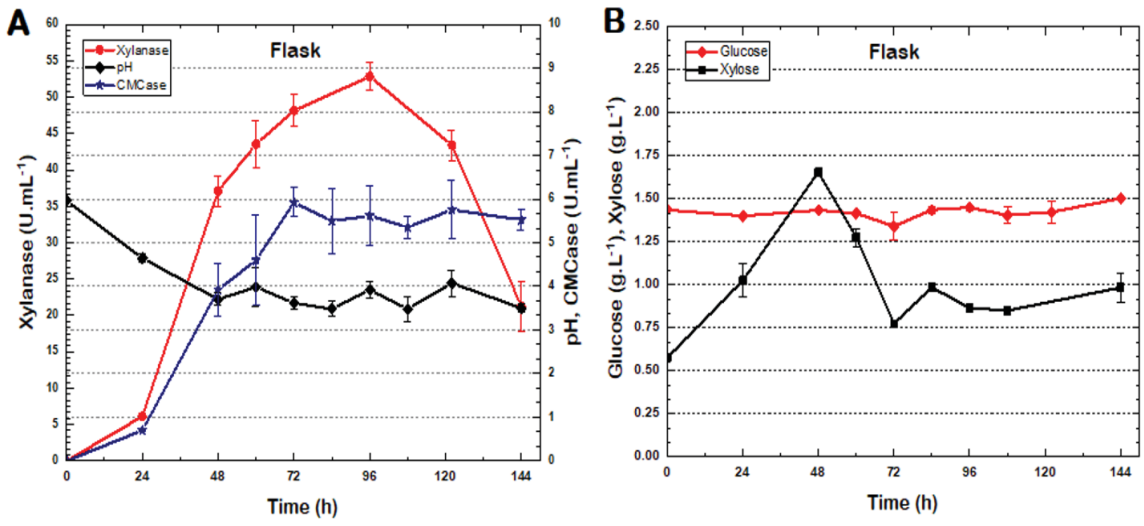


Figure 3. A: Time course profile of enzyme production by *Aspergillus niger* LPB BC in Erlenmeyer flasks; B: Evolution of sugars concentration.

Several authors have worked on the production of xylanases using different fungal and bacterial strains, including recombinant strains. Elegbede and Lateef (2017) used corncob as a carbon source for xylanase production with *Aspergillus fumigatus*, reaching 50.55 $U.mL^{-1}$ with a productivity of 7.22 $U.mL^{-1}.d^{-1}$. Di Marco et al. (2017) obtained a lower production (8.99 $U.mL^{-1}$) using *Paenibacillus sp*. and SB as a substrate with a productivity of 4.49 $U.mL^{-1}.d^{-1}$. In contrast, Hernández-Domínguez et al. (2014) employed a strain of *Stenocarpella maydis* and pure xylan as a carbon source, obtaining a production of 18.02 $U.mL^{-1}$ and 3 $U.mL^{-1}.d^{-1}$. It is clear that the production varies greatly according to the substrate used and the employed strain. These differences may be attributed to the medium composition and process conditions. The production obtained in this work was higher (52.87 $U.mL^{-1}$) with an excellent productivity of 13.22 $U.mL^{-1}.d^{-1}$.

3.3. Xylanase production in different bioreactors

The optimised conditions were employed for xylanase production in a STR and a BCR. In both cases, the forced aeration significantly affected the growth, mycelia formation and, consequently, the xylanase production. The enzyme production profile in both bioreactors showed significant differences (Figure 4). In the case of the STR, the highest xylanase activity (33.73 $U.mL^{-1}$) was obtained after 3 d with a productivity of 11.24 $U.mL^{-1}.d^{-1}$, followed by a sudden decrease in xylanase production to 5 $U.mL^{-1}$. In contrast, in the BCR, the maximum peak of xylanase production was 60.45 $U.mL^{-1}$ at 7 d, with a productivity of 8.64 $U.mL^{-1}.d^{-1}$. As observed in the Erlenmeyer flask fermentation, low CMCase activities were also detected. The protease activity was evaluated with a production of 7.35 $U.mL^{-1}$ in the BCR. Thus, the BCR system exhibited better performance for enzyme production using SB and SM in suspension. This could be

explained by the best homogenisation and lower shear stress originating from the mechanic agitation. The agitation of the BCR is pneumatic, which is promoted by ascending bubbles having a lower impact on filamentous fungi Libardi et al. (2019).

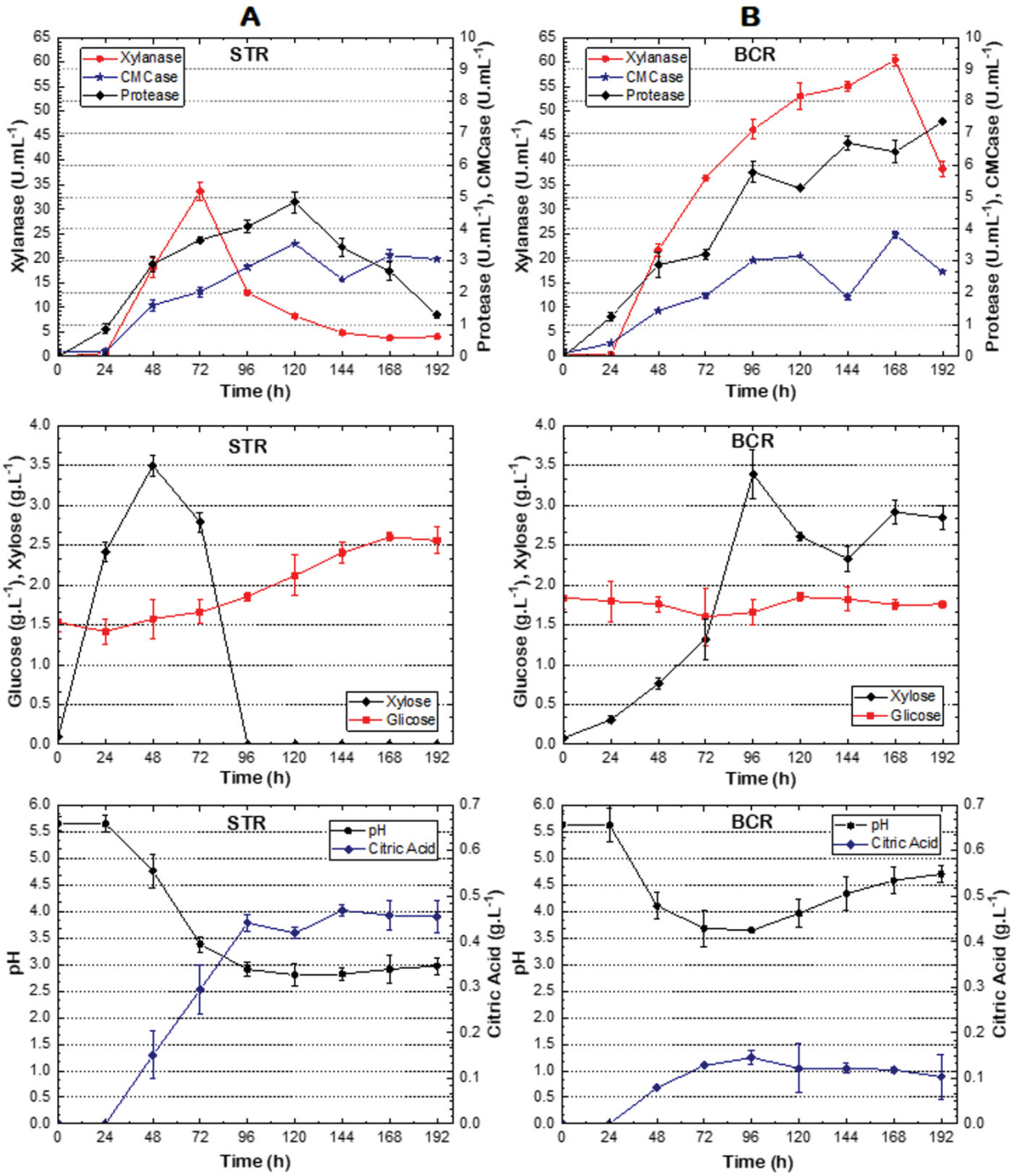


Figure 4. Time course profile of enzymes' production, sugars concentration, and citric acid production and pH variation by *Aspergillus niger* LPB BC. Column A: STR bioreactor and Colum B: BCR bioreactor.

The sugar concentration profile in BCR was similar to that obtained in Erlenmeyer flasks with constant glucose and xylose concentrations for xylanase production until the stationary phase. Alternatively, in the STR system, an increase in glucose concentration was observed compared to the BCR and Erlenmeyer flasks, with the total xylose consumption after 96 h. Therefore, a change in the fungus metabolism can be suggested, which generated a different profile of metabolite and protein secretion, mainly caused by the stress conditions induced by mechanical agitation and forced aeration. In this case, other enzymes were produced from SB with glucose liberation. The HPLC analysis also showed a high citric acid production in the STR, resulting in an accentuated pH decrease to levels below 3. In fact, *A. niger* is one of the largest producers of this organic acid.

Furthermore, an initial production of citric acid was detected in the BCR that stabilised at pH 4. Different reports and studies have showed that pH control is a very important factor during enzyme production and influences the expression of extracellular enzymes Niu et al. (2016) and Peñalva et al. (2002), because it can cause concatenate variations in metabolic pathways. High aeration and stress levels caused by mechanical agitation in the STR, probably induced a higher acid production Ferreira et al. (2016), decreasing the pH, while in the BCR at a stable pH, better enzyme production was observed (Figure 4). These results could explain the high differences in enzyme production in the STR and BCR, which demonstrated that pH control may be the key point in the production of enzymes at a large scale. This shows that the developed process has excellent perspectives to be applied at large scales with a proper choice of bioreactor and process conditions.

3.4. Enzymatic hydrolysis by produced xylanase complex

XOS production from lignocellulosic biomass is generally carried out by physical-chemical and biological methods. Within these methods, the enzymatic hydrolysis with xylanolytic enzymes is one of the most studied due to its high efficiency and specificity Rahmani et al. (2019). The hydrolytic power of the enzyme complex produced by *A. niger* BC was evaluated with commercial xylan and xylan extracted through alkaline methods Sporck et al., (2017).

The enzymatic complex showed excellent efficiency for the enzymatic hydrolysis of xylan for XOS production (Figure 5). The total XOS amounts released from commercial xylan and extracted xylan were 6.9 and 3.1 $g.L^{-1}$, respectively. Xylotriose was the main released oligosaccharide after enzymatic action with 4.3 and 2.3 $g.L^{-1}$ with commercial and extracted xylan, respectively. After the enzymatic hydrolysis of commercial xylan, xylobiose (0.74 $g.L^{-1}$), xylotetrose (0.53 $g.L^{-1}$) and xylohexose (1.23 $g.L^{-1}$) were also released. In contrast, with the extracted xylan from SB, xylobiose (0.33 $g.L^{-1}$) and xylohexose (0.26 $g.L^{-1}$) were also highly produced. In addition, arabinose production was observed, which was not detected in the commercial xylan hydrolysis. These variations in the DP of XOS and monosaccharides released by enzymatic hydrolysis are highly related to the employed enzyme complex, the xylan nature and the hydrolysis condition (the type of enzyme, enzyme loading, substrate, pH and temperature). The different profiles of the produced XOS make the comparison with the results obtained by other authors difficult Brenelli, Lívia B. et al. (2020), Mandelli et al. (2014) and Rahmani et al. (2019).

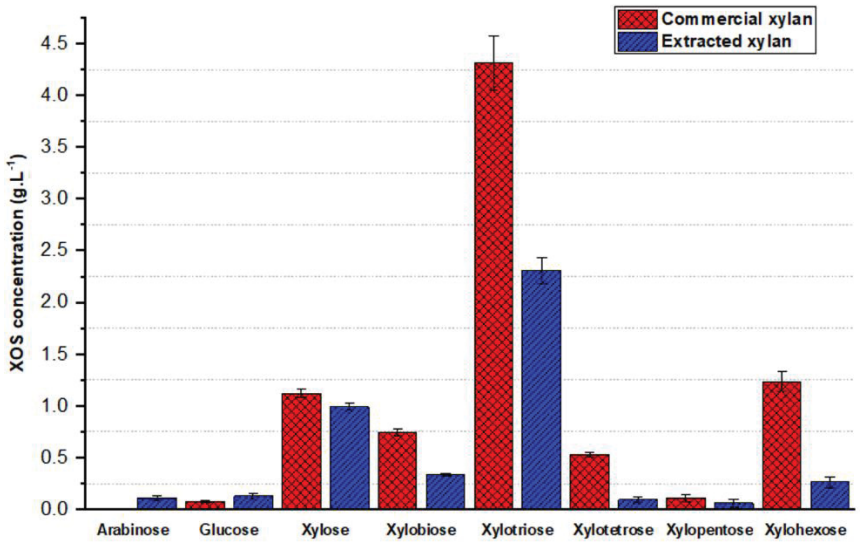


Figure 5. Profile of different XOS produced enzymatic hydrolysis of commercial and extracted xylan from sugarcane bagasse at 1h and 50 °C.

The XOS production from commercial and extracted xylan showed maximum yields of 43.3 and 19.1 g of XOS per 100 g of xylan, respectively. The XOS release values by enzymatic action are quite variable according to the type of substrate used. In the literature, different XOS yields are reported, such as 28.6 g of XOS per 100 g of xylan extracted from corncobs Teng et al. (2010), 17.5 g of XOS per 100 g of xylan extracted from OPEFB Mazlan et al. (2019), 35.3 g of XOS per 100 g of xylan extracted from sugarcane straw Brenelli, Lívia B. et al. (2020) and 31.8 g per 100 g of xylan extracted from SB Bian et al. (2013). Significant differences are mainly due to the characteristics and physicochemical structure of xylan, which significantly depends on the type of treatment used in the extraction. These characteristics have a significant effect on the hydrolytic efficiency and synergistic action of enzymes. Therefore, the low purity of the extracted xylan from SB, with cellulose or lignin residues, may act as a barrier that hinders the action of the enzyme complex Li et al. (2017).

The prebiotic efficacy of XOS is directly related to the DP of the main chain, so smaller chains with a DP of 2–4 are more efficient in the growth of certain probiotics, mainly of the genus *Bifidobacterium* Ho et al. (2018), Lian et al. (2020) and Wang et al. (2010). The results obtained in this work show a significant abundance of XOS with a low DP, where xylobiose and xilotriose (DP 2–3) represent 73% and 86.4% of total XOS produced in the commercial and extracted xylan, respectively.

In contrast, Lian et al. (2020) suggest that XOS has a better performance as prebiotics with the absence or low presence of monosaccharides, such as glucose and xylose. For this reason, the enzyme-catalysed hydrolysis application is more efficient, since the enzymes used, such as β-xylosidase, can be suppressed and avoid monosaccharide release, such as xylose. In fact, in this work, there is a lower release of monosaccharide releases in both types of xylan evaluated, even showing the absence of arabinose release in commercial xylan. The total glucose, xylose and arabinose released by the enzymatic complex only represented 7.5% for each 100 g of xylan hydrolysed. This demonstrates the high potential of the enzymatic complex in XOS production as prebiotics, being able to reduce the steps in downstream processes, such as purification and recovery.

XOS are high value-added prebiotics due to their different properties that include better intestinal function, promote lipid metabolism, better absorption of minerals, such as calcium, and promoting the growth of beneficial intestinal microorganisms. Therefore, the production method of these prebiotics from enzymatic hydrolysis is interesting in the food industry Ho et al. (2018), Lian et al. (2020) and Lou et al. (2016). The results showed excellent performance of the enzymatic complex in XOS release with low DP with the two types of xylan evaluated. These results could be improved with partial purification

of the enzyme complex, better hydrolysis conditions and higher purity of the xylan extracted from SB.

4. Conclusions

An efficient and low-cost enzyme complex production process was developed in submerged fermentation with solids in suspension from SB and SM. Optimization assays led to definition of medium composition and the kinetic of enzyme production show a high enzyme activity (52.87 $U.mL^{-1}$) and productivity of 13.22 $U.mL^{-1}.d^{-1}$. The BCR showed better performance in enzyme complex production, probably due to pneumatic agitation effect on fungal growth and low mycelia stress. Enzyme hydrolysis of xylan led to high production of XOS with mainly xylotriose release. The developed process is promising and may generate high interest with great applicability in the food industry.

5. Imadazole Green Solvent Pre-treatment as a Strategy for Second-Generation Bioethanol Production from Sugarcane Bagasse

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Abstract

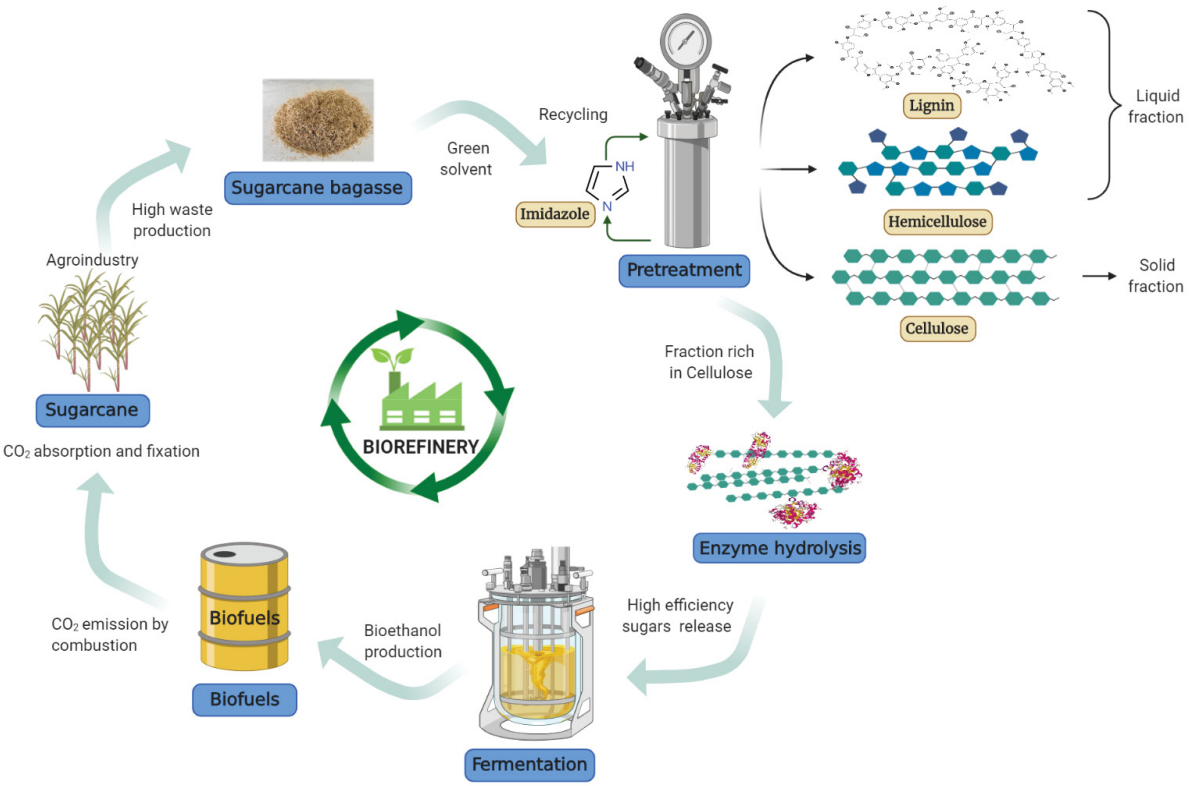
Sugarcane bagasse (SCB) is a potential source for second-generation bioethanol production. However, its recalcitrant structure (carbohydrates and phenolics) hinders the efficient release of sugars. Imidazole is a cheap green solvent that has demonstrated high efficiency in lignocellulosic biomass delignification. The main objective of this work was to study the SCB pre-treatment with imidazole for further enzymatic hydrolysis and bioethanol production. Significant modifications of imidazole-treated biomass were observed in the morphology and composition, such as cellulose enrichment, a great decrease of amorphous compounds (lignin and hemicellulose), and structural disorganization of lignocellulosic fibres. On the other hand, this pre-treatment also shows a substantial improvement of enzymatic conversion performance using Cellic CTec2®/Cellic HTec2® (15 FPU per gram of solid biomass) in all evaluated conditions compared to untreated material. After pre-treatment at 160 °C for 1h, enzymatic conversion efficiency reached 100% for glucose and xylose, significantly decreasing the hydrolysis time to only 8 h. The hydrolysates obtained were used for bioconversion to bioethanol by S. cerevisiae LPB 2705, giving a high production yield (83.7%), which represents a production of 218 L of ethanol per ton of SCB. This means an increase of up to 4 times the ethanol production compared to untreated material. The results obtained showed the great potential of imidazole in SCB pre-treatment, because it increased the release of fermentable sugars, which leads to an efficient and economic use of agro-industrial waste to generate value-added products and reduce the environmental impact under a biorefinery concept.

Keywords: Bioethanol, sugarcane bagasse, imidazole, green solvent, biorefinery

Highlights

- Innovative SCB imidazole treatment with low cellulose loss and high delignification.
- High productivity of enzymatic hydrolysis of imidazole-treated SCB during only 8 hours.
- Highly efficient (100%) in glucose and xylose release were achieved after treatment.
- Imidazole and enzymatically treated SCB for second-generation bioethanol production.
- A 275% improvement of bioethanol production from imidazole-treated SCB.

Graphical abstract



1. Introduction

The projections of population growth worldwide continue to increase with an estimated number of 9.7 billion people by 2050. It is estimated that energy demand will grow by 28% between 2015 and 2040. The need to supply sufficient energy with this growing demand generates the a search for energy sources and a focus on searching for renewable energy Energy (2019). Second-generation bioethanol is emerging as an alternative source of renewable energy, and it can supply the needs of the growing energy market.

Lignocellulosic biomass is a potential source of second-generation bioethanol production due to its large-scale availability. Besides, it can be sustainably produced at low cost, and it does not compete with food production Brodeur et al. (2011), Sindhu; Binod; et al. (2016) and Tan; Lee (2012). Sugarcane bagasse (SCB) is the main subproduct generated in the sugarcane processing. It is an important lignocellulosic biomass produced worldwide. The world sugarcane production was around 1.9 billion tons in 2018. With an average production of 746.8 million tons in Brazil, it is the main product from agroindustry and accounts for approximately 72% of all agricultural production FAO (2020). The chemical composition of SCB consists of cellulose (25%-50%), hemicellulose (20%-40%), and lignin (20%-30%) Ingle et al. (2020) and Laluce et al. (2019). These are complex polymers. For this reason, the hydrolysis and release of fermentable sugars from this lignocellulosic material is very important for the second-generation bioethanol industry. However, the lignocellulosic biomass is highly recalcitrant and requires strong physicochemical pre-treatment Tan; Lee (2012). Among the most common treatments used are steam explosion with dilute acid Ramos et al. (2015), alkali treatment Bartos et al. (2020), and sequential dilute acid and alkali treatment Hemansi et al. (2020). However, conventional use of alkaline or acidic chemicals in lignocellulosic biomass pre-treatments can generate harmful by-products for the environment.

Consequently, novel strategies are being studied in the development of more environmentally friendly solvents also called “green solvents”. Deep eutectic solvents and ionic liquids (ILs) are included in this category. These are relatively safe, pose low contamination risks, and provide high-efficiency delignification of lignocellulosic biomass Tan; Lee (2012), Tan et al. (2020), Usmani et al. (2020) and Xu et al. (2020). The high cost of these solvents currently hinders their use on a larger scale. Imidazole is a reagent that is also considered as a “green solvent”, and it arises as a cheap alternative to eutectic solvents and ILs. The use of imidazole as a solvent in the pre-treatment of lignocellulosic biomass was proposed by Morais et al. (2016) who obtained promising results in the fractionation and delignification of wheat straw. For their part, Toscan et al. (2019) demonstrated that imidazole pre-treatment allows high enzymatic conversions of glucan obtained from elephant grass. Highlights of using imidazole are its low toxicity, low vapor pressure, high boiling point, and its ease of handling as a solvent in biomass pre-treatment compared to other solvents; also, it is recyclable and reusable Morais et al. (2016) and Toscan et al. (2019). So far, imidazole can be an efficient solvent for delignification of biomass. However, it has not yet been evaluated in the treatment of other biomasses such as SCB. Additionally, most of the published works do not include enzymatic hydrolysis nor fermentation steps and focus only on the physicochemical characteristics of the imidazole-treated fractions.

The main purpose of this work was to analyse the effects of imidazole during pre-treatment of SCB on the structure and efficiency of enzymatic hydrolysis to release glucose and xylose as fermentable sugars for second-generation bioethanol production.

2. Material and methods

2.1.Raw materials and chemicals:

Sugarcane bagasse was provided by the Santa Terezinha factory (USACUCAR company) from Paraná, Brazil. Lignocellulosic biomass was dried in an air-circulating oven at 65 °C for 48 h, and milled in a knife mill (Marconi, MA580/E). The particle size used in all experiments was between ASTM No. 20 (0.85 mm) and ASTM No. 45 sieves (0.35 mm). All purchased chemicals and medium components were of analytical grade.

2.2.Compositional analysis of SCB:

The solid content, ash, extractives, acid insoluble lignin (AIL), acid soluble lignin (ASL), and structural carbohydrates were determined according to the National Renewable Energy Laboratory (NREL) procedures Sluiter et al. (2012), Sluiter; Hames; Hyman; et al. (2008), Sluiter; Hames; Ruiz; et al. (2008) and Sluiter; Ruiz; et al. (2008).

2.3.Biomass pre-treatment:

Imidazole was used as a green solvent in pre-treatment for delignification of SCB following the procedure described by Morais et al. (2016), with some modifications. Pre-treatment experiments were carried out in a stainless steel 150 mL reactor (Parr, USA) with mechanical stirring. Reactions were performed with 5 g of dried biomass at a ratio of 1/9 biomass/imidazole ($w.w^{-1}$). Two different reaction temperatures (120 °C and 160 °C) and reaction times (1 h and 3 h) were analysed (Table 1). After each pre-treatment, 135 mL of deionized water was added and mixed for 1 h at 90 °C. The liquid fraction was separated from the solid fractions by vacuum filtration and precipitated with 3 volumes of ethanol for hemicellulose recovery. After that, the soluble lignin fraction was kept for further composition analysis. The precipitated solid fractions, rich in cellulose, were vacuum filtered and washed with 96% $v \cdot v^{-1}$ ethanol. The cellulose-rich fractions recovered after each pre-treatment were dried in an air-circulating oven at 45 °C and further submitted to enzymatic hydrolysis.

2.4.X-ray diffraction (XRD):

The crystalline nature of untreated or pre-treated SCB samples were studied by XRD patterns obtained using an X-ray diffractometer, 700 Maxima (Shimadzu) and Ni-filtered Cu kappa as the X-ray source (CuKα, λ=1.5418 Å). Samples were scanned in the 2θ range of 5°-30° at a rate of $2°\cdot min^{-1}$. The generator was operated at 40 kV and 20 mA.

The crystallinity index (CrI) was calculated using Eq. 1

$$CrI = \frac{I_c - I_a}{I_c} \cdot 100 \qquad (1)$$

where I_c is the peak intensity at a maximum of 2θ between 22° and 23°, and I_a is the intensity peak at a minimum of 2θ around 18°.

2.5.Scanning electron microscopy (SEM):

SEM analysis of untreated and pre-treated SCB was performed to reveal ultra-structural changes. Samples were dried, mounted on aluminium stubs, and sputter-coated with a gold layer. The scanning and acquisition of microphotographs were carried out using a Jeol JSM 6360-LV (Oxford Instruments) scanning electron microscope that was operated at 15kV.

2.6.Fourier transform infrared spectroscopy (FT-IR):

FT-IR spectroscopic analyses were carried out in the transmittance mode and switched to absorbance dice to monitor the relative changes in the biomass functional groups. Discs were prepared by mixing 2 mg dried samples with 100 mg dried KBr. Absorption spectra of untreated or pre-treated SCB samples were monitored between 4000 cm^{-1} and 400 cm^{-1} with a 4 cm^{-1} resolution using a Vertex 70 spectrometer (Bruker).

The bands corresponding to the areas of absorption at 1430 cm^{-1} and 896 cm^{-1} were analysed for cellulose crystallinity index calculation according to Eq. 2 Morais et al. (2016)

$$LOI = A_{1430}/A_{896} \qquad (2)$$

where LOI is the lateral order index and A is the absorbance value of the corresponding band.

2.7.High-performance liquid chromatography (HPLC) analyses:

Organic acids, sugars, and alcohols were analysed by HPLC, samples were filtered through 0.22 μm pore size filter (Millipore Corp., Billerica, MA, USA) and were analysed in an HPLC system equipped with an Aminex Bio-Rad HPX-87H column operating at 50 °C and a refractive index (RI) detector. The mobile phase was aqueous 5 mM H_2SO_4 at a flow rate of 0.6 mL·min^{-1}, and injection volume was 10 μL. The chemical standards were prepared with D-(+)-cellobiose, glucose (>99%), D-(+)-xylose (>99%), D-(-)-arabinose (>99%), D-(+)-mannose (>99%), D-(+)-galactose (>99%), and acetic acid (>99%) from Sigma-Aldrich (USA).

2.8.Enzymatic hydrolysis of pre-treated biomass:

Cellulose-rich solid fractions, after imidazole pre-treatment were subject to enzymatic hydrolysis. Experiments were performed in 125 mL Erlenmeyer flasks containing 2.5% w·v^{-1} biomass (dry weight) in 12.5 mL of 0.05 M sodium citrate buffer solution (pH 4.8) with 0.02% (w.v^{-1}) sodium azide and a 1/9 ratio of Cellic HTec2® / Cellic CTec2® (v.v^{-1}) from Novozymes – Araucaria, Brazil. Cellulase and xylanase loadings were 15 FPU and 1206.7 U per gram of solid biomass, respectively. Samples were incubated in an orbital shaker at 50 °C for 72 h at 150 rpm. Sample aliquots were obtained each 4, 6, and 12 h and then heated up to ~95 °C for 10 min in a water bath for enzyme inactivation. Samples were then filtered through 0.22 μm cellulose acetate membrane syringe filters and analysed by HPLC. All assays were performed at least in duplicate

Glucose and xylose yields obtained after enzymatic hydrolysis of untreated and pre-treated SCB were calculated using Eq. 3 and Eq. 4:

$$\text{Glucose convertion yield (\%)} = \frac{[\text{Glu}] \times \text{V}}{\text{m}_{\text{biomass}} \times \text{f} \times 1.11} \text{x}100 \qquad (3)$$

$$\text{Xylose convertion yield (\%)} = \frac{[\text{Xyl}] \times \text{V}}{\text{m}_{\text{biomass}} \times \text{f} \times 1.13} \text{x}100 \qquad (4)$$

where [Glu] and [Xyl] are glucose and xylose ($g \cdot L^{-1}$) concentrations in the hydrolysate determined by HPLC, respectively; V is the volume (L) of hydrolysate; $m_{biomass}$ is the dry weight of biomass (g); f is the glucan and xylan fraction in the biomass; 1.11 and 1.13 are the glucan to glucose and xylan to xylose conversion factors, respectively.

2.9.Fermentation:

S. cerevisiae LPB 2705 from the culture bank of the Bioprocess Engineering and Biotechnology Laboratory of Federal University of Paraná was tested for bioethanol production. The hydrolysate obtained was fermented in 125 mL Erlenmeyer flasks with the addition of 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 2 g L^{-1} KH_2PO_4 and 1 g L^{-1} $MgSO_4$; pH was fixed at 5.5 with a final volume of 10 mL. Flasks were autoclaved at 121 °C for 15 min. *S. cerevisiae* LPB 2705 (5%, $v \cdot v^{-1}$; 24 h old) with an optical density (O.D.) of 2.4 at 600 nm was transferred to the hydrolysate for fermentation. Flasks were placed in a shaker at 35 °C with agitation of 150 rpm. Samples were withdrawn every 3, 6, and 12 h for up to 24 h and centrifuged (10,000 rpm, 15 min, 25 °C). The chemical composition of the supernatant was then determined by HPLC. Experiments were performed in duplicate.

The theoretical yield of ethanol was calculated using equation Eq. 5.

$$\text{Ethanol yield (\%)} = \frac{[\text{Ethanol}]}{0.511 \times ([Glu_i] - [Glu_r])} \times 100 \qquad (5)$$

where 0.511 is the conversion factor of glucose to ethanol; [Ethanol] is the concentration of ethanol produced by fermentation, and [Glu_i] and [Glu_r] are initial and residual glucose concentrations, respectively.

3. Results and discussion

3.1.Chemical composition of SCB

The average composition of SCB on dry a basis (SCBdry-basis) was determined as 38.72% cellulose, 23.01% hemicellulose, 16.85% lignin, 5.27% extractives, and 3.72% ash (Table 1). These results are in agreement with the average values reported for SCB in other work Ingle et al. (2020), Nascimento et al. (2016) and Rocha et al. (2015). The differences in the chemical composition of SCB are mainly due to the nature of the crop and the type of sugarcane variety used. Among the main factors that influence its composition are the type of soil, geographic location, and harvest season, among others Rocha et al. (2015) and Toscan et al. (2019).

3.2.Composition and solids yield obtained after pre-treatment

The main objective of the pre-treatment step is the delignification and deconstruction of the complex crystalline form of the lignocellulosic material to facilitate and promote significant increases in enzymatic hydrolysis yields. Different solvents and physicochemical conditions have been evaluated for this purpose. Although treatment with imidazole has not yet been reported for SCB, it showed great potential for the pre-treatment of lignocellulosic biomass Morais et al. (2016) and Toscan et al. (2019).

The data for chemical composition and recovery efficiencies of treated biomass are shown in Table 1. It is possible to observe a decrease in solids recovery with an increase in treatment severity. The minimum solid recovery was 55% $w \cdot w^{-1}$ after pre-treatment at 160 °C for 1h. Similar solid recovery values were reported in alkaline pre-treatments (58% $w \cdot w^{-1}$) Li, H. et al. (2019) and aqueous ammonia-glycerol pre-treatment (55% $w \cdot w^{-1}$) Shi et al. (2019). On the contrary, the cellulose percentage of the recovered solid fractions increased with the severity of the treatment, reaching up to 64% $w \cdot w^{-1}$ of cellulose, as shown for the treatment at 160 °C for 1 h compared to 38% $w \cdot w^{-1}$ of cellulose of raw $SCB_{dry\text{-}basis}$. This represents a 1.7-fold increase

of the cellulose proportion in the solid material. At 120 °C, there were minor losses of solid material, and cellulose represented more than 50% $w \cdot w^{-1}$ of the recovered material. It should also be noted that the losses in solid material recovered had almost no affect the cellulose content, with a maximum loss of 7% $w \cdot w^{-1}$ with respect to $SCB_{dry\text{-}basis}$.

Table 1. Chemical composition of raw and pretreated SCB and yields of solid fractions obtained after different applied pretreatment.

Reaction conditions	SCB $_{raw}$	SCB $_{Dry\text{-}basis}$	Pretreated (code)			
			SCB $_{120\text{-}1}$	SCB $_{120\text{-}3}$	SCB $_{160\text{-}1}$	SCB $_{160\text{-}3}$
Temperature (°C)	-	-	120	120	160	160
Time (h)	-	-	1	3	1	3
Composition (wt%)						
Anhydroglucose	35.74 ± 0.81	38.72 ± 0.88	52.08 ± 0.20	60.06 ± 0.41	64.77 ± 0.24	62.60 ± 1.37
Anhydroxylose	15.00 ± 1.00	16.25 ± 1.08	19.22 ± 0.24	20.15 ± 0.10	17.60 ± 0.18	18.57 ± 0.47
Anhydroarabinose	1.99 ± 0.25	2.16 ± 0.27	2.38 ± 0.09	2.24 ± 0.11	Nd	Nd
Acetyl groups	4.25 ± 0.32	4.61 ± 0.34	Nd	Nd	Nd	Nd
Acid-soluble lignin	4.65 ± 0.34	5.03 ± 0.37	7.92 ± 1.12	6.55 ± 0.07	4.65 ± 0.24	4.39 ± 0.20
Acid-insoluble lignin	10.91 ± 0.99	11.82 ± 1.07	6.55 ± 0.07	4.95 ± 0.19	3.13 ± 0.17	2.34 ± 0.63
Extractives-Water	3.12 ± 0.15	3.38 ± 0.16	-	-	-	
Extractives-EtOH	1.74 ± 0.09	1.89 ± 0.10	-	-	-	
Cellulose	35.74 ± 0.81	38.72 ± 0.88	52.08 ± 0.20	60.06 ± 0.41	64.77 ± 0.24	62.60 ± 1.37
Hemicellulose	21.24 ± 0.62	23.01 ± 0.67	21.60 ± 0.18	22.39 ± 0.10	17.60 ± 0.18	18.57 ± 0.47
Lignin	15.56 ± 0.74	16.85 ± 0.80	14.47 ± 0.79	11.49 ± 0.14	7.78 ± 0.21	6.73 ± 0.47
Extractive	4.86 ± 0.12	5.27 ± 0.13	-	-	-	-
Ash	3.44 ± 0.05	3.72 ± 0.05	-	-	-	-
Water	7.70 ± 0.14	-	-	-	-	-
Solid Yield (% $w.w^{-1}$)		100	73.11	63.02	55.92	57.61
Cellulose loss (%)		0	1.66 ± 0.37	2.25 ± 0.67	6.46 ± 0.35	6.85 ± 2.03
Hemicellulose loss (%)		0	31.39 ± 1.03	38.69 ± 1.03	57.24 ± 0.43	53.50 ± 1.18
Lignin loss (%)		0	37.25 ± 0.25	57.03 ± 1.45	74.17 ± 0.23	77.01 ± 1.46

Total lignin was calculated by adding acid soluble and acid-insoluble lignin
Total hemicellulose was calculated by adding anhydroxylose, anhydroarabinose and acetyl group

Nd: not detected

After cellulose, hemicellulose is the second polysaccharide with the highest abundance in SCB. The results showed that this lignocellulosic fraction remains second in proportion under different treatment conditions, decreasing both in the amount and the percentage within the recovered solids as the treatment severity increased. The presence of arabinose in the recovered

solid fraction was only observed with treatments at temperatures above 120 °C, whereas it was not present with treatments at 160 °C. On the other hand, the acetyl groups were totally removed after all applied treatments. The decrease of the hemicellulose content within the solids recovered after imidazole treatment has also been reported in wheat straw and elephant grass Morais et al. (2016) and Toscan et al. (2019).

Lignin is the fraction that most hinders biomass degradation. For this reason, most of the treatments focus on biomass delignification Ramadoss; Muthukumar (2015). The results shown in Table 1 exhibit that biomass delignification occurred for all treatments. The delignification increased proportionally with treatment severity. The highest delignification was obtained after treatment at 160 °C for 1 and 3 h with approximately 75% $w \cdot w^{-1}$ of lignin extracted with respect to $SCB_{dry\text{-}basis}$ lignin composition. A common problem in biomass delignification is the chemical modification of lignin, which increases the proportion of stable lignin and makes its removal difficult. As it is possible to see in the results, the ratio between acid soluble lignin and acid insoluble lignin increased with the severity of the treatment. This means that imidazole treatment promotes the total solubilization of the lignin, and it does not promote lignin regeneration, making its recovery in the native form impossible Morais et al. (2016).

One of the main results observed is the great influence that temperature exerts on the composition and solids yield after imidazole treatment. It was shown that higher temperatures promote higher efficiencies of biomass delignification and increase the amount of cellulose in the recovered solids. This result can be explained by the favourable deconstruction of biomass at higher temperatures, which probably helps to break the ester bonds that bind lignin and hemicellulose and destabilizes the existing hydrogen bonds between lignin, cellulose, and hemicellulose Chaudhary et al. (2012 e Morais et al. (2016). Meanwhile, the processing time did not show a significant influence on the composition and solid yield of pre-treated SCB.

3.3.Crystalline structure analyses by X-ray diffraction patterns (XRD)

The crystallinity index of $SCB_{dry\text{-}basis}$ material and the solids recovered after imidazole treatments were analysed by XRD. Due to the abundance of cellulose, lignocellulosic biomass, especially in SCB, should be uniform and crystalline. However, it is much more complex due to the presence of amorphous polymers such as lignin, hemicellulose, and disordered cellulose. The CrI indicates the action of solvent on biomass. Figure 1 shows the different diffractograms of $SCB_{dry\text{-}basis}$ and the solids recovered under different treatment conditions. The intensity of the signals in the diffractogram of untreated SCB are faint, showing a small increase in the regions near 16° and 22° that represent crystalline cellulose type I. On the other hand, the peak at approximately 18° is associated with the amorphous parts of the biomass that include disordered cellulose, hemicellulose, and lignin Hemansi et al. (2020) and Morais et al. (2016).

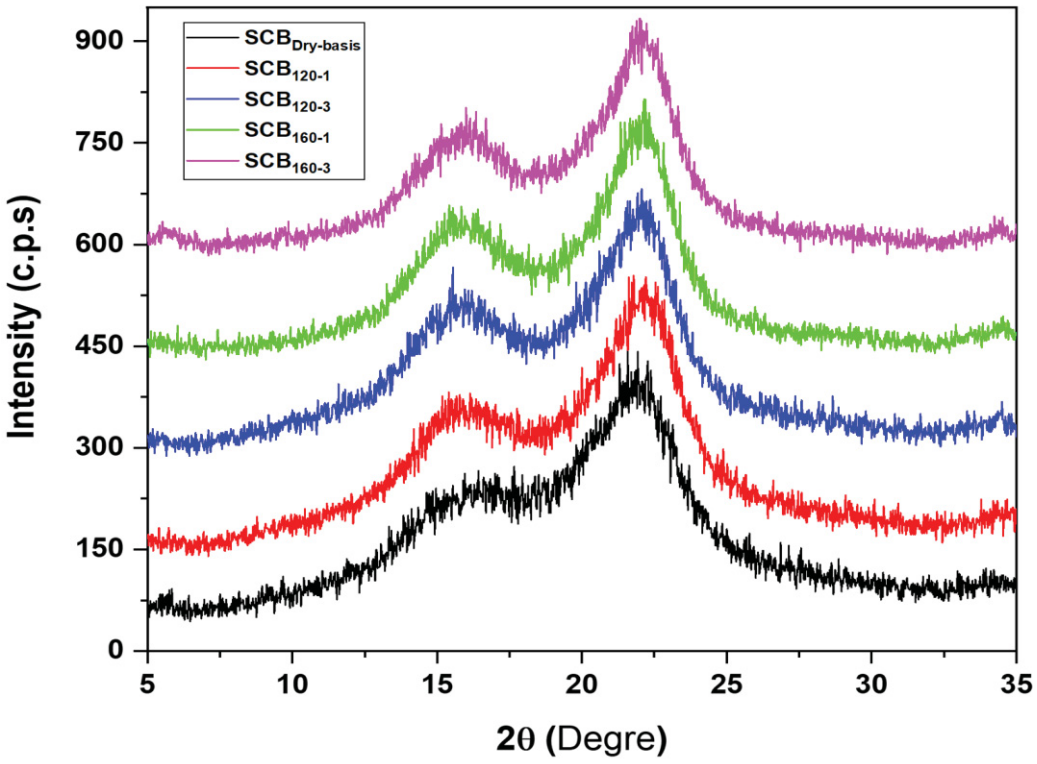


Figure 1. XRD diffractograms for $SCB_{dry\ basis}$, and the different treatments applied to SCB.

The CrI for $SCB_{dry\text{-}basis}$ was 50.5%, which is relatively high due to the abundance of cellulose. CrI of the different treatments were 66.3%, 70.1%, 71.2%, and 73.2% for $SCB_{120\text{-}1}$, $SCB_{120\text{-}3}$, $SCB_{160\text{-}1}$, and $SCB_{160\text{-}3}$, respectively. As observed, the crystallinity values increased with treatment severity. As it was stated, as the severity of the treatment increased, together with the cellulose content in the recovered solids, the crystallinity of the material (CrI) increased. Furthermore, the crystallinity also increased due to removal of amorphous materials such as lignin and hemicellulose, as shown in Table 1.

Similar results were reported for SCB submitted to alkaline treatment. The crystallinity index obtained from XRD analyses increased with the concentration of NaOH used without modifying the crystalline form of cellulose Bartos et al. (2020) and Satlewal et al. (2019). On the other hand, acid treatments produced a decrease in crystallinity, thus affecting the crystalline form of the cellulose Hemansi et al. (2020). Therefore, it is possible to affirm that imidazole would act similarly to an alkaline treatment and change the crystallinity of the material, mainly by the removal of lignin and hemicellulose.

3.4.Morphological structural analysis by scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) helps in the analysis of the topology and morphology of materials. SEM analysis is applied to lignocellulosic biomass to show its roughness and porosity in addition to the surface area present in the fibres. Modifications in the fibres' surface and structural morphology of biomass samples are observed after each applied pre-treatment. This is due to external factors such as heat or chemical destabilization reactions caused by the solvent (imidazole) modifying the rigidity and resistance of the lignocellulosic material Bartos et al. (2020) and Laluce et al. (2019).

As seen in Figure 2, untreated SCB and cellulose-rich solids recovered after the different treatments showed variations in their structure and morphology. These structural and morphological changes were also visible as a clear change in the density and conformation of

the SCB fibers. As the pre-treatment becomes more severe, the biomass becomes less dense and more voluminous. Thus, it shows an increase in the internal fibre surface area.

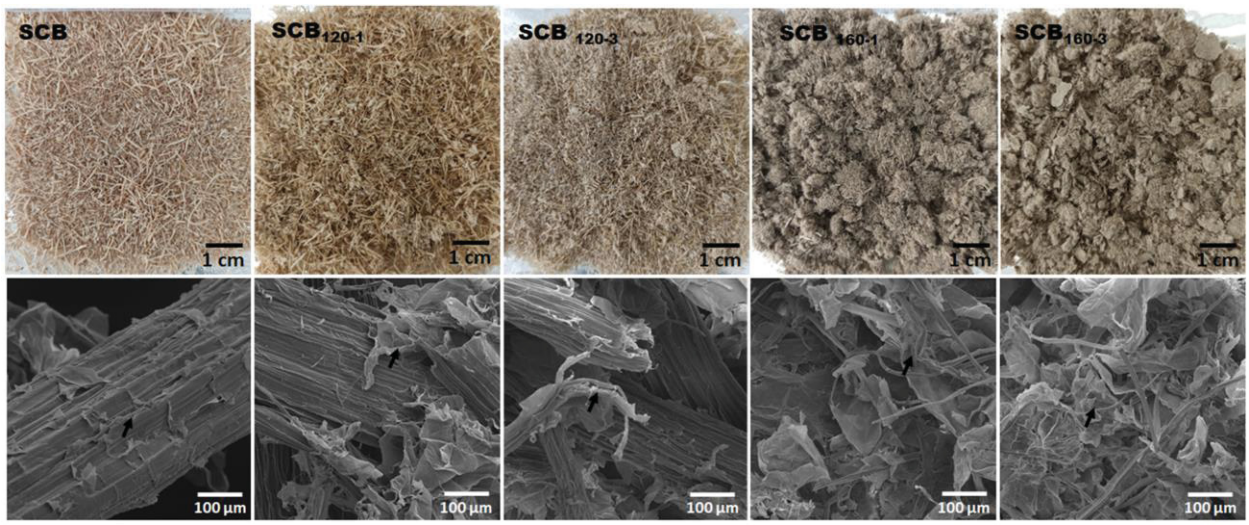


Figure 2. Scanning electron microscopy (SEM) non treated SCB samples and the four cellulosic rich fractions ($SCB_{120\text{-}1}$, $SCB_{120\text{-}3}$, $SCB_{160\text{-}1}$, and $SCB_{160\text{-}3}$), resulting from imidazole pretreatments.

SEM images show that SCB is made up of fibres with a very dense, rigid, and intact surface. This rigid conformation of the fibres decreases the efficiency of enzymatic attack. On the other hand, cellulose-rich fractions recovered after the treatment show a variation in the stiffness of the fibres. This increases progressively with more severe treatment. Treatments performed at 120 °C produced materials with sharper contours and deep grooves with little but notable disorganization of microfibers (black arrows). Materials obtained with treatment at 160 °C showed total fibre disorganization and amorphous agglomerations with a conglomerate texture were formed. Also, there was an abundant release of individual microfibers (black arrows).

The morphological modifications induced by pre-treatment using imidazole would generate an increase in the surface area within the lignocellulosic fibres in addition to a decrease in their rigidity. This facilitates the enzymatic attack that leads to a better release of monosaccharides such as xylose and glucose.

3.5.Fourier transform infrared spectroscopy (FT-IR)

The modification in the spectra and the intensities of the peaks obtained by FT-IR spectrometry show the variations and/or modifications generated by the action of the treatments, which mainly affect the type of bonds of lignocellulosic biomass chemical structures.

In SCB, the peaks at ~1510 cm^{-1} and ~1604 cm^{-1} represent lignin chemical bonds. The signal at 1516 cm^{-1} is related to aromatic vibrations of the lignin phenolic ring (C=C), while the peak at 1604 cm^{-1} is attributed to aromatic compounds Bartos et al. (2020) and Zhu, Z. et al. (2020). As it can be seen in Figure 3, the above-mentioned signals are present in SCB, while the peak at 1510 cm^{-1} disappears in all materials that were treated with imidazole. On the other hand, the peak at 1604 cm^{-1} is present in treatments at 120 °C, but it disappeared in treatments at 160 °C. These data are consistent with the previous discussion about the chemical composition of the treated material (Table 1) where a strong delignification effect is observed with an increase in temperature.

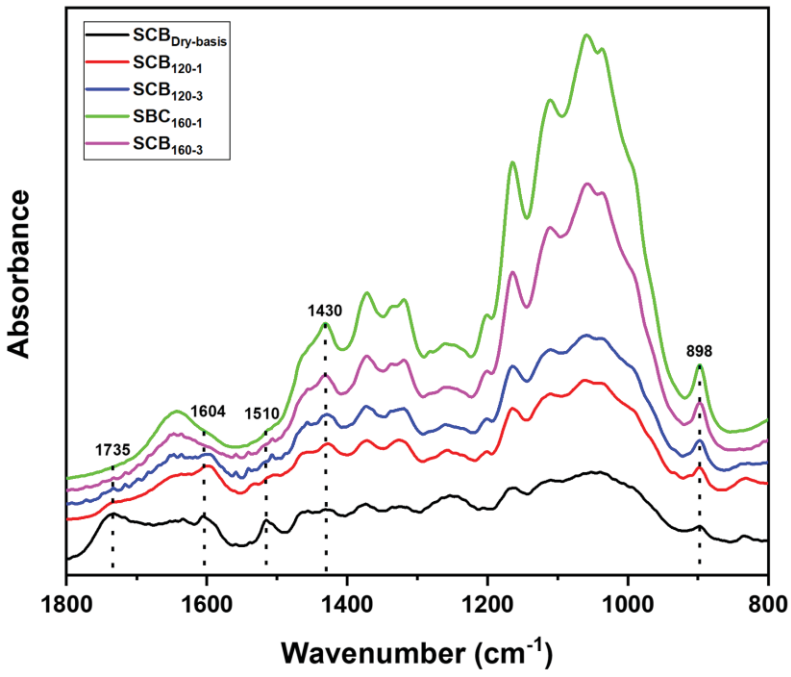


Figure 3. Spectra peaks obtained by FT-IR spectrometry for samples of SCB and the four cellulosic rich fractions

The signal at ~1735 cm^{-1} is related to the stretching vibration of the ester bond (C=O) between hemicelluloses and lignin Bartos et al. (2020) and Zhu, Z. et al. (2020). After imidazole treatment, this signal disappeared, indicating the breakdown of ester bonds that cause a disorganization between lignin and hemicellulose. Ester bonds are also linked to the presence of acetyl groups within hemicellulose Laluce et al. (2019) and Morais de Carvalho et al. (2017). The absence of this peak in the spectra of treated material is consistent with the fact that no acetyl groups were detected in the composition of the treated biomass (Table 1), and this could be directly affected by the decrease in hemicellulose content.

For the cellulose analysis, the signals vary between the 850–1500 cm^{-1} region where the polymorphs of highly crystalline cellulose are characterized Nelson; O'Connor (1964). The peak at ~898 cm^{-1} is attributed to the stretching of the β-glycosidic bond (C-O-C) that is mainly associated with cellulose type II, which is amorphous and disordered. The peak at ~1430 cm^{-1} is assigned to the CH_2 scissor movement. This is a characteristic signal of cellulose type I which is more crystalline and ordered Morais et al. (2016) and Zhu, Z. et al. (2020). As seen in Figure 3, the intensity of the peaks at 898 cm^{-1} and 1430 cm^{-1} are more defined when the severity of treatment is increased, while in untreated SCB, the signals are faint. This would be mainly attributed to the presence of high cellulose content in the treated material (Table 1). An increase in the intensity of the peak is also observed at 898 cm^{-1}, especially in the treatments at a higher temperature (160 °C). However, this increase is almost proportional to the increase in the intensity of the peak at 1437 cm^{-1}.

The calculated LOI values were 1.16; 1.20; 1.29; 1.30, and 1.26 for $SCB_{dry\text{-}basis}$, $SCB_{120\text{-}1}$, $SCB_{120\text{-}3}$, $SCB_{160\text{-}1}$, and $SCB_{160\text{-}3}$, respectively. According to these values, a slight increase in cellulose crystallinity is observed (cellulose type I) when treatment is applied. However, this increase would not be significant. It reaches a maximum of 12% of LOI increase in the treatment at 160 °C during 3 h with respect to SCB dry-basis. LOI values strongly depend on the residue

used, with large variations being found in different lignocellulosic biomasses Kuo; Lee (2009) and Morais et al. (2016). With these results, it can be affirmed that the treatment of SCB using imidazole as a solvent does not generate significant modifications in the variation of cellulose type I or II, but it strongly affects the delignification and removal of hemicellulose.

3.6.Enzymatic hydrolysis of cellulosic rich fractions

The enzymatic hydrolysis efficiency is a crucial parameter in the determination and comparison of the efficiencies of lignocellulosic biomass treatment. For this reason, all recovered solids under different treatment conditions were subjected to enzymatic hydrolysis. To avoid possible inhibition effects from the substrate, a low concentration of solids with a moderate enzyme load was used Toscan et al. (2019).

The enzymatic hydrolysis of materials at different treatment conditions showed significant differences (Figure 4). Untreated SCB promoted low enzyme conversion (EC) efficiency, reaching a maximum glucose conversion of 29.7% and a xylose conversion of 23.6%. In both cases, the enzyme incubation time was 48 h. The low EC efficiency is explained by the complex structure of untreated SCB, which hinders the enzymes' access to the substrate.

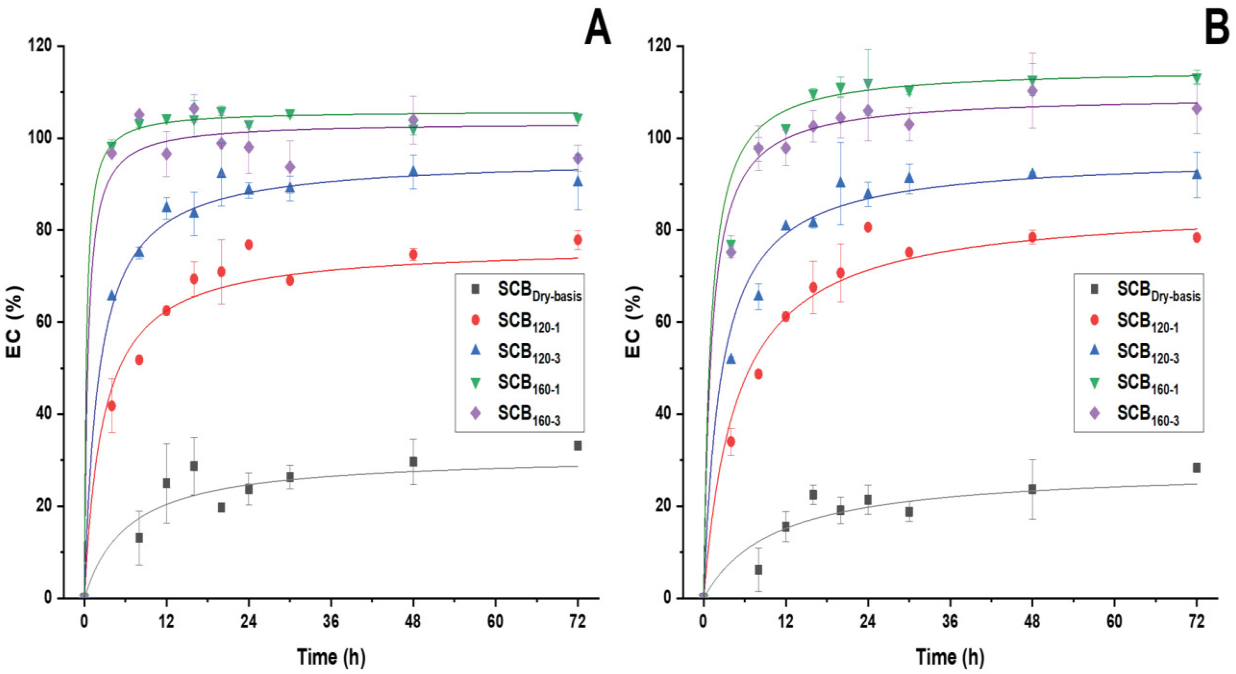


Figure 4. Efficiency of enzymatic conversion (EC): A) Glucan to glucose yields and B) Xylan to xylose yields

On the other hand, imidazole treated biomass presented significantly higher EC than untreated SCB. EC efficiency in glucan conversion to glucose increased with the severity of the treatment (Figure 4). EC for treatment at 120 °C was 70% and 90% for 1 h and 3 h, respectively. In both cases, these efficiencies were reached after 20 h of enzymatic incubation with no significant increases observed after this time. In the case of treatment at 160 °C both at 1 h and at 3 h, the EC reached 100% after 8 h of enzyme incubation, achieving a higher glucose release in much lower incubation times compared to the material treated at 120 °C. These results demonstrate that temperature and imidazole play an important role in lignocellulosic biomass treatment, achieving a total conversion of cellulose to glucose.

Similar behaviour was obtained for xylose release (Figure 4B), where the EC increased with the treatment severity. The EC obtained for biomass treatment at 120 °C during 1 h an 3 h were 85% and 90%, respectively, with 24 h of incubation time in both cases. For treatment at 160 °C, 100% of EC was obtained after 12 h of enzymatic incubation. Unlike EC for glucose,

the EC for xylose presented a slight delay in reaching the maximum efficiency. This may be due to the lower enzyme load of xylanases used in the enzymatic hydrolysis.

The results clearly demonstrate that the treatment of SCB with imidazole generates a highly significant increase in EC efficiency, reaching a complete conversion of both glucan and xylan to fermentable sugars. These results would be strongly linked to SCB delignification and disorganization of its structure Morais et al. (2016), Toscan et al. (2019) and Zhu, Z. et al. (2020). As previously discussed, both delignification and structure disorganization of SCB increased with the treatment severity using imidazole. Consequently, this would generate appropriate conditions for high efficiency in enzymatic degradation.

In comparison to other work found in the literature, EC efficiency in this work was quite high and the incubation time required was low with a moderate enzyme load. These results generate a great perspective on imidazole use as an efficient treatment in the decomposition of SCB not only for the high efficiencies at each process stage but also for its time reduction in treatment process and enzyme incubation. This shows the additional benefits of this environmentally friendly and relatively cheap solvent compared to other green solvents such as ionic liquids Morais et al. (2016).

3.7.Ethanol production from hydrolysate

Based on very satisfactory enzymatic conversion efficiency of the biomass treated with imidazole, the resulting hydrolysates were used to evaluate the fermentation potential for ethanol production. Due to its great production of ethanol and biocompatibility with different hydrolysed sources, *S. cerevisiae* LPB 2705 was used as an inoculum, Pérez de los Ríos et al. (2017). The fermentation profile was analysed for 24 hours by measuring concentrations of fermentable sugars at certain time intervals (Figure 5). As can be observed in Figure 5, most of the variation in the fermentation profile occurred during the first 12 h. Glucose affinity by *S. cerevisiae* LPB 2705 is high; the greatest amount of this sugar was consumed before 9 h. As

for xylose, its consumption was minimal, which is characteristic of this type of yeast Lara-Serrano et al. (2018). Ethanol production increased rapidly, reaching a maximum concentration of 10.0 ± 1.4 $g \cdot L^{-1}$ at 12 h.

The fermentation profile shows a direct relationship between the glucose consumed and the increased concentration of ethanol, while a low consumption of xylose does not induce significant increases in ethanol production. The production yields obtained were also relatively high compared to the literature, producing 0.42 g ethanol/g glucose with a theoretical efficiency of 83.7%, which was calculated at the greatest production time (12 h). On another hand, the highest productivity was obtained at 9 h with a production capacity of 1.11 $g \cdot L^{-1} \cdot h^{-1}$ of ethanol. These results were contrasted with the fermentation of *S. cerevisiae* LPB 2705 in synthetic medium where similar efficiencies and productivities were obtained. This corroborates the absence of inhibitors produced by the process of treating lignocellulosic biomass with imidazole.

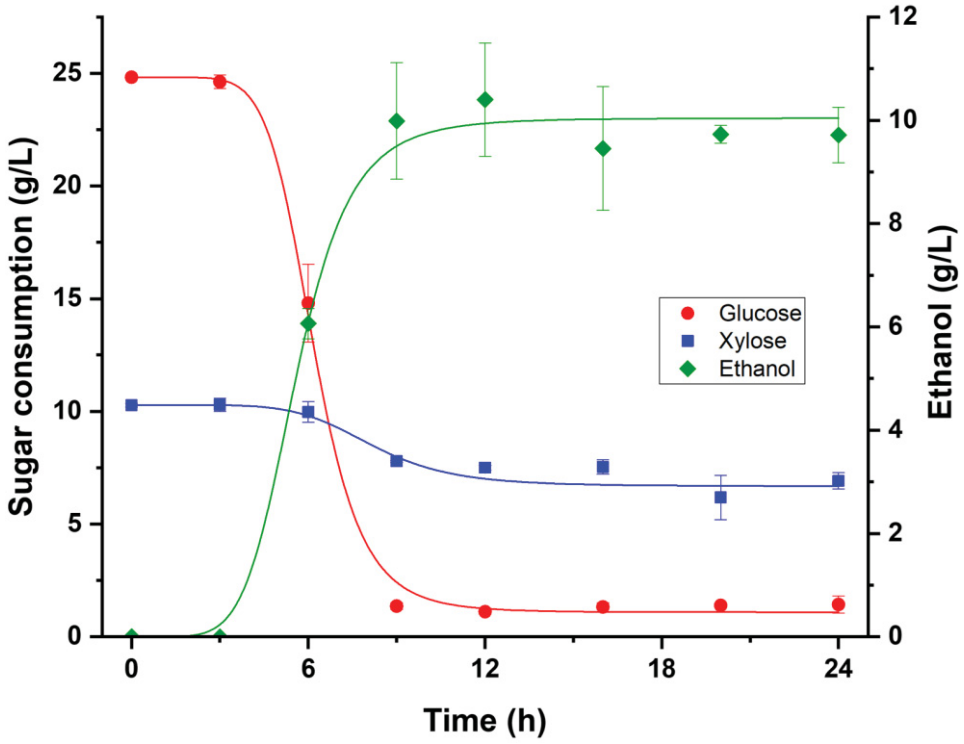


Figure 5. Bioethanol production by *S. cerevisiae* LPB 2705 using hydrolysate from imidazole-treated SCB.

3.8.Mass balance of ethanol production from SCB

Finally, with all the data obtained, a mass balance was performed to analyse the final efficiency of bioethanol production for the four treatments applied to SCB with imidazole (Figure 6). Starting from 1 kg (1000 g) of $SCB_{dry\text{-}basis}$, it is possible to see that the applied treatment drastically reduces the amount of lignin in the solid recovered material from 168.5 g to 38.8 g after treatment at 160°C for 3 h, also mentioned above. In addition to observing an enrichment of cellulose content in the recovered solids, treatments with imidazole have a great influence on enzymatic conversion efficiency. It improved the sugar release from 107 g of glucose in untreated SCB to 402 g of glucose per kg of SCB pre-treated at 160°C for 1 h after 12 h of enzymatic hydrolysis (reaching a 4-fold increase in glucose release). In this case, the bioconversion efficiency of glucose to ethanol by *S. cerevisiae* LPB 2705 reached 83.7%. The ethanol concentration obtained is directly proportional to the amount of glucose released by enzymatic hydrolysis. With the best imidazole treatment condition (160°C, 1 h), 217.9 L of ethanol per ton of SCB can be produced, increasing the production yield by 275.7% compared to untreated SCB. All imidazole treatments showed significant increases of bioethanol production compared to the untreated material (Figure 6).

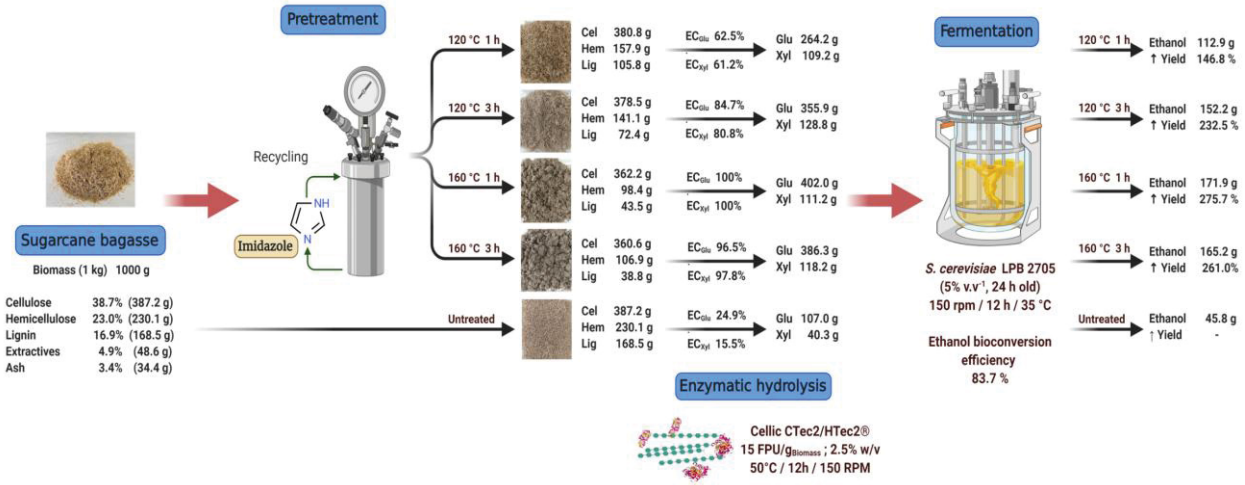


Figure 6. Mass balance for ethanol production from SCB treated with imidazole. EC: Enzyme conversion yield. ↑Yield: Ethanol yield improve respect to SCB untreated.

The best SCB pre-treatment conditions (160 °C and 1 h) led to a high efficiency in subsequent second-generation ethanol production. Other processes that employed different pre-treatment methods showed a large variation in ethanol production (Table 2). Pre-treatment methods such as dilute acid, hydrothermal, enzymatic, and ionic liquid acids were applied to SCB for ethanol production, showing production efficiencies ranging from 84.9 L to 228 L of ethanol per ton of SCB Asada et al. (2020), Lara-Serrano et al. (2018), Mesa et al. (2020) and Prajapati et al. (2020). Under optimum conditions, the results of those works showed ethanol production above the average value reported by other authors with 217.9 L per ton of SCB. This shows the great efficiency of the imidazole pre-treatment process, which can still be improved and optimized. Although pre-treatment plays an important role in the second-generation ethanol production process, the bioconversion efficiency of each strain used in fermentation has to be taken into account. As it is shown in Table 2, the same species of yeast *S. cerevisiae* present different efficiencies in ethanol production, which will influence the final productivity. On the other hand, the residue to be treated greatly influences the final production efficiencies, where the amount of cellulose present in the residue will directly impact the final amount of glucose obtained, whereby the amount of ethanol would also increase.

Another point to highlight is the decrease in the processing time to less than 24 h, strongly reducing the energy costs of the process and greatly improve the ethanol productivity. As future perspectives, the application of simultaneous saccharification and fermentation Bu et al. (2019), Lara-Serrano et al. (2018) and Ojeda et al. (2011) would be interesting to investigate because this would generate a greater decrease in the total processing time. On the other hand, the amount of xylose released after the enzymatic hydrolysis is also considerably high (> 100 g per kg of SCB). Xylose could be also used in bioethanol production using other yeasts like *Pichia* or genetically modified yeast that manage to metabolize xylose Liang et al. (2014) and Wang, Z. et al. (2019a), or a mixture of strains capable of metabolizing these sugars could be used.

Table 2. Second generation bioethanol production from different pretreatment.

Substrate	Pretreatment	Condition	Microorganism	Ethanol theorical yield (%)	Ethanol productivity ($g.L^{-1}.h^{-1}$)	Ethanol production ($L.ton^{-1}$)	References
Sugarcane Bagasse	Dilute acid hydrolysis	$MnSO_4*H_2O$ and ZnO system at 100°C; 30 min and ratio 0.05/10 (w/v) biomass/solvent	*S. cerevisiae*	84.3	0.182	233.2[a]	Ramadoss; Muthukumar (2015)
Barley Straw	Ionic Liquids	1-ethyl-3-methylimidazolium acetate at 105 ºC; 7.5 h and ratio 3/57 (w/w) biomass/Ils	*S. cerevisiae*	97	0.385	290.2[b]	Lara-Serrano et al. (2018)
Sugarcane Bagasse	Pressurized microwave hydrothermal	$MgCl_2$ at 200°C, 5 min 2.45 GHz and ratio: 1/15 (w/v) biomass/solution	*S. cerevisiae* BA11	90	Nr	228.1[a]	Asada et al. (2020)
Sugarcane Bagasse	Enzymatic treatment	*A. tubingensis* enzymatic cocktail with 1 FPU; pH 5,0; 45 °C, 16 h and ratio 0.7/10 (w/v) biomass/solution	*Candida shehatae*	77.9	0.161	84.9[a]	Prajapati et al. (2020)
Sugarcane Straw	Dilute acid hydrolysis	H_2SO_4 at 155°C, 25 min and ratio: 1/10 (w/v) biomass/solvent	*S. cerevisiae*	50	1.096	215.1[a]	Mesa et al. (2020)
Bagasse	Ionic Liquids	Choline acetate at 110°C 21h and ratio 2/3 (w/w) biomass/Ils	*S. cerevisiae* YPH499XU	85	0.625	152.1[a]	Ninomiya et al. (2018)
Sugarcane Bagasse	Sequential pretreatment	Potassium peroxymonosulfate (65 °C, 10h and 175 mmol/L) combined with NaOH (65 °C 1h and 12.5 mmol/L) and ratio 1/20 (w/v) biomass/solution	*S. cerevisiae* SHY07-1	79.01	0.56	135[a]	Bu et al. (2019)
Sugarcane Bagasse	Deacetylation, Liquid hot water pretreatment	NaOH (0.1 M) at 80 °C for 3 h and 1/9 (w/v) biomass/solution ratio. Water /biomass ratio of 1/4 at 70 rpm, 180 °C and 10 min	Genetically modified C5/C6 yeast M11205	Nr	1.42	343.5 [a]	Wang, Z. et al. (2019a)
Sugarcane Straw	Hydrothermally	195°C, 10 min	*S. cerevisiae* Y-904	73.6	0.350	151.0[a]	Pratto et al. (2020)
Sugarcane Bagasse	Steam-assisted sequential salt-alkali	$ZnCl_2$ for 30 min at 121 °C and NaOH at 121°C ratio: 0.97/10 (w/v) biomass/solvent	*S. cerevisiae*	95.9	0.290	62.1[b]	Jugwanth et al. (2020)
Sugarcane Bagasse	Low-temperature sodium hydroxide	NaOH at 50°C; 4h and ratio: 1/9 (w/v) biomass/solvent	*S. cerevisiae* Y2034	67.5	0.932	212.9[a]	Wang, Q. et al. (2019)
Sugarcane Bagasse	Steam explosion	H_3PO_4 (9.5 mg/g of biomass) at 195 °C (18 atm) for 7.5 min		88.9	0.29	174.7 [a]	Neves et al. (2016)
Sugarcane Bagasse	Ionic Liquids	[C_4mim] [OAc] at 120 °C for 24 h and ratio 1/4 (w/w) biomass/Ils	*Schizosaccharomyces pombe*	78	Nr	152.5[a]	Tura et al. (2018)
Sugarcane Bagasse	Aqueous ammonia soaking	NH_4OH (20%) at 50 °C, for 48 h and 1/10 (w/v) biomass/solution	*Candida tropicalis Y-27290*	90.9	1.21	169.5 [a]	Raj; Krishnan (2019)
Sugarcane Bagasse	Green solvent	Imidazole at180°C 1h ratio 1/9 (w/w) biomass/solvent	*S. cerevisiae* LPB 2705	83.7	1.11	217.9[a]	This work

[a]: Calculate from raw biomass

[b]: Calculate from pretreated biomass

Ils: Ionic liquids Nr: Not reported

3.9. Biorefinery approach for economical valorisation of SCB

According to the biorefinery concept, the use of agro-industrial wastes such as SCB to generate value-added products avoids negative environmental impacts and generates economic gains. However, techno-economic analysis needs the validation of several parameters that include some factors of process scale and equipment costs Gubicza et al. (2016), Mandegari et al. (2017) and Pratto et al. (2020). For instance, Gubicza et al. (2016) conducted a techno-economical study to evaluate ethanol production from SCB. The study focused on a co-fermentation process (xylose and glucose) of ethanol with simultaneous production of fertilizer and electricity. The main costs were related to feedstock prices and the annualized capital costs. On the other hand, the ethanol yield greatly influenced the final production cost. The results show the importance of an integrated biorefinery design to obtain the minimum ethanol selling price. Mandegari et al. (2017) discussed techno-economic studies on SCB biorefineries for value-added biochemicals production. The authors showed the importance of the co-production of biofuels and biochemicals for decision making in the development of bio-economy. Recently, the importance of biochemicals such as lactic acid or succinic acid and biopolymers such as poly-hydroxyalkanoates have garnered significant attention. Furthermore, newer second-generation technologies for bioethanol production from lignocellulosic biomass will become commercially feasible in the near future. Moreover, Pratto et al. (2020) proposed the integration of a second-generation ethanol production plant with a first-generation ethanol plant to increase ethanol production. This integration is favourable. It saves capital cost, because the same equipment used in the first-generation ethanol production can be shared for second-generation production. However, the economic evaluation exhibited a negative performance, mainly for the investment cost in the pre-treatment process, because hydrothermal treatment demands more control and technology. The feedstocks and enzymes also showed an increase

in operational expenditure. Thus, for a feasible process, it is necessary to decrease operational expenditure 23.3%. In this way, it is necessary to improve the efficiency of enzymatic hydrolyses and to use new technology to reduce the production costs in the pre-treatment step.

This study presents a process for second-generation bioethanol production from lignocellulosic biomass, which represents a suitable strategy and has a great production efficiency. Furthermore, imidazole has been reported to be a recyclable green solvent Morais et al. (2016). However, further study is specifically needed with SCB to determine the number of times that the solvent can be reused, the total recovery percentage of imidazole from the process, and optimization of the biomass/imidazole ratio used in the treatment.

4. Conclusions

This work clearly demonstrates the potential of imidazole-treated biomass in the production of second-generation bioethanol using SCB as raw material. The pre-treatment mainly induces the delignification of SCB without significant modifications of cellulose characteristics. Additionally, it avoids cellulose mass losses in treated SCB, increases the enzymatic conversion to fermentable sugars, and significantly decreases the entire process time. The results showed encouraging yields and suggest that this new process paves the way for the use of new technologies in the biosynthesis of value-added metabolites using microorganisms and lignocellulosic residues that are abundant worldwide, thus defining an eco-friendly and more efficient process.

6. Integrated sugarcane biorefinery for first- and second-generation bioethanol production using imidazole pretreatment.

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Abstract

Sugarcane bagasse (SB) is a potential source for second-generation bioethanol production. However, its recalcitrant structure hinders the efficient release of sugars. Imidazole is a cheap green solvent that has demonstrated high efficiency in lignocellulosic biomass delignification. The main objective of this work was the use of imidazole-pretreated SB for bioethanol production, proposing the implementation of an integrated sugarcane mills plant. Results showed that SB pretreated with imidazole allowed a cellulose enrichment (167.3%) and a significant decrease of amorphous compounds (lignin and hemicellulose), which led to a 100% conversion of glucan to glucose after enzymatic hydrolysis. Bioethanol production reached 10.4 g.L-1 at 12 h by Saccharomyces cerevisiae and 11.7 g.L-1 after 30 h by Pichia stipitis, which represents a bioconversion yield of 77.6% and 91.5%, respectively. The analysis of the use of SB imidazole-pretreated is proposed by means of implantation of an integrated plant with a total production of 110.3 L of bioethanol per ton of sugarcane, leading to an efficient and economic process with waste valorization, reduction of environmental impacts, and the increase the productivity of bioethanol biorefineries.

Keywords: Bioethanol, Imidazole, Sugarcane bagasse, Integrated plant, Biorefinery

Highlights

- Imidazole pretreatment of SB achieved and enzymatic conversion yield of 100% in 12h.
- Imidazole pretreatment could allow a production of 201 L of ethanol per ton of SB.
- Imidazole pretreatment integrated sugarcane biorefinery increase productivity by 37%.
- Integrated sugarcane biorefinery can produce 110.3 L of ethanol per ton of sugarcane.

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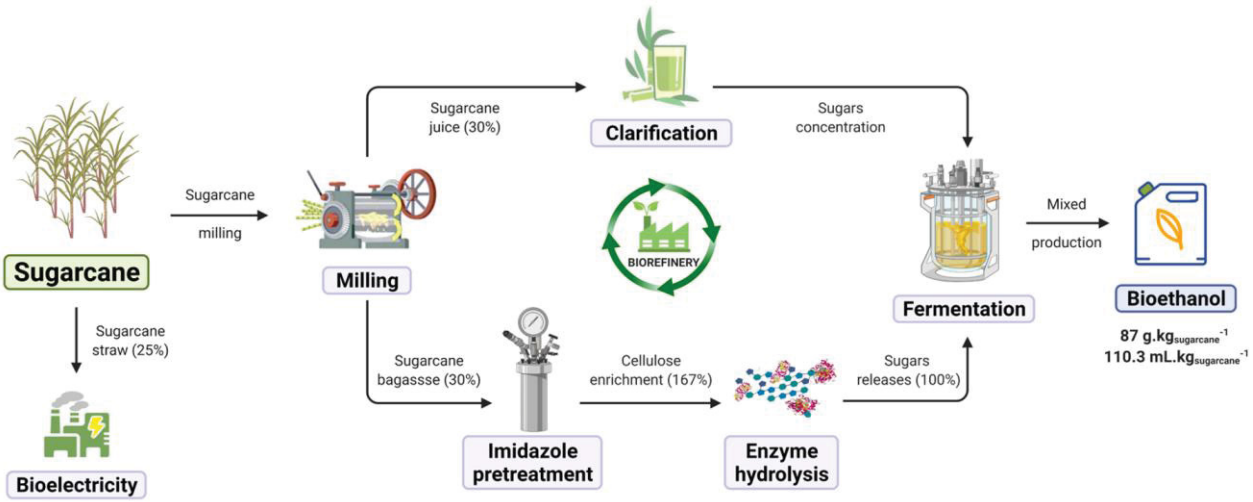
Graphical abstract



1. Introduction

In recent years, the demand for energy has increased by an annual average of 1 to 2%, with an estimated annual global consumption of 580 million terajoules, mainly due to population growth. It is estimated that with this constant growth in 2040 the annual global energy consumption will reach 740 million terajoules, thus increasing its demand by almost 30% The World Counts (2022). Of the total energy produced, 80% is from fossil fuels or non-renewable sources, generating great dependence in most countries due to fluctuations in energy prices influenced mainly by geopolitical aspects. In this sense, the diversification of energy sources is one of the main solutions to avoid dependence on fossil fuels. In recent years, renewable energies have emerged as a response to this problem, having as main characteristics low negative impacts on the environment, capacity for periodic renewal and adaptation to different geographical environments Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). Biofuels are renewable energies are produced from different biomasses from organisms such as plants, fungi or bacteria.

One of the main sources for biofuels production are crops, due to their high content of sugars and lipids that serve as the main source in the production of bioethanol and biodiesel. However, the production of first-generation biofuels generates a competition for the raw material between energy and food industry, causing food prices to rise Kabir et al. (2010). In this sense, second generation biofuels, produced from lignocellulosic biomass, could be a feasible answer to the problem in the "food or fuel" competition Valladares-Diestra; Porto de Souza Vandenberghe; Ricardo Soccol (2021). Brazil is one of the main bioethanol producing countries using sugarcane juice with a production volume in 2021 of 30 million m^3 Statista (2022). In the same way, the production of sugarcane were a total of 10 million cultivated hectares, with an average production of 752.7 million tons in the period of 2018-2020 FAO (2022). The main lignocellulosic by-products in the bioethanol industry from sugarcane are

cane straw and sugarcane bagasse (SB). Together, these by-products represent 55% of sugarcane, with a high content of polysaccharides such as cellulose and hemicellulose Kumar et al. (2020) and Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). The production of second-generation bioethanol (2G) from SB is beneficial because this by-product corresponds to the solid lignocellulosic fraction that is derived from the production of sugar and first-generation bioethanol (1G) production in conventional sugar mills. Approximately 28-30 tons of SB per ton of sugarcane can be produced, and Brazil has an annual production of almost 200 million tons of SB generated per year Barbosa et al. (2017), Kumar et al. (2020) and Santos et al. (2016). For this reason, SB has a high potential in the bioenergy industry for the production of second-generation biofuels.

Although lignocellulosic biomass has a high potential as a raw material in the bioenergy industry, its processing is still expensive due to the need for prior pretreatment to facilitate the release of sugars. In the case of SB, different methods have been studied with the aim of increasing yields of fermentable sugars release. Among the most common pretreatments are chemical and physicochemical methods such as the use of acid and alkaline solutions, which usual glucose conversion efficiencies up to 90% Brienzo et al. (2017). Variations of these pretreatments are reported such as the use of magnetic carbon-based solid acid Lu et al. (2021) and low temperature aqueous ammonia Raj; Krishnan (2020), which increase the cellulose conversion to above 95%. However, the use of these chemical reagents generates by-products that are harmful to the environment, adding additional costs to the process, due to the need for corrosion-resistant equipment. On the other hand, there are new "green solvents" such as ionic liquids and eutectic solvents that emerge as an alternative to be employed in the pretreatment of SB Pin et al. (2019) and Zhang et al. (2020). Although these solvents promote high yields in the conversion of cellulose, their high price makes their use unfeasible at industrial scale. Nevertheless, imidazole is a relatively new low-cost green solvent with ideal characteristics for

biomass pretreatment, presenting high indexes of delignification and low losses of cellulose Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). In addition, their recycling and reuse can be evaluated Morais et al. (2016). Due to these reasons, imidazole has great potential for its application in pretreatment of lignocellulosic biomass and the production of 2G bioethanol Morais et al. (2016), Sayuri Nishida et al. (2021) and Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021).

Biorefineries arise with the aim of using lignocellulosic biomass as a source for medium to high value bioproducts/biochemicals production, Porto de Souza Vandenberghe et al. (2021). In this sense, the emergence of sugarcane biorefineries aims at the full use of the raw material in the production of sugar, bioethanol and bioelectricity. Brazil is one of the countries with the largest production of these products with a total of 422 sugarcane mills in operation, being the pioneer in sugarcane biorefineries NOVACANA (2022). However, the demand for biofuels with a lower environmental impact leads to the search for new technologies that increase productivity without the need to generate new areas of cultivation. For this reason, the implementation of integrated bioethanol production is being studied and developed for the concomitant production of 1G and 2G bioethanol. Vasconcelos et al. (2020). In this way, new alternatives capable of improving the obtaining of fermentable sugars and increasing the global productivity of bioethanol per cultivated area of sugarcane are needed Kumar et al. (2020).

The objective of this work is to study and demonstrate the efficiency of imidazole in the pretreatment of SB to obtain fermentable sugars, evaluating the bioconversion of these sugars in the production of 2G bioethanol, as anew alternative pretreatment in the implementation approach of an integrated sugarcane biorefinery.

2. Material and methods

2.1.Raw materials and chemicals

SB was provided by the Santa Terezinha - USACUCAR company from Paraná State - Brazil. The biomass was dried in an air-circulating oven at 65°C for 48 h, and milled (Marconi, MA580/E). The particle size used in all experiments was between 0.85 mm (ASTM No. 20) and 0.35 mm (ASTM No. 45). All purchased chemicals and culture medium components were of analytical grade.

2.2.Compositional analysis of biomass

The solid content, ash, extractives, acid insoluble lignin (AIL), acid soluble lignin (ASL), and structural carbohydrates of the untreated biomass and after imidazole pre-treatment were determined according to the National Renewable Energy Laboratory (NREL) procedures Sluiter et al. (2012), Sluiter; Hames; Hyman; et al. (2008), Sluiter; Hames; Ruiz; et al. (2008) and Sluiter; Ruiz; et al. (2008).

2.3.Sugarcane bagasse pretreatment

Imidazole was used as solvent in pretreatment for delignification of SB following the optimum procedure described previously by Valladares-Diestra et al., (2020). 5 g of dried SB were added to imidazole at a ratio of 1:9 ($w.w^{-1}$). Pretreatment essays were carried out in a stainless steel 150 mL reactor (Parr, USA) with 1500 rpm of mechanical stirring at 160°C for 1 h. After pre-treatment, 135 mL of deionized water were added to the material and mixed for 1 h at room temperature. The liquid and solid fraction were separated by vacuum filtration. The cellulose-rich solid fraction was washed with 135 mL of 96% $v \cdot v^{-1}$ ethanol for imidazole recovery and then it was dried in an air-circulating oven at 65°C for further analysis.

2.4.Scanning electron microscopy (SEM)

SEM analysis of untreated and pre-treated SB was performed to reveal ultra-structural changes. Samples were mounted on aluminum stubs, and sputter-coated with a gold layer. The scanning and acquisition of microphotographs were carried out using a Jeol JSM 6360-LV (Oxford Instruments) scanning electron microscope that was operated at 15kV.

2.5.High-performance liquid chromatography (HPLC) analyses

Organic acids, sugars, and alcohols were analysed by HPLC. Samples were filtered through 0.22 μm pore size membranes (Millipore Corp., Billerica, MA, USA) and were analysed in an HPLC system equipped with an Aminex Bio-Rad HPX-87H column operating at 60°C and a refractive index (RI) detector. The mobile phase was composed of an aqueous solution of 5 mM H_2SO_4 at a flow rate of 0.6 $mL \cdot min^{-1}$, and injection volume of 15 μL. The chemical standards were prepared with D-(+)-cellobiose, glucose (>99%), D-(+)-xylose (>99%), D-(-)-arabinose (>99%), succinic acid (>99%), acetic acid (>99%) and ethanol (>99%) from Sigma-Aldrich (USA).

2.6.Enzymatic hydrolysis of pre-treated biomass

The recovered cellulose-rich solid fraction after imidazole pre-treatment was subjected to enzymatic hydrolysis. Experiments were performed in 125 mL Erlenmeyer flasks containing 2.5% $w \cdot v^{-1}$ biomass (dry weight) in 12.5 mL of 50 mM sodium citrate buffer solution (pH 4.8) with 0.02% ($w.v^{-1}$) sodium azide and a 1/9 ratio of Cellic HTec2® / Cellic CTec2® ($v.v^{-1}$) from Novozymes – Araucaria, Brazil. The enzymes' loading per gram of solid biomass were: Cellulase 20 FPU; xylanases 2580.7 U and β-glucosidase 35700 U. Samples were incubated in an orbital shaker at 50°C for 96 h at 150 rpm. Sample aliquots were obtained and heated up to ~95 °C for 10 min in a water bath for enzyme inactivation. Then,

samples were filtered through 0.22 μm cellulose acetate membrane syringe filters and analysed by HPLC. All assays were performed at least in duplicate

Glucose and xylose yields obtained after enzymatic hydrolysis of untreated and pre-treated SCB were calculated using Eq. 3 and Eq. 4:

$$\text{Glucose convertion yield (\%)} = \frac{[\text{Glu}] \times \text{V}}{\text{m}_{\text{biomass}} \times \text{f} \times 1.11} \text{x}100 \qquad (3)$$

$$\text{Xylose convertion yield (\%)} = \frac{[\text{Xyl}] \times \text{V}}{\text{m}_{\text{biomass}} \times \text{f} \times 1.13} \text{x}100 \qquad (4)$$

where [Glu] and [Xyl] are glucose and xylose ($g \cdot L^{-1}$) concentrations in the hydrolysate determined by HPLC, respectively; V is the volume (L) of hydrolysate; $m_{biomass}$ is the dry weight of biomass (g); f is the glucan and xylan fraction in the biomass; 1.11 and 1.13 are the glucan to glucose and xylan to xylose conversion factors, respectively.

2.7. Bioethanol and succinic acid production

Two yeast strains of *S. cerevisiae* and *P. stipitis* from the culture bank of the Bioprocess Engineering and Biotechnology Laboratory of Federal University of Paraná were tested for bioethanol. The obtained SB hydrolysate was employed in fermentation procedures using 125 mL Erlenmeyer flasks with the addition of 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 2 g L^{-1} KH_2PO_4 and 1 g L^{-1} $MgSO_4$; pH was fixed at 5.5 with a final volume of 10 mL. Flasks were autoclaved at 121°C for 15 min. *S. cerevisiae* and *P. Stipitis* inoculum cultures (5%, $v \cdot v^{-1}$; 24 h old) with an optical density (O.D.) of 2.4 at 600 nm were employed for fermentation. Flasks were placed in an orbital shaker at 35°C with agitation of 150 rpm. Samples were withdrawn every 3, 6, and 12 h for up to 24 h and centrifuged (10,000 rpm, 15 min, 25 °C). The chemical composition of the supernatant was then determined by HPLC. Experiments were performed in duplicate.

The theoretical yield of bioethanol was calculated using equation Eq. 5.

$$\text{Bioethanol yield (\%)} = \frac{[\text{Bioethanol}]}{0.511 \times ([Glu_i] - [Glu_r])} \times 100 \quad (5)$$

where 0.511 is the conversion factor of glucose to bioethanol; [Bioethanol] is the concentration of bioethanol produced by fermentation, and [Glu_i] and [Glu_r] are initial and residual glucose concentrations, respectively.

3. Result and discussion

3.1.Imidazole pretreatment of SB

The composition of non-treated SB was determined as 38.7% cellulose, 23.0% hemicellulose, 16.9% lignin with other components as shown in Table 1. Cellulose values are very similar to previous studies reported by Ramadoss and Muthukumar, (2015), Wang et al., (2019) and Raj and Krishnan, (2019) (38; 37.3 and 39.6%, respectively). The hemicellulose composition was slightly higher than the results reported by Raj and Krishnan, (2019) (21.04%) and Wang et al., (2019) (22.56). However, the lignin composition obtained in this study was lower in comparison with the average of 20.4 - 27% reported by different authors Raj; Krishnan (2019), Ramadoss; Muthukumar (2015), Tura et al. (2018) and Wang, Z. et al. (2019a). The variation in the reported chemical composition of SB is probably due to different factors where the most important are the variety of the plant, growing conditions, influence of the geographical region, nutritional factors, among others. In particular, the characteristics of SB used in this study show a high potential for its valorization in different processes due to its high content of polysaccharides (61.7% holocellulose) and a low presence of lignin. These characteristics show the profile that is sought in most agro-industrial wastes to maximize the recovery of fermentable sugars Carvalho, D. J. et al. (2020).

Table 1. Chemical composition of non-treated and imidazole-treated SB

Composition (wt%)	**SB** $_{\text{non-treated}}$	**SB** $_{\text{treated}}$
Cellulose	38.72 ± 0.81	64.77 ± 0.24
Hemicellulose	23.01 ± 0.62	17.60 ± 0.18
Lignin	16.86 ± 0.74	7.78 ± 0.21
Extractive	5.27 ± 0.12	-
Ash	3.73 ± 0.05	-
Solid recovery ($w.w^{-1}$ %)	100	55.7 ± 0.14
Cellulose loss (%)	0	6.46 ± 0.35
Hemicellulose loss (%)	0	57.24 ± 0.43
Lignin loss (%)	0	74.17 ± 0.23

Although the lignin amount is relatively low, this polymer, together with hemicellulose (amorphous polysaccharide), is the main barrier for the enzymatic hydrolysis of cellulose (polysaccharide of greater industrial importance). For this reason, physicochemical pretreatment processes are necessary to destabilize and break down these amorphous polymers achieving efficient sugars' release from cellulose fractions. Imidazole showed its high efficiency in the catalyzes lignocellulosic material delignification such as it is shown in Table 1. Significant differences and variations were found between the chemical composition of non-treated SB and imidazole-treated SB. The imidazole pretreatment promoted a cellulose enrichment of 167.3% and the yield of solid fraction recovery that reached an efficiency of 55.7% with an insignificant loss of cellulose (6.5%). These results show that imidazole acts as a promising catalyst in the removal of the amorphous fractions of SB, leading to higher values of delignification (74%) and hemicellulose extraction (57%). In addition, previous studies showed the changes in the increase of crystallinity of pretreated SB, that is mainly due to the

elimination of amorphous polymers Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021).

In addition to the compositional changes, imidazole pretreatment generated structural modifications in SB as it can be seen in Figure 1. The non-treated SB presents rigid and compact structures, showing high resilience (recalcitrance), which is a very common characteristic in plant tissues. This uniformity pattern adopted by plants act as defense barrier that hinders the degradation caused by microorganism such as fungi and bacteria Gibson et al. (2011). However, after imidazole pretreatment it is possible to clearly observe a total destructuring of SB, leaving the initial fibers' uniformity totally disordered (Figure 1). Besides, a spatial expansion, which is generated in the SB biomass after imidazole pretreatment, can also be observed, thus increasing the total surface area of the material. These changes are clearly beneficial for further enzymatic hydrolysis, because pretreatment breaks the physical barriers of lignocellulosic biomass, increases the permeability of the material generating a greater free space that facilitates the coupling of enzymes to biomass.

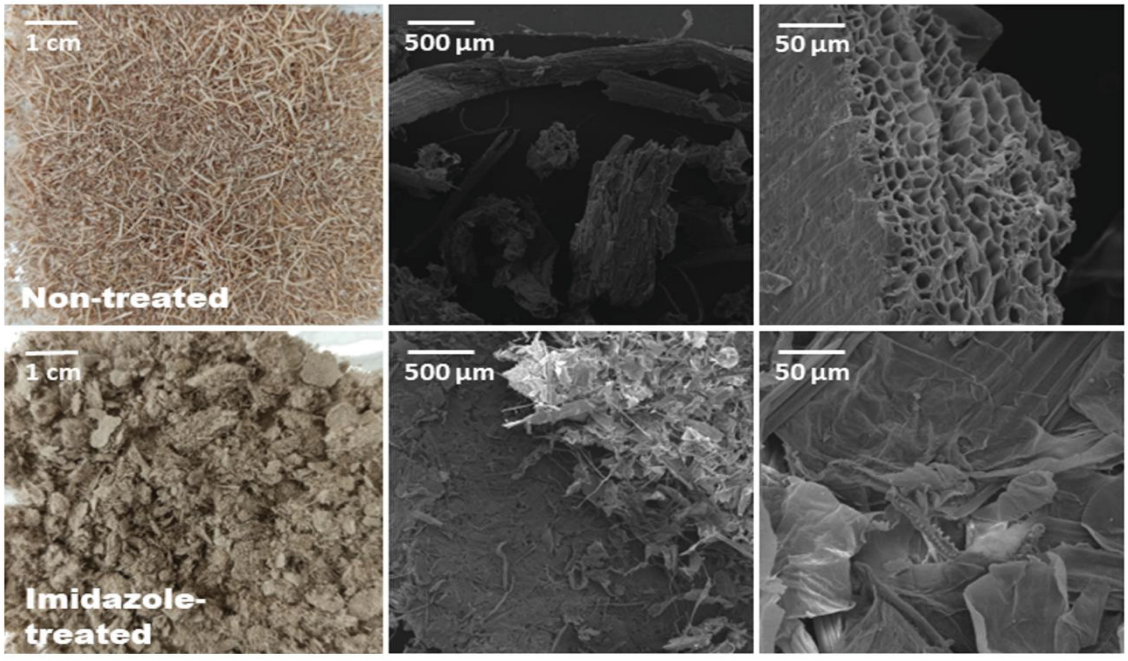


Figure 1. Scanning electron microscopy of non-treated SB and of the cellulose-rich fraction recovered after imidazole pretreatment

3.2.Enzymatic hydrolysis

The enzymatic hydrolysis is the main stage for determining the pretreatment efficiency, which is strongly influenced by different factors, such as the permeability of pretreated material and the employed enzyme complex Ingle et al. (2020) and Raj; Krishnan (2019). In this sense, the cellulose-rich fractions obtained after pretreatment were hydrolyzed with the use of the commercial enzyme complexes Cellic Cetec 2/Cellic Htec 2. These commercial enzyme preparations have several enzymes that act synergistically in the total degradation of the substrate, a set of exo and endo cellulases, hemicellulases and highly specific enzymes such as β-glycosidases and β-xylosidases. The coordinated use of these enzymes generates greater efficiency in the degradation of lignocellulosic material Kim et al. (2014).

As it is shown in Figure 2, after enzymatic hydrolysis of imidazole-pretreated SB sugars' release was clearly more efficient compared to non-treated SB. After 24 h, glucose concentration reached 19.5 g/L and only 2.7 g/L for pretreated SB and non-treated SB, respectively, which represents an increase of up to 7-fold of glucose release (Figure 2A). A significant increase of xylose release was also observed for pretreated SB (5.9 g/L) that was higher than for non-treated SB (1.0 g/L), which means 5-fold more. In the case of pretreated SB, 100% of enzymatic conversion was observed. The low efficiency of enzymatic hydrolysis of non-treated materials can be explained due to natural barriers of the plant biomass that hinder the enzymatic attack Brienzo et al. (2017). A rapid enzymatic conversion of glucan and xylan of pretreated SB can be observed with only 24 h of enzymatic hydrolysis. However, for non-treated SB, the efficiency of conversion of glucan to glucose reached a maximum of 33%; while the efficiency of conversion of xylan to xylose reached only of 28% after 72 h in both cases. This demonstrates the importance of the imidazole pretreatment process, which showed a high efficiency in the release of fermentable sugars, allowing the possible use of all

the polysaccharide content within the lignocellulosic biomass. So, imidazole shows a high potential as solvent for the destructuring of lignocellulosic components for a better performance of SB pretreatment.

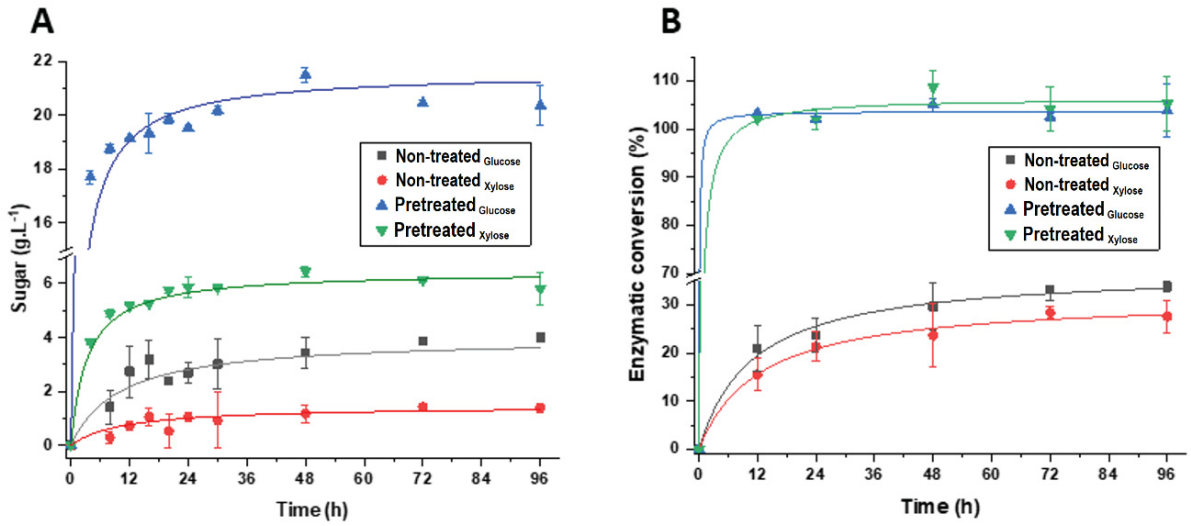


Figure 2. Enzymatic hydrolysis profile of imidazole-treated and untreated SB. A) Sugars' release and B) Enzymatic conversion (EC)

The enzyme loading and time of enzymatic hydrolysis are very important factors that affect the viability of second-generation biofuels production. In this sense, different pretreatment methods were applied to SB to obtain high glucose conversion yield. As it is shown in Table 2, some of the developed SB pretreatments required the use of high amounts of enzymes in order to achieve efficient sugars' release, without achieving 100% of conversion. Magnetic carbon acid pretreatment and low-temperature aqueous ammonia pretreatment evaluated by Lu et al., (2021) and Raj and Krishnan, (2020) promoted above 95% of conversion using 20 FPU per g of pretreated SB, but the enzymatic hydrolysis was carried out during 72 h. On the other hand, Nalawade et al., (2020) applied an alkali pretreatment for SB using 30 FPU per g of pretreated SB, with further enzymatic hydrolysis during 24 h, reached a converion of 75%. New solvents such as Organosolv or ionic liquids presented good performances in the pretreatment of SB achieving, in some cases, a

conversion of above 95% and a reduced time of enzymatic hydrolysis of 48 h Pin et al. (2019) and Zhang et al. (2020). Nevertheless, the cost of these new solvents are too high, which makes their application unfeasible at industrial scale.

In this work the results demonstrated the potential of the use of imidazole in SB pretreatment, showing an efficient sugars' release with relatively low use of enzyme loading and reduced enzymatic hydrolysis times. This fact is could greatly reduce process's costs, with promising and feasible use at larger scales.

Table 2. Efficiencies of enzymatic hydrolysis for SB pretreated with different pretreatment strategies

Pretreatment	Enzymatic loading (FPU.g biomass^{-1})	Biomass loading (w.v^{-1})	Incubation time (h)	Glucose conversion yield (%)	Reference
NaOH-catalyzed organosolv pretreatment	20 FPU Cellic CTec2	10%	24 h	95.1	Zhang et al. (2020)
Magnetic carbon-based solid acid pretreatment	20 FPU Cellulase	5%	72 h	94.26	Lu et al. (2021)
Alkali (NaOH) pretreatment	30 FPU Cellic CTec2	12.5%	24 h	75.8	Nalawade et al. (2020)
Low-temperature aqueous ammonia pretreatment	20 FPU Cellic CTec2	2%/20%	72 h/48 h	98.8/81	Raj; Krishnan (2020)
Protic ionic liquids $[Me(NH_2)(CH_2)_2OH][OAc]$ pretreatment	15 FPU Cellic Ctec2	10%	48 h	72	Pin et al. (2019)
Ferric chloride pretreatment	20 FPU Cellic CTec2	2%	72 h	76.6	Zhang et al. (2017)
Sulphuric acid solution pretreatment	15 FPU Spezyme CP	2%	72 h	88.5	Brienzo et al. (2017)
Imidazole pretreatment	20 FPU Cellic CTec2	2.5 %	24 h	100	This study

3.3.Bioethanol and succinic acid production from SB hydrolysate

Second generation bioethanol is one of the most important renewable biofuels. In this sense, the sugars obtained from imidazole-pretreated SB after enzymatic hydrolysis were employed in submerged fermentation with two yeasts, *S. cerevisiae* and *P. stipitis,* for bioethanol production. S. *cerevisiae* is the most widely applied yeast in the bioethanol industry, and *P. stipitis* is generally used in media with high xylose content, due to its capacity to consume pentoses Agbogbo et al. (2006) and Tura et al. (2018).

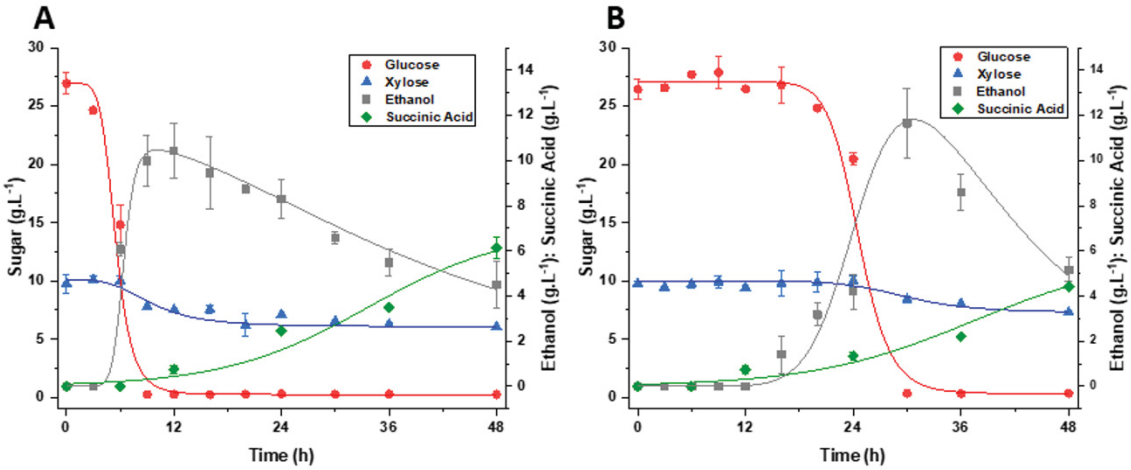


Figure 3. Bioethanol and succinic acid production from hydrolysate of SB pretreated. A) *Saccharomyces cerevisiae* and B) *Pichia stipites*

Figure 3 shows the production profiles of bioethanol and succinic acid by *S. cerevisiae* and P. *stipitis*. As it was expected, *S. cerevisiae* produced a higher concentration of bioethanol with almost total sugar consumption, after only 12 h of fermentation with a production of 10.4 ± 1.2 $g.L^{-1}$ and a productivity of 0.87 $g.L^{-1}h^{-1}$ (Figure 3A). However, xylose consumption was not significant, only 37.8%, after 48h of fermentation. In addition, it was possible to detect the production of succinic acid, a biomolecule of high industrial interest. A total production of 6.13 ± 0.49 g. L^{-1} of succinic acid was obtained after 48 h of fermentation. However, it was observed that succinic acid concentration increased when bioethanol concentration decreased,

which may indicate that succinic acid was produced through the glyoxylate cycle by the oxidation of bioethanol. The glyoxylate cycle is found in yeasts' metabolism where the bioethanol molecule is oxidated by alcohol dehydrogenase enzyme in acetaldehyde and then the acetaldehyde is directed to the glyoxylate pathway for succinic acid production Li et al. (2021). It is very important to highlight that the glyoxylate cycle occurs under aerobic conditions to provide extra NADH that benefits the succinic acid formation, which was the case in the described fermentation process.

In the case of *P stipitis* (Figure 3B) there was a long adaptation phase (lag phase) of approximately 20 h when glucose started to be consumed and bioethanol began to be produced. After this adaptation period, glucose consumption was quite fast till complete consumption at 30 h of fermentation with a maximum of bioethanol production of 11.66. ± 1.53 $g.L^{-1}$, with a low productivity of 0.39 $g.L^{-1}h^{-1}$. A low xylose consumption was also observed, which was not normally expected for this yeast strain. However, some, the metabolism of microorganisms that are able to consume both monosaccharides (glucose and xylose) may preferentially directed to glucose consumption, which could explain the low performance of xylose consumption by *P. stipitis* and *S. cerevisiae* Agbogbo et al. (2006). Similarly, to *S. cerevisiae*, succinic acid production by *P. stipitis* increased with the oxidation of bioethanol, a final succinic acid production of 4.42 ± 0.01 g. L^{-1} was achieved at 48 h of fermentation.

As it is possible to see, the fermentation process occurred satisfactorily, without any interruption, which would suggest that after pretreatment and enzymatic hydrolysis stages, the hydrolyzed material does not present significant inhibitors' concentrations. In the case of *P. stipitis*, the delay in bioethanol production is mainly due to the adaptation time of the strain, which is more susceptible to sudden changes in the environment Agbogbo et al. (2006).

Bioethanol production efficiency were 77.55 and 91.51% for *S. cerevisiae* and *P. stipitis,* respectively. These significant differences may be due to the different metabolisms of each strain. In the case of *S. cerevisiae*, a higher production of biomass was observed (data not shown), which could have decreased a higher synthesis of bioethanol. Even so, bioethanol production was not optimum. However, a better performance of *S. cerevisiae* could have been reached for bioethanol production with a fed-batch mode or simultaneous saccharification and fermentation strategy, mainly due to the low amount of lignin or its degraded components that are present in the material after pretreatment, which are the main inhibitors of enzymatic and fermentative processes Madadi et al. (2022) and Zhu, J. Q. et al. (2020). With this, the substrate (SB hydrolysate) could be fed intermittently to solve the problem of the almost complete consumption of sugars in the first 12 h. Consequently, a higher biomass production could have been reached with also a better bioethanol production Zhu, J. Q. et al. (2020).

3.4.Mass balance of the developed process

The mass balance of developed bioethanol production process was performed. From 1 kg of dry SB it is possible to recover 557 g of pretreated SB with a relatively small loss of cellulose (26.4 g). Furthermore, a lignin reduction of 75% was achieved, from 168.6 g to only 43.3 g of pretreated SB. Pretreatment process was quite successful, which significantly favored the enzymatic hydrolysis step, as already described above, allowing the release of 400.5 g of glucose and 110.8 g of xylose from pretreated SB. The efficient release of fermentable sugars from pretreated SB without the presence of inhibitors is positive for bioethanol production , leading to 158.7 g (201 mL) of bioethanol after only 12 h of fermentation using the *S. cerevisiae* from 1 kg of dry SB. In the case of *P. stipitis*, the efficiency of bioconversion of glucose to bioethanol (91.5%) was higher than that obtained for *S. cerevisiae* (77.6%), resulting in the production of 187.g (237 mL) of bioethanol from of 1 kg of dry SB, as it can

be seen in Figure 4. However, the fermentation process with *P. stipitis* would entail a longer incubation time, causing a decrease in productivity. That is evident when *P. stipitis* productivity of 0.39 $g.L^{-1}h^{-1}$ is compared with *S. cerevisiae* productivity of 0.87 $g.L^{-1}h^{-1}$ even when the final bioethanol production of *P. stipitis* (11.66 $g.L^{-1}$) was higher than *S. cerevisiae* (10.4 $g.L^{-1}$). The longer incubation time and low bioethanol productivity by *P. stipitis* could increase the process costs. For this reason, the adaptation and improvement of this strain is necessary to reduce the lag phase. In addition, the consumption of xylose was not fully exploited, so better strategies need to be studied, such as the use of genetically modified yeast with the ability to efficiently consume xylose Wang, Z. et al. (2019a). Together with simultaneous fermentation and saccharification strategies, is possible produce bioethanol in a shorter time, reducing processes' costs. This would allow a higher final production of bioethanol, generating a better productivity.

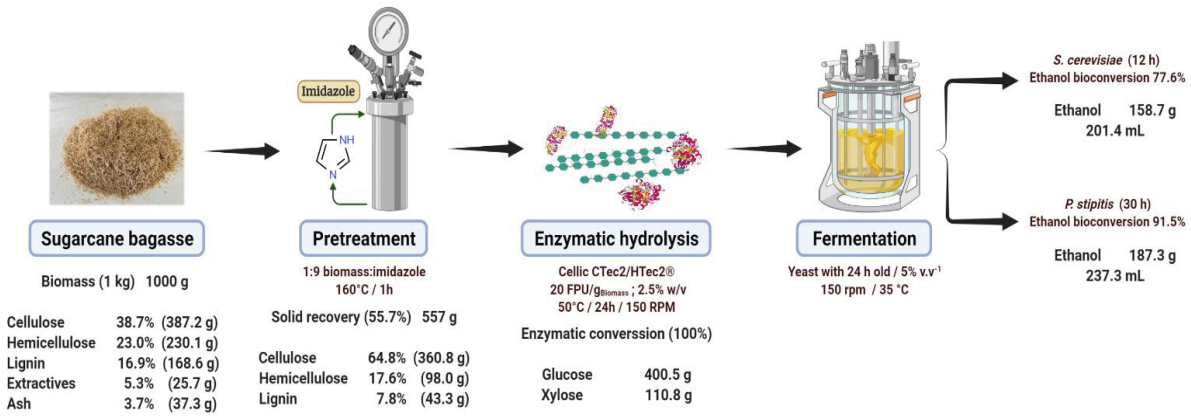


Figure 4: Mass balance of bioethanol production from imidazole-pretreated SB

According to the presented data it is possible to obtain 237.3 L (*P. stipitis*) or 201.4 L (*S. cerevisiae*) of bioethanol from 1 ton of dry SB. This result shows the potential of the second-generation bioethanol production process from SB using imidazole as solvent in the pretreatment stage. Different production values of second generation bioethanol from a ton of

SB have been reported: 233 L of bioethanol using a dilute acid pretreatment and fermentation with *S. cerevisiae* with an bioethanol bioconversion efficiency of 84.3% Ramadoss; Muthukumar (2015); 228 L bioethanol using pressurized microwave hydrothermal pretreatment and *S. cerevisiae* with a biotransformation efficiency of 90% Asada et al. (2020); 169 L of bioethanol with previous soaking treatment in aqueous ammonia using *Candida tropicalis* with a biotransformation efficiency of 90.9% Raj; Krishnan (2019); 152 L of bioethanol using ionic liquids in the pretreatment and *Schizosaccharomyces pombe* with a biotransformation efficiency of 78% Tura et al. (2018); 343 L of bioethanol applying deacetylation, liquid hot water pretreatment and a genetically modified yeast for xylose and glucose consumption Wang, Z. et al. (2019a). As it can be seen, according to the cited literature, the final bioethanol production values are being directly influenced by the type of pretreatment (that affects the enzymatic hydrolysis stage) and the bioconversion efficiency of fermentable sugars to bioethanol, which is a characteristic of each type of yeast.

3.5.Biorefinery approach for an integrated bioethanol production from sugarcane

Although second-generation bioethanol production obtained from imidazole-pretreated SB is quite efficient, its application at industrial scale is not yet possible. This is mainly due to the high costs of pretreatment and enzymatic hydrolysis stages, making the production of 2G bioethanol not yet economically competitive Pratto et al. (2020). An alternative to reduce the impact of these costs, would be the integration of 1G and 2G bioethanol production, which would allow an increase in productivity and reduction of final costs through the use of the same equipment in shared stages of the industrial process.

According to this approach, it is possible to take advantage of both sugars from sugarcane juice and those derived from pretreated SB. The production of 1G bioethanol at industrial scale has been quite applicable and is in continuous development. Productivity

values range from 75 to 85 L of bioethanol per ton of sugarcane, with distillation efficiencies of 99% in sugarcane mills. Brazil is one of the main producers of bioethanol from sugarcane, with a production of 757,1 Mt of sugarcane in a cultivation area of 10 million of hectares in 2020; coming to develop advanced technologies for the processing and production of biofuels. Worldwide, the production of sugarcane was 1,869.7 Mt in 2020, and the compositional characteristics of this raw material are quite variable according to the region of cultivation and cultivated species. In this sense, composition and chemical characteristics of sugarcane are also important in the final productivity. In Table 3 the average of the compositional percentage of sugarcane, as well as the main productivity indexes of the bioethanol industry, according to reported experimental values, are shown Barbosa et al. (2017), De Souza Dias et al. (2015) and Valderrama et al. (2020).

Table 3: Main parameters of sugarcane yields and bioethanol first generation production

Parameter	Value	Unit	Reference
Sugarcane production	1869,7	Mt	FAO (2022)
Area harvested	26,46	Mha	FAO (2022)
Production yield	70,64	$ton.ha^{-1}$	FAO (2022)
Bioethanol yield	80	$L_{ethanol}\ ton_{sugarcane}^{-1}$	Barbosa et al. (2017 e De Souza Dias et al. (2015 e Valderrama et al. (2020)
Distillation yield	99	%	
Sugarcane juice	30	%	Barbosa et al. (2017 e Santos et al. (2016)
Juice moisture content	75	%	
Sugarcane bagasse	30	%	
Bagasse moisture content	50	%	
Sugarcane straw	25	%	
Straw moisture content	50	%	

Based on these parameters, a mass balance was carried out to simulate an integrated production unit of 1G and 2G bioethanol. In this case imidazole-pretreated SB would be employed in the production of 2G bioethanol (Figure 5). It is possible to observe that the final yields of bioethanol production would depend mainly on SB composition characteristics and the type of applied pretreatment. In addition, most of the by-products of this process are used for bioelectricity generation, such as sugarcane straw Sampaio et al. (2019). On the other hand, this approach allows the share of same equipment in common stages for 1G and 2G bioethanol production such as the fermentation and distiller step, reducing the initial costs of implementation for an integrated sugar mills plant in industrial scale.

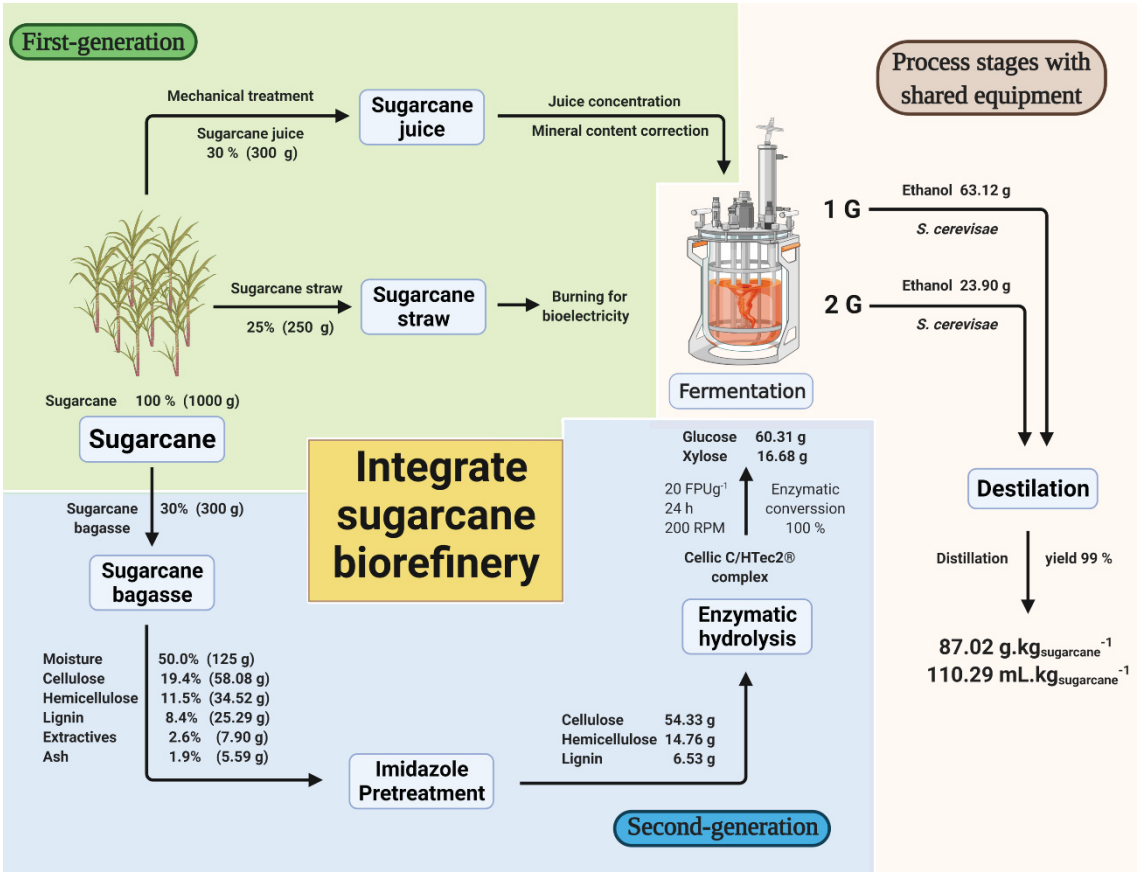


Figure 5: First and second generation bioethanol integrated production simulation using imidazole- pretreated SB

The application of imidazol-pretreated SB in an integrated production of bioethanol could increase the total production of more than 37%, with approximately 110 L per tonne of sugarcane. However, this productivity would be achieved if all SB is employed. This would be one of the main barriers, since high percentages of the SB is applied for bioelectricity production in conventional sugarcane mills, allowing their energy self-sufficiency Watanabe et al. (2020). However, sugarcane straw could replace SB in the bioelectricity production, being a solid by-product with similar composition. Sugarcane straw has similar potential to SB for biofuel production, but differently from SB, an additional step would be necessary for cleaning the soil-derived impurities found in this by-product (such as stone and dirt), which makes biofuel production more expensive. For this reason, sugarcane straw is being used as a raw material with high potential for bioelectricity production supplied in conventional sugarcane mills to replace of SB Sampaio et al. (2019) and Watanabe et al. (2020).

Carpio and Simone de Souza, (2017) analyzed different scenarios for the use of SB in the production of 2G bioethanol and bioelectricity. The results showed that the best scenario with less risks occurred when the cost of bioelectricity production remained at US$50/MWh and the price of bioethanol production was US$0.30/liter. With these previous conditions it was possible to use 84% of SB in the production of 2G bioethanol, with a net return of US$ 20 per ton of SB. Dias et al. (2009) evaluated the energy self-sufficiency of a sugarcane mill with two different distillation systems, with conventional and double effect distillation columns. In both cases, the condition of 50% of sugarcane straw was considered for bioelectricity and steam production. Results showed that with conventional distillation column, it was possible to use 76% of SB for bioethanol production with a final integrated production of 102.5 L of bioethanol per ton of sugarcane. While with double effect distillation column, 90% of SB should be used with a final integrated production of 105.7 L bioethanol per ton of sugarcane. Maximizing energy efficiency within a sugarcane mill would allow the

use of SB for the production of 2G bioethanol while maintaining energy self-efficiency. Finally Barbosa et al., (2017) evaluated the carbon mass balance in sugarcane biorefineries achieving maximum yield of second generation bioethanol using 70% SB. This process strategy led to a significant increase of 29.4% in the yield of bioethanol production per ton of sugarcane, and also represented 22% of the conversion of carbon into bioethanol.

As it can be seen, a sugarcane mill plant can be analyzed through different perspectives, highlighting in recent years the focus on cleaner bioenergy production. This is because the cogeneration of biofuels and bioelectricity are strategically less polluting than energies obtained from fossil sources, promoting a reduction of greenhouse gas emissions Watanabe et al. (2020). In addition, the diversification within integrated plants with the production of different commodities could generate greater fluidity within the market, allowing greater dynamism in production volumes depending on market prices Sampaio et al. (2019). According to Vasconcelos et al., (2020) the benefits and economic returns in the implementation of integrated sugarcane biorefineries not only depend on obtaining higher yields in the final product, but are also strongly linked to the speed of the process and the reuse and valorization of the different by-products generated during the process.

The new advances in 2G bioethanol technology allow the new sugarcane biorefineries to be viable at industrial scale. In addition, the implementation of integrated sugarcane biorefineries for the production of 1G and 2G bioethanol benefits in different aspects such as: a) the increase in the diversity of products, allowing sugarcane biorefineries to have higher diversity of products according to market demand; b) reducing the greenhouse gases emission; C) increase of biofuels productivity without the need for an expansion of cultivation areas, reducing environmental impacts; and d) this approach impulse the use of agro-industrial

by-products for 2G biofuels production avoiding the excessive use of raw materials that are destined to food industry and reducing the discussed agroindustry conflict of "food vs fuel".

4. Conclusion

The pretreatment of SB with imidazole showed excellent results proving to be an optimal catalyst for SB delignification, allowing to achieve an enzymatic conversion efficiency of 100% with an incubation time of only 12 h. This could reduce the process costs at larger scale. *S. cerevisiae* showed a higher productivity (0.87 $g.L^{-1}h^{-1}$) with a biotransformation efficiency of 77.6 % for produce bioethanol from enzymatic hydrolysates. The application of imidazole in the pretreatment of SB would allow a production of 201 L of bioethanol per ton of SB, while that implementation of this technology in an integrated sugarcane biorefinery would increase the production by 37%, reaching 110 L of bioethanol for tone of sugarcane. Finally, with this approach, the emission of greenhouse gases would be reduced, the productivity of bioethanol would increase without increasing the areas of cultivation, and it would diversify the production of sugarcane mills.

7. Xylooligosaccharides production by xylanases complex using xylan extracted from sugarcane bagasse treated with imidazole

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Abstract

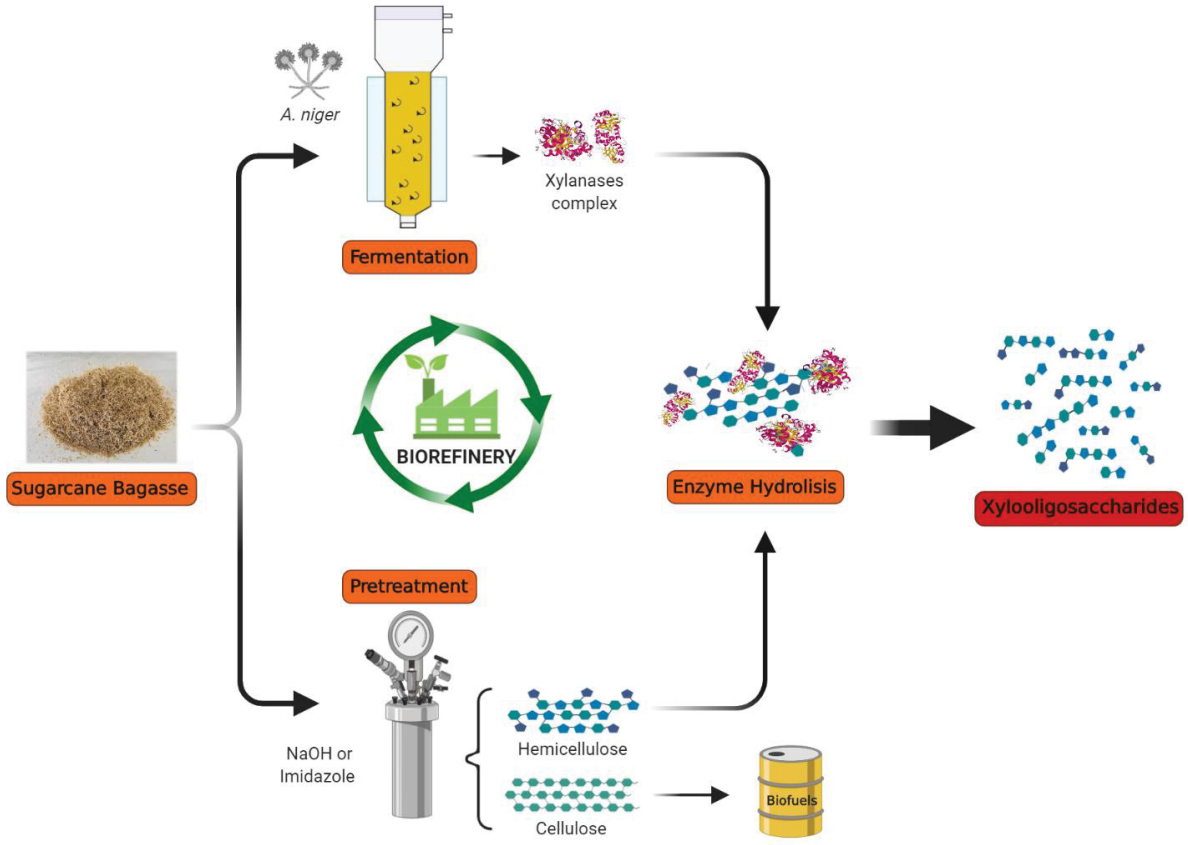
The application of biorefinery concepts to produce different value-added biomolecules such as xylooligosaccharides (XOs) generates economical competitive, sustainable and environmentally friendly processes. The objective of this work was to develop an efficient production of a xylanolytic complex using lignocellulosic biomass for its application in the production of XOs obtained from hemicellulose extracted with imidazole from sugarcane bagasse (SB). Results showed a xylanase production of 53.1 $U.mL^{-1}$ at 120 h of fermentation with *Aspergillus niger*. SB pre-treated with imidazole allowed the recovery of a hemicellulose rich fraction (34% of hemicellulose and 91.2% of delignification). Xylan, which was efficiently extracted with imidazole, allowed the production of 6.06 $g.L^{-1}$ of XOs, where xylotriose represented more than 70%. A high affinity of xylanases was observed for XOs production with a low polymerization degree. Great perspectives are viewed for the implementation of mixed processes to produce biomolecules with concomitant valorization of lignocellulosic material.

Keywords: Xylooligosaccharides, Xylanases, Sugarcane bagasse, Imidazole, Biorefinery.

Highlights

- SB and soybean meal using *A. niger* reached a production of 53.1 $U.mL^{-1}$ of xylanase
- Imidazole pretreatment allowed the recovery of 18.4% $w.w^{-1}$hemicellulose rich in xylan
- Enzymatic hydrolysis of imidazole-extracted xylan can produce 56 g of XOs per kg of SB
- Xylotriose was the main XOs produced representing 72.3% of the total XOs

Graphical abstract



1. Introduction

Xylooligosaccharides (XOs) are oligosaccharides formed by xylose monomers linked through β-(1→4) bonds, which represent an important food product due to their different beneficial characteristics for human and animal health. XOs have the capacity to pass through the upper digestive tract, without being digested, to reach the lower digestive tract and serve as a selective stimulant for the growth and maintenance of beneficial bacteria (probiotics) in the intestinal tract Lan et al. (2021). The main probiotics, which are stimulated by XOs, are bacteria of the genera *Lactobacillus* and *Bifidobacterium*, since these probiotics have anti-allergy and antioxidant properties. They increase high-density lipoprotein levels, has protective activity against cardiovascular diseases, has selective cytotoxic activity, reduces serum triglyceride levels, and lowers cholesterol Dias et al. (2022). In addition, XOs promote calcium absorption, prevent the formation of dental caries, increase immunological, antioxidant and anti-inflammatory activity, being used in the treatment of gastrointestinal infections. Due to the large number of beneficial features, XOs have high market potential, reaching a sales volume of $93 million in 2017 with an expected growth of up to $130 million by 2023 Lan et al. (2021).

XOs can be found naturally in fruits, vegetables, and honey in small concentrations, making it difficult to recover and process at industrial scale. However, lignocellulosic biomass that are rich in polymers, such as carbohydrates and lignin, are emerging as a potential source for large-scale XOs production. Among the different lignocellulosic biomasses, sugarcane bagasse (SB) has a high potential for XOs production, due to its rich xylan content Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021). The sugar-energy industry uses sugar cane as a raw material for the production of edible sugar and bioethanol, being SB the main by-product. Sugarcane cultivation is widely exploited worldwide, especially in countries that are in tropical regions, such as Brazil, where a production of 757.1 million tons was reported in 2020 with a cultivation area of approximately 10 million hectares FAO (2022). Brazil has an

annual production of almost 200 million tons of SB per year and the hemicellulose content within this by-product can represent a percentage of up to 30% Kumar et al. (2020).

The main source of XOs production is hemicellulose, considered the second most abundant polysaccharide of plant origin, which consists of xylan. Xylan is composed of polysaccharide chains made up of xylose monomers, in addition to having branches that can be made up of other sugars such as arabinose, galactose, rhamnose and acetyl residues. Due to its high xylose composition, xylan is emerging as the main source of raw material for the production of XOs. However, this process requires physicochemical and/or biological pretreatment stages due to the recalcitrant nature of lignocellulosic biomass Kundu et al. (2021).

The pretreatments for XOs production are very diverse, ranging from the use of chemical catalysts in severe conditions tobiological enzymes treatments and the combination of these. Hydrothermal pretreatments or assisted with chemical catalysts are the most used in the rapid production of XOs. However, this process results in the production of very diverse XOs with a high degree of polymerization, the production of undesirable sugar monomers, and the by-production of toxic components that lead to the need for additional purification processes, increasing cost of the process Lorenci Woiciechowski et al. (2020) and Valladares-Diestra; Porto de Souza Vandenberghe; Ricardo Soccol (2021). An alternative to hydrothermal and/or catalyst-assisted pretreatment processes is the extraction of xylan for its subsequent hydrolysis with the use of specific enzymes, which generates the obtaining of XOs without the co-production of toxic components. Alkaline methods are among the most used methods in the extraction of xylan, mainly due to the fact that they allow the extraction of xylan chains with a higher degree of polymerization. On the contrary, acid treatments hydrolyze these polymers, releasing monosaccharides, such as xylose, and derivative components such as acetic acid, formic acid or furfural, which, depending on their concentration, can inhibit enzymatic hydrolysis processes Lorenci Woiciechowski et al. (2020).

Imidazole is a relatively new catalyst in the pretreatment process of lignocellulosic material, presenting great potential mainly due to its high capacity to extract lignin. In addition, imidazole has a high alkaline power thanks to its nitrogen atoms that contain a lone pair of electrons due to the effect of delocalization and the behavior of the conjugate acid in the imidazolium ion that generates a resonance efficiency of the positive charge Ouellette; Rawn (2015) and Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). Due to this alkaline behavior, its high level of delignification and its low market price, imidazole can be used in the xylan extraction process.

In the enzymatic hydrolysis of xylan, hydrolytic enzymes of the xylanases type are used. The xylanases (β-1,4-D xylanxylanohydrolase, EC 3.2.1.8) of the GH10 and GH11 family are the enzymes most used in the hydrolysis of xylan for the production of small XOs with low monosaccharide content Morgan et al. (2017) and Porto de Souza Vandenberghe et al. (2020). However, the use of enzymes for the production of XOs on an industrial scale is still economically unfeasible. For this reason, the production of enzymes with the use of low-cost substrates and super producer microorganisms like *Aspergillus* or *Trichoderma*, are an option to reduce the final costs of XOs production Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021). In this sense, the development of biorefineries where a diversity of products can be produced (biofuels, bioenergy and marketable chemicals) and can be used within of the own bioprocesses, decreasing the total costs production and which can also be offered to the market with a competitive price are very important.

Integrated lignocellulosic biomass biorefineries can develop bioprocesses that manage to generate different types of products such as enzymes, phenolic compounds, glucose syrup, ethanol xylose, XOs and others Carvalho, A. F. A. et al. (2020). In addition, these types of biorefineries are friendly to the environment because they use renewable biomass, reduce greenhouse gases, value agro-industrial by-products and generate a closed cycle of production,

which leads to a better development of the bioeconomy or green economy. For this reason, the development of new technologies and the identification of key aspects with greater possibilities of reducing the cost of production within the production bioprocess are essential in the implementation of large-scale biorefineries Lan et al. (2021)

The objective of this work is the production of xylanolytic enzymes complex from low-cost substrates for their application in the production of XOs by hydrolysis of xylan extracted from SB with the use of imidazole as a new pretreatment catalyst with an integrated lignocellulosic biorefinery approach.

2. Material and methods

2.1.Raw materials and chemicals

SB was provided by the Santa Terezinha - USACUCAR company from Paraná State - Brazil. Soybean meal was provided by IMCOPA company from Paraná State - Brazil. The SB and soybean meal were dried in an air-circulating oven at 65°C for 48 h, and milled (Marconi, MA580/E). The particle size used in all experiments was between 0.85 mm (ASTM No. 20) and 0.35 mm (ASTM No. 45). All purchased chemicals and culture medium components were of analytical grade.

2.2.Compositional analysis of biomass

The solid content, ash, extractives, acid insoluble lignin (AIL), acid soluble lignin (ASL), and structural carbohydrates of untreated and imidazole-treated SB were determined according to the National Renewable Energy Laboratory (NREL) procedures Sluiter et al. (2012), Sluiter; Hames; Hyman; et al. (2008), Sluiter; Hames; Ruiz; et al. (2008) and Sluiter; Ruiz; et al. (2008).

2.3. Microorganisms

Aspergillus niger BC strain from the culture bank of the Bioprocess Engineering and Biotechnology Laboratory at UFPR was used for xylanases production. The maintenance of the strain was made by transferred to potato dextrose agar slants that were incubated at 30 ºC for 144 h with periodic renovation. The inoculum preparation was performed in Erlenmeyer flasks (250 mL) with 50 mL of potato dextrose agar and incubated at 30 ºC for 120 h. Then, a solution of 1% ($v.v^{-1}$) of tween 80 was used for spore recovery that would be used as inoculum.

2.4.Xylanase production in bubble column reactor

Kinetics of xylanase production was conducted in a 1.5 L bubble column reactor with a working volume of 1 L and a length-to-diameter ratio of 4.4. Fermentation was carried out according Valladares-Diestra et al., (2021b) with 2.69% ($w.v^{-1}$) substrate concentration (74% SB and 26% soybean meal), $4.33*10^6$ spores.mL^{-1} of inoculum rate, pH 5.96, 3.186 $g.L^{-1}$ of K_2HPO_4 and 0.327 $g.L^{-1}$ of $CuSO_4 \cdot 5H_2O$. Then, the bioreactor was incubated at at 30 °C with an aeration rate of 2 vvm, resulting in a superficial gas velocity of 0.377 cm/s for 192 h. Silicone oil at 5 $mL.L^{-1}$ (Dow Chemical Inc., USA) was added to control foaming. Samples were withdrawn each 24 h.

2.5.Sugarcane bagasse pretreatment

Imidazole was used as solvent in the pretreatment of SB following the optimum procedure described previously by Valladares-Diestra et al., (2020). 5 g of dried SB were added to imidazole at a ratio of 1:9 ($w.w^{-1}$). Pretreatment essays were carried out in a stainless steel 150 mL reactor (Parr, USA) with 1500 rpm of mechanical stirring at 160°C for 1 h. After pre-treatment, 135 mL of deionized water were added to the material and mixed for 1 h at room temperature. The liquid and solid fraction were separated by vacuum filtration. The cellulose-

rich solid fraction was washed with 135 mL of 96% $v \cdot v^{-1}$ ethanol for imidazole recovery and then it was dried in an air-circulating oven at 65°C for further analysis. The liquid content the hemicellulose-rich fraction was concentrated by rotary evaporation (65 °C) until approximately 45 mL. After that, it was mixed with 3 volumes of 96% $v \cdot v^{-1}$ ethanol and kept at 4 °C overnight for hemicellulose precipitation. The precipitated hemicellulose was removed by centrifugation at 2275 g for 25 min, washed with ethanol twice, freeze and lyophilized for further analysis.

2.6. Xylooligosaccharides' production by enzymatic hydrolysis

Commercial xylan (beechwood xylan), imidazole extracted xylan and hydroxy extracted xylan samples were hydrolysed using the produced enzymatic complex. 2% ($w.v^{-1}$) of commercial xylan or extracted xylan diluted in 50 mM with pH 5.8 of citrate-phosphate buffer were employed. Xylanase complex was added at 250 U per gram of biomass. Enzymatic hydrolysis was performed in shake flasks at 50°C and 150 rpm for 1 h. Samples were boiled for 10 min for enzymatic hydrolysis interruption. Then, were centrifuged at 10,000×g for 5 min, filtered and stored at 4°C for further HPLC analysis.

2.7.Determination of enzyme activities

Xylanase and CMCase activities were assayed using 1% beechwood xylan (Megazyme, Denmark) and 2% carboxymethilcellulose (Sigma, USA), respectively, in citrate-phosphate buffer (50 mM and pH 5.8) Bailey et al. (1992). Released reducing sugars were determined with DNS according to Miller (1959). The unit of enzymatic activity was calculated according to the international system of units (SI) as the enzyme amount that produces 1 μmol of monosaccharides sugar (xylose and glucose) per minute.

Protease activity was determined according to Siala et al. (2012) using hemoglobin (Sigma, USA). A volume of appropriately diluted enzyme solution (100 μL) was added to substrate solution (100 μL) composed of hemoglobin 1% ($w.v^{-1}$) and 100 mM glycine-HCl

(pH 3). The reaction was incubated at 60°C for 10 min and interrupted with the addition of trichloroacetic acid saturated (200 µL at 8% $w.v^{-1}$). The, the samples were centrifuged at 10,000×g for 10 min at 10°C, and the absorbance was determined at 285 nm. The standard curve was generated using tyrosine solutions. The necessary amount of enzymes for the release 1 µmol of tyrosine per minute was determined as a unit of enzyme activity according to SI.

The enzymes activities experiments were carried out in triplicate and enzymatic blanks were measured for each essay.

2.8.Analytical procedures

1-Phenyl-3-methyl-5-pyrazolone (PMP) was used for the derivatization of XOs, according by Li et al. (2013). Briefly, 250 µL of hydrolysates, individual or mixed analytical standards of XOs and monosaccharides, were placed in a 2 mL centrifuge tube, followed by the addition of 0.3 M NaOH (250 µL) and 0.5 M PMP in methanol (250 µL). Samples were maintained at 70°C for 30 min and then chloroform (500 µL) were added to remove the excess of PMP. The organic phase was carefully discarded to remove the excess of reagents after vigorous shaking and centrifugation (6000×g), this procedure was repeated for 3 times. Finally, the aqueous phase content the derivatized XOs was diluted with water and filtered through a 0.22 µm membrane. The XOs with DP 2–6 was determined using C_{18} column and a diode-array detector at 245 nm in a HPLC Agilent 1200 series. Sodium phosphate buffer solution composed of a ratio (40 mM, pH 8.0)/acetonitrile (81:19, $v.v^{-1}$) was carried out as elution at 0.5 $mL.min^{-1}$.

The analysed of organic acids, sugars, and alcohols were made by HPLC. Samples were filtered through 0.22 µm pore size membranes (Millipore Corp., Billerica, MA, USA) using a column of Aminex Bio-Rad HPX-87H operating at 60°C with a refractive index (RI) detector. An aqueous solution of H_2SO_4 (5 mM) at a flow rate of 0.6 $mL \cdot min^{-1}$ was used as mobile phase

and the injection samples volume was 15 μL. The chemical standards were prepared with D-(+)-cellobiose, glucose (>99%), D-(+)-xylose (>99%), D-(-)-arabinose (>99%), acetic acid (>99%) and ethanol (>99%) from Sigma-Aldrich (USA).

3. Results and discussion

3.1.Xylanases production in bubble column reactor

A production of 53.1 $U.mL^{-1}$ of xylanase was obtained at 120 h, through fermentation of SB and soybean meal using *A. niger*, with a productivity of 0.44 $U.mL^{1}.h^{-1}$ as it can be seen in Figure 1A. In addition, it is possible to observe that after 120 h, xylanases production reached the stationary phase. These results demonstrate the optimal performance of bubble column bioreactors in the production of enzymes with the use of filamentous fungi. Due to their pneumatic agitation, bubble column bioreactors prevent the rupture and stress of fungal mycelia, withan adequate mixture within the system, facilitating the mass transfer and heat in the fermentation process. That is why hese bubble column reactors are widely used in the production of extracellular enzymes by filamentous fungi such as *A. niger*, allowing a better production of enzymes compared to stirred tank reactors Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021). Besides, *A. niger* is an excellent model microorganism for protein secretion and production of enzymes at industrial scale, being widely used due to its unique characteristics in food safety Li et al. (2020).

The expression of xylanase in *A. niger* is well documented, in this sense it is known that one of the main genes involved in its expression is the *XlnR* gene, which is normally activated in the presence of low amounts of xylose from xylan hydrolysis Moran-Aguilar et al. (2021). However, xylose can also act as a repressor in the gene expression of xylanases through the mechanism of carbon catabolite repression protein (Cre). In the case of the *Aspergillus* genus, CreA mechanism is the main factor for the repression of carbon catalytic enzymes and can be

activated by relatively high concentrations of monosaccharides such as xylan, glucose and fructose. It has been reported that concentrations above 70 mM of xylose lead to the activation of the CreA mechanism, which causes a repression in the protein expression of xylanolytic enzymes in *A. niger* P. de Vries et al. (1999). As it can be seen in Figure 1B, the amount of xylose that was found in the culture medium varied with fermentation time until 96 h, where a maximum concentration of 23.3 mM was obtained. The variation of this monosaccharide in the fermentation process is strongly linked to the action of xylanolytic enzymes, which degrade xylan to obtain monosaccharide sugars as a carbon source for the growth of the fungus. On the other hand, the concentration of free glucose in the culture medium remained stable throughout the fermentation process with approximately 10 mM.

Likewise, the production of enzymes such as cellulases and acid proteases, which are normally produced by *A. niger*, achieved an average of 3.5 and 5.5 $U.mL^{-1}$ for CMCase and protease, respectively (Figure 1A). The production of proteases by *A. niger* is directly influenced by the pH of the fermentation medium, with optimal pH values between 3.5 and 5. As it is known, *A. niger* causes rapid acidification within the culture medium, promoting the drastic decrease of the pH. This is mainly due to the ability of *A. niger* to produce and secrete metabolites such as citric acid, oxalic acid and gluconic acid in the exponential growth phase Niu et al. (2016). The results showed an initial decrease in pH to 3.6 in the first 96h of fermentation that led to highest production of proteases (5.8 $U.mL^{-1}$). However, starting at 120h, a slight increase in pH to 4.5 was observed, at 168h (Figure 1B). These pH variations were mainly caused by the presence of citric acid (data not show), which is low due to the lack of high concentrations of glucose in the medium and, in addition, to its oxidation during the fermentation process Andersen et al. (2009).

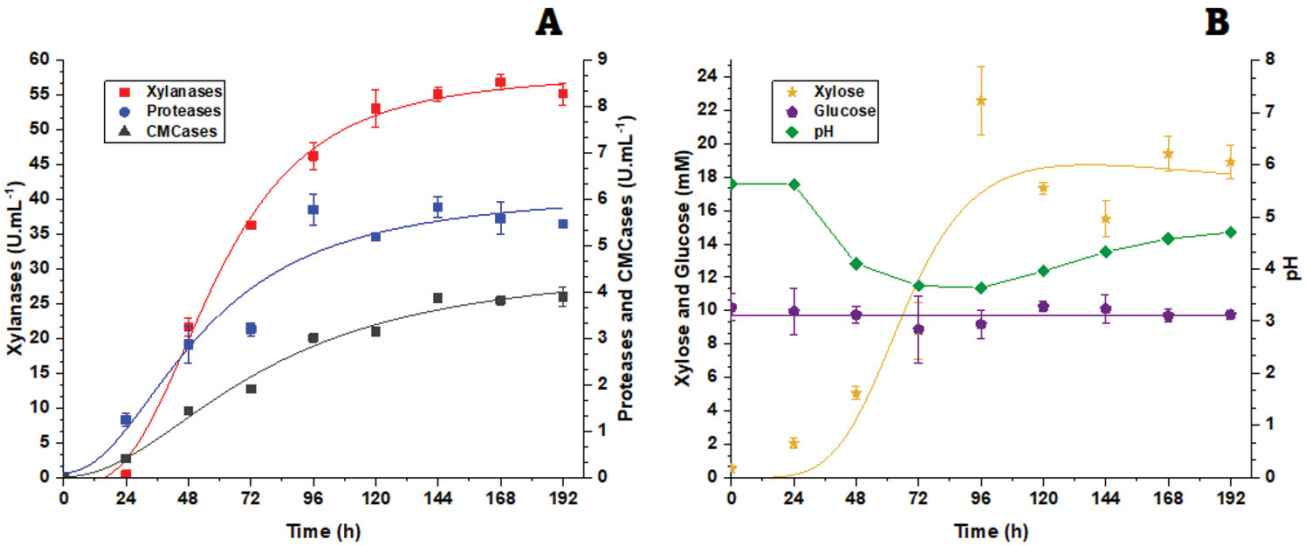


Figure 1. Profile of xylanases production by *A. niger* in bubble column reactor. A) Different enzymes profile produced by *A. niger* and B) Sugar and pH profile in the culture medium

The characteristics of the substrate also play a very important role in the induction of enzyme production. The composition rich in polysaccharides such as cellulose (37.9%) or hemicellulose (23.9%) of SB acts in the induction of lignocellulosic enzymes such as xylanases and cellulases. Compared to cellulose, hemicellulose is the most exposed fraction of lignocellulosic biomass, which facilitates the attack by microorganisms such as fungi. In this sense, during fermentative process, hemicellulose is first degraded because it is more exposed, so this substrate generates a strong induction hemicellulolytic enzymes' production, mainly xylanase due to the high xylan content. On the other hand, soybean meal, which is rich in nitrogenous components and proteins from vegetable source, acts as a nitrogen source for the proper growth of *A. niger* mycelia and as an inducer of proteases' production Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021). In addition, these two substrates also have high contents of micronutrients such as Fe, Zn, Cu, and Mg, which are key in the growth of *A. niger* and, in some cases, cofactors for enzymes such as xylanases Moran-Aguilar et al. (2021) and Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021).

The xylanolytic activity results obtained at 120 h were 1973.6 $U.g^{-1}$ of dry substrate, which represents a productivity of 16.5 $U.g^{-1}.h^{-1}$. These results showed a high productivity in comparison with studies already published such as those obtained by Moran-Aguilar et al., (2021) who used autoclaved SB and *A. niger* CECT 2700, reaching a production of 1365.6 $U.g^{-1}$ with a productivity of 8.1 $U.g^{-1}.h^{-1}$ after 168 h of culture in a solid fermentation system. Ramírez-Lagunes et al., (2021) optimized the production of xylanase in submerged fermentation using alkaline pretreated SB using different *Aspergillus* strains, obtaining an increase of 5.7 times the initial production with *A. tamarii* SCBH2. However, the maximum optimized production only reached 726.7 $U.g^{-1}$ with a productivity of 10.1 $U.g^{-1}.h^{-1}$. Within the optimization processes of xylanase production, also it is also possible to find the use of different strains and substrates, such as the results obtained by Rahnama et al., (2013) who used *Trichoderma harzianum* SNRS3 and rice straw for xylanase production of 433.75 $U.g^{-1}$. Carvalho et al., (2020) evaluated the xylanases' production in submerged fermentation using SB and A. fumigatus obtained a production of 1100 $U.g^{-1}$ with a productivity of 11.5 $U.g^{-1}.h^{-1}$. Other studies presented by Pérez-Rodríguez et al., (2014) and Moran-Aguilar et al., (2021) used the *A. niger* CECT 2700 strain with different substrates such as corn stover and brewery grain, obtaining a total production of 2,279.9 and 2,926 $U.g^{-1}$, respectively in a solid fermentation system.

As it can be seen, the production of xylanases is quite diverse, where the employed strain and substrate play a very important role. However, the physicochemical factors, as well as the fermentation systems have a decisive influence on enzymes' production. Regarding fermentation systems, submerged fermentation processes facilitate the extraction and purification of extracellular enzymes, achieving a rapid separation of biomass and supernatant Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021).

3.2.Sugarcane bagasse pretreatment

Xylan extraction process from SB was carried out using imidazole. Previously, the characterization of SB showed a high content polysaccharides and lignin, with a highest content of cellulose presenting a percentage of 37.9%. Hemicellulose (23.9%) consisting of xylan (17.1%), arabinose residues (2.1%) and acetyl groups (4.8%), . while lignin polymers represented 23.3% with a higher abundance of acid-insoluble lignin (18.8%). The results were in agreement and very similar to the different studies carried out with SB Raj; Krishnan (2019), Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021) and Wang, Z. et al. (2019a). However, small differences of SB composition are reported mainly due to the variety of the crop used as well as the environmental conditions for the growth of sugarcane.

After the SB pretreatment with imidazole, two solid fractions were obtained, which correspond to the cellulose-rich fraction, consisting of glucan, and the hemicellulose-rich fraction consisting mainly of xylan (Figure 2). As it was previously, reported by our research group, imidazole pretreatment generates a higher delignification of the material, thus extracting most of the lignin polymers Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). In addition, as it is shown in Figure 2, the pretreatment generates a complete decrease in the compounds belonging to the extractive fraction, this is not only due to the solvent used, but also to the pretreatment conditions (160°C and 1h) that generate the detachment and hydrolysis of this fraction during the pretreatment process Valladares-Diestra et al. (2022).

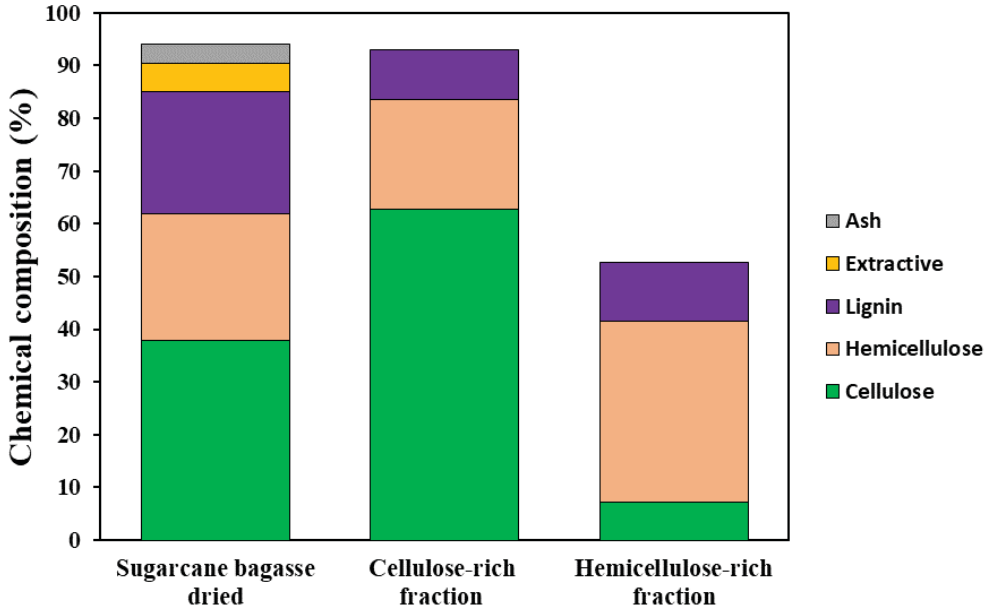


Figure 2. Polymers' composition of sugarcane bagasse and after imidazole pretreatment

The composition of the cellulose-rich fraction presents an enrichment in the percentage of glucan (62.8%) with an insignificant loss of 6.9% of the amount of initial glucan. In addition, a total recovery of solids of 56.2% $w.w^{-1}$ was reached, while the percentage of hemicellulose showed a slight decrease within the pretreated material, with the decrease of arabinose residues and acetyl groups. On the other hand, a strong decrease in both the percentage and the amount of total lignin was observed, reaching only 9.5% of total lignin within the pretreated material, showing a delignification efficiency of 77.2% compared to non-treated SB. These results confirm the high potential of imidazole as a solvent for delignification in pretreatment to obtain cellulose-rich biomass.

On the other hand, a total recovery of solids of 18.4% $w.w^{-1}$of the hemicellulose-rich fraction was reached, with an enrichment in the percentage of hemicellulose of 34.2%, being the xylose residues predominating (28.9%), which mainly consisted of xylan. In addition, the arabinose residues presented a slight enrichment of 5.3%. However, acetyl group residues were not detected in this fraction, this may be due to the strong interaction of the imidazole

solvent with this type of residue, which generates an easy detachment from its initial matrix Valladares-Diestra; Porto de Souza Vandenberghe; Zevallos Torres; et al. (2021). Like the cellulose-rich fraction, this fraction showed a high degree of delignification and a decrease in the percentage of total lignin, obtaining values of 91.2 and 7.6%, respectively. Although the fraction is rich in hemicellulose content, the total recovery of hemicellulose was only 26.3% with respect to the initial content of hemicellulose in the non-treated SB. This is mainly due to the low solid recovery of the hemicellulose-rich fraction (18.4%), compared to the high solids recovery of the hemicellulose-rich fraction (56.2%). This resulted in a higher recovery of hemicellulose in the cellulose-rich fraction, representing a total of 48.9% of the initial hemicellulose. Finally, the hemicellulose-rich fraction presented a lower percentage of glucan (7.3%), which would represent the 3.5% of initial cellulose content of non-treated SB. This means that the total loss of initial glucan represented only 3.4% during the entire pretreatment process of SB with imidazole. The results of chemical composition, percentages of lignocellulosic fractions and solid yields before and after pretreatment of SB with imidazole are shown in Table 1.

Table 1. Chemical composition of dried SB, and the two solid fractions recovered after imidazole pretreatment

Chemical composition (%)	SBdried	Cellulose rich fraction	Hemicellulose rich fraction
Anhydroglucose	37,90 ± 1,00	62,80 ± 1,35	7,25 ± 0,05
Anhydroxylose	17,05 ± 0,75	17,35 ± 0,25	28,90 ± 1,00
Anhydroarabinose	2,05 ± 0,15	1,55 ± 0,05	5,30 ± 0,20
Acetyl groups	4,80 ± 0,00	1,90 ± 0,10	ND
Acid-soluble lignin	4,55 ± 0,45	5,05 ± 0,55	3,55 ± 0,05
Acid-insoluble lignin	18,75 ± 0,15	4,40 ± 0,00	7,55 ± 0,45
Extractives-Water	3,38 ± 0,16	-	-
Extractives-EtOH	1,89 ± 0,10	-	-
Fraction composition (%)			
Cellulose	37,90 ± 1,00	62,80 ± 1,35	7,25 ± 0,05
Hemicellulose	23,90 ± 0,90	20,80 ± 0,30	34,20 ± 1,20
Lignin	23,30 ± 0,60	9,45 ± 0,55	11,10 ± 0,50
Extractive	5,27 ± 0,13	-	-

Ash	3,72 ± 0,05	-	-
Yields (%)			
Solid recovery	100,00	56,18 ± 0,99	18,39 ± 0,40
Cellulose recovery	100,00	93,13 ± 3,65	3,52 ± 0,10
Hemicellulose recovery	100,00	48,88 ± 0,16	26,34 ± 1,49
Lignin recovery	100,00	22,81 ± 1,73	8,77 ± 0,58

The recovery of xylan or arabinoxylan from lignocellulosic materials, such as SB, is very diverse and strongly depends on the employed pretreatment methods. In this sense, the variations in efficiencies of xylan recovery depend not only on the employed lignocellulosic material, but also on the catalysts and pretreatment conditions. For this reason, different authors evaluated several conditions for xylan extraction process such as Khaleghipour et al., (2021) who applied an alkaline pretreatment to SBusing the concentration of 1 M NaOH for 1 h. The results showed a recovery of solids of 8.5% of a xylan-rich fraction, which corresponded to a xylan recovery of 33.3% of the initial xylan of non-treated SB. In addition, they evaluated different concentrations of NaOH, time and temperature, showing that the degree of polymerization of xylan decreased with the increase of NaOH concentration, at a temperature of 121°C. The pretreatment time evaluated (30 and 60 min) did not present significant differences in xylan recovery. The authors proposed a consecutive pretreatment methodology, extracted the residual xylan up to 3 times with a greater yield of xylan dissolution and producing a cellulose-rich byproduct. However, this proposed methodology could generate a higher energy and economic costs due to the high number of operations. Additionally, high contents of by-products with chemical residues that are harmful to health and the environment are generated. Solier et al., (2022) applied an alkali-peroxide pretreatment to SB for xylan extraction by combining NaOH with hydrogen peroxide. The results showed that the effect of peroxide doubled the solubility of hemicellulose compared to just using NaOH, reaching a maximum hemicellulose solubilization of 18.4% of the initial hemicellulose of non-treated SB. However, during ethanol precipitation process, xylan

production decreased to approximately 11% of the initial hemicellulose. On the other hand, the combination of alkaline catalysts (weak and strong bases) resulted in an increase in xylan extraction as shown by Kundu et al., (2021) who used the combination of NaOH and NH_4OH at 10% concentration for SB pretreatment at 120 °C for 1 h, obtaining the recovery of 68 % of initial xylan. As it can be seen, the different techniques and methodologies with alkaline catalysts allow efficient xylan production. Depending on the employed pretreatment, the recovered xylan will have a unique characteristic and composition, which will greatly influence the hydrolysis process to obtain XOs. In this sense, one of the advantages of using imidazole in the pretreatment process to obtain xylan is that this catalyst is highly soluble and easily recovered with the use of ethanol, which avoids the waste and use of large amounts of water or other chemicals such as concentrated acids for the neutralization of pretreated biomass. In addition, imidazole is a low toxicity solvent that has the possibility of being recovered and recycled for use in new processes Morais et al. (2016).

3.3.Xylooligosaccharides production

The obtained enzyme complex, previously semi-purified by ultrafiltration, was used in the hydrolysis of beechwood xylan, xylan extracted by imidazole pretreatment, and xylan extracted by sodium hydroxide to produce XOs. The production of XOs with different degrees of polymerization (DP 2-6) is shown in Table 2. As it can be observed, the profile of XOs production from the different employed substrates is similar, with a strong affinity to produce xylotriose. This means that the enzymatic complex is the most influencing factor of the hydrolysis process for XOs production and their polymerization degree, The highest production of XOs of 8.1 $g.L^{-1}$ was obtained with the beechwood xylan, while the imidazole-extracted xylan promoted a total release of 6.1 $g.L^{-1}$ of XOs followed by 4.2 $g.L^{-1}$ of XOs produced from xylan extracted with sodium hydroxide. The total yield of XOs production was

40.3% for beechwood xylan, 30.3% for imidazole-extracted xylan, and 20.9% for xylan extracted by sodium hydroxide. The higher production and yield of XOs with beechwood xylan is mainly due to its purity (<90%) and the higher xylan content of this material, while the extracted xylan does not present a high purity, containing other components such as cellulose and lignin.

The imidazole-extracted xylan with a composition of 34.2% hemicellulose (Table 1), hydrolyzed with the xylanolytic complex produced 30.3% of XOs, 3.2% of xylose and 1.4% of arabinose from g of biomass hydrolyzed. However, the enzymatic efficiency of the xylanolytic complex for hemicellulose transformation was 88.6% for XOs production, 8.3% for xylose production and 3.6% for arabinose production on basis to real hemicellulose fraction present in the biomass hydrolyzed. These results showed that the hemicellulose fraction within the imidazole-extracted xylan was fully hydrolyzed by the enzyme complex, showing efficient production of XOs and low levels of monosaccharides. Thus, demonstrating the high performance of the enzymatic complex to produce XOs and that the substrate obtained from the imidazole pretreatment generates a high-quality xylan, which is easy to hydrolyze without the presence of significant enzyme inhibitors.

Table 2. Profile of xylooligosaccharides produced using xylanases complex with different xylan source.

Sugars	Xylan Pure	Imidazole extracted Xylan	Hydroxy extracted xylan
Glucose	0,086 ± 0,001	0,283 ± 0,000	0,263 ± 0,036
Arabinose	0,282 ± 0,001	0,280 ± 0,000	0,289 ± 0,035
Xylose	0,595 ± 0,022	0,640 ± 0,117	0,443 ± 0,006
Xylobiose	0,633 ± 0,026	0,580 ± 0,033	0,460 ± 0,081
Xylotriose	6,262 ± 0,096	4,384 ± 0,067	2,512 ± 0,658
Xylotetrose	0,699 ± 0,020	0,513 ± 0,039	0,514 ± 0,063
Xylopentose	0,330 ± 0,002	0,321 ± 0,005	0,356 ± 0,109
Xylohexose	0,133 ± 0,133	0,263 ± 0,002	0,330 ± 0,133
Total XOs	8,057 ± 0,012	6,060 ± 0,054	4,172 ± 0,272
XOs Yield (%)*	40,287 ± 0,059	30,302 ± 0,269	20,859 ± 1,359

* Calculated from the amount of initial biomass hydrolyzed

Xylotriose was the oligosaccharide with the highest production in the different substrates, representing 77.7; 72.3 and 60.2% of the total XOs produced from beechwood xylan, imidazole-extracted xylan, and xylan extracted with sodium hydroxide, respectively. The affinity for xylotriose production could be linked to the structural conformation of the xylanolitic enzyme as well as its catalytic and substrate binding site Morgan et al. (2017). In addition, the degradation of lignocellulosic biomass by enzymatic hydrolysis with extracellular enzymes produced by fungi, normally seeks the production of small oligosaccharides such as XOs with low polymerization degree (xylobiose and xylotriose) that are easier to assimilate. These small XOs are finally hydrolyzed into the fungal cytoplasm, producing xylose that is introduced into the pentose phosphate metabolic pathway to produce energy and other metabolic compounds Huang et al. (2017) and Li et al. (2020). In this sense, this mechanism would also explain the low production of monosaccharides such as xylose, glucose and arabinose, because the enzymes beta-glucosidases and beta-xylosidases, which are responsible for the hydrolysis of glucose and xylose, respectively, are produced intracellularly within the fungal mycelium. Thus,the xylanolytic enzyme complex that is used in this process is entirely composed of extracellular enzymes Huang et al. (2017). On the other hand, the low production of xylose could show that there is no predominance of enzymes of the exo-xylanase type, which are the ones that produce the most xylose due to their mechanism of adhesion to the substrate and hydrolysis. In this sense, it is possible that the used xylanolytic complex presents mostly enzymes of the endo-xylanase type. Finally, the monosaccharides' concentration plays a very important role in the prebiotic compounds made up of XOs, since at low concentrations of glucose and/or xylose the XOs present better prebiotic characteristics, producing an adequate development of probiotics microorganism and reducing the colonization of microorganisms harmful to health within the digestive tract Lian et al. (2020) and Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021).

The production of XOs from SB shows a diverse spectrum in production efficiency such as the results obtained by Khaleghipour et al., (2021) who hydrolyzed xylan (0.5% $w.v^{-1}$) recovered from SB by means of NaOH using the Xyn10A enzyme from *Bacillus halodurans* for 1 h at 62 ºC. The authors obtained an efficiency of 42.3% of XOs. Carvalho et al., (2020) evaluated different parameters for the production of XOs from xylan extracted from SB. The results showed that the catalysts (NaOH and H_2O_2) used in the extraction of xylan, as well as the size of the xylan particle used in the enzymatic hydrolysis, did not show significant differences in the efficiency of XOs production. On the other hand, the evaluated substrate concentration during enzymatic hydrolysis (2; 5 and 10% $w.v^{-1}$) did not show significant differences between 2 and 5% $w.v^{-1}$ (33.4 and 34.6, respectively), while the production efficiency of XOs decreased to 28.5% with a concentration of 10% $w.v^{-1}$. Similarly as in this work, the results obtained by Carvalho et al., (2020) at a concentration of 2% of xylan showed an efficiency of over 30% in obtaining XOs, while the yields obtained was of 5.5% of XOs produced per g of non-treated-SB. However, these results can be improved by optimizing different factors such as enzyme loading, substrate loading, pH, and temperature.

The variations in the composition and difference of xylan structure extracted from lignocellulosic biomass are mainly affected by the conditions of the applied pretreatment, as well as the effects on the synergism of the applied enzymes. Their affinity and catalytic capacity, such as the presence of inhibitory processes within of enzymatic hydrolysis generates a great variation in the production yields and the degree of polymerization of XOs. Due to this fact, the comparison of results of different studies of XOs production is difficult Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021).

3.4.Mass balance and techno-economical approach

According to the obtained results, a mass balance analysis process was carried out for the production XOs from SB xylan extracted with imidazole and hydrolyzed with the xylanolytic enzyme complex produced by *A. niger*.

Starting from 1000 g of dry SB, with a composition of approximately 618 g of holocellulose (279 g of cellulose and 239 of hemicellulose) and 233 g of lignin, it is possible to obtain two solid fractions rich in carbohydrates with a low lignin content. After the pretreatment stage with imidazole at 160 °C for 1 h, the total recovery percentage of solids is 74.6% with an amount of 562 g r cellulose-rich solids and 184 g of hemicellulose-rich solids. The cellulose-rich fraction that is easily digestible by cellulolytic enzymes can generate a high production of sugars, both glucose and xylose for the production of biofuels such as bioethanol, reaching a production efficiency of 171.9 g of ethanol per 1kg of dry SB as already reported by Valladares-Diestra et al., (2021b).

On the other hand, was recovered hemicellulose-rich solid fraction with an approximate composition of 62.9 g of pure hemicellulose, 13.3 g of cellulose and 20.4 grams of lignin. This fraction can be hydrolyzed with the xylanolytic complex produced by *A. niger* reaching a production of 55.7 g of XOs, of which 40.3 g are xylotriose. The results of high enzymatic efficiency (above 95%) obtained from both the cellulose-rich fraction and the hemicellulose-rich fraction are mainly due to the catalytic action of imidazole in the destructuring of lignocellulosic biomass and the removal of lignin, which promoted a high performance of the enzymatic complexes. The removal of lignin constitutes is one of the main challenges of the enzymatic hydrolysis, because this polymer causes a great inhibition of the enzymes due to its easy adherence with proteins, which generates an inactivation of most enzymes that lose their catalytic power Valladares-Diestra et al. (2022).

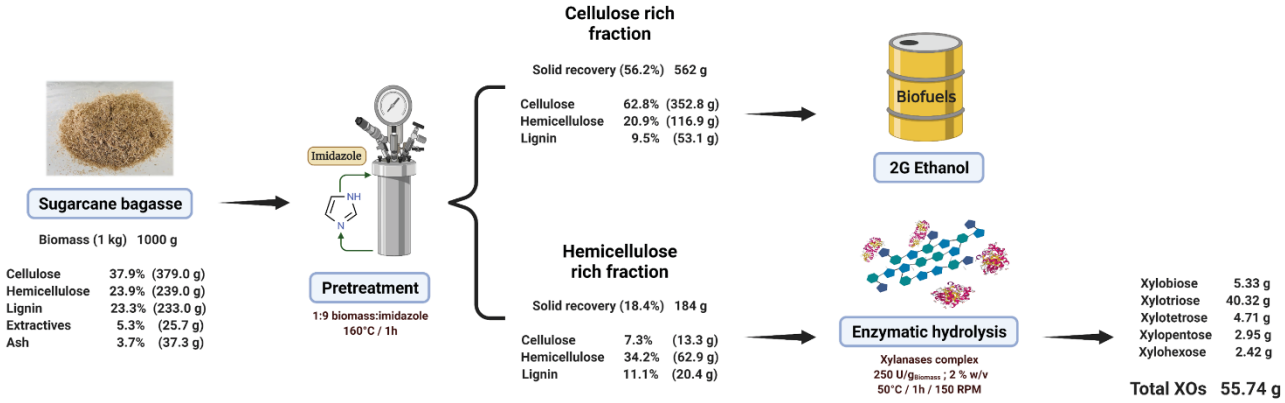


Figure 3. Mass balance of SBpretreated with imidazole for the XOs and second-generation bioethanol production.

The use of XOs as a prebiotic in the food industry has been approved by the European (EFSA) and North American (FDA) agencies, which has generated an exponential growth in its production interest at an industrial level that can also be used as a replacement for antibiotics in food and feed that offer benefits for human and animal health Carvalho, A. F. A. et al. (2020) and Khaleghipour et al. (2021). On the other hand, with respect to the world market, it is estimated that prebiotics represented $4.07 billion in 2017 with a growth of more than 80% for the year 2023 with an approximate %7.37 billion. Within this market, the global financial movement of the XOs had an approximate volume of $93 million in 2017 with an annual growth of 5.3% and a potential growth forecast of up to $130 million, which makes this product to have a high interest within the food market Amorim et al. (2019) and Lan et al. (2021). It is estimated that the average price of XOs is $22-50 per kilogram, which is a relatively high price. However, this price still not make XOs produced from lignocellulosic biomass competitive . The evaluation of the economic efficiency of XOs production has become a fundamental topic of research by different authors seeking the co-concomitant production of XOs with other value-added products that can be produced within a lignocellulosic biorefinery, thus increasing the productive diversity to obtain the highest yield in terms of economic benefits Zhou; Xu (2019). In this sense, identifying the key factors of

production costs , as well as the value-added products that can be produced concomitantly, are essential factors to develop commercial biorefineries at large scale. Lan et al., (2021) carried out a techno-economic study of the production of XOs from miscanthus, evaluating the productive capacity of the biorefinery, as well as the minimum sale price (MSP) of XOs according to the degree of purification (80; 90 and 95%). The results showed that a larger production size of XOs significantly decreased the MSP, while an increase in the purity of XOs would increase the MSP, mainly due to a lower recovery yield, as well as a higher operating and capital cost. The lowest calculated MSP was $3,430 per metric ton of produced XOs. This result was obtained using the production conditions of 80% XOs purity , a production plant with a capacity of 250 oven dry metric tons per day and an internal rate of return of 10%. However, the co-production of cellulose microfiber as a high value-added by-product within the process could result in the MSP of XOs decreasing to as much as $2,460 per metric ton. Thus, demonstrating the competitive potential of a biorefinery with a diverse production of high value-added molecules. Finally, the analysis determined that the content of xylan within the lignocellulosic biomass had a high effect on the decrease of the MSP, where an increase in the 5% of xylan could decrease MSP by up to $1000 per metric ton of XOs Lan et al. (2021).

With all this, the development of new technologies with reduced XOs' production costs, and the application of safe and innovative methods, which are friendly to nature, and use low-cost renewable raw materials compose an important strategy, considering the high nutritional potential of these products Carvalho, A. F. A. et al. (2020). Currently, the best technology to produce prebiotics is not clearly defined, but ,certainly, these results indicated the higher quality of obtained XOs in a bioprocess with a competitive fungal enzyme obtained by using renewable carbon source, such as SB. Also, is is very important to consider the cost and availability of the substrate that is employed in the production of XOs, as well as the specific

laws or regulations of different countries or regions, where they are sought to implement this new type of biorefinery Lan et al. (2021) and Valladares-Diestra; Porto de Souza Vandenberghe; Soccol (2021).

4. Conclusions

An efficient bioprocess for the production of XOs from xylan extracted with imidazole and hydrolyzed with a xylanolytic complex produced *in house* was developed. The high xylanases production of 53.1 $U.mL^{-1}$ with great recovery of hemicellulose of 18.4% $w.w^{-1}$ rich in xylan (28.9%) allow a total XOs production of 6 $g.L^{-1}$ trough enzymatic hydrolysis. These results show that it is possible to obtain 55.7 g of XOs per 1 kg of SB, of which 40.3 g are xylotriose. The development of integrated lignocellulosic biorefineries to produce diverse high-value molecules through bioprocesses is promising and generate high interest in the industry.

8. Considerações finais

A aplicação de imidazol no pré-tratamento de bagaço de cana como nova estratégia na implementação de uma biorefinaria lignocelulosica integrada mostra uma alta eficiência na produção de bioetanol de segunda geração. A produção de xilanases e XOs contribui a aumentar a diversidadee de produção e portfólio de biomoléculas de alto valor agregado em biorrefinarias integradas. Porém são necessáriass análises de ciclos de carbono de todo o processo para avaliar realmente os impactos de uma biorefineria deste tipo. Além disso, deve-se avaliar os aspectos tecno-econômicos para analisar a viabilidade financeira de um projeto em escala piloto ou escala industrial. Finalmente, as análises do número de vezes que o imidazol pode ser reciclado durante a etapa de pré-tratamento é crucial para que se possa avaliar tantos os impactos gerados do solvente, como a viabilidade financeira do seu uso em escala industrial.

9. Recomendações para trabalhos futuros

O imidazol tem apresentado resultados ótimos no pré-tratamento do bagaço de cana, agindo como um solvente altamente satisfatório na deslignificação. A recuperação e análises de lignina obtida após o pré-tratamento pode gerar novas estratégias do seu uso e aplicação. A aplicação do imidazol como catalisador em outros tipos de resíduos agroindústrias para sua valorização em bioprocessos industrial seria importante e de alto interesse, sobretudo em resíduos com alto conteúdo de lignina.

10. Referências

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