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

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA APLICADA

ENZYMATIC AND CHEMICAL PRODUCTION OF XYLOOLIGOSACCHARIDES FROM SUGARCANE BAGASSE AND LEAF

CAROLINA FROES FORSAN

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Microbiologia Aplicada.

Orientador: Prof. Dr. Michel Brienzo

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F732e

Forsan, Carolina Froes
Enzymatic and chemical production of xylooligosaccharides from sugarcane bagasse and leaf / Carolina Froes Forsan. -- Rio Claro, 2020
114 p. : il., tabs., fotos

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências, Rio Claro
Orientador: Michel Brienzo

1. Xilo-oligossacarídeos. 2. Hemicelulose. 3. Hidrólise enzimática. 4. Hidrólise ácida. 5. Auto-hidrólise. I. Título.

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TÍTULO DA DISSERTAÇÃO: **ENZYMATIC AND CHEMICAL PRODUCTION OF XYLOOLIGOSACCHARIDES FROM SUGARCANE BAGASSE AND LEAF**

AUTORA: CAROLINA FROES FORSAN
ORIENTADOR: MICHEL BRIENZO

Aprovada como parte das exigências para obtenção do Título de Mestra em CIÊNCIAS BIOLÓGICAS (MICROBIOLOGIA APLICADA), área: Microbiologia Aplicada pela Comissão Examinadora:

Prof. Dr. MICHEL BRIENZO
Laboratório de Caracterização de Biomassa / Instituto de Pesquisa em Bioenergia do Câmpus de Rio Claro - UNESP

Profa. Dra. SIMONE COELHO NAKANISHI
Laboratório Nacional de Biorrenováveis - LNBR / CNPEM - Centro Nacional de Pesquisa em Energia e Materiais

Prof. Dr. RENATO NALLIN MONTAGNOLLI
Departamento de Ciências da Natureza, Matemática e Educação / UFSCar

Rio Claro, 19 de fevereiro de 2020

DEDICATION

This work is dedicated to my parents, Ana Luiza and José, brothers, Juliana and Humberto and to my fiance, Danilo, for the encouragement, love and affection

ACKNOWLEDGMENT

To my advisor, Professor Michel Brienzo, for the opportunity, trust, dedication, patience and all the teachings.

To my parents, Ana Luiza and José, for supporting me at all times and for everything they always do for me.

To my fiancé, Danilo for the support, encouragement and for always being so present in my life.

To my brothers Juliana and Humberto for all their help and by the example they are for me.

To Jefferson, for their friendship, for all their help and for being so helpful to everyone.

To Alison, for helping with chemical characterization analysis.

To Carol, for providing the enzyme used in this study.

To all my friends who are so important to me.

To Professor Dr. Fernando Masarin, for allowing the use of HPLC for XOS quantification.

To Fernando Paz, Ana Lúcia Nasser and Adriana Moura, for their help with XOS quantification.

To Graduate Program in Applied Microbiology and the Bioenergy Research Institute for the opportunity.

And to all who contributed directly or indirectly to this work.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

ABSTRACT

From biomass, high-value products can be obtained, including xylooligosaccharides (XOS). They act as prebiotics, undigested food components that stimulate the growth of beneficial microorganisms present in the intestine, such as *bifidobacteria* and *lactobacilli*, improving host health. XOS has a range of applications in the food and pharmaceutical industry. The aim of this study was to compare the effectiveness of dilute acid hydrolysis, enzymatic hydrolysis and autohydrolysis methods using sugarcane bagasse and leaf to XOS production. Sugarcane bagasse and leaf were hydrolysate with sulfuric or acetic acid and parameters were defined through a central composite design 2^3 using sulfuric or acetic acid at concentrations between 1 % and 3 % (m/v); temperature from 100 °C to 160 °C and reaction time from 15 to 55 min. In autohydrolysis a central composite design 2^2 was performed using temperatures ranging from 100 °C to 160 °C with a reaction time of 15 to 55 min. XOS were also produced from xylan extracted from sugarcane bagasse and leaf. Alkaline extraction of xylan was performed with 6 % (m/v) hydrogen peroxide, for 4 h at 25 ºC. Xylan was used in hydrolysis with dilute acetic and sulfuric acid and in enzymatic hydrolysis. The parameters of the acid hydrolysis were defined through a central composite design 2^2 with acid concentration from 1.5 % to 4 % (m/v), reaction time from 10 to 30 min, and temperature of 130 °C. Enzymatic hydrolysis was performed using endo-β-1.4-xylanase from *Aspergillus versicolor* and central composite design 2^2 with enzyme activity from 30 to 100 $UI.g^{-1}$, substrate (xylan) concentration from 1 % to 5 % for 24 h. In hydrolysis of bagasse and leaf with dilute sulfuric acid the conversion of xylan into XOS was 90.13 % and 62.18 % under similar conditions (79.55 ºC, 35 min and 2 % m/v acid). The hydrolysis of bagasse and leaf with acetic acid resulted in a yield of 18.41 % (100 ºC, 15 min and 1% m/v acid) and 22.78 % (180.45 ºC, 35 min and 2 % m/v acid). In autohydrolysis the values obtained for bagasse and leaf were 13.67 % (35 min and 172.43 ºC) and 20.71 % (35 min, 130 ºC). In hydrolysis of xylan extracted from sugarcane bagasse and leaf with sulfuric acid the values were 40.16 % and 45.37 % under similar conditions (1.5 % acid concentration, 10 min at 130 ºC). In hydrolysis of xylan extracted from sugarcane bagasse and leaf with acetic acid the values were 56.29 % (1.5 % acid concentration, 30 min at 130 ºC) and 53.65 % (2.75 % acid concentration, 34.14 min at 130 ºC). In enzymatic hydrolysis of xylan extracted from sugarcane bagasse and leaf, the values were 67.43 % and 69.71 % under similar conditions (enzymatic load of 65 $UI.g^{-1}$ and substrate concentration of 0.17 %). The highest results of xylan conversion into XOS were obtained in the hydrolysis of sugarcane bagasse and leaf with sulfuric acid and in the hydrolysis of xylan from sugarcane bagasse and leaf with acetic and sulfuric acid and enzymatic hydrolysis. Except for enzymatic hydrolysis, there was xylose production. The lowest yield was obtained on autohydrolysis due to milder conditions. Xylose is undesirable in the process as it hinders the purification of XOS. Based on the results, enzymatic and acid hydrolysis has shown promise in large scale XOS production.

Keywords: Hemicellulose; Xylooligosaccharides; Enzymatic hydrolysis; Acid hydrolysis; Autohydrolysis.

RESUMO

A partir da biomassa podem ser obtidos produtos de alto valor, entre eles, os xilooligossacarídeos (XOS). Eles agem como prebióticos, componentes alimentares que não são digeridos e estimulam o crescimento de microrganismos benéficos presentes no intestino, como as *bifidobactérias* e os *lactobacilos,* melhorando a saúde do hospedeiro. XOS tem uma variedade de aplicações na indústria alimentícia e farmacêutica. O objetivo deste estudo foi comparar a eficácia dos métodos de hidrólise com ácido diluído e auto-hidrólise com hidrólise enzimática, utilizando bagaço e folha de cana-de-açúcar para produção de XOS. O bagaço e a folha foram hidrolisados com ácido sulfúrico ou acético e os parâmetros foram definidos através de um planejamento composto central 2^3, utilizando ácido sulfúrico ou acético em concentrações entre 1 % e 3 % (m/v); temperatura de 100 °C a 160 °C e tempo de reação de 15 a 55 min. Na auto-hidrólise, um planejamento composto central 2^2 foi realizado com temperaturas variando de 100 °C a 160 °C e tempo de reação de 15 a 55 min. XOS também foram produzidos a partir da xilana extraída do bagaço e da folha de cana-de-açúcar. A extração alcalina da xilana foi realizada com peróxido de hidrogênio 6 % (m/v), por 4 horas a 25 ºC. A xilana foi utilizada na hidrólise com ácido acético e sulfúrico diluído e na hidrólise enzimática. Os parâmetros da hidrólise ácida foram definidos através de um planejamento composto central 2^2 com concentração ácida de 1,5 % a 4 % (m/v), tempo de reação de 10 a 30 min e temperatura de 130 °C. A hidrólise enzimática foi realizada com endo-β-1,4-xilanase de *Aspergillus versicolor* e planejamento composto central 2^2 com atividade enzimática de 30 a 100 $UI.g^{-1}$, concentração de substrato (xilana) de 1 % a 5 % por 24 h. Na hidrólise do bagaço e da folha com ácido sulfúrico diluído, a conversão da xilana em XOS foi de 90,13 % e 62,18 % em condições semelhantes (79,55 ºC, 35 min e ácido 2 % m/v). A hidrólise do bagaço e da folha com ácido acético resultou em rendimento de 18,41 % (100 ºC, 15 min e ácido 1% (m/v) e 22,78 % (180,45 ºC, 35 min e ácido 2 % m/v). Na auto-hidrólise, os valores obtidos para bagaço e folha foram 13,67 % (35 min e 172,43 ºC) e 20,71 % (35 min, 130 ºC). Na hidrólise da xilana extraída do bagaço e folha com ácido sulfúrico, os valores foram de 40,16 % e 45,37 % em condições similares (concentração de ácido de 1,5 %, 10 min a 130 ºC). Na hidrólise da xilana extraída do bagaço e da folha com ácido acético, os valores foram 56,29 % (concentração de ácido de 1,5 %, 30 min a 130 ºC) e 53,65 % (concentração de ácido de 2,75 %, 34,14 min a 130 ºC). Na hidrólise enzimática da xilana extraída do bagaço e da folha, os valores foram de 67,43 % e 69,71 % em condições semelhantes (carga enzimática de 65 $UI.g^{-1}$ e concentração de substrato de 0,17 %). Os maiores resultados da conversão de xilana em XOS foram obtidos na hidrólise do bagaço e folha de cana-de-açúcar com ácido sulfúrico e na hidrólise enzimática e com ácido acético e sulfúrico da xilana do bagaço e da folha de cana-de-açúcar. Exceto pela hidrólise enzimática, houve produção de xilose. O menor rendimento foi obtido na auto-hidrólise devido a condições mais amenas. A xilose é indesejável no processo, pois dificulta a purificação do XOS. Com base nos resultados, a hidrólise enzimática e ácida mostrou-se promissora na produção de XOS em larga escala.

Palavras-chave: Hemicelulose; Xilo-oligossacarídeos; Hidrólise enzimática; Hidrólise ácida; Auto-hidrólise.

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SUMMARY

CHAPTER I: INTRODUCTION AND OBJECTIVES

1.1 Introduction

Brazil stands out as the world's largest producer of sugarcane, used for sugar and ethanol production (MELATI et al., 2017). During the harvesting process and after milling, waste is generated such as straw and bagasse, currently burned to obtain energy for the mill itself. However, a number of researches have been conducted to use these materials to produce second-generation ethanol and other high-value products (FERNANDES et al., 2017). These products include xylan derivatives. Xylooligosaccharides (XOS), xylose oligomers, are compounds of interest in the food industry and can be obtained from xylan hydrolysis (FREITAS; CARMONA; BRIENZO, 2019; LIN et al., 2016).

Among the biomass constituents, hemicellulose, a polysaccharide composed mainly of xylan, has aroused great interest due to its variety of applications in chemical, pharmaceutical and food industries and can also be used for biofuel production (BRIENZO et al., 2016a). When hydrolyzed it may give rise to xylose monomers or xylooligosaccharides (XOS). XOS are considered functional food ingredients that help the body function properly, promoting health (JI et al., 2012).

XOS can be found naturally in foods or industrially obtained and added to products (MENEZES; DURRANT, 2008). They act as prebiotics, that is, are non-digestible food components that stimulate the growth of beneficial microorganisms such as *Bifidobacterium* and *Lactobacillus*, bringing innumerable advantages to the host, such as inhibiting the growth of harmful bacteria, preventing colon cancer, reducing levels. of cholesterol and glycemia, stimulation of the immune system, reduction of infection, among other benefits (LIN et al., 2016).

Lignocellulosic materials have the potential for large-scale production of XOS, because they have advantages such as low cost and abundance. In addition, its use is an alternative for reducing waste in the environment (BARRETO; ZANCAN; MENEZES, 2015; CALDERON; ARANTES, 2019).

XOS can be produced by pretreatments such as autohydrolysis, which uses water heated at high temperatures; chemical treatments such as acid hydrolysis, which uses concentrated or diluted, mineral or organic acids, and enzymatic hydrolysis, which uses enzymes extracted from microorganisms. Lignocellulosic biomass can be used as a raw material but its complex chemical composition makes the hydrolysis process difficult, as an

alternative, it is possible to perform the process in two steps: isolation of xylan with the use of chemical reagents and subsequent acid or enzymatic hydrolysis (QING et al. 2013).

In this context, this study aims to compare XOS production methods such as autohydrolysis, hydrolysis with dilute sulfuric and acetic acid and enzymatic hydrolysis with endo-β-1.4-xylanase from *Aspergillus versicolor* using sugarcane leaf, bagasse and the xylan extracted from these materials.

1.2 Objectives

The objective of this study was to compare enzymatic and chemical pretreatments for xylooligosaccharides production, using sugarcane bagasse and leaf.

1.2.1 Specific Objectives

- Optimize autohydrolysis and hydrolysis with dilute acetic and sulfuric acid of sugarcane bagasse and leaf to produce xylooligosaccharides;
- Extract xylan and Hydrolyze the xylan with dilute acetic and sulfuric acid and endo-β-1.4-xylanase from *Aspergillus versicolor* for XOS production.

CHAPTER II: BIOMASSES FOR XYLAN AND XYLOOLIGOSACCHARIDES PRODUCTION

Abstract

Hemicellulose is a polysaccharide often formed by xylan chains with a wide variety of applications in the industry in obtaining products of high value such as emulsifiers, food additives, thickeners, gelling agents, biopolymers and in its hydrolyzed form may be used in the production of ethanol, xylitol, butanol, xylooligosaccharides (XOS), among others. XOS act as prebiotics, that is, they are food components that are not digested but stimulate the growth of beneficial microorganisms present in the intestine, such as *bifidobacteria* and *lactobacilli*, improving the health of the host. They have great prominence due to their enormous variety of applications in the food and pharmaceutical industry. The raw material for obtaining hemicellulose is biomass a renewable and abundant source in nature, composed mainly of cellulose, hemicellulose and lignin, which can be obtained from industrial waste, agricultural and other sources. Moreover, several dedicated crops such as sorghum, elephant grass, energy cane has aroused great interest in research. These non-wood biomasses are characterized for its short growth and harvest period. This review aims to discuss the biomass, its production process, and characteristics (chemical composition, fiber yield and hemicellulose composition), targeting mainly wood and grasses for the production xylan and XOS.

Keywords: Biomass; Xylooligosaccharide; Wood; Grasses; Non-wood biomass.

2.1 Introduction

Around the world there is a growing concern about health and quality of life, increasing the search for beneficial foods. In this context, functional foods have aroused interest for presenting components that help the body to function properly, promoting health. These compounds can be found naturally in foods or obtained industrially and added to products (MENEZES; DURRANT, 2008; JI et al., 2011).

Among these compounds stand out the xylooligosaccharides (XOS), compounds constituted of xylose units, which have a prebiotic effect, stimulating bacteria present in the intestine with the potential to improve the health of the host (CHITRA; SHARAVANAN, 2015).

XOS can be obtained through various processes such as autohydrolysis, acid or enzymatic hydrolysis of xylan, the main component of hemicellulose, the second most abundant polysaccharide in plant biomass (MENEZES et al., 2010). In addition, hemicellulose has several applications in chemical, pharmaceutical and food industries, and

can be used for the production mainly of biofuels and biofilms (BRIENZO et al., 2016a). Moreover, when hydrolyzed into xylose monomers, it can be used in the production of xylitol (JI et al., 2011).

The use of biomass is advantageous because that can be obtained from sources such as wood, industrial waste, agricultural crops, food, plants and algae (GUEDES et al., 2010) which has the advantages such as low cost, renewability and re-usage of waste (SAIDUR et al., 2011). For this reason, lignocellulosic materials are an interesting alternative to the large-scale production of XOS and high-value products.

The aim of this chapter is a review on biomass and comparing some methods for xylan and XOS production using different sources of biomass.

2.2 Plant cell wall and chemical composition

The plant cell wall is a physical barrier that protects the contents of the cell. The cell wall structure is highly organized in layers and composed of microfibers that give resistance and rigidity. Its chemical composition varies according to plant location, species, fraction/tissue and also by the harvest method. Table 2.1 shows the chemical composition of several biomasses (SANT'ANNA; SOUZA; BRIENZO, 2014).

Table 2.1 - Chemical composition of selected lignocellulosic materials. Content presented as percentage (grams/100 grams of material in dry basis.

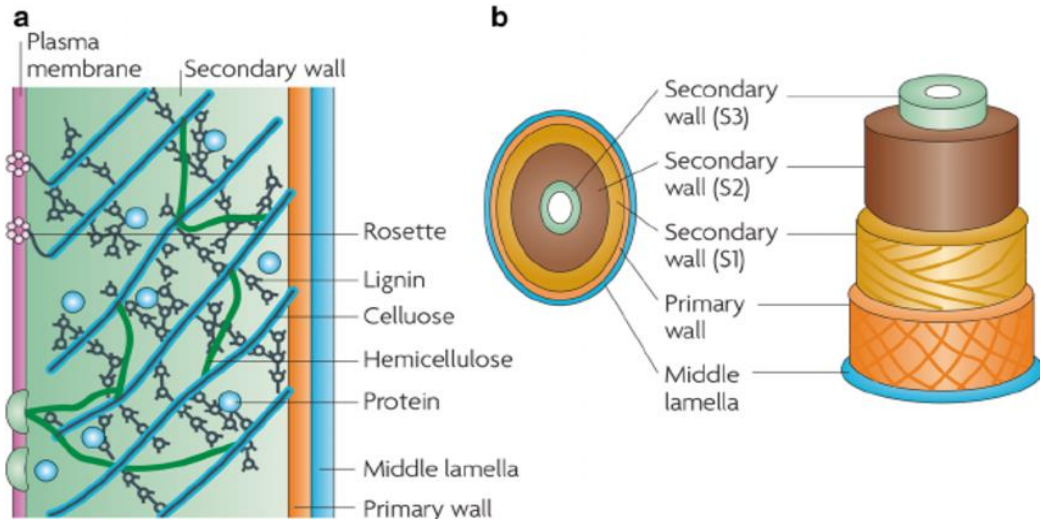
Lignocellulosic biomass	**%Cellulose**	**%Hemicellulose**	**%Lignin**	**References**
Sugarcane straw	40-44	30-32	22-25	Santos et al. (2012)
Sugarcane bagasse	32-48	19-24	23-32	Aguiar et al. (2018)
Hardwood	43-47	25-35	16-24	Santos et al. (2012)
Softwood	40-50	25-35	16-33	Pasangulapati et al. (2012)
Eucalyptus grandis	47.2	15.4	26.8	Vena et al. (2015)
Pinus	44.5	22	28	Hamelinck, 2005
Grasses	25-40	35-50	10-30	Kumar et. al. (2009)
Big bluestem	31.8–36.5	24.9–29.7	14.4–18	Zhang et al. (2012)
Switchgrass	45	31.4	12	Kumar et. al. (2009)
Miscanthus	35–40	16–20	20–25	Galletti; Antonetti (2012)
Elephant grass	40.74	39.94	6.38	Almeida et al. (1999)
Indiangrass	49.8	43.1	6.7	Gupta et al. (2014)
Sorghum	38.5	22.8	23.2	Freita et al. (2016)
Energy cane	43	24	22	Kim; Day, (2011)

The plant cell wall is composed of several layers (Figure 2.1). The first layer is the primary wall, consisting mainly of carbohydrates and some proteins. Below the primary wall is the secondary wall, which is subdivided into layers S1, S2 and S3 (SIQUEIRA, 2015). In

the secondary wall and in the middle lamella (outermost connecting layer between cells) lignin appears as an important component, along with cellulose fibers, thereby providing the plant cell mechanical resistance and stiffening. It is necessary to deconstruct the plant cell wall to have access to the polysaccharides of interest; this process is complex and depends on the intermolecular organization among the main components: cellulose, hemicellulose and lignin (NOVO, 2012; SANT'ANNA; SOUZA; BRIENZO, 2014).

The knowledge of the plant cell wall composition and structure is of fundamental importance so that biomass can be used as a source of renewable energy and for the generation of products with high-value (EMBRAPA, 2011).

Figure 2.1 - Representation of a plant cell wall



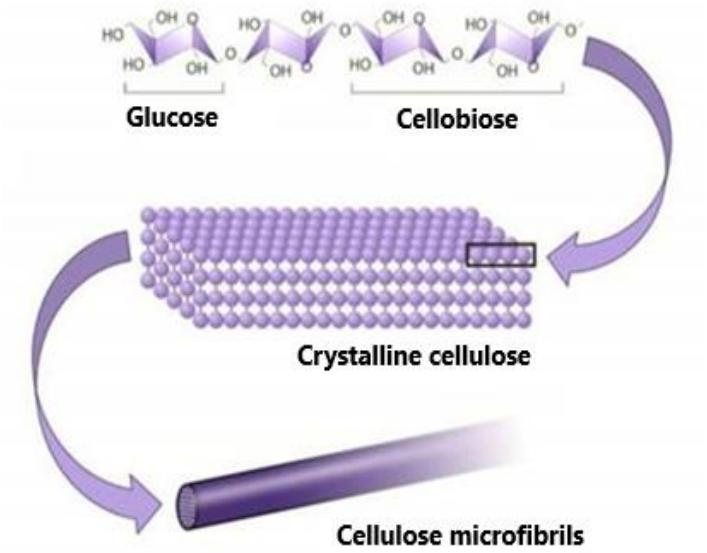
Fonte: Sticklen (2008).

2.3 Primary components of lignocellulosic material

2.3.1 Cellulose

Cellulose is the major constituent of the plant cell wall by weight, which makes it the most abundant polysaccharide biopolymer in the world. Cellulose has a linear chain, presents high molecular weight and is formed by D-anhydroglucose monomers joined by an β-glycosidic linkage (Figure 2.2) (SANTOS, 2008).

Figure 2.2 - Units of β-D-glucose coupled to form the cellulose chain



Source: Fonseca (2015).

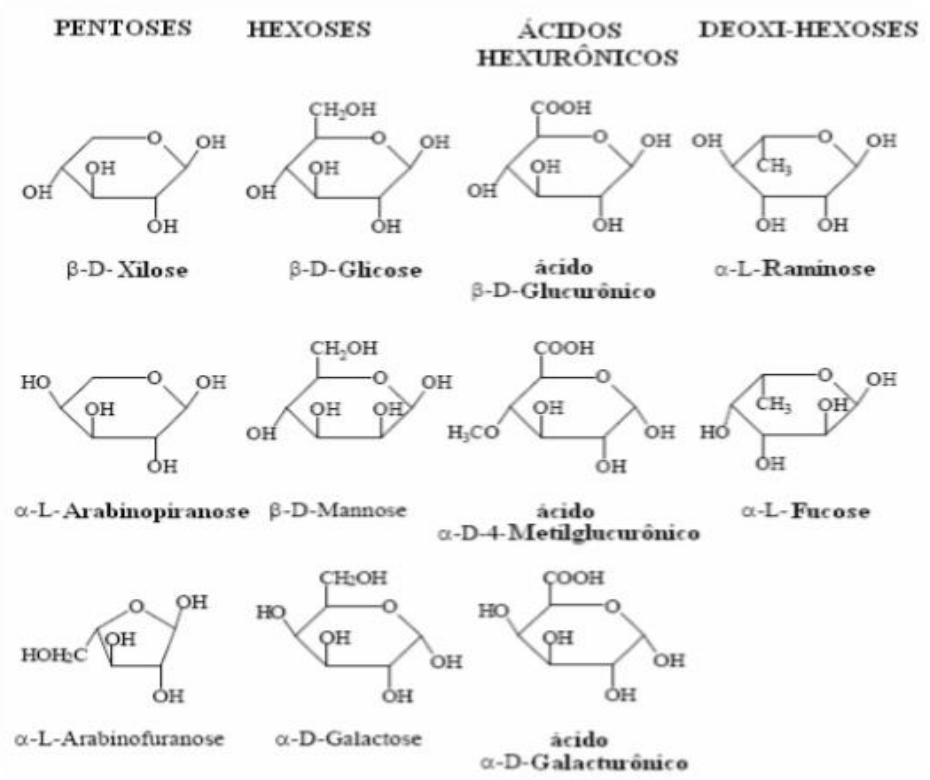
The cellulose polymers form a microfibril structure within which a highly ordered structure denominated as a crystalline region can be found. There are regions with less degree of ordination that are denominated as amorphous regions of microfibrils, where it is possible to find other substances such as lignin which is an aromatic polymer of the plant cell wall and hemicellulose, a polysaccharide. The association among cellulose, hemicellulose and lignin is responsible for the resistance of lignocellulosic materials to thermal, chemical and biological degradation (CORTEZ; LORA; GÓMES, 2008).

2.3.2 Hemicellulose

Hemicellulose is the second major polysaccharide commonly found in the lignocellulosic material. Hemicellulose interlinks with cellulose through hydrogen bonds and with lignin by covalent bonds. In the plant it is responsible for transporting water and nutrients and regulating porosity. It is formed by several types of sugars and has branched and short-chain, is generally formed by 80 to 200 units of sugar residues (SANTOS, 2008; BRIENZO et al., 2016a; OGATA, 2013; OGEDA; PETRI, 2010; CRIVELLARI, 2012; EK; GELLERSTEDT; HENRIKSSON, 2016).

Corresponds to approximately 15 to 35 % of the lignocellulosic material, and is composed of pentoses (sugars of 5C), which are composed mainly of D-anhydroxylose and L-anhydroarabinose and hexoses (sugars of 6C), constituted by D-anhydroglucose, D-anhydrogalactose and D-anhydromanose and uronic acid constituted by 4-O-methyl-D-glucuronic and D-galacturonic acids (Figure 2.3) and acetic acid (OGATA, 2013; CRIVELLARI, 2012).

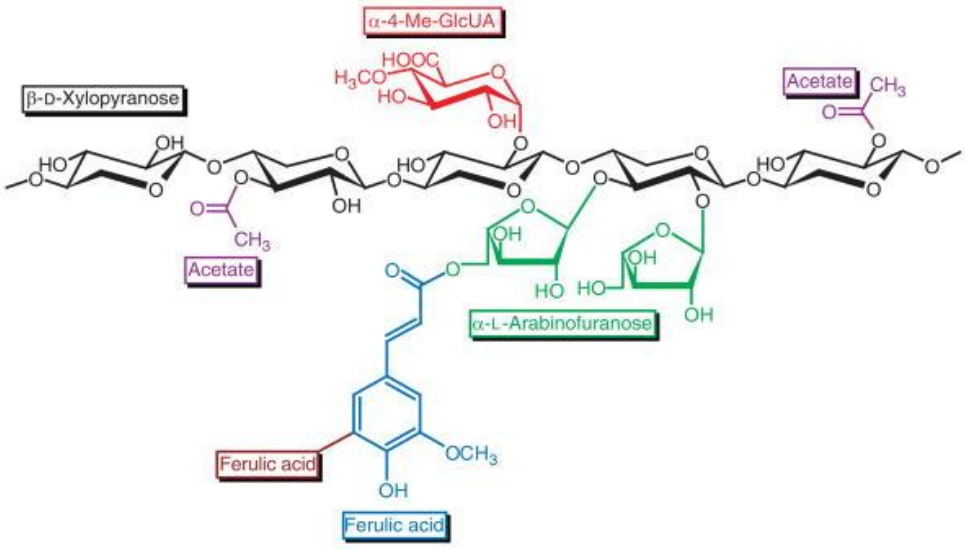
Figure 2.3 - Main components of hemicellulose



Source: Fengel; Wegener (1984).

The main hemicellulose is xylan (Figure 2.4), and there are other polysaccharides such as glucomannan, arabinogalactan, xyloglucan and galactoglucomannan (ALHAJ, 2013).

Figure 2.4 - Structure of hemicellulose, with backbone linear chain represented by xylan



Source: Dodd; Cann (2009).

Some characteristics such as solubility, three-dimensional conformation, physical interaction with other molecules and degradability by enzymes are determined according to the nature of the monosaccharide, bonds between the structural units and pendant groups (SPIRIDON; POPA, 2008). Due to its low degree of polymerization and its amorphous character, it has low thermal and chemical stability, in order to be more easily hydrolyzed by reagents, such as acids, compared to cellulose (FARDIM; DURÁN, 2004).

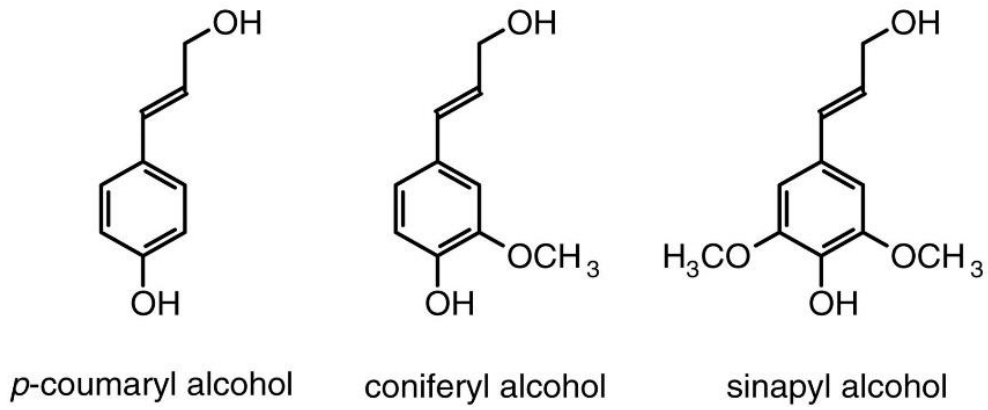
Its composition varies with the type of plant and tissue. For example, hardwood contains hemicellulose with a greater amount of xylan (corresponding to about 20-30 % of the biomass) whereas hemicellulose of softwood contains mostly glucomannans. The amount of xylan present in the tissue of some grasses and cereals may correspond to 50 % and in herbaceous plants the amount is similar to that found in hardwoods (ASIF, 2009; JI et al., 2011).

Hemicellulose or their oligomers and monomers have a wide variety of applications in industry. Can be used in the production of emulsifiers, food additives, thickeners, gelling agents, biopolymers like polyhydroxyalkanoates (PHA) and polylactates (PLA), formic acid, furfural, 5-hydroxymethylfurfural, butanediol, ethanol, xylitol, butanol, xylooligosaccharides (XOS), among others (CANILHA et al., 2013).

2.3.3 Lignin

Lignin is one of the major components of the plant cell wall. Lignin reinforces cell walls, which is responsible for the transportation of water and nutrients in plants vascular system and provides mechanical resistance and protection against microorganisms. Lignin chemical composition and structure is complex and varies between different plant species and even between similar species and different parts of the plant. The lignin is mainly composed of the p-coumaryl, coniferyl and sinapyl alcohols (Figure 2.5) interlinked by various types of bonds (WATKINS et al., 2015).

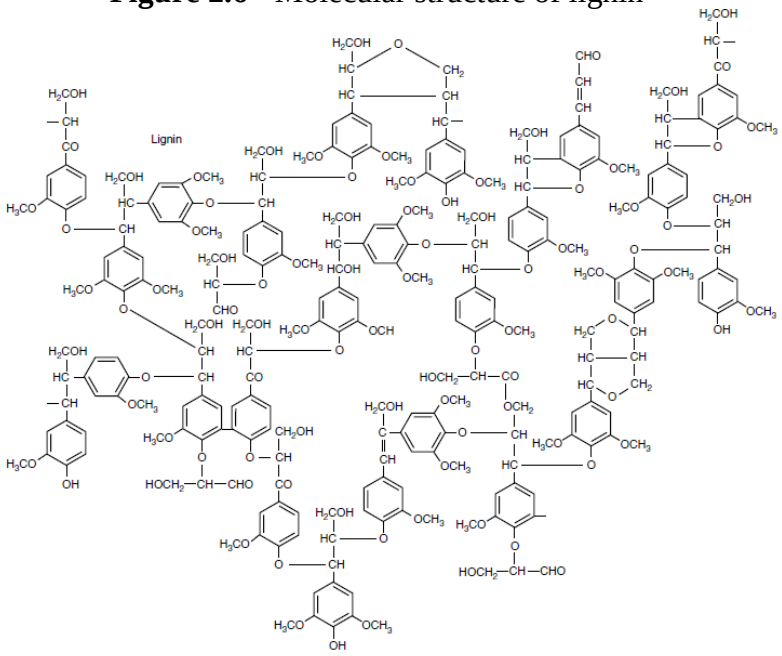
Figure 2.5 - Alcohols precursors that form lignin



Source: Fengel; Wegener (1984).

Lignin content varies approximately 18 to 25 % in hardwoods; 21 to 29 % in softwoods and 17 to 20 % in grasses (SCHUTYSER et al., 2017). Several studies have been carried out aiming to produce high-value materials using lignin. It can be used as fuel for energy production, in the production of nanoparticles, dispersant, biodegradable polymers and added to fabrics to improve UV barrier properties (CHIO; SAIN; QIN, 2019; WELKER et al., 2015). The lignin can be isolated as a residue, through the addition of sulfuric acid, hydrochloric or other mineral acids for the hydrolysis of polysaccharides in the lignocellulosic material. Through this method, acid lignin is obtained that cannot be used to determine their structure due to modifications that occurred during acid treatment (KLOCK; ANDRADE, 2013). Figure 2.6 shows a representation of the molecular structure of lignin.

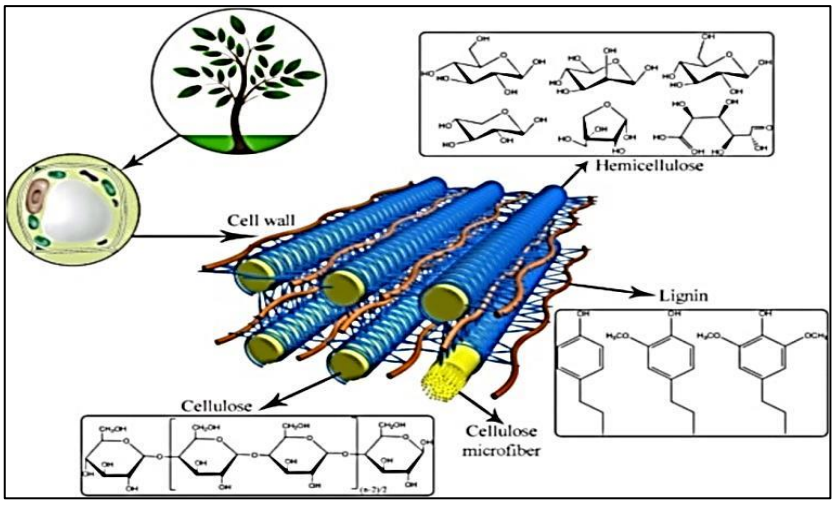
Figure 2.6 - Molecular structure of lignin



Source: Watkins et al. (2015).

Lignin plays an important role in the biomass recalcitrance. The cellulose chains are joined together by intra and interchain hydrogen bonds, forming long fibers. In the plant cell wall, cellulose is associated interlinked with hemicellulose and lignin as shown in figure 2.7 (OGEDA; PETRI, 2010). The combination of the cellulose, hemicellulose and lignin in the plant cell wall creates a material resistant to the tension and insoluble in diverse types of solvents (OGATA, 2013).

Figure 2.7 - Cellulose chain interlinked with hemicellulose and lignin macromolecule



Source: Song et al. (2017).

2.3.4 Extractives

Extractives are organic substances, which generally have low molecular mass and are mainly composed of aromatic compounds, terpenes, flavonoids, fatty acids, proteins and polysaccharides of low degree of polymerization. Extractives of biomass are usually associated with properties such as color, smell and taste (CARVALHO et al., 2009).

There may be variations in their amount in the biomass according to the time of harvest and are found in greater quantity in the foliage and in the bark of the trees compared to the wood. In coniferous wood the values vary between 3.5 and 5.4 % and in hardwoods between 4.4 and 7.5 % (BRAND, MUÑIZ , 2010).

Most of the research on extractives was based on the problems that they can cause in the production of paper and cellulosic pulp, changing its quality and some of these compounds contribute to the effluent toxicity in this industry (NOVO, 2012).

2.3.5 Ash

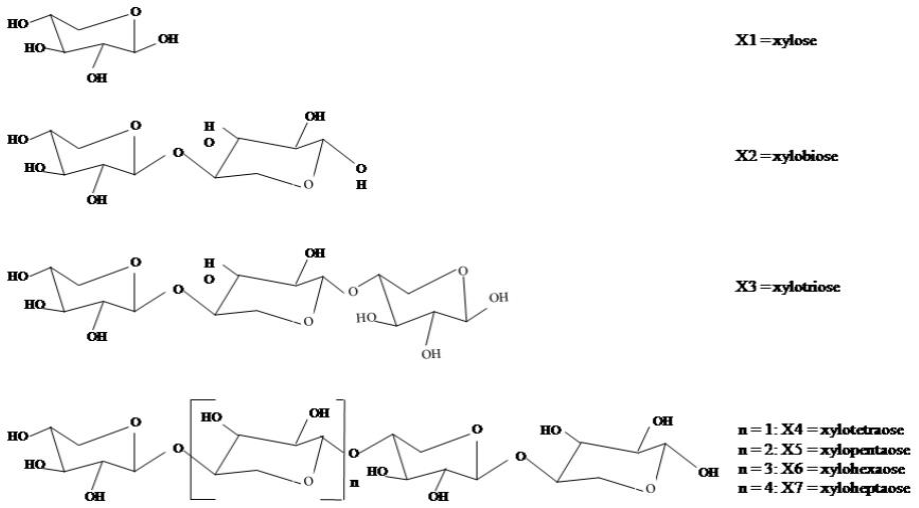
Apart from major cell wall polymers and extractives, ash corresponds to the inorganic part of the lignocellulosic material (KLOCK; ANDRADE, 2013). In plants, there are many elements indispensable to plant cells besides carbon (C), oxygen (O) and hydrogen (H), such as iron (Fe), calcium (Ca), phosphorus (P), potassium (K), manganese (Mn), zinc (Zn), nitrogen (N), sulfur (S), copper (Cu), among others. When plant samples are dried and heated to high temperatures, several chemical elements disappear as gaseous compounds and the remainder is ash, a residue rich in minerals in the form of an oxide (CHEN, 2014). The burning of sugarcane bagasse for the generation of energy generates the ashes as waste, which is usually used as fertilizer, but due to its composition rich in silica, can also be used as feedstock for the production of ceramics, concrete and mortar (MELATI et al. 2017). The percentage of ash varies according to plant species, the type of soil and nutrients present and the distribution of the metals varies in each part of the plant according to their different functions (CHEN, 2014).

The main constituents of wood ash are: potassium (K), calcium (Ca), magnesium (Mg) and its percentage varies. In wood from the temperate zone, the value found is 0.2 to 0.9 % and for wood from the tropical zone approximately 5 % (OGEDA; PETRI, 2010). Ash content in the biomass of some grasses: sugarcane bagasse – 1 to 8 %, switchgrass – 8.3 %, miscanthus – 2.3 % (PLATAČE, R., ADAMOVIČS, 2014).

2.4 Xylooligosaccharides (XOS)

They are xylose polymers having from 2 to 10 units (Figure 2.8) and are naturally present in milk, honey, fruits and vegetables and can be obtained industrially through different processes such as autohydrolysis, enzymatic hydrolysis and acid hydrolysis of xylans present in biomass. When incorporated into the diet can bring numerous health benefits. The oligosaccharides can be applied in the areas of food, animal feed, pharmaceuticals, cosmetics, immunomodulatory agents and prebiotic (MITMESSER; COMBS, 2017; BARRETO; ZANCAN; MENEZES, 2015; VÁZQUEZ et al., 2000; GIESE et al., 2011).

Figure 2.8 - Schematic structure of xylose and xylooligosaccharides



Source: Brienzo et al. (2016).

Prebiotics are food components that are not digested, but stimulate the growth of beneficial microorganisms present in the intestine, improving host health (BARRETO; ZANCAN; MENEZES, 2015). Among the microorganisms that have the growth stimulated by the use of xylooligosaccharides are *bifidobacteria* and *lactobacilli*. Prebiotics must be resistant to stomach acid, digestive enzymes in the gastrointestinal tract and capable of stimulating the growth of bacteria that are beneficial to health and fermented by them. The use of xylooligosaccharides can also bring innumerable benefits when used in animal feeding, making them more resistant to diseases and thus reducing the need for antibiotics, which make microorganisms more resistant and virulent (VÁZQUEZ et al., 2000; BRIENZO et al., 2016b).

Other advantages of XOS are the resistance to acid present in the gastrointestinal tract and digestive enzymes and the fact that they are not used by most of the pathogenic microorganisms in the intestinal flora, lower glycemic indexes and cholesterol in the blood; reduction of pro-carcinogenic enzymes, improved absorption of minerals, such as calcium, fighting osteoporosis, immune system stimulation, antioxidant activity, anti-inflammatory properties and mildly laxative effect (BARRETO; ZANCAN; MENEZES, 2015; MOURE et al., 2006).

They are also capable of improving the nutritional and sensory properties of food because they are slightly sweet and stable over a wide pH range (2.5 to 8.0) and temperatures up to 100 °C. This resistance makes them more advantageous in relation to fructooligosaccharides (FOS), allowing, for example, their use in acidic juices and carbonated drinks (AKPINAR et al., 2007; VÁZQUEZ et al., 2000).

They are used in the production of biodegradable plastics and films, chitosan-xylan capsules and hydrogel, which has the main advantage of causing less environmental impacts due to the shorter time for biodegradation (MOURE et al., 2006).

During the production of XOS, by chemical or enzymatic processes, many undesirable compounds such as acetic acid, monosaccharides, furfural, soluble inorganic components, and extractives among others are produced, necessitating the purification step (GIESE et al., 2011; MOURE et al., 2006).

The choice of method for purification depends on the desired degree of purity and a sequence of various physicochemical processes may be required. Some of the most used methods are: vacuum evaporation, which removes acetic acid and aromatic compounds and concentrates the oligosaccharides in the solution; solvent extraction, uses reagents such as ethanol, acetone, and isopropanol to separate polysaccharides from other substances and chromatography, which separates the compounds according to the molar mass and produces XOS fractions with high purity (VÁZQUEZ et al., 2000; JAIN; KUMAR; SATYANARAYANA, 2015).

Nanofiltration membranes are being used successfully for oligosaccharides separation, concentration and purification. These methods, however, are expensive and complex and hinder the production of XOS on a large scale (ALTERTHUM et al., 2019; AKPINAR et al., 2007).

2.5 Pretreatment methods

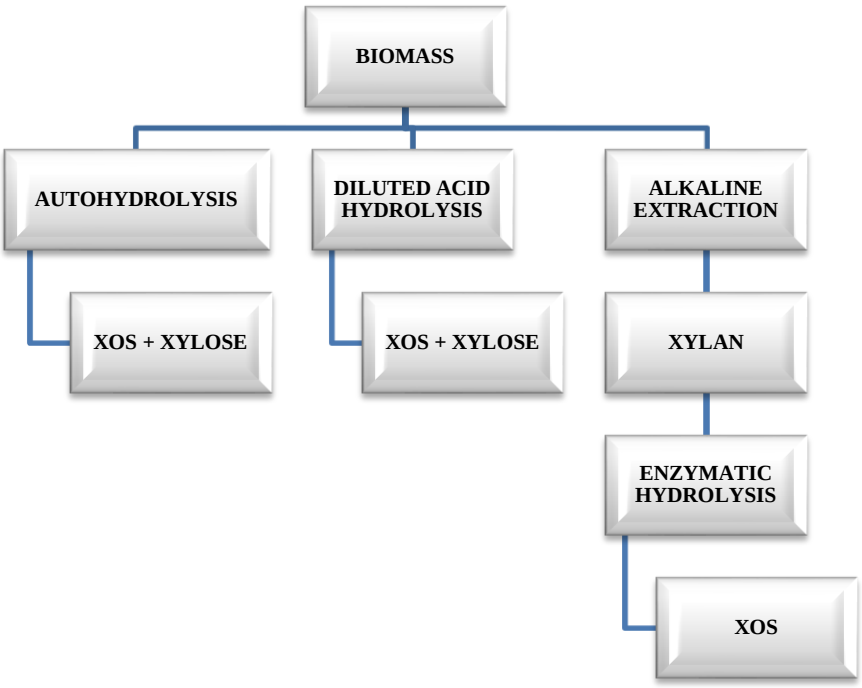
Pretreatment of lignocellulosic materials aims to break the interactions between the components of the cell wall and separate them. The choice of the most appropriate method depends on the composition and structure of the material and the evaluation of some parameters such as interest component and whether or not there is interest in maintaining its integrity, low generation of degradation products, absence of solid waste, reduced cost, low energy consumption, improved sugar formation, lower carbohydrate breakdown, reusability of reagents and recovery of solubilized products for the generation of others with high added value (GUILHERME et al., 2017; KUMAR et al., 2009).

For pretreatment, physical, chemical, biological, physicochemical or combination of them may be used (AMIN et al., 2017). Physical methods by pulverizing and grinding fragment the lignocellulosic material into small particles and reduce the degree of polymerization and crystallinity. Disorganization of the material facilitates further chemical processes (KUMAR et al., 2009). Chemical pretreatments may use concentrated or dilute acids (more commonly diluted sulfuric acid) or bases such as sodium hydroxide for separation of cell wall components. It has as a disadvantage the possibility of the formation of fermentation inhibitors such as furfural and hydroxymethylfurfural (HMF), reducing the efficacy of the method (BRODEUR et al., 2011). Biological pretreatments use microorganisms or enzymes extracted from fungi or bacteria that solubilize lignin, removing it from biomass, making the cellulose more accessible to hydrolysis. In this process there is no need to add chemicals or high temperatures, there is a high lignin removal rate and the method is a good choice for materials with a lot of carbohydrate. As a disadvantage, the delay in the reaction, the consumption of a part of the carbohydrates by the micro-organisms and the high cost (HAGHIGHI MOOD et al., 2013; CARVALHO et al., 2009; OGEDA; PETRI, 2010).

2.6 Xylooligosaccharides (XOS) production using biomass as feedstock

XOS can be produced by several processes such as autohydrolysis, diluted acid and solubilization of xylan followed by enzymatic hydrolysis (Figure 2.9). These methods are described in the following sections in detail.

Figure 2.9 - Methods for obtaining xylooligosaccharides (XOS).



Source: Author.

2.6.1 Autohydrolysis

In the autohydrolysis or hydrothermal process, the only reactants are biomass and hot water compressed between 150 and 230 ºC and the time varies from seconds to hours. There is a formation of degradation products of monosaccharides such as formic acid, levulinic acid, furfural, HMF and some lignin degradation products, however in lesser amount due to the absence of corrosive solvents. The absence of chemicals in this pretreatment avoids the formation of waste, reducing environmental impacts (MANRICH, 2012; CARVALHEIRO et al., 2016; CHEN, 2015).

In this process, there is a need for specific equipment operating at high temperatures. The reaction occurs through the formation of hydronium ions from the self-ionization of the water that acts by hydrolyzing polysaccharides from the biomass and forming acetic acid by breaking the bonds between the hemicellulose and the acetyl groups accentuating the hydrolysis of the plant biomass in a similar way to that occurs during acid diluted hydrolysis. During this process the xylan is partially hydrolyzed into xylose and XOS which dissolve in the solution, while the cellulose and lignin remain in the solid fraction (CARVALHEIRO et al., 2016; AKPINAR et al., 2007; CHEN, 2015).

2.6.2 Dilute acid

This pretreatment is considered effective for XOS production and economically viable due to the low acid concentration used. The main hydrolyzed component is hemicellulose, giving rise to a liquid fraction rich in XOS and xylose (JEONG; LEE, 2015; WEN et al., 2007). This method can break ether bonds in the polysaccharide chain, forming sugar monomers, unlike some other methods, which hydrolyze the hemicellulose, making it more soluble and resulting in a greater amount of oligosaccharides which are non-fermentable (JI et al., 2017).

For the production of XOS, sulfuric acid in concentrations between 0.1 and 0.5 $mol.L^{-1}$ is the most commonly used. The yield depends on the structure and composition of the hemicellulose, concentration of the acid used, temperature and reaction time (QING et al., 2013). During the process also occur breaks down lignocellulosic complex bonds providing several compounds such as furfural and HMF, which are products of the degradation of pentoses and hexoses respectively; acetic acid originated from the deacetylation of hemicellulose; formic and levulinic acids, sugar degradation products; phenolic compounds, formed mainly by the partial degradation of lignin (MARTIN et al., 2007). Some of these products formed as furfural, formic and glycolic acid are widely used by the chemical and pharmaceutical industries (OLIVEIRA, 2016). These compounds harm the fermentative processes. Alternatives to reduce the formation of inhibitors during the hydrolysis are: the use of dilute acid, lower temperatures, shorter reaction times and the rapid removal of hydrolytic agents (JI et al., 2011). Sulfuric acid is the most used, because it is easier to hydrolyze carbohydrates and lower cost about to with concerning other acids such as phosphoric and nitric. It has as a disadvantage the corrosive power and the sulfate ions released in the medium inhibit fermentative processes (OLIVEIRA, 2016).

The pretreatments using dilute inorganic acid have received more attention about to with concerning pretreatments with diluted organic acid, as for example acetic acid, but these have desirable characteristics as less formation of degradation products like furfural and HMF; greater removal of lignin, allowing the cellulose to be digested by enzymes; hydrolysis of hemicellulose and recovery of xylose. At the end of the process the acetic acid can be recovered (JEONG; LEE, 2015; OLIVEIRA, 2016).

2.6.3 Alkaline extraction of hemicellulose

Through the use of alkali it is possible to separate hemicellulose and lignin from cellulose without degrading polysaccharides, with moderate temperature or alkalinity conditions. The most used bases are: potassium, sodium, ammonium and calcium hydroxide (BRIENZO; SIQUEIRA; MILAGRES, 2009; BRODEUR et al., 2011). This type of pretreatment removes lignin and acetyl groups leaving the cellulose more accessible to the action of enzymes. With the addition of hydrogen peroxide, which is an oxidizing agent the removal of lignin is favored. At pH 11.6 the hydrogen peroxide reacts with the hydroxyl of the base (generally NaOH) forming peroxydryl HOO^- and superoxide O^{2-} ions highly reactive that oxidize the lignin and dissolve the hemicellulose, according to the reactions below (BRIENZO; SIQUEIRA; MILAGRES, 2009; SUN et al., 2000).

$$H_2O_2 + HO^- \leftrightarrow H_2O + HOO^-$$
$$H_2O_2 + HOO^- \leftrightarrow HO + O_2^- + H_2O$$

The obtained hemicellulose can be used for subsequent enzymatic hydrolysis to obtain xylose and XOS.

2.6.4 Enzymatic hydrolysis of hemicellulose

Enzymes are produced with microorganisms like bacteria, fungi, yeasts, mainly filamentous fungi, among others, inoculated on substrates such as bagasse, leaves, bran, peels and fruits previously sterilized. When the microorganism can metabolize the substrate, under ideal conditions of temperature, pH and nutrients, specific enzymes used in the pretreatments are obtained (POLIZELI et al., 2005; CASCIATORI; FRASSATTO, 2018).

The hydrolysis of lignocellulosic materials through this method shows specificity of the reaction, absence of secondary reactions causing loss of yield, absence of byproducts such as furfural, HMF and acetic acid and do not require high pressures, temperatures or chemicals that cause corrosion in equipment (RODRIGUES; GUIRARDELLO, 2008).

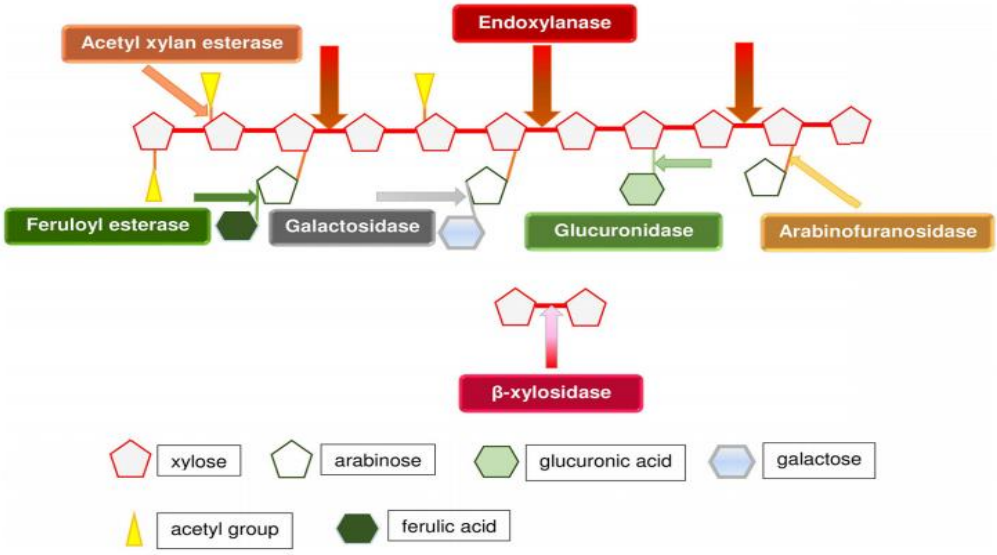
This process, however, is slow and expensive due to the complexity of the cellulose, lignin and hemicellulose binding. Moreover their efficiency can be decreased in the presence of metals and by the capillary structure of the fibers that form the cellulose (TSAO et al., 1978). The enzyme must be in contact with the substrate for hydrolysis to occur. If the

substrate has a high lignin content, is highly crystalline or depending on the heterogeneity of the lignocellulosic material the enzyme will not be very effective and the time for hydrolysis to occur will be high (CHANG; HOLTZAPPLE, 2000).

Due to complex composition, heterogeneity of the molecule, branching in its structure and different types of bonds between the constituents, several enzymes are required to degrade or modify hemicelluloses (JI et al., 2011). For the hydrolysis to occur, an enzyme complex is required. The enzyme endo-1-4-β-xylanase (EC 3.2.1.8) acts by breaking down glycosidic bonds present in the main chain of xylan (main class of hemicellulose), reducing its degree of polymerization and generating the XOS, which serve as a substrate for the enzyme β-xylosidase (EC 3.2.1.37) generate xylose through the non-reducing terminal (SHALLOM; SHOHAM, 2003; POLIZELI et al., 2005; BRIENZO; ARANTES; MILAGRES, 2008).

Branches of the xylan chain are hydrolyzed by α-glucuronidase (EC 3.2.1) which removes glucuronic acid, by α-L-arabinofuranosidase (EC 3.2.1.55), which removes arabinose and acetyl xylan esterase (EC 3.1.1.6), which removes acetyl groups (DE VRIES; VISSER, 2001). In Figure 2.10, it is possible to see a representation of how the enzymes act in the hemicellulose chain.

Figure 2.10 - Representation of xylan plant structure and sites of microbial xylanolytic enzyme performance



Source: Ergün; Çalik (2016).

Enzymes have gained increasing prominence in the industry due to the growing demand for natural products in the substitution of chemicals and toxicants. Xylanases have an

important role due to their variety of applications such as bioconversion of agricultural waste, cellulose pulp pre-bleaching for paper production, food, beverage, feed, ethanol, xylitol, xylo-oligomers, to clarify juices and wines and has been especially prominent in baking, as well as cellulases and glucanases, as they improve bread quality, flexibility and volume, reduce hardening during storage and increase shelf life (BUTT et al., 2008; HARRIS; RAMALINGAM, 2010; POLIZELI et al., 2005).

2.7 Biomass sources and composition

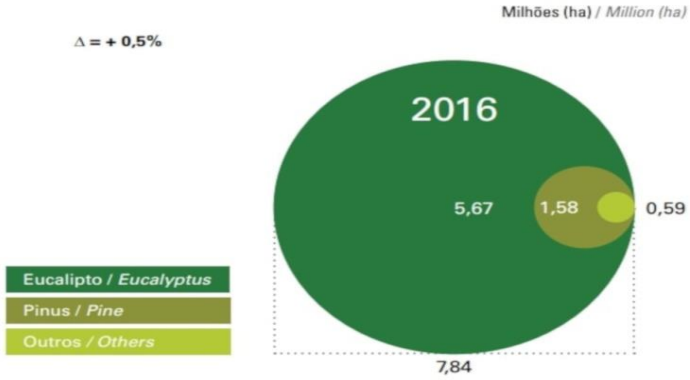
2.7.1 Wood biomass

A biomass resource that has been widely used is wood, which can be obtained from forest residues and wood industries residues, for example (SONG et al., 2017). The woods are divided into two categories: softwoods such as conifers, which belong to the group of gymnosperms, which do not produce fruits and have needle-shaped leaves; and the hardwoods, which belong to the group of angiosperms, which have seeds containing fruits and broader leaves (CARVALHO et al., 2009). The average composition of cell wall polymers of softwoods is 42 % of cellulose, 27 % of hemicellulose, 28 % of lignin and 5 % of extractives whereas in hardwoods 45 % of cellulose, 30 % of hemicellulose, 20 % of lignin and 3 % of extractives (KLOCK; ANDRADE, 2013). The biomass of the wood includes the trunk, roots, bark and leaves of the tree and accounts for approximately 13.9 % of all energy generated in Brazil and 8.7 % in the world. The precise management of the wood production either obtained primarily through reforestation based wood or forest byproduct and strictly not through deforestation, is considered to be a sustainable method of obtaining wood based feedstock energy. Through the carbon cycle, the plants capture CO_2 from the atmosphere generated by the combustion of wood or fossil fuels and use it to carry out photosynthesis that ultimately drives the wagon wheel of the entire biomass generation process (BNDES, 2011). In addition to energy generation, biomass from wood can be a promising source for XOS production.

In Brazil two wood species that adapted very well were *Pinus* and *Eucalyptus*, due to the climate, soil and favorable territorial extension. The productivity is at least ten times higher than in many temperate countries, due to the advanced technologies in its cultivation (VALVERDE et al., 2004). In the year 2016 the total number of trees planted in Brazil represented approximately 7.84 million hectares, of which 5.7 million hectares are occupied

by *Eucalyptus* and 1.6 million hectares by *Pinus* (Figure 2.11) (INDÚSTRIA BRASILEIRA DE ÁRVORES, 2017).

Figure 2.11 - Total area of trees planted in Brazil



Source: Indústria Brasileira De Árvores (2017).

2.7.1.1 Eucalyptus

The *Eucalyptus* genus originated in Australia, Indonesia and other islands of Oceania (EMBRAPA, 2014). The most common wood in Brazil for cellulose and coal production is *Eucalyptus* (SARAIVA; KONIG, 2013). The forests with this type of trees correspond to 60.7% of the total reforested area of Brazil and can fix on average 100-400 tons per hectare of CO_2 (GATTO et al., 2010).

Wood is used for various purposes worldwide. Young trees are used for producing coal, paper, and fuel. Mature trees are used for furniture production, and can be used for the production of volatile oils, used in medicaments and the chemical industry (EMBRAPA, 2001).

Eucalyptus is capable of producing up to 20 tons of dry biomass per hectare per year. It has a disadvantage of delay in reaching the appropriate size for the first cut, which is on average seven years (SARAIVA; KONIG, 2013). *Eucalyptus* plantations have several advantages: help in the capture and retention of CO_2; adapt to diverse conditions due to the great diversity of species; produce many seeds and propagate easily; protect the soil due to vegetation cover; allow the growth of other plants inside the forest; do not require large amounts of fertilizers and have greater resistance to pest attack (SILVA; ROCHA, 2010).

Hemicellulose was extracted from *Eucalyptus* byproduct using a 2 M sodium hydroxide solution and 6 % (v/v) hydrogen peroxide was performed after the delignification

process with sodium chlorite at the temperature of 25°C. Thus, 62.6 % of the hemicellulose from *Eucalyptus* byproduct was extracted. Hemicellulose derived from *Eucalyptus* byproduct presented xylan content of 55 %, which was similar to 58.5 % found in commercial Birchwood hemicellulose. The enzymatic hydrolysis of extracted from *Eucalyptus* byproduct and Birchwood hemicelluloses presented 30 % as the maximum conversion of xylan into XOS (MAFEI et al., 2019).

Chemical composition is approximately 47.2 % of cellulose, 14.9 % of xylan, 0.5 % of arabinose and 26.8 % of lignin. About 60.2 % of xylan (47.4 % in the form of monomers and 12.8 % in the form of oligomers) present in wood of *Eucalyptus grandis* can be extracted with dilute sulfuric acid 0.7 % (v/v) at 140 ºC for 30 min (VENA et al., 2015).

2.7.1.2 Pinus spp.

Approximately 90 species are part of the genus *Pinus*, all native to the northern hemisphere. The wood is of low density and primarily used for the manufacture of pencils, shoes and boxes. The cellulose extracted from its fibers is widely used for the manufacturing brown paper and paperboard. Some *Pinus* species produce resins that can be used in the pharmaceutical industries and the manufacture of paints (REYES, 2015).

Due to their ease of adaptation, *Pinus* can be cultivated around agricultural plantations, adding value to the production of wood and forming a cover that protects the soil. In addition, trees of this genus have a great capacity to absorb atmospheric CO_2, contributing to a decrease in the greenhouse effect and absorb many nutrients from the soil to accumulate large amounts of biomass (UNIVERSIDADE ESTADUAL DO SUDOESTE DA BAHIA, 2016).

Lignocellulosic material obtained from softwood is considered most recalcitrant for hemicellulose extraction and enzymatic hydrolysis due to the highly lignified structure. The chemical composition of the *Pinus* is 43.1 % of cellulose, 23.3 % of hemicellulose and 29.9 % of lignin. The amount of xylan in the material corresponds to 7 %. Hemicellulose was extracted from *Pinus* using a 10 % sodium hydroxide solution before and after the delignification process with sodium chlorite or peracetic acid. Without delignification only 3.4 % of hemicellulose was extracted and after, more than 50 % of the hemicellulose was extracted (GENG, 2018).

2.7.2 Grass biomass

Grasses or fast-growing shrubs that have several advantages in the production of large-scale biomass for biofuels. The grasses roots can establish symbiotic interactions with bacteria, allowing greater fixation of mineral nutrients and nitrogen that increase the life expectancy of the plant and the production of biomass; decrease of soil erosion (ROCHA; SOUZA; DAMASCENO, 2017).

Grasses have a wide geographic distribution, important scientific and economic role and are an important source of food for herbivorous animals (LOPES, 2004). It has the potential to be used in the production of biofuels preserving native forests and some grasses also have the greatest potential to obtain biomass for XOS production as described in the next topics (MARAFON; SANTIAGO, 2016).

2.7.2.1 Saccharum officinarum (Sugarcane)

The origin of the sugarcane is attributed to New Guinea and has spread to India and later throughout Europe. By the year 1532, sugarcane was introduced into Brazil by the Portuguese (MACHADO, 2003). From sugarcane, sugars are obtained and used to produce fermented alcohol which is used as fuel, in alcoholic beverages and cleaning products. The resulted bagasse from the sucrose extraction can be used to produce XOS, cardboard, animal feed, fertilizer, bioplastic, cosmetics, biogas and civil engineering products MELATI et al., 2017; FERNANDES et al., 2017; UNISALESIANO, 2011).

The bagasse, together with the straw, is also used to generate energy for the mill (co-generation) making the company self-sufficient in energy, and the production of cellulosic ethanol (2nd generation ethanol) (DANTAS, 2010). Sugarcane is currently the most used grass in Brazil and has an average productivity of 85 tons per hectare, generating approximately 12 tons of straw and 12 tons of bagasse. With the use of all parts of sugarcane in the production of ethanol, the yield could increase from 7000 liters per hectare to approximately 14000 liters per hectare, without the need to increase the cultivated area (SANTOS et al., 2012).

Sugarcane bagasse and straw are promising sources for production of XOS because it contents of xylan. The chemical composition of sugarcane bagasse is in the range of 42.4 % of cellulose, 25.2 % of hemicellulose and 19.6 % of lignin (BRIENZO; SIQUEIRA; MILAGRES, 2009). In this study the extraction of hemicellulose from sugarcane bagasse

with hydrogen peroxide was performed, varying parameters such as concentration, time and temperature of the reaction. The best yield was 86 % hemicellulose extracted, with a low concentration of lignin (5.9 %), with hydrogen peroxide (H_2O_2) 6 % for 4 h at 20 °C. The xylose concentration ranged from 73.1-82.6 % in all extracted hemicellulose samples. Other compounds such as arabinose, glucose and glucuronic acid were found in low amounts. The low lignin content favors the use of hemicellulose for subsequent enzymatic hydrolysis.

2.7.2.2 Sorghum spp. (Sorghum)

Sorghum biomass especially hybrid varieties are genetically improved with high biomass yield and calorific value (EMBRAPA, 2013). It has calorific power similar to that of sugarcane, *Eucalyptus* and elephant grass. *Sorghum* is a plant capable of adapting to tropical and temperate climates, to use water effectively, tolerate drought and capable of producing large amounts of lignocellulosic biomass. *Sorghum* biomass can reach productivity of 150 tons per hectare, fully mechanized growth, and in a cycle of 5 months. In addition, sorghum can be propagated by seeds, reducing operational costs (EMBRAPA, 2015).

Sorghum originated from Africa and parts of Asia and it has a great developmental program (reaches up to 6 meters in 180 days) in many agricultural regions around the world (EMBRAPA, 1982). In Brazil four main types are cultivated: grain, used for grain production; fodder, for silage production; saccharine, for the production of ethanol and sorghum biomass, used for the generation of energy by burning in boilers of power plants and thermoelectric plants (EMBRAPA, 2015). In addition, after pretreatments, sorghum can be used as a raw material for high added-value products.

Chemical composition of Sorghum bagasse is: 42.8 % of cellulose, 26.3 % of hemicellulose and 20.2 % of lignin. The Maximum yield of hemicelluloses from sorghum bagasse was approximately 33.3 %, with NaOH 12.33 % (w/w), time and temperature of reaction of 3.91 h and 86.1 ºC respectively. Hemicellulose obtained through process revealed a composition of xylose (79.0 %), arabinose (5.3 %), glucose (1.7 %) and lignin (5.6 %) (WEI et al., 2018).

2.7.2.3 Energy cane

Energy cane was obtained through crossing species of *Saccharum spontaneum* with hybrids of sugarcane. In this way the energy cane has a high yield of biomass rich in fibers

and can adapt to marginal agricultural areas and limited environmental conditions in relation to conventional sugarcane (MARAFON; SANTIAGO, 2016).

Energy cane is able to develop in soils with little fertilizer, high salinity, with low water availability and extreme temperatures. Due to its high resistance, it does not require the use of large amounts of fertilizers and pesticides. In addition, it can control soil erosion and harvesting can be carried out several times a year (SANTOS; BORÉM; CALDAS, 2010).

Chemical composition of energy cane is approximately 43 % cellulose, 24 % hemicellulose, and 22% lignin (KIM; DAY, 2011). Through the auto-hydrolysis using Energy cane bagasse, Bazetto (2018) obtained an XOS concentration of 1.2 $g.L^{-1}$ at 170 °C for 5 min and 2.0 $g.L^{-1}$ when the time increased to 15 min. At the temperature of 190 °C for 5 min the yield was 6.5 $g.L^{-1}$ and 8.6 $g.L^{-1}$ at the same temperature for 15 min.

2.7.2.4 Panicum virgatum L. (Switchgrass)

Switchgrass is native to North America and later introduced to Europe and many other parts of the world. Switchgrass has easy adaptation to variable climates and yields approximately 8-14 tons of dry mass per hectare per year. The growth is maximum in good quality soils, but it also grows in poor soils with lower water levels (WAGENINGEN UNIVERSITY & RESEARCH, 2013).

It is a renewable source with the potential to obtain carbohydrates for use in the production of XOS and bioethanol, due to its excellent productivity, ease of adaptation to different types of climates and soils. Switchgrass it is composed by approximately 39.1-45 % of cellulose, 30-32.8 % of hemicellulose and 12-20.1 % of lignin being that 25 % of the raw material corresponding to xylan (BOWMAN et al., 2015; SAMUEL et al., 2011; GENG, 2018; JI et al., 2011).

Generally switchgrass has less lignin and more hemicellulose in relation to softwoods. The lower percentage of lignin makes it easier to extract hemicellulose (PASANGULAPATI, 2012).

Geng (2018) also extracted hemicellulose from Switchgrass with the same methodology used for *Pinus.* Extraction with 10 % sodium hydroxide solution before and after the deslignification process with sodium chlorite or peracetic acid. Without deslignification 55.5 % of hemicellulose was extracted and after approximately 65 % of the hemicellulose was extracted.

2.7.2.5 Miscanthus spp. (Miscanthus)

Miscanthus is native in East Asia and it can grow on a wide variety of soils and is able to use water and solar radiation efficiently. So far most of the data obtained comes from research in Europe, where the most studied genotype is *Miscanthus giganteus*. The dry biomass yield per hectare per year in northern and central Europe is on average 10-25 tons, reaching 30 tons in the southern region, where temperatures are higher (REIS; BERNARDES; SIQUEIRA, 2014).

Miscanthus can grow on poorly fertile soil and can produce up to twice as much biomass as Switchgrass. *Miscanthus giganteus* produces high amounts of lignocellulose biomass, which differs from other biomass crops. In the United States it is able to produce more biomass per year than other important species, except sugarcane (EXTENSION FARM ENERGY, 2019).

The chemical composition of Miscanthus according to (LIGERO et al., 2011) is 38 % of cellulose, 17.5 % of hemicellulose of which 14.9 % correspond to xylose and 21.7 % of lignin. A high yield of XOS, with values close to 65 % of the dissolved xylan (based on the initial amount of xylose), could be obtained by an autohydrolysis process with a temperature of 160 °C for 60 min. In this condition, there was little formation of degradation products.

Chen (2014) found a similar chemical composition: 35.9 % of cellulose, 19.5 % of xylan and 19.6 % of lignin. In this study the XOS were also produced by autohydrolysis under conditions of temperature and time of 160 ºC for 60 min, 180 °C for 20 min and 200 °C for 5 min. All three conditions generated similar results. The maximum conversion of xylan in XOS and xylose was 69.2 % (m/m).

2.7.2.6 Pennisetum (sp) (Elephant grass)

Elephant grass has characteristics similar to the sugarcane bagasse, and is capable to produce biomass rich in fibers and lignin. Elephant grass has several main advantages such as high productivity per hectare, lower production cycle and the possible use of mechanization to produce the crop (MARAFON; SANTIAGO, 2016).

The elephant grass originated in Africa and then introduced in Brazil in 1920, which spread to all regions of the country. This is a perennial grass, which reaches from 3-5 meters in height and has leaves with light or dark green coloration (LOPES, 2004). The grass produces 30-40 tons of dry biomass per hectare per year on average. Due to its rapid growth,

it reaches an adequate size for cutting 180 days after planting, thus offering two cuts per year (SARAIVA; KONIG, 2013).

Chen et al. (2017) determined its chemical composition as 44 % cellulose, 25.8 % hemicellulose and 15.8 % lignin. In the same study, hemicellulose was solubilized from *Pennisetum* using 0.5 % of hydrogen peroxide solution and 4 % of sodium hydroxide. The solution was heated at 50 °C for 30 min in a water bath and then treated with microwave for 4 h. The yield of hemicellulose was 84.7 %, after hydrolysis of the hemicellulose with dilute sulfuric acid 1 % at 105 °C for 4 h, a yield of xylose of 86.5 % was obtained.

The following grasses are subtilized for hemicellulose solubilization but due to their composition are promising sources.

2.7.2.7 Sorghastrum nutans (Indiangrass)

Indiangrass is native in North America (Quebec, Manitoba and southern Mexico) and this grass is more adaptable than *Panicum virgatum* and *Andropogon gerardii*. Indiangrass is widely used for floral arrangements due to its reddish-brown flowers and soil erosion control. The ideal cultivation condition is in moist soils and sunny locations but is very drought tolerant (BLUESTEM NURSERY, 2018). Indiangrass is used as food for some herbivorous mammals such as bison and cattle. It also provides coverage for bird protection due to plant height and its tendency to remain erect (ILLINOIS WILDFLOWERS, 2018).

2.7.2.8 Andropogon gerardii (Big bluestem)

Big bluestem is native in North America, from central Mexico to southern Canada (BLUESTEM NURSERY, 2018). Big bluestem is highly distributed between these two countries and the United States. The high productivity is due to its ability to efficiently utilize soil nutrients, producing twice as much biomass as Switchgrass and Indiangrass (ZHANG, 2014). Big bluestem is considered to be the best choice for soil erosion control and is highly used as forage for cattle. This grass adapts to conditions of low pH and soil fertility (UNITED STATES OF DEPARTMENT AGRICULTURE, 1995). The biomass yield is approximately 29.7 tons per hectare and it is one of the grasses with the highest yield and in some conditions yields almost five times more biomass than the switchgrass (ZHANG, 2014).

2.8 Conclusion

XOS has the potential to bring numerous health benefits and this justifies the growing interest in research to produce and understand its effects. Biomass represents an abundant and renewable source, which can be used for XOS production and numerous high-value products for industry. Agricultural and industrial waste can be reused in order to generate profit for the bio-based industries that produce them and avoid contamination of the environment with its incorrect disposal. Among all the woods and grasses addressed in this study, all are promising sources for XOS production, due to the ability to adapt to different types of soils, climates, conditions and amount of biomass generated per hectare, but grasses generally have less lignin and a greater amount of xylan in its composition in relation to wood, which makes the solubilization process of hemicelluloses easier and the quantity obtained greater. Grasses grow faster and their harvest is easier compared to wood. Among the grasses, sugarcane is prominent, because of the advantage of being generated in a great amount in the sugar and alcohol industry.

CHAPTER III: XYLOOLIGOSACCHARIDES PRODUCTION BY AUTOHYDROLYSIS AND DILUTED ACID HYDROLYSIS OF SUGARCANE BAGASSE

Abstract

From the lignocellulosic biomass can be obtained products of added value, among them xylooligosaccharides (XOS). XOS acts as prebiotic, a food component that is not digested but stimulates the growth of beneficial microorganisms present in the intestine, such as *bifidobacteria* and *lactobacilli*, improving the health of the host. This study obtained XOS by acid hydrolysis and autohydrolysis of sugarcane bagasse. The acid hydrolysis process varied the parameters applying a 2^3 factorial design using 1 to 3 % (m/v) sulfuric or acetic acid; a temperature of 100 to 160 ºC and reaction time of 15 to 55 min. The autohydrolysis was performed by a 2^2 factorial design using the same range of temperatures and reaction time of acid hydrolysis. Hydrolysis of the bagasse with acetic acid resulted in the highest yield of xylan conversion in XOS of 18.41 % with 1 % acid at 100 °C for 15 min, with xylopentaose/xylohexaose predominance. The hydrolysis of bagasse with sulfuric acid resulted in 90.13 % of XOS with 2 % of acid at 79.55 ºC for 35 min, with xylobiose prevalence. Autohydrolysis of bagasse resulted in 13.67 % xylan conversion into XOS at 172.4 °C for 35 min, with a high concentration of xylotetraose. The results demonstrated that the most effective pretreatment for XOS production was hydrolysis with dilute sulfuric acid.

Keywords: Biomass conversion; Hemicellulose; Xylan; Autohydrolysis; Acetic acid; Sulfuric acid.

3.1 Introduction

Increasingly people are concerned with health and quality of life and this causes changes in lifestyle and increased demand for healthy foods, as a way to prevent obesity and chronic diseases. In this scenario, the nutraceuticals have attracted attention. This term emerged in the late 1980s and is a combination of the words nutrition and pharmaceutical, and is used to define foods or parts of foods capable of promoting health improvement (DAS et al., 2012), preventing disease, slowing the aging of the body in order to increase life expectancy (NASRI et al., 2014). Among the products that fit into this definition are probiotics, antioxidants, dietary fibers, plant-derived foods and prebiotics, which are non-digestible components that, when consumed, stimulate the growth of some desirable bacteria present in the colon (DAS et al., 2012; SAMANTA et al., 2015).

Several compounds act as prebiotics, among them xylooligosaccharides (XOS), which are oligomers formed by 2 to 10 xylose units (HUANG et al., 2019). XOS consumption brings many benefits to human and animal health, such as controlling the proliferation of harmful bacteria to prevent infections; improves absorption of some metals like calcium, preventing osteoporosis (AMORIM; SILVÉRIO; RODRIGUES, 2019); antioxidant and anti-inflammatory activity, prevention of colon cancer, decreased blood glucose and cholesterol levels; reduction of pro-carcinogenic enzymes and laxative effect (SINGH; BANERJEE; ARORA, 2015). Besides that, they can be used in agriculture as a ripening agent and growth stimulator (VÁZQUEZ et al., 2000).

XOS are naturally present in some foods like honey, milk, fruits and vegetables (SINGH; BANERJEE; ARORA, 2015) and can be produced industrially from xylan-rich lignocellulosic materials, such as agro-industrial and food waste, wood, plants, among others, through different pretreatments such as autohydrolysis, a process that uses only hot water to break the xylan chain into oligomers (JAIN; KUMAR; SATYANARAYANA, 2015; SAMANTA et al., 2015). However, this process also generates xylose and some degradation products, but in less quantity compared to dilute acid hydrolysis, a more efficient process that most commonly uses sulfuric and hydrochloric acid in concentrations up to 10 % and high temperatures. Organic acids such as acetic acid has gained prominence due to effective hydrolysis, a higher rate of formation of oligomers and lower formation of degradation products (LENIHAN et al., 2010; ZHAO et al., 2014).

Considering the importance of XOS as a market product, this study aimed to compare the pretreatments autohydrolysis and hydrolysis with dilute sulfuric and acetic acid using a factorial design. Sugarcane bagasse, an abundant waste rich in xylan was used to evaluate xylose and XOS production.

3.2 Material and methods

3.2.1 Sample preparation

Sugarcane bagasse (from the São João Plant, Araras-São Paulo, Brasil) was oven-dried at 60 ºC, ground in a knife mill and selected by sieve at 20 mesh.

3.2.2 Determination of extractives content

Extractives content was determined with 1 g of sugarcane bagasse in an oven-dried filter paper bags at 105 °C. The filter paper bag was inserted into the Soxhlet and extraction was performed for 8 h with ethanol, the same procedure was performed with distilled water. The filter paper bag was oven-dried at 105 °C for at least one night and placed in a desiccator until it reached a temperature of 25 °C and mass determined.

3.2.3 Chemical characterization of biomass

About 300 mg of bagasse extractive-free was hydrolyzed with the addition of 1.5 mL of 72 % (m/m) sulfuric acid at 45 °C for 7 min. The reaction was stopped with the addition of 45 mL of distilled water and the solution was autoclaved at 121 °C for 30 min. After cooling the hydrolyzed solution was filtered through a previously tared crucible porous plate (ROCHA, 2000; GOUVEIA et al., 2009). The liquid fraction obtained was quantified by High-Performance Liquid Chromatography (HPLC), according to item 3.2.4 to determine glucose, xylose, arabinose and acetic acid.

To determine the acid soluble lignin content, the liquid fraction was diluted with 4 % (v/v) sulfuric acid until the absorbance was between 0.2-1.0 and quantified by UV-Vis spectrophotometry at 215 and 280 nm. The solid residue was washed with distilled water, oven-dried at 105 °C to determine insoluble lignin.

3.2.4 Sugars and acetic acid determination

The concentrations of glucose, xylose, arabinose and acetic acid were determined by HPLC, using Bio-Rad Aminex HPX-87H (300 x 7.8 mm) column maintained at 45 ºC, WATERS 2414 refractive index detector, a mobile phase of sulfuric acid 0.005 $mol.L^{-1}$, flow rate of 0.6 $mL.min^{-1}$, the volume of the injected sample of 20 μL. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size.

The cellulose, xylan, arabinosyl and acetyl groups content were calculated by multiplying the percentage of glucose, xylose, arabinose and acetic acid by its hydrolysis factors, which are 0.9; 0.88; 0.88 and 0.72 respectively.

3.2.5 Determination of ash content

About 1 g of sugarcane bagasse was placed in a muffle-dried porcelain crucible at 105 °C and heated in a muffle at a temperature of 575 °C for 4 h. After this time, it was placed in a desiccator until it reached a temperature of 25 °C (approximately 1 h) and mass determined. The dry crucible mass was discounted and the ash content was calculated (NBR ABNT, 2016).

3.2.6 Diluted acid for XOS production

About 5 g of bagasse from sugarcane milled to particles 20 meshes was added to the stainless steel reactor containing 50 mL of acetic or sulfuric acid, which was then hermetically sealed, culminating in a mass/water ratio of 1:10, respectively. The assays were performed according to a central composite design 2^3 star rotational with triplicate at the central point to evaluate the influence of independent variables such as temperature (ºC), acid concentration (m/v, mass acid/volume solution) and reaction time (min) to obtain XOS. Independent variables were established with three levels: maximum, corresponding to 160 °C, 55 min time and 3 % acid concentration; minimum, 100 °C, 15 min and 1 % acid and the center point, 130 °C, 35 min and 2 % acid (Table 3.1).

Table 3.1 - Central composite design 2^3 to evaluate the acid hydrolysis process (sulfuric and acetic) of bagasse from sugarcane to xylooligosaccharides (XOS) production

	Temperature (ºC)	**Time (min)**	**H_2SO_4 or CH_3COOH (% m/v)**
Assay			
1	100	15	1
2	160	15	1
3	100	55	1
4	160	55	1
5	100	15	3
6	160	15	3
7	100	55	3
8	160	55	3
9	79.55	35	2
10	180.45	35	2
11	130	1.36	2

Continued from Table 3.1			
12	130	68.64	2
13	130	35	0.32
14	130	35	3.68
15*	130	35	2

** Experiments performed in triplicate, corresponding to the central point*.

After completion of the reaction, the heating was stopped and the system was cooled in an ice bath. The hydrolysate and the solid residue were vacuum filtered using filter paper. The hydrolysate was recovered, filtered with syringe filter 0.22 µm and xylose and XOS quantified by HPLC according to item 3.2.8. Conversion of xylan into byproducts were calculated based on the amount of XOS and xylose released related to the xylan content in the material. The results were analyzed by software STATISTICA version 7.0

3.2.7 Autohydrolysis for XOS production

About 5 g of sugarcane bagasse, milled at 20 mesh was added to the stainless steel reactor containing 50 mL of distilled water, which was then hermetically sealed, culminating in a mass/water ratio of 5:50, respectively. A central composite design 2^2 with three replicates at the central point and axial points (star) was carried out to evaluate the influence of the variables: temperature (ºC) and time (min) to XOS production. Independent variables were established with three levels: maximum, corresponding to 160 °C and 55 min time; minimum, 100 °C and 15 min and the center point, 130 °C and 35 min (Table 3.2).

Table 3.2 - Central composite design 2^2 to evaluate the process of autohydrolysis of bagasse from sugarcane to XOS production.

	Temperature (ºC)	**Time (min)**
Assay		
1	100	15
2	160	15
3	100	55
4	160	55
5	87.57	35
6	172.43	35
7	130	6.72
8	130	63.28

Continued from Table 3.2		
9*	130	35

** Experiments performed in triplicate, corresponding to the central point.*

After the reaction, the reactor was cooled in an ice bath. After cooling, the hydrolysate was vacuum filtered using filter paper. The liquid phase was filtered using a syringe filter with a 0.22 μm pore size and was characterized by the concentration of xylose and XOS by HPLC according to item 3.2.8. Conversion of xylan into bioproducts was calculated based on the amount of XOS and xylose released related to the xylan content in the material. The results were analyzed by software STATISTICA version 7.0.

3.2.8 Determination of XOS concentration

Concentrations of XOS were determined by HPLC on equipment (SHIMADZU, model NEXERA XR) under the following conditions: BIO-RAD Aminex HPX-87C (300 x 7.8 mm) column; temperature: 80 °C; eluent: ultrapure water with flow 0.6 $mL.min^{-1}$; sample volume: 20 μL; detector: refractive index at 80 °C (Shimadzu, RID model) with an analysis time of 15 min. The pH of the samples was adjusted between 5-9 with 1 $mol.L^{-1}$ sodium hydroxide (NaOH). Xylose (X_1) (Sigma), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5) and xylohexaose (X_6) (Megazyme-Ireland) solutions were used as standards. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size (MAFEI et al., 2019).

3.2.9 Determination of furfural and HMF concentration

Three samples from each pretreatment were analyzed, namely: the ones with the highest and lowest conversion into XOS and the center point. Concentrations were determined by HPLC under the following conditions: C_{18} (150 x 4.6 mm) column maintained at 25 ºC, a mobile phase of acetonitrile, water and acetic acid, a flow rate of 0.8 $mL.min^{-1}$, volume of injected sample of 10 μL, with an analysis time of 20 min. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size.

3.3 Results and discussion

3.3.1 Chemical composition of bagasse

The chemical composition of bagasse was 38.7 % of cellulose, 32.8 % of hemicellulose (27.1 % xylan, 2.6 % arabinosyl groups and 3.0 % acetyl groups), 26.1 % of lignin and 3.2 % of ashes (Table 3.3).

Table 3.3 - Chemical composition of sugarcane bagasse* from different studies.

Chemical composition of sugarcane bagasse (%, dry mass)					
Cellulose	**Hemicellulose**	**Lignin**	**Ashes**	**Extractives**	**References**
38.7	32.8	26.1	3.2	6.8	Present study
38.5	27.9	17.8	8.8	2.7	Guilherme et al., 2017
44.3	30.3	23.9	2.5	2.5	Ávila; Forte; Goldbeck, 2018
40.5	29.0	30.6	2.3	-	Brienzo et al., 2016a
37.7	27.2	20.6	6.5	-	Szczerbowski et al.,2014
36.1	23.6	17.8	2.0	6.1	Martin et al., 2007

(-) not determined.
(*) bagasse extractive-free

All values are close to those described in the literature, reported 36.1-44.3 % of cellulose, 23.6-30.3 % of hemicellulose, 17.8-30.6 % of lignin and 2-8.8 % of ashes (Table 3.3). The extractive content of bagasse was 6.8 %, close to that found by Martin et al. (2007). The content of these compounds varies depending on the location, plant growth conditions, climate, soil type, type of tissue analyzed (MOKHENA et al., 2018).

Sugarcane has a high percentage of xylan in its composition, which makes it a promising source for XOS production. Another advantage is the fact of sugarcane bagasse is abundant and produced in several countries.

3.3.2 Diluted acetic acid pretreatment for XOS production

In assay 1 there was greater production of XOS (5.0 $g.L^{-1}$) using sugarcane bagasse, with acetic acid 1 % (m/v), a reaction time of 15 min at 100 ºC. The xylan conversion into XOS was 18.41 %, with higher xylopentaose/xylohexaose formation (Table 3.4). Xylan conversion into xylose was only 3.92 % (Table 4).

Table 3.4 - Xylooligosaccharides (XOS) and xylose yield from the hydrolysis with acetic acid of sugarcane bagasse, using a central composite design 2^3 with variation of time (min), temperature (ºC) e concentration (%, m/v).

	Temperature (ºC)	Time (min)	CH_3COOH % (m/v)	Conversion into xylose and XOS (%)						XOS ($g.L^{-1}$)
Assay				**X_1**	**X_2**	**X_3**	**X_4**	**X_5/X_6**	**XOS**	
1	100	15	1	3.92	0.81	0	5.40	12.20	18.41	5.01
2	160	15	1	3.47	1.07	0.63	2.73	4.82	9.25	2.52
3	100	55	1	0	0	0	0.27	0,0	0.27	0.07
4	160	55	1	3.36	1.26	0.55	1.07	0.27	3.15	0.86
5	100	15	3	0	0	0.16	0.19	0	0.35	0.09
6	160	15	3	6.66	0	0	2.03	10.96	13.0	3.54
7	100	55	3	0	0	0.01	0	0.96	0.97	0.26
8	160	55	3	0	0.59	0.50	0.09	1.04	2.23	0.61
9	79.55	35	2	0	0	1.06	0.47	0.39	1.91	0.52
10	180.45	35	2	15.91	6.43	2.87	1.97	2.22	13.49	3.67
11	130	1.36	2	0	0.01	0	0.28	0	0.29	0.08
12	130	68.64	2	6.31	0.33	0	2.98	6.55	9.87	2.68
13	130	35	0.32	0	0.14	0	1.07	0	1.21	0.33
14	130	35	3.68	5.32	0.02	0.46	0.52	0	1.0	0.27
15	130	35	2	7.13	0.95	0	0	11.83	12.78	3.45
16	130	35	2	5.92	0.82	0	0.38	10.11	11.31	3.05
17	130	35	2	5.22	0.79	0.21	0	11.18	12.18	3.28

X_1: xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose; X_5/X_6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=$X_2+X_3+X_4+X_5/X_6$.

During pretreatment degradation products such as furfural and hydroxymethylfurfural were generated. Table 3.5 shows the values obtained in the assays with higher and lower XOS yield and in the central point. The application of XOS as an additive to any food or supplement requires control of toxic compounds, for this reason it is important to decrease the generation of degradation products.

Table 3.5 - Yield of degradation products generated in hydrolysis with acetic acid of sugarcane bagasse.

	Temperature (ºC)	Time (min)	CH_3COOH % (m/v)	XOS yield (% m/m)	Yield of degradation products (% m/m)		
Assay*					**Furfural**	**HMF**	**Siringil**
1	100	15	1	18.41	0.163	0.014	0.005
3	100	55	1	0.27	0.017	0.001	0.007

Continued from Table 3.5							
15	130	35	2	12.78	0.029	0.003	0.016

*assay related to table 3.1.

Xylan conversion into XOS equivalent to 45.86 % was reported using xylan obtained from the processing of viscose fiber with 20 % (m/v) acetic acid for 20 min at 140 °C. Xylan conversion into xylose was 8.35 % and furfural 0.13 % (m/m). In this study, temperature was the statistical influence factor (ZHANG et al., 2017). In the present study, the maximum xylan conversion of bagasse into XOS was 18.41 % (m/m) with low formation of degradation products.

In pretreatment of the sugarcane bagasse with sulfuric acid was obtained XOS yield of 36.76 %, xylose of 9.29 % and furfural of 0.11 % using acid concentration of 0.5 % (m/m) for 5.5 min at 170 °C (MARCONDES, 2018). This higher yield is probably because sulfuric acid is more efficient than organic acids in polysaccharide hydrolysis (QIN et al., 2012).

Analysis of variance (ANOVA) with a 95 % confidence level ($p < 0.05$) showed that the temperature, time and concentration of acid had a significant effect on XOS production (Table 3.6).

Table 3.6 - Analysis of variance (ANOVA) for the independent variables in the production of Xylooligosaccharides (XOS) using sugarcane bagasse.

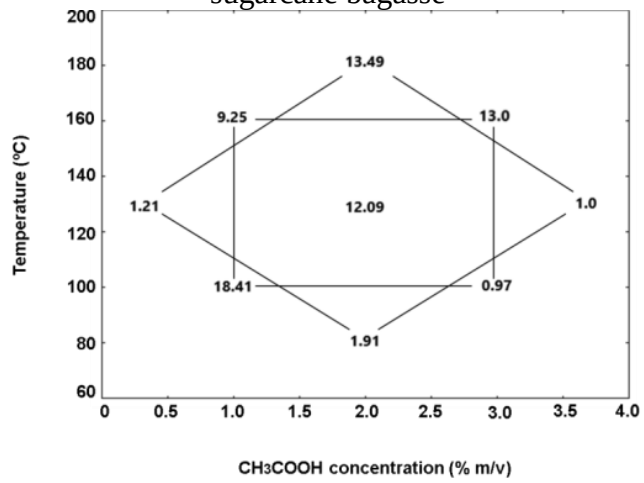
Factor	SS	DF	MS	F	*p*
Temperature (ºC) (L)	53.83	1	53.83	98.54	0.0099
Temperature (ºC) (Q)	15.06	1	15.06	27.57	0.0344
Time (min) (L)	24.43	1	24.43	44.72	0.0216
Time (min) (Q)	48.88	1	48.88	89.48	0.0110
CH_3COOH (% m/v) (L)	16.25	1	16.25	29.75	0.0320
CH_3COOH (% m/v) (Q)	137.18	1	137.18	251.11	0.0040
Temperature by Time (L)	0.05	1	0.05	0.09	0.7884
Temperature by CH_3COOH (L)	51.01	1	51.01	93.36	0.0105
Time by CH_3COOH (L)	24.85	1	24.85	45.49	0.0213
Lack of Fit	278.73	5	55.75	102.04	0.0097
Pure Error	1.09	2	0.55		
Total	601.30	16			
$R^2 = 0.53$					

SS: sum of squares; DF: degree of freedom; MS: mean of squares.

The polynomial model presented no significance, due to a significant lack of fit and low R^2 of 0.53, only 53 % of the experimental data adjusted to the model for acetic acid hydrolysis of bagasse.

Considering XOS xylan conversions obtained in the region of study, the better conversion is in the region where the temperature is 100 °C and the acid concentration of 1 % (Figure 3.1).

Figure 3.1 - Xylooligosaccharides (XOS) yield on the hydrolysis with acetic acid using sugarcane bagasse



Source: Author

3.3.3 Diluted sulfuric acid pretreatment for XOS production

The best condition for XOS production was in assay 9, with 2 % sulfuric acid (m/v), reaction time of 35 min at 79.55 ºC. Xylan conversion into XOS was 90.13 % and the concentration of XOS was 25.85 $g.L^{-1}$, with higher xylobiose formation. Xylan conversion into xylose was 7.15 % (Table 3.7).

The yield of furfural, HMF and siringil in samples with higher and lower XOS yield and a center point sample are shown in table 3.8.

Table 3.7 - Xylooligosaccharides (XOS) and xylose produced in the hydrolysis with sulfuric acid of sugarcane bagasse, using a central composite design 2^3 with variation of time (min), temperature (ºC) and concentration (% m/v)

	Temperature (ºC)	**Time (min)**	**H_2SO_4 % (m/v)**	**Conversion into xylose and XOS (%)**						**XOS ($g.L^{-1}$)**
Assay				**X_1**	**X_2**	**X_3**	**X_4**	**X_5/X_6**	**XOS**	
1	100	15	1	0	0.48	38.27	0	0	38.75	10.54

				Continued from Table 3.7						
2	160	15	1	9.71	0	25.87	0	0	25.87	7.04
3	100	55	1	0.72	0.26	31.25	0	3.92	35.42	9.63
4	160	55	1	5.93	3.18	0	8.19	0	11.37	3.09
5	100	15	3	1.49	72.98	0	0	0	72.98	19.84
6	160	15	3	28.57	73.03	0	0	0	69.38	19.86
7	100	55	3	6.96	75.11	0	0	0	75.11	20.43
8	160	55	3	4.15	0.02	44.46	0	0	44.48	12.10
9	79.55	35	2	7.15	90.13	0	0	0	90.13	24.51
10	180.45	35	2	2.62	0	52.81	0	0	52.80	14.36
11	130	1,36	2	0.26	0	59.80	0	0	59.80	16.27
12	130	68.64	2	36.98	61.95	0	0	0	61.95	16.85
13	130	35	0.32	5.72	1.71	0.87	7.36	0	9.94	2.70
14	130	35	3.68	37.49	40.83	0	0	0	40.83	11.10
15	130	35	2	18.39	76.50	0	0	0	76.50	20.81
16	130	35	2	19.91	78.67	0	0	0	78.67	21.40
17	130	35	2	16.52	80.51	0	0	0	80.51	21.90

X_1: xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose; X_5/X_6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=$X_2+X_3+X_4+X_5/X_6$.

Table 3.8 - Yield of degradation products generated in hydrolysis with sulfuric acid of sugarcane bagasse

	Temperature (ºC)	**Time (min)**	**H_2SO_4 % (m/v)**	**XOS Yield (% m/m)**	**Yield degradation products (% m/m)**		
Assay					**Furfural**	**HMF**	**Siringil**
9	79.55	35	2	90.13	0.312	0.046	0.007
13	130	35	0.32	9.94	0.011	0.002	-
17	130	35	2	80.51	0.152	0.052	0.012

*assay related to table 3.1.
(-): not determined.

Recently the literature has reported a pretreatment in bagasse with 0.5 % sulfuric acid (m/v) aiming at the production of XOS. This study showed a conversion of xylan into XOS of 36.7 % at 170 °C for 30 min. Besides, showed a conversion of xylan into xylose and furfural of 20.2 % and 0.23 % (m/m), respectively (MARCONDES, 2018). This XOS and furfural values were lower than those obtained in the present study, probably due to the fact of the low acid concentration used.

For XOS production using rice straw, with optimal conditions using sulfuric acid at 2 % (m/v) concentration at 100 ºC for 30 min, resulted in 27.8 % xylan to XOS conversion (SOPHONPUTTANAPHOCA et al., 2018). With increasing time to 1 h under the same

conditions, 65.4% of xylan was converted into xylose. In the present study, under similar conditions, at a temperature of 79.55 ºC, a time of 35 min and a sulfuric acid concentration of 2 % (m/m), using sugarcane bagasse it was possible to convert 90.13 % of xylan into XOS and 7.2 % into xylose.

Xylose concentration was reported as 20.6 $g.L^{-1}$ using sugarcane bagasse pretreated with 10 % solids load (m/v), 2 % sulfuric acid (m/v) at 121 ºC for 30 min (PASSARINHO, 2018). In the present study under similar conditions, with 2 % acid (m/v) at 130 ºC for 35 min, the value found was 5.4 $g.L^{-1}$ of xylose, being more advantageous as it facilitates future purification processes of the produced XOS.

Analysis of variance (ANOVA) with 99 % confidence ($p < 0.01$) showed that the concentration of sulfuric acid, temperature and time showed a significant effect for XOS production (Table 3.7).

Table 3.7 - Analysis of variance (ANOVA) for the dependent variables in the production of xylooligosaccharides (XOS) using sugarcane bagasse

Factor	SS	DF	MS	F	*p*
Temperature (ºC) (L)	1243.01	1	1243.01	308.51	0.0032
Temperature (ºC) (Q)	127.31	1	127.31	31.60	0.0302
Time (min) (L)	120.89	1	120.89	30.00	0.0318
Time (min) (Q)	568.99	1	568.99	141.22	0.0070
H_2SO_4 (% m/v) (L)	3112.62	1	3112.62	772.54	0.0013
H_2SO_4 (% m/v) (Q)	4358.40	1	4358.40	1081.73	0.0009
Temperature by Time (L)	218.93	1	218.93	54.34	0.0179
Temperature by H_2SO_4 (L)	5.04	1	5.04	1.25	0.3797
Time by H_2SO_4 (L)	9.22	1	9.22	2.29	0.2694
Lack of Fit	580.31	5	116.06	28.81	0.0339
Pure Error	8.06	2	4.03		
Total	9775.02	16			
$R^2 = 0.94$					

SS: sum of squares; DF: degree of freedom; MS: mean of squares.

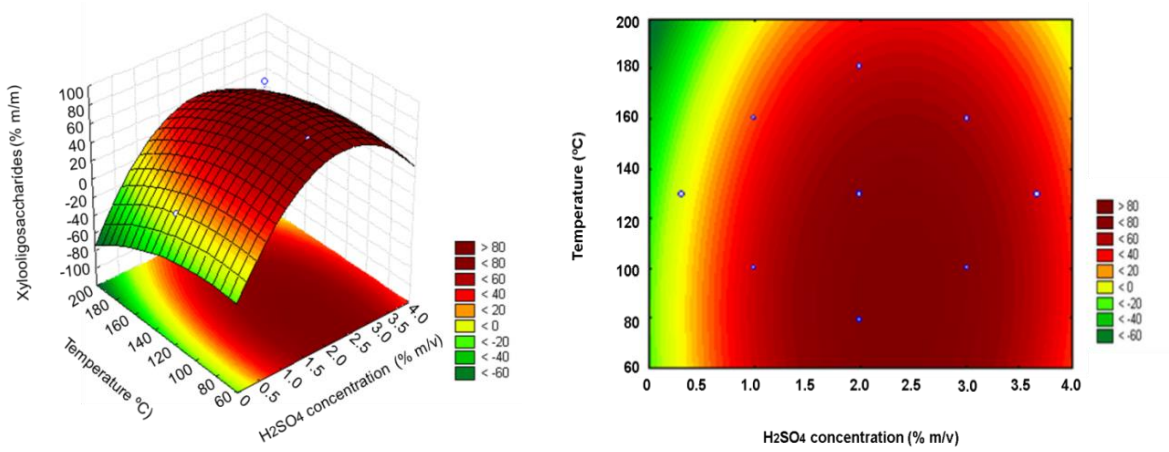
The polynomial model presented significance, with 94 % of the experimental data adjusted to the model for acid hydrolysis of bagasse. The statistical model that describes XOS conversion is represented by Equation 1.

$$XOS\ (\%) = -105.029 + 0.905x - 0.004x^2 + 2.335y - 0.018y^2 + 92.324z - 19.695z^2 - 0.009xy + 0.026xz - 0.054yz \quad (1)$$

Where:
X = temperature (°C)
Y = time (min)
Z = H_2SO_4 concentration (m/v)

Through the analysis of the response surface, it was possible to observe that the highest XOS yield using bagasse (Figure 3.2) is in the region where the temperature is between about 60 and 120 °C and acid concentration between 1.5 % and 3 % (m/v).

Figure 3.2 - (A) Response surface and (B) Contour plot for xylooligosaccharides (XOS) yield produced on the hydrolysis with sulfuric acid using sugarcane bagasse



A **B**

Source: Author

3.3.4 Autohydrolysis for XOS production

The best yield of XOS using sugarcane bagasse was obtained in assay 6, with a time of 35 min and a temperature of 172.4 °C, resulting in a conversion of xylan into xylobiose (0.16 %), xylotriose (0.06 %) and xylotetraose (13.47 %) culminating in a total of 3.72 $g.L^{-1}$ XOS and conversion of 13.67 % xylan into XOS and 0.29 % into xylose (Table 3.8).

Table 3.8 - Xylooligosaccharides (XOS) and xylose yield in the autohydrolysis of sugarcane bagasse, using a central composite design 2^2 with time variation (min) and temperature (ºC)

	Time (min)	Temperature (ºC)	Conversion into xylose and XOS (%)						XOS ($g.L^{-1}$)
Assay			**X_1**	**X_2**	**X_3**	**X_4**	**X_5/X_6**	**XOS**	
1	15	100	0	0	0.01	1.19	0	1.20	0.33
2	15	160	0	0.01	0.31	1.87	3.28	5.47	1.49
3	55	100	0	0.03	0.06	0.69	0	0.77	0.21
4	55	160	0.97	0.01	0	9.45	0.01	9.47	2.58
5	35	87.57	0	0.04	0.05	0.36	0	0.46	0.13
6	35	172.43	0.29	0.16	0.06	13.46	0	13.67	3.72
7	6.72	130	0	0.03	0.04	0.45	0	0.51	0.14
8	63.28	130	0	0.04	0.05	1.08	0.08	1.26	0.34
9	35	130	0	0.17	0.72	1.76	1.49	4.14	1.13
10	35	130	0	0.20	0.64	1.69	0.93	3.47	0.94
11	35	130	0.03	0.13	0.80	1.83	2.05	4.81	1.31

X_1: xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose; X_5/X_6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=$X_2+X_3+X_4+X_5/X_6$.

Table 3.9 shows the values of the byproducts formed in the autohydrolysis of sugarcane bagasse. The samples used were with higher and lower XOS yield and a center point sample.

Table 3.9 - Yield of degradation products generated in autohydrolysis of sugarcane bagasse

	Temperature (ºC)	Time (min)	XOS yield (% m/m)	Yield degradation products (% m/m)	
Assay				**Furfural**	**HMF**
5	35	87.57	0.46	0.010	0.001
6	35	172.43	13.67	0.011	0.003
11	35	130	4.81	0.011	0.002

*assay related to table 3.2.

At similar temperature (170 °C), but with 90 min time has been reported in the literature a conversion of hemicellulose in monomers and oligomers of 61.7 % (SANTUCCI et al., 2015). This result higher can be justified by the longest reaction time and by the sum of monomers and oligomers.

In the present study, XOS were separated in xylobiose, xylotriose, xylotetraose and a xylopentaose/xylohexaose mixture.

Compared with the present study, Brienzo (2010) obtained a lower concentration of XOS corresponding to 2.06 $g.L^{-1}$ (8.22 % conversion) from sugarcane bagasse, with a solid load of 10 % m/v and predominance of xylobiose at 190 ºC for 60 min of reaction, but there was a greater conversion of hemicellulose to xylose (31.63 %) e higher formation of furfural (0.59 % m/m) and HMF (0.12 % m/m). In the study of Bazetto (2018) the concentration of XOS obtained using sugarcane bagasse (solid load of 10 % m/m) at 170 ºC was lower than that presented in this study at a similar temperature, a fact justified possibly by the shorter reaction time employed. The yield obtained by the author was 1.2 $g.L^{-1}$ at 170 °C for 5 min and 2 $g.L^{-1}$ when the time increased to 15 min. When the temperature increased, XOS yields were higher. At 190 °C for 5 and 15 min the concentration was 6.5 $g.L^{-1}$ and 8.6 $g.L^{-1}$, respectively.

The conversion of xylan into xylose was low in comparison to the XOS, which is desirable in this study. This is due to the fact that in this type of pretreatment there is more formation of oligosaccharides, unlike treatments like steam explosion, whose monosaccharide yields are usually higher (CARVALHEIRO et al., 2016). In addition, for oligomers production, treatments with low severity are better and for xylose production, treatments with higher severity are required (BAZETTO, 2018).

The increase in temperature is a determinant factor in the removal of hemicellulose from the lignocellulosic material, because hydrolysis of the acetyl groups release acetic acid in the reaction medium and lowering the pH of the solution, further potentiating the hydrolysis of hemicellulose (LIU et al., 2015). Most hemicellulose is solubilized between 150-230 °C. At temperatures above 260 °C approximately 20 % of the cellulose and 60 % of the lignin can also be solubilized (SANTOS et al., 2014).

Longer reaction times are also significant in the solubilization of lignocellulosic compounds (SANTUCCI et al., 2015). However, conditions of high severity such as excessive temperature and time may cause more formation of degradation products such as furfural and HMF (SARIP et al., 2016).

Analysis of variance (ANOVA) showing the effect of the independent variables (temperature and time) on XOS yield (dependent variable) using sugarcane bagasse, with a confidence level of 95 % ($p < 0.05$) showed temperature and time significant effect (Table 3.9).

Table 3.9 - Analysis of variance (ANOVA) for the independent variables in the production of XOS using sugarcane bagasse

Factor	SS	DF	MS	F	*p*
Time (min) (L)	2.68	1	2.68	5.97	0.1345
Time (min) (Q)	13.82	1	13.82	30.78	0.0310
Temperature (ºC) (L)	125.23	1	125.23	278.97	0.0036
Temperature (ºC) (Q)	13.15	1	13.15	29.29	0.0325
Time by Temperature (L)	4.91	1	4.91	10.93	0.0806
Lack of Fit	4.99	3	1.66	3.71	0.2197
Pure Error	0.90	2	0.45		
Total	176.90	10			
R^2 = 0.97					

SS: sum of squares; DF: degree of freedom; MS: mean of squares

.

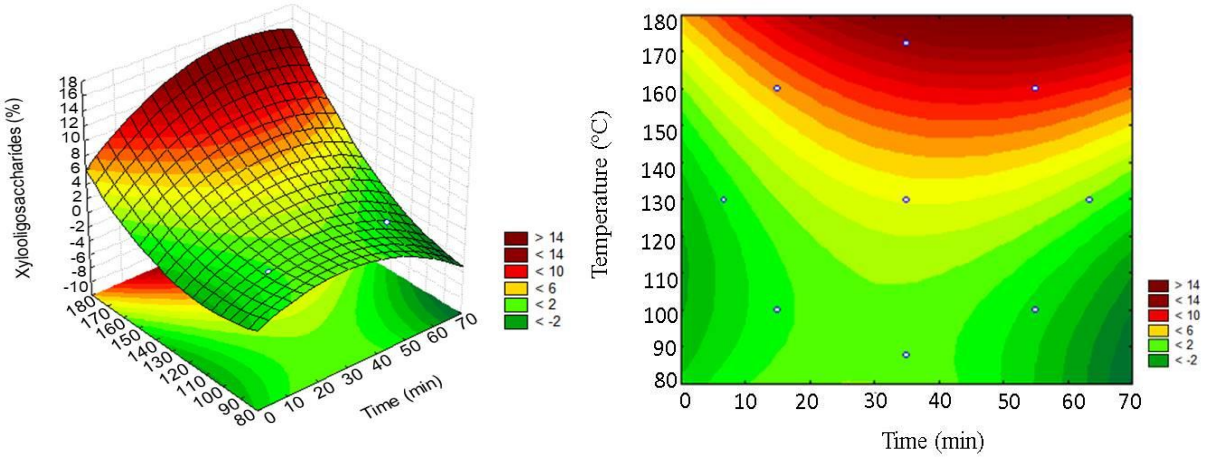
The model proved to be significant since 97 % of the experimental data they adjusted to the polynomial model when bagasse was used. The statistical model that describes the conversion of XOS is represented by Equation 2.

$$XOS\ (\%) = 18.234 + 0.063x - 0.004x^2 - 0.373y + 0.002y^2 + 0.002xy \quad (2)$$

Where:
X = time (min)
Y = temperature (°C)

Figure 3.3 shows the response surface and the contour plot for the variable XOS conversion obtained in the autohydrolysis of sugarcane bagasse. The highest conversion of XOS occurs at temperatures above 170 °C and reaction time between 30 and 60 min.

Figure 3.3 - (A) Response surface and (B) Contour plot for XOS yield produced on the autohydrolysis using sugarcane bagasse



A **B**

Source: Author.

3.4 Conclusion

In the autohydrolysis of sugarcane bagasse the yield of conversion of xylan to XOS was of 13.67 %, with higher xylotetraose formation. In the hydrolysis with dilute acetic acid the biggest yield conversion was 18.41 %, with xylopentaose/xylohexaose prevalence. With dilute sulfuric acid, the conversion of xylan into bioproducts was of 90.13 %, with xylobiose and xylotriose prevalence and high xylose formation.

In hydrolysis with sulfuric acid, the highest conversion of xylan into XOS was obtained and with a lower degree of polymerization, a desirable feature for better probiotic effect, however, there was greater formation of xylose and degradation products, that makes it difficult future purification processes.

In acetic acid hydrolysis and autohydrolysis, conversion of xylan into XOS, xylose and byproducts were lower, probably because they are less severe pretreatments, which tend to form chain oligomers with more than 6 xylose units, which are not detected by HPLC. A post hydrolysis process could increase the amount of short-chain XOS.

CHAPTER IV: XYLOOLIGOSACCHARIDES PRODUCTION USING SUGARCANE LEAF BY AUTOHYDROLYSIS AND DILUTED ACID HYDROLYSIS

Abstract

Straw is an abundant byproduct generated after sugarcane harvest with potential for the generation of various products by chemical and biological pretreatments. Straw is composed of leaf and top of sugarcane culm. The content of xylan in this material is interesting to produce xylooligosaccharides (XOS), oligomers composed of xylose, which provides numerous health benefits. XOS production was performed in this study through two types of pretreatment using sugarcane leaf: autohydrolysis and hydrolysis with dilute sulfuric and acetic acids. For the hydrolysis processes, a central composite design with axial points was performed to evaluate the effects of the independent variables on the production of XOS. Hydrolysis with sulfuric or acetic acid concentrations ranged from 1 and 3% (m/v), temperature of 100 to 160 ºC and reaction time of 15 to 55 min. Autohydrolysis temperature was from 100 to 160 ºC and reaction time from 15 to 55 min. Hydrolysis with acetic acid resulted in the conversion of xylan into XOS of 22.78 % with 2 % of acid at 180 °C for 35 min. Hydrolysis with sulfuric acid resulted in 62.18 % of XOS with 2 % of acid at 79.55 ºC for 35 min. Autohydrolysis of leaf resulted in 20.71 % of XOS at 130 °C for 35 min. The most effective pretreatment for XOS production was hydrolysis with dilute sulfuric acid, resulting in higher yield.

Keywords: Straw; Biomass; Hemicellulose; Xylan; Hydrothermal treatment; Prebiotic.

4.1 Introduction

Sugarcane is of great importance in the economy of Brazil and other tropical countries, due to the numerous products that can be obtained as sugar, alcohol, rapadura and aguardente (LUI et al., 2011). Also besides, byproducts such as vinasse, formed during the production of alcohol, can be used as fertilizer or as an animal feed ingredient as well as bagasse, generated after the grinding of the culm (FERNANDES et al., 2017), which is also reused for production of energy, paper, second-generation ethanol, among others products (ALBUQUERQUE, 2005; SILVA, 2016). Another byproduct obtained from sugarcane is the straw, formed mainly by dry leaves, green leaves and a culm pointer (TROMBETA; CAIXETA FILHO, 2017). Straw is usually left in the fields after harvesting, with potential risk of combustion, producing ashes that pollute the atmosphere, destroying the soil microbiota and causing environmental damage. The amount generated varies according to the productivity and the type of sugarcane cultivated, corresponding to approximately 15 tons per

hectare (CHANDEL et al., 2012). Recent studies suggested that around 50 % of the straw could be recovered from the field without prejudice the soil properties (DE AQUINO et al., 2018).

Straw has a large amount of xylan in its composition, which can be depolymerized into monomers, which can be used for xylitol and ethanol production through fermentation; and into oligomers, called xylooligosaccharides (XOS) (CANILHA et al., 2013). XOS acts as prebiotics due to stimulate the growth of beneficial bacteria in the gut, preventing infections by pathogenic bacteria, have antioxidant properties, prevent osteoporosis, improve intestinal functions, decrease of blood glucose and cholesterol levels, among other benefits (BHATIA et al., 2019; JAIN; KUMAR; SATYANARAYANA, 2015). XOS are stable under various temperature and pH conditions and have a sweet taste, characteristics that facilitate their incorporation in foods (MHETRAS; MAPRE; GOKHALE, 2019).

Pretreatments such as autohydrolysis and dilute acid hydrolysis are widely studied for XOS production. In the autohydrolysis pretreatment only high-temperature water is used, able to break the bonds of acetyl groups present in the xylan chain, acidifying the medium and enhancing the hydrolysis of polysaccharides (SUREK; BUYUKKILECI, 2017; JEONG; LEE, 2015). In the acid pretreatment are usually applied diluted acids such as sulfuric, hydrochloric and nitric, that act by breaking glycosidic bonds between polysaccharides, resulting in formation mainly of monomers and a greater tendency for generation of monosaccharide degradation products (CHEN, 2015).

XOS can be produced by enzymatic hydrolysis of xylan, requiring previously xylan isolation (BRIENZO et al., 2016b). However, it is a necessary purified enzyme to reach high conversion yield. Alternatively, this study was dedicated to use sugarcane leaf for XOS production evaluating acid and autohydrolysis. Pretreatments were evaluated applying experimental design considering the most important variables of acid concentration, temperature and reaction time. Conditions used in the pretreatments were moderate, aiming low formation of xylose and sugar degradation products.

4.2 Materials and methods

4.2.1 Sample preparation

The sugarcane leaf was supplied by Usina São João, located in the city of Araras-SP, Brazil. The sample was oven-dried at 60 °C for 48 h and ground in a knife mill and selected with a 20 mesh sieve.

4.2.2 Chemical characterization of biomass

Glucose, xylose, arabinose, acetic acid and lignin contents were determined by the method of Rocha et al. (2000) and validated by Gouveia et al. (2009).

About 300 mg of sugarcane leaf extractive-free were hydrolyzed with the addition of 1.5 mL of 72 % (m/m) sulfuric acid at 45 °C for 7 min. To the reaction was added 45 mL of distilled water and the flask was autoclaved at 121 °C for 30 min. After cooling the hydrolysate was filtered through a previously tared crucible porous plate. The liquid fraction obtained was analyzed by high-performance liquid chromatography (HPLC) according to item 4.2.3. To determinate the acid soluble lignin content the liquid fraction was diluted with 4 % (m/m) sulfuric acid until the absorbance was between 0.2 and 1.0 and quantified by UV-Vis spectrophotometry at 215 and 280 nm. The solid residue was washed with distilled water, oven-dried at 105 °C to determine insoluble lignin.

4.2.3 Sugars and acetic acid determination

Glucose, xylose, arabinose and acetic acid contents were determined by HPLC, using Bio-Rad Aminex HPX-87H (300 x 7.8 mm) column maintained at 45 ºC, WATERS 2414 refractive index detector, a mobile phase of sulfuric acid 0.005 $mol.L^{-1}$, a flow rate of 0.6 $mL.min^{-1}$, volume of injected sample of 20 μL. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size. Percentage of glucose, xylose, arabinose and acetic acid was multiplied by the hydrolysis factor, 0.9; 0.88; 0.88 and 0.72 respectively for determining the percentage of glucan/cellulose, xylan, arabinosyl and acetyl groups.

4.2.4 Determination of ash content

Total ash content was calculated according to the method proposed by the NBR ABNT (2016). About 1 g of sugarcane leaf was heated in a muffle furnace at 575 °C for 4 h in porcelain crucibles previously oven-dried at 105 °C. The crucible was then desiccated to room temperature (approximately 1 h) and weighed. The ash content was calculated based on the mass determined related to leaf mass used.

4.2.5 Determination of extractives content

About 1 g of sugarcane leaf was inserted into a filter paper bag previously oven-dried for one night at 105 °C. Extractives were removed in Soxhlet with ethanol for 8 h, followed by distilled water for the same period. The filter paper bag it was again oven-dried at 105 °C for one night and cooled in a desiccator. This procedure was repeated until constant mass. The extractives content was determined by the difference between the initial and final mass of filter paper bags multiplied by 100.

4.2.6 Diluted acid for XOS production

About 5 g of leaf from sugarcane at 20 mesh was hydrolyzed with 50 mL of acetic or sulfuric acid in stainless steel reactor. The tests were performed with variation of temperature (ºC), time (min) and acid concentration (% m/v, mass acid/volume solution) according to a central composite design 2^3, star rotational and triplicate at the central point (Table 4.1). The independent variables were divided into three levels: maximum, with a temperature of 160 °C, time of 55 min and acid concentration of 3 %; minimum, 100 °C, 15 min and 1 % acid and the center point, 130 °C, 35 min and 2 % acid.

After completion of the reaction, the reactor was cooled to room temperature with the aid of an ice bath. Liquid fraction containing XOS and xylose was separated from the solid fraction by vacuum filtration using filter paper, filtered with syringe filter 0.22 µm and quantified by HPLC according to item 4.2.8. XOS and xylose contents were calculated relative to the amount of xylan in the material. The results were analyzed by software STATISTICA version 7.0.

Table 4.1 - Central composite design 2^3 to evaluate the acid hydrolysis process (sulfuric and acetic) of leaf from sugarcane to XOS production

	Temperature (ºC)	**Time (min)**	**H_2SO_4 or CH_3COOH (% m/v)**
Assay			
1	100	15	1
2	160	15	1
3	100	55	1
4	160	55	1
5	100	15	3
6	160	15	3
7	100	55	3
8	160	55	3
9	79.55	35	2
10	180.45	35	2
11	130	1.36	2
12	130	68.64	2
13	130	35	0.32
14	130	35	3.68
15*	130	35	2

* Experiments performed in triplicate, corresponding to the central point.

4.2.7 Autohydrolysis for XOS production

In autohydrolysis the assays were performed according to a central composite design 2^2, star rotational and triplicate at the central point, varying temperature (ºC) and time (min). These independent variables were divided into three levels: maximum, with a temperature of 160 °C and time of 55 min; minimum, 100 °C and 15 min and the center point, 130 °C and 35 min (Table 4.2).

About 5 g of sugarcane leaf at 20 mesh and 50 mL of distilled water were added to the reactor. After completion of the reaction, the reactor was cooled in an ice bath and the material was vacuum filtered using filter paper. Liquid fraction was filtered using a syringe filter with a 0.22 μm pore size and quantified by HPLC according to item 4.2.8 for the determination of the XOS and xylose contents. XOS content was calculated relative to the amount of xylan in the material.

Table 4.2 - Central composite design 2^2 to evaluate the process of autohydrolysis of leaf from sugarcane to XOS production

	Temperature (ºC)	Time (min)
Assay		
1	100	15
2	160	15
3	100	55
4	160	55
5	87.57	35
6	172.43	35
7	130	6.72
8	130	63.28
9*	130	35

* Experiments performed in triplicate, corresponding to the central point.

4.2.8 Determination of XOS contents

XOS contents were determined by HPLC on equipment (SHIMADZU, model NEXERA XR) under the following conditions: BIO-RAD Aminex HPX-87C (300 x 7.8 mm) column; temperature: 80 °C; eluent: ultrapure water with flow 0.6 $mL.min^{-1}$; sample volume: 20 μL; detector: refractive index at 60 °C (Shimadzu, RID model) with an analysis time of 15 min. The pH of the samples was adjusted between 5.0 and 9.0 with 1 $mol.L^{-1}$ sodium hydroxide (NaOH). Xylose (Sigma), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose/xylohexaose (X5/6) (Megazyme-Ireland) solutions were used as standards. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size (MAFEI et al., 2019).

4.2.9 Determination of furfural and HMF contents

Three samples from each pretreatment were analyzed, one with the highest yield of XOS, the second with the lowest yield and one sample from the central point.

HMF and furfural contents were determined by HPLC under the following conditions: C18 (150 x 4.6 mm) column maintained at 25 ºC, a mobile phase of acetonitrile, water and acetic acid, flow rate of 0.8 $mL.min^{-1}$, volume of injected sample of 10 μL, with an analysis time of 20 min. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size.

4.3 Results and discussion

4.3.1 Chemical composition of leaf

The leaf chemical composition was 37.9 % of cellulose, 33.2 % of hemicellulose, 25.2 % of lignin, 3.5 % of ashes and 17.6 % of extractives (Table 4.3). For the straw (top part of the culm plus leaf) the literature data reported were in the range of 31.7-39.4 % of cellulose, 27-29.9 % of hemicellulose, 21.3-31.1 % of lignin and 1.5-6.2 % of ash. This study used the leaf, excluding the top part of the culm. The chemical composition determined to leaf showed to be in the range reported in the literature to straw (Table 4.3). An exception was observed for the extractive content, found in leaf was 17.6 %, higher than those content reported in other studies. The advantage of performing a study with a material as the leaf is the lower heterogeneity compared to straw, which is a mix of materials. Fractionation or isolation of biomass is an advantage to evaluate process or material influence in the recalcitrance (BRIENZO et al., 2016b; 2014).

Table 4.3 - Chemical composition of leaf and sugarcane straw from different studies

Chemical composition of sugarcane straw* (%, dry mass)					
Cellulose	**Hemicellulose**	**Lignin**	**Ashes**	**Extractives**	**References**
37.9	33.2	25.2	3.5	17.6	Present study
38.1	29.2	24.7	3.4	4.7	*Miléo et al., 2011
33.3	27.4	26.1	2.6	10.6	*Costa et al., 2015
33.7	27.4	21.3	6.2	-	*Szczerbowski et al.,2014
39.4	29.9	21.8	4.1	4.6	*Ávila; Forte; Goldbeck, 2018
31.7	27	31.1	1.5	-	*Hernández-Pérez; de Arruda; *Felipe, 2016
36.1	28.3	26.2	2.1	5.3	*Canilha et al., 2013
38.1	32.1	24.6	5.05	-	*Mendes et al., 2015

(-) not determined
***** Study performed with straw: the top part of the culm plus leaf

4.3.2 Diluted acetic acid pretreatment for XOS production

The maximum XOS yield obtained in the pre-treatment of the leaf with acetic acid was in the assay 10, resulting in 6.20 $g.L^{-1}$, with an acid concentration of 2 %, 180 ºC and 35 min (Table 4.4). This condition resulted in higher xylotriose formation. The conversion of xylan into XOS was 22.78 %, and into xylose was 4.28 %. It is possible that during reactions with

acetic acid oligomers with more than 6 units were formed, but could not be quantified. A post-hydrolysis reaction could reveal an amount of XOS higher

Conversion of xylan into XOS equivalent to 55.8 % was reported on the hydrolysis with acetic acid of poplar. Conditions used were 30 min of reaction time, 170 ºC and 5 % acid concentration (WEN et al., 2019). For poplar hydrolysis, an acid concentration above that used in the present study was more effective. The hydrolysis of corncob with acetic acid resulted in a 45.91% conversion of xylan into XOS, however produced 24.53 % of xylose (ZHANG et al., 2017). The authors used an acid solution with a pH adjusted to 2.7, temperature 150 °C and time 30 min of reaction. High xylose production together XOS is a disadvantage due to a requirement of the purification process.

Sugarcane bagasse was pretreated with 10 % acetic acid at 150 °C for 45 min resulting in an XOS yield of 39.1 %, and xylose yield of 8.95 % of xylose, 0.09 % of HMF and 0.46 % of furfural (ZHOU; XU, 2019). In the present study, the highest XOS yield was obtained with 2% acid concentration and there was less formation of furfural and HMF.

A literature comparison suggested that processes with low severity tend to produce a higher amount of XOS and lower concentration of xylose. On the other hand, high acid concentration tends to xylose production (GARROTE; DOMINGUEZ; PARAJÓ, 1999).

Table 4.4 – Xylooligosaccharides (XOS) and xylose yield from the pretreatment with acetic acid of sugarcane leaf, using a central composite design 2^3 with variation of time, temperature e concentration

	Temperature (ºC)	**Time (min)**	**CH_3COOH % (m/v)**	**Conversion of xylan into xylose and XOS (%)**						**XOS ($g.L^{-1}$)**
Assay				**X_1**	**X_2**	**X_3**	**X_4**	**$X_{5/6}$**	**XOS**	
1	100	15	1	2.72	0	1.04	1.53	4.20	6.76	1.84
2	160	15	1	0	0	0.32	1.12	3.40	4.84	1.32
3	100	55	1	0	0	0.01	0.60	0	0.61	0.17
4	160	55	1	2.35	0.37	1.78	1.48	16.31	19.93	5.42
5	100	15	3	0	0	1.16	1.83	0	3.00	0.82
6	160	15	3	0	0	0.07	1.50	3.98	5.55	1.51
7	100	55	3	0	0	1.48	1.55	4.74	7.77	2.11
8	160	55	3	0.91	0.45	0.08	0	5.74	6.27	1.71
9	79.55	35	2	0	0	0.11	0.82	0	0.93	0.25
10	180.45	35	2	4.28	1.69	18.89	2.20	0	22.78	6.20
11	130	1.36	2	0	0	0.02	1.70	0	1.71	0.47
12	130	68.64	2	0	0	0.07	1.67	0.92	2.66	0.72

Continued from Table 4.4										
13	130	35	0.32	3.17	0	1.92	0.92	14.67	17.50	4.76
14	130	35	3.68	2.53	0	0.14	0.62	0	0.76	0.21
15	130	35	2	0.73	0	0.91	1.90	4.32	7.13	1.21
16	130	35	2	0	0	1.02	2.01	5.79	8.82	2.40
17	130	35	2	0	0	0.80	2.11	4.41	7.32	1.69

X_1: xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose; X_5/X_6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=$X_2+X_3+X_4+X_5/X_6$.

Table 4.5 shows the values of furfural, HMF e siringil formed in the hydrolysis with acetic acid of sugarcane leaf. The samples used were with higher and lower XOS yield and a center point sample.

Table 4.5 - Yield of degradation products generated in hydrolysis with acetic acid of sugarcane leaf

	Temperature (ºC)	**Time (min)**	**CH_3COOH % (m/v)**	**XOS yield (% m/m)**	**Yield degradation products (% m/m)**		
Assay*					**Furfural**	**HMF**	**Siringil**
3	100	55	1	0.61	0.010	0.002	0.004
10	180.45	35	2	22.78	0,195	0,021	0,008
16	130	35	2	8.82	0.047	0.005	0.006

*assay related to table 4.1.

Analysis of variance (ANOVA) with 99 % confidence ($p < 0.01$) showed that the acid concentration and temperature had a significant effect on the acetic acid pretreatment for XOS production (Table 4.6).

Table 4.6 - Analysis of variance (ANOVA) for the independent variables in the production of xylooligosaccharides (XOS) using sugarcane leaf

Factor	**SS**	**DF**	**MS**	**F**	***p***
Temperature (ºC) (L)	223.08	1	223.08	260.30	0.0038
Temperature (ºC) (Q)	17.71	1	17.71	20.66	0.0451
Time (min) (L)	18.81	1	18.81	21.95	0.0427
Time (min) (Q)	52.88	1	52.88	61.71	0.0158
CH_3COOH (% m/v) (L)	104.02	1	104.02	121.37	0.0081
CH_3COOH (% m/v) (Q)	0.94	1	0.94	1.10	0.4039
Temperature by Time (L)	36.94	1	36.94	43.10	0.0224
Temperature by CH_3COOH (L)	33.42	1	33.42	38.99	0.0247

Continued from Table 4.6					
Time by CH_3COOH (L)	1.49	1	1.49	1.74	0.3183
Lack of Fit	195.77	5	39.15	45.69	0.0216
Pure Error	1.71	2	0.86		
Total	715.58	16			
$R^2 = 0.72$					

SS: sum of squares; DF: degree of freedom; MS: mean of squares

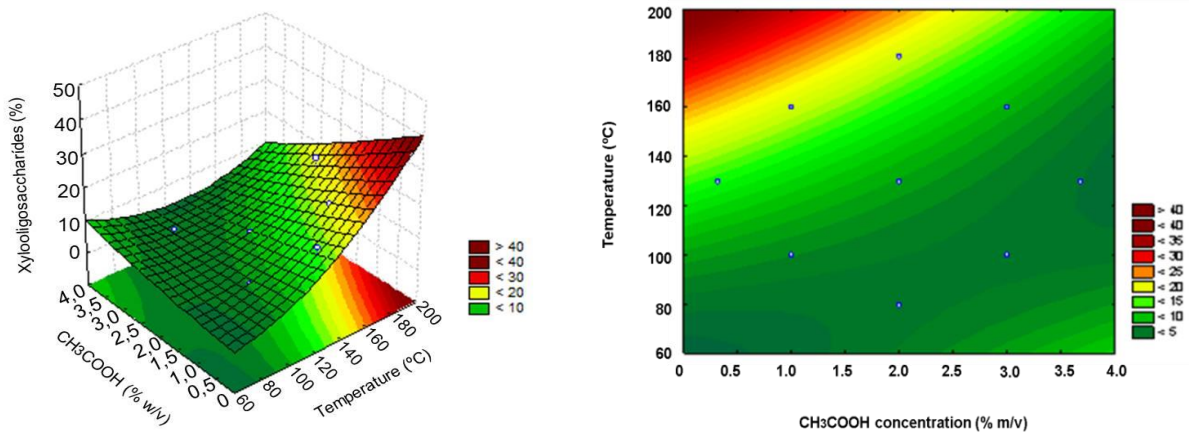
Polynomial model presented significance, 72 % of the experimental data adjusted to the model for acid hydrolysis of leaf. The statistical model that describes xylan conversion into XOS with acetic acid is represented by Equation 1.

$$XOS\ (\%) = 8.905 - 0.216x + 0.001x^2 + 0.015y - 0.005y^2 + 5.690z + 0.290z^2 + 0.004xy - 0.068xz - 0.022yz \quad (1)$$

Where:
X = temperature (°C)
Y = time (min)
Z = CH_3COOH concentration (m/v)

Through the analysis of the response surface, it was possible to observe that the biggest conversion of xylan into XOS is in the region with acid concentration up to 2 % and temperature above 160 ºC (Figure 4.1).

Figure 4.1 - (A) Response surface and (B) Contour plot for conversion of xylan into xylooligosaccharides (XOS) produced on the hydrolysis with acetic acid using sugarcane leaf



A **B**

Source: Author

4.3.3 Diluted sulfuric acid pretreatment for XOS production

Maximum conversion of xylan into XOS was 62.18 %, concentration of 16.91 $g.L^{-1}$ and occurred in assay 9, with a temperature of 79.55 ºC, time of 35 min and 2 % sulfuric acid (m/v). There was a greater formation of xylotriose and conversion of xylan into xylose of 1.30 % (Table 4.7).

Under the conditions of the present study, there was a greater tendency for oligomer formation with 2 or 3 xylose units. The size of the xylan chain after hydrolysis depends on the method used and its severity (BRIENZO et al., 2016a).

In hydrolysis with sulfuric acid of liquor of Kraft pulping process XOS concentration was 11.63 $g.L^{-1}$ achieved with 0.3 % acid at 120 °C for 2 h (CHEN et al., 2018). Acid concentrations and high temperatures are responsible for the formation of unwanted products such as furfural (MOURA, 2015).

Table 4.7 - Xylooligosaccharides (XOS) and xylose produced in the hydrolysis with sulfuric acid of sugarcane leaf, using a central composite design 2^3 with variation of time (min), temperature (ºC) and concentration (% m/v)

	Temperature (ºC)	**Time (min)**	**H_2SO_4 % (m/v)**	**Conversion of xylan into xylose and XOS (%)**						**XOS ($g.L^{-1}$)**
Assay				**X_1**	**X_2**	**X_3**	**X_4**	**$X_{5/6}$**	**XOS**	
1	100	15	1	0.60	0	0.01	15.15	0	15.16	4.11
2	160	15	1	37.02	0	27.07	0	0	27.07	7.36
3	100	55	1	5.38	1.32	32.17	0	3.68	37.17	10.11
4	160	55	1	15.74	0	21.95	0	0	21.95	5.97
5	100	15	3	0.23	0.13	57.40	0	0	57.53	15.65
6	160	15	3	40.26	46.95	0	0	0	46.95	12.77
7	100	55	3	3.51	2.15	20.58	0	0	22.74	6.18
8	160	55	3	14.18	51.44	0	0	0	51.44	13.99
9	79.55	35	2	1.30	0	62.18	0	0	62.18	16.91
10	180.45	35	2	3.70	0	58.75	0	0	58.75	15.98
11	130	1.36	2	0	49.78	0	0	0	49.78	13.54
12	130	68.64	2	43.11	50.32	0	0	0	50.32	13.69
13	130	35	0.32	1.12	1.07	0.90	4.54	0	6.50	1.77
14	130	35	3.68	3.55	0.09	0	28.58	0	28.67	7.82
15	130	35	2	43.10	55.21	0	0	0	55.21	15.01
16	130	35	2	35.41	54.04	0	0	0	54.04	14.70
17	130	35	2	33.48	56.87	0	0	0	56.87	15.47

X_1: xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose; X_5/X_6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=$X_2+X_3+X_4+X_5/X_6$.

Table 4.8 shows the values of furfural, HMF e siringil formed in the hydrolysis with sulfuric acid of sugarcane leaf. The samples used were with higher and lower XOS yield and a center point sample.

Table 4.8 - Yield of degradation products generated in hydrolysis with sulfuric acid of sugarcane leaf

	Temperature (ºC)	**Time (min)**	**H_2SO_4 % (m/v)**	**XOS yield (% m/m)**	**Yield degradation products (% m/m)**		
Assay*					**Furfural**	**HMF**	**Siringil**
9	79,55	35	2	62,18	0.120	0.015	0.009
13	130	35	0.32	6.50	0.149	0.012	0.009
16	130	35	2	54.04	0.146	0.020	0.033

*assay related to table 4.1.

XOS with a lower degree of polymerization act better as probiotics because they are consumed faster by intestinal bacteria (SUREK; BUYUKKILECI, 2017).

Analysis of variance (ANOVA) with 99 % confidence ($p < 0.01$) showed that the acid concentration and the interaction between acid and time presented a significant effect for XOS production (Table 4.9).

Table 4.9 - Analysis of variance (ANOVA) for the independent variables in the production of xylooligosaccharides (XOS) using sugarcane leaf

Factor	**SS**	**DF**	**MS**	**F**	***p***
Temperature (ºC) (L)	5.99	1	5.99	3.45	0.2044
Temperature (ºC) (Q)	0.95	1	0.95	0.55	0.5370
Time (min) (L)	11.44	1	11.44	6.59	0.1241
Time (min) (Q)	129.72	1	129.72	74.72	0.0131
H_2SO_4 (% m/v) (L)	961.76	1	961.76	553.93	0.0018
H_2SO_4 (% m/v) (Q)	2496.18	1	2496.18	1437.70	0.0007
Temperature by Time (L)	18.45	1	18.45	10.63	0.0826
Temperature by H_2SO_4 (L)	57.41	1	57.41	33.06	0.0289
Time by H_2SO_4 (L)	278.36	1	278.36	160.33	0.0062
Lack of Fit	772.78	5	154.56	89.02	0.0111
Pure Error	3.47	2	1.74		
Total	4876.24	16			
$R^2 = 0.84$					

SS: sum of squares; DF: degree of freedom; MS: mean of squares

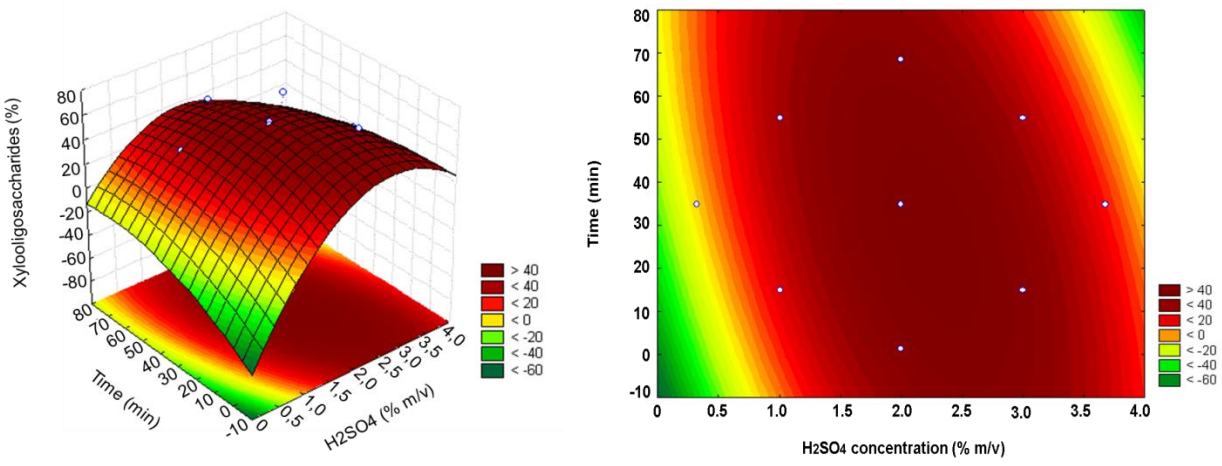
Polynomial model presented significance, with 84 % of the experimental data adjusted to the model for hydrolysis with sulfuric acid. The statistical model that describes xylan conversion into XOS using leaf is represented by Equation 2.

$$XOS\ (\%) = -12.776 - 0.329x + 0.0003x^2 + 0.809y - 0.009y^2 + 66.731z - 14.905z^2 + 0.003xy + 0.089xz - 0.295yz \quad (2)$$

Where:
X = temperature (°C)
Y = time (min)
Z = H_2SO_4 concentration (m/v)

Through the analysis of the response surface, it was possible to observe that the biggest conversion of xylan into XOS is in the region where the acid concentration is between 1.5 e 3.5 % and time up to 60 min (Figure 4.2).

Figure 4.2 - (A) Response surface and (B) Contour plot for conversion of xylan into xylooligosaccharides (XOS) produced on the hydrolysis with sulfuric acid using sugarcane leaf



A **B**

Source: Author

4.3.4 Autohydrolysis for XOS production

The highest values were obtained under the experimental conditions of central point, with a temperature of 130 ºC for 35 min, corresponding to 1.63 % xylobiose, 6.68 %

xylotriose, 12.40 % xylopentaose/xylohexaose with a total of 5.59 $g.L^{-1}$ XOS. Xylan conversion into XOS was 20.71 % and into xylose was 0.60 % (Table 4.10).

Autohydrolysis of various lignocellulosic materials resulted in the maximum yield of XOS at temperatures and time in the range of 220 °C and 35 min. Conversion based on material (mass XOS/mass raw material) were of 27.1 % for barley husks, 24.8 % for corncobs, 18 % for rice husks and 15.4 % for *Eucalyptus globulus* wood (PARAJÓ et al., 2004). Yields were higher than the obtained in the present study probably due to the higher temperature.

Table 4.10 - Xylooligosaccharides (XOS) and xylose produced in the autohydrolysis of sugarcane leaf, using a central composite design 2^2 with time reaction and temperature

	Time (min)	**Temperature (ºC)**	**Conversion into xylose and XOS (%)**						**XOS ($g.L^{-1}$)**
Assay			**X_1**	**X_2**	**X_3**	**X_4**	**$X_{5/6}$**	**XOS**	
1	15	100	0.99	0	4.54	4.50	3.06	12.10	3.27
2	15	160	0.45	1.36	6.20	0	11.76	19.31	5.22
3	55	100	0.58	0.45	4.95	0	7.66	13.06	3.53
4	55	160	1.29	0.93	4.19	4.50	4.94	14.56	3.93
5	35	87.57	1.33	0	4.27	3.09	5.54	12.90	3.48
6	35	172.43	0	1.06	4.91	3.57	5.01	14.55	3.93
7	6.72	130	0	0	4.24	1.76	4.68	10.68	2.89
8	63.28	130	0.05	0	3.54	0	6.32	9.86	2.66
9	35	130	0.03	1.59	6.65	0	12.38	20.67	5.58
10	35	130	0.60	1.63	6.68	0	12.40	20.71	5.59
11	35	130	0.63	1.61	6.73	0	12.35	20.69	5.58

X_1: xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose; X_5/X_6: xylopentaose/xylohexaose; XOS: xylooligosaccharides=$X_2+X_3+X_4+X_5/X_6$.

Table 4.11 shows the values of furfural e HMF formed in the autohydrolysis of sugarcane leaf. The samples used were with higher and lower XOS yield and a center point sample.

Table 4.11 - Yield of degradation products generated in autohydrolysis of sugarcane leaf

	Temperature (ºC)	**Time (min)**	**XOS yield (% m/m)**	**Yield degradation products (% m/m)**	
Assay*				**Furfural**	**HMF**
8	130	63.28	9.86	0.018	0.003

Continued from Table 4.11					
9	130	35	20.67	0.003	0.006
10	130	35	20.71	0.002	0.005

*assay related to table 4.2.

High severity factor of the reaction, which is determined by the influence of temperature and reaction time, greater the hydrolysis of acetyl groups of xylan, causing an increase in the acidity of the medium and consequently improve the breakdown of xylan chain into XOS. However, the high severity can result in sugars degradation products is such as furfural and HMF (GARROTE, DOMÍNGUEZ, PARAJÓ, 1999). Autohydrolysis with low severity can generate XOS containing more than 6 xylose units. For the production of a smaller chain of XOS additional treatment with enzymes or reagents may be required (FIGUEIREDO, 2016).

Analysis of variance (ANOVA) showing the effect of the independent variables (temperature and time) on conversion xylan into XOS (dependent variable), with a confidence level of 99 % ($p < 0.01$) (Table 4.12). For the sugarcane leaf autohydrolysis reaction time and temperature presented significant effect.

Table 4.12 - Analysis of variance (ANOVA) for the independent variables in the production of XOS using sugarcane leaf

Factor	SS	DF	MS	F	*p*
Time (min) (L)	11.62	1	11.62	32.85	0.0291
Time (min) (Q)	38.33	1	38.33	108.37	0.0091
Temperature (ºC) (L)	94.45	1	94.45	266.99	0.0037
Temperature (ºC) (Q)	1.71	1	1.71	4.83	0.1590
Time by Temperature (L)	0.17	1	0.17	0.48	0.5618
Lack of Fit	7.04	3	2.35	6.63	0.1339
Pure Error	0.71	2	0.35		
Total	152.60	10			
R^2 = 0.95					

SS: sum of squares; DF: degree of freedom; MS: mean of squares

The model proved to be significant since 95 % of the experimental data they adjusted to the polynomial model. The statistical model that describes the conversion of xylan into XOS using leaf is represented by Equation 3.

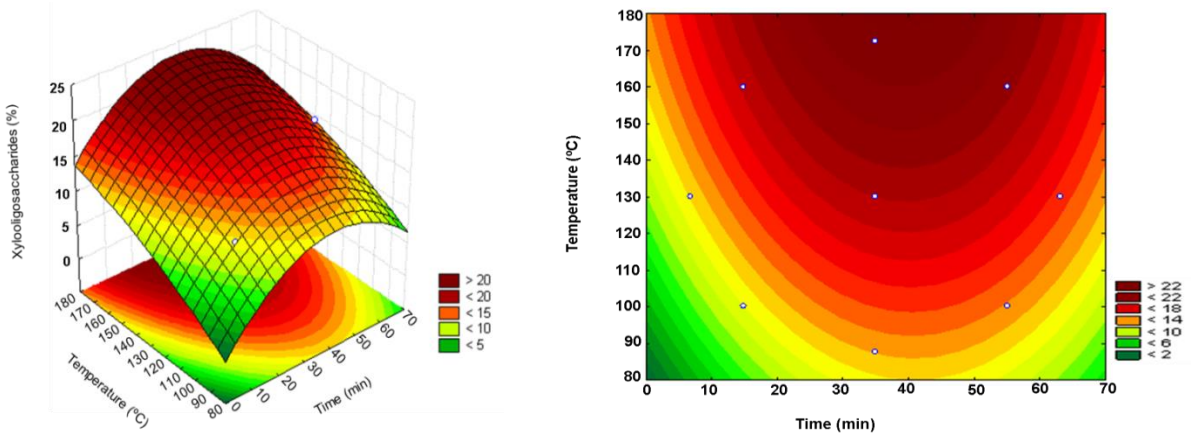
$$XOS\ (\%) = -17.841 + 0.561x - 0.007x^2 + 0.285y - 0.001y^2 - 0.0003xy \quad (3)$$

Where:

X = time (min)
Y = temperature (°C)

Figure 4.3 shows the response surface and the contour plot for the variable XOS obtained in the autohydrolysis of the sugarcane leaf. The best yield can be obtained with a temperature above 160 ºC and a time interval between 25 and 50 min.

Figure 4.3 - (A) Response surface and (B) Contour plot for conversion of xylan into XOS produced on the autohydrolysis using sugarcane leaf



A **B**

Source: Author

4.4 Conclusion

Sulfuric acid hydrolysis was more efficient than acetic acid hydrolysis and autohydrolysis in the production of XOS, with a conversion of 62.18 % of xylan to XOS and formation of short-chain oligomers, with up to 3 xylose units, which act better as a prebiotic. There was the formation of xylose, furfural, HMF and siringil, making the XOS purification process more difficult and expensive. In autohydrolysis, the highest yield was 20.71 % and in the hydrolysis with acetic acid was 22.78 %. In both cases, the lower concentration can be explained by mild process conditions that break xylan into long-chain oligomers that are not quantified by HPLC. Temperature increase or a post hydrolysis reaction has the potential to increase the concentration of short-chain XOS, between 2 and 6 xylose units. Autohydrolysis it was the pretreatment that generated the least amount of degradation products.

CHAPTER V: XYLOOLIGOSACCHARIDES PRODUCTION FROM SUGARCANE BAGASSE AND LEAF USING *ASPERGILLUS VERSICOLOR* ENDOXYLANASE AND DILUTED ACID.

Abstract

Xylooligosaccharides (XOS) are xylose oligomers that when incorporated into the diet bring numerous health advantages, arousing the interest of the food and pharmaceutical industries. XOS can be produced by acid or enzymatic hydrolysis of xylan-rich materials. The use of enzymes has great potential because unlike acids does not form undesirable products and does not require the use of expensive equipment, but obtaining them is still expensive and complex. The aim of this study was to produce XOS by hydrolysis of xylan extracted from sugarcane bagasse and leaf with *Aspergillus versicolor* endoxylanase and diluted acid. Xylan was extracted with 6 % (m/v) hydrogen peroxide in alkaline medium, for 4 h at a temperature of 25 ºC. For enzymatic hydrolysis a central composite design 2^3 was performed with axial points, ranging enzyme concentration from 30 to 100 $UI.g^{-1}$, substrate concentration (xylan) from 1 to 5 % and reaction time of 24 h. For acid hydrolysis a central composite design 2^2 was performed with axial points, varying concentration of acetic or sulfuric acid from 1.5 to 4 %, reaction time from 10 to 30 min at 130 °C. The extraction yield of xylan was 65.15 % e 69.7 % using bagasse and leaf, respectively. The highest conversion of xylan from bagasse into XOS using acetic acid hydrolysis was 56.29 %, with 1.5 % (m/v) of acid for 30 min at 130 ºC. Xylan from leaf was used as raw material and the highest yield was 53.65 %, with an acid concentration of 2.75 % (m/v), a reaction time of 34 min at 130 ºC. In the hydrolysis with sulfuric acid of xylan from bagasse and leaf, the XOS yield was 40.16 % and 45.37 % respectively, with 1.5 % (m/v) of acid for 10 min at 130 ºC for both. In enzymatic hydrolysis the highest conversion yield was 67.43 % and 69.71 % for xylan from bagasse and leaf respectively, with enzymatic loading of 65 $UI.g^{-1}$ and substrate concentration of 0.17 % (m/v). The results showed that acid and enzymatic hydrolysis resulted in high yields of XOS, however, acid hydrolysis has the disadvantage of furfural and hydroxymethylfurfural (HMF) formation, which does not occur in enzymatic hydrolysis, but in this process, there is a need for enzyme purification, which makes the pretreatment expensive and complex.

Keywords: Biomass; Xylan; Acid hydrolysis; Enzymatic hydrolysis; Xylooligosaccharides.

5.1 Introduction

Xylooligosaccharides (XOS) are xylose oligomers that are commonly used as prebiotics due to their ability to stimulate the growth of some desirable bacteria for healthy gut functioning (GUIDO; SILVEIRA; KALIL, 2019). In addition they increase the calcium absorption, protect against cardiovascular disease and reduce the risk of colon cancer,

improve the immune system and have antioxidant, anti-inflammatory and anti-allergic activity (CARVALHO et al., 2015).

Xylan-rich materials such as forest, industrial and agricultural byproducts can be used as raw materials for industrial production. The use of these materials is environmentally beneficial as it prevents accumulation in unwanted places. Moreover, it is economically advantageous because it is an abundant and renewable resource (KUMAR; PUSHPA; PRABHA, 2012).

Due to its numerous applications, XOS has aroused interest from industries to develop new technologies to produce it with high purity and a lower degree of polymerization but its production is still expensive and complicated and its market value very high (GUIDO; SILVEIRA; KALIL, 2019). XOS can be produced by a variety of processes, including enzymatic or acid hydrolysis of xylan. In lignocellulosic biomass xylan binds to lignin making it resistant to hydrolysis, as an alternative XOS production can be performed in two steps: alkaline extraction of xylan present in lignocellulosic biomass followed by acid or enzymatic hydrolysis (AKPINAR; ERDOGAN; BOSTANCI, 2009).

The use of enzymes for XOS production is considered a promising alternative when compared to chemical processes, since the final product is highly pure and there is less formation of unwanted byproducts, but the yield is generally low, the process is time-consuming and the costs are high (RASHAD et al., 2016; CARVALHO et al., 2015). Xylanolytic enzymes used in XOS production act by breaking the xylan chain into oligomers and can also be used in hydrolysis of lignocellulosic material and bioconversion of sugars to ethanol, in baking, in juice and beer clarification and in pulp bleaching (CARMONA et al., 1998; CARVALHO et al., 2015).

In the present study the enzymatic hydrolysis of xylan from sugarcane bagasse and leaf was performed with enzyme extracted from *Aspergillus versicolor*, a slow-growing filamentous fungus that is widely found in food and humid environments and can be used in production industrial enzyme (NADUMANE; VENKATACHALAM; GAJARAJ, 2016).

Acid hydrolysis breaks the glycosidic bond in the polysaccharides by the addition of mineral or organic acids (HILPMANN et al., 2016). Dilute acid hydrolysis is advantageous over concentrated acid hydrolysis because using a low concentration of acid, there is less environmental impact, reagent waste and equipment corrosion. However, this pretreatment requires the use of high temperature and pressure, making it necessary to use special equipment to support these conditions (CHEN, 2015). In this process polysaccharides can be degraded into byproducts such as furfural and hydroxymethylfurfural (HMF) that can also be

degraded into other substances like formic and levulinic acids (KANCHANALAI et al., 2016).

In the present study the xylan used was solubilized from sugarcane bagasse and leaf, using hydrogen peroxide in alkaline medium. The xylan was hydrolyzed using endo-β-1.4-xylanase extracted from *Aspergillus versicolor* and with dilute sulfuric and acetic acid, varying several parameters to produce XOS and low concentration of xylose and degradation products.

5.2 Materials and methods

5.2.1 Sample preparation

Sugarcane bagasse and leaf (from the São João Plant, Araras - São Paulo, Brazil) was oven-dried at 60 ºC, ground in a knife mill and selected by the sieve of 20 mesh.

5.2.2 Xylan solubilization

Sugarcane bagasse and leaf were washed with 0.2 % (m/v) ethylenediaminetetraacetic acid (EDTA) for 1 h at 90 ºC to remove metal cations. About 10 g of material was treated with 200 mL of 6 % hydrogen peroxide. The pH of the solution was adjusted to 11.6 with 5 $mol.L^{-1}$ sodium hydroxide (NaOH) (BRIENZO; SIQUEIRA; MILAGRES, 2009). The suspension was stirred on a rotary shaker at 110 rpm for 4 h at 25 ºC. The solid fraction was separated from the liquid fraction by filter paper filtration. The pH of the liquid fraction was adjusted to 6.0 with 6 $mol.L^{-1}$ hydrochloric acid (HCl) and concentrated to about one-third of its volume under air circulation at 45 ºC. The concentrated sample was poured into three volumes of 95 % ethanol to precipitate xylan and washed four times with 70 % ethanol. The decanted hemicellulose was dried by air circulation at 45 ºC. The extraction yield was calculated based on the relation between the solubilized xylan mass and the xylan mass contained in the bagasse or leaf used.

5.2.3 Xylan solubility

The solubility of xylan was determined according to the method of Bailey; Biely; Poutanen, 1992 with modifications. Was prepared a 1 % xylan solution in 50 mM sodium

phosphate buffer, pH 6.0. The solution was heated to boiling point in a microwave; was cooled and stirred at 80 rpm for one night at 25 ° C and centrifuged at 10000 xg for 15 min to separate the insoluble material that was dry at 60 ° C until a constant mass

5.2.4 Determination of extractives content

Extractives content was determined with 1 g of sugarcane bagasse or leaf in an oven-dried filter paper bags at 105 °C. The filter paper bag was inserted into the Soxhlet and extraction was performed for 8 h with ethanol, the same procedure was performed with distilled water. The filter paper bag was oven-dried at 105 °C for at least one night and placed in a desiccator until it reached a temperature of 25 °C and the mass was determined by gravimetry.

5.2.5 Chemical characterization of xylan and biomass

A sample containing 300 mg of extractive free biomass or solubilized xylan (bagasse or leaf) was hydrolyzed by the addition of 1.5 mL of 72 % (m/m) sulfuric acid at 45 °C for 7 minutes. The reaction was stopped by the addition of 45 mL of distilled water and the solution was autoclaved at 121 °C for 30 min. After cooling the hydrolyzed solution was filtered through a previously tared crucible porous plate (ROCHA, 2000; GOUVEIA et al., 2009). The solid residue was washed with distilled water, oven-dried at 105 °C to constant weight for the determination of insoluble lignin by gravimetry. The acid soluble lignin content was determined by UV-Vis spectrophotometry at 215 and 280 nm using a solution of 4 % sulfuric acid as a blank. The total lignin content was calculated by the sum of lignin insoluble and soluble in sulfuric acid. The soluble fraction was quantified by High-Performance Liquid Chromatography (HPLC), according to item 5.2.5.

5.2.6 Sugars and acetic acid determination

The contents of glucose, xylose, arabinose and acetic acid were determined by HPLC, using Bio-Rad Aminex HPX-87H (300 x 7.8 mm) column maintained at 45 ºC, WATERS 2414 refractive index detector, a mobile phase of sulfuric acid 0.005 $mol.L^{-1}$, flow rate of 0.6 $mL.min^{-1}$, sample injection volumes of 20 μL. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size.

The glucan, xylan, arabinosyl and acetyl side-groups content were calculated by multiplying the percentage of glucose, xylose, arabinose and acetic acid by its hydrolysis factors, which are: 0.9; 0.88; 0.88 and 0.72 respectively.

5.2.7 Determination of ash content

Sample containing 1 g of sugarcane bagasse was placed in a muffle-dried porcelain crucible at 105 °C and heated in a muffle at a temperature of 575 °C for a period of 4 h. After this time, it was placed in a desiccator until it reaches a temperature of 25 °C (approximately 1 h) and mass determined. The dry crucible mass was discounted and the ash content was calculated (NBR ABNT, 2016).

5.2.8 Enzymatic activity

The method of Bailey et al. (1992) modified was used to determine xylanase activity. 750 µl of 1 % (m/v) Birchwood xylan (Sigma) was added to a tube containing 0.05 mol.L^{-1} sodium phosphate buffer, pH 6 and 10 to 250 µl of enzyme at 55 °C. (CARMONA et al, 1998). 250 µL aliquots were collected at certain time intervals. 250 µL of 3.5-dinitrosalicylic acid (DNS) reagent was added to the tubes to stop the reaction and determine the concentration of reducing sugars using xylose as standard. Absorbance reading was performed on a spectrophotometer at 540 nm (MILLER, 1959). Enzyme activity was expressed in units per volume (U.mL^{-1}) of the enzyme.

5.2.9 Enzymatic hydrolysis for XOS production

The enzymatic hydrolysis of xylan solubilized from sugarcane bagasse or leaf with hydrogen peroxide was performed with *Aspergillus versicolor* endoxylanase produced in wheat bran and purified (FREITAS, 2019). The tests were conducted in a Falcon tube containing 5 mL of 50 mM sodium phosphate buffer (pH 6) in a Shaker at 55 °C with 120 rpm agitation. Assays varied in substrate concentration (1-5 % m/v) and enzymatic load (30-100 UI.g^{-1}), with a reaction time of 24 h. A central composite design 2^2 with three replicates at the central point and axial points (star) was carried out to evaluate the influence of the variables: enzymatic load (UI.g^{-1}) and substrate concentration (%, m/v) to XOS production. Independent variables were established with three levels: maximum, corresponding to 100

UI.g^{-1} and 5 % (m/v); minimum, 30 UI.g^{-1} and 1 % (m/v) and the center point, 65 UI.g^{-1} and 3 % (m/v) (Table 5.1).

Table 5.1 - Central composite design 2^2 to evaluate the enzymatic hydrolysis process of xylan extracted from sugarcane bagasse or leaf

Assay	Enzyme (UI.g^{-1})	Substrate (% m/v)
1	30	1
2	100	1
3	30	5
4	100	5
5	15.5	3
6	114.5	3
7	65	0.17
8	65	5.83
9*	65	3

* Experiments performed in triplicate, corresponding to the central point.

After reaching the reaction time the tubes were heated in boiling water for 5 min. The hydrolysate was centrifuged and the liquid fraction was filtered using a syringe filter with a 0.22 μm pore size and was characterized by the concentration of xylose and XOS by HPLC according to item 5.2.10. The conversion of xylan was calculated based on the amount of XOS and xylose released related to the xylan content in the material. The results were analyzed by software STATISTICA version 7.0.

5.2.10 Dilute acid pretreatment for XOS production

A sample of 0.1 g of the xylan solubilized from sugarcane bagasse or leaf with hydrogen peroxide was added to the borosilicate tube containing 5 ml of an acetic acid solution or sulfuric acid in concentrations ranging from 1.5 to 4.0 %. The tubes were sealed and heated in an oil bath at 130 °C. The reaction time ranged from 10 to 30 min. The assays were performed according to central composite design 2^2 with three replicates at the central point and axial points (star) to evaluate the influence of the variables: acid concentration (% m/v) and time (min) to XOS production. Independent variables were established with three levels: maximum, corresponding to 4 % (m/v) and 30 min time; minimum, 1.5 % (m/v) and 10 min and the center point, 2.75 % (m/v) and 20 min (Table 5.2).

Table 5.2 - Central composite design 2^2 to evaluate the acid hydrolysis process (sulfuric and acetic) of xylan from sugarcane bagasse or leaf under temperature of 130 ºC

	Time (min)	**H_2SO_4/CH_3COOH (% m/v)**
Assays		
1	10	1.5
2	10	4
3	30	1.5
4	30	4
5	20	0.98
6	20	4.52
7	5.86	2.75
8	34.14	2.75
9*	20	2.75

* Experiments performed in triplicate, corresponding to the central point.

After the reaction, the tubes were cooled in an ice bath. The hydrolysate was centrifuged and the liquid fraction was filtered using a syringe filter with a 0.22 μm pore size and was characterized in terms of xylose and XOS concentration by HPLC according to item 5.2.10. Conversion of xylan into byproducts was calculated based on the amount of XOS and xylose released related to the xylan content in the material. The results were analyzed by software STATISTICA version 7.0.

5.2.11 Determination of XOS contents

The concentrations of XOS were determined by HPLC on equipment (SHIMADZU, model NEXERA XR) under the following conditions: BIO-RAD Aminex HPX-87C (300 x 7.8 mm) column; temperature: 80 °C; eluent: ultrapure water with flow 0.6 $mL.min^{-1}$; sample volume: 20 μL; detector: refractive index at 60 °C (Shimadzu, RID model) with an analysis time of 15 min. The pH of the samples was adjusted between 5-9 with 1 $mol.L^{-1}$ sodium hydroxide (NaOH). Xylose (X_1) (Sigma), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5) and xylohexaose (X_6) (Megazyme-Ireland) solutions were used as standards. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size.

5.2.12 Determination of furfural and hydroxymethylfurfural (HMF) contents

Samples of acetic acid and sulfuric acid pretreatments were analyzed. In enzymatic hydrolysis no degradation products are formed. The samples chosen were obtained at the minimum and maximum levels and central point. Concentrations were determined by HPLC under the following conditions: C_{18} (150 x 4.6 mm) column maintained at 25 ºC, [a] mobile phase of acetonitrile, water and acetic acid, flow rate of 0.8 $mL.min^{-1}$, volume of injected sample of 10 μL, with an analysis time of 20 min. Samples were pre-filtered on a syringe filter with a 0.22 μm pore size.

5.3 Results and discussion

5.3.1 Chemical composition of biomass

The content of cellulose, hemicellulose, lignin, ashes and extractives present in the bagasse was of 38.7 %, 32.8 %, 26.1 %, 3.2 % and 6.8 %, successively and for the leaf 37.9 %, 33.2 %, 25.2 %, 3.5 % and 17.6 %, successively. The amount of xylan present in the bagasse was 27.14 % and on the leaf 27.05 %. The values are close to those reported in other works. For bagasse the values are approximately: 35.2-40.8 % of cellulose, 24.5-35.8 % of hemicellulose, 22.2-33.7 % of lignin, 0.7-20.9 % of ashes and 2.7-14.1 % of extractives (REZENDE et al., 2011; PHILIPPINI et al., 2019; SASAKI; ADSCHIRI; ARAI, 2003; BRIENZO et al., 2016a). Regarding the straw the values are approximately: 38.8-43 % of cellulose, 15-30.8 % of hemicellulose, 23-28.6 % of lignin and 2.2-6 % of ashes. The amount of extractive found on the leaf in the present work was higher than that described in the literature which is approximately 3-10.6% (MORETTI et al., 2016; CANDIDO; MORI; GONÇALVES, 2019; MOUTTA et al. 2012; MILÉO et al. 2011; COSTA et al., 2015).

5.3.2 Solubilization and chemical composition of xylan

The condition for xylan solubilization used in the present study was chosen according to an optimized method in the literature (BRIENZO; SIQUEIRA; MILAGRES, 2009).

Solubilization yields were: 65.15 % and 69.7 % for xylan from bagasse and leaf respectively. The yield was above those obtained by Carvalho et al. (2017), Brienzo; Siqueira; Milagres (2009) e Banerje et al. (2014) (Table 5.3).

Xylan solubilized from leaf presented higher xylose concentration (72.23 %) in its composition in relation to xylan extracted from bagasse (61.68 %), similar results to those obtained by Brienzo; Siqueira; Milagres (2009) under the same conditions and higher than those obtained by the same authors using a lower concentration of hydrogen peroxide.

In the present study, xylose contents were close to those obtained by Banerje et al. (2014) and Sporck et al. (2017) but the yield was higher compared to the first and was not reported by the second. In the study by Carvalho et al. (2017) the xylose content was higher, but the solubilization yield was lower compared to the present study.

Table 5.3 - Chemical composition of xylan extracted from sugarcane bagasse and straw* obtained from different studies

Chemical composition of xylan (% dry mass)								
Xylose	**Arabinose**	**Acetyl**	**Uronic acids**	**Glucose**	**Lignin**	**Extraction Yield**	**Conditions**	**Reference**
61.68 ± 1.88	5.73 ± 1.02	0.79 ± 0.06	-	3.26 ± 0.41	8.13 ± 1.20	65.15 ± 1,98	Sugarcane bagasse washed with EDTA 2 % (m/v). Pretreated with H_2O_2 6 % (m/v) in alkaline medium, for 4 h, at 20 ºC	Present study[1,*]
72.23 ± 2.73	8.04 ± 0.52	0.28 ± 0.20	-	3.52 ± 0.27	13.54 ± 1.46	69.7 ± 2.46	Sugarcane leaf washed with EDTA 2 % (m/v). Pretreated with H_2O_2 6 % (m/v) in alkaline medium, for 4 h, at 20 ºC	Present study[2]
80.9	3.8	-	3.9	4.2	5.9	86.0	Sugarcane bagasse washed with EDTA 2 % (m/v). Pretreated with H_2O_2 6 % (m/v) in alkaline medium, for 4 h, at 20 ºC	Brienzo; Siqueira; Milagres (2009)[1]
50.4	4.4	-	4.4	3.9	10	52	Sugarcane bagasse: H_2O_2 in alkaline medium (2%, m/v), 20 °C, 4h.	Brienzo; Siqueira; Milagres (2009)[1]
83.8	11.3	-	1.4	3.1	-	-	Ethanol/Toluene Treatment 8 h; Sodium Chloride In Acid Medium 75 °C, 2 h.. 10 % KOH 30 ºC, 10h, liquid fraction neutralized with acetic acid and precipitated with 95 % ethanol.	Bian et al. (2014)[1]
88.0	4.7	8.72	-	5.0	-	19.3	Ethanol/Toluene Treatment 12 h; 0.2 % (m/v) EDTA 90 ºC for 1 h.10 % Peracetic acid, 85 ºC for 30 min. Dimethyl sulfoxide 24 ºC for 24 h. Liquid fraction: ethanol (pH=3.5 with formic acid) for 12 h at 4 ºC. Hemicellulose washed with methanol.	Carvalho et al. (2017)[1]
61.7	7.7	-	6.5	5.23	8.4	-	Sugarcane bagasse delignified with acid-chlorite. Xylan extraction with 40% NaOH (m/m) at 25 °C for 16 h	Sporck et al. (2017)[1]

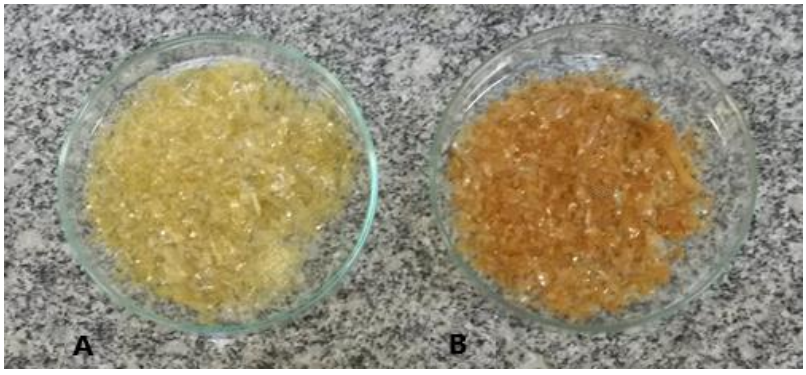
Continued from Table 5.3

78.8	4.6	-	3.2	3.1	7.2	14.9	Hot water 15 min, 170°C, and 3% H_2O_2, 0.25% magnesium sulfate, 40°C, 12h.	Banerje et al. (2014)[1]
82.0	6.3	2.42	-	8.6	-	21.7	Ethanol/Toluene Treatment 12 h; 0.2 % (m/v) EDTA 90 ºC for 1 h.10 % Peracetic acid, 85 ºC for 30 min. Dimethyl sulfoxide 24 ºC for 24 h. Liquid fraction: ethanol (pH=3.5 with formic acid) for 12 h at 4 ºC. Hemicellulose washed with methanol.	Carvalho et al. (2017)[3]

**assays performed in triplicate*
(-): not reported
(1): Xylan solubilized from sugarcane bagasse.
(2): Xylan solubilized from sugarcane leaf.
(3): Xylan solubilized from sugarcane straw.

Figure 5.1 shows xylan extracted from sugarcane bagasse and leaf in the present study. Xylan solubilized using alkaline solvents usually has a brown coloring that can difficult some uses in industry as a food additive, for example. Hydrogen peroxide in an alkaline medium can be used for lighter xylan production. During the reaction hydroxyl and superoxide radicals are generated which are efficient in delignification and solubilizing hemicellulose, with less damaging carbohydrates (SUN et al., 2000; WANG et al., 2018; ZHANG et al., 2019). After the reaction the peroxide leaves no residue in the medium as it decomposes into oxygen and water (BRIENZO; SIQUEIRA; MILAGRES, 2009; ZHANG et al., 2019). In xylan extraction by this method, it is necessary to use a chelating agent such as ethylenediamine tetra-acetic acid (EDTA) to remove metal ions avoiding decomposition of hydrogen peroxide (CARVALHO et al., 2017).. .

Figure 5.1 - (A) Xylan solubilized from sugarcane leaf (B) Xylan solubilized from sugarcane bagasse



Source: Author

5.3.3 Xylan Solubility

The solubility of xylan solubilized from sugarcane bagasse and leaf were 76 % and 84 %, respectively. Xylan extracted with hydrogen peroxide tends to be more branched compared to xylan extracted with alkaline reagents such as NaOH, being more soluble. This is because generally the alkali concentrations employed are lower when peroxide is used, avoiding degradation reactions. Solubility is an important factor as it may interfere with enzymatic hydrolysis (FANG et al., 1999).

5.3.4 Acid hydrolysis of xylan for XOS production

The maximum yield of XOS obtained in the hydrolysis with acetic acid of xylan extracted from sugarcane bagasse was 56.29 %, obtained with 1.5 % acetic acid, at 130 ºC and reaction time of 30 min. At the central point the conversion was close, corresponding to approximately 53.37 %, using a higher concentration of acetic acid (2.75 %), shortest time (20 min) and same temperature (130 ºC) (Table 5.4). With the leaf the highest xylan conversion into XOS was 53.65 %, using 2.75 % acid concentration, reaction time of 34.14 min and at 130 ºC. This value was close to that obtained with the xylan from bagasse at the central point (Table 5.5).

In the hydrolysis with sulfuric acid of xylan extracted from sugarcane bagasse and leaf the highest yields of xylan to XOS conversion were obtained in assay 1 for both, using an acid concentration of 1.5 %, time of 10 min and temperature of 130 ºC. The results obtained were 40.16 % and 45.37 % respectively (Table 5.6 e 5.7).

Table 5.4 – Xylooligosaccharides (XOS) and xylose produced in the hydrolysis with acetic acid of xylan from sugarcane bagasse, using a central composite design 2^2 with variation of time (min) and concentration (% m/v) and temperature of 130 ºC

	Time (min)	**CH_3COOH (% m/v)**	**Conversion of xylan into xylose and XOS (%)**			**XOS ($g.L^{-1}$)**
Assays			**X_1**	**X_2-X_6**	**XOS**	
1	10	1.5	11.89	45.76	45.76	9.16
2	10	4	28.97	22.38	22.38	4.48
3	30	1.5	13.17	56.29	56.29	11.26
4	30	4	35.81	17.84	17.84	3.57
5	20	0.98	0.56	39.85	39.85	7.97

Continued from Table 5.4						
6	20	4.52	55.69	12.46	12.46	2.49
7	5.86	2.75	12.52	32.94	32.94	6.59
8	34.14	2.75	43.94	31.68	31.68	6.33
9	20	2.75	38.71	51.29	51.29	10.25
10	20	2.75	41.85	53.37	53.37	10.67
11	20	2.75	38.12	52.74	52.74	10.54

X_1: xylose; X_2-X_6 (X_2+X_3+X_4+X_5/X_6): xylobiose; xylotriose; xylotetraose; xylopentaose/xylohexaose; XOS: xylooligosaccharides=X_2+X_3+X_4+X_5/X_6.

Table 5.5 – Xylooligosaccharides (XOS) and xylose produced in the hydrolysis with acetic acid of xylan from sugarcane leaf, using a central composite design 2^2 with variation of time (min) and concentration (% m/v) and temperature of 130 ºC

	Time (min)	**CH_3COOH (% m/v)**	**Conversion of xylan into xylose and XOS (%)**			**XOS ($g.L^{-1}$)**
Assays			**X_1**	**X_2-X_6**	**XOS**	
1	10	1.5	11.74	36.93	36.93	7.39
2	10	4	30.51	44.76	44.76	8.95
3	30	1.5	14.89	43.85	43.85	8.77
4	30	4	42.57	20.19	20.19	4.04
5	20	0.98	2.35	34.33	34.33	6.87
6	20	4.52	51.71	9.41	9.41	1.88
7	5.86	2.75	20.23	38.02	38.02	7.60
8	34.14	2.75	41.01	53.65	53.65	10.73
9	20	2.75	46.19	38.14	38.14	7.62
10	20	2.75	47.59	40.77	40.77	8.15
11	20	2.75	45.20	39.51	39.51	7.89

X_1: xylose; X_2-X_6 (X_2+X_3+X_4+X_5/X_6): xylobiose; xylotriose; xylotetraose; xylopentaose/xylohexaose; XOS: xylooligosaccharides=X_2+X_3+X_4+X_5/X_6.

Table 5.6 - Xylooligosaccharides (XOS) and xylose produced in the hydrolysis with sulfuric acid of xylan from sugarcane bagasse, using a central composite design 2^2 with variation of time (min) and concentration (% m/v) and temperature of 130 ºC

	Time (min)	**H_2SO_4 (% m/v)**	**Conversion of xylan into xylose and XOS (%)**			**XOS ($g.L^{-1}$)**
Assays			**X_1**	**X_2-X_6**	**XOS**	
1	10	1.5	30.67	40.16	40.16	8.04
2	10	4	37.25	24.44	24.44	4.89
3	30	1.5	35.84	31.23	31.23	6.25
4	30	4	57.12	16.18	16.18	3.24
5	20	0.98	14.39	32.54	32.54	6.52

Continued from Table 5.6						
6	20	4.52	65.27	15.14	15.14	3.03
7	5.86	2.75	25.76	26.51	26.51	5.31
8	34.14	2.75	36.14	20.97	20.97	4.19
9	20	2.75	34.84	21.52	21.52	4.30
10	20	2.75	33.51	20.24	20.24	4.05
11	20	2.75	33.39	20.51	20.51	4.10

X_1: xylose; X_2-X_6 (X_2+X_3+X_4+X_5/X_6): xylobiose; xylotriose; xylotetraose; xylopentaose/xylohexaose; XOS: xylooligosaccharides=X_2+X_3+X_4+X_5/X_6.

Table 5.7 - Xylooligosaccharides (XOS) and xylose produced in the hydrolysis with sulfuric acid of xylan from sugarcane leaf, using a central composite design 2^2 with variation of time (min) and concentration (% m/v) and temperature of 130 ºC

	Time (min)	**H_2SO_4 (% m/v)**	**Conversion of xylan into xylose and XOS (%)**			**XOS ($g.L^{-1}$)**
Assays			**X_1**	**X_2-X_6**	**XOS**	
1	10	1.5	35.17	45.37	45.37	9.41
2	10	4	42.31	2252	22.52	4.50
3	30	1.5	36.55	32.39	32.39	6.48
4	30	4	64.41	21.32	21.32	4.26
5	20	0.98	27.82	37.43	37.43	6.68
6	20	4.52	56.77	16.59	16.59	2.96
7	5.86	2.75	26.41	28.99	28.99	5.17
8	34.14	2.75	45.32	30.11	30.11	5.37
9	20	2.75	48.29	33.44	33.44	6.69
10	20	2.75	47.97	34.14	34.14	6.83
11	20	2.75	49.53	34.98	34.98	7.00

X_1: xylose; X_2-X_6 (X_2+X_3+X_4+X_5/X_6): xylobiose; xylotriose; xylotetraose; xylopentaose/xylohexaose; XOS: xylooligosaccharides=X_2+X_3+X_4+X_5/X_6.

During pretreatment with acid, degradation products such as furfural, hydroxymethylfurfural and siringil were generated. The tables 5.8; 5.9; 5.10 and 5.11 shows the values obtained in the assays with maximum and minimum levels and central point.

Table 5.8 - Yield of degradation products generated in hydrolysis with acetic acid of xylan from sugarcane bagasse

	Time (min)	**CH_3COOH (% m/v)**	**XOS yield (% m/m)**	**Yield of degradation products (% m/m)**		
Assay*				**Furfural**	**HMF**	**Siringil**
1	1,5	10	45,76	1.26	0.34	0.01

Continued from Table 5.8						
4	4	30	17,84	0..72	0.07	0,01
10	2,75	20	53,37	1.49	0.26	0.02

*assay related to table 5.2.

Table 5.9 - Yield of degradation products generated in hydrolysis with acetic acid of xylan from sugarcane leaf

	Time (min)	**CH_3COOH (% m/v)**	**XOS yield (% m/m)**	**Yield of degradation products (% m/m)**		
Assay*				**Furfural**	**HMF**	**Siringil**
1	1,5	10	36,93	1.41	0.34	0.01
4	4	30	20,19	0.81	0.29	-
10	2,75	20	40,77	1.46	0.50	0.01

*assay related to table 5.2.
(-): not detected

Table 5.10 - Yield of degradation products generated in hydrolysis with sulfuric acid of xylan from sugarcane bagasse

	Time (min)	**H_2SO_4 (% m/v)**	**XOS yield (% m/m)**	**Yield of degradation products (% m/m)**		
Assay*				**Furfural**	**HMF**	**Siringil**
1	1,5	10	40,16	1.76	0.38	0.01
4	4	30	16,18	0.91	0.06	-
10	2,75	20	20,24	1.47	0.29	0.01

*assay related to table 5.2.
(-): not detected

Table 5.11 - Yield of degradation products generated in hydrolysis with sulfuric acid of xylan from sugarcane leaf

	Time (min)	**H_2SO_4 (% m/v)**	**XOS yield (% m/m)**	**Yield of degradation products (% m/m)**		
Assay*				**Furfural**	**HMF**	**Siringil**
1	1,5	10	45,37	1.87	0.45	0.01
4	4	30	21,32	0.90	0.33	0,01
10	2,75	20	34,14	1.07	0.18	-

*assay related to table 5.2.
(-): not detected

In the acid hydrolysis of xylan extracted from sugarcane bagasse, using 0.25 $mol.L^{-1}$ (2.46 % m/v) sulfuric acid for 20 min at 121 ºC, were obtained concentrations of 2.014, 2.106 e 1.228 $g.L^{-1}$ of xylose, xylobiose and xylotriose, successively (KAUR; UPPAL; SHARMA, 2018). In the present study the center point tests were closer, with an acid concentration of

2.75 %, time of 20 min and temperature of 130 ºC and the amount of XOS obtained were: 4.30 $g.L^{-1}$ and 10.67 $g.L^{-1}$ and in the hydrolysis of xylan from bagasse with sulfuric and acetic acid and 7 $g.L^{-1}$ e 8.15 $g.L^{-1}$ in the hydrolysis of xylan from leaf with sulfuric and acetic acid.

An XOS yield of 290.2 $mg.g^{-1}$ and xylose of 85.5 $mg.g^{-1}$ were obtained in acid hydrolysis of xylan from sugarcane bagasse using 0.24 $mol.L^{-1}$ sulfuric acid heated in a microwave to 90 °C for 31 min with a solid to liquor ratio of 1:25 ($g.ml^{-1}$). In this study degradation products such as furfural and HMF were not detected by HPLC under the conditions used, probably due to the low temperature (BIAN et al., 2014).

Xylan solubilized from wheat straw containing 85.17 % of xylose in its composition was hydrolyzed to XOS production using 0.25 $mol.L^{-1}$ (2.46 % m/v) sulfuric acid for 30 min at 100 °C. The conversion of xylan into XOS was 10.23 %, into xylose was 9.59 % and furfural was 0.05 % (AKPINAR; ERDOGAN; BOSTANCI, 2009). In the present study higher conversion values were obtained under similar conditions of acid concentration (2.75 %) and time (34 min however with a higher temperature of 130 ºC (assay 8). The values obtained from XOS and xylose in the hydrolysis of xylan extracted from bagasse were: 20.97 % and 36.14 % (sulfuric acid) and 31.68 % and 43.94 % (acetic acid). In the hydrolysis of xylan extracted from the leaf the results were: 30.11 % and 45.32 % (sulfuric acid), 53.65 % and 41.01 % (acetic acid).

A high yield of XOS conversion could be obtained by hydrolysis with acetic and sulfuric acid, but there was a large formation of xylose, which makes the purification process difficult and expensive. High acid concentrations increase xylose formation and decrease XOS formation.

During the acid hydrolysis process, polysaccharide degradation products such as furfural, HMF and acetic acid are formed depending on reaction conditions such as acid concentration, high temperature and reaction time (CHEN, 2015; HILPMANN et al., 2016).

An analysis of variance (ANOVA) was performed for acid hydrolysis with a 99 % confidence level ($p < 0.01$). In the hydrolysis with acetic acid using xylan from sugarcane bagasse the independent variables acid concentration and reaction time were significant for XOS production. For leaf the variables acid concentration and your interaction with the reaction time were significant (Table 5.12). The models showed no lack of fit and a high R_2 of 0.93 and 0.82 for xylan from sugarcane bagasse and leaf, respectively. In the hydrolysis with sulfuric acid using xylan from sugarcane bagasse the independent variables acid concentration and reaction time were significant for XOS production. For leaf only the variable acid

concentration was significant (Table 5.13). The models showed no lack of fit and a high R2 of 0.90 and 0.93 for xylan from sugarcane bagasse and leaf, respectively.

Table 5.12 - Analysis of variance (ANOVA) for the independent variables in the production of XOS in hydrolysis with acetic acid using xylan from sugarcane bagasse and leaf

	Xylan from sugarcane bagasse					**Xylan from sugarcane leaf**				
Factor	**SS**	**DF**	**MS**	**F**	***p***	**SS**	**DF**	**MS**	**F**	***p***
CH_3COOH (L)	1263.81	1	1263.81	1110.91	0.0009	326.20	1	326.20	174.73	0.0057
CH_3COOH (Q)	756.88	1	756.88	665.31	0.0015	374.42	1	374.42	200.56	0.0049
Time (L)	2.21	1	2.21	1.95	0.2977	2.48	1	2.48	1.33	0.3685
Time (Q)	408.12	1	408.12	358.75	0.0028	83.11	1	83.11	44.52	0.0217
CH_3COOH by Time (L)	56.78	1	56.78	49.91	0.0195	247.91	1	247.91	132.79	0.0074
Lack of Fit	154.18	3	51.39	45.18	0.0217	257.59	3	85.86	45.99	0.0214
Pure Error	2.28	2	1.14			3.73	2	1.87		
Total	2396.36	10				1452.75	10			
R^2	0.93					0.82				

SS: sum of squares; DF: degree of freedom; MS: mean of squares

Table 5.13 - Analysis of variance (ANOVA) for the independent variables in the production of XOS in hydrolysis with sulfuric acid using xylan from sugarcane bagasse and leaf

	Sugarcane bagasse xylan					**Sugarcane leaf xylan**				
Factor	**SS**	**DF**	**MS**	**F**	***p***	**SS**	**DF**	**MS**	**F**	***p***
H_2SO_4 (L)	383.28	1	383.28	841.94	0.0012	502.28	1	502.28	844.83	0.0012
H_2SO_4 (Q)	37.91	1	37.91	83.28	0.0118	52.88	1	52.88	88.94	0.0111
Time (L)	78.28	1	78.28	171.96	0.0058	19.84	1	19.84	33.36	0.0287
Time (Q)	36.58	1	36.58	80.36	0.0122	18.10	1	18.10	30.44	0.0313
H_2SO_4 by Time (L)	0.11	1	0.11	0.25	0.6687	34.69	1	34.69	58.35	0.0167
Lack of Fit	51.32	3	17.11	37.58	0.0260	42.50	3	14.17	23.83	0.0405
Pure Error	0.91	2	0.46			1.19	2	0.59		
Total	571.45	10				658.26	10			
R^2	0.90					0.93				

SS: sum of squares; DF: degree of freedom; MS: mean of squares

The polynomial model presented significance, with 93 % of the experimental data adjusted to the model for hydrolysis with acetic acid using xylan from sugarcane bagasse. Relative to xylan from sugarcane leaf 82 % of the experimental data adjusted to the model.

The statistical model that describes XOS conversion using bagasse and leaf is represented by Equations 1 and 2 respectively.

$$XOS\ (\%) = -\ 27.469 + 36.653x - 7.395x^2 + 4.283y - 0.085y^2 - 0.301xy \quad (1)$$

$$XOS\ (\%) = -\ 6.254 + 36.098x - 5.201x^2 + 0.252y + 0.038y^2 - 0.630xy \quad (2)$$

Where:
X = CH_3COOH concentration (% m/v)
Y = time (min)

The hydrolysis of xylan from sugarcane bagasse and leaf with sulfuric acid the polynomial model presented significance with 90 % e 93 % of the experimental data adjusted respectively. The statistical model that describes XOS conversion using bagasse and leaf is represented by Equations 3 and 4 respectively.

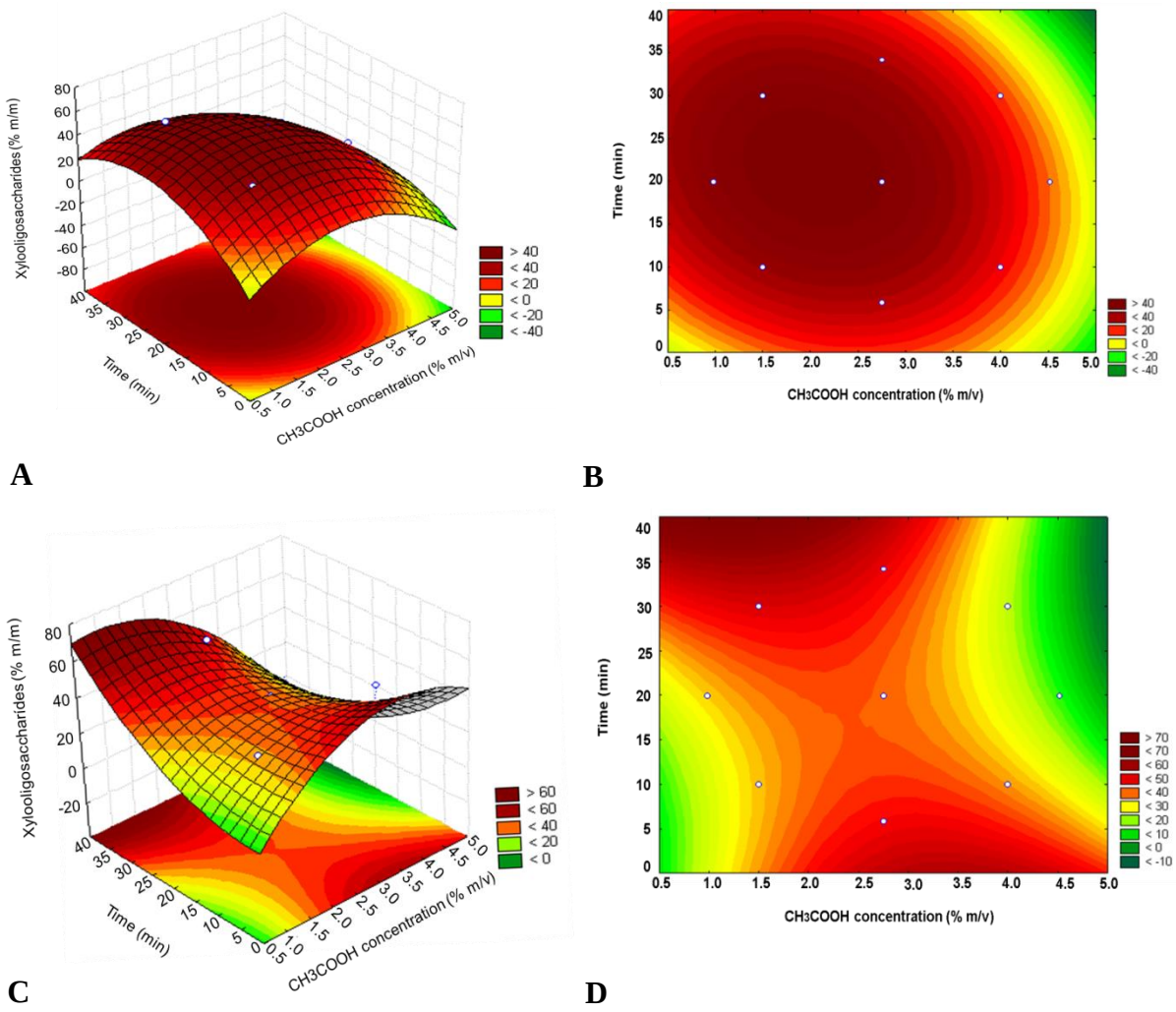
$$XOS\ (\%) = 65.674 - 14.905x + 1.655x^2 - 1.368y + 0.026y^2 + 0.013xy \quad (3)$$

$$XOS\ (\%) = 45.770 - 0.296x - 1.955x^2 - 0.089y - 0.018y^2 + 0.236xy \quad (4)$$

Where:
X = H_2SO_4 concentration (% m/v)
Y = time (min)

In the response surface and contour plot of acetic acid hydrolysis, it is possible to observe that the highest yield of XOS conversion occurs for xylan from sugarcane bagasse at acid concentrations between 1.5 % and 2.5 % and reaction time from 15 to 30 min; for xylan from sugarcane leaf there are two regions with higher yields. Acid concentration between 2.5 % and 4 % with time up to 5 min or lower concentrations (0.5 to 2 %) and time above 35 min (Figure 5.2).

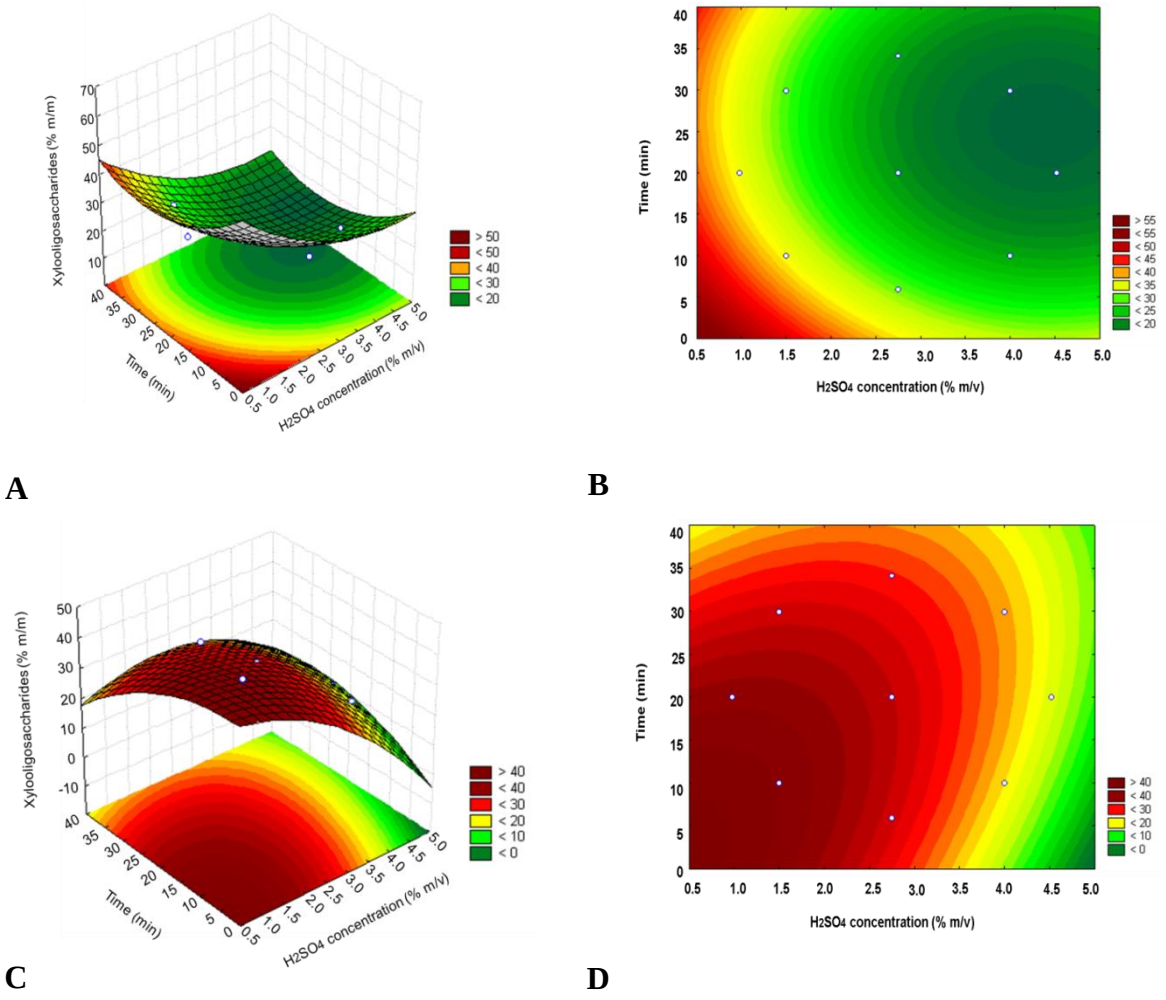
Figure 5.2 - (A) e (C): Response surface and (B) e (D): Contour plot for XOS yield produced on the hydrolysis with acetic acid of xylan of sugarcane bagasse and leaf respectively



Source: Author

In hydrolysis using sulfuric acid the highest yield of XOS was obtained similarly for both xylans with acid concentration below 1.5 % and reaction time between 10 and 15 min (Figure 5.3).

Figure 5.3 - (A) e (C): Response surface and (B) e (D): Contour plot for XOS yield produced on the hydrolysis with sulfuric acid of xylan of sugarcane bagasse and leaf respectively



Source: Author

5.3.5 Enzymatic hydrolysis for XOS production

In the present study a purified endoxylanase from *Aspergillus versicolor* was used because of its efficiency in the production of XOS. In the hydrolysis of the xylan from bagasse and leaf it was possible to obtain conversion of xylan into XOS of 67.43 % and 69.71 % (assay 7) (Table 5.14 e 5.15) but with a low substrate concentration (0.17 %) and moderate enzyme load (65 $UI.g^{-1}$). At the center point with the same enzyme load and substrate increased to 3 %, it was obtained a similar yield, equivalent to 65.48 % and 63.41 % (Table 5.14 and 5.15). There was no production of xylose, which is desirable, as it facilitates the purification of the product of interest, making the process cheaper.

Table 5.14 – Xylooligosaccharides (XOS) and xylose produced by the enzymatic hydrolysis of xylan from sugarcane bagasse, using a central composite design 2^2 with a variation of enzyme load ($UI.g^{-1}$) and substrate concentration (% m/v)

	Enzyme ($UI.g^{-1}$)	Substrate (% m/v)	Conversion of xylan into xylose and XOS (%)				XOS ($g.L^{-1}$)
Assays			**X_1**	**X_2**	**X_3- X_6**	**XOS**	
1	30	1	-	2.40	36.39	38.79	4.03
2	100	1	-	1.78	54.45	56.23	5.62
3	30	5	-	1.60	34.65	36.25	18.42
4	100	5	-	1.67	37.43	39.10	19.78
5	15.5	3	-	0.64	14.50	15.14	4.60
6	114.5	3	-	3.16	61.29	64.45	19.59
7	65	0.17	-	3.17	64.26	67.43	1.19
8	65	5.83	-	1.35	26.81	28.16	16.39
9	65	3	-	3.74	61.74	65.48	19.91
10	65	3	-	1.75	59.95	61.70	18.76
11	65	3	-	2.97	60.24	63.21	19.21

X_1: xylose; X_2: xylobiose; X_3-X_6 (X_3+X_4+X_5/X_6): xylotriose; xylotetraose; xylopentaose/xylohexaose; XOS: xylooligosaccharides=X_2+X_3+X_4+X_5/X_6.
(-): not detected

Table 5.15 – Xylooligosaccharides (XOS) and xylose produced by the enzymatic hydrolysis of xylan from sugarcane leaf, using a central composite design 2^2 with a variation of enzyme load ($UI.g^{-1}$) and substrate concentration (% m/v)

	Enzyme ($UI.g^{-1}$)	Substrate (% m/v)	Conversion of xylan into xylose and XOS (%)				XOS ($g.L^{-1}$)
Assays			**X_1**	**X_2**	**X_3- X_6**	**XOS**	
1	30	1	-	2.45	50.40	52.85	5.60
2	100	1	-	3.29	57.65	60.94	6.46
3	30	5	-	2.59	28.68	31.27	15.76
4	100	5	-	1.89	40.71	42.6	21.48
5	15.5	3	-	1.91	15.97	17.88	5.47
6	114.5	3	-	2.41	59.94	62.35	19.07
7	65	0.17	-	23.2	46.51	69.71	1.23
8	65	5.83	-	1.71	22.93	24.64	14.39
9	65	3	-	5.12	55.9	61.02	18.29
10	65	3	-	4.15	59.26	63.41	19.01
11	65	3	-	3.77	58.60	62.37	18.70

X_1: xylose; X_2: xylobiose; X_3-X_6 (X_3+X_4+X_5/X_6): xylotriose; xylotetraose; xylopentaose/xylohexaose; XOS: xylooligosaccharides=X_2+X_3+X_4+X_5/X_6.
(-): not detected

The enzyme used in this study was previously used for XOS production in the enzymatic hydrolysis of banana pseudostem (FREITAS, 2019). With an enzymatic load of 30 $UI.g^{-1}$, substrate concentration of 2 % and time of 24 h it was possible a conversion of 61 % of xylan in XOS. In the present study, a higher yield of XOS production was obtained for both xylans with enzymatic load of 65 $UI.g^{-1}$ and substrate concentration of 3 %.

A yield of 36 %, lower than the one found in the present study, was obtained with xylan extracted from sugarcane bagasse and hydrolyzed by *Aspergillus fumigatus* M51 xylanase, with enzymatic load of 500 $U.mL^{-1}$ per g of substrate for 48 h at 50 ºC (CARVALHO et al., 2015). In the hydrolysis of sugarcane bagasse with xylanases from *Thermoascus aurantiacus* it was obtained XOS yield of 39.70 % with enzymatic load of 60 $U.g^{-1}$ and 2 % (m/v) substrate for 96 h at 50 ºC. In this case, there was 23.37 % xylose yield (BRIENZO; CARVALHO; MILAGRES, 2010). In the present study the yield of XOS was higher, in a shorter time (24 h) and there was not a formation of xylose because it was used a purified endoxylanase.

Xylan extracted from sugarcane bagasse, wheat bran, rice straw and corn cobs using NaOH were hydrolyzate by crude xylanase from *Bacillus amyloliquifaciens* NRRL B-14393 for 72 h at 50 ºC for XOS production. The XOS yield was 69 %, 85.5 %, 84.6 % and 58 % respectively (RASHAD et al., 2016). In another study with xylan extracted from sugarcane bagasse hydrolyzed by recombinant endoxylanase from the recombinant *Pichia pastoris* using enzymatic load of 800 $U.g^{-1}$, substrate concentration of 35 $g.L^{-1}$ for 24 h at 36 ºC, the conversion amount into XOS was 57.36 % (XUE et al., 2016). The production of XOS requires the use of endoxylanase enzymes that hydrolyze β-1.4 glycosidic bonds in the xylan chain and low or no activity of β-xylosidase, enzyme responsible for xylose formation (GUIDO; SILVEIRA; KALIL, 2019). Enzymatic hydrolysis has the advantages of the absence of undesirable products such as furfural and HMF, no need for equipment resistant to high temperatures, pressure or corrosive reagents. However, it is important the purification of enzymes to avoid xylose release (WAHLSTRÖM; SUURNÄKKI, 2015).

XOS with a low polymerization degree, between 2 and 3 xylose units have better prebiotic activity as they are consumed faster by intestinal bacteria, having a beneficial effect on intestinal flora (ÁVILA et al., 2019). In the present study it was not possible to quantify separately the amount of X_3 to X_6.

An analysis of variance (ANOVA) was performed for enzymatic hydrolysis with a 99 % confidence level ($p < 0.01$). The independent variables enzymatic load and substrate

concentration were significant in the hydrolysis of xylan from sugarcane bagasse and leaf for XOS production (Table 5.16). The models showed no lack of fit.

Table 5.16 - Analysis of variance (ANOVA) for the independent variables in the production of XOS using xylan from sugarcane bagasse and leaf

	Xylan from sugarcane bagasse					**Xylan from sugarcane leaf**				
Factor	SS	DF	MS	F	*p*	SS	DF	MS	F	*p*
Enzyme ($UI.g^{-1}$) (L)	1017.55	1	1017.55	281.07	0.0035	846.89	1	846.89	589.74	0.0017
Enzyme ($UI.g^{-1}$) (Q)	835.30	1	835.30	230.73	0.0043	594.44	1	594.44	413.94	0.0024
Substrate (% m/v) (L)	707.18	1	707.18	195.34	0.0051	1343.31	1	1343.31	935.43	0.0011
Substrate (% m/v) (Q)	372.87	1	372.87	102.0	0.0096	255.76	1	255.76	178.10	0.0056
Enzyme by Substrate (L)	53.22	1	53.22	14.70	0.0618	2.62	1	2.62	1.83	0.3090
Lack of Fit	471.41	3	157.14	43.40	0.0226	328.20	3	109.40	76.18	0.0130
Pure Error	7.24	2	3.62			2.87	2	1.44		
Total	3219.64	10				3203.41	10			
R^2	0.85					0.89				

SS: sum of squares; DF: degree of freedom; MS: mean of squares

The polynomial model presented significance, with 85 % of the experimental data adjusted to the model for enzymatic hydrolysis of xylan from bagasse and 89 % for enzymatic hydrolysis of xylan from leaf. Statistical models describing the conversion of xylan into XOS in the hydrolysis of xylan from sugarcane bagasse and leaf are represented by Equations 5 and 6.

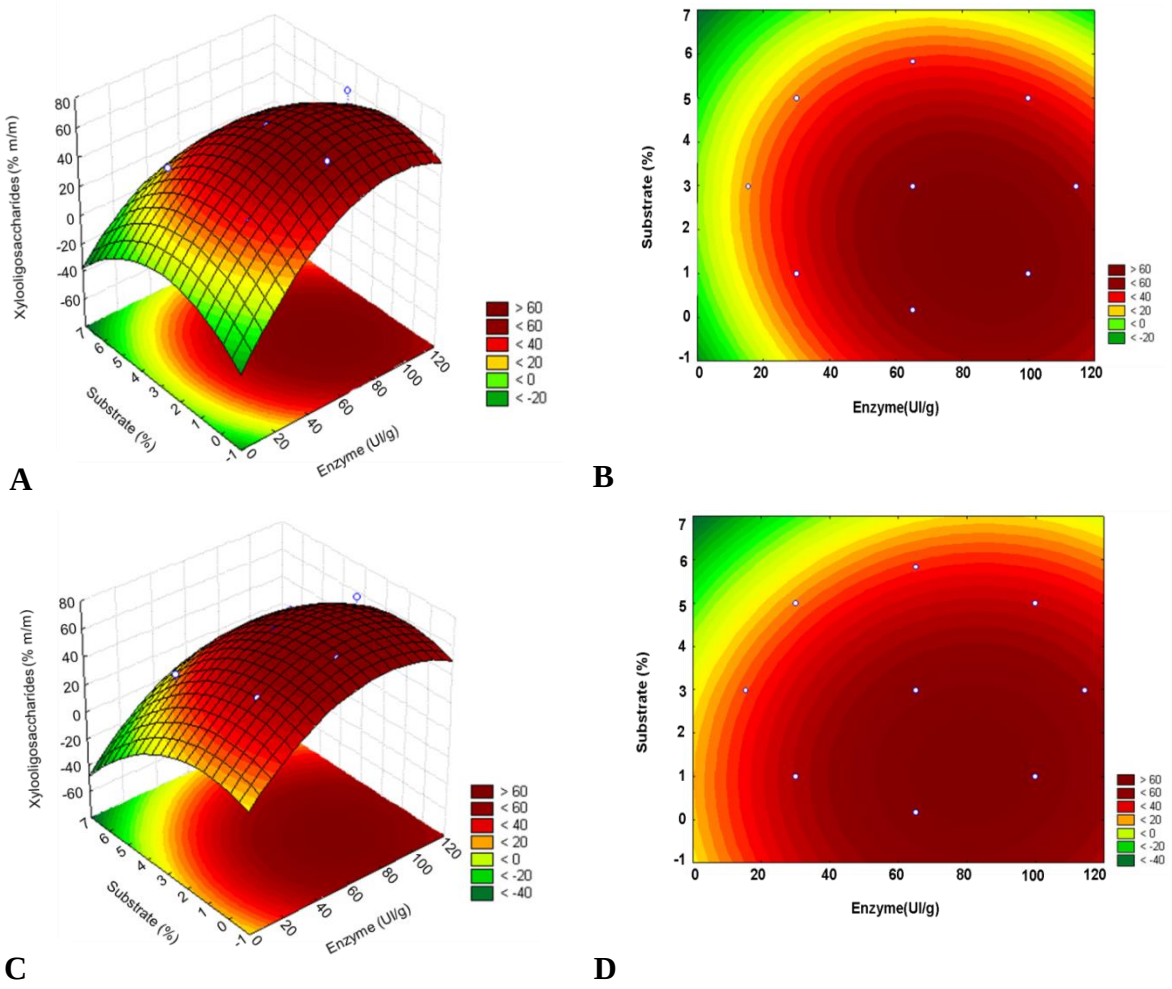
$$XOS\ (\%) = -13.758 + 1.769x - 0.010x^2 + 10.866y - 2.030y^2 - 0.052xy \quad (5)$$

$$XOS\ (\%) = 14.333 + 1.348x - 0.008x^2 + 2.857y - 1.681y^2 + 0.012xy \quad (6)$$

Where:
x = Enzyme ($UI.g^{-1}$)
y = Substrate (% m/v)

The analysis of the response surface showed that the xylan from bagasse and leaf behaved similarly. In both cases the highest conversion of xylan into XOS was obtained in the region of enzymatic load 60 $UI.g^{-1}$ to 100 $UI.g^{-1}$ and substrate concentration until 2 % (m/v) (Figure 5.4).

Figure 5.4 - (A) Response surface and (B) Contour plot for XOS yield produced on the enzymatic hydrolysis with *Aspergillus versicolor* endoxylanase using xylan from sugarcane bagasse



Source: Author

5.4 Conclusion

Xylan solubilized by hydrogen peroxide in alkaline medium showed high xylose content and high solubility. In all pretreatments using bagasse and leaf xylan, high yields of conversion to XOS were obtained. In hydrolysis with sulfuric acid the XOS yield for bagasse and leaf xylan was 40.16 % and 45.37 %, respectively. When acetic acid was used, the XOS yield increased to 56.29 % and 53.65 % for bagasse and leaf xylan, respectively. In both methods, there was formation of xylose, furfural, HMF and siringil, especially when sulfuric acid was used, probably because it is a strong acid, with lower pH and higher efficiency in polysaccharide hydrolysis, so low concentrations break the xylan chain in XOS and higher concentrations generate xylose and degradation products. Milder conditions of acid

concentration and temperature can be an alternative to solve this problem. In enzymatic hydrolysis, the highest yield of XOS was obtained, corresponding to 67.43 % and 69.71 % for bagasse and leaf xylan, without the presence of xylose and degradation products.

CHAPTER VI: CONCLUSION

The xylan extracted with hydrogen peroxide showed high solubility and xylose content and was used in enzymatic hydrolysis with endo-1-4-β-xylanase from *Aspergillus versicolor* and in hydrolysis with dilute acetic and sulfuric acid. The highest yield of xylooligosaccharides was obtained in enzymatic hydrolysis, followed by hydrolysis with acetic acid and lastly by hydrolysis with sulfuric acid. The results were similar for xylan extracted from bagasse and leaf. Regarding xylose and degradation products, no formation occurred in enzymatic hydrolysis, because purified endo-xylanase was used and the yield was high in acid hydrolysis, especially when sulfuric acid was used and this is due to the fact that it is a stronger acid than acetic acid and can break the xylan chain more intensely. Using sugarcane bagasse and leaf the highest yield of XOS was obtained in the hydrolysis with sulfuric acid, followed by hydrolysis with acetic acid and autohydrolysis. The highest values were obtained with the leaf, except in the sulfuric acid assay. There was a greater formation of xylose and degradation products in acid hydrolysis compared to autohydrolysis. In the hydrolysis of solubilized xylan, there was a greater formation of degradation products compared to biomass hydrolysis.

Hydrolysis with sulfuric acid, using biomass *in natura* or solubilized xylan and enzymatic hydrolysis and acetic acid hydrolysis using xylan showed promise in the production of XOS considering the optimum conditions determined in this study.

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