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USE OF NON-THERMAL PLASMA ASSOCIATED WITH ENZYMATIC HYDROLYSIS TO OBTAIN XYLO-OLIGOSACCHARIDES (XOS) FROM BREWERY BY-PRODUCT

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Doctoral thesis for the degree of Doctor in Food Engineering presented to the Postgraduate Program in Food Engineering at the Federal University of Santa Catarina.
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GABRIELA NAYANA PEREIRA

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RESUMO

A valorização de resíduos tem sido muito explorada nos últimos anos, permitindo a obtenção de produtos com valor agregado. Nesse sentido, os xilo-oligossacarídeos (XOS) se apresentam como uma proposta prebiótica, que pode ser obtida através de materiais lignocelulósicos (LCMs). Diversas estratégias podem ser adotadas para converter um LCM em XOS, dentre elas a combinação de pré-tratamento seguido de hidrólise enzimática. Neste trabalho, primeiramente, é feita uma revisão dos principais tópicos que envolvem a obtenção de xilo-oligossacarídeos. Posteriormente, foi realizado um estudo para determinar as melhores condições de aplicação de plasma não-térmico na deslignificação de resíduo cervejeiro, sendo avaliadas as principais alterações químicas, físico-químicas e morfológicas que ocorreram no material após a utilização deste pré-tratamento. As condições de hidrólise enzimática também foram estudadas para avaliar a conversão da xilana, contida na hemicelulose, em XOS. Por fim, foi avaliada a viabilidade de ampliação de escala e reutilização da enzima. A realização de uma revisão bibliográfica permitiu-nos identificar a inovação deste trabalho. Ao estudar a influência do plasma não-térmico como pré-tratamento do resíduo de cervejaria, foi possível concluir por meio da cinética exploratória que um tempo de apenas 5 minutos foi suficiente para deslignificar o material em cerca de 50% quando comparado ao material in natura, com algumas perdas nos teores de celulose e hemicelulose. A capacidade da enzima em converter hemicelulose em XOS foi explorada e, verificou-se que nas condições ótimas da enzima para pH e temperatura (6,0 e 50 ºC) o tempo de 48 horas, 1% de substrato e 2% de enzima foram as condições otimizadas, levando a produção a 750 mg XOS/g xilana. Neste trabalho, foi possível também aumentar em 25 vezes a escala laboratorial e reutilizar a enzima por duas vezes, por meio de membranas de ultrafiltração. Com isso, o presente trabalho apresenta um processo bastante promissor quando se pensa no enriquecimento da cadeia de biorrefinaria do subproduto da cervejaria.

Palavras chave: Plasma não-térmico, pré-tratamento, resíduo cervejeiro, hidrólise enzimática, reuso de xilanases, XOS.

RESUMO EXPANDIDO

Introdução

Cada vez mais há uma busca acentuada e intensa por produtos saudáveis pela parte dos consumidores. Os alimentos com propriedades funcionais vêm, nesse sentido, como uma alternativa de alimentos que além de fornecer a nutrição básica, auxiliam na saúde quando aliados a hábitos saudáveis de vida. As substâncias biologicamente ativas encontradas nos alimentos funcionais podem ser classificadas em probióticas e prebióticas, sendo a principal diferença que probióticos são microrganismos que conferem benefícios à saúde do hospedeiro e os prebióticos são carboidratos que fornecem substratos para as bactérias benéficas do cólon (BIELECKA, 2006). Alguns oligômeros têm potencial prebiótico, dentre eles pode-se destacar os xilo-oligossacarídeos (XOS), os quais são oligômeros de carboidratos gerados por unidades de xilose e podem ser produzidos por meio de materiais lignocelulósicos (LCMs), sendo estes providos de base rica em xilana (JAYAPAL *et al.*, 2013).

O material lignocelulósico para obtenção de XOS pode ser advindo de uma variedade de matérias-primas, tais como palha de trigo (BOONCHUAY *et al.*, 2018), bagaço de cana de açúcar (BRENELLI *et al.*, 2020), resíduos florestais (HENRIQUES *et al.*, 2021), entre outros, sendo que estes possuem semelhanças na composição química, isto é, celulose, hemicelulose e lignina. Um material lignocelulósico de bastante repercussão nos últimos tempos é o resíduo da indústria cervejeira, sendo o bagaço do malte, o subproduto mais abundante gerado no processo de fabricação de cerveja, representando aproximadamente 85% do total de subprodutos obtidos (MUSSATTO, 2014). A quantidade de resíduos geradas neste processo é alta, pois para cada 100 litros de cerveja são obtidos aproximadamente 20 kg do resíduo (ALIYU; BALA, 2011a), sendo parte deste resíduo destinada à ração animal.

As biomassas de uma maneira geral são consideradas recursos abundantes e renováveis, podendo ser convertidas em produtos de valor agregado, como biocombustíveis e energia, além de fontes baratas para fermentação microbiana. No entanto, a biomassa lignocelulósica necessita de uma etapa que auxilie a diminuir a recalcitrância do material, fazendo-se necessária uma etapa de pré-tratamento.

Diversos métodos de pré-tratamento são encontrados na literatura, porém a maioria dos procedimentos tradicionais requer utilização de grandes quantidades de compostos químicos, além da formação de subprodutos indesejáveis. Baseado neste princípio, um método que vem sendo explorado ao longo dos anos é o uso do plasma não-térmico como pré-tratamento em materiais lignocelulósicos (PEREIRA *et al.*, 2021a). O plasma é considerado o quarto estado da matéria, sendo um método onde é gerado o ozônio (O_3) à pressão atmosférica e à temperatura ambiente, além de espécies reativas de nitrogênio e oxigênio e UV, as quais contribuem auxiliando na degradação da lignina (SCHULTZ-JENSEN *et al.*, 2011). Neste método há algumas vantagens como ser um pré-tratamento de relativo baixo custo e não causar efeitos estruturais na celulose e hemicelulose, além da possibilidade de ser realizado à pressão atmosférica, usando o ar como gás para formação do plasma, o que reduz o custo operacional pela ausência de câmara de vácuo (ŽIGON; PETRIČ; DAHLE, 2018).

A estratégia mais comum para obtenção de xilo-oligossacarídeos é aquela que combina um pré-tratamento de eficiência com outro que possa converter a xilana exposta pelo pré-tratamento em XOS. Nessa linha, o uso de enzimas, é uma abordagem interessante, pois é ecologicamente correta, operando sem a aplicação de produtos químicos, além de apresentar alta eficiência e especificidade, o que garante menor quantidade de xilose e subprodutos indesejáveis (WAN AZELEE *et al.*, 2016).

Objetivo

O presente trabalho visou avaliar a tecnologia de plasma não-térmico como pré-tratamento na deslignificação do resíduo cervejeiro, e a partir disso, explorar condições de hidrólise enzimática, além de ampliação de escala laboratorial e reuso de enzima para a obtenção de xilo-oligossacarídeos (XOS).

Metodologia

A primeira etapa deste trabalho envolveu uma discussão crítica dos principais tópicos de revisão da literatura como resíduo cervejeiro, hidrólise enzimática e tipos de pré-tratamento, além de uma revisão sistemática para evidenciar o ineditismo do pré-tratamento utilizado neste trabalho para obtenção de XOS. Para isso, foram selecionadas as palavras chaves: plasma não-térmico, pré-tratamento e material lignocelulósico, realizando buscas em plataformas como *Science Direct*, *Scopus* e *Web of Science*, no período entre os anos de 2010 e 2019, onde foi elaborado um artigo de revisão sobre o uso da tecnologia do plasma não-térmico como técnica de pré-tratamento em materiais lignocelulósicos.

Na segunda etapa, baseado nos poucos estudos encontrados na literatura, foi explorada a técnica de plasma não-térmico como pré-tratamento em resíduo cervejeiro. Este estudo foi composto pela avaliação da composição química do resíduo cervejeiro antes e após pré-tratamento por plasma não-térmico. Foram avaliados aspectos como a influência do gás de plasma no processo (ar e mistura ar/argônio), cinética para avaliação do melhor tempo em relação à deslignificação do material nos tempos de 2,5, 5, 15, 30 e 60 minutos, além da avaliação da influência da concentração de sólidos (7,5, 10 e 12,5%) na deslignificação do material. Várias técnicas foram utilizadas para avaliar as modificações causadas pelo plasma no material, dentre elas a Microscopia Eletrônica de Varredura (MEV), Espectroscopia de Infravermelho por Transformada de Fourier (FTIR), Varredura Diferencial de Calorimetria (DSC), Difração de Raios-X (DRX) e Raman.

Na terceira etapa, foi avaliada a hidrólise enzimática do resíduo cervejeiro pré-tratado com plasma não-térmico (obtidos da etapa anterior). Foram avaliados o efeito de diferentes concentrações de enzima (0,1, 0,5, 1, 2, 4 e 8%) e diferentes concentrações de substrato (0.5, 1, 2 e 3%) na produção de XOS. As cinéticas foram realizadas nos tempos 3, 6, 9, 12, 24, 48, 72 e 96 horas, com condições de temperatura e pH ótimos da enzima (50 ºC e 6.0). Nesta etapa foram também realizados estudos prévios de ampliação de escala laboratorial onde foram realizados estudos com um aumento de 25 vezes (de 2 mL para 50 mL) e medido o rendimento de XOS ao final de 48 horas, além do reuso da enzima por meio de concentração por membranas, onde a solução contendo enzima foi concentrada e reutilizada, e sua atividade medida a fim de verificar a permanência de atividade enzimática ao final dos ciclos.

Resultados e Discussão

Na primeira etapa, além de uma revisão dos tópicos sobre resíduo cervejeiro, hidrólise enzimática, tipos de pré-tratamento, diferença entre prebióticos e probióticos, a revisão detalhada do plasma não-térmico como técnica de pré-tratamento em materiais lignocelulósicos foi realizada, onde os principais tópicos abordados foram: plasma, plasma não-térmico, uma visão geral de aplicações potenciais, uso de plasma não-térmico em materiais lignocelulósicos (LCMs), efeitos do uso de plasma não-térmico como pré-tratamento em LCMs e principais alterações observadas nos LCMs após o uso da técnica de plasma não-térmico, tendo relevância científica no que diz respeito à inovação em se trabalhar com esta tecnologia em biomassas em geral. Esta revisão foi publicada na revista Trends in Food Science & Technology com o título "*Use of non-thermal plasma in lignocellulosic materials: A smart alternative*", onde mostra-se que o único trabalho que utilizou plasma não-térmico como técnica de pré-tratamento em resíduo cervejeiro foi para a obtenção de bioetanol, mostrando, portanto, o ineditismo do presente trabalho.

Na segunda etapa da pesquisa, ao avaliar a influência do pré-tratamento por plasma não-térmico no resíduo de cervejaria, verificou-se que o nível de deslignificação obtido foi muito semelhante para os dois gases de plasma utilizados (ar e ar/argônio) atingindo uma redução de aproximadamente 42% no teor de lignina. Quando se avaliou a influência do tempo de aplicação do plasma observou-se uma redução acentuada da lignina solúvel e insolúvel nos primeiros 5 min de tratamento, em torno de 45 e 40%, respectivamente, em comparação com o controle (sem tratamento), mostrando uma grande redução em um tempo muito curto. Quando foi avaliada a influência do PNT nas diferentes concentrações de sólidos avaliadas (7,5; 10,0 e 12,5%) foi possível observar que a utilização de até 12,5% não altera o percentual de deslignificação de resíduo cervejeiro. Por meio de análises físico-químicas (MEV, DSC, FTIR, DRX e RAMAN) foi possível concluir que o plasma não-térmico afetou pequenas quantidades dos componentes lignocelulósicos. Pelo MEV, por exemplo, observou-se que as amostras apresentaram uma superfície mais "lisa" após tratamento, provavelmente devido à remoção de componentes mais solúveis. FTIR mostrou pequenas alterações em vários picos que podem indicar celulose, hemicelulose e lignina, já o DSC mostrou o comportamento térmico do material pré-tratado, onde as forças intermoleculares das moléculas (celulose, hemicelulose e lignina) existem e podem ser mais fortes ou mais fracas dependendo da modificação que o material sofreu após o tratamento. O difractograma DRX para o resíduo cervejeiro mostra um pico de difração a 21,8°, representando os picos de celulose. Já o Raman mostrou pequenas alterações nos picos em torno de 120 a 130 cm^{-1} no resíduo sólido, os quais indicam ligações de hidrogênio, por exemplo e na corrente líquida, indicando alterações nas estruturas de lignina.

Na terceira etapa, ao avaliar a cinética enzimática no rendimento de XOS nas condições fixas de temperatura, agitação, pH e concentração de substrato (50 ºC, 180 rpm, 6,0 e 1%, respectivamente), observou-se resultados otimizados (750 mg XOS/ g xilana) com apenas 2% de enzima (0,64 U/mL), não havendo diferença significativa nos tempos de 48, 72 e 96 horas. Na sequência, avaliou-se a influência da concentração de substrato, baseado na condição otimizada da cinética, confirmando que com apenas 1% de substrato ocorreram as maiores conversões de XOS a partir do resíduo cervejeiro (750 mg XOS/ g xilana). Finalmente, foi avaliada a ampliação de escala em 25 vezes, onde observou-se uma pequena diferença dos teores de XOS quando comparados aos experimentos feitos em escala reduzida, essas diferenças observadas durante o aumento de escala laboratorial podem ocorrer como resultado de diferentes fenômenos de agitação e transferência de calor. A avaliação do reuso da enzima também foi realizada neste trabalho, onde por meio de membrana de ultrafiltração a enzima foi concentrada e constatou-se que a atividade enzimática permaneceu praticamente a mesma ao longo de dois ciclos, comprovando a viabilidade de reuso de xilanase livre na obtenção de XOS a partir do resíduo cervejeiro pré-tratado com plasma não-térmico.

Considerações Finais

A partir da revisão da literatura confirmou-se que o plasma não-térmico é uma tecnologia promissora para ser utilizada em materiais lignocelulósicos, possuindo as vantagens de não ser tóxico, não poluente, com eficácia comprovada na retirada de lignina, que é um dos problemas enfrentados em outros métodos convencionais.

Os resultados obtidos na etapa de pré-tratamento apontam soluções promissoras em relação ao pré-tratamento com plasma não-térmico, pois, além de ser um método

ambientalmente correto, o tempo de uso é muito pequeno quando comparado a métodos tradicionais, os quais podem auxiliar na diminuição do teor de lignina de materiais lignocelulósicos, retirando aproximadamente 50% da lignina presente no resíduo cervejeiro em apenas 5 minutos de tratamento. Já a etapa de hidrólise enzimática onde foram investigados melhores tempos de reação, concentração de enzima e concentração de substrato, observa-se as melhores condições no tempo de 48 horas, 2% de enzima (64 U/g) e 1% de substrato, onde o rendimento satisfatório na produção de xilo-oligossacarídeos (XOS) (750 mg XOS/g xilana) é obtido. Posteriormente, foram realizados estudos de ampliação da escala laboratorial, bem como reutilização da enzima, onde verificou-se a possibilidade de ampliação de escala laboratorial em até 25 vezes e reuso da enzima livre em até 2 ciclos. Portanto, este trabalho mostra de maneira inédita questões como reuso de xilanase na obtenção de XOS, ampliação da escala laboratorial, além do uso de um pré-tratamento promissor e ainda pouco explorado em materiais lignocelulósicos, enriquecendo com isso a cadeia de biorrefinaria.

Palavras-chave: Plasma não-térmico, pré-tratamento, resíduo cervejeiro, hidrólise enzimática, reuso de xilanases, XOS.

ABSTRACT

The recovery of waste has been much explored in recent years, allowing the production of value-added products. In this sense, xylooligosaccharides (XOS) are a prebiotic proposal obtained through lignocellulosic materials (LCMs). Several strategies can be adapted to convert an LCM to XOS, among them the combination of pretreatment followed by enzymatic hydrolysis. This work reviews the main topics involving obtaining xylooligosaccharides from brewer's residue pretreated with non-thermal plasma. Additionally, a study to determine the best application conditions of non-thermal plasma in the delignification of brewer's residue was conducted, evaluating the main chemical, physical-chemical and morphological changes that occurred in the material after the use of this process. Enzymatic hydrolysis conditions were also studied to assess the conversion of xylan contained in hemicellulose into XOS. Finally, the feasibility of scaling up and reusing the enzyme under optimized conditions was evaluated. First, performing a literature review allowed us to identify the novelty of this work. When studying non-thermal plasma as a pretreatment in brewery waste, exploratory kinetics allowed us to verify that in a time of just 5 minutes, it was enough to delignify the material by about 50% through chemical characterization of the material. After exploratory kinetics, to evaluate the ability of the enzyme to convert hemicellulose into XOS, it was found that the time of 48 hours, in the conditions of 1% substrate, 2% enzyme, pH 6.0, and temperature of 50 °C was the optimized condition leading production to 750 mg XOS/g xylan. In this work, it was possible to scale up a 25-fold increase in laboratory scale and reuse the enzyme by 2-fold. With that, this work presents a good process when considering the enrichment of the biorefinery chain of the brewery by-product.

Keywords: Non-thermal plasma, pretreatment, brewer's residue, enzymatic hydrolysis, xylanase reuse, XOS.

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Chapter 1 – Introduction

In recent years there has been an intense search for healthier products. Foods with functional properties are an alternative in addition to providing essential nutrition and help in health. Biologically active substances found in functional foods can be classified into probiotics and prebiotics, the difference being that probiotics are microorganisms that provide health benefits to the host, and prebiotics are carbohydrates that offer substrates for beneficial colonic bacteria (BIELECKA, 2006; SANDERS, 2003). Some oligomers have prebiotic potential, including xylooligosaccharides (XOS), which are carbohydrate oligomers generated by xylose units and can be produced using lignocellulosic materials (LCMs), which are provided with a rich base in xylan. The lignocellulosic material for obtaining XOS can come from various raw materials, such as wheat straw, sugarcane bagasse, forest residues, among others. In this sense, a lignocellulosic material with considerable repercussion is the waste from the brewing industry, with malt bagasse being the most abundant by-product generated in the brewing process, representing approximately 85% of the total by-products obtained (MUSSATTO, 2014). The supply and availability of this residue in geographic terms are of great interest, as the brewing industries are expanding widely in Brazil, with 20 kg of residue being obtained for every 100 liters of beer (ALIYU; BALA, 2011b). The most abundant compounds in these grains are hemicellulose, lignin and cellulose in amounts of approximately 21-28%, 11-27% and 16-25%, respectively, with the remaining portions being quantified as proteins, starch and others.

Lignocellulosic biomass is considered the most abundant and renewable resource on Earth and can be converted into value-added products such as biofuels and energy, in addition to cheap sources for microbial fermentation (MICHELIN *et al.*, 2011). However, lignocellulosic biomass needs a step that helps to reduce the material's recalcitrance. In this sense, a pretreatment step must be carried out, which assumes a fundamental role in the economy of the processes. Thus, energy-efficient and cost-effective pretreatment methods are needed to make the process sustainable and economically viable (WRIGHT *et al.*, 2018).

Conventional pretreatment methods, such as acidic chemicals, for example, are interesting. In this method, the acid used releases protons that break the ether bonds between sugars in the polymer chain formed by hemicelluloses and cellulose. With the breaking of these bonds, there is the release of several compounds, mainly xylose, arabinose and glucose (AGUILAR *et al.*, 2002). Alkaline treatment is considered an effective method, with the main effect being delignification, increasing the porosity of the biomass (NASCIMENTO *et al.*, 2016). Despite assisting in the pretreatment step, these conventional methods require a large amount of energy, in addition to not being ecologically correct, as they lead to the formation of undesirable compounds such as formaldehyde, aliphatic acid and others (RAVINDRAN; JAISWAL, 2016).

These characteristics motivated the replacement of these conventional pretreatments by sustainable treatments (SAHA *et al.*, 2016). Based on this principle, a method that has been explored over the years is the use of non-thermal plasma as a pretreatment in lignocellulosic materials (RAVINDRAN *et al.*, 2019a). Plasma is considered the fourth state of matter (MERCHE; VANDENCASTEELE; RENIERS, 2012), a method where ozone (O_3) is generated at atmospheric pressure and room temperature, in addition to reactive nitrogen and oxygen and UV species, as which contribute by helping in the degradation of lignin. In this method there are some advantages such as being an economical pretreatment and not causing structural effects on cellulose and hemicellulose (SCHULTZ-JENSEN *et al.*, 2011), in addition to the possibility of being carried out at atmospheric pressure, using air as gas to form the plasma, which reduces the operating cost due to the absence of a vacuum chamber (ŽIGON; PETRIČ; DAHLE, 2018).

Lignocellulosic materials such as wheat straw (SCHULTZ-JENSEN *et al.*, 2011), coconut fibers (PRAVEEN *et al.*, 2016), coffee beans (RAVINDRAN *et al.*, 2017), grass varieties (GAO *et al.*, 2018), sugarcane bagasse (MIRANDA *et al.*, 2019), in addition to the beer residue itself (RAVINDRAN *et al.*, 2019a), have already been pretreated with non-thermal plasma, in order to verify the effectiveness of the method to decrease the recalcitrance of lignocellulosic material. The use of non-thermal plasma was investigated in brewer's waste as a pretreatment strategy. However, the pretreated material was submitted to enzymatic hydrolysis to produce bioethanol (RAVINDRAN *et al.*, 2019a).

The most common strategy for obtaining xylo-oligosaccharides is one that combines an efficient pretreatment with one that can convert the xylan exposed by the pretreatment into XOS. In this line, the use of enzymes is an interesting approach, as it is ecologically correct, operating without the application of chemicals (WAN AZELEE *et al.*, 2016), in addition to presenting high efficiency and specificity, which guarantees a lower amount of xylose and undesirable by-products (AKPINAR *et al.*, 2007). Generally, the complete deconstruction of xylan occurs due to the presence of two enzymes (BIELY; SINGH; PUCHART, 2016), the endo-xylanases (endo-1,4-β-xylanase, EC3.2.1.8), which cleave the β-1,4-xylose bonds within the heteroxylan and the β-xylosidases (xylan-1,4-β-xylosidase, EC3.2.1.37), which release monomeric xylose from the non-reducing ends of xylooligomers and xylobiosis produced by the action of endo-xylanase on xylan (WALIA *et al.*, 2017). Accessory enzymes, which can be hydrolases or esterases, act on the side chains of xylans or on the spinal chains of different types of xylans (POLETTO *et al.*, 2020a).

1.1 Hypothesis

The valorization of agro-industrial waste has been growing in recent times. New types of treatments are being explored, as well as different materials. Xylo-oligosaccharides are carbohydrates formed by xylose units and can be obtained from lignocellulosic residues. Thus, the main hypotheses of this work are:

(i) Non-thermal plasma can be efficient in the delignification of lignocellulosic material;

(ii) There is a possibility of obtaining XOS from brewer's waste pretreated with non-thermal plasma;

(iii) Scale-up of enzymatic hydrolysis step and reuse of xylanase enzymes becomes feasible.

1.2 Aim of the work

This work aims to obtain xylooligosaccharides (XOS) by enzymatic hydrolysis of brewery by-product, exploring the non-thermal plasma as a pretreatment alternative method removing lignin from the material.

Specific objectives

- Evaluate, through a critical review, the feasibility of non-thermal plasma as an innovative method of pretreatment for lignocellulosic materials;
- Assess the influence of plasma gases (air and air and argon mixture) and solids concentration (7.5, 10 and 12.5%) on the delignification of brewing residue;
- Study the alterations caused in the lignin content by the non-thermal plasma as a pretreatment in brewer's residue through SEM, FTIR, DSC and RAMAN techniques;
- Evaluate possible sugar losses through mass balance;

- Assess the optimal time to obtain xylooligosaccharides (XOS) through enzymatic kinetics;
- Verify the influence of enzyme concentration and substrate concentration on XOS production;
- Evaluate the possibility of scaling up the production of XOS in an optimized time;
- Verify the possibility of reusing the xylanase enzyme through ultrafiltration membranes to obtain XOS.

1.3 Structure of the thesis

This thesis is divided into chapters, and the main points are presented below:

The current Chapter (1) presents a general contextualization and objectives.

Chapter 2 presents the theoretical foundations on the main topics studied in the construction of this work. The literature review will address items such as lignocellulosic materials, grains used in the brewery, importance and difference between prebiotics and probiotics, types of pretreatments, enzymatic hydrolysis and xylooligosaccharides. Also, in this chapter on the topic of pretreatments, a literature review article published in the Trends in Food Science & Technology journal on non-thermal plasma as a pretreatment method is presented. In this review, a critical assessment of the current state of the art and future trends in the use of non-thermal plasma as a pretreatment technology in lignocellulosic materials was performed, showing its potential in relation to traditional methods.

Chapter 3 presents the results of the use of non-thermal plasma as a pretreatment of brewing waste, the results were published in the journal Innovative Food Science & Emerging Technologies. This chapter covers a study of pretreatment times, influence of different plasma gases and solids concentration, where responses were obtained through chemical, physical-chemical and morphological characterization techniques. As a result, the viability of non-thermal plasma as an alternative pretreatment of lignocellulosic residues is confirmed.

Chapter 4 presents the research work that is in the process of writing and reviewing and will be submitted to a prestigious international journal. This chapter consisted of the study of the enzymatic hydrolysis of brewery residue pretreated with non-thermal plasma, evaluating the kinetics, with optimal conditions of temperature and pH of the enzyme, in addition to evaluating the effect of different concentrations of enzymes and substrates in obtaining xylooligosaccharides. A study on laboratory scale-up and enzyme reuse was also carried out. Finally, Chapter 5 presents a summary of the main conclusion, including future perspectives on the subject of this work. The following Figure 1 shows the conceptual diagram of this work.

Plasma (4th State of Matter) delignifies material
Conciliated to enzymatic hydrolysis

Direct fermentation (Amorim et al., 2019)
Starch extraction + auto hydrolysis (Beatriz et al., 2015)
Enzymatic (Sajib et al., 2018)
Self hydrolysis (Carvalheiro et al., 2004)
Non-thermal plasma (Ravindran et al., 2019)

- ✓ Do beer residues contain an amount of hemicellulose that is close to the amounts of materials already used to obtain XOS?
- ✓ Is non-thermal plasma pretreatment efficient for lignin removal and hemicellulose exposure?
- ✓ Is it possible to scale up and reuse the xylanase enzyme?
- ✓ Is plasma-pretreated beer biomass sufficiently hydrolysable by endoxylanases of the GH11 family?

- ✓ Xylanases can be reused
- ✓ Non-thermal plasma as a pre-treatment is efficient in the delignification of brewer's waste
- ✓ Possibility of scaling up
- ✓ Is it possible to get XOS from brewer's waste
- ✓ XOS from brewer residue has biological activity

Figure 1. Conceptual diagram of work.

Chapter 2 - Literature review

This chapter aims to give the reader an overview of the main topics covered within the theme of this doctoral thesis. Starting from the assumption of generalization of lignocellulosic materials, knowing that the grains used in breweries are potential lignocellulosic biomasses, which they need pretreatment steps so that their different fractions (cellulose, hemicellulose and lignin) are broken down. Among the pretreatments, there is non-thermal plasma, and this topic will be addressed in the form of a review article already published in the journal Trends in Food Science & Technology (DOI: 10.1016/j.tifs.2021.01.047). Subsequently, an enzymatic hydrolysis step is necessary, which will in fact convert the xylan exposed by the pretreatment into products with prebiotic properties such as xylooligosaccharides (XOS).

2.1 Lignocellulosic Material

Lignocellulosic materials are the most abundant organic materials in the biosphere, representing 60% of plant biomass. These materials present a resistant and complex network composed of lignin (10% to 30%), hemicelluloses (15% to 35%) and cellulose (30% to 50%), and the composition depends on the raw material in question, age and vegetative stage (BISWAS; PERSAD; BISARIA, 2014a). The main component of lignocellulosic materials is cellulose, a polysaccharide formed by glucose molecules joined by β-1,4-glycosidic bonds. These fibrils are joined to each other through hemicelluloses, the second most abundant component of lignocellulose, which is an amorphous polysaccharide, composed of several five- and six-carbon sugars, such as arabinose, galactose, glucose, mannose and xylose (YANG *et al.*, 2015a). These structures are covered by lignin, an aromatic macromolecule formed by three basic phenolic structures, p-coumaryl alcohol, coniferyl alcohol and synapyl alcohol and its derivatives (ANWAR; GULFRAZ; IRSHAD, 2014).

Each of these portions can be used for a purpose, for example, the cellulose and hemicellulose portions can be hydrolyzed to various sugars and then fermented. Lignin can be degraded to smaller molar mass fractions, being used in several chemical processes, such as in the manufacture of polyurethane foams, phenolic and epoxy resins, in the production of phenol and ethylene (LORA; GLASSER, 2002).

This type of material can be used as raw material for the production of food, fuel, chemical inputs, enzymes, various consumer goods, in addition to obtaining oligomers with prebiotic properties such as xylo-oligosaccharides. However, for this purpose, it is known that the portion of great interest in the chemical structure of lignocellulosic material is the amount of hemicelluloses (POLETTO *et al.*, 2020b). There are several residues already used for this purpose, such as sugarcane bagasse (CARVALHO *et al.*, 2015), wheat straw (CHEN *et al.*, 2018), corn cobs (BOONCHUAY *et al.*, 2018), among others.

2.2 Grains used in Brewery

An example of lignocellulosic material that has been explored in recent times is brewer's bagasse. It has been of great interest in Brazil, as the Brazilian beer market is the third-largest in the world, behind only the USA and China, having reached a production of 14.1 billion liters in 2016, according to CervBrasil (2017). This sector has been growing every year and, consequently, producing more waste, with malt bagasse being the waste with the greatest impact in production and volume (FAO, 2013). The malt bagasse comes from the process of obtaining the wort, by boiling the ground malt and the adjuncts. After filtration, results in a residue that is destined for animal feed (AQUARONE, 2001).

Research shows amounts of 14-20 kg of residue for every 100 liters of beer produced (CORDEIRO; EL-AOUAR; GUSMÃO, 2012), and the chemical composition of this residue varies according to the barley variety and harvest season, conditions malt grinding and type of adjuncts (corn, rice, wheat and sorghum) incorporated in the brewing process (SANTOS *et al.*, 2003).

The use of malt bagasse in biotechnological processes in the production of enzymes and as a substrate for the cultivation of microorganisms has been reported. Carvalheiro *et al.* (2004) used malt bagasse to obtain a mixture of oligosaccharides with different molecular weights after subjecting the malt bagasse to the process of autohydrolysis at temperatures of 150, 170 and 190°C.

In general, the grains used in breweries contain a percentage of approximately between 21-28% hemicellulose, 11-27% lignin and 16-25% cellulose (MUSSATTO; DRAGONE;

ROBERTO, 2006; SANTOS *et al.*, 2003), which make them attractive in the possibility of obtaining xylo-oligosaccharides (XOS).

Amorim *et al*. (2019), using brewer's residue as substrate, obtained satisfactory results in obtaining XOS, through direct fermentation using Trichoderma species, where a production yield of 38.3 ± 1.8 mg/g was obtained (xylose equivalents/g of malt bagasse).

Spent brewery grain was evaluated as raw material to obtain a mixture of arabinoxylooligosaccharides (AXOS) suitable for use as prebiotics in the elderly, when subjected to a two-step aqueous processing (starch extraction and autohydrolysis) (BEATRIZ; PARAJO; LU, 2015). Some studies, therefore, prove the possibility of this residue as a raw material in obtaining XOS.

2.3 Types of pretreatments

2.3.1 Thermochemical production (via autohydrolysis or hydrothermal)

It is one of the most traditional methods used to solubilize hemicelluloses and produce pulps rich in cellulose and lignin. This method for obtaining XOS is characterized by the autohydrolysis of the biomass being pressurized at a temperature between 150 and 230 ºC. This procedure leads to the ionization of water molecules generating hydronium (H_3O^+) and hydroxyl (OH^-) ions (RUIZ *et al.*, 2013). Acetic and uronic acids are released from heterocyclic bonds, acidifying the pH of the medium and producing new hydronium ions. This increase in the acidity of the medium promotes the hydrolysis of hemicellulose chains, releasing oligomeric fragments of different degrees of polymerization (RUIZ *et al.*, 2013).

The hydrothermal process has the advantage of not requiring reagents other than water (CARVALHEIRO; DUARTE; GÍRIO, 2008). In fact, these processes selectively hydrolyze the hemicellulose fraction and, therefore, are influenced by the hemicellulose composition (GARROTE; DOMÍNGUEZ; PARAJÓ, 2002; RUIZ *et al.*, 2013), in contrast to acid-catalyzed processes. Therefore, autohydrolysis promotes less degradation of cellulose and hemicellulose, in addition to less release of lignin-derived compounds, which favor bioconversion processes, due to the low levels of microbial inhibitors generated during this pretreatment (GARROTE; DOMÍNGUEZ; PARAJÓ, 2001).

2.3.2 Chemical methods (Acids and Alkalines)

Chemical methods involve the use of acids, bases and organic solvents. Acid hydrolysis is one of the most promising and extensively studied and employed pretreatments. The efficiency of obtaining xylose from the hydrolysis of hemicellulose can vary between 75 and 90%, which is the main monosaccharide obtained, other sugars, such as glucose and arabinose, are obtained in smaller amounts (HUANG *et al.*, 2009).

One of the disadvantages of this method is the formation of by-products such as HMF and furfural, which can cause problems in the process as they are inhibitory agents in the fermentation processes (KIM *et al.*, 2012; PANAGIOTOPOULOS *et al.*, 2011). Industrially, diluted acid and mild temperature conditions are used, up to 120ºC, because under these conditions a smaller amount of these fermentation inhibitors is produced. Sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), phosphoric acid (H_3PO_4) are used, with H_2SO_4 being the main one (SAHA *et al.*, 2005; ZHANG *et al.*, 2012).

Another chemical method is alkaline pretreatment, the main effect of which on biomass is delignification (lignin solubilization), making hemicelluloses and cellulose more accessible to subsequent hydrolysis processes. The use of alkaline agents promotes swelling in the fibers, impacting the bonds of hemicelluloses and cellulose, thus increasing the porosity of the biomass (HAGHIGHI MOOD *et al.*, 2013; WANG *et al.*, 2010b).

The most commonly used alkaline agents are: sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$) and ammonia. Of these, the most studied is NaOH (MORADI *et al.*, 2013; WEI *et al.*, 2013) however, $Ca(OH)_2$ has been shown to be effective as a pretreatment agent and is cheaper than NaOH. Other is to alkaline hydrogen peroxide, which leads to high glucose yields and can be carried out under moderate temperature and pressure conditions (RABELO *et al.*, 2014).

2.3.3 Non-thermal plasma

This topic will be presented in the form of a review article already published in the journal Trends in Food Science & Technology, DOI: 10.1016/j.tifs.2021.01.047.

2.3.3.1 Use of non-thermal plasma in lignocellulosic materials: A smart alternative

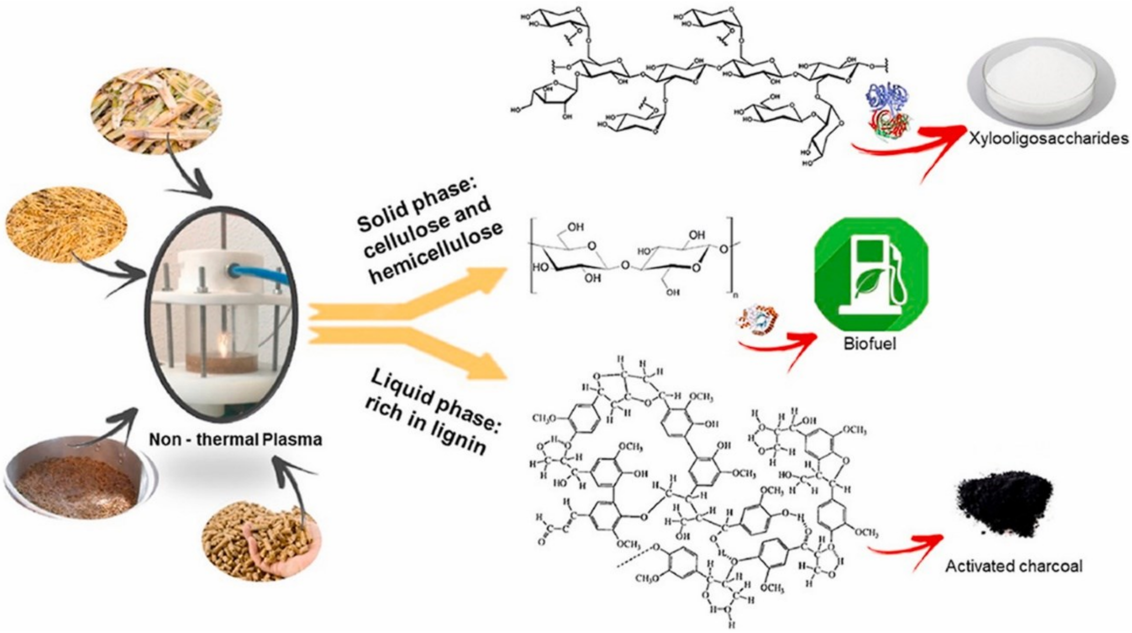


Figure 2. Graphical abstract of article.

In the last few years, there has been a great effort to value agro-industrial wastes and their respective reuse. In this sense, lignocellulosic materials have been highly considered, making possible to obtain several value-added products such as biofuels, renewable chemicals, and renewable materials (LOPES *et al.*, 2016). Lignocellulosic materials are the most abundant organic materials in the biosphere, representing 60% of plant biomass. They are composed of lignin (10%–30%), hemicellulose (15%–35%) and cellulose (30%–50%), the composition of which depends on the raw material in question, age and vegetative stage (BISWAS; PERSAD; BISARIA, 2014b). In the conversion of lignocellulosic biomass into biofuels or other products, a pretreatment step is necessary, taking a fundamental role in the economics of processes. Therefore, energy-efficient and cost-effective pretreatment methods are required to make the process sustainable and economically viable (WRIGHT *et al.*, 2018).

Conventional pretreatments require large amounts of energy and are not environmentally friendly, leading to the formation of numerous undesirable compounds, such as aliphatic acids, formaldehyde, and others (RAVINDRAN; JAISWAL, 2016). This reason encouraged the replacement of conventional unsustainable pretreatments (for example, chemical and physical-chemical ones) with sustainable pretreatments called "green" (for example, biological pretreatments) (SAHA *et al.*, 2016).

Biological pretreatments, using microorganisms, are considered sustainable. However, they require an extensive treatment period, low yield, and consumption of carbohydrates by the organisms, being considered one of the main challenges of this technique (SAHA *et al.*, 2016). Enzymatic hydrolysis is also a promising way because it does not generate effluents (ecologically correct), but exclusively enzymatic depolymerization has low efficiency due to the high natural recalcitrance to material, due to the complexity of the chemical interactions between cellulose, hemicellulose and lignin in the plant cell wall (OTIENO; AHRING, 2012). These obstacles encouraged the investigation of other pretreatments for lignocellulosic biomass. One of the not so conventional technologies that are in an increasing application, in the pretreatment of raw materials, is the use of non-thermal plasma (RAVINDRAN *et al.*, 2019b). This method has been explored over the last years and has excellent potential in the most diverse applications, as in the treatment of mammalian cells (KALGHATGI *et al.*, 2011), degradation of pharmaceuticals in wastewater (KRISHNA *et al.*, 2016), treatment of biofilms (GUPTA; AYAN, 2019), treatment of water and sewage (CUBAS *et al.*, 2019; HASHIM *et al.*, 2016), aid in the degradation of p-nitrophenol in water (SHANG *et al.*, 2019), degradation of dye in water degradation of dye in water (JO; MOON; MOK, 2015), pretreatment of lignocellulosic waste (MIRANDA *et al.*, 2019; RAVINDRAN *et al.*, 2019b), among others.

In the non-thermal plasma technique, plasma is produced using a dielectric barrier (DBD) discharge, to improve a later stage of access of the enzyme complex to the lignocellulosic matrix (KRIEGSEIS *et al.*, 2011). This method has proved to be interesting, as the process can be carried out at atmospheric pressure and use atmospheric air as exhaust gas, making the cost of the operational process and maintenance low due to the absence of a vacuum chamber (ŽIGON; PETRIČ; DAHLE, 2018). This technology can be considered a promising substitute for conventional and aggressive techniques aiming to obtain value-added products, such as biofuels, renewable chemicals, and renewable materials from different lignocellulosic materials.

In this context, the present review aims to present the main concepts and mechanisms of action of non-thermal plasma in different materials, in addition to the main characterization techniques used so far. It is worth noting that this is the first review exposing the primary studies of the use of this promising and eco-friendly technique in residual biomass, particularly for lignocellulosic materials.

2.3.4 Plasma

The concept of plasma emerged in 1879, when William Crooks used and defined it as the fourth state of matter. It was only in 1928 that the term "plasma" was used by the American chemist Irving Langmuir (KALIA *et al.*, 2013). Plasma is a diversified and unique medium for modifying the surface of plant fibers, as it contains a mixture of reactive species such as free radicals, electrons, and massive particles, being defined as a gaseous environment composed of charged and neutral species with a total density zero load. The transformations of the physical states of matter happen with the gain of energy (Fig. 3). Therefore, plasma is a partially or fully ionized gas made up of electrons, ions, excited and neutral molecules, reactive species, and photons (FRIDMAN, 2008; JIANG *et al.*, 2014).

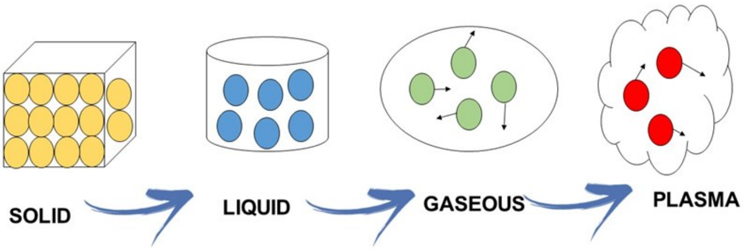


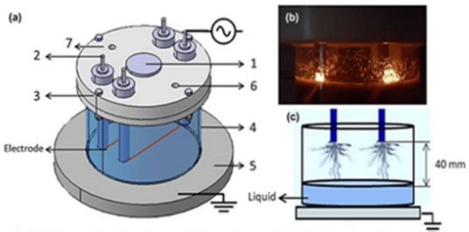
Figure 3. Illustration of the physical transformation of the state of matter with energy gain.

Plasma is classified according to the temperature, density and, energy level of the species that make it up (FRIDMAN, 2008). Thus, we can consider high-temperature plasma or thermal plasma that with temperatures above 3000 K and low temperature or non-thermal plasma that with temperatures below 1000 K (GAO *et al.*, 2018). In the literature, there are different configurations of plasmas used in lignocellulosic materials. Dielectric barrier plasmas, for example, employ a conventionally coaxial electrode configuration. The high voltage electrode is sealed in a quartz tube, submerged in the treated liquids, so that the liquid serves as an additional dielectric barrier layer and the coolant for the discharge (RAVINDRAN *et al.*, 2019b). Other configurations of the NTP reactor are the dielectric discharge of a plane, consisting of a Perspex glass and two steel plates, where studies used this plasma configuration in the deconstruction of lignocellulosic material (LIM; ZULKIFLI, 2018). Plasma configurations of weakly ionized oxygen have also been tested for lignocellulosic materials (PRAVEEN *et al.*, 2016). In general, it is known that the electrical discharge plasma in contact with water, by generating many types of reactive species and molecular species, in addition to UV irradiation and shock waves, is capable of efficiently destroying recalcitrant organic compounds, mainly due to its efficient generation of the strongest electron radical oxidant OH (SHANG; LI; MORENT, 2019). The most common discharge regimes for the generation of NTP in the laboratory are: corona discharge, dielectric barrier discharge (DBD), spark discharge (from English spark discharge), streamer, light discharge (from English glow discharge) etc. (JIANG *et al.*, 2014; ZHANG *et al.*, 2017a), these being characterized by an energetic electron temperature much higher than that of bulk gas molecules. In this review, we will only address the non-thermal plasma technology as it is a technology that does not use high temperatures.

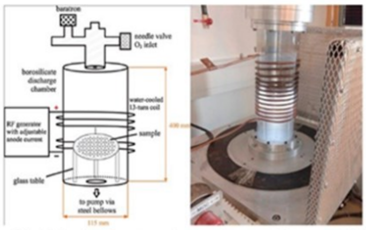
2.3.4.1 Non-thermal plasma (NTP)

Non-thermal plasma (NTP) is a partial electrical discharge initiated at sufficient voltages. Partial electrical discharges cause the electron temperature to be higher than the temperature of the surrounding gas molecules. Electrons interact with gas molecules to generate active radicals, reactive species and, ions, which have higher levels of energy, increasing the rate of reactions (WU *et al.*, 2013). Plasma is technologically generated by electric or electromag- netic fields and is generally indicated as discharge plasma (GILBERT CARLSSON; STRÖM, 1991). These dielectric barrier discharges can be generated in many configurations (Wolf, 2012). It generally consists of metallic conductive electrodes, mainly refrigerated, connected to a high voltage source of alternating polarity (KRÁL *et al.*, 2015). An electric field with sufficient field forces is generated by applying a high voltage to the space between the electrode and the material, where the electrical break occurs (WOLF, 2012) and a stable plasma state is formed (ZANINI *et al.*, 2008). The total charge is approximately zero, that is, the number of electrons is equal to the sum of the positive and negative ions multiplied by their respective costs (ESTEVES MAGALHÃES; FERREIRA DE SOUZA, 2002). The positive electrode accelerates free electrons. After impact, they dissociate gas molecules producing ionization and fragmentation. Ions and electrons continue to collide with other atoms and gas molecules and promote an avalanche of electrons (WAGNER *et al.*, 2003).

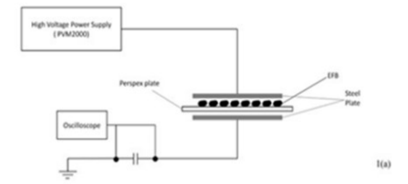
Figure 4 shows different types of plasmas. In 4.1 (a) shows a schematic drawing of a liquid plasma reactor, with (1) sample port (2) electrodes (3) Teflon cover (4) glass chamber (5) grounded metal plate (6) inlet gas (7) gas inlet (7) gas outlet. In (b), there is the plasma discharge, in which the yellowish color of the discharge is due to the sodium environment inside the reactor and in (c) there is a schematic drawing showing the distance between the plasma discharge and the surface of the liquid in the reactor. In 4.2, Weakly ionized oxygen plasma. In 4.3 Plane to plane dielectric barrier discharge reactor, consisting of a Perspex glass and two steel plates and in 4.4 Plasma enhanced fluid bed reactor (PEFBR).



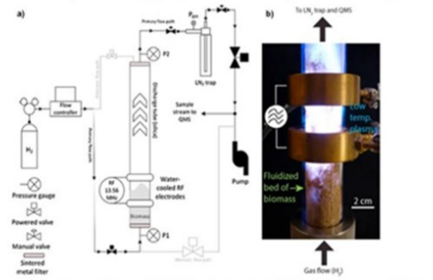
4.1 Schematic drawing of the plasma-liquid reactor. Source: (Miranda et al., 2019).



4.2 Weakly ionized oxygen plasma. Source: (Praveen, 2016).



4.3 Plane to plane dielectric barrier discharge reactor, consisting of a Perspex glass and two steel plates. Source: (Lim & Zulkifli, 2018).



4.4 Plasma enhanced fluid bed reactor (PEFBR). Source: (Gao, 2018).

Figure 4. Schematic drawing of the plasma-liquid reactor. Source: Miranda et al. (2019).

2.3.4.2 Non-thermal plasma: an overview of potential applications

The growing number of publications that use non-thermal plasma (Fig. 5) highlights the importance of this technology, which has increased in recent years. These surveys show an average advance of approximately 17 times more publications related to the topic in 2019 (376 documents) when compared to the 2000s (22 papers), generating a total of about 2930 article documents in that period, when using the words "nonthermal plasma "or" non-thermal plasma ". This interest is due to the NTP technology, as it presents characteristics such as clean technology, with an enormous advantage to the drastic reduction of pollutants and a corresponding reduction of costs in the treatment of effluents, in addition to being easy to handle.

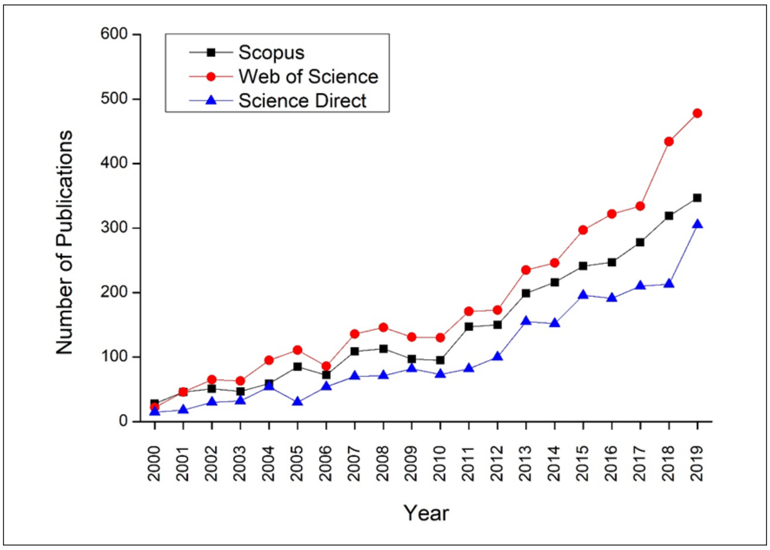


Figure 5. Number of publications related to non-thermal plasma between the years 2000 and 2019. Source: Scopus, Web of Science and Science Direct, key- word "non-thermal plasma" OR "nonthermal plasma".

Plasma has potential applications in several research areas, with works for example that include the use of NTP technology in fabrics, where this technique helps in improving the ability to print on fabrics (MORENT *et al.*, 2008). In the treatment of water and wastewater (HASHIM *et al.*, 2016), in the treatment of mammalian cells, proving the inter-action of plasma with cells at the molecular level (KALGHATGI *et al.*, 2011), in the degradation of pharmaceuticals in wastewater (KRISHNA *et al.*, 2016), in the treatment of biofilms, where the promising nature of technology to eradicate biofilms in the medical field is clearly shown (GUPTA; AYAN, 2019), in the decomposition of volatile organic compounds (VOCs) (SCHMIDT *et al.*, 2015), use of cold-atmospheric plasma in oncology (DUBUC *et al.*, 2018), among others. Among the documents found in this review, we will use the eight main articles on the topic "use of NTP in lignocellulosic materials", because despite the amount of work has increased over the years, only a small number of studies are focused on this use. Some research studies have investigated the effects of plasma application on different lignocellulosic materials, such as bunches of empty palm fruits (LIM; ZULKIFLI, 2018), coffee grain spent (RAVINDRAN *et al.*, 2017), lignocellulosic natural coir fibers (PRAVEEN *et al.*, 2016), spent brewery grain (RAVINDRAN *et al.*, 2019b), sugar cane bagasse (MIRANDA *et al.*, 2019) to verify its effectiveness as a pretreatment to obtain value-added products.

2.3.4.3 Use of non-thermal plasma in lignocellulosic materials

Lignocellulosic materials are found in almost all materials derived from plants, from wood, grass to agricultural waste and municipal solid waste (YANG *et al.*, 2007). Examples of these materials are Switchgrass, Hardwood (hybrid poplar), Softwood (pine), Corncob, Rice straw, Bagasse, among others (HAMELINCK; VAN HOOIJDONK; FAAIJ, 2005). Nowadays, these materials as being wheat straw, rice straw, cane bagasse, corn cobs, among others are used, for example, for the production of lignin-modifying enzymes. Many significant efforts are devoted to converting these lignocellulosic products into value-added products others, including too compounds, fine chemicals, animal feed, cellulose and paper, biofuels (BILAL *et al.*, 2017). The composition of the lignocellulosic material in the residues may change depending on the genotype and environmental conditions. This type of content is generally composed of a large fraction of cellulose (40–50%), followed by hemicellulose (25–30%) and lignin (15–25%) (BISWAS; PERSAD; BISARIA, 2014b). A representation of the structures of the cell wall of lignocellulosic materials and their chemical structures is presented in Figure 6.

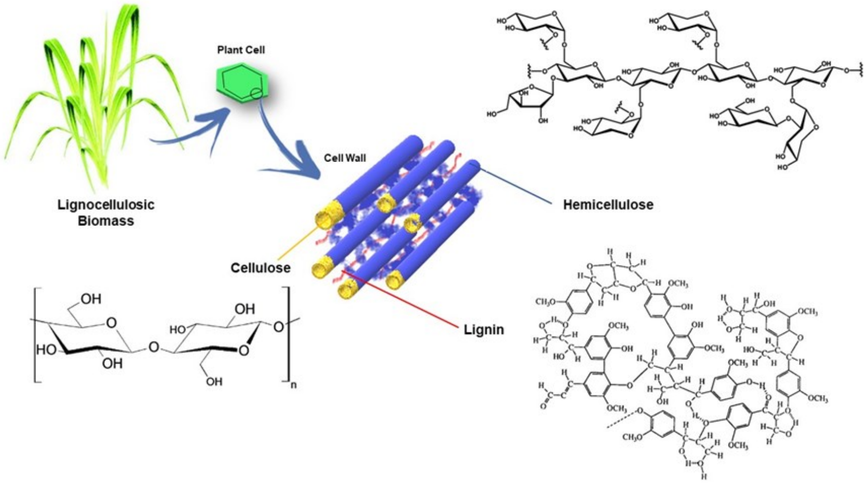


Figure 6. Representation of the structures that form the plant cell wall.

The main component of lignocellulosic materials is cellulose, a polysaccharide formed by glucose molecules joined by β-1,4-glycosidic bonds. These fibrils are joined to each other through hemicellulose. Hemicellulose is the second most abundant polysaccharide in plants (YANG *et al.*, 2015b). Hemicellulose molecules are present by several polymers consisting of pentoses (D-xylose and D-arabinose) and hexoses (D-mannose, D-glucose, and D-galactose) with xylose as the most abundant sugar (KUMAR; SINGH; SINGH, 2008). Also, uronic acids (D-glucuronic, D-galacturonic, and methylgalacturonic acids) may also be present in branched chains (LIMAYEM; RICKE, 2012). Hemicellulose polymers are often interconnected by covalent and hydrogen bonds. Lignin is a heterogeneous amorphous polymer, insoluble in water, and composed of three aromatic alcohols, also called monolignols, corresponding to propyl phenol units: p-coumaric alcohol, coniferyl alcohol and synaphyl alcohol (ANWAR; GULFRAZ; IRSHAD, 2014).

Non-thermal plasma can assist as a pretreatment of biomass, the central aspect when using this technology is the delignification of lignocellulosic material. With this, portions of lignin, cellulose, and hemicellulose can be obtained separately, each of which is a source of value-added products. Lignin, for example, can be used to get products of commercial value such as activated carbon (HAYASHI *et al.*, 2000) and vanillin (which applied in the production of herbicides, antifoaming agents, and antimicrobial agents (WALTON; MAYER; NARBAD, 2003)). The delignified part, that is, rich in cellulose and hemicellulose, can be also used to obtain products with added value. Hemicellulose is a source of xylose, from which xylitol comes, which is used as a sweetener (WÖLNERHANSSEN; MEYER-GERSPACH; ISLAM, 2019), in addition to dental use (JOSEPH, 2017) and furfural, used in the manufacture of plastics, solvents) (MAMMAN *et al.*, 2008), soil improvers (CHUN'AI DAI *et al.*, 2010), cellulose can also be used to obtain second-generation ethanol (bioethanol) (ZHANG *et al.*, 2019) and pharmaceutical applications (KULKARNI; DIXIT, 2011).

The non-thermal plasma technique has the advantage of attacking the lignin of the lignocellulosic material (MIRANDA *et al.*, 2019). This is a very important aspect when, for example, the final product of interest are xylo-oligosaccharides, where the portion of interest is hemicellulose (VÁZQUEZ *et al.*, 2000). Hydrothermal treatment has the disadvantages of forming inhibitors depending on the severity of the process and solubilizing hemicellulose (NANDA *et al.*, 2014), which depending on the desired final product, would not be ideal.

Therefore, this NTP technology becomes extremely interesting, for use in the pretreatment phase of the most varied raw materials, seeing the amount of products that can be obtained from the different portions of the LCMs. One aspect that must be emphasized when dealing with non-thermal plasma, is that despite its numerous advantages, it can consume electrical energy. In this sense, several homogeneous and heterogeneous catalysts are used for the catalysis of ozone and hydrogen peroxide and to cooperate with UV effect to improve the degradation of pollutants in water, for example. A range of homogeneous ($Fe^{2+,}$ Fe^{3+}) and heterogeneous (non-doped metal oxides) catalysts obviously improves pollutant degradation compared to plasma alone (SHANG *et al.*, 2019).

Table 1 shows the different types of lignocellulosic raw materials that have been pretreated with non-thermal plasma. Among the biomasses explored with the plasma technique to date we have the wheat straw (SCHULTZ-JENSEN *et al.*, 2011), coffee grain spent (RAVINDRAN *et al.*, 2017), lignocellulosic natural coir fibers (PRAVEEN *et al.*, 2016), waste bunches of empty palm fruit (LIM; ZULKIFLI, 2018), used grains from brewing industries (RAVINDRAN; JAISWAL, 2016), grasses (GAO *et al.*, 2018), grass (WRIGHT *et al.*, 2018) and also the sugarcane bagasse (MIRANDA *et al.*, 2019). Some more recalcitrant biomasses were investigated, for example, sawdust was used as one of the raw materials in a study, but the intention was the plasma liquefaction of lignocellulosic biomass in order to obtain maximum energy yield and not with focus in a pretreatment to assess the structure after using plasma (MEI *et al.*, 2020).

These are some of the few studies that used lignocellulosic materials and sought to evaluate the efficiency of non-thermal plasma technology as a different pretreatment method, since it has the advantages of not being toxic, non-polluting, with proven efficacy in lignin extraction, which is one of the problems faced in other conventional pretreatment methods tested for these materials.

When compared to pretreatment with conventional steam blast, some studies reported that the efficiency of 90% enzymatic hydrolysis was achieved in 24 h, but due to the high temperature achieved in the process, some sugars were degraded (GROUS; CONVERSE; GRETHLEIN, 1986). Studies with alkaline hydrolysis in beer residue reached 75 g/L of reducing sugar after enzymatic hydrolysis. This study involved the use of concentrated alkalis to digest lignin fibers. The alkaline treatment was preceded by the removal of hemicellulose with sulfuric acid (LIGUORI *et al.*, 2015).

A thermomechanical pretreatment was developed for brewing waste by (PIERRE *et al.*, 2011) where a DIC reactor was used, where Using a variety of processing pressures, the researchers were able to obtain a maximum glucose yield of 24 g per 100 g of waste. The reducing sugar produced by the new dielectric barrier discharge plasma was comparable to the values obtained for different pretreatment strategies (RAVINDRAN *et al.*, 2019b).

Table 1. Use of non-thermal plasma in different lignocellulosic materials.

Raw material	**Why the plasma was used?**	**Main results**	**Authors**
Wheat straw	Potential for the production of bioethanol by fermentation	Extent of plasma lignin removal over time, thereby reducing recalcitrance	(SCHULTZ-JENSEN *et al.*, 2011)
Lignocellulosic natural coir fibers	Investigations of plasma induced effects on the surface properties	Changes in surface topography have been confirmed after treatment with plasma	(PRAVEEN *et al.*, 2016)
Coffee spent waste	Evaluation of different techniques such as pretreatment	Conservation of a large part of the hemicellulose fraction along with the removal of acid-insoluble lignin	(RAVINDRAN *et al.*, 2017)
Empty fruit bunches	Investigatation of NTP effects on surface reactivity, morphology, changes in associated functional groups and chemical bonds, chemical elimination reactivity and surface morphology	Changes in residue morphology, increased surface reactivity, changes in lignin structure	(LIM; ZULKIFLI, 2018)
Switchgrass (grasses)	Evaluation of the deconstruction of lignocellulosic biomass	Significant hydrocarbon yield, where the biomass carbon generated methane	(GAO *et al.*, 2018)
Miscanthus grass	Efficacy of plasma as an alternative pretreatment method for biofuel production	Reactive species produced by plasma attacked biomass and released lignin in the solution	(WRIGHT *et al.*, 2018)
Wasted grain brewer	Improve the subsequent step of enzymatic hydrolysis for bioethanol production	Increased efficiency in enzymatic hydrolysis by removing lignocellulose recalcitrance	(RAVINDRAN *et al.*, 2019b)
Sugarcane bagasse	Evaluation of biomass delignification efficiency caused by plasma treatment	Positive effect on lignin solubilization, which contributed to the enzymatic hydrolysis process and favored a high carbohydrate solubilization yield	(MIRANDA *et al.*, 2019)

According to Table 1, we can see that the central studies to date are related to the use of lignocellulosic materials for later production of biofuels. Much can still be explored due to the extensive list of high added value products that can be obtained from different fractions from biomass (cellulose, hemicellulose, and lignin).

2.3.4.4 Effects of using non-thermal plasma as pretreatment on LCMs

Atmospheric pressure plasma is a source of highly reactive species, such as ozone (O_3), hydroxyl radical (OH), hydronium ions (H_3O^+), among others. Ozone, for example, is a powerful oxidizer, with an oxidation potential of 2.08 V that can cleave carbon double bonds that are abundant in lignin, whereas hydronium ions are the basis for determining the pH of aqueous solutions (GARCÍA-CUBERO *et al.*, 2009). In fact, the higher its concentration, in contrast to the concentration of hydroxyl ions, the more acidic the solution is, and the lower the concentration of hydronium ions in favor of the concentration of hydroxyl ions, the more alkaline (basic) is the solution. These species react with chemical structures of nearby lignocellulosic materials to effectively disintegrate certain parts of these chemical structures. The ozone molecule, typically generated in abundance in air plasmas, can specifically attack the lignin of the treated material (Fig. 7), leaving cellulose and hemicellulose intact, which is the direct degradation (RAVINDRAN *et al.*, 2019b). Besides, it is also possible to carry out the indirect degradation of the lignocellulosic matrix through UV light and shock waves (BRUGGEMAN *et al.*, 2007). These chemical species (O_3) produce UV light between the liquid plasma interfaces and, consequently, photochemical reactions could take part in the activation of hydrogen peroxide environment breaking the form of O and OH.

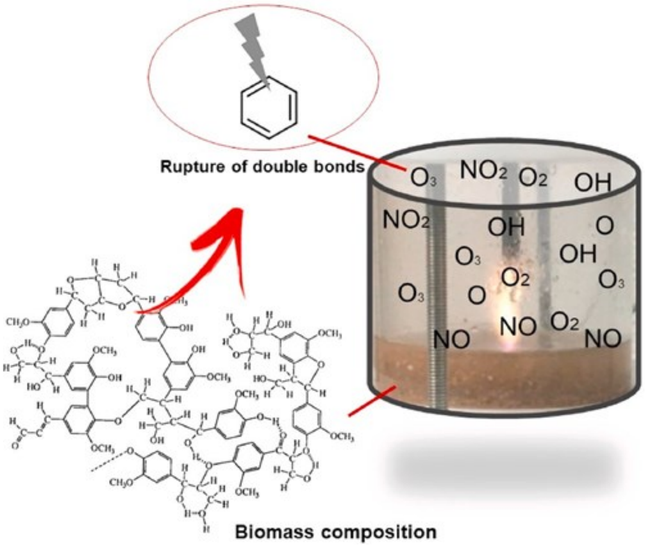


Figure 7. Schematic drawing of the air environment in the plasma forming ozone, which helps in the cleavage of double bonds of lignocellulosic materials.

Oxygen (O) and hydroxyl groups (OH), being proton acceptors when in a plasma reactor, cause the chemical interaction between the Brønsted bases, resulting from air discharge and the lignin structure of the lignocellulosic biomass to occur, which causes deprotonation of lignin. Deprotonated lignin molecules are soluble at pH higher than 7 due to the interaction of the binding (WRIGHT *et al.*, 2018). During plasma treatment, the buffered solution has an amphoteric behavior, that is, it can behave as an acid or as a base, depending on the other reagent present, using hydrolysis of the bicarbonate ion to restore pH and provides pH balance during all plasma treatments, as described in Equations (1) and (2) with reversible reactions.

$$HCO^{+3} + H_2O \leftrightarrow CO_2^{+3} + H_3O^{+} \text{ (1)}$$

$$HCO^{+3} + H_2O \leftrightarrow H_2CO_3{}^{+} OH^{-} \text{ (2)}$$

Miranda *et al*. (2019), using plasma technology combined with pH buffered solution (pH 12) had a positive effect on the lignin solubilization (about 58.5%), which helped in enzymatic hydrolysis and provided a high solubilization yield of acetyl groups present in the hemicelluloses (MIRANDA *et al.*, 2019).

The initial pH of the solution exposed to the plasma plays an essential role in the process of degradation of the chemical structures that make up the lignocellulosic material. Direct reactions (O_3 attacking lignin) are prevalent in acidic environments (low pHs) and are efficient processes for compounds with functional groups, aromatic (phenolic) systems, or with double bonds (as is the case with lignin in LCMs) (SARANGAPANI *et al.*, 2018). When the nitrogen atmosphere is used for the formation of NTP, important reactive nitrogenous species (RNS) are formed (CADORIN *et al.*, 2015). When air ($N_2 + O_2$) is used to form the NTP, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed (JIANG *et al.*, 2014). From the secondary nitrogen and oxygen species, other species are produced according to Eqs. (CREMA *et al.*, 2020):

$$H_2O_2 + NO^{-2} + H_3O+ \longrightarrow \mathrm{ONOOH} + H_2O_2 \quad (3)$$

$$ONOOH + H_2O \leftrightarrow \mathrm{ONO}O^{-} + H_3O+ \quad (4)$$

$$ONOOH \rightarrow \mathrm{HN}O_3 \quad (5)$$

However, some studies indicate that a low pH environment leads to reduced efficiency of plasma-induced lignin. This is attributed to the formation and accumulation of strong acids, such as nitric acids, nitrous acid and other carboxylic acids (SU *et al.*, 2002).

At higher pH, indirect oxidation through O_3 and H_2O_2 predominates and results in the formation of OH radicals. In the pH range between 6.5 and 8.5, for example, the radicals O_3 and OH predominate in the disintegration process of the lignocellulosic matrix, with the oxidative capacity of O_3 interrupting the carbon double bonds present in the aromatic rings (GRABOWSKI *et al.*, 2007). This probably leads to better lignin removal efficiencies in the pH range between 6.5 and 8.5, in which the oxidation potential of the OH radical (2.80 V) is significantly greater than that of O_3 (2.08 V). Secondary species (OH, NO_2^- , NO_3^- , H_2O_2, HNO_3, H_3O^+) are also responsible for decreasing the pH and increasing the conductivity of the medium (CUBAS *et al.*, 2019). This is due to the dissociation of water that occurs under the plasma, which leads to acidification of the medium and formation of oxidizing species. Depending on the radical formed, there is an increase in conductivity with the plasma treatment time and this increase results from the production of ions, demonstrating that the active ions were generated due to the activation of the plasma. That is, these consequences occur depending on the radical formed and its oxidizing potential.

The reaction of the hydroxyl radical contributes more to the change in pH (to some extent, $pH < 8$), which increases the efficiency of lignin removal (SARANGAPANI *et al.*, 2018). However, at pH values higher than 8.5, the formation of HO_2^- occurs, which can eliminate a certain percentage of OH radicals, consequently reducing the efficiency of the pretreatment process (RAVINDRAN *et al.*, 2019b).

Another aspect in relation to the use of ozone as a pretreatment is that although it is widely reported to not produce inhibitory/toxic products that can affect fermentation enzymes or bacteria, the nature of the degradation products depends on the type of biomass and the products of plasma dissolved in the pretreatment liquid. Ozonation studies of wheat straw showed that among the inhibitory compounds produced, significant increases in the concentrations of oxalic acid and acetovanilone were observed (SCHULTZ-JENSEN *et al.*, 2011).

2.3.5 Main changes observed in LCMs after using the NTP technique

Several changes occur in the structure of a lignocellulosic material after applying a pretreatment technique, it is no difference with plasma. These changes can be observed by different techniques such as Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Differential Scanning Calorimeter (DSC), and Atomic Force Microscopy (AFM) (Fig. 8). These techniques can be addressed together to analyze better the changes that have occurred.

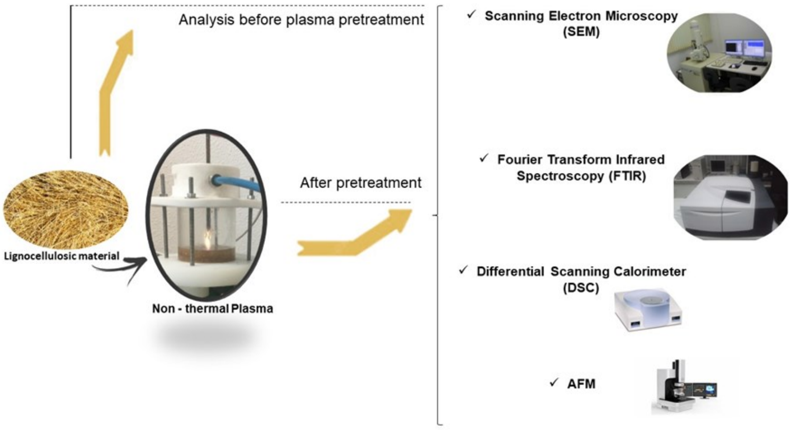


Figure 8. Schematic drawing of the Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimeter (DSC), and Atomic Force Microscopy (AFM) analyzes, which can be performed before and after pretreatment using.

Fourier Transform Infrared Spectroscopy (FTIR), for example, is a qualitative technique that indicates compositional changes in biomass based on the presence or absence of functional groups belonging to cellulose, hemicellulose, and lignin (RAVINDRAN *et al.*, 2019b). Physical methods such as FTIR have also been used to characterize natural constituents of biomass, such as lignin, extractives, hemicellulose, methoxy, and aromatic hydroxyl groups, among others (LU *et al.*, 2017). Characteristic peaks usually related to lignocellulosic raw materials in this technique are related to glycosidic bonds, double bonds between carbons, arylalkyl ether (C–O–C) bonds, where a decrease in the peak after plasma use, indicates changes in main structure material (RAVINDRAN *et al.*, 2019b). This decrease was observed in brewery by-product after pretreatment with plasma (RAVINDRAN *et al.*, 2019b). Changes in the syringil and guaiacyl components of lignin and in the carboxyl and carbonyl groups were also verified by this technique in empty fruit (LIM; ZULKIFLI, 2018), where the decrease in absorbance at the syringil and guaiac peaks was verified. By the same technique (FTIR), the conservation of hemicellulose in spent coffee beans was observed after the use of plasma. This was verified by the peak value before and after the remain the same (RAVINDRAN *et al.*, 2017).

Another technique that assesses changes caused by pretreatments in the physical properties of biomass is Differential Scanning Calorimeter (DSC), being a non-specific diagnostic tool to obtain in a single step the entire pattern of phase transitions that occur during a temperature scan (BRYS, 2016). Changes of this nature were observed in brewery residue, after treatment with NTP, with a decrease in melting temperature from 361.05 ºC to 309.9 ºC (RAVINDRAN *et al.*, 2019b). As lignin has specific characteristics due to its high degree of association between its macromolecules, it decomposes at higher temperatures due to its compacted and interconnected propane units in a complex amorphous matrix (PISHNAMAZI *et al.*, 2019). Consequently, that treatment with NTP altered the structure of lignin, decreasing its stability, and consequently decreasing its melting temperature.

Scanning Electron Microscopy (SEM), on the other hand, is a technique that aims to obtain information about the morphology and surface roughness of lignocellulosic materials (OKENWA *et al.*, 2020). After plasma treatment in biomass of empty palm fruit clusters, changes in the raw material surface morphology were observed (LIM; ZULKIFLI, 2018). These changes may have occurred because NTP induces lignin exposure and crystalline portions of cellulose (XIE *et al.*, 2015). Some "flaws" were observed in the sugar cane bagasse after 2 h of treatment, which the authors stated would be an effect of the plasma on the fibers of the material, with the removal of the layers that covered the fibers (this layer, with a glue-like structure, composed of fibril structures connected by a xyloglucan) (Fig. 9 (b) and (c)) (MIRANDA *et al.*, 2019). The biomass of *Mischantus* grass, treated under different operating conditions (2 and 4 h) of plasma, was analyzed by SEM. Minor structural damage, probably to the lignin structure, was observed after 2 h of dry pretreatment, and after 4 h of treatment, it presented more significant localized damage (WRIGHT *et al.*, 2018). In coconut lignocellulosic fibers, after 10 s of exposure to plasma, changes in morphology were observed for technical SEM, as the surface appeared to be etched by reactive oxygen species and became softer (PRAVEEN *et al.*, 2016).

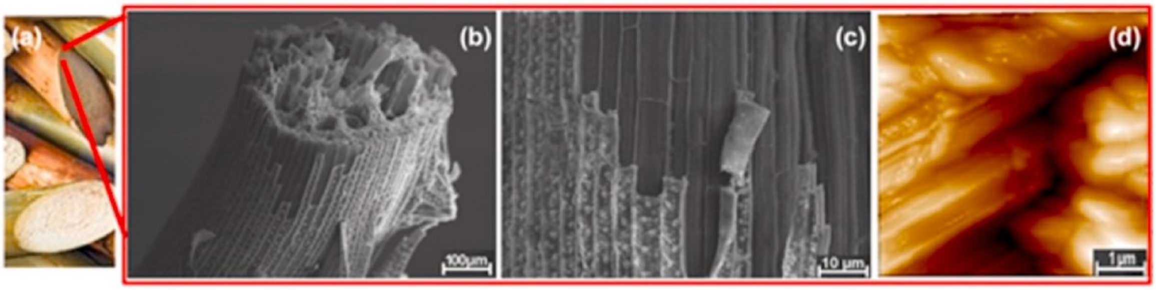


Figure 9. (a) Cane bagasse in nature; (b) and (c) SEM images of cane fibers after plasma pretreatment and (d) AFM image of the cane fiber structure after plasma treatment, which shows the structure of cellulose and microfibrils bound by xyloglucans. Source: Miran

Another technique that has the function of detecting morphological changes resulting from the pretreatment is the Atomic Force Microscopy (AFM). This technique provides three-dimensional images of the surface (GARCÍA, 2002). AFM analysis of coconut lignocellulosic fibers for example, allowed to observe differences in surface topography and viscoelastic properties at the nanoscale (PRAVEEN *et al.*, 2016). Taken together, the SEM and AFM analysis confirmed that the plasma had a marked effect on the pretreated sugarcane bagasse biomass, removing the layers that covered the fibers of the material. The effect of plasma on the liquid was not limited to the sample surface, but also changed the volume of sugarcane bagasse, a fact has proven after enzymatic hydrolysis (Fig. 9(d)) (MIRANDA *et al.*, 2019). The fact of the change in the volume of the structures of lignocellulosic materials, is due to the fact of the exposure of the material in a liquid next to the plasma, because the contact of the lignocellulosic material with water causes the swelling of the material to occur, increasing the surface area, which allows a greater penetration of reactive species in the material. This fact was also verified in *Michantus grass* (WRIGHT *et al.*, 2018), and proves the importance of using plasma in a liquid medium. Figure 9 presents an example of images that can be obtained by the SEM and AFM techniques of sugarcane bagasse to highlight the images captured by these two different techniques for the same raw material.

2.3.6 Trends on pretreatment

The growing concern with the final destination of lignocellulosic residues (LCMs) has increasingly attracted the attention of researchers in making possible to transform this type of material into products that can add value until they reach the consumer. These materials have high recalcitrance due to the chemical composition, therefore the need for pretreatment steps that help to reduce resistance. Many alternatives are tested however, it is known of a large amount of toxic residues and solvents that are used in conventional techniques. In this line of reasoning, it appears that in recent times there has been a significant advance in research involving the use of non-thermal plasma (NTP), as evidenced by the increase in the number of publications. In addition, this technique has proved, by means of few studies, to be effective when used as a pretreatment in lignocellulosic materials. Understanding the modes of action of NTP specifically in LCMs is of utmost interest because through different characterization techniques such as SEM, FTIR and DSC, the main changes caused in biomass can be proven when using this technique. The NTP technique is considered adequate, non-polluting, and non-toxic, which makes it attractive and a tendency to be further explored. Based on the few studies found in the literature using NTP in lignocellulosic materials, an important direction in the future would be the use of this technique as a pretreatment in LCMs, focused on a larger range of value-added products, after the total separation of cellulose, hemicellulose, and lignin. An up-and-coming trend is the branch of biorefinery, where all extracted parts (cellulose, hemicellulose and lignin) could be used separately. For example, by concentrating the amount of hemicellulose in the biomass, the use of specific enzymes such as xylanases could be efficient in obtaining xylooligosaccharides, which are xylose oligomers with prebiotic properties, which are increasingly attracting interest in the food area. The liquid part (rich in lignin) can be explored when isolated from black liquor to obtain various products of commercial interest, such as activated carbon, vanillin, phenols, benzene, fertilizers, polymers, among others. Cellulose, on the other hand, enriches this biorefinery chain in obtaining biofuels, which has been seen in some studies so far. Therefore, knowing that the pretreatment stage is one of the most important stages of a process, it is necessary to search for a practical and economical method, with the minimum or nonexistent use of chemicals and that preserves the maximum amount of cellulose and hemicellulose. With this, it is suggested that NTP technology be further explored, as conventional lignocellulose separation technologies, and thereby enriching productive chains of great potential today.

2.4 Enzymatic hydrolysis

Enzymatic hydrolysis consists of removing protons from water molecules to form oligosaccharide molecules and hydroxide ions from xylan, by the catalytic action of xylanase enzymes. The enzyme-catalyzed synthesis represents an interesting alternative to classical chemical methods. It allows the control of regioselectivity and stereochemistry of the final reaction products (PERUGINO *et al.*, 2004).

The enzymatic hydrolysis method is considered a promising way to produce XOS, because it is a high-efficiency strategy that does not generate effluents (eco-friendly). An advantage when using this method to obtain XOS would be the small degree of impurities obtained, due to the specificity of the enzymes, but the exclusively enzymatic depolymerization (without pretreatment of the material previously) presents low efficiency due to the high natural recalcitrance of the material, which it is derived from the complexity of the chemical interactions between cellulose, hemicellulose and lignin in the plant cell wall (OTIENO; AHRING, 2012).

The main groups of enzymes used in the hydrolysis of xylan are glycosyl hydrolases, which hydrolyze glycosidic bonds and the group of carbohydrate esterases, which cleave ester bonds between phenolic residues and xylose units, which may be Endo-1 ,4-β-xylanases (EC 3.2.1.8), α-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), feruloyl esterases (EC 3.1.1.73) and ρ-coumaric acid esterases (EC 3.1.1.) (BIELY; SINGH; PUCHART, 2016).

Some studies show the efficiency in the use of xylanases to obtain XOS in waste materials. The sugarcane straw residue was submitted to enzymatic treatment with different extracellular fungal xylanases, these being different recombinant GH11, recombinant GH10 and commercial GH11, after hydrothermal pretreatment under ideal conditions (190°C; 20 min). In order to verify the best enzyme in obtaining XOS with DP 2 and 3. The results showed that GH10 produced more X2 and X3 with minimal xylose formation, while GH11 produced more xylose (BRENELLI *et al.*, 2020).

Sugarcane bagasse was also evaluated in relation to the potential for obtaining XOS with the use of a genetically modified xylanase XynZ from *C. thermocellum* ATCC 27405 in the enzymatic hydrolysis, proving the biotechnological potential for the use of xylanase (XynZ) from *Clostridium thermocellum* ATCC (MANDELLI *et al.*, 2014).

2.5 Importance and difference between prebiotics and probiotics

Probiotics, according to one of the most accepted definitions, are live microorganisms, administered in adequate amounts, that confer health benefits on the host (SAAD, 2006; SANDERS; KLAENHAMMER, 2001).

The probiotic activity is based on the fact that the colon flora can be selectively stimulated by indigestible carbohydrates through fermentation, such as *bifidobacteria* and *lactobacilli* considered beneficial to human health (CUMMINGS; MACFARLANE; ENGLYST, 2001). A microorganism will be considered a probiotic if it inhabits the gastrointestinal tract and survives passage through the stomach, in addition to maintaining viability and activity in the intestine (FAO-WHO, 2002).

The term prebiotic refers to carbohydrates identified as non-digestible including lactose, inulin and the oligosaccharide line, where they provide fermentable substrates for beneficial colonic bacteria (FLOCH, 1999).

According to Fooks *et al.* (1999), the criteria for classifying prebiotics as food ingredients include: being neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract, promoting selective fermentation by bacteria beneficial to the colon, altering the composition of the colonic microflora in favor of a healthier composition, produce beneficial effects for the health of the host.

Some oligosaccharides can be synthesized naturally in fruits and vegetables, while others are commercialized through the production via hydrolysis of polysaccharides, such as dietary fibers and starch, in addition to being generated by enzymatic breakdown (PANESAR; BALI, 2015). Some oligomers are classified with potential prebiotic activity, such as lactulose, fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides. Oligosaccharides are the food ingredients that fulfill all prebiotic functions, where the most used in the food industry are fructo-oligosaccharides (FOS), gluco-oligosaccharides (GOS), transgalacto-oligosaccharides (TOS), isomalto-oligosaccharides (IMO), inulin and xylo-oligosaccharides (XOS) (PANESAR; BALI, 2015; VÁZQUEZ *et al.*, 2000).

The synthesis of oligosaccharides can be done by several combinations between the monomeric units of sugars and the stereo-specific introduction of glycosidic bonds (BARRETEAU; DELATTRE; MICHAUD, 2006; V.B.TOLSTOGUZOV, 1991). Bioactive oligosaccharides can be obtained through synthesis (chemical or enzymatic) or depolymerization of polysaccharides (physical, chemical or enzymatic).

The chemical synthesis of oligosaccharides requires multiple steps of protection and deprotection of specific molecular groups, having a complexity that causes chemically directed synthesis, unattractive for industrial applications. To overcome this challenge, new strategies for the continuous production of oligosaccharides in innovative enzyme membrane bioreactors have already been developed (CZERMAK *et al.*, 2004; ENGEL *et al.*, 2007). Thus, the enzymatic synthesis of oligosaccharides and their derivatives is a promising alternative to chemical synthesis.

Studies indicate that the ingestion of AXOS obtained from brewer's residue could improve the intestinal microbiota and its functions and, consequently, the health of the elderly (BEATRIZ; PARAJO; LU, 2015).

2.6 Xylo-oligosaccharides

Xylo-oligosaccharides (XOS) are carbohydrate oligomers generated by xylose units, which naturally exist in fruits, vegetables, milk and honey. Industrial production can be obtained through lignocellulosic materials (LCMs), which are used for various purposes, including food applications (VÁZQUEZ *et al.*, 2000). They are a promising class of oligosaccharides and they stimulate the increase of bifidobacterial levels to a greater extent than fructooligosaccharides (TUOHY *et al.*, 2005) or other oligosaccharides (SAMANTA *et al.*, 2012).

XOS structures vary in degree of polymerization (DP), monomer units and bond types depending on the various xylan sources used for XOS production. Generally, XOS are mixtures of oligosaccharides formed by xylose residues linked through β - ($1 \rightarrow 4$) bonds. The number of xylose residues involved in its formation can vary from 2 to 6 and are known as xylobiose, xylotriose and so on. For food applications, xylobiose (DP = 2) is considered an interesting xylo-oligosaccharide. The presence of different side groups results in branched XOS with different biological properties (VÁZQUEZ *et al.*, 2000, 2005). XOS are obtained from xylans by means of hydrolysis.

XOS is considered non-digestible oligosaccharides (NDOs), non-cariogenic in humans having biological properties. They are used as dietary sweeteners in low-calorie diet foods and for consumption by individuals with diabetes (CHOQUE DELGADO *et al.*, 2011; RIVERO-URGELL; SANTAMARIA-ORLEANS, 2001; VÁZQUEZ *et al.*, 2000).

They are marketed as a white powder containing between two to six xylose molecules linked by β 1-4 bonds (VÁZQUEZ *et al.*, 2000). The stabilities of these oligomers can differ greatly depending on the types of oligosaccharide and sugar residues, bonds, ring shapes, and anomeric configurations.

In addition to prebiotic properties, XOS also have advantages about the moderate degree of sweetness they have, effects on starch retrogradation and better sensory and nutritional properties of foods (VORAGEN, 1998). With all these properties, XOS become suitable compounds for incorporation into foods (AYYAPPAN *et al.*, 2016).

An interesting topic in enzymatic hydrolysis to obtain XOS is laboratory scale-up. The scale up is considered of extreme importance in biotechnological processes to direct the way in which the reaction will be carried out in larger amounts of substrate (PEREIRA *et al.*, 2018). Usually, problems related to scaling up arise from the fact that the times required for the mechanisms to occur increase with the increase in scale. The challenge is to produce large quantities with high productivity and product quality (ZIZHUO XING *et al.*, 2009).

Another very relevant aspect is the reuse of enzymes, this step is extremely important for industrial application, because the greater the possibility of reusing the biocatalyst, the lower the expenses and, consequently, the greater the chances that the industry will be interested in replacing traditional catalysts with biocatalysts (PEREIRA *et al.*, 2018).

2.7 Considerations regarding the state of the art

In the literature, there are few works (4) using brewer's residue to obtain xylo-oligosaccharides, and the main studies use different pretreatment techniques. Studies on brewer's residue also investigated the best conditions for obtaining XOS through autohydrolysis. Longer reaction times led to lower production of oligosaccharides and higher concentrations of monosaccharides, with the maximum yield of XOS (61% of the xylan raw material) being obtained at 190 °C after 5 minutes of reaction (CARVALHEIRO *et al.*, 2004).

BEATRIZ *et al.* (2015) used the brewer's residue to obtain arabinoxylooligosaccharides (AXOS) with a two-step process, the first being starch extraction, followed by autohydrolysis. In this study, the residue first underwent a hydrothermal treatment and then the AXOS were refined by ion exchange membrane filtration and modified by enzymatic hydrolysis. AXOS were evaluated for their prebiotic activity by in vitro fermentation assays. As experimental results, fermentation with AXOS resulted in an increase in the populations of *bifidobacteria* and *lactobacilli* in relation to negative control cultures, enabling the improvement of the intestinal microbiota and its functions (BEATRIZ; PARAJO; LU, 2015).

Other studies focused on the development of techniques to extract arabinoxylan-rich portions of brewer's bagasse using various xylanases, in addition to in vitro experiments to evaluate the fermentability of the products. It was found that the pure xylanases of the GH family 10 and 11 are more efficient in the production of short oligosaccharides than the more complex mixtures available commercially. In vitro fermentation experiments confirmed that xylanase-catalyzed hydrolysis promoted the subsequent growth of *B. adolescentis* (SAJIB *et al.*, 2018).

AMORIM *et al.* (2019), for example, studied the optimization of AXOS (arabinoxylooligosaccharides) production from unpretreated brewery waste in a single step using *Trichoderma* species (*T. reesei* and *T. viride*). In this study, there was a comparison to the use of commercial enzymes, and as a conclusion, they proved that direct fermentation is an attractive and advantageous approach to hydrolyze brewery residue and produce AXOS (AMORIM; SILVÉRIO; RODRIGUES, 2019).

Other studies have focused on the use of non-thermal plasma as a pretreatment of brewing residue for later obtaining bioethanol through enzymatic hydrolysis. An important aspect suggests that subjecting lignocellulose to plasma discharges can increase the efficiency of enzymatic hydrolysis, in addition to obtaining a high amount of ethanol after the fermentation of the hydrolyzate (RAVINDRAN *et al.*, 2019b).

In view of what has been exposed in this chapter, with regard to the state of the art, the importance and originality of this work is evident, where a large amount of lignocellulosic material in the country (brewery waste) will be explored with an environmentally less aggressive technology (plasma) to the future production of value-added products, named xylo-oligosaccharides (XOS) via the enzymatic route. The differential of this study is in obtaining XOS from brewer's residue, through the non-thermal plasma technique as pretreatment and subsequent enzymatic hydrolysis, in addition to the study of the possibility of expanding the laboratory scale and reuse of the xylanase enzyme.

Chapter 3: Pretreatment step with non-thermal plasma

Non-thermal plasma as an innovative pretreatment technology in delignification of brewery by-product

This chapter aims to give the reader a look at non-thermal plasma as a pretreatment in brewery waste. An exploratory plasma study was carried out, evaluating different types of plasma gases (air and air/argon mixture), different times and different solid concentrations. The results were discussed in terms of different chemical (characterization) and physicochemical (SEM, FTIR, DSC, XRD and Raman) evaluations. This study was written in the form of an article already published in the Innovative Food Science & Emerging Technologies Journal, DOI: 10.1016/j.ifset.2021.102827.

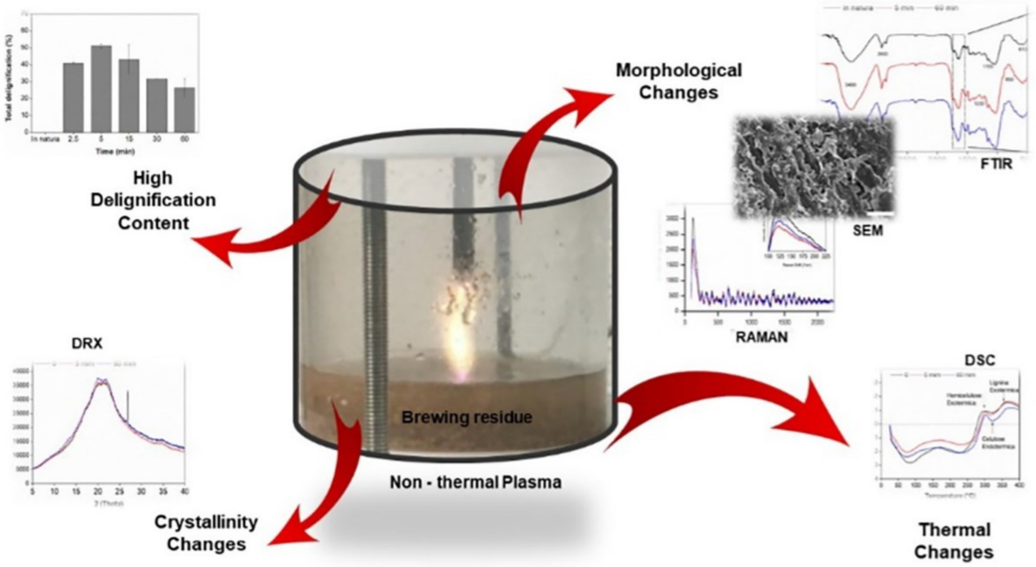


Figure 10. Graphical abstract of article.

Abstract

Non-thermal plasma was applied as an innovative pretreatment for the delignification of brewery by-products. The influence of different plasma gases (pure air or air/argon), time and solids concentration (7.5, 10 and 12.5%) was evaluated during the delignification tests. The levels of cellulose, hemicellulose and lignin were monitored before and after treatment. The reduction in lignin content was approximately 50% after 5 min using pure air in all tested solids concentrations. Longer pretreatment times (60 min) influenced the removal of hemicellulose (50%) and cellulose (12%), also indicated by morphological changes. After 5 and 60 min of plasma application, the physical-chemical characteristics showed minor changes in its chemical composition, mainly affected by the losses of lignocellulosic compounds. Non-thermal plasma is considered an attractive alternative for the pretreatment of brewery by-products, resulting mainly in removing lignin, assisting in subsequent processes such as the enzymatic hydrolysis of the spent solid.

Keywords: Lignocellulosic material, Lignin, Carbohydrate fractionation, Brewing by-product.

3.1 Introduction

The valorization of industrial waste and its reuse are very important issues nowadays (MIRABELLA; CASTELLANI; SALA, 2014). The brewery by-product is a lignocellulosic material generated during the beer-making process, representing approximately 85% of the total by-products obtained. The most abundant compounds in these grains are hemicellulose, lignin and cellulose in the range of 28, 28 and 17%, respectively (MUSSATTO, 2014).

These compounds can be converted into different value-added products. However, a pretreatment step is often required to degrade the structural network of lignin and improve the enzymatic hydrolysis of hemicellulose and cellulose (CHEN *et al.*, 2017). Pretreatments are generally used to remove hemicellulose and lignin fractions from biomass and promote cellulose enrichment in pretreated solids.

Several pretreatment methods are used to improve the digestibility of lignocellulosic biomass. These methods range from traditional chemical treatments, mainly because the reagents are accessible and inexpensive (JAMBO *et al.*, 2016), such as alkaline (NASCIMENTO *et al.*, 2016) and acid (PANAGIOTOPOULOS *et al.*, 2012), physical-chemical treatment under subcritical conditions (MONTEIRO *et al.*, 2021) in addition to new processes, such as the use of non-thermal plasma (RAVINDRAN *et al.*, 2019b).

Plasma is the fourth state of matter (MERCHE; VANDENCASTEELE; RENIERS, 2012). Non-thermal plasma (NTP) is considered a partial electrical discharge initiated with sufficient voltages. These partial discharges cause the electron's temperature to rise, becoming higher than the temperature of the surrounding gas molecules. The interaction between electrons and gas molecules generates active radicals, reactive species and ions, which have higher energy levels, increasing the rate of reactions (WU *et al.*, 2013). It is a non-toxic and sustainable method and can minimize lignocellulose biomass recalcitrance by removing lignin (RAVINDRAN *et al.*, 2019b). In general, the application of non-thermal plasma (NTP) technology is a desirable and environmentally friendly alternative, operating at low temperatures and atmospheric pressure is relatively simple and low operating cost installations (LEAL VIEIRA CUBAS *et al.*, 2020). The NTP process can produce reactive species and simultaneously induce chemical processes such as ionization, dissociation and excitation, in addition to inducing physical processes such as electric field, UV light and shock waves (VIEIRA CUBAS *et al.*, 2021). The most common discharge regimes for the generation of NTP in the laboratory are: corona discharge, dielectric barrier discharge (DBD), spark discharge (from English spark discharge), streamer, light discharge (from English glow discharge) etc. (JIANG *et al.*, 2014; ZHANG *et al.*, 2017b), these being characterized by an energetic electron temperature much higher than that of bulk gas molecules.

Despite this, few studies used non-thermal plasma technology as a pretreatment step for lignocellulosic materials (PEREIRA *et al.*, 2021a). So far, a single study evaluated the effect of NTP on brewery by-products to improve the enzymatic hydrolysis of cellulose for post-production of bioethanol (RAVINDRAN *et al.*, 2019b). In that study, the authors evaluated the consequences of voltage variation (22 kV, 25 kV and 28 kV), the type of solvent (acid, alkali and water), and time (5, 10 and 15 min) on delignification levels, using air as a plasma gas, but did not mention the assessed solids concentration. After reaching 36% delignification, a 2.14-fold increase in reducing sugar yield was obtained compared to the control (RAVINDRAN *et al.*, 2019b). In this context, the present study aims to evaluate the application of non-thermal plasma as a pretreatment to decrease the lignin content of the brewery by-product by assessing the influence of plasma gases (pure air and air/argon mixture), the time of pretreatment (2.5 to 60 min) and the solids concentration (7.5, 10 and 12%). We also discussed how complementary characterization analyzes (SEM, FTIR, DRX, DSC and RAMAM) could be used to verify possible effects of plasma on the physical-chemical characteristics of brewery by-product after treatment. The fractionation obtained by the NTP can help in the conversion of the different constituents of the lignocellulosic material into value-added products.

3.2 Material and methods

3.2.1 Materials

The spent grain of Vienna-type beer was obtained from a brewery located in São Josá -SC, Brasil. The bagasse was first washed with tap water to remove residues from the brewing process, dried in an electric oven at 60 ºC for 24 h and stored for use in the following steps. Other materials used in this study are listed: sulfuric acid (Sigma-Aldrich), deionized water and the following standards xylose, glucose and arabinose from Sigma-Aldrich.

3.2.2 Application of non-thermal plasma (NTP)

The apparatus used in this study is composed of an NTP reactor made of quartz walls and Teflon caps, coupled to a 17 kV power source. 30 mA alternating current was used to generate the plasma using flat-tip electrodes operating under atmospheric pressure (VIEIRA CUBAS *et al.*, 2021).

The first test was performed to evaluate the influence of the plasma gas containing only air (nitrogen (78%) and oxygen (21%)) or air (50%)/argon (50%) mixture, maintaining the pretreatment time and solid concentration in 15 min and 10% (m/v), respectively. The samples were exposed to NTP acting with 220 V, under constant agitation of 700 rpm. Subsequently, a kinetic study was performed at times 2.5, 5, 15, 30 and 60 min, using 10% solids under the plasma gas selected in the first assay. Finally, different solids concentrations, 7.5, 10 and 12.5% (m/v), were evaluated over the selected time.

After the pretreatment step, the samples were centrifuged at 1493.8 x*g* for 10 min. The chemical composition (lignin, cellulose and hemicellulose) and physical-chemical characteristics (SEM, FTIR, DRX, DSC and RAMAN) of the spent solid were analyzed. In the liquor, the sugar content was measured by HPLC and the RAMAN spectra were evaluated.

3.2.2.1 Determination of lignocellulosic content

The lignocellulosic composition of the solid samples was determined according to National Renewable Energy Laboratory (NREL). Sequential extraction with ethanol and water in raw material was used to determine extractives content according to the procedure NREL/TP-51042,619. Cellulose, hemicellulose, lignin and ashes were quantified according to the protocols of (SLUITER *et al.*, 2016). After acidic biomass treatment (300 mg material in 72% H_2SO_4 for 1 h at 30 ºC followed by 4% dilution (84 mL water) and autoclaved at 121 ºC for 1 h), the solid was separated from the liquid. The liquid was filtered using a gravity filtration apparatus and the acid-soluble lignin was measured at 240 nm in a quartz cuvette. The solid was washed and placed in an oven at 100 ºC overnight to obtain the insoluble lignin value. The dry solid was subsequently taken to a muffle furnace at 575 ºC for up to 24 h to determine the ash content. The quantification of xylose, glucose and arabinose was carried out by High-Performance Liquid Chromatography (HPLC) (Knauer, Germany) equipped with a Knauer-RI detector and an Aminex HPX 87H column (300 mm × 7.8; Biorad, USA). The 40 µL injection was eluted with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.7 mL/min at 35ºC. Sugar concentrations were determined from standard glucose, xylose and arabinose curves.

3.2.2.2 Physical-chemical characterization of solid material

The raw and plasma pretreated brewery by-product were analyzed by different techniques. Scanning electron microscopy (SEM) was performed in a JEOL JSM-6390LV (JEOL, Tokyo, Japan) with a tungsten filament and an accelerated voltage of 10 kV. Fourier transform infrared (FTIR) spectra were recorded on a Cary 600 Series device (Agilent Technologies, St. Clara, United States), in the wavelength in between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and accumulation of 16 scans in attenuated total reflectance (ATR) mode. Differential scanning calorimetry (DSC) was used to evaluate thermal behavior. All runs were performed between 25 and 400 ºC at a heating rate of 10 ºC/min under nitrogen atmosphere in a Shimadzu DSC-60 (Waltham, EUA) coupled with the TA-60WS software. X-ray diffractometry (XRD) was performed on MiniFlex600 (Rigaku, Tokyo, Japan), using CuKα radiation, 40 kV voltage, 1.5 mA filament emission. Each sample was scanned from 5º to 50º in the 2θ range with a scan speed of 0.05º/step. Raman spectroscopy was performed using Anton Paar Cora 5200 Raman equipment, in the spectral range of 100 to 2000 cm^{-1}, with laser power at 450 mW and single wavelength at 785 nm.

3.2.2.3 Statistical analysis

Origin software was used in the statistical analysis. The results were evaluated by analysis of variance (ANOVA) and the significant differences were determined by the Tukey test at a probability level below 5% ($p < 0.05$).

3.3 Results and discussion

3.3.1 Effect of plasma gas on delignification of the brewery by-product

Initially, the influence of plasma gas (pure air or air/argon mixture) on the removal of lignin was evaluated. The conditions used were 15 min, 700 rpm and 10% solid. Table 2 shows the lignocellulosic composition of raw and pretreated brewery by-products. The raw sample showed 27.6% ± 1.4 cellulose, 35.0% ± 1.7 hemicellulose and 38.7% ± 0.1 total lignin. High lignin content in the brewery by-product was also showed by (Ravindran *et al.*, 2019), about 30.5%.

Table 2. Cellulose, hemicellulose, lignin and ash contents in brewery by-product before and after treatment with pure air or air/argon mixture in non-thermal plasma (15 min and 10% solid).

Brewery by-product (%)	Cellulose	Hemicellulose	Lignin	Ashes	Others
Raw	27.6±1.4	35.0±1.7	38.7±0.1	0.9±0.3	-
Pure air	27.3±0.4	20.1±0.3	22.5±0.1	4.6±0.3	25.5
Air/Argon mixture	17.2±1.7	12.6±0.6	22.2±0.3	7.4±0.3	40.6

The level of delignification obtained was very similar under the two plasmas gas reaching a reduction of approximately 42% in the lignin content. It was also observed that the air/argon mixture had a more significant influence on the decrease of cellulose and hemicellulose contents, which is not attractive when it is desired to concentrate these components in the solid for later hydrolysis. This result can be explained because, when argon is used, its molecules (Ar*) are excited to produce radical species in water, for example, hydroxyl radicals. The hydroxyl radical OH• is a highly active species due to its high oxidative potential ($E^\circ = 2.85$ V). However, it has a short half-life and is quickly converted to H_2O_2 (BENETOLI *et al.*, 2012). Several synergistic effects occur during plasma discharge, such as the direct chemical interaction between the cell membrane of endosperm and reactive oxygen (ROS) and nitrogen (RNS) species. Such species (O_2, O_3, OH, NO and NO_2) together with charged particles can damage cellular components (cellulose and hemicellulose). These results corroborate the findings of (VIEIRA CUBAS *et al.*, 2021) for reactive species (O_2, O_3, OH, NO, NO_2), along with ultraviolet (UV) radiation and charged particles, which interact chemically with the endosperm membrane of ground coffee cells, causing their rupture and facilitating the extraction of oil. It is known that the initial pH of the solution exposed to the plasma plays a very important role in the process of degradation of the chemical structures that make up the lignocellulosic material. Direct reactions (O_3 attack lignin) prevail in acidic environments (low pHs) and are considered efficient for compounds with functional groups, aromatic systems (phenolic) or with double bonds (as is the case with lignin in LCMs) (SARANGAPANI *et al.*, 2018).

Considering that the same decrease in lignin content was obtained applying air or air/argon mixture, and mainly undesirable reduction in cellulose and hemicellulose contents, the subsequent tests were performed only with air. The use of pure air makes the process less costly due to the high commercial value of argon.

3.3.2 Kinetic assays

The evolution of delignification and the effect of NTP on the contents of cellulose and hemicellulose (xylose and arabinose) was evaluated in a kinetic assay under the conditions selected in the previous test (700 rpm and 10% solid) (Fig. 11).

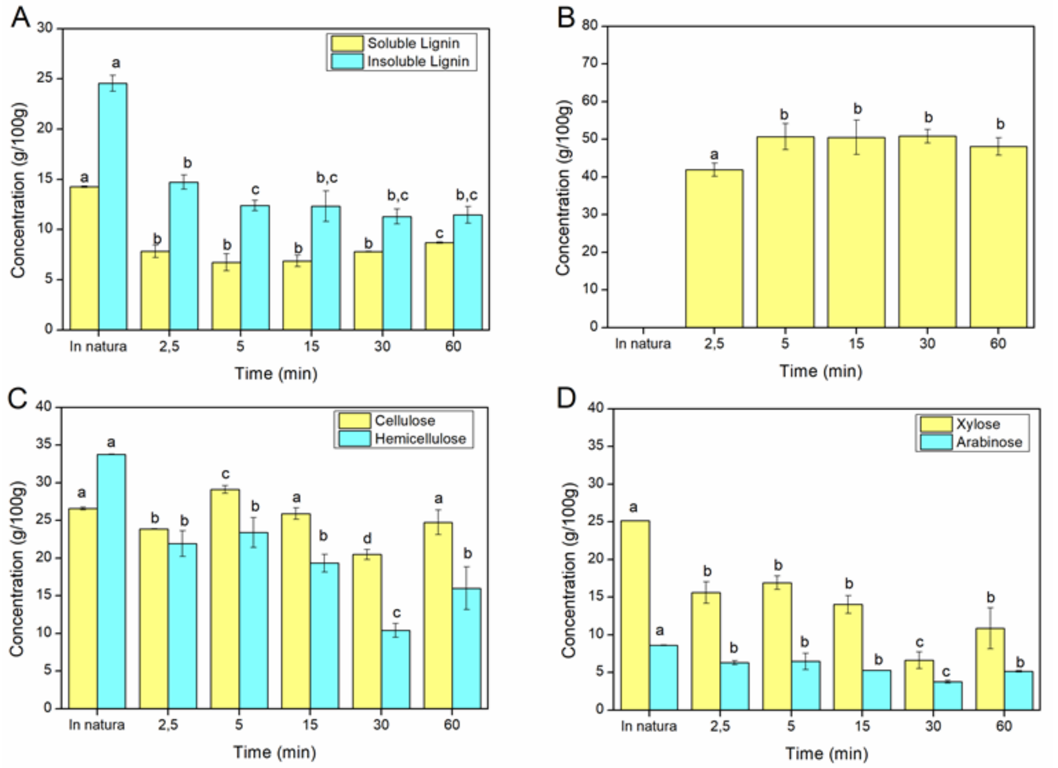


Figure 11. Kinetic evaluation of the non-thermal plasma treatment applied to the brewing by-product (10% solid content). (A) Soluble and insoluble lignin content; (B) Total delignification (C) Cellulose and hemicellulose content; (D) Xylose and arabinose.

There was a marked reduction in soluble and insoluble lignin (Fig. 11A) in the first 2.5 min of treatment, were reduced by 45% and 40% respectively compared with control. In fact, a high reduction in a very short time was observed. After that time, there was a slight increase in the soluble lignin content. This effect can be caused when part of the depolymerized lignin released in the initial minutes of pretreatment binds again to the fiber surface and remains accumulated as globular lignin. This adverse effect was described by (LI *et al.*, 2014) when studying the deposition of lignin on cellulose during hydrothermal pretreatment and its effect on cellulose hydrolysis. Our data show that the supposed deposition of globular lignin increases due to the time of exposure to plasma discharge. In this case, washing the pretreated solids may be necessary to reduce the undesirable effects of the globular lignin deposited on the material.

Total delignification (Fig. 11B) reached approximately 43 g/100 g after 2.5 min of treatment, and the maximum delignification of 50 g/100 g was achieved in 5 min. The increase in treatment time did not result in higher removal of lignin.

The values found for different biomasses pretreated with NTP showed 36 g/100 g delignification in brewery by-product after 10 min (RAVINDRAN *et al.*, 2019b), 58 g/100 g delignification in sugarcane bagasse after 2 h , and 74% delignification in wheat straw after 7 h (SCHULTZ-JENSEN *et al.*, 2011). The difference between lignocellulosic biomasses, especially recalcitrance, may have caused these variations in the treatment times. Other parameters such as plasma power, plasma gas and particle size of the solid can also contribute to different results. The result obtained in this study is very promising since in just 5 min a reduction of 50 g/100 g in the lignin content was obtained, making the brewing by-product an interesting material to be pretreated by this technique.

The cellulose content decreased in the first 2.5 min and increased after 5 min. The hemicellulose content (Fig. 11C) also decreased with the rise in the pretreatment time, according to Figure 11C and Figure 11D. This content reduced approximately 32% after 5 min, losing 34% xylose and 25% arabinose (Fig. 11D). The reduction in hemicellulose reached 50% in 60 min of NTP treatment. The differences in cellulose and hemicellulose losses may be due to cellulose's inherent crystalline nature, which is more resistant to chemical attacks. Meanwhile, hemicellulose is amorphous structure and can deteriorate more quickly in the presence of reactive species (CHUNDAWAT *et al.*, 2010). In conventional pretreatment methods, such as alkaline, (WILKINSON; SMART; COOK, 2014) observed a 25 g/100 g delignification in brewery by-product using 5% NaOH after 12 h. In addition, a decrease of approximately 45 g/100 g in arabinose was observed, resulting in a loss of 32.9 g/100 g in the hemicellulose content. It is known that the main effect of alkaline depolymerization is to break the ester bonds intertwined between lignin and hemicellulose (xylose and arabinose) and the hydrogen bonds with cellulose, releasing more lignin than acid treatments (RAI; PANDEY; PANDEY, 2019). On the other hand, plasma acts as a highly reactive source that breaks the carbon double bonds abundant in lignin (GARCÍA-CUBERO *et al.*, 2009). An aspect observed in this work is that plasma also affects hemicellulose and cellulose during treatment. However, the environmentally friendly character, added to the very low pretreatment time, are advantages that should be highlighted in relation to conventional pretreatment methods.

3.3.3 Effect of solids concentration in the delignification

The effect of the solids concentration (7.5, 10 and 12.5%) on the delignification of the brewery by-product is shown in Figure 12.

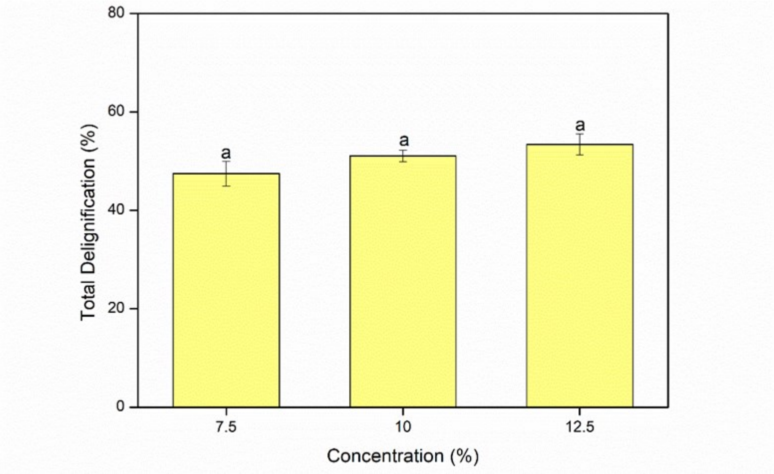


Figure 12. Delignification after 5 min varying the solids concentration (7.5, 10 and 12.5%).

It is observed that, despite the slight tendency to increase delignification (47.5 ± 0.3; 51.1 ± 0.1 and 53.5 ± 0.1%) with the increase in the solids' concentration (7.5, 10 and 12.5%), there was no significant difference ($p < 0.05$) after 5 min of exposure to NTP. This demonstrates that the system is not saturated since even increasing the mass of material in the reactor, the lignin removal rate did not differ. Therefore, the proposed pretreatment removed about 50% of total lignin using 12.5% of solids, which guarantees a high amount of material treated by batch. Cellulose content also remained unchanged, with values of 26.6% ± 1.6; 25.4 ± 4 and 27.6% ± 1.9 for the solids' concentration of 7.5, 10 and 12.5%, respectively. Hemicellulose content showed 11.8% ± 3.2; 15.26 ± 4.47 and 15.1% ± 1.3 for the same conditions, respectively. Brewery by-product conventionally pretreated with 5% NaOH using 25% solids concentration showed 25% delignification after 12 h (WILKINSON; SMART; COOK, 2014). It is worth remembering that these conventional methods requires a lot of energy, in addition to not being environmentally friendly, as they lead to the formation of undesirable compounds such as formaldehyde, aliphatic acid and others (JAMBO *et al.*, 2016).

3.3.4 Physical-chemical characterization

To better understand the changes in the physical-chemical characteristics of the brewery by-product after exposure to NTP, some characterizations are presented in Figure 13 and Figure 14.

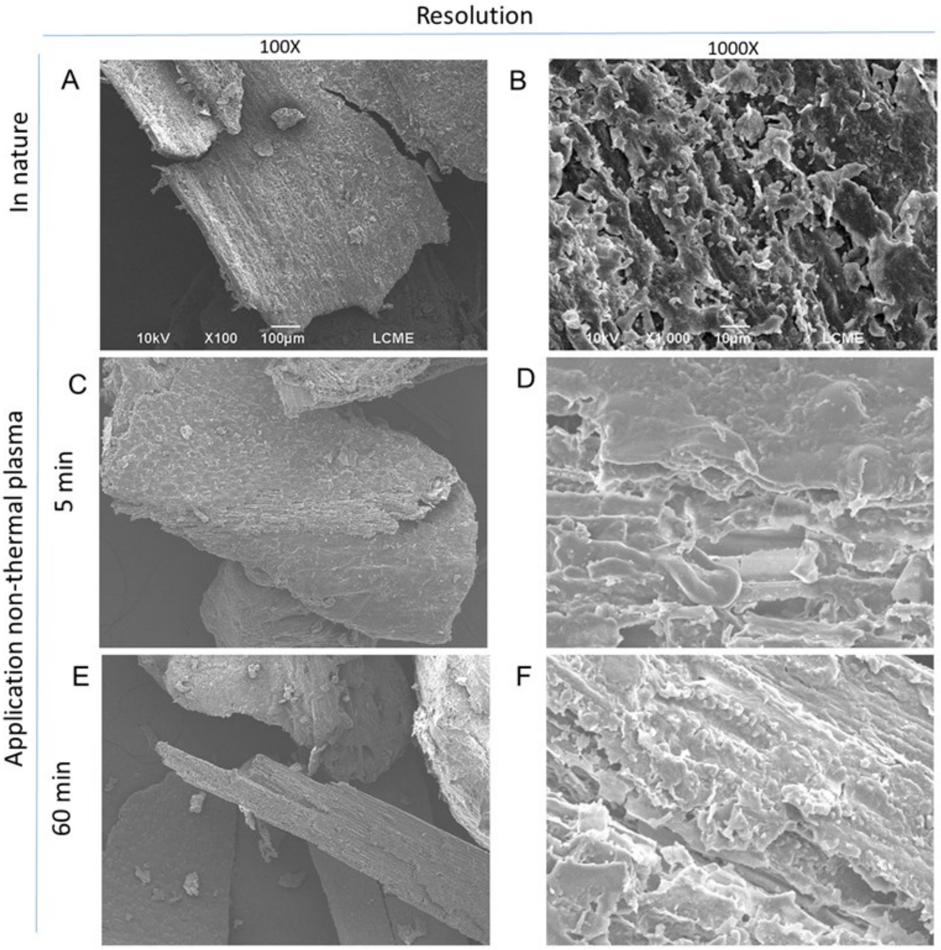


Figure 13. Scanning electron microscopy of the brewer's spent grain. Raw material (A, B) and after 5 (C, D) and 60 min (E, F) of plasma pretreatment. Magnification views of 100 and 1000×.

First, the morphology of samples was evaluated by SEM. It was observed that the brewer's spent grain is a material with a rigid structure and slightly irregular surface (Fig. 13B). In addition, pores in the cross-section of brewer's spent grain and regular channels that make up the plant's vascular system were described by (FONTANA; PETERSON; CECHINEL, 2018).

After NTP pretreatment, the samples showed a more "smoother" surface (Fig. 13C and F) due to the removal of more soluble components. A scanning electron micrograph of a sugarcane bagasse fiber revealed that a layer of glue on the material structure was removed after plasma treatment (MIRANDA *et al.*, 2019). In this work, after 5 min, it is possible to see species of "holes" that can be better observed in Figure 13D. These holes are the plant's conducting vessels (FONTANA; PETERSON; CECHINEL, 2018), commonly in vegetal tissues composed of cells with thick and lignified secondary walls. In the Figure 13F, a globular structure can be seen, which is described as lignin redeposited on the surface of the material, creating the pseudo-lignin (FONTANA; PETERSON; CECHINEL, 2018), indicating that after a specific pretreatment time, the lignin is redeposited, which is in accordance with the results obtained previously (Fig. 13A).

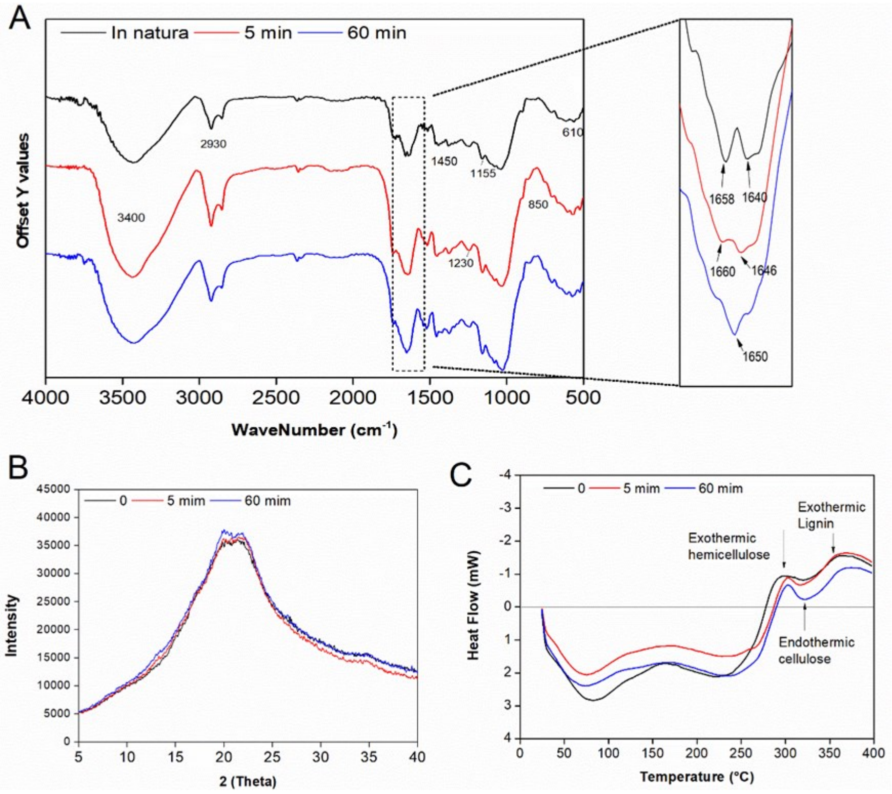


Figure 14. FTIR (A), DRX (B) and DSC (C) characterization of the raw and non-thermal plasma pretreated brewery by-product at 5 and 60 min.

Fourier transform infrared spectroscopy (FTIR) is commonly used to indicate compositional changes in cellulose, hemicellulose and lignin according to the assessment of the functional groups belonging to each component. Figure 14A shows the FTIR of the raw brewery by-product and after 5 and 60 min of NTP treatment.

The differences were identified mainly in the peaks 1658 and 1640 cm^{-1} (Fig. 14A). The peak at 1660 cm^{-1} in the raw sample corresponds to the double bonds between disubstituted alkene carbons (two groups other than H linked to C–C). After 5 min, the peak ranges from 1640 to 1646 and that of 1660 cm^{-1} remains unchanged. This small change caused by the plasma may represent double bonds between monosubstituted carbons, indicating only one group other than H connected the double bond between carbons (DARMAWAN *et al.*, 2016). After 60 min, the two initial peaks (1640 and 1660 cm^{-1}) are transformed into a single rise at

1650 cm^{-1}, this peak represents double bonds between cyclic alkene and carbonyl groups (PAPP *et al.*, 2005). Aromatic rings of oxidized lignin were described for peaks 1450, 1580 and 1600 cm^{-1} (MAZIERO *et al.*, 2012). However, low prominence peaks were observed in the brewer's spent grain. It is essential to highlight that at 3400 cm^{-1}, a slight variation was observed after pretreatment, which proves the preservation of functional groups in this range, such as amino groups (N–H) (FONTANA; PETERSON; CECHINEL, 2018). Small peaks also appear at approximately 2930 cm^{-1}, which may indicate the C–H bond's vibrations in aliphatic chains (single bond CH_2 and single bond CH_3) belonging to cellulose, lignin, or hemicellulose (FERRAZ *et al.*, 2015). Peaks close to 1240 cm^{-1} refer to C–O–C in the cellulose chain. Bands close to 1155 cm^{-1} are related to the asymmetric C–O–C deformation of cellulose and hemicellulose (MOTHÉ; DE MIRANDA, 2009). At 850 cm^{-1}, another band related to lignin was observed, which indicates the C–H bond for 1,3,5-trisubstituted benzene rings (DONOHOE *et al.*, 2008).

The crystallinity of lignocellulosic materials is considered one of the main factors influencing the efficiency of acid/enzymatic hydrolysis (ZHANG *et al.*, 2008). Figure 14B shows the DRX diffractogram for raw and pretreated brewer's spent grain. It is possible to observe a diffraction peak at 21.8º for all samples, representing the cellulose peaks (REDDY *et al.*, 2018). An interesting aspect is related to the extended profile of the peaks, representing amorphous regions since this material can be considered semi-crystalline (FERREIRA *et al.*, 2017). Peaks of intensity at the diffraction angles of 2θ at approximately 19.6◦ and 20.6º have been identified for the samples (5 and 60 min.) and are related to processing-induced crystallinity (CORRADINI *et al.*, 2005). In lignocellulosic materials such as pinewood, Mangium wood and Candlenut bark, the cellulose peaks were identified at 16.5º and 22.6º (DARMAWAN *et al.*, 2016).

Differential Scanning Calorimetry (DSC) was used to evaluate the thermal behavior of brewery-spent grain. Figure 14C shows the thermogram for raw and pretreated samples at 5 and 60 min. During the elevation of the temperature, the event around 100 ºC is related to moisture removal. In addition, two exothermic peaks (290–315 ºC and 360–370 ºC) and one endothermic peak (325–330 ºC) were observed, which distinguishes the interaction of the main components of cellulose, hemicellulose and lignin (YANG *et al.*, 2007). The peaks obtained refer to the components thermal decomposition and provide information about the interaction that occurs within them. Lignin molecules, for example, decompose at higher temperatures due to their interconnected packaged propane units in a complex amorphous matrix (PISHNAMAZI *et al.*, 2019). The difference between the peaks of approximately 81.6, 163.7, 294.3 and 361.3 ºC shows that the molecules intermolecular forces (cellulose, hemicellulose and lignin) exist and can be stronger or weaker depending on the modification that the material underwent after treatment.

Raman spectroscopy is a vibrational technique in which photons are measured after generating during the interaction between light and matter (LUPOI; GJERSING; DAVIS, 2015), providing information about the molecular composition, structure and intermolecular interactions in the sample. Figure 15 shows the RAMAN spectra for the raw and pretreatment brewery by-product.

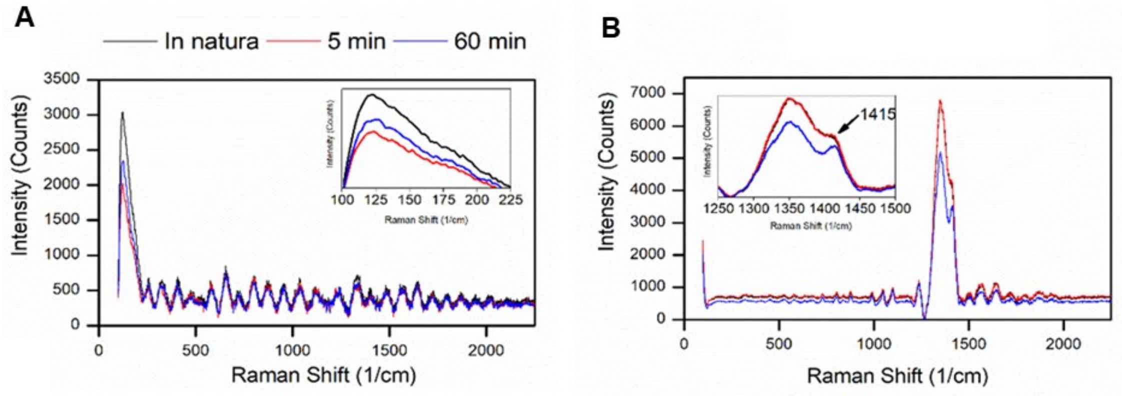


Figure 15. RAMAN spectroscopy for raw and non-thermal plasma pretreated brewery by-product at 5 and 60 min (A) and liquid sample (B).

Minor changes were observed in peaks around 120 to 130 cm^{-1} for the treated solid (Fig. 15A). The region of low frequency or low vibrational energy around 10 to 200 cm^{-1}, observed in this work, is known as terahertz. In this spectral band, the bands refer to the phonon modes of crystals' vibrations, intermolecular vibrational modes, such as the hydrogen bonds and Van der Waals interactions (MAIA *et al.*, 2020). Peaks between 200 and 2000 cm^{-1} were also detected but with lower intensities. In the liquid stream (Fig. 15B), the most intense peaks were between 1230 cm^{-1} to 1440 cm^{-1}. As with FTIR, many peaks stand out as characteristic of lignocellulosic components. For example, Raman spectra of guaiacila, siringila and hydroxyphenyl lignins showed peaks at 2940, 1455, 1331, 1156, 1037, 597, 531, 522, 503, 472, 447, 431, 417 and 369 cm^{-1} (AGARWAL *et al.*, 2011). In addition, the molecular structures of hemicellulose are similar to those of cellulose and, therefore, in the Raman spectra, the peaks overlap (ZHANG *et al.*, 2017b). Some glucose peaks were reported as 1275, 1331, 1347 and

1460 cm^{-1} (ZHANG *et al.*, 2017b) and xylose as 1244, 1339 and 1394 cm^{-1} (ZHANG *et al.*, 2017b). Other peaks in 1336, 1476 cm^{-1} were described as cellulose (BERTEAU; STENUTZ, 2004) and 1247 and 1378 cm^{-1} as xylan (AGARWAL; RALPH, 1997). Thus, the comparison of the Raman spectra and the data present in the literature made it possible to confirm the compounds' presence in the treatment liquor.

3.3.5 Mass balance

Figure 16 illustrates the mass balance of the lignocellulosic components of brewery by-product after 5 min (Fig. 16A) and after 60 min (Fig. 16B) under NTP treatment, calculated for 20 g of dry mass.

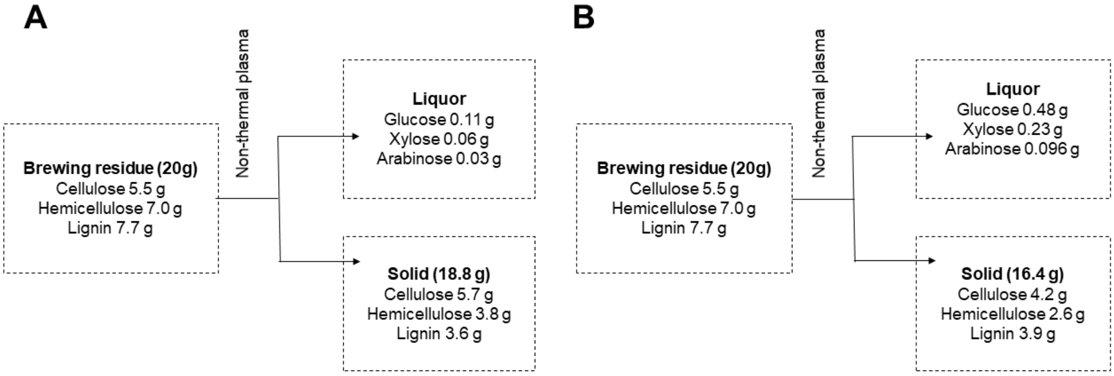


Figure 16. Mass balance of the brewery by-product pretreated after (A) 5 min and (B) 60 min under non-thermal plasma.

The yield of solids after 60 min indicated that more cellulose and hemicellulose were extracted. In addition, the missing hemicellulose may suggest that part of the hemicellulose is still in the form of oligosaccharides. The additional glucose content can be derived from the residual starch present in the brewery by-product, also observed by (RAVINDRAN *et al.*, 2019b). The mass balance confirmed that longer times lead to more significant losses of sugars. Therefore, this study shows that shorter pretreatment times are more interesting for the delignification of brewery by-product, preserving large amounts of the constituent sugars, which is very attractive at the industrial level.

3.4 Conclusions

The use of non-thermal plasma as pretreatment of a brewery by-product proved to be attractive in reducing the lignin content. Using air as a plasma gas yielded 50% delignification after 5 min of pretreatment. The different concentrations of solids showed that it is possible to use up to 12.5% without causing a loss in the percentage of delignification. Through physical-chemical analyzes, it was possible to conclude that the non-thermal plasma affected small amounts of the lignocellulosic components. This work showed that non-thermal plasma is an efficient alternative in the delignification of brewery by-product, opening possibilities for obtaining products with high added value.

Chapter 4: Enzymatic hydrolysis step to obtain XOS

Obtaining xylo-oligosaccharides (XOS) through enzymatic hydrolysis of pre-treated brewery by-product by non-thermal plasma

This chapter presents the results related to the study of enzyme kinetics, as well as the main influences of different concentrations of enzyme and substrate in obtaining xylo-oligosaccharides (XOS) from the brewery by-product. It also presents a study of the possibility of expanding the laboratory scale in obtaining XOS, as well as a perspective of enzyme reuse through the concentration of enzymes through membranes. This study was written in the form of an article that will be submitted to a journal of scientific relevance.

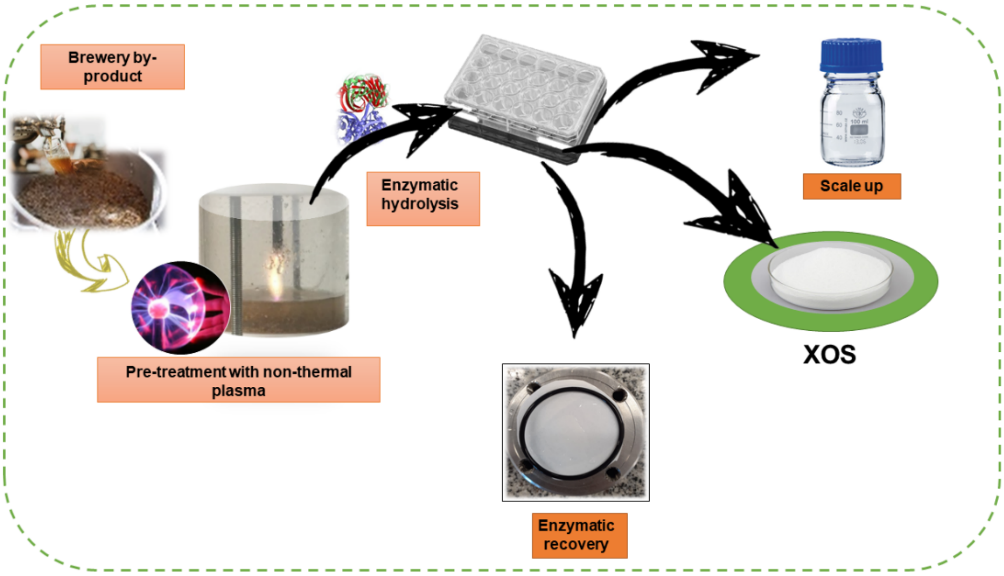


Figure 17. Graphical abstract of article.

Abstract

Xylo-oligosaccharides (XOS) are carbohydrates generated by xylose units, which can be obtained from lignocellulosic materials. In this work, brewery by-product delignified with non-thermal plasma was evaluated concerning the potential for the production of XOS. First, an exploratory kinetics was carried out, where the influence of different concentrations of commercial enzyme endo-1,4-β-Xylanase M3 (*Trichoderma longibrachiatum*) (0.1, 0.5, 1, 2, 4 and 8%) in relation to the mass of delignified brewery by-product (1%) was evaluated, under optimal enzyme conditions (50 °C and pH 6.0). As a result, it was found that 48 h and an enzyme concentration of 2% resulted in higher levels of XOS (750 mg XOS/g xylan). Afterwards, a study was carried out to evaluate the influence of solids concentration on XOS production, where 0.5, 1, 2 and 3% of solids were studied under optimized conditions of time and enzyme concentration, where it was found that 1% of residues was sufficient for the highest conversion to XOS. The enzymatic reuse of xylanase was explored in this work, showing the perspective of reuse through ultrafiltration membranes, because after 2 cycles of hydrolysis the activity of the enzyme remained the same. The laboratory scale up was studied, which proved the possibility of obtaining XOS on a scale of up to 25 times larger, making it attractive to obtain XOS from brewer's waste on larger scales.

Keywords: Xylooligosaccharides, laboratory scale-up, xylanase reuse, ultrafiltration.

4.1 Introduction

Environmental concerns related to the depletion of petroleum resources and public awareness have led to increased scientific interest in developing strategies in biorefineries, converting renewable raw materials into conventional or new chemicals (AXELSSON *et al.*, 2012). The conversion of lignocellulosic materials, consisting of cellulose, hemicellulose and lignin into biomaterials and biofuels is of great interest today. The conversion of these different lignocellulosic fractions into high value products is possible as long economic process technologies are developed (YANG *et al.*, 2011).

Xylan is at hemicellulosic polysaccharide, the second most abundant biopolymer in the plant kingdom. Obtaining xylo-oligosaccharides (XOS) from xylan recently attracted attention due to their functional properties (YANG *et al.*, 2011). Xylo-oligosaccharides (XOS) have excellent prebiotic properties and are one of the few nutraceuticals that can be produced from lignocellulosic biomass (ÁVILA *et al.*, 2020). As lignocellulosic biomass is cheap and abundant, producing XOS from agricultural waste offers an excellent opportunity for the food and pharmaceutical industries (SAMANTA *et al.*, 2015).

Obtaining XOS is usually carried out in two steps, a xylan fractionation step and an enzymatic hydrolysis step. Several methods have been developed for producing XOS, including chemical processes, autohydrolysis, direct enzymatic hydrolysis, and various pretreatments with enzymatic hydrolysis (AACHARY; PRAPULLA, 2009). New methods have been explored among the pretreatments, such as non-thermal plasma (NTP).

Non-thermal plasma can be defined as partial electrical discharges initiated at sufficient voltages. Partial electrical discharges cause the temperature of the electrons to be higher than the temperature of the surrounding gas molecules. Electrons interact with gas molecules to generate active radicals, reactive species, and ions, which have higher energy levels, increasing the rate of reactions (WU *et al.*, 2013). Some research studies have investigated the effects of plasma application on different lignocellulosic materials as a pretreatment method for various applications (PEREIRA *et al.*, 2021a).

After the pretreatment, the structure of the natural xylan branch requires the coordination of several hydrolases to decompose the pretreated biomass, such as the 1,4-β-d-xylanase (known as endoxylanases), which hydrolyze the 1,4-β-d-xylosidic bonds (BELKACEMI; HAMOUDI, 2003). The efficiency of enzymatic hydrolysis is closely related to the substrate structure, enzyme specificity, pH value, incubation time and temperature (AACHARY; PRAPULLA, 2009), in addition to the xylan source. When selecting enzyme media for XOS production, enzyme preparations should have lower exoxylanase activity to minimize xylose production in the XOS mixture (ÁVILA *et al.*, 2020).

In the present study, we used non-thermal plasma technology for pretreatment of brewer's residue and subsequent enzymatic hydrolysis to obtain xylo-oligosaccharides (XOS). Exploratory kinetics using different concentrations of enzyme in its optimal conditions of pH and temperature was carried out to select the best dosage. Later, there were performed studies varying the concentrations of solids in the reaction. Additionally, we tested the possibility of enzyme reuse and process scaling up.

4.2 Material and methods

4.2.1 Material

The spent grain of Vienna-type beer was obtained from a brewery in São José-SC, Brasil. The bagasse was first washed with tap water to remove residues from the brewing process, dried in an electric oven at 60 ºC for 24 h and stored for use in the following steps. Other materials used in this study are listed: sulfuric acid (Sigma-Aldrich), deionized water and the following standards xylose, glucose and arabinose from Sigma-Aldrich. A commercial endo-1,4-β-xilanase from *T. longibrachiatum* M3 (GH11 and 20 kDa) and the Birchwood standard were purchased from Megazyme.

4.2.2 Application of non-thermal plasma (NTP)

Brewery by-product was pretreated with non-thermal plasma, according to the conditions found by PEREIRA *et al.* (2021b) (12.5% solids, 700 rpm, 5 min.) and then washed with approximately 300 mL of distilled water, centrifuged at 1493.8 x g for 10 min, and oven dried at 100 °C for 12 h.

4.2.3 Enzymatic activity

The enzymatic activity of *T. longibrachiatum* endo-1,4-b-Xylanase was based on the Megazyme protocol, where 0.5 mL of buffered enzyme preparation pH 6.0 (pre-equilibrated at 40 °C) was added to 0.5 mL of pre-equilibrated substrate solution (1% w/v Birch Azo-Xylan) on a vortex mixer. The mixture was immediately taken to the water bath and incubated at 40 °C for 10 min. The reaction was terminated by adding 2.5 mL of ethanol (95% v/v) with vigorous stirring in a vortex mixer. The tubes were stored at room temperature for 5 min, shaken, centrifuged at 1,000 g (approx. 3,000 rpm for 10 min) and absorbance read in a spectrophotometer at 590 nm against water.

4.2.4 Enzymatic hydrolysis

Enzyme kinetics were performed in 24-well plates containing 1% residue delignified, sodium phosphate buffer (100 mM) pH 6.0 and an enzyme solution at different concentrations to fill the total volume of 2 mL. Different enzyme concentrations (0.5; 1, 2, 4 and 8%) were tested concerning the substrate. This kinetics was performed at 3, 6, 9, 12, 24, 48, 72 and 96 h at 50 °C and 180 rpm. Subsequently, it was worked with the condition 2% of enzyme varying the concentration of substrate in 1, 2 and 3%, in the times of 6, 12, 24 and 48 h. After hydrolysis, the samples were boiled at 100 °C for 5 minutes to deactivate the enzymes, then placed in an ice bath and, then centrifuged, so that the supernatants were frozen until further analysis.

4.2.5 Determination of lignocellulosic content

The lignocellulosic composition of the solid samples was determined according to National Renewable Energy Laboratory (NREL). Sequential extraction with ethanol and water in raw material was used to determine extractives content according to NREL/TP-510−42619. Cellulose, hemicellulose, lignin and ashes were quantified according to the protocols of (SLUITER *et al.*, 2016) and as described in previous study by the group (PEREIRA *et al.*, 2021b).The quantification of xylose, glucose and arabinose was carried out by High-Performance Liquid Chromatography (HPLC) (Knauer, Germany) equipped with a Knauer-RI detector and an Aminex HPX 87H column (300 mm x 7.8; Biorad, USA). The 40 µL injection was eluted with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.7 mL/min at 35 °C. Sugar concentrations were determined from standard glucose, xylose and arabinose curves.

4.2.6 Quantification of XOS

The quantification of xylose and XOS was performed by high- performance anion-exchange (HPLC-PAD) using the supernatants resulting from enzymatic hydrolysis, according to methodologies of (ÁVILA *et al.*, 2020). Chromatographic analysis was performed on a Dionex DX-500 (Sunnyvale, CA, USA) with a CarboPac PA1 column (4 mm x 250 mm), a CarboPac PA1 guard column (4 mm x 50 mm), and an electrochemical detector, adopting a linear gradient of A (100 mM NaOH) and B (100 mM NaOH; 300 mM NaOAc). The integrated peak areas were adjusted based on standards purchased from Megazyme® (Bray, County Wicklow, Ireland): xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6).

4.2.7 Scale up of the hydrolysis step

In this step of the study, the amount of substrates was increased by about 25 times, from 2 mL wells to 50 mL flasks to verify the influence of the laboratory scale increase in obtaining XOS from the brewer's residue. For this procedure, a reaction volume of 50 mL was used exposed under different concentrations of enzymes (1, 2, and 3%) for a fixed time of 48 hours, under the agitation of 150 rpm, temperature of 50 °C and pH 6.0.

4.2.8 Enzyme reuse

This step consisted of the reuse of xylanase (Fig. 18), in order to verify the possibility of reuse of the enzyme throughout the process. A commercial UP010 ultrafiltration membrane from the manufacturer Microdyn-Nadir, made of PES material – Polyethersulfone, with MWCO 10 (kDa) was used. The experimental procedure for conditioning began with the immersion of membranes in water for 30 minutes to remove preservatives and fill the pores with the solvent. This step consisted of the concentration and reuse of xylanase to verify the possibility of reuse of the enzyme throughout the process. A commercial UP010 ultrafiltration membrane from the manufacturer Microdyn-Nadir, made of PES material – Polyethersulfone, with MWCO 10 (kDa). The permeation tests to determine the membrane's permeability and permeate flux were carried out in a dead-end filtration cell made of steel stainless steel with a volume of 250 mL. The force for permeation was the pressure exerted by the injection of nitrogen at the top of the cell, regulated by a pressure gauge (0-20 bar) attached to the cylinder (99.0%, White Martins, Brazil) of gas. The cell was placed on a magnetic stirrer with a magnetic bar inside to carry out the stirring to simulate tangential filtration. Still in the upper part of the cell, the needle valve (Swagelok, model SS-31RS4) is installed to allow the depressurization of the system. The tests were performed in an air-conditioned environment at an average temperature of 22 °C. The permeate was collected by an on-off micrometer valve (Swagelok, model SS-42GS4) located at the bottom of the cell in a beaker with compatible graduation, the enzymatic activity in the sequence being measured, according to the methodology described in item 4.2.3. The experiments were carried out under an atmosphere of nitrogen, and the required pressure was applied by adjusting the Nitrogen cylinder pressure regulator.

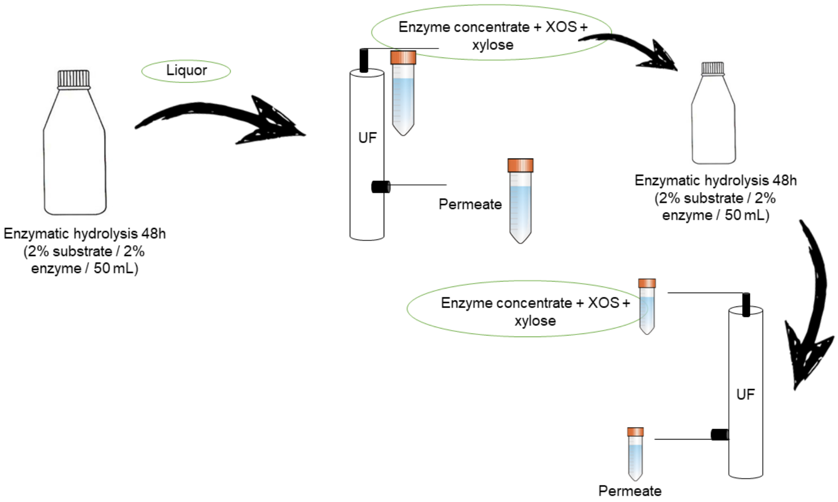


Figure 18. Schematic drawing of enzymatic reuse through ultrafiltration membranes.

4.2.9 Statistical analysis

Origin software was used in the statistical analysis. The results were evaluated by analysis of variance (ANOVA) and the significant differences were determined by the Tukey test at a probability level below 5% ($p < 0.05$).

4.3 Results and Discussion

4.3.1 Brewery by-product characteristics

The brewery residue pretreated by non-thermal plasma, according to the optimization reported in Chapter 3 of this work, was used. This pretreated residue was then washed with approximately 300 ml of pure water, dried in an oven at 100 °C for 12 hours, and taken to the enzymatic hydrolysis step. It was then verified that the time of 5 minutes of contact with the non-thermal plasma, in the molar ratio of 1:12.5, 200V, 700 rpm, and air as gaseous plasma were the best conditions reported by the research group (PEREIRA *et al.*, 2021b). These conditions were considered because, in addition to the great delignification (50%) found in just

5 minutes, it was the condition with the lowest losses in hemicellulose content (18.3%), which is very important when one wants to obtain xylo-oligosaccharides.

4.3.2 Enzymatic Hydrolysis

At this stage of the work, a 96-hour kinetics was first performed to evaluate the influence of different enzyme concentrations in obtaining XOS, using optimal enzyme pH and temperature (6.0 and 50 ºC, respectively), 1% of the substrate and 180 rpm of agitation. Figure 19 shows the yields of XOS (mg XOS/g xylan) obtained during the enzyme kinetics assay (96 h) for enzyme concentrations of 0.1, 0.5, 1, 2, 4 and 8%.

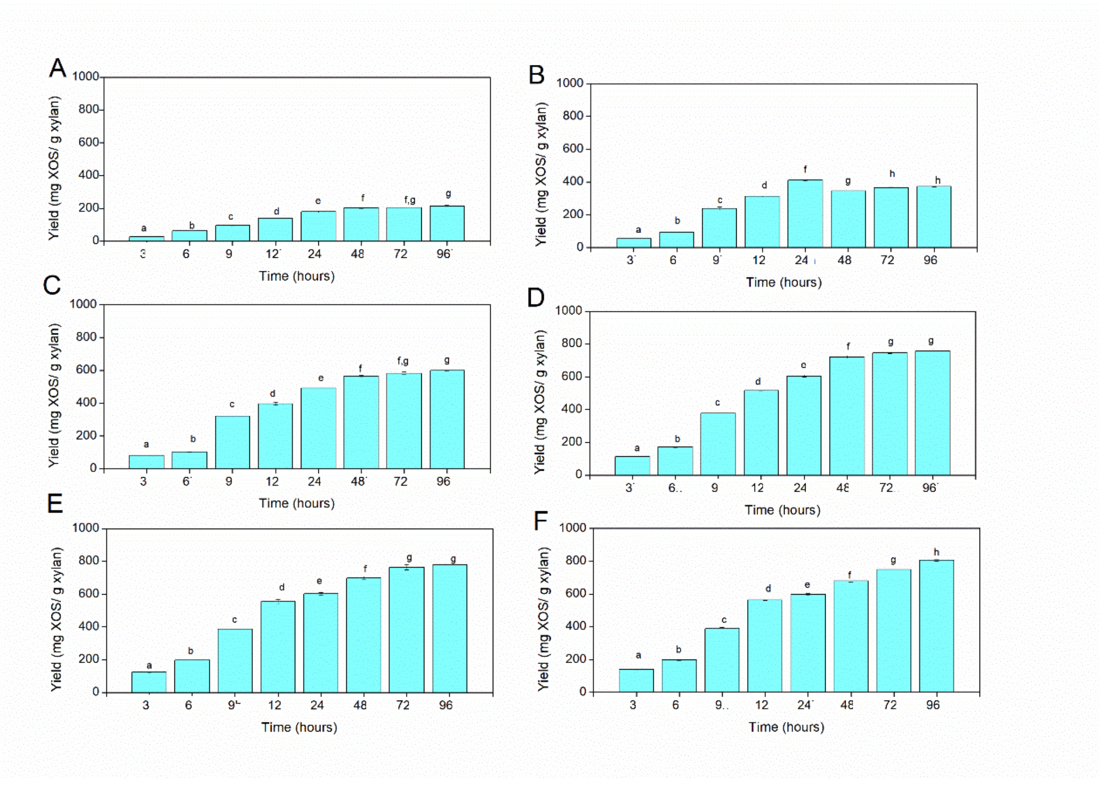


Figure 19. Yield of XOS at different enzyme concentrations (A) 0.1%; (B) 0.5%; (C) 1%; (D) 2%; (E) 4% and (F) 8%, with fixed conditions of substrate concentration of 1%, temperature 50°C and 180 rpm.

In Figure 19, one can observe the increase in yield over time for different enzyme concentrations. At an enzyme concentration of 0.1% (0.032 U/mL) (A), the higher concentration of 215 mg XOS/g xylan was obtained at 72 hours, whereas at 0.5% (0.16 U/mL) (B) 370 mg XOS/g xylan was also observed at 72 hours. At enzyme concentrations of 1% (0.32 U/mL) (C), 2% (0.64 U/mL) (D), 4% (1.28 U/mL) (E) and 8% (2.56 U/mL) (F) yields of 590, 750, 760 and 750 mg XOS/g xylan were obtained in 72 hours, with no significant difference at 72 and 96 hours in A, B, C, D and E. brewer residue, this work is promising, because for example (AMORIM; SILVÉRIO; RODRIGUES, 2019) obtained 444.3 mg XOS/g xylan and 12 hours of reaction with about 2 U/mL of enzyme, using the same enzyme.

Other residues are also used to obtain XOS, among them, we have corn husk that when hydrothermally treated and subsequent enzymatic hydrolized, using approximately 36 U of an endo-xylanase from *Trichoderma reesei*, for 48 hours had a total of 299 mg XOS /g xylan (HUANG *et al.*, 2017). Pineapple peel treated with alkaline extraction and enzymatic hydrolysis with *Trichoderma viridea* endo-xylanase (15 U) had a yield of 257 ± 0.4 mgXOS/g xylan in 48 hours (BANERJEE *et al.*, 2019), coconut husk using crude xylanase obtained about 242 mg XOS/g xylan, for 18 hours with 4.5% enzyme. The materials most studied in the literature, such as sugarcane bagasse, obtained up to 677 mg XOS/g xylan (JNAWALI *et al.*, 2018), corn husk with 750 mg XOS/g xylan (LIU *et al.*, 2018).

A relevant aspect to be considered is the amount of XOS of different degrees of polymerization (X2 – X6) that are obtained by the action of the enzyme. Thus, in Figure 20, we observe the concentrations of XOS (mg/L) with different degrees of polymerization (X2-X6) at different concentrations of enzymes.

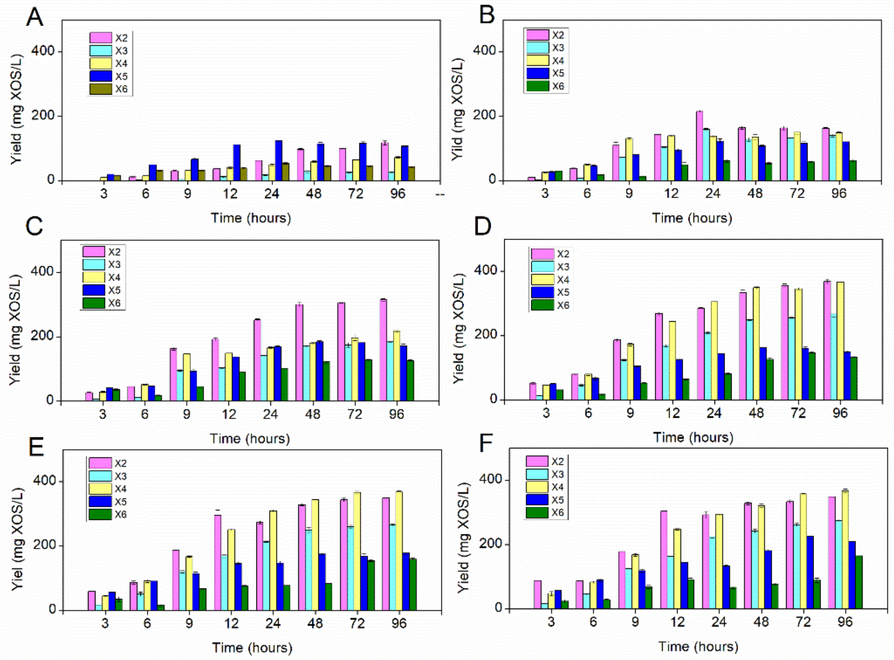


Figure 20. Yield of XOS at different enzyme concentrations (A) 0.1%; (B) 0.5%; (C) 1%; (D) 2%; (E) 4% and (F) 8%, with fixed conditions of substrate concentration of 1%, temperature 50°C and 180 rpm.

Figure 20 shows the different degrees of polymerization from X2 to X6 for different enzyme concentrations. According to the literature, XOS with a low degree of polymerization (2–4) are preferred for food-related applications (REILLY; WELLS, 2006), as they are more easily metabolized by probiotic bacteria and have increased prebiotic activity. The preference of beneficial microorganisms for low degree polymerization (DP) XOS was evaluated in in vitro fermentation, where for example wheat bran XOS with DP 3 were preferred followed by DP 2 (HO *et al.*, 2018), while XOS with DP 4, 7 and 14 of empty fruit bunches were consumed in this order (WANG *et al.*, 2010a).

Based on this first kinetics, it was verified that between 48 hours and 72 hours a significantly less evident difference was observed between these points than the others by the Tukey test, in the same way as between the times of 72 and 96 hours. Regarding the enzyme concentration, there was no significant difference between 2, 4 and 8% of enzyme, so based on these facts and on the choice for the lowest consumption of time and input (enzyme), it was decided to start with a new kinetics in the time of 48 hours and 2% enzyme, varying substrate concentrations.

4.3.3 Influence of different substrate concentrations

This work stage involved investigating the influence of different concentrations of pretreated solids in obtaining XOS. For this, the enzyme concentration of 2% (0.64 U/mL or 64 U/g) and a time of 48 hours were used, keeping the temperature at 50 ºC, pH (6.0) and agitation of 180 rpm. Figure 21 shows the graph with the different solid concentrations over time.

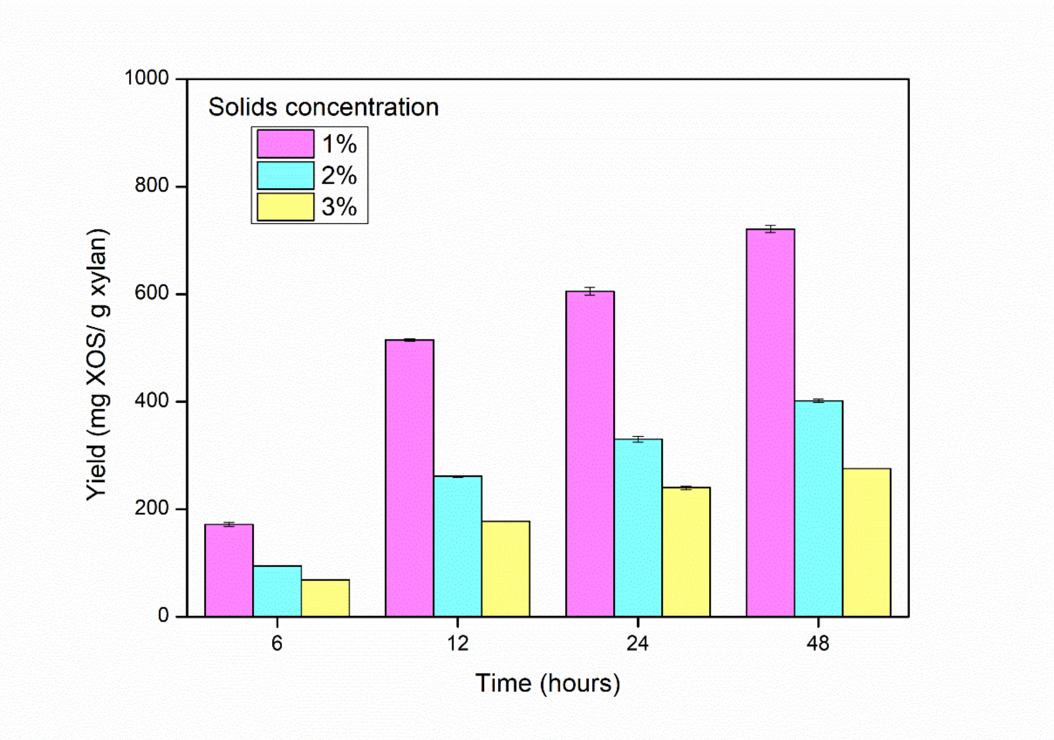


Figure 21. Yield in XOS for different substrate concentrations (1.0, 2.0 and 3.0) under fixed conditions of 50 °C, 180 rpm, pH 6.0 and enzyme concentration 2% (0.64 U/mL).

From Figure 21 one can observe product concentrations around 750, 400 and 275 mg XOS/g xylan in 48 hours for substrate contents of 1.0, 2.0 and 3.0 %, respectively. Some studies show that substrate concentration has a highly significant effect on XOS production, indicating that there is an optimal amount of substrate to be degraded to maintain a certain level of XOS production (BRIENZO; CARVALHO; MILAGRES, 2010). For many lignocellulosic substrates, working at higher concentrations of solids can lead to significant decreases in hydrolysis yield (CARVALHO *et al.*, 2013), a fact observed in this work where concentration of solids where the highest yield of XOS was obtained (750 mg XOS/g xylan) was at a concentration of 1% of substrate.

Studies on the production of arabino-xylooligosaccharides from spent brewery grain using *Trichoderma* species evaluated the influence of different solid concentrations (5, 10, 20 and 60 g/L) and as a result verified similar XOS yields for 5, 10 and 20 g/L (29.9 ± 1.9, 29.4 ± 1.1 and 27.9 ± 0.7 mg/g, respectively), substrate concentrations greater than 20 g/L had a negative impact on yield (AMORIM; SILVÉRIO; RODRIGUES, 2019). This could be due to possible inhibition by the substrate, in addition to mass transfer and aeration problems, possibly caused by an increase in the viscosity and density of the reaction mixture when higher substrate concentrations are present (AKPINAR *et al.*, 2007).

4.3.4 Scale up the XOS production

In this study, a 25-fold laboratory scale up step was performed to verify the reproducibility of enzymatic hydrolysis at two different substrate concentrations. Figure 22 shows the increase in laboratory-scale at 48 hours of reaction, using a fixed concentration of 64 U/g of enzyme and varying solid concentrations by 2.0 and 3.0%, with the letter A in the figure representing the magnification in the two different concentrations of solid.

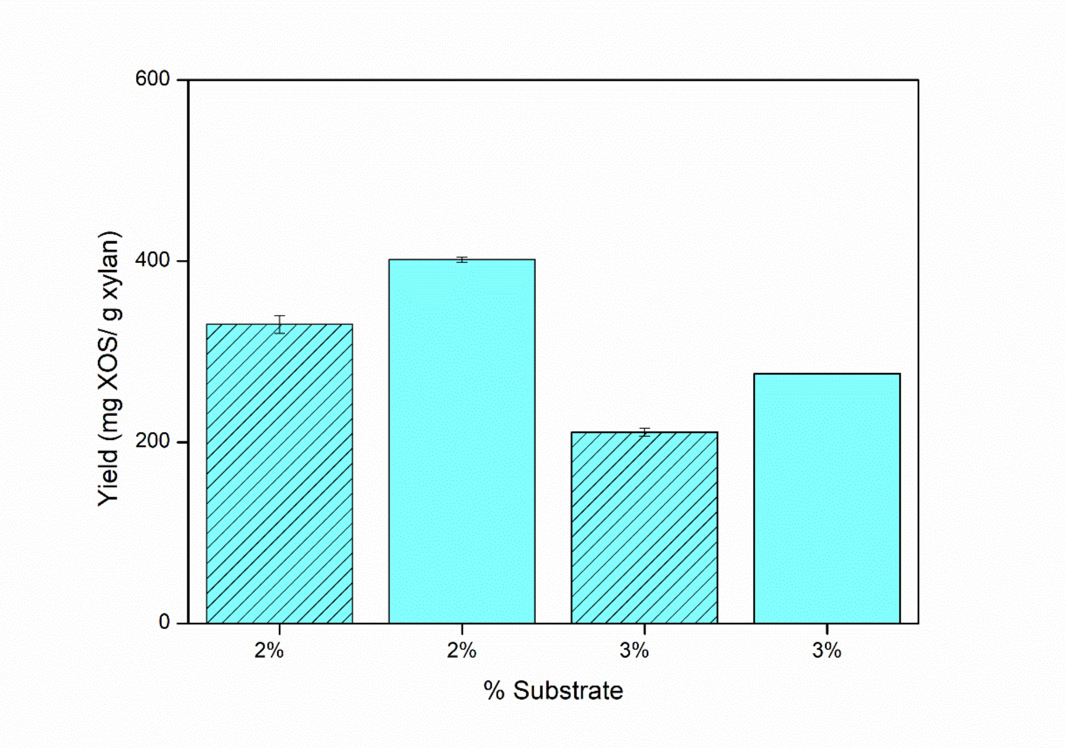


Figure 22. XOS yield in 48h time varying solids concentrations (2 and 3%) in scale differents under fixed conditions of 50 °C, 180 rpm, pH 6.0 and enzyme concentration 2% (64 U/g).

Through Figure 22, values of 330.0 ± 9.0 and 211.2 ± 5.0 mg XOS/g xylan are observed at solid concentrations of 2 and 3%, respectively on the enlarged laboratory scale. When these values are compared to the results of analyzes performed in 2 mL wells, 401.6 and 275.7 mg XOS/g xylan are obtained. Table 4 shows the XOS yields (mg XOS/g xylan) in the two situations (2 mL and 50 mL), in addition to the percentage of reproducibility of the two scales used.

In studies using sugarcane straw to obtain XOS, laboratory scale and pilot scale were tested, and from the bench process to the pilot scale, the solubilization of cellulose, arabiloxylan and lignin decreased about 2.7, 1.3 and 1.4 times, respectively. These differences observed in the solubilization of the components during the scale up are probably the result of different phenomena of agitation and heat transfer (BRENELLI *et al.*, 2020).

A view of XOS (mg XOS/L) at different degrees of polymerization (X2-X6) at the two different solids concentrations is shown in Figure 23.

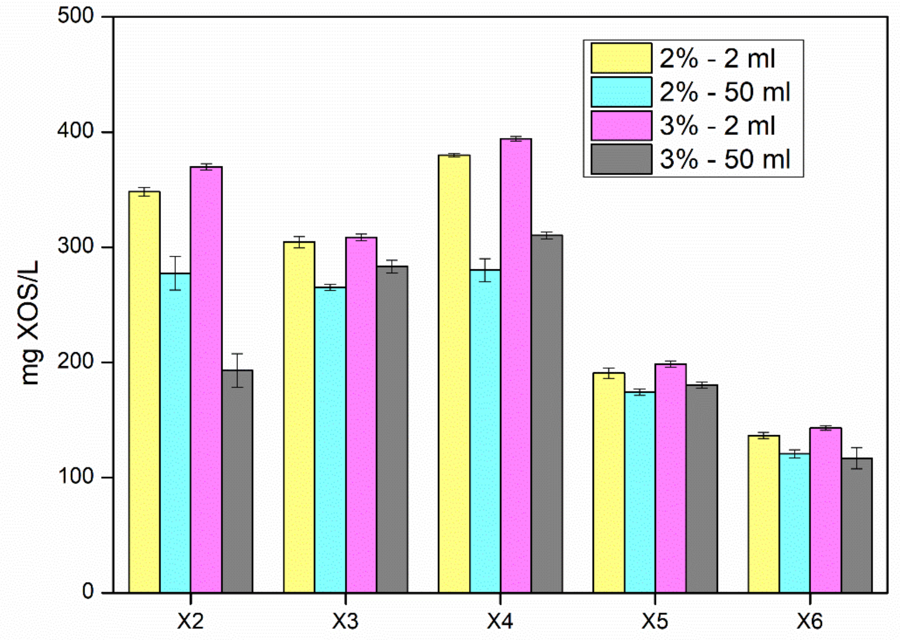


Figure 23. Profile of X2, X3, X4, X5 and X6 in mg XOS/L at 2 and 3% solid concentrations on two different scales (2 and 50 mL).

It can be seen that the contents of X2, X3, X4, X5 and X6 decrease on the enlarged laboratory scale for the two different solids concentrations. This can be explained by the different agitation and heat transfer phenomena that change with increasing laboratory scale. It is interesting to verify that under these conditions again the profile of X2 and X4 is superior in relation to X3, X5 and X6.

Subsequently, we increased the scale to 1000 mL under the conditions 1% residue, 2% enzyme, 50 ºC, 180 rpm and pH 6.0 (Figure 24).

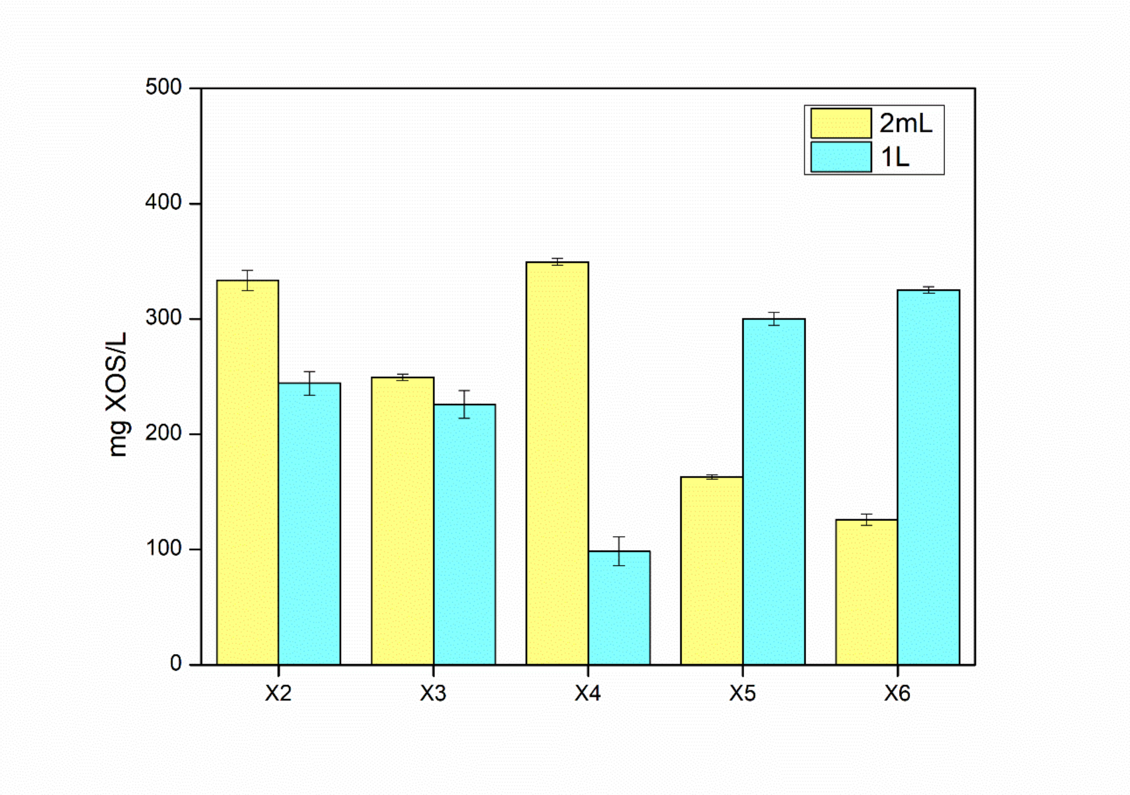


Figure 24. XOS profile of different degrees of polymerization X2, X3, X4, X5 and X6 (mg XOS/L) in the 2 mL and 1000 mL scales.

Through Figure 24, it can be seen that there was a change in the degrees of polymerization prevail in 2mL and 1000 mL. While in 2 mL the XOS of degrees of polymerization 2 and 4 predominate, in 1000 mL the profile changes to X5 and X6. These data reinforce that an expansion of laboratory scale consists of changes in the profiles of sugars released during the enzymatic hydrolysis reaction.

4.3.5 Solids concentration and enzyme reuse

After the experiment was scaled up, the enzyme reuse step was carried out, according to item 4.2.8. Through Table 4, it can be seen that the enzymatic activity remained 99.2% in concentrate 2 in relation to concentrate 1. An aspect of great relevance here is that no further tests were carried out for the amount of liquor obtained and used, but it is It is highly relevant to evaluate the reuse of commercial enzymes due to their high cost. Some works investigated the reuse of xylanases for other purposes, such as the biobleaching process of acetosolv pulp from sugarcane bagasse (LUÍS R. M. OLIVEIRA, 2006) however, there are still no studies in the literature that report the reuse of xylanases to obtain xylo-oligosaccharides.

Table 3. Enzymes recovered by the Nadir UP010 membrane and their respective enzymatic activities.

Sample	Enzymatic activity (mU/mL)	Conditions
Experiment 1	1.42 ± 0.2	Enzyme activity after 48 h of reaction
Concentrate 1	1.45 ± 0.1	Activity after first membrane concentration and dilution
Experiment 2	1.24 ± 0.1	Activity after 48 h of reaction
Concentrate 2	1.10 ± 0.4	Activity after second enzyme concentration

The results of this work are quite promising, as we work with a free xylanase, which makes recovery more difficult. Some studies already use ultrafiltration membranes to fractionate XOS, therefore, xylanase immobilization steps become attractive in order to increase the number of reuse cycles in obtaining XOS, since the immobilized enzymes are more easily recovered and reused.

4.4 Conclusions

The results presented in this work prove the possibility of using brewer's residue pretreated with non-thermal plasma to obtain XOS. By exploratory kinetics, a time of 48 hours and 2% of the enzyme were verified to obtain the amount of 750 mgXOS/g xylan, which is equivalent to a 43.9% yield. The concentration of solids that showed the highest yield in XOS was 1%. The possibility of xylanase reuse was also demonstrated, maintaining 99.2% of the activity after two reuses, a fact that is practically unprecedented when it comes to xylanases used to obtain XOS, in addition to the scale-up study, which proves the reproducibility of approximately 98 % when increased by 25 times the laboratory scale, thus valuing the brewery waste and the biorefinery chain.

Chapter 5 - Conclusions and future perspectives

In this work, we conclude through a critical review of the literature that non-thermal plasma can be an innovative method of pretreatment for lignocellulosic materials, which we proved through experimental data with brewery residue. Regarding the experimental results, during the exploration of plasma as a pretreatment, we found that only air as plasma gas was more efficient in the delignification of the brewing biomass, obtaining delignification of 50% in a time of 5 minutes. The evaluation of different solids concentrations showed that it is possible to use up to 12.5% without causing a loss in the delignification percentage. Through physical-chemical analyses, it was possible to conclude that the non-thermal plasma affected small amounts of lignocellulosic components. The mass balance confirmed that longer pretreatment times lead to more significant sugar losses. Regarding the enzymatic hydrolysis step, shows the results where, first, from the kinetics, results were obtained that allow considering the ideal time of 48 hours in this process. The evaluation of the influence of enzyme concentration shows that with only 2% enzyme and 1% substrate, levels of 750 mgXOS/gxylan, which is equivalent to a 43.9% yield can be obtained under optimal conditions of pH and temperature for the enzyme. In the exploration stage of substrate concentrations, the use of 1% of residue is confirmed to obtain the highest content of XOS. In this step, the possibility of increasing the scale of the process by up to 25 times was also proven, without significant losses in the final content of XOS, in addition to the feasibility of reuse of the enzyme, and in this work, it was verified, in an unprecedented way, 2 consecutive cycles reuse to obtain XOS. Thus, these results confirm the hypotheses that non-thermal plasma is a suitable pretreatment technique for the delignification of lignocellulosic materials such as brewery by-product, being possible to obtain XOS when this material is enzymatically treated by xylanases, as well as the expansion of the laboratory scale is possible, even with small losses in the final yield of XOS. Therefore, based on the results obtained, this work presents a way of valuing the biorefinery chain from the brewery by-product, where it is reused, as well as the valorization of the same from obtaining value-added products such as XOS.

For future works (Figure 25), it is indicated to evaluate the liquid part of the pretreatment with non-thermal plasma, evaluating its potential to obtain XOS from this fraction, as well as evaluating and reusing the solid after enzymatic hydrolysis, in addition to verifying the number of cycles of use that the enzyme used in this work is able to achieve. These future proposals are already being done in our lab and can be better visualized in the flowchart presented below.

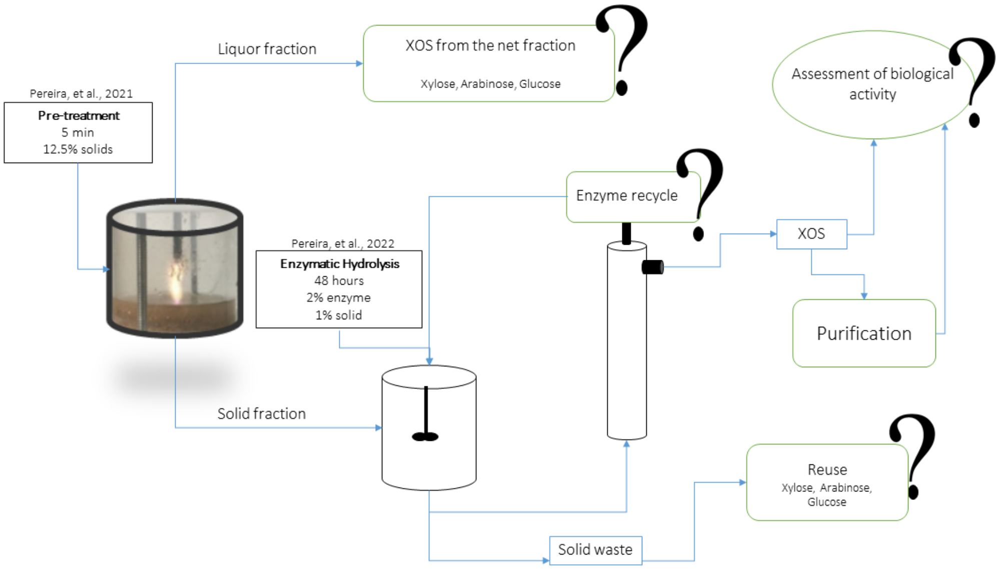


Figure 25. Scheme of future perspectives.

Another future proposal would be the immobilization of the commercial xylanase endo-1,4-β-xylanase from *T. longibrachiatum* M3, which would facilitate its recovery and consequently its reuse.

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