UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

DEVELOPMENT OF ENZYMATIC COCKTAILS FOR MODIFICATION OF XYLAN AND PRODUCTION OF DERIVATIVES

DANILO BUENO

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Orientador: Prof. Dr. Michel Brienzo
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Dedico este trabalho à minha família, em especial ao meu filho Fernando.

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“Study what interests you most in the most undisciplined, irreverent and original way possible.” (Richard Feynman)

ABSTRACT

The recalcitrance of lignocellulosic biomass has been indicated as a barrier to the technical-economical feasibility of the process of macromolecules extraction and production of high-added value products. Naturally, the lignocellulosic materials are resistents to enzymatic and microbian degradation, which occurs due to the organization of its components, where the cellulose is embedded in a matrix composed of hemicellulose and lignin. Therefore, extracting the hemicellulose from sugar cane bagasse without residual lignin is a great challenge. The xylan has several applications, depending on its structure: polymeric, oligomeric, monomeric, with or without branching/pending groups (xylooligosaccharides, xylose, hydrogels, packaging, artificial skin, etc.). An alternative approach to the chemistry pathway is to develop an enzymatic cocktail capable of: (1) complete hydrolysis of xylan, containing enzymes that cleave linkages of both the main chain and its pendant groups; (2) cleaving the bonds with the pendant groups, generating a free xylan chain of pendant groups; (3) cleaving different bonds in the main chain generating products with different degree of polymerization; (4) cleaving bonds between the xylan chain and the residual lignin. In this context, this project developed two enzymatic cocktails capable to lead xylan for complete hydrolysis removing the main chain and pendent groups with the use xylanase, β-xylosidase, and accessory enzymes. Through this study was possible to obtain XOS in higher content of concentration 19.6 g/L and with yield of 93%. Also, with this study it was possible to obtain a delignified xylan that presented interesting results as a substrate for xylanase activity (700 IU/mL) and for the growth of *Aspergillus versicolor*, which produced the xylanase enzyme with high activity (1350 IU/mL) in the presence of this substrate over 10 days. The bioplastics produced in this study using xylan modified via enzymatic hydrolysis (B2-lacase and B4-arabinofuranosidase) and chemical treatment (B3-H2O2) with the addition of starch, glycerol, and bis-acrylamide, presented as an interesting approach due to the observed improvements in the properties of the bioplastic when compared to original xylan (B1). The results indicated low opacity, moisture retention, solubility, and major tensile stress compared to the B1 bioplastic, possibly due to the removal of lignin and arabinose pendant groups, including the use of bis-acrylamide in the formulation of the bioplastics. Therefore, this project is a pioneer in production of value-added products derivates from xylan of sugarcane bagasse, such as two robust enzymatic cocktails to XOS production. In addition, obtaining bioplastics derived from delignified xylan and without arabinose, the xylan modification promotes an substrate for the growth of *A. versicolor* and also for determining the enzymatic activity of xylanase.

Key-words: enzymatic extracts, xylanases, auxiliary enzymes, heterologous expression, xylooligosaccharides, hydrogels.

RESUMO

A recalcitrância da biomassa lignocelulósica é uma barreira à viabilidade técnico-económica do processo de extracção de macromoléculas e produção de produtos de alto valor agregado. Naturalmente, os materiais lignocelulósicos são resistentes a degradação enzimática e microbiana, que ocorre devido a organização dos seus componentes, onde a celulose está embutida numa matriz composta de hemicelulose e lignina. Portanto, a extração da hemicelulose do bagaço de cana de açúcar sem lignina residual é um grande desafio. A xilana tem várias aplicações, dependendo da sua estrutura: polimérica, oligomérica, monomérica, com ou sem grupos ramificados/pendentes (xiloligossacarídeos, xilose, hidrogel, embalagens, pele artificial, etc.). Uma abordagem alternativa a via química é desenvolver um coquetel enzimático capaz de: (i) completa hidrólise da xilana, contendo enzimas que clivam as ligações tanto da cadeia principal como os seus grupos pendentes; (ii) clivagem das ligações com os grupos pendentes, gerando uma xilana livre de grupos pendentes; (iii) clivagem de diferentes ligações na cadeia principal gerando produtos com diferentes graus de polimerização; (iv) clivagem das ligações entre a cadeia xilana e a lignina residual. Neste contexto, este estudo desenvolveu dois coquetéis enzimáticos capazes de levar a xilana a uma completa hidrólise removendo a cadeia principal e grupos pendentes com o uso de xilanase, β-xilosidase e enzimas acessórias. Através deste estudo foi possível obter XOS com concentração de 19,6 g/L e rendimento de 93%. Além disso, foi possível obter uma xilanase deslignificada que apresentou resultados interessantes como substrato para a atividade de xilanase (700 UI/mL) e para o crescimento de *Aspergillus versicolor*, que produziu a xilanase com alta atividade (1350 UI/mL) na presença deste substrato durante 10 dias. Os bioplásticos produzidos neste estudo utilizando xilana modificada por hidrólise enzimática (B2-lacase e B4-arabinofuranosidase) e tratamento químico (B3-H_2O_2) com adição de amido, glicerol e bis-acrilamida, apresentaram-se como uma abordagem interessante devido as melhorias observadas nas propriedades do bioplástico quando comparado com a xilana original (B1). Os resultados indicaram baixa opacidade, retenção de humidade, solubilidade, e grande tensão de tração em comparação com o bioplástico B1, possivelmente devido à remoção de lignina e dos grupos pendentes de arabinose, incluindo a utilização de bis-acrilamida na formulação dos bioplásticos. Portanto, este estudo é pioneiro na produção de produtos de alto valor agregado derivados da xilana do bagaço da cana de açúcar, tais como dois robustos coquetéis enzimáticos para a produção de XOS. Além disso, a obtenção de bioplásticos derivados de xilana delignificada mostraram-se eficazes como substrato para o crescimento de *A. versicolor* e também para determinar a atividade enzimática da xilanase.

Palavras-chave: extractos enzimáticos, xilanases, enzimas auxiliares, expressão heteróloga, xilooligossacarídeos, hidrogéis.

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CHAPTER 1: INTRODUCTION

Lignocellulosic materials represent a source of feedstock forbiotechnological processes. Lignocellulosic materials are used mainly based on the generation of energy through burning and animal feed, however, new applications are emerging and providing better use of these materials. There are numerous studies with the objective of using the macromolecules of this biomass, cellulose, hemicellulose, and lignin. Hemicellulose, represented in sugarcane bagasse by xylan, is being used by the industry for the production of lactic acid by xylose fermentation. Xylan has been used for the development of bio-products for industrial aplication, in the manufacture of packaging, and recently, xylan has been used for the production of furfural by hydrolysis and dehydration of xylose, and production of xylitol by hydrogenation of xylose. Furthermore, producing a xylan with different characteristics such as with or without pending groups, with or without residual lignin, could differentiate the applications for example as a substrate for microorganisms, as a substrate for xylanase activity determination, and bioplastic production.

Among the bioproducts obtained from xylan, bioplastic stands out, a product with value-added due to its properties. It has the ability to respond to external stimuli, such as pH, temperature, ionic strength, among others (QIU and PARK, 2001). The hemicellulose-based bioplastic/hydrogel has gained prominence for its wide possible industrial use, as a biological skeleton for tissue engineering, as a biosensor, as a barrier to the regulation of biological adhesions, and it can also be used in the encapsulation of living cells and drug encapsulation (SUN et al. al., 2013). Among many biotechnological applications, hemicellulose can be used as an additive in papermaking. In its hydrolyzed form, it is applied as oligosaccharides for therapeutic and analytical purposes or in the form of monomers for the production of xylitol. The search for a healthy lifestyle through food care has driven the interest in functional foods, that is, those that help the body function properly. Among these components, xylooligosaccharides (XOS) stand out, to which several beneficial effects are attributed, such as preventing caries, reducing serum cholesterol levels, and stimulating the growth of bifidobacteria in the gastrointestinal tract. Their beneficial health effect is related to their physicochemical properties, as they are moderately sweet, stable over a wide range of pH and temperature, and confer organoleptic characteristics to foods.

Hemicellulose can be extracted/solubilized in an alkaline medium. On the other hand, this hemicellulose extracted in an alkaline medium presents some undesirable characteristics for the application. Residual lignin content and main chain pendant groups make it difficult, for example, to form a film. They also hinder the complete hydrolysis of hemicellulose, as the endoxylanase does not act on the xylose adjacent to the pending group. Thus, it would be important to develop enzymatic cocktails/extracts with different actions on the xylan chain, allowing to obtain different types of products or derivatives of interest for industrial and commercial applications. It would be desirable for the effective industrial application of hemicellulose in the production of value-added materials to remove residual lignin. This residual lignin could be performed with the application of ligninolytic enzymes.

The xylan extraction techniques and the identification of technological needs in its application, determined in this study, were based on the knowledge obtained by our research group at IPBEN-Unesp Rio Claro (BRIENZO et al., 2009; ALVES et al., 2021; MELATI et al., 2021). The strategies for purification and concentration of enzymes were based on studies performed specifically for endoxylanse from *Aspergillus versicolor,* developed at Unesp Rio Claro (CARMONA et al. 1997; FREITAS et al. 2021). The selection of microorganisms for this project was based on a literature survey of microorganisms that produce the enzymes of interest (xylanase, α-glucuronidase, α-arabinofuranosidase, acetyl-xylan esterase, and feruloyl esterase). This study collaborates with the development of value-added products, such as a high-quality xylan, desirable for industrial applications, and also derivatives, such as XOS with different degrees of polymerization and pending groups. Additionally, with this project, it will be possible to produce a bioplastic, a product in great demand by the industry that allows the encapsulation of slow-release drugs.

1.1 Problem statement

Among the products with value-added derived from hemicellulose are: xylooligosaccharides (XOS), xylitol, hydrogel, dressings and substitutes for human skin, films for biodegradable packaging, drug encapsulation, fat substitute in cheeses, additives in the production of paper, substitute for gelatine as in food gums, additives in the textile and cosmetics industry, production of new biomaterials such as glucagel, etc (EBRINGEROVÁ, 2006). Hemicellulose applications are constantly being studied, such as high-purity XOS (REDDY and KRISHNAN, 2017), XOS for fermentation (CHEN et al., 2017), adhesive mucus film (HANIF and ZAMAN, 2017), xylan-based thermoplastic – lactate copolymer (ZHANG et al., 2017) and also desirable for biomedical applications (CANTU-JUNGLES et al., 2017).

Considering that sugarcane bagasse is currently one of the main raw materials, it is important that the hemicellulose of this biomass, xylan, is used as a substrate for determining xylanase activity and in the cultivation of microorganisms for the production of xylanases. Many studies with sugarcane bagasse present hemicellulose with high extraction yield but with residual lignin content (BRIENZO et al., 2009; XU et al., 2006). Even hemicellulose with low yield still presents residual lignin (BANERJE et al., 2014; SUN et al., 2004). The use of ligninolytic enzymes can contribute to the removal of residual lignin, and thus produce a xylan with greater purity.

Some applications of hemicellulose are only interested in the composition of polysaccharides and their enzymatic digestibility. For these applications, a robust enzyme cocktail capable of hydrolyzing hemicellulose (pending groups and main chain) could meet this need. Thus, the polysaccharide composition and maximum digestibility of hemicellulose could be determined by an enzyme cocktail.

It is important to highlight that a robust enzymatic cocktail would lead to maximum digestibility of xylan as a result of the action of enzymes that hydrolyze both the main chain and the pendant groups of this macromolecule. Once a robust enzyme cocktail has been obtained, the application of this cocktail will lead to different raw materials. It will be possible, for example, to obtain xylose, debranched xylan, and branched or unbranched XOS. The debranching of xylan, removing the pendant groups, will produce a raw material of great industrial interest, as well as XOS, branched or not, and xylose, which can be used for conversion into industrial bioproducts.

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CHAPTER 2: OBJECTIVES

The objective of this study was produce enzymatic extracts with diversified action on the xylan chain, leading to different degrees of hydrolysis of the main chain and acting on pendant groups. The study evaluated the application of the enzymatic extracts in the production of XOS and the removal of pendant groups for bioplastic formation.

2.1 Specific objectives

- Develop an enzymatic extract capable of leading to complete hydrolysis of xylan;

- Develop an enzymatic extract capable of removing pendant groups from xylan;

- Develop enzymatic extract capable of removing residual lignin present in the xylan chain;

- Develop enzymatic extract for the production of XOS with different degrees of polymerization;

- Evaluate xylan with different modifications such as residual lignin content and degree of branching regarding its use as a substrate for enzymatic activity, microorganism growths and hydrogel production.

- Evaluate xylan with different modifications such as residual lignin content and without arabinose pendent groups to production of xylan-based bioplastics and evaluation of their properties.

2.3 Thesis format and organization

This study was organized into chapters, which were designed considering independent publications such as book chapters and papers. The important sections of the thesis were separated into chapters, for example, the bibliographic review was compiled in a book chapter. The results were organized into two papers, respecting the scientific journal structure, however, in the thesis the repetition was avoided indicating methods already used in the previous chapter.

The organization was like Chapter 1: General introduction and novelty statement, Chapter 2: Objectives, followed by a chapter that corresponds to a book chapter published in Springer publishing - Chapter 3: Bibliographic review - Enzymatic approach on structural modification of hemicellulose chain and production and purification of the main enzymes, and the following chapters (4 and 5) that correspond to scientific papers - Chapter 4: Enzymatic cocktail formulation for xylan hydrolysis into xylose and xylooligosaccharides, Chapter 5: Chemical and enzymatic modifications for the production of xylan as substrate and also xylan-based bioplastics, and lastly the final chapter which corresponds to conclusions of the study - Chapter 6: Conclusion.

2.4 Thesis rational research line

The Chapter 3 presented a literature review involving enzymatic modifications in the hemicellulose chain, both in the main chain and in the pendant groups, such as acetylations, esterifications, and oxidations. Additionally, the chapter presents data from the literature on the production and purification processes for xylanolytic enzymes.

The Chapter 4 was composed of an article presenting the formulation of two enzymatic cocktails that were developed in order to produce xylooligosaccharides and also to obtain a complete hydrolysis of the sugarcane bagasse xylan. The enzymatic cocktails were evaluated through experimental design, seeking to identify the enzymatic loads that would contribute to the highest yield and also the concentration of XOS and xylose.

The Chapter 5 consists of a paper on enzymatic modifications of xylan extracted via alkaline treatment from sugarcane bagasse. This xylan was subjected to two different treatments: (i) an enzymatic hydrolysis using laccase to remove residual lignin and (ii) a second alkaline treatment, both to remove residual lignin, and (iii) a third treatment envolving remotion of pendent groups of arabinose from xylan. Delignified xylans generated were compared with the original to determine which of the three would present potential as a substrate for the growth of *Aspergillus versicolor* and also to determine the xylanase activity. Then, all xylans generated were compared to the original xylan extracted from sugarcane bagasse and used to formulate bioplastics containing starch, glycerol, and bis-acrylamide. The four distinct bioplastics formed had their properties evaluated for opacity of the material, moisture, solubility, and mechanical strength.

CHAPTER 3: BIBLIOGRAPHIC REVIEW - ENZYMATIC APPROACH ON THE HEMICELLULOSE CHAIN STRUCTURAL MODIFICATION AND THE MAIN ENZYMES PRODUCTION AND PURIFICATION

ABSTRACT

Hemicellulose, one of the main components of lignocellulosic biomass, has great potential as a raw material to produce high value-added bioproducts such as hemicellulose-based films/bioplastic and biomaterials. Xylan is the major hemicellulose in grasses, and the second most abundant polysaccharide found in nature, corresponding to approximately one-third of the planet's renewable carbon source. Xylan bioplastic can respond to external stimuli, such as pH, temperature, ionic strength, among others. For this reason, these films/bioplastics are highlighted because of their wide industrial utility as an oxygen barrier in packaging, an adhesive or additive in plastics, food additive, and stabilizer, a hydrogel in medical applications such as artificial skin and drug encapsulation. However, to improve its applicability and versatility, the molecular structure of xylans can be modified by the action of accessory enzymes such as *α-glucuronidase* and *α-L-arabinofuranosidase*. These enzymes hydrolyze pending groups in the xylan chain, decreasing their solubility and helping in the formation of aggregates, also increasing its adsorption in relation to cellulosic substrates. Structural modification can be performed by esterification, stearation, acylation, and oxidation, resulting in xylan derivative with specific properties. This chapter showed possible changes in the xylan structure through the application of enzymes for properties improvement.

Keywords: xylan, xylanase, esterification, stereation, acylation, oxidation.

3.1 Introduction

Biomass is composed basically of cellulose, hemicellulose, and lignin. There are many examples of biomass types around the world, such as softwood, hardwood, grasses, food waste, among others. Worldwide, energy is produced mainly from non-renewable sources such as oil and coal, which makes lignocellulosic biomass an important renewable source for bioenergy production. Moreover, bioproducts from polymers (biopolymers) represent a feedstock source for value-added products. However, lignocellulosic biomass represents a raw material source little exploited in industrial-scale biotechnological processes. Therefore, researches about new applications are emerging and can provide a better use of these materials. In this context, it is known that besides the importance in biofuel production, biomass hemicelluloses can still be used to obtain various products of industrial interest, such as furfural, hydrophilic acid, among others (TAKKELLAPATI et al., 2018). In addition to sugarcane biomass, which is the main raw material for biofuels production in Brazil, several other biomasses such as corn/stalk cob, soybean/stems, rice straw, wheat straw, cotton, and coffee, have potential use in obtaining biofuels and high value-added products.

For each ton of sugarcane processed for juice extraction, 140 kg (dry basis) of bagasse are generated (FERNANDES et al., 2020). It is estimated that for the 2020/2021 sugarcane harvest in Brazil, there will be a total production of 665,105 million tons of sugarcane, which is about 3.5% higher than the previous harvest (2019/2020) (AGUIAR, 2020). In the southeast region of Brazil, around 420.71 million tons of sugarcane are produced. From this amount, it is estimated a production of 93.115 ton of bagasse, which has available around 23.278 ton of hemicellulose.

Hemicellulose is widely available considering sources such as agro and industrial wastes. Hemicellulose can be a raw material for several industrial products via biotechnological and chemical routes. Based on the importance of this polysaccharide and its products, this chapter aims to describe the enzymatic structural modifications of hemicellulose to obtain products with high value-added. The production and purification of the main enzymes (xylanases) responsible for chain modification were presented.

3.1.1 Hemicellulose and other components in biomass

The morphological characteristics attributed to cell wall components of the lignocellulosic biomass are responsible for the difficulty in using them as raw material. Naturally, lignocellulosic materials are resistant to enzymatic or microbial degradation, which occurs due to the organization of the components, where cellulose is embedded in a matrix composed of hemicellulose and lignin. Hemicellulose is chemically linked to lignin (ZAMORA et al., 2020), while its association with cellulose occurs through physical interactions in the fiber surface, presenting chains between cellulose fibers.

Cellulose is the main constituent of the plant cell wall. Cellulose is composed of a linear chain of 7000 to 15000 glucose residues joined by β-(1,4) glycosidic bonds, without branches. The β-type bond causes a rotation (180 degrees) alternating the glucose unit. This rotation provokes a linear chain structure (FENGEL and WEGENER, 1984). Lignin is another molecule that appears together with cellulose and lignin in the plant cell wall. The lignin collaborates with the resistance and recalcitrance of the lignocellulosic material.

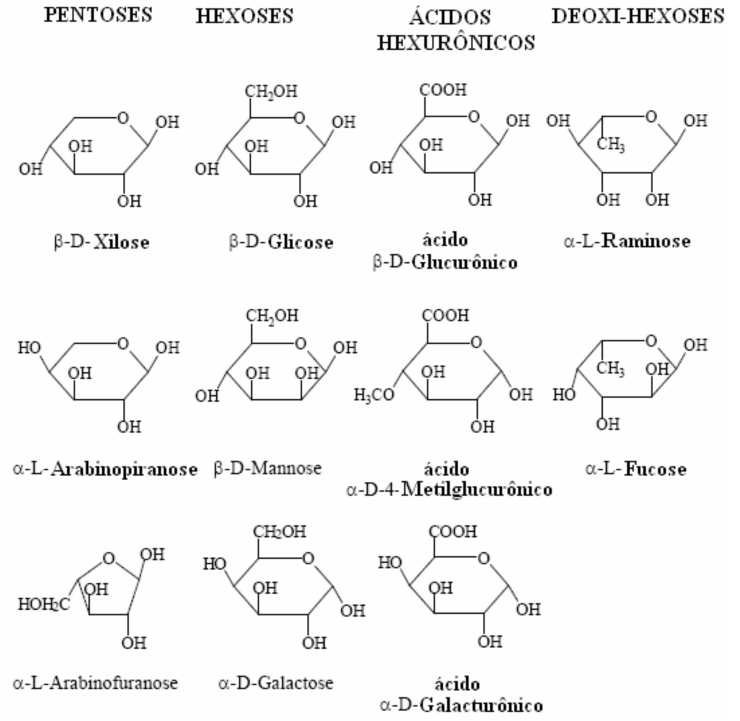
Lignin is a macromolecule composed of phenylpropane units: guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units. The coupling of the phenylpropane units does not occur in a regular and repetitive way, due to the biosynthesis mechanism, which is processed via the radical from the reaction of three precursor cinnamic alcohols, forming a three-dimensional and amorphous macromolecule (SCHMATZ et al., 2020). Its composition varies between different groups of plants: in gymnosperms, type G (guaiacyl) predominates, in angiosperms, GS (guaiacyl-syringyl) and in grasses such as sugarcane bagasse, GSH-type lignin (guaiacyl) stands out (HIGUCHI, 2006). Cellulose-hemicellulose-lignin complex presents recalcitrance that limits the enzymatic hydrolysis of cellulose and hemicellulose. Recalcitrance occurs since hemicellulose difficult access to cellulose, which presents rigidity and crystallinity, and lignin is the main recalcitrant component due to plant defense and prevention to phytopathogens and their hydrolytic enzymes (HOUFANI et al., 2020).

Hemicelluloses, in general, consist of 80 to 200 units of sugar residues, of which pentoses (D-xylose, L-arabinose, and L-rhamnose), hexoses (D-glucose, D-mannose, and D-galactose), and uronic acids (4-O-methyl-D-glucuronic and D-galacturonic acids) (XU et al., 2013) (FENGEL and WEGENER, 1984) (Figure 3.1). The hemicellulose chain can consist of a single monosaccharide (xylans) or two or more units (xyloglucans, 4-O-methyl-glucuronoxylan) (Figure 3.2). Despite presenting an amorphous structure, hemicellulose is capable of forming hydrogen bonds and tends to crystallize after removing the side chains. The substituting groups, arabinosil and acetyl, decrease the adsorption of hemicellulose to cellulose (KABEL et al., 2007). This change in the pending group interferes with the properties and is the key in hemicellulose biological modification to improve its application.

Over the years, assessments have been made regarding the property of hemicellulose in forming films when combined with small concentrations of chitosan (5 to 10%). It has been reported that the addition of these levels of chitosan to hemicellulose increases the crystallinity of the film due to interactions between xylan acid groups and chitosan amino groups (GABRIELI et al., 2000).

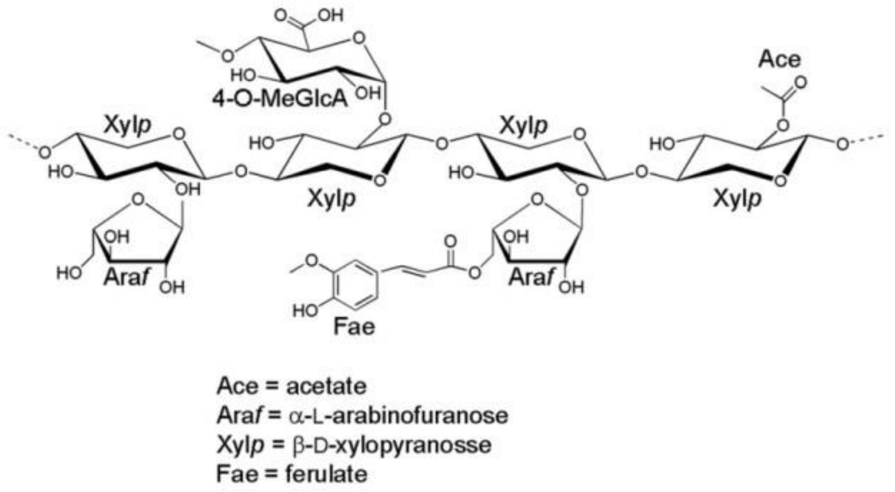
Xylan is the hemicellulose present in a greater proportion in the sugarcane bagasse, around 25% (ALVES et al., 2020; BRIENZO et al., 2016). In the industry, the use of xylan to obtain bioproducts has gained prominence through the manufacture of packaging, hydrogels, production of ethanol and lactic acid through the fermentation of xylan monomeric sugars by microorganisms, and additionally, furfural production by xylan hydrolysis and dehydration of xylose monomers, and finally, obtaining xylitol via xylose hydrogenation pathways (NAIDU et al., 2018).

Figure 3.1 - Structure of hemicellulose components: pentoses, hexoses, hexuronic acids and deoxi-hexoses.



Source: Xu et al. (2013).

Figure 3.2 - Xylan structure composed by a main chain of xylose and pendent groups of acetate, ferulate, α-L-arabinofuranose and α-L-xylopyranose.



Source: Correia et al. (2012).

Hemicellulose can be obtained through chemical, physical, physicochemical and biological pretreatments (MELATI et al., 2017) from different sources, such as sugarcane bagasse and straw, softwoods and hardwoods, soybean, algae, macaúba, babaçu, buriti, sunflower, spent coffee ground, elephant grass and sorghum (SHIMITZU et al., 2020; SANTOS et al., 2018; CHIYANZU et al., 2014), among others. Xylan is the main type of hemicellulose and is also the most abundant biopolymer found in these agroindustrial wastes (URTIGA et al., 2020).

3.1.2 Hemicellulose bioproducts

Hemicellulose is a raw material for several industrial products such as xylooligosaccharides (XOS), xylitol, hydrogel, dressings, and substitutes for human skin, films for biodegradable packaging, drug encapsulation, substitute for fat in cheeses, additives in paper production, substitute for gelatin in food gums, additives in the textile and cosmetics industry (FREITAS et al., 2019; EBRINGEROVÁ, 2006). Hemicellulose is constantly focusing on the study with the aim of improving its applications, such as XOS with high purity (FORSAN et al. 2021; FREITAS et al., 2019; REDDY and KRISHNAN, 2017), films (HANIF and ZAMAN, 2017), xylan-based thermoplastic (lactate copolymer) (ZHANG et al., 2017) and also for biomedical applications (CANTU-JUNGLES et al., 2017). Among the high value-added bioproducts obtained from hemicellulose, hydrogel, a product with high added value due to its properties, stands out. Hydrogels have the ability to respond to external stimuli, such as pH, temperature, ionic strength, etc (QIU and PARK, 2001). In the literature, hydrogels are defined as 3D polymer networks with a high degree of flexibility and high capacity of retaining water or biological fluids (ULLAH et al., 2015).

Since first reports in the literature about hemicellulose films (SMART and WHISTLER, 1949), hemicellulose-based hydrogels have gained prominence for their wide industrial utility, as a biological skeleton for tissue engineering, a biosensor, as barriers for the regulation of biological adhesions, and it can also be used in the encapsulation of living cells and drugs (SUN et al., 2013). In the literature, there are reports of many xylan-based hydrogels obtained by conversion of different biomasses, such as corn cob xylan, oat spelt xylan, rye xylan, aspen wood xylan, corn hull xylan, barley husk xylan, cotton stalk xylan, birchwood xylan, and beechwood xylan (URTIGA et al., 2020). These products have different physicochemical properties since hemicellulose from which the biofilms were obtained are a heterogeneous macromolecule.

Hemicellulose modification is desirable for film and biomaterial improvement. There are different approaches in the search for better conditions of resistance, elasticity, and hydrophobicity of the film, such as (I) additions of plasticizer agents such as xylitol and glycerol to the hemicellulose film (GRÖNDAHL et al., 2004); (II) mixtures of natural polymers to the film, such as starch (ZHANG and WHISTLER, 2004); (III) chemical modifications of hemicellulose film by introducing pendent groups to main chain (esterification, etherification, acetylation, among others).

Hemicellulose presents a wider potential in industrial applications. In this context, hemicellulases have a key factor in hemicellulose conversion to high added value products, both xylanases, and β-xylosidase that hydrolyze xylan main chain and accessories enzymes that hydrolyze xylan pendent groups, described in more details in the next topic.

3.1.3 Enzymatic modifications of xylan

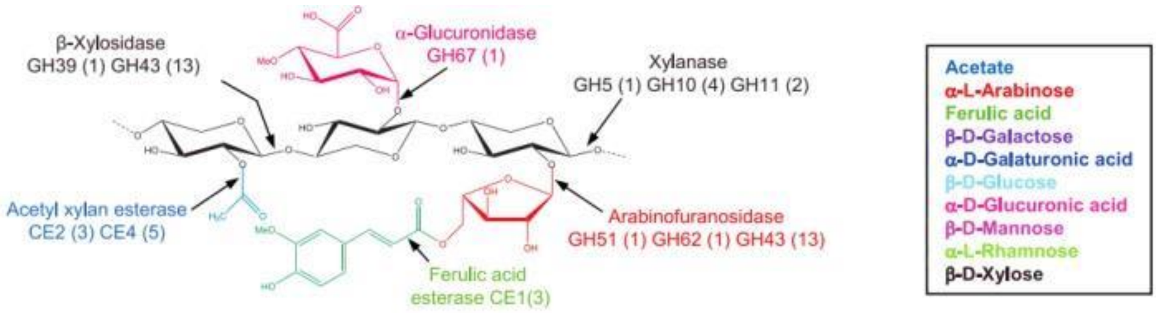
Xylan application can be empowered by modification of its chain, and as result improve its properties. Structural modifications of the xylan chain can be done by enzymatic action. Enzymes can hydrolyze xylan into oligosaccharides and monosaccharides, important carbohydrates in the biotechnology industry. Endo-β-1,4-D-xylanase (EC 3.2.1.8) acts on the xylan backbone and generates a low degree of polymerization chain, the xylooligosaccharides. XOS are substrates for β-xylosidase, which acts through the non-reducing terminal releasing D-xylose (FREITAS et al., 2019).

These enzymes are responsible for the modification of the main chain, and in addition there are enzymes that hydrolyze the pendant/side groups. The enzymes responsible for the hydrolysis of side groups are defined as accessory enzymes. Once the accessory enzymes release the side groups, the xylanases can efficiently act on the xylan backbone. Arabinose side groups are removed by the action of α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.131) removes glucuronic acids, and acetyl-xylan esterase (EC 3.1.1.72) removes the acetyl groups (JUHÁSZ et al., 2005). Ester bonds between hydroxycinnamic acids and sugars are breakdown by the action of feruloyl esterase (MALGAS et al., 2019; DEBOY et al., 2008) (Figure 3.3). The accessory enzyme action can produce a debranched xylan, which means no side groups are attached. This debranched xylan chain can be from interest of biomaterial production as well as for xylooligosaccharides that will not present side groups.

The α-glucuronidases are classified into two families, the GH 67 and GH 115. The GH 67 α-glucuronidases cleaves uronic acids linked to the non-reducing terminal of xylosil residues of small xylooligosaccharides. The GH 115 α-glucuronidases hydrolyzes 4-O-D-methylglucuronic acids linked to non-reducing terminals xylopropanosyl residues and those linked to internal xylosyl residues. Bacteria and fungi are known as producers of the α-glucuronidase families GH

67 (about 70 kDa) and GH 115 (about 90 kDa), respectively (YEOMAN et al., 2010; DIMAROGONA and TOPAKAS, 2016).

Figure 3.3 - Xylan hydrolysis with xylanases and accessories enzymes. Xylan main chain is formed by xylose monomers may contain pendent groups of acetate, α-L-arabinose, ferulic acid and others.



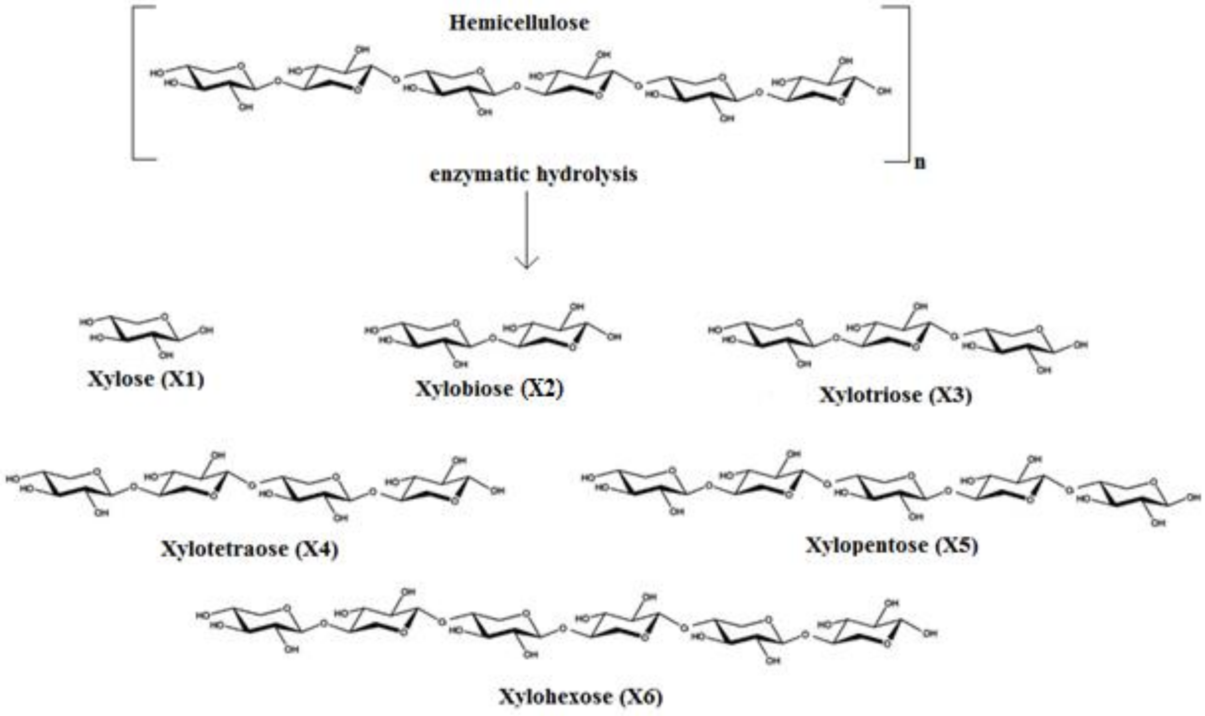
Source: Deboy et al. (2008).

Both bacteria and fungi produce the accessory enzyme acetyl-xylan esterases. Acetyl groups from heteroxylans are cleaved by the action of the acetyl-xylan esterases. The microorganisms producing these enzymes are capable of degrading acetylated xylans. Feruloyl esterases cleave bonds between esterified hydroxycinnamic acids in arabinoxylans. This enzyme is produced by both fungi and bacteria, with an important role in lignocellulosic material decay. Feruloyl esterase production can be induced according to the presence of certain substrates in microbial medium growth (KOMIYA et al., 2017; HETTIARACHCHI et al., 2019).

Debranched xylan, without side groups, is an important bioproduct in industrial applications such as xylooligosaccharides (XOS) (PASTEN et al., 2018), sugar oligomers made with xylose units that have great prebiotic potential and nutrient benefits, promoting the growth of probiotic bacteria in the intestinal tract (FREITAS et al., 2019). XOS are naturally present in foods, such as fruits and vegetables, however, they can be obtained by acid or enzymatic hydrolysis of xylan (FORSAN et al., 2021; BRIENZO et al., 2016) (Figure 3.4). In addition to prebiotic effects on humans, XOS contribute to human health in the reduction of cardiovascular symptoms, alleviation of dental decaying, activation of calcium absorption, and also on bowel function improvement (GROOTAERT et al., 2017).

The XOS produced can vary in degree of polymerization and amount of monomeric units (FREITAS et al., 2019), taking into account that there are different sources of xylan with different chemical compositions due to the varied resources of lignocellulosic biomass. Since xylan's accessory enzymes can hydrolyze their pending groups (DEBOY et al. 2008), it is known that it is possible to obtain branched XOS or not, therefore, accessory enzymes play a key role in the production of XOS.

Figure 3.4 – Hemicellulose and xylooligosaccharides (XOS) structure. XOS are produced through enzymatic hydrolysis what generates xylose (xylanase and β-xylosidase action), and xylotriose (X3), xylotetraose (X4), xylopentose (X5) and xylohexose (X6) (xylanase action)



Source: Author.

There are many reports in the literature of obtaining XOS via enzymatic hydrolysis with different xylanases from bacteria and fungi. A xylanase from *Bacillus halodurans* S7 (XynA) was applied to obtain XOS from wheat straw xylan (FARIAR et al., 2015). Using the bacteria *Clostridium thermocellum* ATCC 27405 xylanase (XynZ), it was possible to obtain XOS from sugarcane xylan (MANDELLI et al., 2014).

Besides bacteria, fungi also produce xylanases important for obtaining XOS. The fungus *Penicillium funiculosum* produces xylanase (XynD) that was used to obtain XOS from wheat xylan (LAFOND et al., 2011). Another example, *Thermomyces lanuginosus* IMI 84400, IMI 96213 and *Erwinia chrysanthemi* produce the XynA (GH11 family) and XynA (GH30 family) xylanases, which were successively used to obtain XOS from beechwood glucuronoxylan (PUCHART and BIELY, 2008).

Recently, literature reports about the development of enzyme mixtures, known as enzymatic cocktails, obtained from microorganisms, for XOS production, is increasing. In these studies, there is a debranching of xylan obtained through different biomasses by chemical pretreatment processes followed by enzymatic hydrolysis to obtain XOS (REDDY and KRISHNAN, 2015; COSTA et al., 2018). The composition and structure of XOS depend on the biomass used as raw material and also on the production processes. Obtaining XOS by the action of accessory enzymes, which hydrolyze the pending groups of xylan, is an attractive process due to the non-production of byproducts, since the action of such enzymes is specific for each pending group, as shown previously (COSTA et al., 2018).

Auxiliary enzymes are important in obtaining several products with high added value. The *α-L-arabinofuranosidase*, for example, is an accessory enzyme that hydrolysis the pendent groups α-L-arabinose from xylan structure, being produced by both fungi and bacteria groups (PORIA et al., 2020). This enzyme has wide industrial applications, such as increase digestibility in animal feed industries (COZANNET et al., 2017), enhance bread quality and texture (ZHOU et al., 2019) in the food industry, increase reducing sugar content (CHADHA et al., 2017) in biofuel industries, increase pulp delignification (NUMAN and BHOSLE, 2006).

The feruloyl esterase is another accessory enzyme, which hydrolysis ferulic acid pendent groups from xylan. This enzyme, for example, has a great potential in several industrial areas, such as chemical, animal feed, pulp and paper, food and agriculture, and also pharmaceutical industries due to the production of ferulic acid. In pulp and paper industries, feruloyl esterase has a key function by removing substitutions and linkages between polymers during pulping (FAZARY and JU, 2008).

The α-glucuronidase is an accessory enzyme that hydrolysis xylan glucuronic acid pendent groups. This enzyme has been used with a mix of enzymes (glucooligosaccharide oxidase) to produce an important acid for industrial application, called 4- O-methyl-D-glucaric acid, a dicarboxylic acid, that offers several applications in detergent builder and biopolymer fields (VUON and MASTER, 2020).

Acetyl-xylan esterase is responsible for the deacetylation of xylan, that is, the removal of pending groups of acetic acid. Recently, this enzyme was applied to aspen xylan, and this application contributed to the reduction of recalcitrance and increased saccharification of this biomass (WANG et al., 2020), which for the fuel production sector are key factors in the process of obtaining biofuel.

Xylanases and accessory enzymes are produced and commercialized for different industrial applications. The mainly commercialized products are for the pulp and paper, and food industries (CHAKDAR et al., 2016; GOSWAMI and PATHAK, 2013; WALIA et al., 2017). These industrial products, producing company, and industrial application are summarized in

Table 1.1.

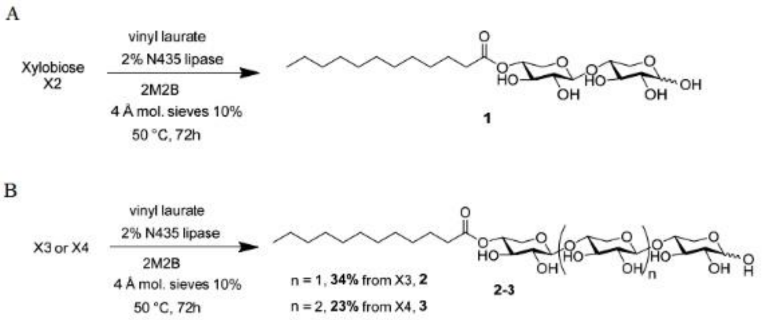
Table 3.1 - Commercial xylanases based products. Here, the enzymes were classified according to product name, company and country and also by industrial application, such as for food, bakery and pulp bleaching.

Product name	**Company**	**Industrial application**
Albazyme-10[a]	Ciba Giegy, Swiss	Food
Bacterial Xylanase XBK BX9	Leveking, China	Bakery
Belfeed B1100	Agrimex, Belgium	Food additives
Cartazyme	Sandoz,Charlotte,N.C. e Basel,Swiss	Pulp bleaching
Ecopulp	Alko Rajamaki, Finlland	Pulp bleaching
Ecozyme	Thomas Swan, UK	Pulp bleaching
Luminase	Verenium, USA	Pulp bleaching
Nutri Xylanase Enzyme	Ultra Biologic Inc., USA	Food additives
Pulpzyme (HA, HB, HC)	Novo Nordisk, Denmark	Pulp bleaching
Sanzyme X	Sankyo, Japan	Food
VAI Xylanase	Voest Alpine, Austria	Pulp bleaching

Source: Author.

Different from hydrolysis by xylanases and accessory enzymes, several other enzymatic modifications can be applied to the xylan chain, such as xylan esterification. Fatty esters of XOS were produced from xylobiosis (X2), xylotriose (X3), and xilotetraose (X4) using a commercial lipase enzyme (Novozymes) as a catalyst in the presence of vinyl laurate (Gérard et al., 2020). It was obtained monoesters with no acyl chain at the OH-4 non-reducing terminal of xylose units (Figure 3.5).

Figure 3.5 - Fatty ester of xylooligosaccharides. A: synthesis of 4’-O-laurylxylobiose 1 from xylobiose (X2). B: synthesis of 4’’-O-laurylxylotriose 2 and 4’’’-O-laurylxylotetraose 3 from xylotriose (X3) and Xylotetraose (X4), successively.



Source: Gérard et al. (2020).

The commercial lipase Novozym 435 (*Candida antarctica*) (Novo Nordisk A/S) was applied as a catalyst in the formation of xylose caproate ester (ABDULMALEK et al. 2016). The reaction was performed by xylose condensation, an aldopentose, and caproic acid in an organic solvent, showing high capacity and stability in esterification reactions. The study optimized the condensation of xylose and caproic acid in organic solvents (DMSO and acetone 1:10 (v/v)) using lipase as a catalyst in an incubation time of 24 h, at 60 °C and pH 10-11.

An increase in the superficial hydrophobicity of the films was observed with enzymatic modifications of xylan (STEPAN et al., 2013). A combination of different commercial lipases (L1754 from *C. rugosa*, 86012 from *R. oryzae*, L9031 from *Mucor miehei*, and L8906 from *M. javanicus*) (Sigma – Aldrich) with cutinases (ARAÚJO et al., 2007) was used to promote acetylation with vinyl acetate and stearation with vinyl stearate in rye arabinoxylan films. The hydrophobicity of the surface increased with the acylation, what is desired for certain application such as resistance to humidity.

Xylan chain (grafted) was oxidized through enzymatic modifications (PEI et al., 2015). Oxidation of the xylan chain was promoted using phenols such as 4-coumaric acid (PCA), ferulic acid (FA), syringaldehyde (SD), and vanillin (VA) in a reaction catalyzed by a commercial laccase (Novozymes). The reaction improved the molecular weight of xylan through these structural modifications using laccase, which led to better applicability of xylan when used as a film barrier. The film presented higher strength using xylan derivative and antibacterial effect to *Escherichia coli*, *Corynebacterium glutamicum*, and *Bacillus subtilis*.

In addition to xylans enzymatic modifications, esterification can be applied to polysaccharides in general, such as lignocellulose. Through esterifications, lignocellulose can be converted into high added-value chemicals such as fatty acid esters (SIEBENHALLER et al., 2018). In this study, lipase B from *Candida antarctica* (Sigma-Aldrich) was used as a catalyst for the deep eutectic solvent (DES) reaction system for the sugar esterification with fatty acids. Through this approach, this study synthesized sugar esters based only on lignocellulosic biomass as raw material.

Several studies in the literature evaluate the use of lignocellulosic biomass as raw material for obtaining sugar esters via enzymatic esterification. A study similar was prepared by Siebenhaller et al. (2017), in which it was possible to obtain glycolipids from glucose and xylose, previously generated by the acid pretreatment of lignocellulosic biomass. The glucose and xylose fractions were used to generate deep eutetic solvent (DES) in a reaction catalyzed by a lipase B (from *Candida antarctica*) (Sigma-Aldrich). Glycolipids are considered sustainable surfactants with great industrial potential.

3.1.4 Xylanases production from fungi and bacteria

Xylanolitic enzyme production is induced by xylan presence in the growth medium, which most of the time it is the best inducer (BUENO et al., 2020; CALORE et al., 2020; FERNÁNDEZ-ESPINAR et al., 1994). However, some studies demonstrated the inducing effect of fragments from xylan hydrolysis (BRIENZO et al., 2012; ARO et al., 2005). Xylanase repression was demonstrated by the presence of readily assimilable sugars such as glucose, lactose, and xylose (GASPAR et al., 1997). Lignocellulosic materials (sugarcane bagasse, wood sawdust, etc) can be used to obtain xylanolitic enzymes, with low substrate cost in submerged or solid culture (NANJUNDASWAMI and OKEKE, 2020).

Several microbial species produce specific xylanases when growing in a medium containing xylan, with little or no cellulose production. However, cellulases are produced together with xylanases when microorganisms growing in a culture medium containing cellulose or cellulose-based material. One strategy used to obtain the cellulosic-free xylanolytic complex is the cultivation of microorganisms in a culture medium containing xylan not contaminated with cellulose (BAILEY et al., 1992).

Xylanolitic microorganisms are described in marine and terrestrial environments, including in extreme conditions such as hot springs, Antarctic environments, and saline lakes. Comparing bacterial and fungal xylanases, it is noted that bacterial xylanases belong to the GH10 family, whereas fungal xylanases belong to the GH11 family. In addition to the classification, the biochemical properties of endoxylanases produced from bacteria and fungi can vary. Among the main biochemical properties are molecule size (8.5 to 85 kDa) and isoelectric point (4.0 to 10.3 pI) (CHAKDAR et al., 2016). The optimal pH and temperature of the enzymes and their respective molecular sizes are presented in Table 3.2, for some microorganisms xylanase producers (CHAKDAR et al., 2016; GOSWAMI and PATHAK, 2013; WALIA et al., 2017).

Xylose is the most abundant pentose in nature (LEE et al., 2015), and it represents a suitable carbon source in microbial fermentation, is obtained by breaking the main xylan chain by the action of xylanases, as previously demonstrated. Moreover, xylose is an important source to obtain a great variety of high-added products of importance to different industrial sectors (Table 3.3), such as the pharmaceutical industry.

Table 3.2 - Microbial xylanases microorganism producers and properties. The enzymes were classified according to microorganism of origin, optimal performance conditions such as pH, temperature (°C) and molecular weight (kDa).

Microorganism	**Conditions**		**Molecular Weight (kDa)**	**Reference**
	pH	**Temperature (°C)**		
Bacteria				
Bacillus pumilus SSP 35	6	50	20	Subramaniyan (2012)
Bacillus halodurans	9	78	40	Kumar and Satyanarayana (2013)
Cellulosimicrobium cellulans CKMX1	8	60	58	Walia et al. (2014)
Paenibacillus macerans IIPSP3	4.5	60		Dheeran et al. (2012)
Paenibacillus sp. NF1	6	60	37	Zheng et al. (2014)
Fungi				
Aspergillus niger ANL-301 9	5.5	45	13.5-14	Okafor et al. (2010)
Geotrichum candidum	4	50	60-67	Radionova et al. (2000)
Trichoderma reesei	5 a 5.4, 4 to 4.5	45.4	20, 19	Tenkanen et al. (1992)
Penicillium capsulatum	5 a 5.5, 4 to 4.5	48	22	Ryan et al. (2003)
Penicillium sp. CGMCC 1669	3.8	40	21	Liu et al. (2010)

Source: Author.

Table 3.3 - High-added products obtained from xylose conversion and their applications such as biofuels, food industry, dispersant, solvent and polymer precursor.

Product	**Industrial application**	**Reference**
Ethanol	Biofuel	Matsushika et al. (2009)
Lactic acid	Food	Turner et al. (2015)
Xylonic acid	Dispersant	Raposo et al. (2017)
Succinic acid	Drug	Salvachuá et al. (2016)
Xylitol	Food	Rao et al. (2016)
Furfural	Solvent	Peleteiro et al. (2015)
Levulinic acid	Polymer precursor	Wang et al. (2018)

Source: Author.

3.1.5 Improvement of xylanase production and purification

Xylanases can be produced by submerged fermentation (SmF) techniques or via solid-state fermentation (SSF). The fermentation process used will depend on the type of microorganism. Since fungi can grow at relatively low water activities, SSF conditions are more suitable for them than for bacteria Submerged fermentation is well-known for the production of xylanase and the control of the fermentation conditions is easier in this method. However, enzyme productivity is usually higher in SSF (MOTTA et al., 2013).

To obtain a satisfying xylanase production, the choice of substrate is of great importance. Depending on the substrate, the quantity and quality of the fermentation product can vary. Purified xylan (commercial product) is an excellent substrate because they increase xylanase yield by selective inducing xylanase without inducing cellulase production. The disadvantage is that this substrate has a very high cost, therefore, for the process to be economically viable, alternative substrates from agroindustrial waste such as sugarcane bagasse, wheat bran, corn cob, rice bran, etc., have been used (CHAKDAR et al., 2016; BHARDWAJ et al., 2019b).

In filamentous fungi, substrates containing xylan induce the expression of the xylanolytic system. When xylanases are produced by the fungi, xylooligosaccharides are release and they induce the expression of other xylanolytic enzymes. The effect of oligosaccharide degree of polymerization on the induction of xylan-degrading enzymes by *Fusarium oxysporum* f. sp. *lycopersici* was evaluated. Results showed that compared to other xylanases production inducers such as sophorose, lactose, and cellooligosaccharides, the xylooligosaccharides are the best inducers of endoxylanase and β-xylosidases. A correlation was found between the degree of polymerization and enzyme induction efficiency since xylotetraose was the best substrate to induce endoxylanase production and xylobiose was the best inducer of cell-bound β-xylosidase (NAJJARZADEH et al., 2020).

Different strategies can be employed to improve the yield production of the desired enzyme. The process can be optimized by the nutrient concentration in media, regulation of physical growth parameters (temperature, pH, agitation, incubation period), and use of biotechnology to improve producing-xylanase strains (SHARMA, 2017). A common approach for the selection of these important parameters is the one factor at a time (OFAT), in which just one parameter is variable and the others are kept constant (BHARDWAJ et al., 2019b). Moreover, a response surface methodology (RSM) is used to optimize and understand parameter interaction.

The effect of substrate composition and temperature parameters were evaluated to improve xylanase production under solid-state fermentation by *Aspergillus fumigatus*. Using the OFAT approach, the highest xylanase activity was achieved using a combined substrate of sugarcane bagasse and wheat bran (1:1 w/w) at 45 º C (GOMES et al., 2016). An improvement of 3.2-fold was obtained after optimizing conditions for *Fusarium sp*. xylanase production. Different substrate and concentrations, the addition of carbon and nitrogen sources on the medium and physical parameters were evaluated by the OFAT approach and it was possible to reach a high xylanase yield of 4200 U/mL (RAMANJANEYULU et al., 2017).

Media optimization using OFAT enhanced xylanase from *Aspergillus oryzae* extracellular production by 5.7 times. Different media were tested by altering the concentration of the inorganic components. The non-optimized media presented xylanase production of 225 IU/mL, whereas after optimization xylanase production was up to 1290 IU/mL (BHARDWAJ et al., 2018). Evaluation of several nutritional and physical parameters for xylanase by *Aspergillus fumigatus* strain was performed using the OFAT approach. The highest xylanase activity was obtained by sorghum hemicellulose as substrate at 2% (w/v), incubation temperature of 30 º C, agitation of 150 rpm, 1% (v/v) inoculum concentration, 50 mL volume of medium in 250 mL flask, and initial pH 7 (RAVICHANDRA et al., 2016).

Optimization using OFAT approach requires a large number of experiments and, consequently, more time to accomplish. Considering this fact, the statistical approach has been increasingly used. With a reduced number of design experiments taking into account different nutritional and physical parameters as variables and studying their interaction, this approach has presented satisfactory results for xylanase production optimization (BHARDWAJ et al., 2019).

After preliminary studies by OFAT, response surface methodology (RSM) was employed to optimize the production of xylanase, β-xylosidase, and β-glucosidase by *Colletotrichum graminicola*. Good levels of all enzymes were produced simultaneously using wheat bran as substrate at temperature and pH of 65 º C and 5.0, respectively (ZIMBARDI et al., 2013). The optimization of two nutritional and five physical parameters to improve xylanase from *Aspergillus niger* production was reported using the RSM approach. Only the parameters of substrate concentration and moisture content showed a statistically significant effect on xylanase production. Initially, xylanase activity was 2.93 U/g and after optimized conditions, it reached 5.23 U/g, which means that production increased 785 % (TAI et al., 2019).

Optimization of some fermentation parameters such as media composition, pH, and temperature was performed by response surface methodology. Before optimization, *Aspergillus niger* ADH-11 produced at most 22.14 U/g of α-L-arabinofuranosidase. After performing the experiments, the results indicated that a temperature of 55 °C, pH 4, and wheat bran as substrate were the optimal conditions for maximum enzyme production. The yield of α-L-arabinofuranosidase was increased by 2.34-fold after optimization of fermentative parameters (PATEL et al., 2015).

For industrial production and application of xylanases, a high yield of enzymes with specific properties such as stability over a wide range of pH and temperature, high substrate specificity, and resistance to chemicals and metal cations is necessary. However, most native xylanases reported are produced in low quantities and do not present these important characteristics required by industry. Therefore, a biotechnological approach can be applied to enhance xylanase production and add desirable characteristics to the enzyme. Technologies involving genetic manipulation such as mutations and recombinant DNA can be used for these purposes (MOTTA et al., 2013; SELVARAJAN and VEENA 2017; BHARDWAJ et al., 2019).

Strains of *Aspergillus brasiliensis* and *Penicillium digitatum* were submitted to physical and chemical mutagenesis to enhance the fungus ability to produce xylanase. After physical mutagenesis with UV irradiation for 50 min and chemical mutagenesis with 150 µg/mL of N-methyl-N'-nitro-N-nitrosoguanidine or ethyl methanesulfonate, xylanase activity of both fungal strains improved at least 30% (POPA et al., 2017). A protoplast fusion of two *Trichoderma* strains resulted in fusant presenting a 4.7-fold increase in xylanase activity compared to the parents. Results of a qPCR analysis showed that relative expression of the xylanase encoding gene increased by 4.9 fold (ZAHRA et al., 2018).

To improve xylanase activity from *Streptomyces* L10608, four amino acid residues were replaced by site-directed mutagenesis. Enzyme hydrolytic activity was improved 1.25-fold and presented a higher affinity for beechwood xylan as substrate (XIONG et al., 2018). After site-directed mutagenesis, the mutants of *E. coli* β-glucuronidase producer strain showed between 3- and 5-fold increases in enzyme activity compared to wild type. Structural analysis showed that the mutation stabilized enzyme conformation, therefore facilitating effective binding on the substrate (ZHANG et al., 2018).

Site-directed mutagenesis was performed to improve the activity of an alkaline stable and high-temperature tolerant xylanase from *Bacillus pumilus*. The mutant was expressed in *E. coli* and presented xylanase activity 1.5-fold higher at 60 º C. The mutant expressed in *Pichia pastoris*, which remained stable for 30 min at 60 º C, was optimized based on codon usage bias in order to further increase expression level. After codon optimization, strain Y16 showed an increase of 7.5-fold of enzyme activity. A qPCR analysis revealed that this strain presented 9 copies of the recombinant gene in the genome (LU et al., 2016). By the same technique, endoxylanase gene from *Aspergillus usamii* was optimized and expressed in *P. pastoris*. Xylanase activity of the optimized gene was 59% higher than wild-type gene (WANG et al., 2016).

Catalytic performance and thermostability of a GH10 xylanase were enhanced by the fragment-replacement-based technique. Substitution of some fragments and their combinations improved catalytic efficiency up to 4-fold and increased stability temperature up to 4.7 ºC (YOU et al., 2019). Deletion of *Aspergillus nidulans* genes with predicted function enhanced homologous GH10 xylanase. The knockout of glycosyltransferase (pbnA), thioredoxin (trxA), cyclophilin (cypA) and hsp40co-chaperone (ydjA) genes improved homologous production of xylanase by 78, 105, 125 and 171%, respectively. Total protein secretion was affected by the gene deletions and decreased, indicating that it occurred a specifical alteration for the production of homologous xylanase (ZUBIETA et al., 2020).

Two GH11 xylanase genes from thermophilic fungus *Myceliophthora thermophila* were cloned and expressed in *Pichia pastoris*. Both enzymes presented yield in 7.5 L fermentor, optimal activity at 60 ºC with pH between 6.0-7.0, and their activities were not affected by EDTA and metal ions, meaning that they have important properties for use in industry (BASIT et al., 2018). GH11 xylanase gene isolated from *Bacillus brevis* was expressed in *E. coli*. The recombinant enzyme was active under a wide range of temperature and pH, however optimal activity at 55 º C and pH 7. In addition, the xylanase recovered 90% of activity when returned to 55 º C after boiling for 5 min, demonstrating thermostability (GOSWAMI et al., 2014). A xylanase encoding gene was retrieved from a metagenomic library constructed from DNA extracted from compost-soil and expressed in *E. coli*. The recombinant xylanase presented activity over a wide range of temperature and pH and optimal conditions at 80 º C and pH 9 (VERMA et al., 2013).

For their growth and development, microorganisms produce several enzymes and metabolites along with the enzyme of interest. Therefore, a purification process is necessary to remove impurities and obtain a pure enzyme. Different strategies can be applied for xylanase purification, such as ammonium sulfate precipitation followed by dialysis, ion-exchange chromatography, gel filtration chromatography, affinity chromatography, aqueous phase chromatography, and ultrafiltration. In order to obtain a good purification fold, these techniques are often combined (BHARDWAJ et al., 2019b).

For enzyme precipitation, an adequate ammonium sulfate concentration (30-90%) needs to be selected. After precipitation, the enzyme is subjected to dialysis for salt removal. Xylanase produced by bacteria from sea sediment was partially purified by ammonium sulfate precipitation followed by dialysis. Precipitation of crude xylanase occurred with 40% ammonium sulfate saturation and dialysis was performed with sodium phosphate 0.05 mol/L pH 6 buffer. The specific xylanase activity improved after partial purification and the enzyme was characterized for optimum temperature and pH (KUMAR et al., 2018).

A multi-step process where enzyme precipitation is followed by chromatography techniques is usually observed. Xylanase produced by *Bacillus licheniformis* was purified by a two-step process. Ammonium sulfate precipitation, the crude xylanase presented 72.53 IU/mg specific activity. After anion exchange chromatography using DEAE-cellulose, a yield of 15.97 % and increased 5.86-fold specific activity was obtained (KUMAR et al., 2017). Thermostable alkaline xylanase from *Anoxybacillus kamchatkensis* NASTPD13 was precipitated by 80% ammonium sulfate and after dialysis, the specific activity of 5.96 U/mg and 2-fold purification was obtained. The concentrated product from dialysis was submitted to gel filtration using a Sephadex G-100 column. After this process, specific activity and purification fold were 32.98 U/mg and 11, respectively (YADAV et al., 2018).

A three-step process was necessary to purify xylanase from *Bacillus amyloliquefaciens*. Ammonium sulfate precipitation, ultrafiltration, and ion-exchange chromatography were applied to obtain a specific activity of 217.39 U/mg and 5-fold purification (KUMAR et al., 2017). However, each step in a purification process reduces enzyme recovery. Besides, the downstream process for enzyme production is approximately 60-70% of the total processing cost. Therefore, a single-step purification is more advantageous for an industrial enzyme production process (BHARDWAJ et al., 2019b).

Crude xylanase from *Aspergillus oryzae* LC1 was submitted to different purification methods separately, in order to evaluate the purification fold of a single-step purification for each technique. Purification after ammonium sulfate precipitation, anion exchange chromatography, and gel filtration chromatography resulted in purification fold of 4.1, 3, and 6.6, respectively. Moreover, the yield obtained was 21, 31.9, and 78.7% for each single-step purification technique, respectively. Single-step aqueous two-phase system (ATPS), a liquid-liquid fractionation technique, resulted in 13-fold purification and 86.8% yield, which was much higher than the other purifications techniques (BHARDWAJ et al., 2019a). A GH11 xylanase from *Aspergillus tamarii* was submitted to a one-step chromatography using a CM-cellulose column. The specific activity of approximately 1.215 U/mg, 7.43-fold purification, and 36.72% was achieved (HEINEN et al., 2018). Immobilized metal affinity chromatography (IMAC) has been also used as a single-step process for enzyme purification. The specific activity of *Bacillus* sp from soil termite abdomen xylanase increased from 0.56 to 5.45 U/mg and purification using this single technique resulted in a 10-fold purification (TADDIA et al., 2021).

Biomass hydrolysis enzymes such as xylanase, cellulase, and pectinases, correspond to approximately 20% of total global enzyme production. A cost analysis of each step involved in xylanase industrial-scale production is not available in the public domain. However, a techno-economic analysis of a recombinant β-glucosidase industrial production revealed that major cost is facility dependent (45%), which are included costs not directly associated with the process. Raw materials (25%) and consumables (23%) also represent a large cost. Glucose and IPTG (isopropyl-β-D-thiogalactoside) represent 47 and 41% of raw materials cost, respectively. In addition, the significant cost of consumables is due to the ultrafiltration membranes, which correspond to 80% of total consumables cost. Labor-dependent (4%), utilities (2%), and waste disposal (1%) generate little expense in β-glucosidase production (FERREIRA et al., 2018; BHARDWAJ et al., 2019).

3.1.6 Biosynthesis of xylan

Around 10 years ago, new investigations started being developed to produce engineer plants for biofuel production that has modified xylan improving its properties through the regulation of enzymes from Golgi apparatus (such as *glycosyltransferase* and *xylosyltransferase*), since xylan is synthesized in plants by the process that occurs in the Golgi apparatus (RENNIE and SCHELLER, 2014). Xylan is mainly produced in the secondary cell wall (SCW) during xylem development, which requires massive up-regulation of hemicellulose (e.g. glucuronoxylan) biosynthesis in the Golgi apparatus (MEENTS et al., 2019).

On the other hand, metabolic changes on xylan biosynthesis and modification are poorly understood (WIERZBICKI et al., 2019). To date, there are several reports in the literature about the enzymes that are involved in the xylan biosynthesis and their respective functions, such as (i) *Xylan xylosyltransferases* (GT47), involved in the stretching of the xylan skeleton; (ii) *Xylan glucuronyltransferases* (GT8), responsible for catalyzing the addition of GlcA in xylan, (iii) *Xylan acetyltransferase*, catalyst in xylan monoacetylation, (iv) *Glucuronoxylan methyltransferases* (DUF579), catalyst for GlcA methylation, among others (ZHONG and YE, 2014). In any case, regulation of xylan biosynthesis has the potential to be a future alternative to obtaining products with high added value.

CONCLUDING REMARKS

In addition to being one of the most abundant raw materials in nature, hemicellulose can be obtained from agroindustrial and waste. Therefore, hemicellulose is a renewable and sustainable source for converting high value-added products and biofuels. This bioconversion involves the action of enzymes that hydrolyze the main chain and the pendant groups of hemicelluloses from different biomasses. Such enzymes are produced from bacteria and fungi, whether wild or via heterologous expression, and purified by different methodologies. These enzymes can be applied in the modification of the xylan chain, generating a polysaccharide with specific a pending group or degree of polymerization. The enzymatic modification can improve the xylan properties such as solubility or hydrophobicity. Via different routes xylan can be converted into products with high value-added (e.g. xylitol, lactic acid, levulinic acid, succinic acid, furfural acid, and xylooligosaccharides). Therefore, knowing and producing both xylanases and accessory enzymes, acting separately or in enzymatic cocktail formulations, makes biorefinery and bioeconomics key processes to enrich technological and scientific advances for obtaining new products.

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CHAPTER 4: ENZYMATIC COCKTAILS FORMULATION FOR XYLAN HYDROLYSIS INTO XYLOSE AND XYLOOLIGOSACCHARIDES

ABSTRACT

In the context of biorefinery, lignocellulosic materials represent an important source of raw material for the bioconversion of cellulose, hemicellulose, and lignin into value-added products, such as xylose for fermentation, oligosaccharides, bioplastics for packaging. Among the most abundant lignocellulosic materials in Brazil, sugarcane bagasse biomass stands out, rich in cellulose and hemicellulose. In this context, through an experimental design, this study developed a robust enzyme cocktail containing xylanases and accessory enzymes to complete the hydrolysis of xylan from sugarcane bagasse, obtaining a low xylose yield and concentration (9%, 1.8 g/L using enzyme loads equivalent to 130, 10 and 6 IU/g for xylanase, β-xylosidase and auxiliary enzymes), a fermentable sugar that is important in the production of second-generation ethanol, and high xylooligosaccharides (XOS) yield and concentration (93.1%, 19.6 g/L, obtaining from xylooligosaccharides production assay), which in general have prebiotic activities, that favor an improvement in intestinal functions, with immunological, antimicrobial action and other benefits to human health. In addition to completely hydrolyzing sugarcane bagasse xylan, this enzymatic cocktail has great potential to be applied possibly in other sources of lignocellulosic biomass for the conversion of xylan into xylose and XOS due to its enzymes content, involving both main chain and pendent groups hydrolysis of hemicelluloses.

Keywords: biorefinery; high added-value products; enzymatic hydrolysis; biomass conversion; bioactive compounds

4.1 Introduction

Hemicellulose represents a raw material in biotechnological processes since a large surplus of lignocellulosic industrial residues from agricultural products is generated annually. These residues represent a source for bioconversion into many compounds of industrial interest, such as antioxidant compounds and oligosaccharides with bioactive potential (AKPINAR et al., 2010). Xylooligosaccharides can be obtained through the bioconversion of hemicellulose from different lignocellulosic biomasses, such as sugarcane bagasse (ZHOU and XU, 2019), corn husk (QIAN et al., 2020), banana pseudostem (FREITAS et al., 2021), among others.

Xylooligosaccharides (XOS) present several beneficial effects on human health, such as preventing caries, reducing serum cholesterol levels, and stimulating the growth of bifidobacteria in the gastrointestinal tract. Their beneficial health effect is related to their physicochemical properties, as they are moderately sweet, stable over a wide range of pH and temperature, and confer organoleptic characteristics to foods. Among the benefits of oligosaccharides in human health are the reduction of cholesterol levels, maintenance of gastrointestinal health, increased calcium bioavailability, reduced risk of colon cancer, cytotoxic effects on human leukemia cells, and the beneficial effect on type 2 diabetes mellitus (ANDO et al., 2004).

XOS are oligomers formed by xylose units joined by β-1,4 glycosidic bonds and are classified according to the number of repeats of xylose molecules. They are considered non-digestible oligosaccharides, and when present in the intestine they promote the proliferation of beneficial microorganisms for the intestinal microbiota, for this reason, they are considered prebiotics. XOS can be obtained by the hydrolysis of hemicellulose via chemical reagents, temperature, or biological agents, such as enzymatic hydrolysis (FREITAS et al., 2019). These carbohydrates participate in the constitution of dietary fiber and are partially digested by humans, in which the non-digestible portions serve as food for bacteria that are part of the natural flora such as *Bifidobacteria sp.* and *Lactobacillus sp.* (GIESE et al., 2011), acting in the following ways: stimulating selectively the growth and activity of one or more beneficial intestinal bacteria, improving the health of the host (SINGH et al., 2015), preventing their adhesion to gastrointestinal epithelial cells. (HOLZAPFEL and SCHILLINGER, 2002; SHIMIZU et al., 2011).

Oligosaccharides can be obtained through the bioconversion of different lignocellulosic biomasses, such as sugarcane bagasse (ZHOU and XU, 2019), corn husk (QIAN et al., 2020, banana pseudostem (FREITAS et al., 2021), among others. Different approaches can be used for the conversion of lignocellulosic material into XOS, however, enzymatic hydrolysis stands out for its effectiveness, in addition to being used concomitantly with other techniques (HE et al., 2014). In the process of obtaining xylooligosaccharides via enzymatic hydrolysis, the importance of xylanases stands out by hydrolyzing the main chain of xylan into smaller fragments containing from two to six xylose residues, which are named xylobiose, xylotriose, xylotetraose, xylopentose and xylohexose, respectively. The fungus *Aspergillus versicolor* (CARMONA et al., 1998) is a wild-type microorganism that produces xylanase with a molecular mass of 19 kDa with p*I* equivalent to 5.4 and presents a specific activity of 1440 U/mg and Km of 6.5 mg/mL when purified via ion exchange and subsequent gel filtration.

There are literature reports of studies that seek to obtain the deconstruction of xylan by combining the synergistic action of xylanases and accessory enzymes (YI et al., 2014; ÁVILA et al., 2020). However, in these studies, the xylanolytic cocktails were not robust enough to completely hydrolyze the xylan of one or more biomasses, considering that hemicelluloses are heteropolysaccharide. The option of producing xylose or XOS is of great interest since both products are for industrial/commercial uses. In this context, this study aimed to develop an enzymatic cocktail composed of hemicellulases, both xylanases (endo-1,4-β-xylanase and β-xylosidase), which cleave the xylan main chain, and accessory enzymes (α-glucuronidase, α-L-arabinofuranosidase, acetyl xylan-esterase, and feruloyl-esterase), which hydrolyze the pendant groups of the xylan. The xylanase and β-xylosidase enzymes were produced and purified from *Aspergillus versicolor*, while the accessory enzymes were purchased commercially. Subsequently, the enzymatic cocktails were evaluated in two main approaches: (i) complete hydrolysis of sugarcane bagasse xylan producing xylose and XOS; (ii) hydrolysis of xylan for the production of XOS.

4.2 Methodology

4.2.1 Hemicellulose extraction

Hemicellulose extraction was carried out with sugarcane bagasse treated with 0.2% (m/v) ethylenediamine tetraacetic acid (EDTA) solution for 1 h at 90 °C to remove metals. Extraction was performed with conditions optimized for bagasse, 6% H_2O_2 (m/v) at 25 °C for 4 h (BRIENZO et al., 2009). Ten grams of bagasse were placed in 1 L flasks, followed by the addition of the prepared reagents in a volume of 200 mL, the pH was adjusted to 11.6 with 5 mol/L NaOH, and the medium was stirred at 80 rpm. After the reaction, the material was filtered through filter paper. The pH of the filtrate was corrected to 6 with the addition of 6 mol/L HCl. In the xylan solution was added 3 vol ethanol. After the xylan precipitation, the liquid fraction (75% ethanol) was changed 3 times for washing it. The xylan was separated from the liquid fraction and oven dried at 45 °C.

4.2.2 Evaluation of lignin residual content

The methodology for bagasse, which was applied to hemicellulose (BRIENZO et al., 2009), consisted of hydrolyzing approximately 300 mg of biomass, adding 1.5 mL of 72% H_2SO_4 (m/m), the reaction occurred at 45 °C for 7 min. The reaction was stopped with the addition of 45 mL of distilled water. This mixture was autoclaved at 121 °C for 30 min (GOLVEIA et al., 2009). The contents were filtered through a porous plate filter nº 4, previously tared. The solid residue was washed with distilled water, and dried in an oven at 105 °C until constant weight for the determination of insoluble lignin (Klason).

4.2.3 Purification of xylanase and β-xylosidase from *A. versicolor*

The fungus *A. versicolor* was initially cultivated for 7 days at 30 °C in petro plates containing a solid medium from Vogel (1965). Then, 1.0 mL of the spore suspension ($5x10^7$ spores/mL) were inoculated for 5 days in a Vogel (1965) liquid medium containing 1% wheat bran following the methodology established (CARMONA et al., 1998). The material was filtered on Whatman No. 1 paper and was dialyzed against 50 mmol/L Tris-HCl buffer to remove impurities. Then the crude extract was subjected to ion exchange purification (GOLVEIA et al., 2019). Thus, 15 mL of SEPHADEX A-50 resin (GE HEALTHCARE) and 150 mL of the enzymatic extract were used in 250 mL Erlenmeyer submitted to 10 °C under light agitation for a period of 24 h in a refrigerated incubator (MARCONI MA 830/A). After a period of 24 h, the mixture was filtered through filter paper (7 cm of diameter, pores of 14 µm) with the aid of a vacuum pump (MARCONI MA057). The gel filtration was performed using SEPHADEX G-75 resin (GE HEALTHCARE), completing the purification process.

4.2.4 Determination of enzymatic activity and protein quantification

The xylanase activity of *A. versicolor* was determined following the method established (MILLER, 1959), in a reaction at 50 °C for 5 min, using 1% xylan as substrate. The β-xylosidase activity of *A. versicolor* was determined following an established methodology (KUMAR et al., 1996), in a reaction at 50 °C for 5 min using Nitrophenyl-β-D-xylopyranoside (Megazyme) as substrate. The auxiliary enzymes used in this study were purchased from the company Megazyme and their information was gathered in Table 4.1. To enzyme cocktail application, the temperature, reaction volume and time were fixed and different enzyme loads were evaluated. The optimal temperature for each enzyme that composes the enzyme cocktail produced and applied in this study varies from enzyme to enzyme, therefore the temperature of 50 °C was fixed, otherwise it would be necessary to apply other experimental designs.

Table 4.1 - Description of the commercial auxiliary enzymes used in the present study such as microorganism of origin, optimum temperature of action as well as optimum pH and respective activity and specific activity.

Enzyme	**Microorganism**	**Activity (IU/mL)**	**Specific activity (U/mg)**	**Temperature (º C)**	pH
acetil xylan esterase	*Orpinomyces* sp.	1000	36	40	6.7
a-glucuronidase	*Geobacillus stearothermophilus*	100	40	70	7
a-L arabinofuranosidase	*Bacteroides ovatus*	1000	575	40	6.5
feruloyl esterase	Rumen microorganisms	400	30	40	6.5

Source: Author.

4.2.5. Complete xylan hydrolysis and xylooligosaccharides production

Enzyme cocktails were evaluated to determine the enzyme load (IU/g of xylan/substrate) required for the complete hydrolysis of hemicellulose. An experimental design was carried out by varying the enzymatic loads of xylanase, β-xylosidase, and auxiliary enzymes (α-glucuronidase, α-L-arabinofuranosidase, acetyl xylan esterase, and feruloyl esterase) in order to obtain complete hydrolysis of the xylan (Table 4.2).

A second experimental design was carried out to obtain xylooligosaccharides, excluding the use of the β-xylosidase enzyme (Table 4.3). Parameters such as reaction time (24 h), temperature (50 °C), stirring (100 rpm) and reaction volume (1 mL) were fixed for all reactions in both approaches. Samples were filtered through 0.22 μm syringe filters and evaluated by HPLC using the Aminex HPX-87C BIO-RAD column (300 × 7.8 mm) (FREITAS et al., 2020). Finally, another assay was performed where it was applied only to xylanase enzymes with the same enzyme loads (IU/g) used for XOS production (Table 4.3), for comparison with the approach where xylanase and auxiliary enzymes were used together.

Table 4.2 - Experimental design for determination of complete hydrolysis of xylan. (*) auxiliary enzymes were added with the same enzymatic load. The experimental design indicated 17 experiments containing central and axial points according to the three observed variables (enzyme loading of xylanase, β-xylosidase and auxiliary enzymes).

Experiment	*Xylanase* (IU/g)	*β-xylosidase* (IU/g)	Auxiliary enzymes (IU/g)*
1	60	5	4
2	200	5	4
3	60	15	4
4	200	15	4
5	60	5	8
6	200	5	8
7	60	15	8
8	200	15	8

9	12.27	10	6
10	247.73	10	6
11	130	1.59	6
12	130	18.41	6
13	130	10	2.64
14	130	10	9.36
15	130	10	6
16	130	10	6
17	130	10	6

Source: Author.

Table 4.3 - Experimental design for xylooligosaccharides production. (*) auxiliary enzymes were added with the same enzymatic load. The experimental design indicated 11 experiments containing central and axial points according to the two observed variables (enzyme loading of xylanase and auxiliary enzymes).

Experiment	*Xylanase* (IU/g)	Auxiliary enzymes (IU/g)*
1	20	2
2	80	2
3	20	10
4	80	10
5	7.57	6
6	92.43	6
7	50	0.34
8	50	11.66
9	50	6
10	50	6
11	50	6

Source: Author.

4.2.6. Statistical analyses

Statistical analysis of the effects of each variable to obtain complete hydrolysis of xylan and the production of xylooligosaccharides were performed using the Statistica 8 software, considering the confidence level of 95% ($p>0.5$), with ANOVA statistical analysis, and the respective response surface graphics to both complete hydrolyses of xylan and production of xylooligosaccharides assays were generated.

The results of the second-order ANOVA statistical analysis for the experimental design of complete hydrolysis of xylan indicated that it would be appropriate to perform a first-order analysis (Supplementary table 1), while for the results of the experimental design of obtaining XOS the second-order ANOVA statistical analysis was more appropriate (Supplementary table 2).

4.3 Results and discussion

4.3.1 Hemicellulose extraction and determination of residual lignin content

A total of 200 g of xylan were extracted through an alkaline medium pretreatment under mechanical agitation, resulting in an extraction yield of about 50%, taking into account that 10 L of extraction solution were prepared and 500 g of bagasse was initially applied. The experiments on a small scale were carried out using 10 g of biomass in a reaction volume of 200 mL. After chemical characterization, the results indicated that an average of 5% corresponds to residual lignin. The content of residual lignin observed with this approach was higher than the 5% of residual lignin resulting from the chemical characterization performed right after the alkaline pretreatment, following the methodology published elsewhere (BRIENZO et al., 2009), where it was reported a good yield of xylan extraction from sugarcane in alkaline pretreatment (86%) with low residual lignin content (5,9%).

4.3.2 Xylanase and β-xylosidase from *A. versicolor*

The xylanase from *A. versicolor* showed an enzymatic activity of 1000 IU/mL, and a protein concentration equivalent to 1.98 mg/mL. Compared to data in the literature, *Aspergillus ficuum* AF-98 xylanase shows an activity of 485 IU/mL after purification with gel filtration using beechwood xylan as a substrate (FENGXIA et al., 2008). As in this study, the *Aspergillus niger* ANL 301 xylanase evaluated by Okafor et al. (2007) was produced using wheat bran as a carbon source in the culture medium. However, a low xylanase activity of *Aspergillus niger* ANL 301 was observed, equivalent to 6.47 IU/mL, different from what was obtained in this study (1000 IU/mL). Additionally, *Aspergillus niger* ANL 301 xylanase showed a protein concentration of 1.14 mg/mL, a little lower when compared to the xylanase concentration observed in this study (1.98 mg/mL).

A third study investigated the xylanase activity of *Aspergillus foetidus* in the presence of beechwood xylan as a substrate for enzymatic reaction (1%), observing a xylanase activity corresponding to 210 IU/mL (SHA and MADAMWAR, 2005). Here, using sugarcane bagasse xylan (1%) extracted via alkaline pretreatment, an *A. versicolor* xylanase activity equivalent to 1000 IU/mL was determined, which again was much higher than observed in the literature.

The β-xylosidase that was produced and purified from *A. versicolor* showed an enzyme activity equivalent to 4.8 IU/mL in nitrophenyl-β-D-xylopyranoside substrate, and a protein concentration of 0.55 mg/mL. As for the purification of β-xylosidase from *A. versicolor*, there are no data for enzyme activity in the study published elsewhere (CARMONA et al., 1998).

In the literature, there are reports of specific activity equivalent to 51.30 (IU/mg protein) for β-xylosidase from *A. versicolor* when cultivated with 1% xylan as a carbon source. For the microorganism *Aspergillus nidulans* in Nitrophenyl-β-D-xylopyranoside substrate, the total enzymatic activity (IU/mL) of β-xylosidase observed was equivalent to 44.2, 22.3, 20, 0, and 12 in different purification steps (crude extract, Q sepharose, Mono-Q and Superdex-200, successively), values higher than the results reported in this study (KUMAR et al., 1996).

4.3.3 Complete hydrolysis of xylan

In the present study, a combination of enzymes was applied aiming to maximize the xylan hydrolysis. The highest yield and highest concentration of xylose observed were 9% and 1.8 g/L, with concomitant production of 90.1% and 18.22 g/L of xylooligosaccharides (Table 4.4). The results of the experimental design indicate that both xylose yield and concentration were low but high for xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentose, and xylohexose) (93.1% and 19.64 g/L) in the study region observed.

The results of the response surface analysis for both xylose yield (%) and concentration (g/L) indicate that the best results were obtained when 130 IU/g of xylanase and 6 IU/g loads of auxiliary enzymes (Figure 4.1a and b). When the variables β-xylosidase (IU/g) and accessory enzymes (IU/g) were evaluated, the best results were observed with 10 IU/g of β-xylosidase and 6 IU/g of auxiliary enzymes (Figure 4.1c and d). The variables xylanase (IU/g) and β-xylosidase (IU/g) produced a higher xylose yield using 130 (IU/g) and 10 (IU/g) of enzyme loads, respectively (Figure 4.1e, and f).

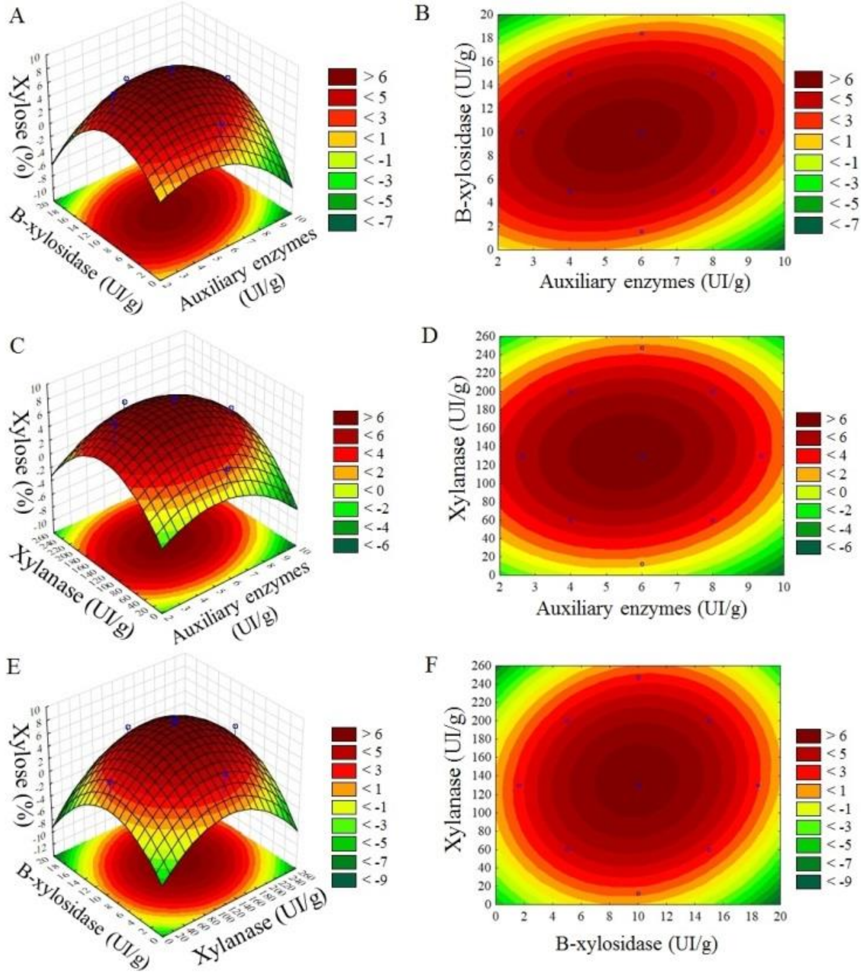
In the experimental design of the complete xylan hydrolysis were also observed high yield and concentration of xylooligosaccharides (93.1% and 19.6 g/L, respectively) (Table 4.4). It is important to highlight that even in the presence of β-xylosidase, this cocktail presented better results in obtaining xylooligosaccharides than the experimental design itself, which used an enzyme cocktail with the absence of β-xylosidase since the production of xylose was not the target of the analysis. Hypothetically a higher β-xylosidase load could lead to higher xylose production (in yield and concentration), thus reducing XOS production (yield and concentration). On the other hand, the xylose was not increased and probably a higher amount of β-xylosidase could be necessary.

In the experimental design to obtain the complete hydrolysis of xylan, the highest yield and concentration of xylooligosaccharides observed were 93.1% and 19.6 g/L, respectively, with concomitant production of 4% and 0.8 g/L of xylose. According to the response surface, the highest value for XOS yield (>90%) was observed when 130 IU/g of xylanase, 20 IU/g of β-xylosidase, and 7 IU/g of accessory enzymes were used.

Through the results, it was also possible to observe that the maximum xylanase load (247.7 IU/g) generated the highest yield and XOS concentration results (93.1% and 19.6 g/L, respectively), while the lower xylanase load (12.2 IU/g) generated low yield and XOS concentration results (76.6% and 15.3 g/L). Additionally, the maximum load of β-xylosidase (15 IU/g) generated XOS yields ranging from 40 to 74.5% and concentrations from 12 to 17,7 g/L, while the lower β-xylosidase load (1,5 IU/g) generated a high yield of XOS (93%) and also a high concentration (19 g/L). The intermediate auxiliary enzymes load (central point) generated higher XOS yield in combination with xylanase (Figure 4.2). Probably there was a positive interaction between these enzymes resulting in better XOS production.

In relation to the results obtained for xylose, a study in the literature showed that 22.5% xylose was obtained when sugarcane bagasse was submitted to an alkaline pretreatment and subsequent enzymatic hydrolysis for 24 h using 5 IU/g of xylanase (Quintero et al., 2020). In the present study, to obtain 9% of xylose (better result), 130 IU/g of xylanase were used but other enzymes were added, namely β-xylosidase (10 IU/g) and accessory enzymes (6 IU/g) in a 24 h reaction with xylan as a substrate also submitted to alkaline pretreatment. However, despite the low xylose yield observed, high xylooligosaccharides yields were obtained in the same experiment, as described above. Possibly, higher loads of β-xylosidase than applied in this study (10 IU/g) could contribute to higher yield and xylose concentration than observed by the present study.

Figure 4.1 - Response surface of xylose yield (%) with xylanase (IU/g), β-xylosidase (IU/g), and auxiliary enzymes (IU/g) variables. (a) and (b): β-xylosidase (IU/g) and auxiliary enzymes (IU/g) variables. (c) and (d): xylanase (IU/g) and auxiliary enzymes (IU/g) variables. (e) and (f): xylanase (IU/g) and β-xylosidase (IU/g) variables. For all the surfaces, the fixed variable was employed at the intermediate level (central point level).



Source: Author.

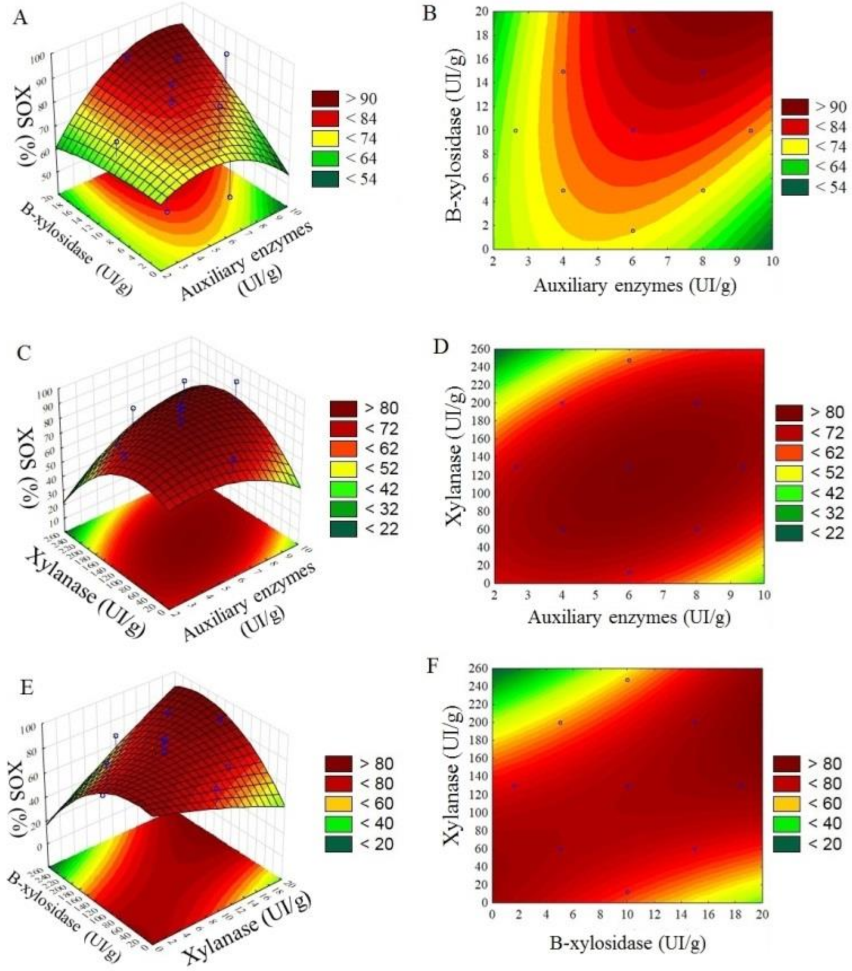
A second study produced 4.26% of xylose also with sugarcane bagasse biomass submitted to alkaline pretreatment and subsequently hydrolyzed using 500 U/g of xylanase for periods longer than 24 h of reaction in a bioreactor (RAJ and KRISHNAN, 2020). For comparison purposes, this present study produced more than double this xylose yield (9%) using a much lower enzyme load (130 IU/g), in a shorter reaction time (24 h).

Table 4.4 – Experimental design to obtain the complete hydrolysis of xylan. * *α-L-arabinofuranosidasse*, *α-glucuronidase*, *acetil xylan-esterase* e *feruloil esterase*, X2: xylobiose; X3: xylotriosis; X4: xylotetratose; X5+X6: Xylopentose e xylohexose.

Assay	*Xylanase* (IU/g)	*β-xylosidase* (IU/g)	Auxiliary enzymes (IU/g)*	X2 (%)	X3 (%)	X4 (%)	X5+X6 (%)	XOS (%)	XOS (g/L)	Xylose (%)	Xylose (g/L)	Total sugars (%)	Total sugars (g/L)
1	60	5	4	9.9	28.1	13.4	19	70.4	14.08	1.5	0.3	71.9	14.3
2	200	5	4	5	22.2	11.6	11.1	49.9	10	1.4	0.03	51.3	10
3	60	15	4	8	29	15.5	22	74.5	14.9	0.2	0.004	74.7	14.9
4	200	15	4	3	30.1	12.2	17.5	62.8	12.5	1.1	0.2	63.9	12.8
5	60	5	8	14	-	21.5	30.7	66.2	13.2	0.2	0.04	66.4	13.3
6	200	5	8	1.4	-	17.2	24.5	43.1	8.6	0.5	0.1	43.6	8.7
7	60	15	8	14.2	-	18.8	26.8	59.8	12	1	0.2	60.8	12.2
8	200	15	8	39.5	-	20.2	28.8	40	17.7	3.6	0.7	43.6	18.4
9	12.2	10	6	31.6	-	18.5	26.5	76.6	15.3	3.7	0.7	80.3	16
10	247.7	10	6	25.6	33.5	14	20	93.1	19.6	4	0.8	97.1	19.7
11	130	1.5	6	22.1	35.8	15.2	21.7	93	19	5.4	1	98.4	20
12	130	18.41	6	24.2	35.2	12.6	18	90	19.3	3.3	0.6	93.3	20
13	130	10	2.6	14	28.8	14.9	21.2	78.9	15.7	8.9	1.7	87.8	17.5
14	130	10	9.3	30	-	16.9	24	70.9	14.1	4.1	0.8	75	15
15	130	10	6	15	33.2	15.9	22.6	85	17.3	8	1.6	94.3	18.9
16	130	10	6	16	33.3	16.8	24	90.1	18.2	9	1.8	99.1	20
17	130	10	6	14	32.2	15	21.3	83	16.5	7.1	1.4	89.6	17.9

Source: Author.

Figure 4.2 - Response surface of xylooligosaccharides yield (%) to experimental design to obtain a complete hydrolysis of xylan using xylanase (IU/g), β-xylosidase (IU/g), and auxiliary enzymes (IU/g) variables. (a) and (b): β-xylosidase (IU/g) and auxiliary enzymes (IU/g) variables. (c) and (d): xylanase (IU/g) and auxiliary enzymes (IU/g) variables. (e) and (f): xylanase (IU/g) and β-xylosidase (IU/g) variables.



Source: Author.

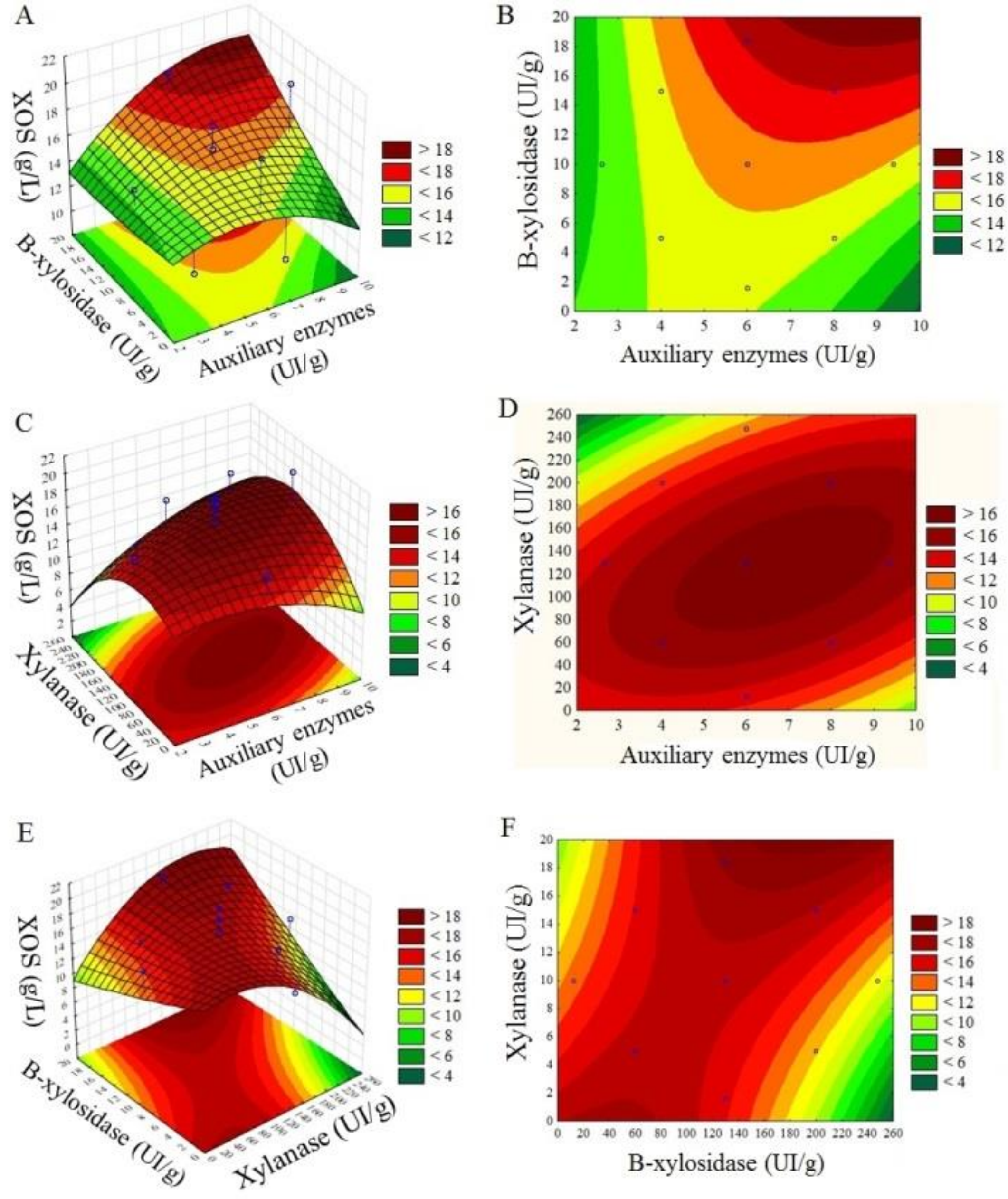
4.3.4 Xylooligosaccharides production

The best results observed for both yield (around 84%) and concentration (16.8 g/L) of xylooligosaccharides occurred when low enzymatic loads of auxiliary enzymes (2 IU/g) were used, with xylanase loads ranging from 20 IU/g to 80 IU/g, respectively (Table 4.5). The results of response surface analysis for both xylooligosaccharide yield (%) (Figure 4.3) and its concentration (g/L) indicate that the ideal xylanase enzymatic load corresponds to 100 (IU/g), that is, higher than the study region observed in this study (7.47 to 92.43 IU/g), while low loads of auxiliary enzymes would be ideal to obtain better results.

The maximum xylanase load (92.4 IU/g) generated a yield of 81.2% XOS and a concentration of 16.2 g/L, while the lowest xylanase load (7.5 IU/g) generated a yield and an XOS concentration equivalent to 80.3 % and 16 g/L, respectively. For accessory enzymes, the maximum load (11.6

IU/g) generated a yield of 68.3% and a concentration of 13.6 g/L of XOS, while the lower load (0.3 IU/g) presented a yield and concentration of XOS equivalent to 80.6% and 16.1 g/L of XOS. Here, through the response surface plot below it is possible to highlight that optimal maximum xylanase loading for maximum yield and XOS concentration is above that evaluated in this study (equivalent to 92.4 IU/g), but this data could be obtained by evaluating higher loads of xylanase.

Figure 4.3 - Response surface of xylooligosaccharides yield (%) and concentration (g/L) with xylanase (IU/g) and auxiliary enzymes (IU/g) variables. (a) and (b): xylooligosaccharides yield (%) with xylanase (IU/g) and auxiliary enzymes (IU/g) variables. (c) and (d): xylooligosaccharides concentration (g/L) with xylanase (IU/g) and auxiliary enzymes (IU/g) variables.



Source: Author.

Table 4.5 – Experimental design for xylooligosaccharides production. * *α-L arabinofuranosidasse, α-glucuronidase, acetil xylan-esterase* e *feruloil esterase*. X2: xylobiose; X3: xylotriosis; X4: xylotetratose; X5+X6: Xylopentose e xylohexose.

Experiment	*Xylanase* (IU/g)	Acessory enzymes (IU/g)*	X2	X3	X4	X5+X6	Total XOS (%)	Total XOS (g/L)
1	20	2	9.9	23.1	21	30	84	16.8
2	80	2	5.5	39.6	16	22.8	83.9	16.8
3	20	10	8.8	19.6	17.8	25.4	71.6	14.3
4	80	10	5.5	18.6	16	15.3	55.4	11.1
5	7.5	6	5.6	23.3	21.2	30.2	80.3	16
6	92.4	6	7	23.2	21	30	81.2	16.2
7	50	0.3	3.1	33.1	18.3	26.1	80.6	16.1
8	50	11.6	6.8	19.2	17.4	24.9	68.3	13.6
9	50	6	6.1	18.9	17.1	24.5	66.6	13.3
10	50	6	3.8	42.6	14.2	13.6	74.2	14.8
11	50	6	3.1	39.4	17.4	16.7	76.6	15.3

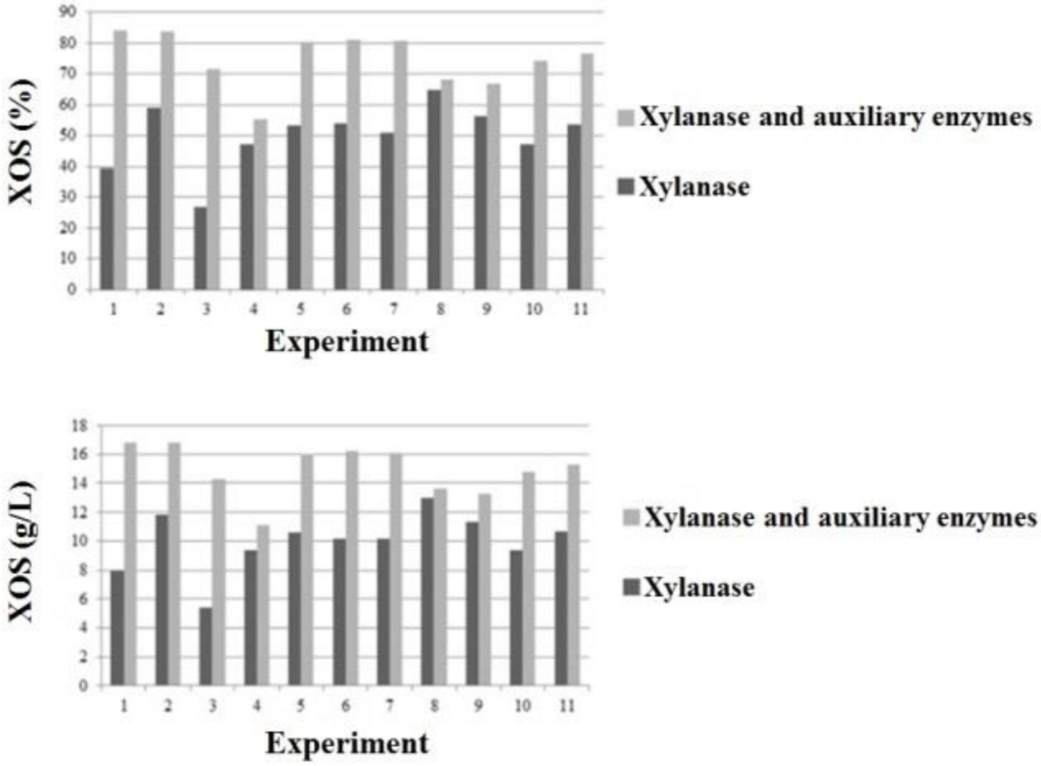
Source: Author.

A study also used *A. versicolor* xylanase (FREITAS et al., 2020), the same used in this study, to obtain xylooligosaccharides from banana pseudostem biomass. Various xylanase enzyme loads were used (10, 30, 60, 100 and 200 IU/g) and 52.27, 60.98, 58.0, 57.14 and 62.91 (%) of xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentose and xylohexose) were produced, with concentrations (g/L) equivalent to 9.44, 11.02, 10.48, 10.32 and 11.36, respectively. In this present study, all enzyme loads evaluated for xylanase (IU/g) led to yields ranging from 55.4 to 84% and concentrations from 11.1 to 16.8 g/L, considering that this study used a complete enzyme cocktail with xylanase and also auxiliary enzymes. Regarding the polymerization degree for xylooligosaccharides produced, in this study, yields equivalent to 9.9% (xylobiose), 39.6% (xylotriose), 21.2% (xylotetraose), and 30.2% (xylopentose and xylohexose) were observed, a yield that was mostly higher compared to that observed in the study (FREITAS et al., 2021), where yields of 0.55% (xylobiose), 0.53% (xylotriose), 3.75% (xylotetraose), and 6.97% (xylopentose and xylotetraose) were reported.

A second study (FORSAN et al., 2021) used the same xylanase and *A. versicolor* (FREITAS et al., 2020) and this present study for the production of xylooligosaccharides from sugarcane bagasse and leaf biomass. Enzymatic loads of xylanase ranging from 15.5 to 100 IU/g were used, obtaining a maximum XOS yield of 67.43% for bagasse and 69.71% for sugarcane leaf. The maximum XOS concentration was 19.91 g/L for sugarcane bagasse and 21.48 g/L for sugarcane leaf. The polymerization degree of xylooligosaccharides described was 3.74% for xylobiose, and 64.26% for xylotriose, xylotetraose, xylopentose and xylohexose together, also mostly lower than those observed in this study.

With *Bacillus subtilis* (using xylanase and β-xylosidase not purified) a XOS yield of 3.25% was observed in a 72 h growing with wheat middlings as substrate (REQUE et al., 2019). Also, were observed a polymerization degree of 0.37% for xylobiose, 1.78% for xylotriose, 1.47% for xylotetraose, and 0.9% for xylopentose and xylohexose, also lower than observed in this present study. In this context, it is noteworthy that in the present study the use of the auxiliary enzymes α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan-esterase, and feruloyl esterase was essential to obtain better results in the production of xylooligosaccharides. The same xylanase loads (IU/g) were evaluated in the absence and presence of auxiliary enzymes (Figure 4.4).

Figure 4.4 - XOS production by xylanase and auxiliary enzymes approach (IU/g) and only xylanase approach (IU/g) elucidating that the presence of the auxiliary enzymes contributed to the higher yields and concentrations of XOS obtained.



Source: Author.

The biomass is recalcitrant and required a series of strategies to convert it into value-added molecules (HOU et al., 2021). A biorefinery approach appears as a solution for better use of biomass components, resulting in a different process such as xylose for furfural (NIE et al., 2019) or fermentation, XOS and even using the xylan solubilization residue for briquette (energy densification) (NDUMBO et al., 2022). Xylose and XOS can be produced from different types of waste biomass (PEREIRA et al., 2022), by applying an enzymatic or acid approach (FORSAN et al., 2022; FREITAS et al., 2022). However, the present study showed the advantage of using combined hemicelluloses hydrolysis enzymes for XOS production.

4.4 Conclusion

This study is a pioneer in using a robust enzyme cocktail containing both the enzymes that cleave the hemicellulose main chain and most of the existing auxiliary enzymes that hydrolyze the pendant groups of hemicellulose, such as arabinose, glucuronic acid, acetic acid, and ferulic acid. A complete hydrolysis of xylan from sugarcane bagasse is carried out and still produces xylooligosaccharides, one of the xylan derivatives with added value. High yields (93.1%) and concentrations (19.6 g/L) of xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentose and xylohexose) were observed in this study. It is important to emphasize that this enzyme cocktail was developed taking into account that hemicellulose is a heteropolysaccharide, that is, its content may vary from biomass to biomass. Therefore, in addition to being used in the bioconversion of xylan from sugarcane bagasse into xylose and xylooligosaccharides, this enzyme cocktail may also be used for the bioconversion of hemicellulose from other sources of lignocellulosic raw material.

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Supplementary table 4.1 – ANOVA statistical analysis of XOS yield (%) from experimental design of complete hydrolysis of xylan (R-sqr=.98934).

Factor	SS	Df	MS	F	p
Curvature	1653.502	1	1653.502	110.2090	0.008952
(1)Xylanase	705.001	1	705.001	46.9896	0.020625
(2)β-xylosidase	7.031	1	7.031	0.4686	0.564295
(3)Auxiliary enzymes	294.031	1	294.031	19.5977	0.047426
1 by 2	18.301	1	18.301	1.2198	0.384496
1 by 3	14.311	1	14.311	0.9539	0.431737
2 by 3	87.781	1	87.781	5.8508	0.136722
1*2*3	3.781	1	3.781	0.2520	0.665469
Error	30.007	2	15.003		
Total SS	2813.747	10			

Supplementary table 4.2 - ANOVA statistical analysis of XOS yield (%) from experimental design of xylooligosaccharides obtaining (R-sqr=.75176).

Factor	SS	Df	MS	F	p
(1)Xilanase(L)	28.3371	1	28.3371	0.74060	0.428794
Xilanase(Q)	56.5161	1	56.5161	1.47707	0.278484
(2)Auxiliary enzymes(L)	424.2537	1	424.2537	11.08807	0.020782
Auxiliary enzymes(Q)	0.0012	1	0.0012	0.00003	0.995669
1L by 2L	65.0442	1	65.0442	1.69996	0.249091
Error	191.3108	5	38.2622		
Total SS	770.6558	10			

CHAPTER 5: CHEMICAL AND ENZYMATIC MODIFICATIONS FOR THE PRODUCTION OF XYLAN AS SUBSTRATE AND ALSO XYLAN-BASED BIOPLASTICS

ABSTRACT

This study aimed to promote modifications in xylan extracted from sugarcane biomass via alkaline pretreatment with the objective to obtain for determination of xylanase activity and for the cultivation of the fungus *Aspergillus versicolor* through two approaches of delignification of xylan, and a second approach where the original xylan has had its pendant arabinose groups removed. Firstly, the original xylan was subjected to enzymatic hydrolysis using 1.67 IU/g of laccase and was also subjected to an alkaline treatment using 6% H2O2. Then, the same original xylan was submitted to another enzymatic hydrolysis using α-L-arabinofuranosidase to remove the pendent groups of arabinose. The results obtained indicated that original xylan (containing 5% residual lignin) and xylan delignified with lacase are interesting substrates for the determination of xylanase activity (700 IU/mL for both) and for the cultivation of *A. versicolor* (producing xylanase at 1350 and 1320 UI/mL, respectivelly). Additionally, this study pointed to the improvement in the quality of the bioplastic properties (such as opacity, moisture, solubility, and stress tension) when the xylan was delignified and also when arabinose was removed, and that the presence of bis-acrylamide probably contributed to the water absorption resistance of the material. The results for original xylan (opacity 4.7 ± 0.2%, moisture 7%, solubility 35% and tensile stress 2.7 MPa) demonstrate that the presence of residual lignin increases the opacity of the material, moisture retention, higher water solubility and decreased the tensile stress. The search for more accessible and cheaper substrates that can be obtained by easily reproducible methods represents an advantageous approach in relation to the acquisition of commercial substrates such as beechwood xylan widely used in studies involving fungi of the genus *Aspergillus* sp., which although generally present scientific performance was actually discontinued, needing a protocol evaluated for such application. Moreover, the data described in this study show that the modifications into xylan promoted improvement in the quality of bioplastics, integrity and resistance to breakage, and that the use of bis-acrylamide may also contribute to the results observed.

Keywords: biomass conversion, delignified xylan, low-cost substrate, xylan-based biofilm, sugarcane bagasse.

5.1 Introduction

Lignocellulosic materials represent a source of raw material in biotechnological processes since a large surplus of industrial residues of agricultural products is generated annually. A large part of these residues are used in animal feed or are burned for alternative disposal and energy generation. However, such residues represent a potential source for many compounds of industrial interest, such as antioxidant compounds and oligosaccharides with bioactive potential (AKPINAR et al., 2009).

Hemicellulose is the second largest polysaccharide in lignocellulosic biomass. Its structure supports the cellulose microfibrils by hydrogen bonds and binds to the lignin by covalent bonds. Unlike cellulose, hemicellulose is classified as a heteropolysaccharide, composed of different carbohydrates, and presents an amorphous structure with a degree of polymerization of approximately 200 units (HOLTZAPPLE, 2003). The composition of hemicellulose presents monosaccharides such as pentose, hexoses, acetylated sugars, and uronic acids. Its composition usually varies in different plant species (Saha, 2003). The xylan main chain is formed by a homopolymer of xylose or a heteropolymer of glucomannans, with the presence of arabinose, galactose, and 4-O-methylglucuronic acid that bind to each other through β-(1→4) glycosidic bonds, or also of the type β-(1→3), β-(1→6), α-(1→2), α-(1→3), and α-(1→6) (Limayem and Ricke, 2012).

The hemicellulose is the second most abundant polysaccharide found in nature, corresponding to approximately one-third of the renewable carbon source of the planet (COLLINS et al., 2005). In industry, the use of xylan to obtain bio-products has gained prominence through the making of packaging, hydrogels, ethanol, and lactic acid production through the fermentation of monomeric sugars from xylan by microorganisms, and additionally, furfural production by xylan hydrolysis and dehydration of xylose monomers, and finally, obtaining xylitol by xylose hydrogenation routes (NAIDU et al., 2018). Hemicellulose has been used for the production of different high value-added derivatives, such as XOS (REDDY and KRISHNAN, 2017) and XOS for fermentation (CHEN et al., 2017), films (HANIF and ZAMAN, 2017), xylan-lactate copolymers (CHEN et al., 2017), and yet such derivatives have been widely employed in medical applications (CANTU-JUNGLES et al., 2017) and bioplastic formulations (MACEDO et al., 2022).

Bioplastics are biobased materials composed of total or partial biomass and are biodegraded in the environment due to the action of microbial enzymes (IMRE et al., 2019; LACKNER, 2015). Bioplastics based on xylan and starch have been described in the literature, and their properties such as opacity, moisture, solubility and tensile stress have been evaluated (MACEDO et al., 2022). Besides starch, xylan-based bioplastics can also be formulated using polysaccharides, such as glycerol, a plasticizing agent that contributes to an improvement in flexibility and elongation of the biofilm, but on the other hand, it can reduce the mechanical strength (BERTUZZI et al., 2012; IMRE al., 2013; XU et al, 2019).

Considering that sugarcane bagasse is now one of the main raw materials, it is important that the hemicellulose of this biomass, xylan, is used as a substrate for the determination of xylanase activity and in the cultivation of microorganisms for xylanase production. Normally hemicellulose extraction yield is high and show the presence of residual lignin (BRIENZO et al., 2009; XU et al., 2006), on the other hand, the hemicellulose with low yield still presents residual lignin (BANERJE et al., 2014; SUN et al., 2004). The use of lignolytic enzymes, such as laccases could contribute to the removal of residual lignin and obtaining a xylan with a higher degree of purity.

In this context, this study aimed to promote modifications in the xylan extracted from sugarcane bagasse using peroxide in alkaline treatment. Such modifications include the removal of residual lignin with alkaline treatment and also enzymatic hydrolysis. After promoting the removal of residual lignin, the delignified and original xylans obtained in this study were evaluated as substrate for the growth of the fungus *Aspergillus versicolor* and also as a substrate for determining the enzymatic activity of xylanase produced by this same fungus. All the xylan modificated were used together with starch, glycerol, and bis-acrylamide to formulate bioplastics. The properties of the four bioplastics formulated such as opacity, moisture, solubility and mechanical strength were evaluated.

5.2 Methodology

5.2.1 Hemicellulose extraction

Xylan extraction essay was applied according to section 4.2.1 described at Chapter 4.

5.2.2 Evaluation of lignin residual content

The residual of lignin in the xylan was determined as described at section 4.2.2 from Chapter 4.

5.2.3 Chemical and enzymatic modifications of xylan for removal of residual lignin

The xylan extracted according to 5.2.1 item was again subjected to an alkaline treatment using 6% H2O2 (m/v) at 25 °C for 4 h (BRIENZO et al., 2009), then the material was dried in an oven at 105 °C for 24 h, and then 100% ethanol was added, and after drying again for 24 h, 75% ethanol was added, and after another drying cycle for 24 h the material was removed from the oven and stored. Additionally, in parallel to the chemical treatment, an enzymatic hydrolysis was performed using 1.67 U/g of laccase solubilized in 0.05 mol/L sodium tartrate buffer pH 6.5 in a reaction containing 2% xylan at 30 °C for 4 h as Rabia et al (2018).

In addition, the original xylan (2%) in this study was also subjected to enzymatic hydrolysis using 10 IU/g α-L-arabinofuranosidase (Megazymes) in a reaction at 50 °C for 24 h to remove arabinose pendant groups. To facilitate the understanding, the different xylans obtained through chemical and enzymatic modifications are described here as X1: untreated original xylan; X2: xylan treated with 6% H2O2; X3: xylan hydrolyzed with lacase; X4: xylan hydrolyzed with α-L-arabinofuranosidase.

5.2.4 Fourrier transform infrared spectroscopy (FTIR) analysis

Here, original xylan and modified xylanas (through enzymatic and chemical trials using lacase and arabinofuranosidase enzymes and 6% H2O2, successively) were directly placed onto ZnSe crystal. Then, were produced pelets containing 0.001 g of sample and 0.25 g of KBr. For this analysis were observed lignin bands such as 1510 and 1700 cm^{-1} and arabinose bands between 980 to 1100 cm^{-1} using a Fourier transform infrared spectrometer (Shimadzu IRAfnity-1S).

5.2.5 Cultivation of *Aspergillus versicolor*

A 2% concentration of the three xylans generated in the study were evaluated as a substrate for the growth of *A. versicolor* in minimal medium (MM) (BARRATT et al., 1965) for 10 days at 37 °C without agitation. Collections were performed at 48, 96, 144, 192 and 240 h. The samples were centrifuged at 2000 x*g* for 5 min to remove cells and obtain the supernatant, which was then submitted to a determination of xylanase activity (IU/mL) according to Miller (1959).

5.2.6 Determination of enzymatic activity of xylanase

The enzymatic activity of the xylanase purified from Aspergillus versicolor was evaluated as described in Chapter 4, item 2.3, following the methodology of Miller (1959). The enzymatic reaction was prepared using 1% of each modified xylan (w/v) and 100µl of xylanase in a final volume of 1 mL for 5 min at 50 °C. All reactions were performed in triplicate.

5.2.7 Xylan-based bioplastics

The formation of the bioplastics in this study followed the methodology of Macedo et al. (2022), with modifications such as the addition of 0.5% (w/v) bis-acrylamide in the formulation of the bioplastics, following the methodology of Sun et al. (2013). All bioplastics in the study were composed of 5% total polysaccharides (w/w total mass of the filmogenic solution), including xylan (X1, X2, X3, and X4), starch and 20% glycerol (w/w of polysaccharides), in addition to the use of bis-acrylamide as described previously. The gelatinization process of the bioplastic was performed at 80 °C for 2 min. The content was arranged in Petri plates and submitted at 30 °C for 24 h for drying of the films.

5.2.8 Evaluation of biofilm properties

I) Opacity

The opacity test was realized in triplicates with dimensions of 3.0 × 0.9 cm, supported on the wall of a glass cuvette, and samples were read at a 450nm wavelength at spectrophotometer (Biospectro, SP-22). The opacity was determined by the division between the absorbance and the thickness of biofilms (GOUNGA et al., 2007).

II) Moisture and solubility

Samples (X1 to X4) in triplicate of 0.9 × 1.5 cm were weighed and oven-dried at 105 °C for 24 h. The procedure was repeated until a constant mass had been obtained, and its moisture percentage was then determined from the difference in masses before and after drying, which represents the water content present in the bioplastic (JARAMILLO et al., 2017). The samples were inserted in a different beaker containing 50 mL of distilled water and then stirred at 110 rpm at room temperature for 24 h. After this period, they were again oven-dried at 105 °C until the obtaining of the final dry mass (JARAMILLO et al., 2017; AZEREDO et al., 2015). After leaving the oven and before being weighed, the samples were kept in a desiccator to cool.

III) Mechanial test

Tensile stress and elongation at break of samples (including X1 to X4 xylan-based bioplastics) were measured by an Engco texturometry machine. The thickness of specimens of 2 cm in length and 0.6 cm in width were previously measured in different and random regions by a digital 0–25 mm micrometer of 0.001 mm resolution (Mitutoyo 293-230). The test was conducted at a 1 mm.min^{-1} displacement speed.

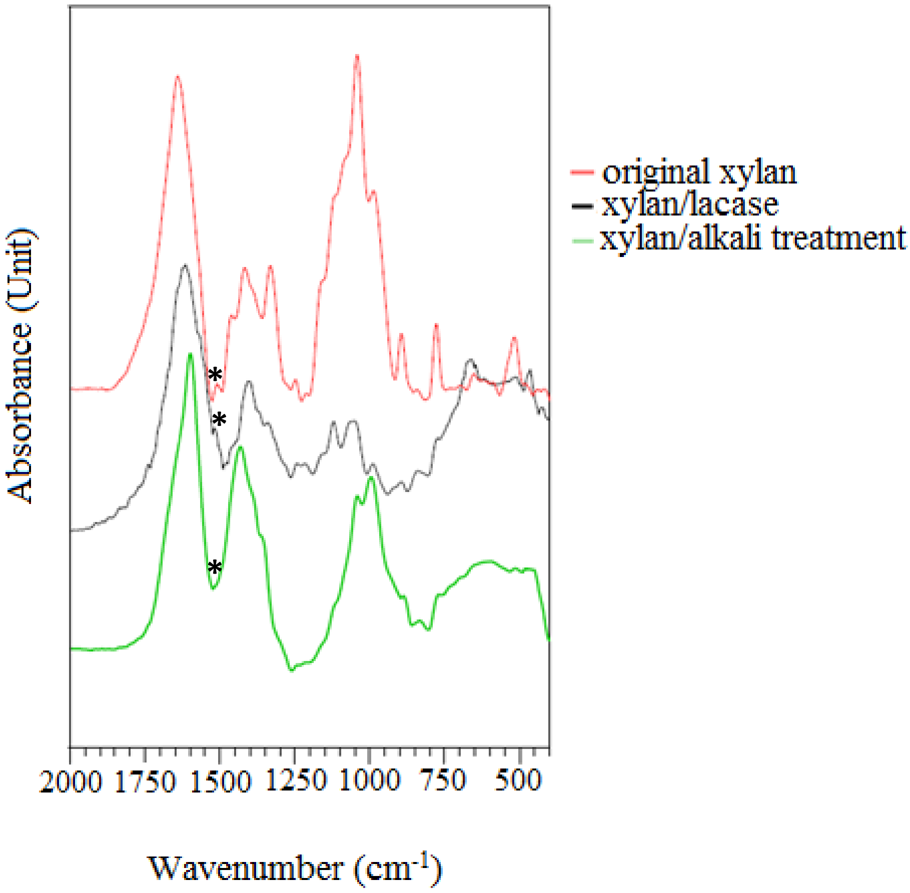
5.3 Results and discussion

The results obtained in the study are summarized in figures 5.1 to 5.3. The first analysis of the work refers to the comparison via FTIR of the composition of the three xylans obtained in the study (original, xylan modified with lacase and xylan modified with alkaline treatment), with emphasis on the removal of residual lignin in search of greater purity of xylan (Figure 5.1).

Through the results of FTIR analysis it is observed that there was a delignification of xylan when comparing the presence of the band at 1562 cm^{-1} corresponding to the aromatic skeleton of the lignin molecule (MAZIERO et al., 2012) between the original xylan, still with residual lignin content, with the xylans modified with lacase and via alkali treatment (Figure 5.1). The xylan modified with alkali treatment showed the lowest lignin content, with an absent band at 1562 cm^{-1}. The xylan modified via enzymatic hydrolysis using lacase also showed some delignification when comparing its band at 1562 cm^{-1} with this same band of the original xylan, however, it is observed that this band at 1562 cm^{-1} is still higher than that observed for xylan modified via alkaline treatment, reinforcing that the alkaline treatment of xylan showed the best delignification effect. Additionally, through the FTIR it is possible to observe the bands between 1166 and 1000 cm^{-1} that are typical for sugarcane bagasse xylan (JAYAPAL et al., 2013).

After the analyses of lignin removal from xylan through FTIR, the determination of the xylanase activity of *Aspergillus versicolor* was started using the three xylans obtained in the study as substrate at the final concentration of 1% (Figure 5.2). In these enzymatic reactions, 100 microliters of xylanase were used under equal conditions for all samples analyzed (50 °C for 5 min).

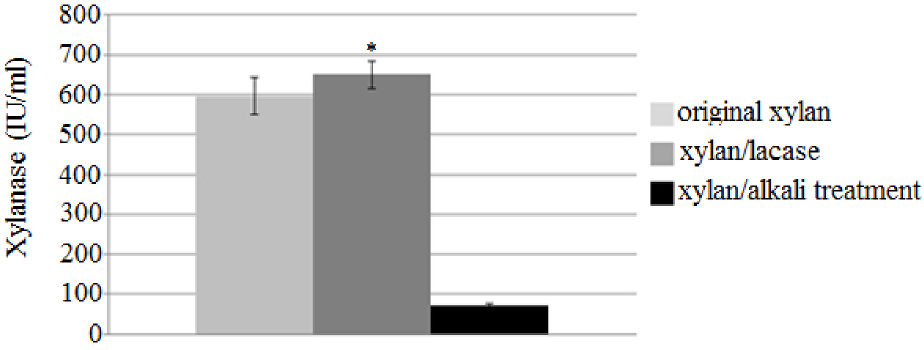
Figure 5.1 – Comparison of FTIR analysis of xylan modifications. The symbol (*) indicates the band corresponding to aromatic skeleton of the lignin molecule according to Maziero et al. (2012). In this way, the illustration shown that the lignin is present in the original xylan samples (red line), xylan hydrolyzed with lacase (black line) and absent in the sample of xylan submitted to alkaline treatment (green line).



Source: Author.

The results indicate that the highest activity of xylanase from *A. versicolor* was observed when xylan previously hydrolyzed with laccase was used as substrate. The alkaline-treated xylan was not an ideal substrate for xylanase activity since the results obtained were low, with a xylanase activity about five times lower than that observed for the unprocessed xylan. The hypothesis is that the alkaline treatment to which the unprocessed xylan was subjected was probably severe and damaged the integrity of the xylan molecule, probably causing the xylanase to not perform as expected.

Figure 5.2 – Determination of xylanase activity in three different substrates. Original xylan: xylan that was initially extracted from sugarcane bagasse by alkaline pretreatment. Xylan/laccase: xylan submitted to enzymatic hydrolysis in a reaction containing 1.67 IU/g of laccase. Xylan/alkali treatment: xylan submitted to a second chemical treatment with hydrogen peroxide. * indicates that although the values between original xylan and xylan/lacase are close, the media of xylan/lacase is higher and its standard deviation reaches values greater than the standard deviation of original xylan.

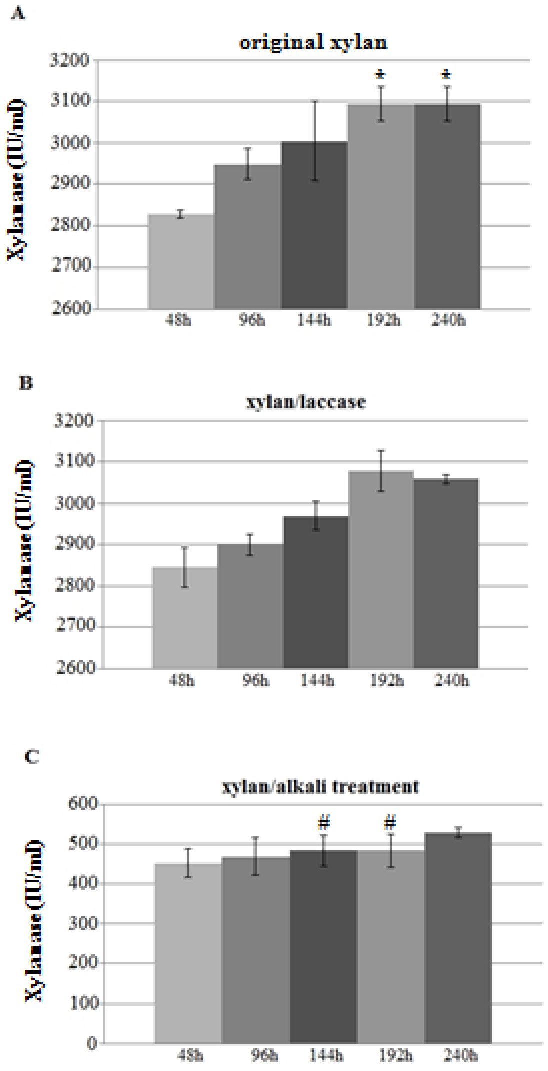


Source: Author.

Aspergillus niger was genetcaly modified to obtain a higher thermostability xylanse. The different mutants generated were evaluated for xylanase activity with beechwood xylan (1%) as substrate, and the highest specific activity observed in the study was close to 200 IU/mg (LI et al., 2022). A second one induced mutations in the fungus *Aspergillus niger* through UV radiation in the quest to enhance xylanase production, and xylanase activity in the presence of beechwood xylan (0.5%) was determined, and it was observed that the highest xylanase activity observed in *A. niger* mutants was 9.36 IU/mL (IRE et al., 2021). In the present study, the wild-type *A. versicolor* fungus showed a xylanase activity close to 700 IU/mL in the presence of 1% xylan delignified with laccase, the best treatment obtained in this study.

The xylans obtained in this study besides being used as a substrate to determine the xylanase activity were also evaluated as a carbon source for the growth of *A. versicolor* over 10 days. In this growth, which was performed in a minimal medium, the soly carbon source available was xylan. The three xylans obtained in this study were compared to determine which one would be an ideal carbon source for the highest xylanase activity observed over time (Figure 5.3). It was possible to observe through the maximum result of original xylan observed was equal to 3150 IU/mL (Figure 5.3a) and 3120 IU/mL for xylan previously hydrolyzed with laccase at 192 h of cultivation (Figure 5.3b). For the substrate composed of xylan submitted to alkaline treatment, the highest activity of xylanase from *A. versicolor* was observed after 240 h of growth (540 IU/mL). The results of the growth of *A. versicolor* on different xylans (substrates) indicate that the xylan delignified with laccase presents itself as a substrate of closed quality when compared to the original xylan (containing 5% of residual lignin).

Figure 5.3 – *Aspergillus versicolor* growth over 10 days on three different substrates (xylans) and evaluation of xylanase activity over time. A- Original xylan: xylan that was initially extracted from sugarcane bagasse through alkaline pretreatment. B- Xylan/laccase: xylan submitted to enzymatic hydrolysis in a reaction containing 1.67 IU/g of laccase. C- Xylan/alkali treatment: xylan submitted to a second chemical treatment with hydrogen peroxide. * the values of the medium and standard deviations for 192 h and 240 h are statistically equal for the xylanase activity (IU/mL) of the original substrate xylan. # the values of the medium and standard deviations for 192 h and 240 h are statistically equal for the xylanase activity (IU/mL) of the xylan/alkali treatment.



Source: Author.

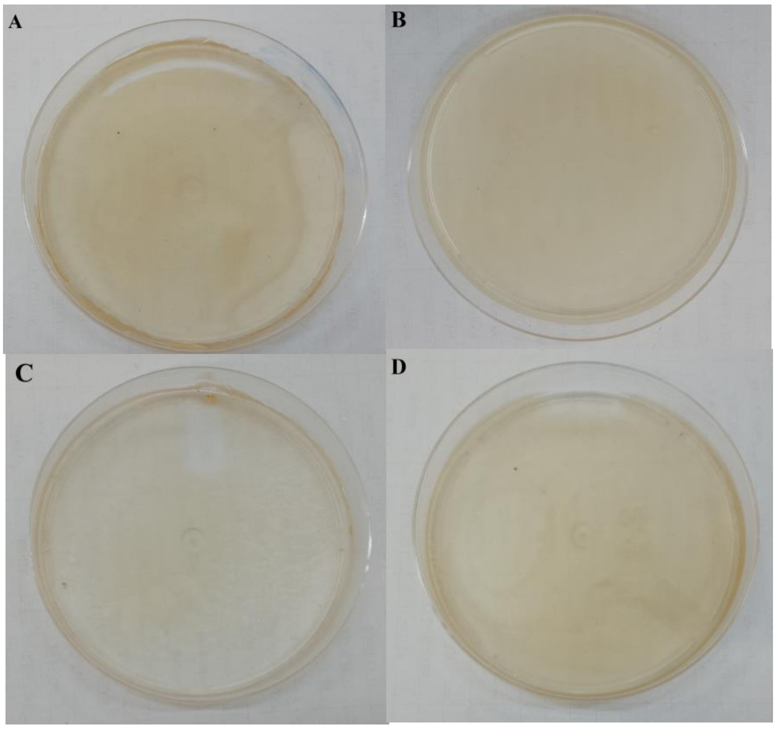
The xylan submitted to a second alkaline treatment presented itself as the worst substrate among the three evaluated. The hypothesis is that a second alkaline treatment on the xylan may possibly have modified its polymerization degree, and yet the alkaline treatment reaction possibly left NaOH residues on the xylan, and both situations probably hindered the action of xylanase.

A study in the literature investigated the xylanase activity of *Aspergillus tamarii* over 120 h using 1% sugarcane bagasse xylan as substrate, and the results indicated that the highest xylanase activity (1.91 U/mL) occurred after 72 h of growth (LAGUNES et al., 2021). Another study investigated the xylanase activity of *Aspergillus heteromorphus* in the presence of rice straw (1-5%) as substrate over 5 days, and at the end of this fermentation period the highest xylanase activity identified was 11.6 IU/mL (BAJAR et al., 2020). The results of the present study for the three xylans evaluated as substrate were higher than those observed in the literature, even when comparing the xylan submitted to alkaline treatment that here presented the lowest values of xylanase activity (540 IU/mL after 10 days of cultivation).

All bioplastics obtained in the study (B1, B2, B3 and B4) presented an intact structure, without breaks and light brown color (Figure 5.4). Among the four bioplastics formed, the bioplastic formulated with original xylan (B1) presented the darkest brown coloration (Figure 5.4a), probably due to its higher content of residual lignin (5%), while the bioplastic formulated with xylan previously hydrolyzed with laccase presented the lightest coloration (Figure 5.4c), possibly due to the removal of the lignin content of the xylan. It is known that the presence of lignin influences the coloration of the bioplastic (ZHANG et al., 2020). The bioplastics obtained in this study showed lighter brown coloration than the bioplastics formulated with 25 and 50% xylan in relation to the total mass of polysaccharides and with 12.87% residual lignin content described by Macedo et al. (2022).

The results for the opacity of the xylan-based bioplastic original (B1, 3.2%) (Table 5.1) were close to the data described in the literature, as close to 3% for the bioplastic made from xylan in a proportion of 5% (w/v) of polysaccharides (MACEDO et al., 2022), since in this study this proportion was also followed. Compared to the study of Abe et al. (2022), where the opacity described was 2.54% for this same concentration of xylan in the bioplastic formulation, the data of the present study indicate that the bioplastic B1 presents higher opacity (3.2%).

Figure 5.4 – Xylan-based bioplastics. Bioplastics composed of modified xylans. In A: B1, B: xylan B2, C: B3 and D: B4, all containing starch, glycerol and bis-acrylamide in their composition.



Source: Author.

For the bioplastics prepared with delignified xylan (B2 and B3), which presented opacity of 2 and 2.3% (Table 5.1), it is noteworthy that the opacity of both were lower than the values described in the works of Macedo et al. (2022) and Abe et al. (2022), with values close to 3 % and exactly 2.54%, respectively. The data corroborate that the delignification of xylan can contribute to the reduction of the brown coloration and the transparency of the biofilm, because as previously described, lignin contributes to the coloration of the film (ZHANG et al., 2020).

Regarding the moisture content of the bioplastic, the bioplastics B1, B2, B3 and B4 showed values equivalent to 7, 4, 4 and 4%, respectively (Table 5.1). The bioplastic based on unprocessed xylan (B1) presented a higher percentage of moisture in relation to the other bioplastics formulated with delignified xylan (B2 and B3) and without arabinose pendent groups (B4). Relative to the literature, all bioplastics obtained in this work presented results for moisture below that observed in the study of Macedo et al. (2022), where the moisture was equivalent to 13.34% for the bioplastic made from xylan under the same concentration conditions as those used in the present study.

The bioplastics B1, B2, B3 and B4 showed solubility equal to 15, 12.3, 12.9 and 14%, respectively (Table 5.1). In the literature, the results for solubility of xylan and starch based bioplastics formulated using 5% polysaccharides were equivalent to 16.40% (ABE et al., 20) and

20.25% (MACEDO et al., 2022). The hypothesis of the higher values found for solubility in the present study is that bis-acrylamide may have contributed in decreasing water abosorption, since cross-linking agents reduce the solubility of hemicellulose in liquid and increase water resistance (ZHAO et al., 2020).

Table 5.1 – Xylan-based bioplastics and its respective composition and evaluation of the properties of bioplastics such as opacity, moisture, solubility and tensile stress.

Sample	Content	Opacity (%)	Moisture (%)	Solubility (%)	Tensile Stress (MPa)
B1	Original xylan, starch, glycerol and bis-acrilamida.	3.2 ± 0.2	7 ± 0.3	15 ± 0.3	1.7 ± 0.1
B2	Delignified xylan with 6% of H_2O_2. starch. glycerol and bis-acrilamida	2 ± 0.1	4 ± 0.7	12.3 ± 0.1	2.5 ± 0.2
B3	Delignified xylan with lacase, starch, glycerol and bis-acrilamida	2.3 ± 0.1	4 ± 0.2	12.9 0.3	2.3 ± 0.05
B4	Xylan without arabinose pendent groups, starch. glycerol and bis-acrilamida	2.7 ± 0.2	4 ± 0.5	14 ± 0.5	1.8 ± 0.1

Source: Author.

As for mechanical strength, the bioplastics formulated in this study (B1, B2, B3 and B4) showed a tensile stress of 1.7, 2.5, 2.3 and 1.8 MPa, respectively. The original xylan-based bioplastic (B1) showed higher tensile stress, since the residual lignin makes the bioplastic more mechanically vulnerable due to the tendency of lignin to form aggregates (YANG, et al. 2019; MACEDO et al., 2022). Starch is also known to be a reinforcing agent and contributes to the improvement in the mechanical properties of films (ZHAO et al., 2020). In comparison with literature data, bioplastic B2 showed tensile stress higher than 2.35 MPa as described by Macedo et al. (2022), where the same concentrations of polysaccharides and sugarcane bagasse xylan (12.87% residual lignin) were used, on the other hand, all other bioplastics in this present study showed tensile stress lower than 2.35 MPa.

Regarding the composition of bioplastics, the modifications in xylan extracted from sugar cane bagasse promoted in this study, such as delignification and arabinose removal, led the bioplastics to present better quality in their properties, such as reduction in moisture (%), solubility (%) and opacity (%), and contributed to a higher mechanical strength compared to the bioplastic B1. The presence of starch contributed to the mechanical strength of all bioplastics since it is a reinforcing agent, while bis-acrylamide probably promoted water resistance and this led to reduction in the solubility of the bioplastic compared to literature data (ZHAO et al., 2020), also including the presence of glycerol as a plasticizing agent.

5.4 Conclusion

This study aimed to evaluate the xylan extracted from sugarcane bagasse (original) promoting three modifications in this same xylan. The original xylan was modified via enzymatic hydrolysis with the application of laccase, α-L-arabinofuranosidase and alkaline treatment moditication. The original xylan and delignified xylans were evaluated as a substrate for growth of *A. versicolor* and determination of xylanase activity. It was possible to observe in this study that the xylan delignified with laccase presents itself as an interesting substrate since in its presence a high xylanase activity is detected (700 IU/mL) and in the cultivation of *Aspergillus versicolor*, after 192 h a xylanase activity equivalent to 3150 IU/mL is observed, generally higher than the data present in the current literature. Although the delignification of xylan makes it purer, it was also observed in this study that original xylan (containing 5% residual lignin) is also an interesting substrate for the determination of xylanase activity and for the cultivation of *A. versicolor*. Moreover, the bioplastics formulated with delignified xylan showed lower opacity, moisture and solubility (%), and major tensile stress than the bioplastic containing original xylan. The presence of starch and bis-acrylamide contributed to the mechanical strength of the material and resistance to water absorption and also integrity of the bioplastic, respectively. Therefore, this study is pioneering in demonstrating that providing the removal of lignin and arabinose from xylan and adding a cross-linker such as bis-acrylamide can contribute to the quality properties of xylan-based bioplastics, and also, the substrates generated from delignification of xylan possibly may be used instead of using commercial xylans such as beechwood and beechwood xylans, representing an easy lab protocol.

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CHAPTER 6: CONCLUSION

In the present study, the xylan was extracted from sugarcane biomass via hydrogen peroxide in alkaline medium, resulting in high yield but with residual lignin presence. Additionally, both xylanase and β-xylosidase enzymes from *A. versicollor* were produced and purified by ionic change chromatography and gel filtration. Two enzymatic cocktails containing the purified endoxylanases and four accessory enzymes purchased commercially were produced and evaluated, namely α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan-esterase, and feruloyl esterase. The enzyme cocktails developed in this study were able to completely hydrolyze xylan and also to produce xylooligosaccharides. Due to its wide composition in enzymes, these cocktails could possibly be used successfully in other types of lignocellulosic biomass, considering the heterogeneous character of the hemicellulose from different biomass. Additionally, this study aimed the delignification of xylan using laccase and treatment with hydrogen peroxide (6%) in alkaline medium, evaluating the effect of residual lignin in the xylan. The xylanase activity of *A. versicolor* was determined with both substrates to evaluate the influence of residual lignin in the enzymatic activity. The results showed that the delignification of xylan using laccase led to high enzymatic activities of xylanase and also this same xylan presented itself as an interesting substrate for the growth of *A. versicolor* with high xylanase activity.

The same original xylan and the delignified xylans (X2 and X3), in addition to the xylan without arabinose (X4) were used in the formulation of bioplastics containing also starch, glycerol, and bis-acrylamide. The results obtained in the study indicated that the modifications promoted in xylan (X2, X3 and X4) culminated in an improvement in the properties of these bioplastics, such as opacity, moisture, solubility, and tensile stress. This study is pioneering in promoting various modifications in xylan via enzymatic hydrolysis and chemical treatment, using enzyme cocktails, laccase, α-L-arabinofuranosidase, and hydrogen peroxyde, respectively, obtaining xylan derivatives with value-added compounds such as XOS, bioplastics, and substrates for cultivation of microorganisms.