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DEVELOPMENT OF BIOPROCESS FOR FIBROLYTIC FUNGAL ENZYMES PRODUCTION FROM LIGNOCELLULOSIC RESIDUES AND ITS APPLICATION ON KRAFT PULP BIOBLEACHING AND XYLOOLIGOSACCHARIDES PRODUCTION

Tania Sila Campioni

Tese apresentada ao Instituto de Pesquisa em Bioenergia de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências.

Orientador(a): Pedro de Oliva Neto

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TÍTULO DA TESE: DEVELOPMENT OF BIOPROCESS FOR PRODUCTION OF FIBROLYTIC FUNGAL ENZYMES FROM LIGNOCELLULOSIC MATERIALS AND ITS APPLICATION ON KRAFT PULP BIOPULPING AND XOS PRODUCTION

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Dedico

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Resumo

Desejando ao final do trabalho obter um bioprocesso integrado usando palha de cana-de-açúcar (PC), este trabalho teve início com a utilização desse substrato para produção de enzimas fribrolíticas, xilanases e celulases, em culturas axênicas, incluindo espécies de *Trichoderma* e *Aspergillus*. A triagem para o melhor produtor foi realizada em "shaker" em fermentação submersa. A cultura do fungo *T. reesei* QM9414 alcançou a melhor produção de enzimas, e em tanque agitado, utilizando um biorreator de 3 L, mostrou o mesmo perfil de produção (~90 U/mL, 0.6 FPU/mL para xilanase e celulases, respectivamente). Em relação a este resultado, a produção de enzimas para as misturas binárias e ternárias destes fungos foi menor, sendo que a melhor combinação, *T. reesei* QM 9414+*A. fumigatus* M51, alcançou 60 U/mL e 0.08 FPU/mL respectivamente. Com intuito de otimizar a produção de enzimas utilizando um mix de substratos: palha de cana, como principal componente, e o farelo de trigo e a polpa cítrica, como supostos indutores de atividade enzimática, foi realizado um delineamento de misturas do tipo D-optimal. O resultado da otimização da mistura dos substratos mostrou que o trigo e a polpa cítrica não tiveram um efeito indutivo na produção das enzimas tendo a palha de cana como principal substrato. A enzima xilanase foi caracterizada em seu pH e temperatura ótimos (pH 5, e 50 ºC respectivamente), bem como a estabilidade da enzima nestes parâmetros. Alguns íons e EDTA foram aplicados para determinar a estabilidade da enzima nessas condições, sendo o melhor indutor o Mn^{2+} com 49% de aumento de atividade (10 mM). O extrato contendo xilanases, produzido nas condições previamente otimizadas foi aplicado no biobranqueamento da polpa Kraft. A otimização da biobranqueamento mostrou uma diminuição do índice Kappa, 12.5% (30 U/g e 30 min), bem como houve a liberação de açúcares e compostos cromóforos. Este tratamento na polpa foi responsável por diminuir em 10% a quantidade de dióxido de cloro utilizado no branqueamento químico, uma vez que sua alvura foi a mesma que o controle sem tratamento enzimático. A xilana presente na PC foi extraída com NaOH por meio de tratamento termo-químico. Após este processo a xilana foi hidrolisada, para a produção de xiloligossacarídeos (XOS) por duas diferentes rotas, com enzimas (utilizando o extrato contendo xilanases), e com ácido fosfórico (95 ºC e 120 ºC). Os melhores ensaios que produziram XOS nas duas rotas não apresentaram diferença significativa, 5.34 e 5.94 g/L correspondendo a 16 e 17.45% de rendimento em XOS. A produção de XOS por via enzimática não formou furfural, entretanto, a hidrólise ácida de XOS é uma alternativa mais rápida. XOS e xilose foram produzidos por meio da hidrólise enzimática da xilana, foram assimilados por

bactérias probióticas e por uma levedura produtora de xilanase e celulase. Assim, os resultados mostram que a PC pode ser usada em bioprocessos utilizando microrganismos especiais, visando a produção de enzimas, açúcares fermentecíveis, aproveitamento de resíduos e produção de moléculas nobres tais como o XOS, dentro de um conceito moderno de biorrefinaria desde que outros componentes presentes na PC possam ser utilizados em outros bioprocessos, como produção de bioenergia.

Palavras-chave: Palha de cana-de-açúcar. Enzimas fibrolíticas. Biobranqueamento. Produção de XOS. Biorrefinaria.

Abstract

In order to obtain an integrated bioprocess using Sugarcane Straw (SS), this work began with the use of this substrate for the fibrolytic enzymes production, xylanases and cellulases, in axenic fungal cultures, including *Trichoderma* and *Aspergillus* species. The screening for the best producer was performed in shaker under submerged fermentation. The *T. reesei* QM9414 culture achieved the best enzyme production, and in a stirred tank using a 3 L bioreactor showed the same production profile (~90 U/mL and 0.6 FPU/mL for xylanase and cellulase, respectively). Regarding this result, the enzyme production by binary and ternary mixtures of these fungi was lower, as example the best combination *T. reesei* QM 9414+*A. fumigatus* M51, reached 60 U/mL and 0.08 FPU/mL, respectively. Aiming optimize the enzyme production by a mix of substrates using SS as the main substrate, and wheat bran and citrus pulp as supposed enzyme inductors, a D-optimal mixture design was performed. The mixture substrates optimization showed that wheat bran and citrus pulp did not have an inductive effect on the enzymes production. The enzyme xylanase was characterized by its optimal pH and temperature (pH 5 and 50 ºC, respectively, as well as the stability of the enzyme in these parameters. Some ions and EDTA were applied to determine the xylanase stability under these conditions, and the ion Mn^{2+} was the best inductor, 49% (10 mM). The extract containing xylanases, produced under previous optimized conditions was applied in the Kraft pulp biobleaching. The biobleaching optimization showed a decrease in the Kappa number, 12.5% (30 U/g e 30 min), as well as well as the release of sugars and the presence of chromophores compounds were also observed. This treatment performed in the pulp was responsible the decrease in 10% the chlorine dioxide amount used in the chemical bleaching, since its brightness was the same found in the sample that have no enzymatic treatment. The xylan present in the SS was extracted with NaOH by thermo-chemical treatment. After this, the xylan was hydrolyzed, for the production of xylooligosaccharides (XOS), by two different routes, enzymatic (using the crude extract produced) and acid (95 ºC and 120 ºC). The best tests that produced XOS in both routes did not present significant difference, 5.34 and 5.94 g/L corresponding to 16 e 17.45% of XOS yield. The enzymatic XOS production did not produce furfural, but the acid route is a faster alternative. As products of xylan enzymatic hydrolysis, XOS and xylose, were assimilated by probiotic bacteria and a fibrolytic yeast. Thus, the results showed that SS can be used in bioprocesses using special microorganisms, aiming the production of enzymes, fermentable sugars, waste utilization and noble molecules production,

such as XOS in a modern biorefinery concept since other components of the PC can be used in other bioprocesses, such as bioenergy production.

Keywords: Sugarcane straw. Fibrolytic enzymes. Biobleaching. XOS production. Biorefinery.

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GENERAL INTRODUCTION

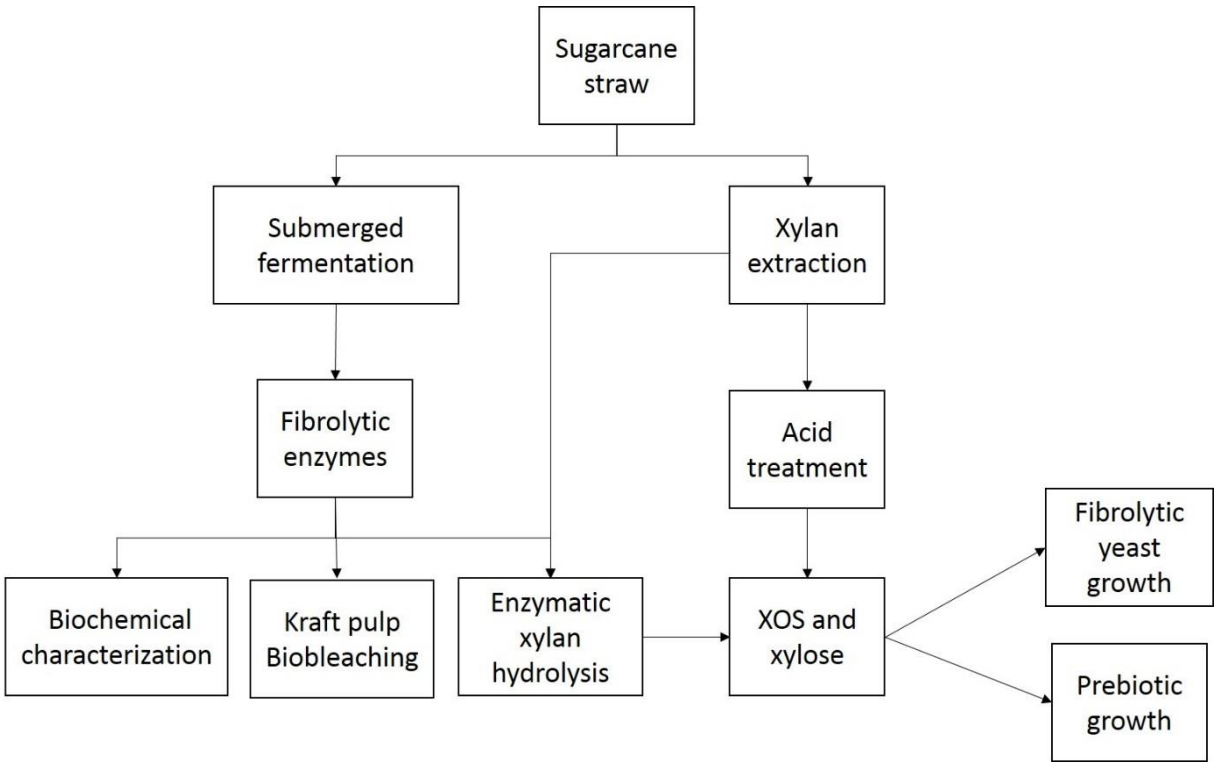
Biotechnology processes are an important route to produce many types of biomolecules in different areas. Investments in research and development of new bioprocesses, mainly focused on industrial enzyme production, are recently highlighted due to the great diversity of their application. Enzymes are being used in a wide range of sectors, especially in the food, paper, biofuel, textile, animal and pharmaceutical industries, with a promising and growing market. In addition, enzymes have other advantages such as the high reaction specificity, which contributes to the process efficiency, are biological products and can have its activity regulated, and still act in low concentrations under mild conditions of pH and temperature. On the other hand, the enzyme cost, based in a total cost of the bioprocess, is relevant. For example, considering the ethanol production from lignocellulosic materials, in an industrial plant that uses enzymatic hydrolysis, the use of cellulases enzymes represents about 18% of the total operational cost. Thus, the improvement of technologies for the fibrolytic enzymes production and application, mainly xylanases and cellulases, in economical bioprocess, represents the key to increase the productivity and the economic viability of the enzymatic route for several applications. These approaches were debated in this work.

Currently, studies are being done to increase the hydrolytic enzymes production by fermentation of agricultural residues (lignocellulolytic materials – LCM) through biotechnological processes resulting in promising yields, and enabling the use of these residues to production costs decrease. There are some microorganism species used in fibrolytic enzymes production, but *Trichoderma* and *Aspergillus* species are the main ones. A variety of agricultural residues can also be used to produce enzymes responsible for degradation of their cell wall since these cultures simulate the natural growth of the fungi and bacteria that degrades the LCM in the environment. The use of LCM by microorganisms is related to the production of cellular proteins, enzymes, organic acids, important secondary metabolites, and also prebiotic oligosaccharides. Pretreatments in these materials also can be used to obtain a bioprocess yields improvement since their objective is to decrease the LCM recalcitrance.

Sugarcane straw is a new residue that is accumulating in the Brazilian fields due to the mechanical harvesting is been applied. The straw composition seems to the other residue from sugarcane crop which has been studied for a long time, sugarcane bagasse. The bagasse is now known as feedstock for the production of several samples of value added.

In this work, sugarcane straw was used as the main LCM substrate and give some products, as xylanases, xylan, that were used to kraft pulp biobleaching, and xylooligosaccharides (XOS) production respectively. XOS were used to fed probiotic microorganisms and fibrolytic yeast, as demonstrated in the Fig. 1:

Fig. 1 - Flowchart with the main processes developed in this work beginning with the residue sugarcane straw.



GENERAL OBJECTIVE

The general objective of this work was to study an integrated bioprocess, using mainly sugarcane straw as feedstock for several bioprocesses: fibrolytic fungal enzymes production, including the selection of substrates and microorganisms, biochemical characterization of xylanases and its application in biobleaching and XOS production. XOS fermentability tests are also performed.

Specific objectives:

- Evaluation of different fungi and substrates (agroindustrial residues: sugarcane straw, citric pulp and wheat straw) in a submerged axenic and mixed cultures for fibrolytic enzymes production;
- Scale-up the enzyme production in the optimized condition;
- Biobleaching of kraft pulp with fungal xylanases, evaluation of the chlorine consumption and other tests on paper mills in laboratorial scale;
- Xylan extraction from sugarcane straw by the alkaline method;
- XOS production from sugarcane straw hemicellulose by acid and enzymatic route;
- Growth of probiotic cultures and a fibrolytic yeast using produced XOS as carbon source.

CHAPTER 1 – GENERAL REVIEW: Use of different lignocellulolosic residues in the fibrolytic enzyme production and its application

1. LIGNOCELLULOSIC MATERIALS, USES AND CHARACTERIZATION

The energy and environmental crisis that the world is currently experiencing, due to its dependence on oil and rapidly development of economy and society, added with the environmental problems that it generates, obliges us to think in alternatives sources as natural renewable ones. These actions include the use of lignocellulosic materials (LCM) as a clean technology for the production of biofuels and other value-added products [1-3].

Agroindustrial wastes are low-cost raw LCM that can be used for a variety of processes and can be acquired in regions located near to where the material is produced, besides that, their use no needs of any extra land or interference on food and feed crop production [3; 4]. These materials accumulate every year in large quantities, causing environmental problems. Wastes are produced by several sectors including industries, forestry, agriculture and municipalities, and this accumulation results in several environmental problems, such as health issues and safety hazards, and prevent sustainable development. Then, actions that aiming to recycle these materials are very important [5].

Brazil produces a large sum of tons of LCM per year with potential use in renewable fuels, chemical products in general, and energy [6]. It is possible to mention the main ones (millions tons/year): sugarcane (166.7-straw and bagasse), wood (50.7-hulls, stumps, chips), wheat (8.6-stem, leaves), coffee (2.6-shell, leaves), bean (1.6-shell, straw), and peanut (78.4-shell), taking into account the data published by Embrapa [7] based on the residues generated by the 2010 harvest. Thus, the country is a strategic target for the most diverse lignocellulosic materials used as feedstock to promote the biotechnological production of some Brazilian valued-add products as: enzymes [8-10], functional foods [11; 12], biofuels[13-15], polyhydroxyalkanoates (PHA) [16], among others.

The LCM have a recalcitrant structure due to their components conformation. The composition is basically divided among cellulose (35-50%), hemicellulose (20-35%), lignin (15-20%), ash and others (15-20%) [17-19]. The use of LCM is mainly related to animal feeding and burning for energy generation, the lowest raw material added value, but other uses can be

suggested depending on their composition and availability [4; 5]. As reported before LCM are rich in sugars, present in cellulose and hemicellulose, and it can be assimilated by microorganisms and transformed in relevant components produced by fermentation process as ethanol, food additives, organic acids and enzymes [20; 21].

The Figure 1 depicts the LCM structure composition reported below. Cellulose and hemicellulose are the LCM polysaccharide portion. Cellulose is present in the cell wall of plants, being the major component of the lignocellulosic structure. It is a linear homopolymer of a long chain with a variable molecular mass and empirical formula $(C_6H_{10}O_5)n$ [1]. Hemicellulose, which accounts for 20-45% of the total dry matter in lignocellulosic wastes, is a short heteropolymer branched with pentoses and hexoses. The components of this heteropolymer include xylose, arabinose, mannose, galactose, and glucose, as well as uronic acids, and glycosidic bonds of type 1-3, 1-4 and 1-6 [22]. Lignin is the non-polysaccharide element in lignocellulosic materials, which fills the spaces between cellulose and hemicellulose [23]. The Tab. 1 depicts different uses of these three components in a biorefinery.

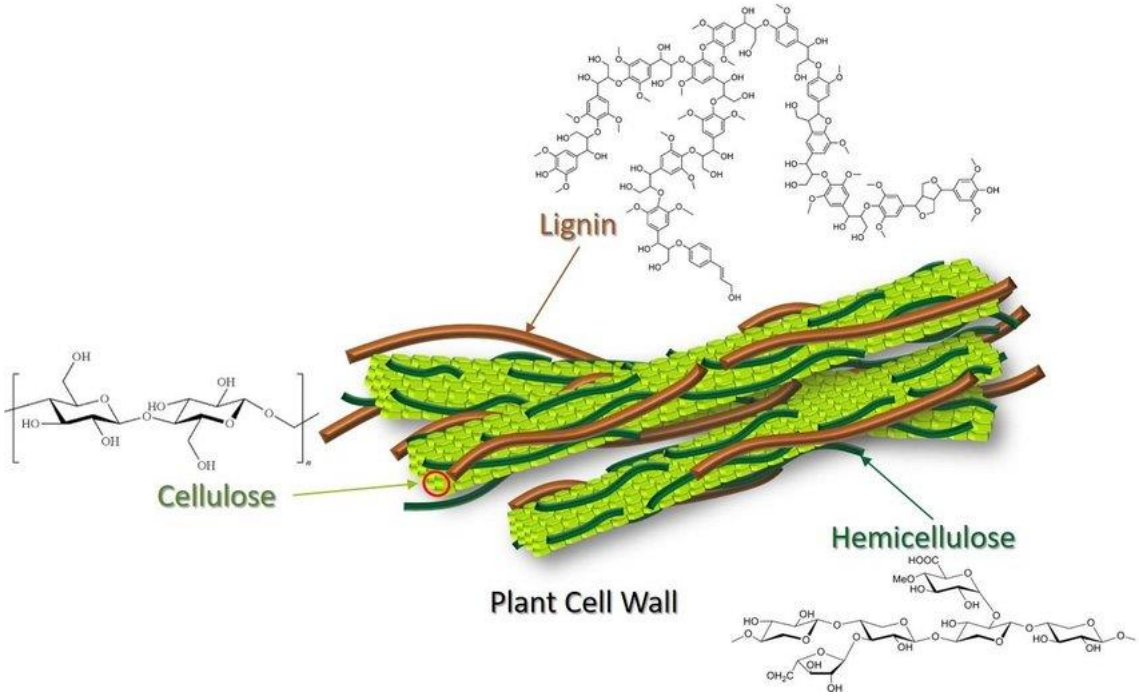


Fig. 1 Schematic representation of lignocellulosic biomass with the structure of cellulose, hemicellulose and lignin [24].

Table 1 – Some examples of LCM compounds use in biorefinery.

Component	Uses	Reference
Cellulose	Bioethanol	25
Hemicellulose	Xylitol	26
	XOS	27;28
Lignin	Fine chemicals and raw material to fuel	29

The recalcitrance is observed in the LCM hydrolysis by enzymes due to the strong union between its components, high crystallinity, and difficulties in the adsorption with the substrate. The LCM use in biochemical processes, involving enzymes and microorganisms, needs receive a pretreatment to reaches the following requirements: (1) improve the sugars formation or the ability to subsequently release free sugars by enzymatic hydrolysis; (2) avoid the carbohydrates degradation or loss; (3) avoid the by-products formation, that can inhibit the subsequent hydrolysis and fermentation processes; and (4) be cost-effective [18]. In an integrated biorefinery the purpose of the pretreatments is to separate lignin, cellulose, and hemicellulose, to allow that these isolated components be used in an appropriated issue. Depend on the finality of the process a specific pretreatment is proposed, and it will differ from each other about the mode of action, reaction conditions and overall outcomes. Physical, physicochemical, chemical and biological processes are examples of LCM pretreatments [3; 17; 20; 30].

Considering the biological use of LCM as a carbon source to microorganism growth, the pretreatment also helps the sugars availability. In the work of Zhang [31] the lignocellulosic residue corn straw was used in the of xylanases and cellulases production by *T. reesei,* after different pretreatments (ammonia fiber expansion, diluted acid, and diluted alkali). The research also showed that the cellulases production occurred independently of the pretreatment residue used, whereas for xylanases the substrate pretreatment was important. In addition, the enzymatic performance, as measured by degradation of cellulose and xylose, was influenced by the mixture of the type of enzymes. In the study by Bandikari [32], the alkaline pretreated substrate also produced more xylanases than the untreated using the fungus *T. koeningi.*

1.1 Sugarcane straw

The sugarcane cultivation started in Brazil colony and today is one of the main cultures of the Brazilian economy. Only in Brazil, 635.6 million tons of sugarcane were produced in 2017/2018 crop [33]. Brazil is not only the largest producer of sugarcane, it is also the world's leading sugar and ethanol producer, and has increasingly achieved the international market with the use of biofuel as an alternative energy [34]. The sugarcane cultivated area in the Brazilian´s soils corresponded to approximately 9 million hectares in 2017/2018 crop [33].

For each ton of sugarcane harvested and processed in the alcohol mills, 280 kg of waste is produced. Half of this quantity corresponds to the bagasse left after the juice has been extracted, and the other one corresponds to the straw that must be removed before grinding [35; 36]. The amount of straw that, results from sugar cane harvesting, depends on some factors, such as age and sugarcane variety, as well as the environmental factors in which they are planted (climate, soil) [14]. The sugarcane bagasse is practically consumed through by the burning in the mills to ensure energy. In addition, there are still other fibers in the leaves (straw), which were usually burnt in the preharvest [6; 14].

The practice of cane residues burning in the field was used in the past to facilitate manual harvest operations (facility for cutting and to protect against venomous animals). However, the environmental problems caused the attention of the authorities that prohibited the sugar cane fields burning, so the harvest is mechanized now. Emissions from sugarcane burning are known to cause impact on the respiratory health the local populations, due to the increase of particulate matter present in the air [37]. An important pre-harvesting burning problem is related to the health, Mauro and co-workers [38] studied the school absence of children due to respiratory problems in the period and concluded that it improves a lot, related to a greater soot concentration in the air. Mechanized harvesting is the most modern option available, and should contemplate the straw recovery too, at least partially, with low cost and conservation of this material for possible reuse [36; 39]. This harvest substitution is still undergoing studies to ensure the quality of the process, and the straw is extremely important due to its energetic characteristics, so left it in the field is not interesting. A CTC (Centro de Tecnologia Canaviera - Brazil) study showed that if left in the field straw can yield benefits such as soil protection against erosion; reduction of soil temperature variation and protection against direct radiation; increase in biological activities; better water infiltration; better water availability due to lower evaporation; and better control

of weeds. However, some disadvantages also highlighted: fire risk; difficulties of doing the mechanical cultivation during or between the seasons of the year; delay in sprouting and consequent productivity decrease; and mainly pest population increased (insects) [40; 41].

In Brazil, a state law approved by São Paulo state, the major sugarcane producer, in 2002 - Law number 11.241, established that after 2017, 80% of sugarcane harvesting should be mechanized, and after 2021 no more burning will be allowed to mechanizable areas. Sugarcane straw accounts for approximately one-third of the total primary energy contained in sugarcane in the field, however, its use is currently low because it is either burned in the sugarcane pre-harvest or ends up in the soil to decompose due to the mechanized harvest. Its chemical composition resembles that of bagasse, which could be reused as a source of energy (burning), as substrate for the microorganisms to fibrolytic enzymes production, and also for the action of these enzymes in bagasse degradation for the sugars production [15].

The study by Szczerbowski [19] showed that the sugarcane bagasse and straw composition are basically the same, that is, straw can be used for the same purposes as bagasse, which is widely studied. The cane straw is mainly composed by cellulose (35.1%), hemicellulose (27.08%), and lignin (20.3%), based on the media of the detailed values observed in Table 2.

Table 2 – Sugarcane straw composition.

Component % (w/w)	Cellulose	Hemicellulose	Total Lignin	Ash	Extractives	Reference
	39.8	28.6	22.5	2.4	6.2	36
	38.1	29.2	24.2	2.5	5.9	37
	33.77	27.38	21.28	6.23	-	18
	37	29	21	4.1	10	41
	31.7	27	31.1	1.5	-	26
	30.3	21.3	27.5	10.8	-	Chapter 4 of this thesis

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2. ENZYMES APPLIED IN BIOPROCESS

The enzymes have gained a prominent place in the world´s technological development due to their operational characteristics that ensure advantages over conventional chemical processes. They can be applied in several sectors, such as food processing, animal nutrition, cosmetics, medicines, biofuels, among others. [42- 45]

Enzymes are biodegradable, have high specificity, which leads to low by-product formation, reduce the downstream processes and energy costs, and enable that the process is carried out under mild pH and temperature with high yields [5; 46]. Among LCM enzymatic hydrolysis advantages, the main is the absence and/or decrease of toxic compounds (by-products). The process conducted in the traditional way with chemical agents can cause the equipment corrosion [47-49], as well as the toxic compounds are generated, which affect the next steps, such as the sugar fermentation [50].

It is known that there are approximately 400 known enzymes, of which 200 are produced by microbial sources and are used commercially. However, only 20 ones are industrially produced in a large scale. With the increasing interest in researches to understand the biochemistry related to the enzyme production, involving fermentation processes, and new downstream methodologies, there also may be a considerable increase in the number of enzymes available [42].

Table 3 – Commercial fibrolytic fungal enzymes and their respective producers.

Commercial enzyme	**Source and characteristics**	**Producer**
Celluclast 1.5L	Cellulase; *Trichoderma reesei* ATCC 26921	Novozymes ®
Novozymes 188	Cellobiase from *Aspergillus niger*	Novozymes ®
Cellic CTec2	Multi-enzyme product for cellulose degradation	Novozymes ®
Cellic HTec 2	Pool of hemicellulases enzymes	Novozymes
Cellulase AP3	Cellulase from *Aspergilus niger*	AMANO ENZYME
Accellerase Trio	Endo and exo-glucanase, and β-glucosidase; *Trichoderma reesei* (genetically modified)	Genencor ®
β-xylosidase	1,4-β-D-xylan hydrolase; *Bacillus pumilus* (recombinant)	Megazyme

Source: The site of the companies.

On the other hand, Brazil lacks national industries to produce lignocellulolytic enzymes. The main industries are abroad, for example, Novozymes and Danisco / DuPont (Denmark), Genecor (USA), Basf (Germany) and Roche (Switzerland). Together, Danisco / DuPont, Novozymes and Roche, lead 75% of the world's enzyme production [42; 44] (Table 3). The need for enzymes importation increases the costs of the bioprocesses, so new technologies of internal production are extremely important for the viability of these processes [42; 51].

2.1 Fibrolytic enzymes

The LCM conversion by fibrolytic enzymes is an important strategy for the sustainability and advancement of several industries. These materials can be used by microorganisms as a carbon source and results in the production of cellular proteins, enzymes, organic acids, important secondary metabolites, and also prebiotic oligosaccharides [21; 52; 53]. Despite the widespread use of natural cellulosic wastes, there are still abundant quantities of raw materials that are not exploited or could be used more efficiently [54].

Many studies are being carried out to reduce the enzyme production cost, among them is possible to highlight: screening of new or more efficient enzymes microorganisms producers, genetic improvement of existing industrial strains with enzymatic engineering, and implantation of production and operation models of important factors, such as substrate selection, culture conditions, enzyme recycling and process remodeling [55; 56].

Filamentous fungi, bacteria, and yeasts are used to xylanolytic enzymes production [57]. The filamentous fungi ability to produce large amounts of extracellular enzyme has given them an important highlight in the enzyme industrial production. Some of the most commonly used filamentous fungi for xylanase production are *Penicillium sp*., *Trichoderma viride*, *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus sp*. etc. The xylanases use for industrial purposes increases and accelerates the research for better production yields and faster processes. As previously mentioned, agroindustrial wastes, if used correctly as substrate for enzyme production, are promising sources to decrease the production cost [47- 49].

2.2 Xylanases

Xylanases are genetically characterized to be glycoproteins chains of 6-8 kDa weight, and chemically by have an optimum pH activity spectrum, 4.5-6.5, in temperatures between 40-60 ºC. However, this range could change for the enzymes according to the organism producer and production mode [58]. These enzymes are the main responsible to xylan hydrolysis.

Due to the complex composition of the natural xylan chemical structure (the main component of hemicellulose), its complete breakdown requires the action of a set of hydrolytic enzymes with different specificities and action modes. The most important xylanolytic enzymes are the endo-β-1,4-xylanase, which hydrolyze the insoluble xylan chains in smaller soluble xylooligosaccharide (XOS), decreasing its polymerization degree, and β-xylosidases, which hydrolyze the soluble xylan. β-xylosidases are also effective in degradation of monosaccharides in xylose units [8; 59].

In recent years the interest in xylan degradation enzymes has increased greatly due to its biotechnological potential. The fungal xylanases are being applied in biopulping process in paper and pulp industries [8], hydrolysis of lignocellulosic materials [31; 60], xylitol production [26], and functional foods [11; 61].

The xylan polymer can be converted into β-D-xylanopyranosyl group, and its oligosaccharides, through two types of hydrolysis: chemical (acidic) or enzymatic route. The acid pathway has the advantage to be a fast process, but this is accompanied by the formation of toxic products that may subsequently affect the sugars microbial fermentation. In addition, in the long term, acid contact with metal equipment can cause corrosion. Some industries have shown interest in efficient enzymatic hydrolysis processes to replace acid hydrolysis in the treatment of materials containing hemicellulose [55].

2.3 Cellulases

Cellulases are the main enzymes involved in the microbial hydrolysis of cellulosic residues with by-products production. They can be produced by different microorganisms and have different enzymatic classifications. Cellulases hydrolyze β-1,4-D-glucan bonds and produce primary products like glucose, cellobiose, and cellu-oligosaccharides. The enzymatic complex of cellulases is one of the most studied and comprises endo and exo-glucanases, and

β-glycosidases. These enzymes act synergistically to convert crystalline cellulose to glucose [1; 18; 44]. Specifically, they can be classified into three groups of cellulases that are involved in cellulose hydrolysis: I) endoglucanase (EG; endo 1,4-β-D-glucanases or endo -1,4-glucanohydrolase; EC 3.2.1.3) randomly attacks regions of low crystallinity of the cellulose fiber, generating free ends (reducing and non-reducing) and there it decreases the polymerization degree; II) exoglucanase or cellobiohydrolase (CBH I and II; 1,4-β-glucan-cellobiohydrolase or exo 1,4-β-D-glucanase; EC 3.2.1.91), which degrades the molecule by removal of the cellobiose units and / or glucosidase inhibitors (CBH I) and non-CBH reductase inhibitors; III) β-glycosidases (BG-EC 3.2.1.21) that hydrolyzes cellobiose (cellobiases) to produce glucose, they act synergistically with CBH and EG, to reduce the inhibitory effect of cellobiose on endo/exo-cellulases [1; 14; 18].

Currently, cellulases represent the third largest industrial production in the world and their applications can be found in cotton processing, paper recycling, juice extraction, detergent and animal feed. The production of these enzymes may substantially increase if the enzymatic route of ethanol production of 2nd generation be implanted in the world [62]. Cellulases are a key part in the 2nd generation ethanol production from agroindustrial residues via the enzymatic pathway [18; 63].

2.4 Xylanases and Cellulases fungal production

The researches on fibrolytic enzymes production, cellulases and xylanases, are mostly carried out through the genera *Trichoderma* and *Aspergillus*, by different species of these microorganisms with different substrates, or the mixture of them, as can be observed in Table 4.

The concentration of substrates is important in the optimization of the production of enzymes, cellulases and xylanases, for example in the Das [64] work, the statistical planning was able to increase the enzyme production after optimizing the amount of each substrate wheat straw and rice straw in a mixture (wheat straw and rice straw). In the work of Guimarães [8], wheat bran was the substrate that excelled in the xylanase production by *A. niger* and *A. flavus* among other substrates such as rice bran, glucose, sugarcane bagasse and corn cob. However, the simple mixture of wheat bran and corn cob also showed good results.

Table 4 – Xylanases and cellulases production from LCM by *Aspergillus* and *Trichoderma* species on isolated or mixed cultures.

Substrate (residue)	Microorganism	Xylanase Activity	Cellulase Activity	Reference
Wheat bran + corn cob	*A. flavus* (SmF)	11.92 (U/mL)	-	8
Wheat bran + rice straw (PT)	*A. fumigatus* ABK9 (SSF)	1130.4 (U/g)	102.5 U/g (FPase)	64
Corn stover (PT)	*T. reesei Rut* C-30 (SmF)	2.03 (U/mL)	0.93 (U/mL)	31
Sugarcane straw + wheat bran (PT)	*A. fumigatus* (SmF)	821 (U/g)	5.1 (FPU/g)	65
Wheat bran	*A. terricola* Marchal (SmF)	4.63 (U/mL)	-	9
Sugarcane bagasse (PT)	*T. hazianum* (SmF)	36.96 (IU/mL)	0.78 (FPU/mL)	66
Rice straw+wheat bran	*T. reesei*+*A.fumigatus*	2859.6 (IU/gds)	30.9 (IU/gds)	67
Prosopis juliflora pods	*Trichoderma reesei NCIM 1186*	422.16 U/L	3055.65 U/L	68
Sugarcane bagasse	*T. harzianum* 1 (PS1-G06) + *A. niger*	0.58 U/mL	6.12	69
Sugarcane bagasse	*T. harzianum* 1 (PS1-G06) + *T. harzianum* 2 (PS3) + *A. niger*	0.46 (U/mL)	3.3 (U/mL-endo)	69
Cellulose (commercial medium)	*T. reesei* Rut C30+*A.niger* LMA	-	7.1 (U/mL)	70

PT= pretreated; SmF = submerged fermentation; SSF = solid state fermentation

Considering biological process, the advantages of submerged fermentation (SmF) should be highlighted due to: the mix of the medium is easy and allows uniform conditions for the growth of microorganisms; the modification of cultivation conditions such as pH, dissolved oxygen, temperature, agitation and nutrient concentration is also easy and fast; the temperature control is favored by high specific heat and thermal conductivity, and by the possibility of automating the process with modern and diversified equipment. During the microorganism growth, the bioactive compounds are secreted into the culture medium making easier the secondary metabolites extraction and subsequent purification. Due to these characteristics of easy monitoring and handling, approximately all large-scale enzymatic production processes use SmF technology [21; 71].

3. MIXED CULTURE TO IMPROVE THE ENZYME PRODUCTION

As previously mentioned in Table 4, not only axenic cultures but mixed cultures can be applied in enzymes production using LCM, resulting in a high enzymatic activity, and a synergistic enzymatic pool for the role substrate bioconversion in soluble sugars. Thus, a detailed study of the microorganism and the most appropriate conditions of the mixed culture for enzyme production is necessary [70; 72].

The complete cellulose hydrolysis, as said before, in the 2.3 Section, is due to a cellulase complex, in that β-glucosidase is the main important enzyme that can reduce the cellobiose inhibitory effect of cellobiose on cellulase activity. To overcome the deficiency of β-glucosidase in a *T. reesei*-derived enzyme system, *T. reesei* can be co-cultivated with another fungus from *Aspergillus* genera [67; 69; 70]. The *Trichoderma* specie is known to be low β-glucosidase producer, and *Aspergillus* ones produced it easily. In the work of Haq, Javed and Khan [73], the cultivation with *A. niger* and *T. viride* in submerged fermentation increased the production of lignocellulolytic enzymes in the range of 30-50% in relation to axenic cultures.

In another work, the co-cultures system of *T. reesei* and *A. niger* increased the number of cellulases when compared to the *A. niger* axenic culture production. In addition, the microorganisms together were responsible for degrading the cellulose present in the culture medium by 89.4% [70]. *T. reesei* and *A. phoenicis* were also co-cultivated, and this study showed that the cellulolytic enzymes production in these processes was effective, and that these enzymes can be used in the cellulosic residues degradation [72].

Despite the various succeeds reports some contradicted ones can be cited, as example, the decrease of cellulase production in mixed cultures systems was also observed with *T. reesei* RUT-C30 and *A. phoenics* QM 329 when compared with its axenic cultures. The authors explained that the involved species can have started a competition, blocking the enzyme production [72; 74]. Some explanations for the no-improvement in enzyme production can be also related to the carbon source, an important parameter to a succeed mixed culture [31; 67]. The authors also reported a little improvement on cellulase production, 22.89 - 24.17 IU/g, to axenic and mixed cultures, respectively in SSF, while the substrate consumption was better in the second one. The mixed cultures can also be improved by genetic mutation as proved in an experiment using *T. reesei* mutant specie the results from improvement in enzyme production using mixed cultures with *A. niger* was different from the non-mutant strain, that no presents improvement [75].

4. INDUSTRIAL APPLICATION OF XYLANASES AND CELLULASES

As previously mentioned, the applications of the enzymes xylanases and cellulases are diverse, however, the performance in the paper and pulp industry, xylooligosaccharides (XOS) production, and in second-generation ethanol production are the most relevant.

4.1 Kraft pulp bleaching

Currently, in many countries, including Brazil, the process used in the pulp and paper industry uses chemicals rather than enzymatic hydrolysis. The most commonly used methodology is the Kraft pulping. Three species of Eucalyptus (*E. grandis, E. saligna and E. urophylla*) are preferred as feedstock for papermaking. Pulp and paper mills process huge quantities of woody materials every year. During the paper manufacturing is important that lignin be removed from all woody plants and agroresidues used as raw materials, to obtain a whiter paper and one improvement of paper properties, ex: crystallinity and brightness [76; 77; 78; 79]. The break and subsequent removal of all lignin present are based on the following steps: pulping processes, in which lignin is removed during the heating process; and/or the residual lignin that remains blocked on the cellulose fiber surface, mainly linked to short-chains xylan, is then removed by a multistep bleaching process using large amounts of elemental chlorine (Cl_2) and chlorine dioxide (ClO_2) chemicals [80; 81].

Unfortunately, by-products generated from chemicals uses are chlorinated organic substances, some of which are toxic, mutagenic, persistent, and bio-accumulative, causing numerous harmful disturbances in biological systems [81]. Due to these effluent-based problems, legislative and environmental pressures have been suggested that these mills should change some parameters in bleaching technologies, or wastewater treatment technologies, making it greener [82-84]. One of the aspects of in-situ process modification is the replacement of toxic elemental chlorine with bleaching chemicals like elemental chlorine free (ECF) (using chlorine dioxide) and total chlorine free (TCF) (using oxygen and ozone) processes [84]. Recently, studies along the replacement of Cl_2 bleaching with ClO_2 is the better available techniques for organic pollutants, as adsorbable organic halides (AOX), reduction in non-wood pulp and paper mills, considering the lower environmental pollution compared to Cl_2 bleaching and the superior bleaching effect [85; 86; 87]. Regarding these interventions, some modifications, and/or capital investments should be done. A biotechnological route for pulp bleaching using xylanases is one

suitable alternative to be used in the pulp and paper industry to reduce and/or eliminate the use of chlorine compounds, and other chemical bleaching chemicals, as well as the operational costs related to water, electricity, fuels [78; 81; 83; 88].

Biobleaching process is a technological fungal xylanase application in that these enzymes act on pulp, before chemical bleaching, due to cleavage of linkage of residual lignin to hemicellulose, prominent to increased accessibility of the pulp to bleaching chemicals and thereby enhanced the lignin extrability [89] during subsequent bleaching stages. Some microorganisms already produced xylanases that showed effectivity on kraft pulp biobleaching and chlorine decrease as *Bacillus subtilis*, *Pichia pastoris, Bacillus pumilus* and *Arpergillus niger and Aspergillus flavus* [8; 83; 88; 90]. Overall, major advantages of biobleaching are: reduced consumption of bleaching chemical, reduced absorbable organic halogen compounds, improved pulp and paper quality, improved brightness, reduced effluent toxicity and pollution load [84].

4.2 Bakery industry

Xylanases also have great potential for use in the baking industry that increase processing and products quality. The application of xylanases in baking results in volume increase, viscosity and the hardening decrease, and longer shelf life. The enzyme can replace the addition of different emulsifiers and other chemical additives used in bread production. However, for the best results, various enzymes should be used at optimal levels, since overdose has adverse effects on the final product. Moreover, it is a good strategy to use xylanase in combination with other enzymes because the synergistic effects of xylanase with other enzymes provide better results as compared to its exclusive use [58; 59].

The beneficial role of xylanases in bakery process is generally attributed to their property to hydrolyze polysaccharides present in the flour. The monomers and oligomers resulting from enzyme activity affect the water balance and modify the protein-starch interaction during bread storage. The use of xylanases also improves dough properties, enhance bread quality and reduce the staling xylanase decreased dryness and stiffness of the dough whereas, resulted in increased elasticity, extensibility and coherency and increase in volume and decrease in bread density [91-93].

Xylanases are important enzymes improving quality of bread and can be produced by different microorganisms, such as bacteria, fungi, and yeasts. Species of *Trichoderma, Streptomyces, Aspergillus, Fibrobacter, Phanerochetes, Chytridiomycetes, Ruminococcus, Clostridia, and Bacillus* are major xylanase producers [91]. Xylanases from *Pichia pastoris* produced results related to a decrease in firmness, stiffness and consistency, and improvements in specific volume and reducing sugar content were recorded [94]. During storage at different temperatures, bread containing xylanase from *Penicillium citrinum* MTCC 9620 exhibited less firmness but larger volume with whiter crumb color longer shelf life as compared to control bread, with no xylanase, and better sensory features [95].

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4.3 XOS production

The natural sources of XOS can be fruits, vegetable and honey [96] or it can be produced from structural hemicellulose polysaccharide xylan from LCM, by enzymatic or acid hydrolysis [27; 97; 98]. During the acid hydrolysis, the xylan depolymerization is formed randomly by the attach in the glycosidic bonds between adjacent xylose units. However, in enzymatic route the breaks occurred generally by endo-β-1, 4-xylanases degrade that xylan by attacking the b-1, 4-bonds between xylose units to produce XOS, and β-xylosidase converts lower-DP (degree of polymerization) XOs into monomeric xylose. Both treatments show difficulty to control the XOS´s type and DP produced, but the enzymatic process does not produce furfural from pentoses degradation [12; 98; 99]. In addition, other aspects influence XOS yield and the composition of oligosaccharides produced by enzymatic reactions, such as xylanase type, xylan composition, the type of pretreatment to xylan extraction, time and other parameters of the reaction. Thus, more studies of production and application of xylanases to improve XOS yields are necessary [11].

5. CONCLUSION

LCM – lignocellulose materials previously only consider a waste, currently they are being considered an important feedstock which can be used in interesting applications. Enzymes, important biomolecules, can be produced by microorganisms using LCM as a carbon source to grow, so helping the decrease in these wastes accumulation. Another LCM uses is related to its hydrolysis in sugars that can be transformed in ethanol or special sugars as XOS, as well as in the paper mills and bakery industries. Sugarcane straw is a new LCM feedstock in Brazil since the mechanical harvesting was implemented but it was not so well studied as bagasse was. However, sugarcane straw shows similar characteristics of the bagasse. Thus, more studies using sugarcane straw for different applications should be done to include this residue in the bio-refinery scenario.

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CHAPTER 2: Sugarcane straw as a carbon source for fungal xylanases and cellulases production in submerged culture

Abstract

Sugarcane straw (SS) was evaluated as substrate to fibrolytic enzymes production by different axenic fungal cultures: *Trichoderma reesei* QM9414, *T. reesei* 2768, *T. harzianum* N51, *T. harzianum* FS09, *Aspergillus fumigatus* M51 (CCT7732), and *A. fumigatus* U2370. The screening of the best enzyme producers among these microorganisms was performed in shaker flasks (submerged fermentation). *T. reesei* QM9414 culture reached the maximum enzyme production in pH 4.5, at 28 ºC, and 180 rpm, and in a stirred tank, 3 L bioreactor, showed the same yield production profile (~90 U/mL and 0.6 FPU/mL). Regarding this result, the enzyme production by binary and ternary mixtures of these fungi was lower, as example the best combination *T. reesei* QM 9414+*A. fumigatus* M51, reached 60 U/mL and 0.08 FPU/mL, respectively. The improvement of enzymatic yield through the supplementation of SS was performed by a D-optimal design, this revealed that wheat bran and citric pulp did not show an inductive effect on the enzymes production. The xylanase was characterized by the pH (5.0) and temperature (50 ºC) profiles, and stability. Thermal stability was not observed at temperatures above 50 ºC and pH 5-6 was established as the enzyme pH stability range. Some ions and EDTA were added to the enzymatic reaction medium for determining the xylanase stability on these conditions. Cu^{+2} and Ag^{1+} (10 mM) resulted in a strong inhibition of xylanase, while Mn^{2+} (5 and 10 mM) had a stimulatory effect on it. These results indicate SS as a possible substrate for fungal fibrolytic enzymes production and that it is possible to employ this enzymatic complex in industrial processes.

Keywords: Sugarcane straw. D-optimal design. Mixed cultures. Fibrolytic enzymes. Filamentous fungi. Biochemical xylanase characterization.

1. INTRODUCTION

The use of the renewable sources, including organic wastes mainly produced by agricultural countries, is currently considered to produce biofuels or other value-add products by cleaner technologies and also represents an alternative to decrease the environmental pollution due to the petroleum fuels use [1-4]. Only in Brazil, 646.5 million tons of sugarcane were produced in 2017-2018 [5]. In Brazil, a state law approved by São Paulo state, the major sugarcane producer, in 2002 - Law number 11.241, established that after 2017 80% of sugarcane harvesting should be mechanized, and after 2021 no more burning will be allowed to mechanizable areas. As a consequence of this new system implantation, almost 15 Mg ha^{-1} dry mass has been left in the field yearly, mainly sugarcane straw, available for further processing to increase the sector´s energy efficiency [6]. In the burn harvesting system, almost 27 Kg of carbon dioxide was released into the atmosphere per ton of sugarcane processed, related to burn (40%), fertilizers (20%) and fossil fuels use (18%), so this quantity can decrease using no-burning system [7]. Besides that, emissions from sugarcane burning are known to cause impact on the respiratory health the local populations, due to the increase of particulate matter present in the air [8; 9].

Straw represents around one-third of the total primary energy of the sugarcane crop, with a composition very similar to the widely used bagasse, mainly cellulose, hemicellulose and lignin, 30, 30 and 25%, approximately [10-12]. The straw left in the soil range from positive impacts, such as increase in the macrofauna (mainly worms and ants), nutrient recycling, water storage, carbon accumulation, promotion of the control of soil erosion and weed infestation, to negative impacts, such as increase in pest populations and biomass loss production [11; 13]. In fact, a recent work shows that 50% of SS left in the soil is necessary to improve the yield of sugarcane crop but the other 50% should be recovered to be used in eco-friendly process [14]. Depending on the amount and characteristics, this residue can be collected to produce energy or co-products such as enzymes and [15; 16] or xylitol [17].

In addition, the enzyme-based process has continuously substituted traditional chemical processes in many areas, especially fine chemical and pharmaceutical industries, and the development of new technologies can turn it easier and industrially applied [18]. For example, the BCC Group Research analyzed the global market for industrial enzymes and reported that it reached nearly $4.6 - $4.9 billion in 2014 and 2015, respectively. This market is expected to increase from nearly $5.0 billion in 2016 to $6.3 billion in 2021 with a compound annual growth

rate (CAGR) of 4.7% for 2016-2021 [19]. In the same study, the main enzymes companies were pointed: Novozymes, BASF Corp., Associated British Foods plc (AB Enzymes), Danisco/DuPont (Genencor Industrial Biosciences), Specialty Enzymes and Biotechnology Ltd., DSM, Maps Enzymes Ltd., and Chr. Hansen A/S.

The importance of enzymatic technology includes the knowledge of the biochemistry, fermentation and downstream process, that results in a high number of available enzymes and applications [20]. In this sense, the use of agroindustrial residues as a carbon source to microorganisms' enzymes producers, considering an "in situ" production, could decrease the production costs and the final price of enzymes [21-23]. Currently, cellulases represent the third high industrial enzyme production, and their applications were found in cotton, paper recycling, juice extraction, detergent and feed industry [24]. Other important fibrolytic enzymes are the xylanases. This class of enzymes is composed by glycoprotein chains, with a molecular weight ranging from 6-8 kDa, working in pH 4.5-6.5, and at 40-60 ºC. However, these data can change due to the production process mode and the microorganism producer.

Filamentous fungi, bacteria, and yeast were used to produce xylan-degrading enzymes [24]. The ability of these microorganisms to produce extracellular enzymes is the main interest to industrial purposes due to it decreases the production cost and the final price of the product [21; 25]. Mesophilic fungus as the genera *Aspergillus* and *Trichoderma* have a remarkable importance in xylanase and cellulases production, and when cultivated in mixed culture were also reported to improve enzymes production [26-28]. As example xylanases and cellulases production of xylanases using lignocellulosic wastes was observed by *A. fumigatus* ABK9, that grow using wheat bran and rice straw [29] and *T. reesei Rut* C-30 with corn stover [30].

Although the efficiency of SS as a feedstock and inducer for cellulolytic enzyme production by some microorganisms were reported to *Streptomyces sp* SLBA-08 [31] and *T. citrinoviride* [32], there are no studies of SS as feedstock for xylanase and cellulase production in submerged culture for *T. reesei, T. harzianum* and *A. fumigatus*

In the present work, a bioprocess for some fibrolytic enzymes production was conducted considering the formulation of the culture medium with SS as main feedstock and some special fungi from *Trichoderma* and *Aspergillus* genera, in axenic and mixed cultures. In addition, the biochemical characterization of the xylanases produced in the best conditions was performed considering future applications.

2. MATERIALS AND METHODS

2.1 Microorganisms and substrates

The microorganisms tested in axenic cultures were: *T. reesei* (Coleção de Culturas Tropicas André Tosello CCT -2768), *T. reesei* QM9414, *T. harzianum* N51, *T. harzianum* FS09, *A. fumigatus* M51 and *A. fumigatus* U2370. They were cultured in plates with PDA medium for 7 days, at 28 ºC, and stored at 4 ºC. For experimentation purposes, spore suspension was prepared by incubating the cultures on PDA plates at 28 ºC for about 10 days, until sufficient sporulation was observed. The spores were harvested using 0.1% Tween 80 solution (v/v) and the spore count of about 1×10^6 cells/ml was used for fermentation medium inoculation purposes. Lignocellulosic substrates were used as carbon source in the culture medium. The sugarcane straw was obtained from Água Bonita Mill, Tarumã-SP, Brazil, pretreated (autoclave 121 ºC, 15 min, 1 atm), and milled (14 mesh). The citrus pulp was milled (14 mesh-from Citrovita, Catanduva-SP, Brazil), and wheat bran was used without any previous treatment (from Moinho Nacional, Assis-SP, Brazil).

2.2 Selection of microorganisms in axenic and mixed cultures

The axenic and mixed strains were cultivated in Erlenmeyer flasks (250 mL) by submerged fermentation (SmF) containing 80 mL medium (m/v): 3.0% pretreated sugarcane straw, 0.1% $(NH_4)_2SO_4$, 0.0017% $MgSO_4{\cdot}7H_2O$, 0.1% K_2HPO_4, 0.0028% $ZnSO_4$, 0.1% $NH_4H_2PO_4$, 0.06% KCl, 0.1% yeast extract and 0.1% sucrose at pH 4.5 [33]. The flasks were inoculated and incubated at 28 ºC, in an orbital shaker (Tecnal mod. 421, Piracicaba, Brazil) at 180 rpm for 360 h. The biomass was separated by 15 min centrifugation at 4 ºC, and 2900 x *g* (Megafuge, 16 R, Heraeus, Thermo Fisher, Massachusets, USA). The liquid fraction was used as a crude extract of enzymes.

Mixed cultures were performed with the best xylanase and cellulase enzymes producers, $1x10^6$ cells/mL for each one, organized binary and ternary mixtures: *T. harzianum* FS09 + *A. fumigatus* M51; *T. harzianum* FS09 + *T. reesei* QM9414; *T. reesei* QM9414+ *A. fumigatus* M51; *T. harzianum* FS09 + *T. reesei* QM9414+ *A. fumigatus* M51.

2.3 Formulation of culture medium with mixtures of agroindustrial wastes for fibrolytic enzymes production

The submerged culture of *T. reesei* QM9414 was performed in Erlenmeyer flasks (250 ml, with 80 mL of the described medium in item 2.2) during 288 h of incubation in a shaker at 28 ºC and 180 rpm. D-Optimal mixture design was performed in order to define the influence of individual substrates and the interactions among them in ternary mixtures on specific xylanase and cellulase activities [34; 35]. The number of experimental combinations in each experimental design was enough to fit special cubic models for response variables. The parameters and restrictions of the mixtures were: sugarcane straw (60–100% w/w range), citrus pulp (0–40% w/w range) and wheat bran (0–20% w/w range) (Table 1). A control experiment using 100% (w/v) of each substrate was performed at the same conditions.

Table 1 - D-optimal experimental design for defining substrate influence on fibrolytic enzyme production. The ternary mixtures of agroindustrial wastes were composed of wheat bran, citrus pulp and sugarcane straw.

Run	Sugarcane Straw (% m/m)	Citrus Pulp (% m/m)	Wheat Bran (% m/m)
1	80.0	0.0	20.0
2	75.0	15.0	10.0
3	60.0	20.0	20.0
4	60.0	40.0	0.0
5	60.0	20.0	20.0
6	90.0	0.0	10.0
7	82.5	7.5	10.0
8	66.67	20.0	13.33
9	60.0	40.0	0.0
10	100.0	0.0	0.0
11	100.0	0.0	0.0
12	80.0	20.0	0.0
13	70.0	10.0	20.0
14	67.5	27.5	5.0
15	80.0	20.0	0.0

The D-optimal experimental design for mixture problems with constraints was set and analyzed using Design-Expert software (Design-Expert® software, version 10, Stat-Ease, Inc., Minneapolis, MN, USA). The statistical decisions were taken considering a significance level of 0.05. The strength of linear relationships between actual and predicted values by different models was assessed using the linear correlation coefficient (R^2). The xylanolytic activity in ternary mixtures of agroindustrial wastes D-Optimal experimental design were optimized using a desirability function. The optimization criterion was to maximize xylanolytic activity according to a fitted polynomial for this variable.

2.4 Stirred tank bioreactor culture

The enzyme production by *T. reesei* QM9414 was scaled-up in 3 L BioFlo 115 fermenter (New Brunswick, New Jersey, USA) using a medium and inoculation described on the item 2.2, with usual volume of 1.5 L, and Rushton impeller. The bioreactor´s culture conditions were 28 °C, 1.7 vessel volume per minute (vvm) of air supply, and pH 4.5. Dissolved oxygen was measured by an oxygen electrode (Mettler Toledo AG, Greifensee, Switzerland) and pH was measured with a glass electrode (Mettler Toledo AG, Greifensee, Switzerland) and controlled with 1.0% (v/v) H_2SO_4 and 1.0 M NaOH.

2.5 Biochemical characterization of fungal xylanase

The biochemical characterization of xylanases produced from *T. reesei* QM9414 using sugarcane straw as substrate was performed following protocols [36; 37].

2.5.1 Optimum pH and stability

The optimum pH was evaluated by measuring enzyme activity at 50 °C using different buffers: sodium citrate (pH 3.0-6.0), sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0), and glycine-NaOH (pH 9.0-11.0) and a reaction mixture containing 0.65 mL 0.5% (w/v) xylan in 0.25 M buffer and 0.10 mL crude enzyme. For pH stability crude enzyme was dissolved (1:1)

in all buffers and maintained at 25 °C for 20, 40 and 60 min. An aliquot was used to determine the remaining activity (2.5).

2.5.2 Optimum temperature and thermostability

The optimal temperature was determined by incubating the reaction mixture at 20-70 °C (10 min) and assaying the activity at the optimum pH, in the same reaction mixture (2.5.4). For thermostability assay, the enzyme solution was incubated at various temperatures (20-70 °C) for 20, 40 and 60 min, at pH 5.0 in sealed tubes to prevent evaporation. The enzyme solution was maintained at these temperatures and times and aliquots were removed and placed on ice before assaying for residual enzyme activity at the optimum pH and temperature.

2.5.3 Effect of ions and EDTA

The effects of ions (Cu^{+2}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, Ag^{1+}) and EDTA (Ethylenediamine tetra-acetic acid) on the activity of the xylanase were evaluated. At concentrations of 5 and 10 mM, the solutions were added to the reaction mixture at the concentration of 0.2% (v/v). The calculation of the percentage of activity was performed based on the reference sample without any ion.

2.6 Enzymes activity assay

Endoxylanase activity was assayed at 50 °C in a reaction with 0.1 mL raw extracts and 0.65 mL of 0.5% (m/v) xylan Birchwood solution (Sigma-Aldrich) in 250 mM sodium acetate buffer, at pH 5 [38]. The reducing sugar concentration was quantified by the dinitrosalicylic acid (DNS) method [39]. One unit (U) of xylanase activity was defined as the amount of enzyme to release 1 µmol of reducing sugar per minute per mL of reaction. The cellulase activity was determinate by Ghose protocol [40]. One FPU here is defined as µmoles glucose equivalents released from Whatman nº. 1 per minute averaged over 60 minutes, considering the low enzyme concentration in the raw enzymatic extract.

3. RESULTS AND DISCUSSION

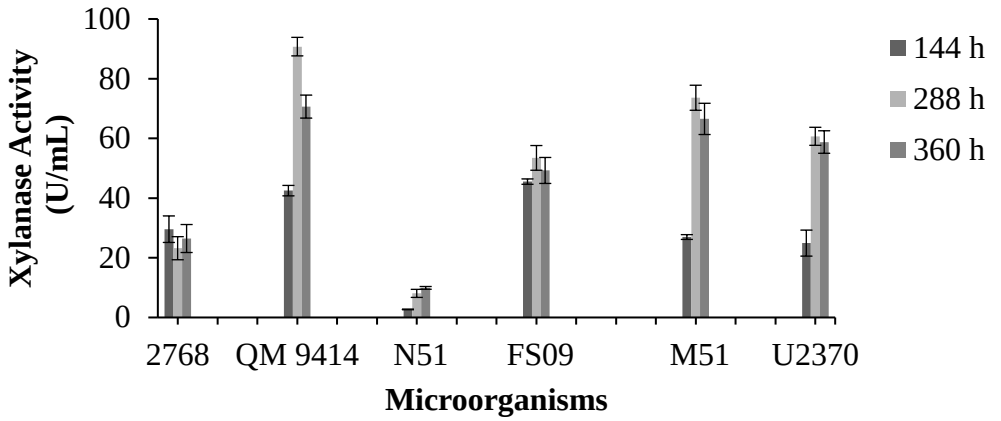
3.1 Selection of the fungi for fibrolytic enzymes production in axenic and mixed cultures using SS as a carbon source

3.1.1 Axenic fungal cultures

All tested microorganisms showed the fibrolytic enzymes production, xylanases and cellulases (Fig. 1-2), using Sugarcane Straw (SS) substrate as the only carbon source. These results show the ability of this substrate on enzyme production and also an important way to give an eco-friendly destination to SS. The *T. reesei* QM9414 strain stood out in relation to the other fungi tested, reaching the highest production in 288 h of culture, 90 U/mL for xylanase and 0.56 FPU/mL for cellulase. The culture time of 288 h was the best period to xylanase production in axenic cultures, after that the decrease in activity is probably due to protease presence in the extract [33; 41]. A higher level of xylanase was obtained when compared with other works: 3.38 U/mL in 120 h of culture with *Trichoderma inhamatum* [42], but similar to Carvalho [43] : 43.7 U/mL in 144 h of culture with *T. reesei* CCT2768, 35 U/mL with *A. fumigatus* M51 and 28 U/mL with for *A. fumigatus* U2370, using a similar carbon source, sugarcane bagasse. The fungi *A. fumigatus* M51 and *A. fumigatus* U2370 also showed good results for xylanases production, approximately 70 U/mL (Fig. 1), as reported in the literature [43].

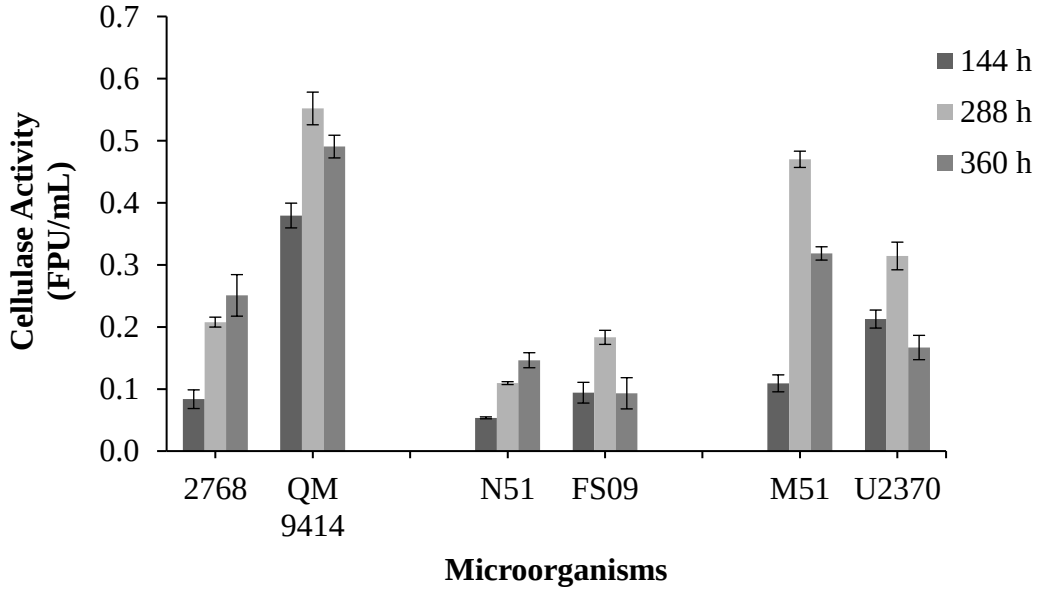
The fungi *T. harzianum* FS09, *A. fumigatus* M51 and *T. reesei* QM 9414 were the best cellulase producers, 0.2, 0.4 and 0.6 FPU/mL in 288 h respectively. The results obtained with cellulases were lower than those found in other works as Zhang [30] (0.93 FPU/mL, 96 h) and Xiong [44] (2.33 FPU/mL, 144 h) also produced by *Trichoderma* species. These authors used different substrates as pretreated corn stover and a synthetic medium, Yeast glucose medium (YG), for the growth respectively.

Figure 1 - Profile of xylanase enzyme production by the fungi: *T. reesei* 2768 (2768), *T. reesei* QM9414 (QM9414), *T. harzianum* N51 (N51), *T. harzianum* FS09 (FS09), *A. fumigatus* M51 (M51) and *A. fumigatus* U2370 (U2370), in SmF using SS as substrate (28 ºC, pH 4.5, 180 rpm)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Figure 2 - Profile of cellulase enzyme production by the fungi: *T. reesei* 2768 (2768), *T. reesei* QM9414 (QM9414), *T. harzianum* N51 (N51), *T. harzianum* FS09 (FS09), *A. fumigatus* M51 (M51) and *A. fumigatus* U2370 (U2370), in SmF using SS as substrate (28 ºC, pH 4.5, 180 rpm)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

3.1.2 Mixed fungal cultures

The mixed fungal culture was evaluated in the present work in comparison with the axenic ones. Since the *Trichoderma* + *Aspergillus* co-culture system is reported in the literature, the followed mix were proposed with some cultures previously selected: *T. reesei* QM9414, *A. fumigatus* M51, and *T. harzianum* FS09. Xylanase and cellulase production profile by mixed cultures during 360 h of culture can be seen in Figures 3 and 4 respectively.

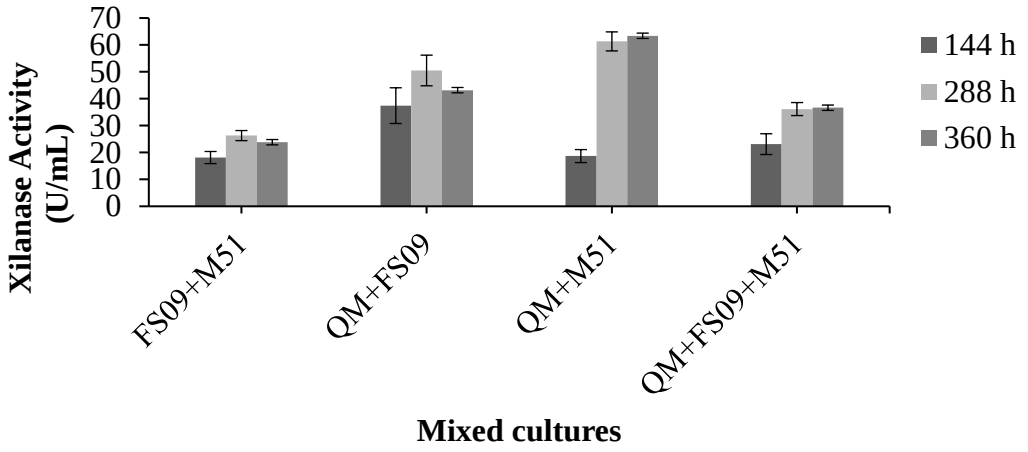
Analyzing the Figures 1-4 is evident that mixed cultures were not interesting to improve xylanase and cellulase production using the mix proposed. When these mixed cultures were compared with the monoculture experiment, the enzyme activities were lower. This result was not expected since the mixed cultures with *Trichoderma* and *Aspergillus* genera resulted in a complete enzymatic pool that acts synergistically better in the substrate degradation than the respective extract from axenic culture [26-28], but maybe there was a competition and inhibition of their metabolisms.

For the xylanase production, since in the mixed culture *T. reesei* QM 9414+ *A. fumigatus* M51, reached the maximum value of 60 U/mL, while to cellulase the best results were achieved by *T. harzianum* FS09 + *A. fumigatus* M51 and *T. reesei* QM 9414+ *A. fumigatus* M51, almost 0.08 FPU/mL (Fig. 3 and 4).

The decrease of cellulase production in mixed cultures systems was also observed with *T. reesei* RUT-C30 and *A. phoenics* ATCC329 when compared with its axenic cultures. The authors explained that the involved species can have started a competition, blocking the enzyme production [45].

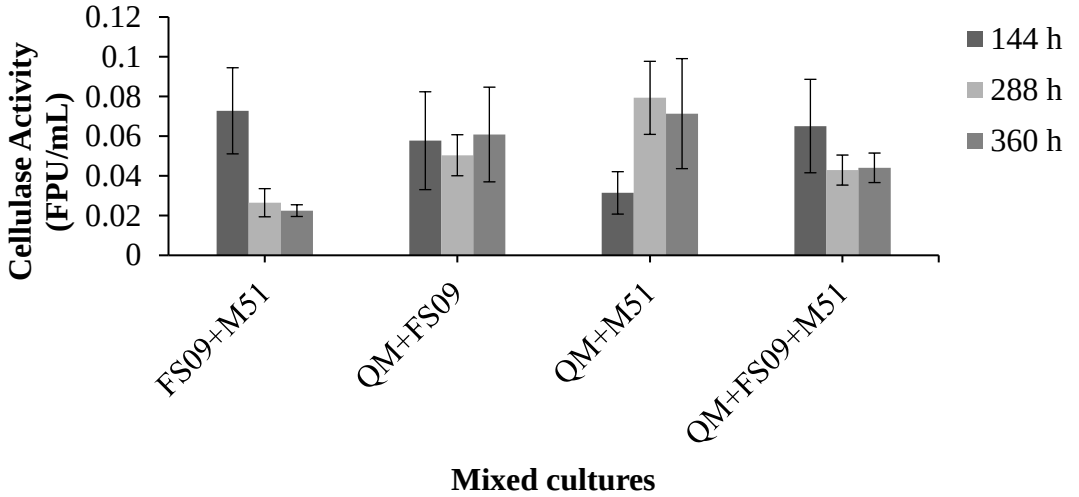
These results were not expected since there are some examples of mixed cultures with *Trichoderma* and *Aspergillus* resulting in a synergistic enzymatic pool for the role substrate degradation [26]. Even the mixed cultures of these genera reported in the literature for a positive effect on the fibrolytic enzymes production, our best result with this system of xylanase production with *T. reesei* QM 9414 and *A. fumigatus* M51 (60 U/mL) was better than that found by Zhang [30] (2.5 U/mL), using another very studied strain (*T. reesei* Rut C-30).

Figure 3 - Profile of xylanasae production using the mixed cultures: *T. harzianum* FS09 + *A. fumigatus* M51 (FS09+M51); *T. harzianum* FS09 + *T. reesei* QM 9414 (QM+FS09); *T. reesei* QM 9414+ *A. fumigatus* M51 (QM+M51); *T. harzianum* FS09 + *T. reesei* QM 9414+ *A. fumigatus* M51 (QM+FS09+M51), in SmF using SS as substrate (28 ºC, pH 4.5, 180 rpm)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Figure 4 – Profile of cellulases production, using the mixed cultures: *T. harzianum* FS09 + *A. fumigatus* M51 (FS09+M51); *T. harzianum* FS09 + *T. reesei* QM 9414 (QM+FS09); *T. reesei* QM 9414+ *A. fumigatus* M51 (QM+M51); *T. harzianum* FS09 + *T. reesei* QM 9414+ *A. fumigatus* M51 (QM+FS09+M51), in SmF using SS as substrate (28 ºC, pH 4.5, 180 rpm)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Some explanations for the no-improvement in enzyme production can be also reported by other works. The carbon source is reported to be an important parameter to a succeed mixed culture [28]. The authors also reported a little improvement on cellulase production, 22.89 - 24.17 IU/g, to axenic and mixed cultures, respectively in SSF, while the substrate consumption was better in the second one. Using *T. reesei* mutant specie the results from improvement in enzyme production using mixed cultures with *A. niger* was different from the non-mutant strain, that no presents improvement [46]. The separated enzymes production is preferred to achieve the better substrate degradation from its synergic effect, despite the mixed culture improves the of cellulase and β-glucosidades production by *T. reesei* QM9414 and *A. terreus* SUK-1 [27].

Therefore, regarding to enzymes production is possible to affirm that the mixed cultures in submerged fermentation were not effective, probably due to two important elements: microorganism affinity and culture medium, where these strains competed each other to the same nutrients, that were reported by other works as important factors to effective yields to mixed cultures to fungi and bacteria [26; 45; 47]. For these reasons, just *T. reesei* was selected for the next steps of this work with emphasis for xylanases.

3.2 The effect of the mixture of agroindustrial wastes in formulated media for fibrolytic enzyme production by *T. reesei* QM 9414

For the fibrolytic enzymes production, 3% (m/v) of the substrates Sugarcane Straw (SS), Citrus Pulp (CP) and Wheat Bran (WB) were evaluated isolated by *T. reesei* QM9414 in a culture medium (Table 2). The culture medium formulated only by SS as substrate showed higher performance for xylanases biosynthesis (90 U/mL) than the others. For cellulases production, the cultures of *T. reesei* QM9414 also showed a higher preference for sugarcane straw (0.6 FPU/mL) (Table 2). The use of wheat bran was proposed since its use promote higher xylanase production in the solid-state fermentation [28; 48], but this result was not confirming in SmF. The substrates composition cited in Tab. 2 suggested that citrus pulp and wheat bran should be more easily hydrolyzed due to the low lignin content.

Table 2 - Comparison of some agroindustrial wastes as a carbon source for fibrolytic enzymes production by *T. reesei* QM 9414 (pH 4.5, 28 ºC, 288 h), and its composition*.

Substrate**	Xylanase activity (U/mL)	Cellulase activity (FPU/mL)	Cellulose (%) w/w	Hemicellulose (%) w/w	Lignin (%) w/w	Reference
Sugarcane straw	90.6±7.04	0.56±<0.1	33.77	27.38	21.28	10
Citrus pulp	31.0±5.87	0.10±<0.1	24.52	7.57	7.51	49
Wheat bran	37.7±4.23	<0.10±<0.1	22.3	32	4	50

*The results are related with the average and standard deviation of three experiments.

**(3% w/v).

In the second set of experiment, a D-Optimal mixture experimental design was used to determine the synergistic or antagonistic effects of mixed carbon sources in the culture medium to fibrolytic enzymes production by *T. reesei* QM 9414 in 12 days (Table 3). When xylanase and cellulase activities, for ternary mixtures of these substrates, were modeled in D-optimal design, cubic models were satisfactorily fitted to the experimental data (model significance tests, $p<0.05$ and lack of fit tests, $p>0.05$). The equations for xylanase and cellulase activities (Equations 1-2 for actual values) in conjunction with contour Graphs (Fig. 5 A and B) showed the major contribution of wheat bran for higher values of fibrolytic enzymes activities.

The Figures 6 A and B depicts the substrates interactions, for both responses the three substrates interact each other, and it also can be noted in the equations 1 and 2. For SS influence in the xylanase activity is notable that the activity decreases with the substrate concentration, while for CP the high concentration increases the enzymatic activity still a limiting value. The substrate WB was not interesting for this purpose in high concentration. Regarding to cellulase production, SS high concentration results in great activities, and for WB there is a point that after this the enzymatic activity stain constant. For citrus pulp its comportment should be done in an ample range to try define the interaction. Wheat bran, when used in combination with other substrates, did not represent a synergic effect with any other substrate. However, these results are in disagreement with some authors that found an improvement on enzymes

production in the optimization of mix-substrates. In Das and co-workers study, the cellulolytic enzymes production increased 1.3 folds after the medium optimization containing wheat bran+rice straw, using *A. fumigatus* ABK9. Wheat bran also performed a positive effect, 21%, on the xylanase production, using *A. flavus* [48].

Table 3 - Results derived from D-optimal experimental design for ternary mixtures of wheat bran, citrus pulp, and sugarcane straw as carbon sources in SmF by *T. reesei* QM9414 (pH 4.5, 28 ºC, 288 h).

Run	Xylanase Activity (U/mL)	Cellulase Activity (FPU/mL)
1	69.4	0.3
2	83.9	0.4
3	70.0	0.3
4	81.8	0.4
5	71.8	0.3
6	67.3	0.2
7	93.3	0.4
8	61.2	0.2
9	78.4	0.4
10	90.2	0.5
11	88.1	0.5
12	83.9	0.4
13	84.7	0.4
14	88.2	0.4
15	83.5	0.5

The math models are expressed in Eq. 1-2, with the coded variables showing the enzymatic activities in the function of: A = Sugarcane straw (w/w), B = Citrus pulp (w/w), and C = Wheat bran (w/w). According to ANOVA, for each activity response desired, xylanolytic and cellulolytic activities produced a significant ($p<0.05$) answer, respectively, for the cubic math models with high Regression coefficient (R^2_{adj}= 0.95, 0.93).

Xylanase activity (U/mL) = 89.18*A+80.18*B+1408.6*C-3.97*AB-2716.56*AC-2693.27*BC+3926.94*ABC+269.98*AB(A-B)+1683.22*AC(A-C) +1.798*BC(B-C) **Eq.(1)**

Cellulase activity (U/mL) = 0.52*A+0.43*B+10.87*C-0.11*AB-21.73*AC-21.4734*BC+30.88*ABC+1.88*AB(A-B)+11.89*AC(A-C) + 13.45*BC(B-C). **Eq.(2)**

Figure 5 – Contour plots with the responses generated by the interactions of the A= sugarcane straw (w/w); B= citrus pulp (w/w); C= wheat bran (w/w), in the enzymatic activity. A) Xylanase activity and B) Cellulase activity, produced by SmF of *T. reesei* QM9414 using SS as substrate (28 ºC, pH 4.5, 180 rpm).

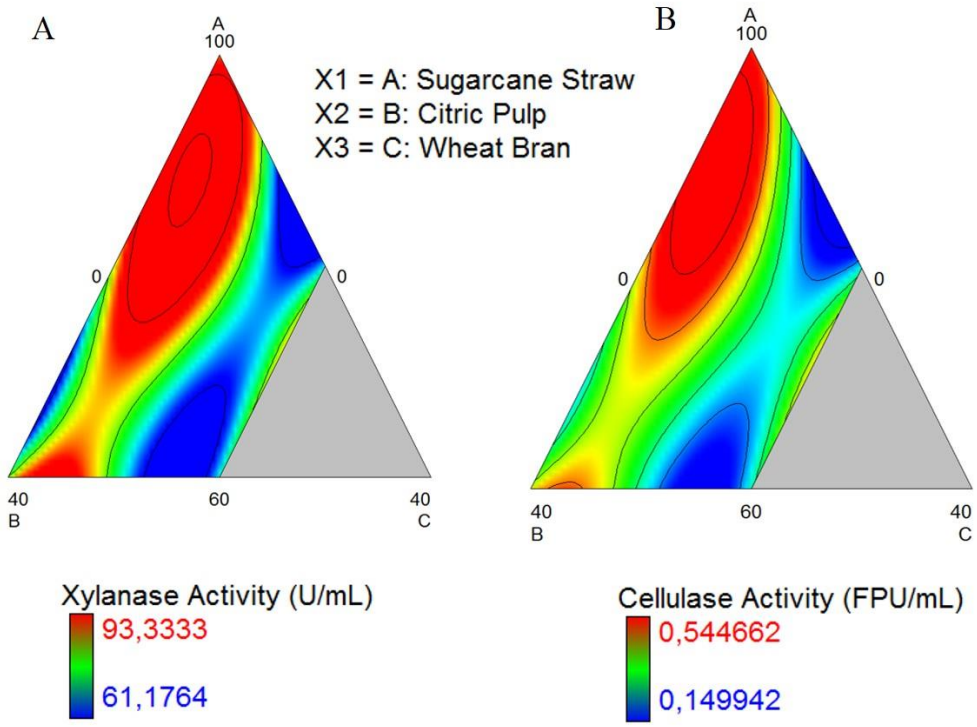
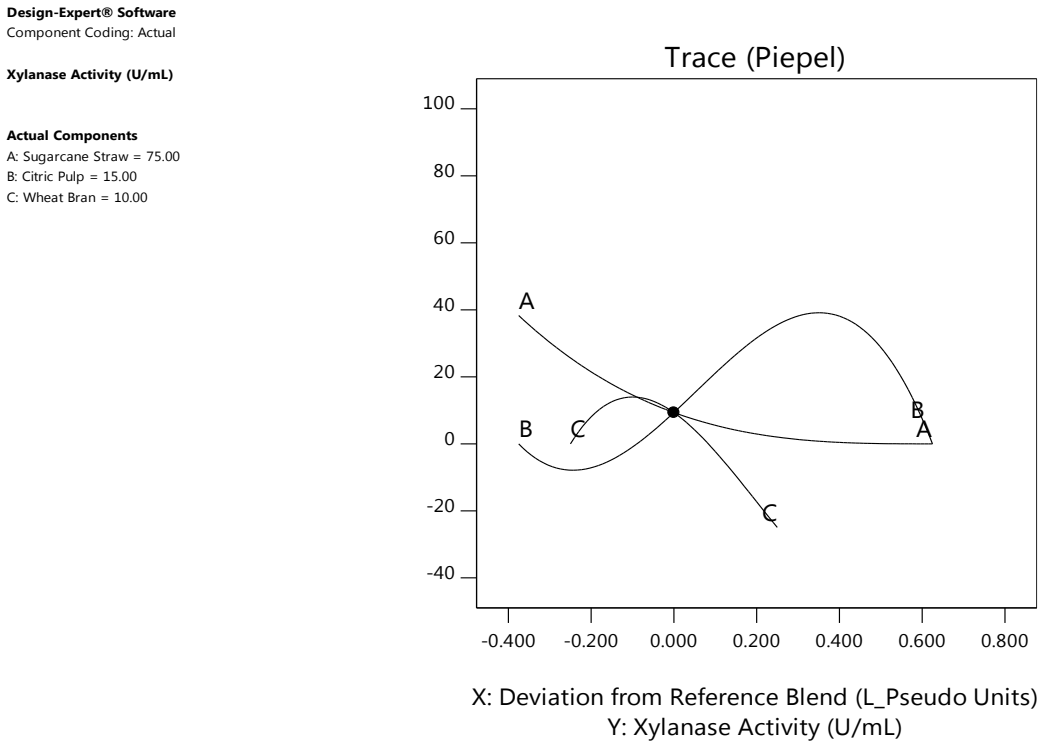
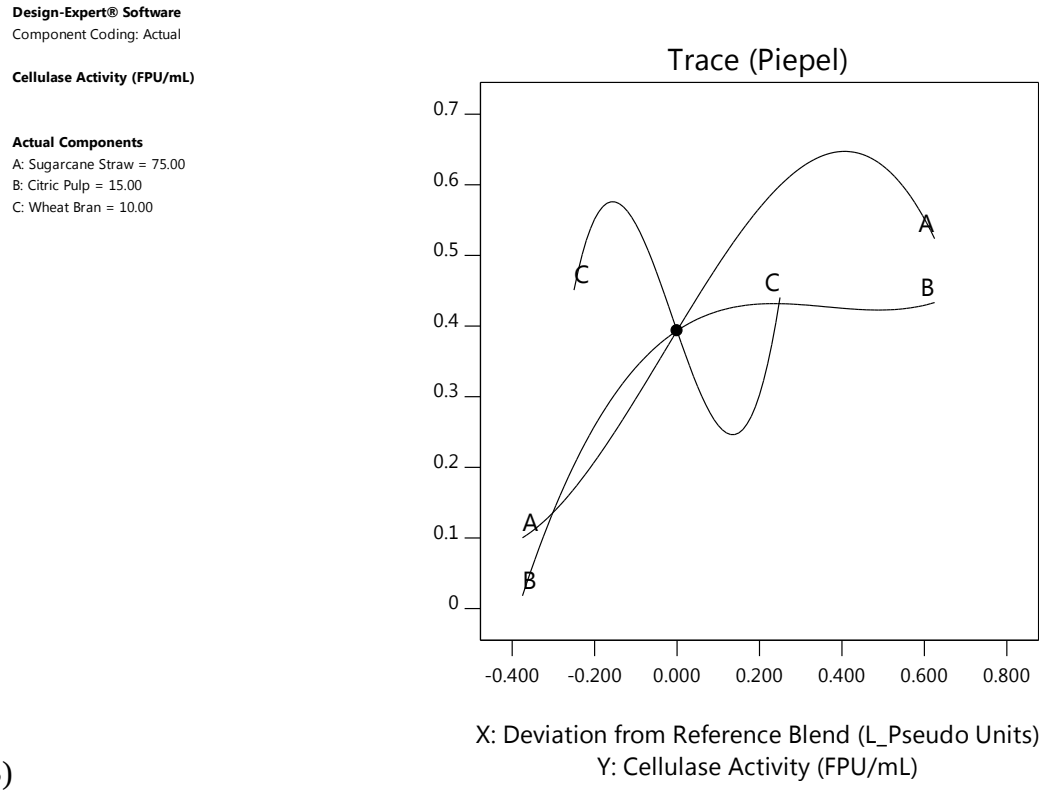


Figure 6 - Perturbation graphs associated to the polynomial models adjusted for describing xylanase (A) and cellulase (B) activity from sugarcane straw, citrus pulp and wheat straw when fermented in mixtures by *Trichoderma reesei* QM9414. A)





B)

Considering the final purpose of use the crude enzymatic extract rich in xylanases and poor in cellulases, an important characteristic to biobleach kraft pulp [48; 51] the optimization was adjusted on the software to reach a maximum of xylanases, and the minimum cellulases production. The optimal set of factors to maximize xylanase production, 100% SS, was observed experimentally at the same combination of factors for xylanase production by *T. reesei* SS fermentation. And the optimal factor values defined by desirability function were the same 100% SS. The most significant results are achieved with 100% (m/m) of SS with desirability predicted for the model was 0.92 respectively. This resulted was not validated once is the same condition that the control experiment with (100% of SS). The predicted result from the desirability function was 89.2 U/mL and the result obtained, 90.6 Um/mL, present no significant difference (Anova+Tuckey, $p>0.05$). The crude extract in this condition was rich in xylanases and poor in cellulases, a ratio of 1:0.005 U/mL, respectively.

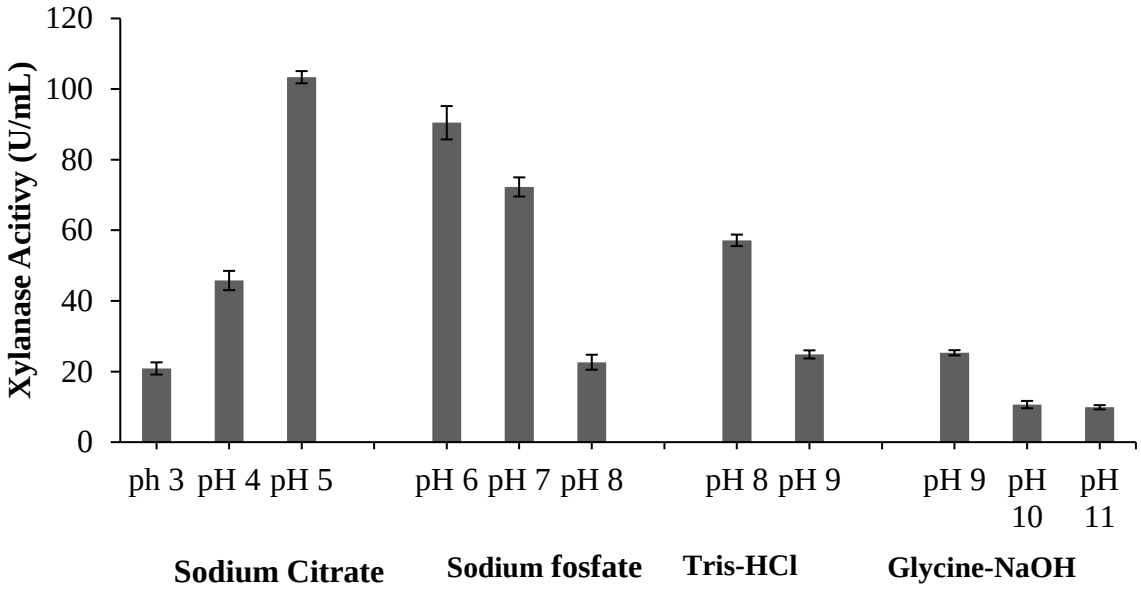
After the selection of microorganism and agroindustrial wastes used as carbon source, the enzymatic production was scaled up in bioreactor using 1.5 L and controlled conditions, resulting in 88.02±4.54 U/mL and 0.41±<0.1 FPU/mL, for xylanase and cellulase respectively, proving that xylanase production from sugarcane straw and *T. reesei* QM9414 can be used in industrial scale without losses. On the other hand, the enzyme production losses were detected in scaling-up of *T. harzianum* P49P11in SmF using sugarcane bagasse in the same stirred tank bioreactor [52].

3.3 Xylanases biochemical characterization

The activity of xylanase from different sources shows distinctive characteristics. In particular, as the active protein, they are sensitive to temperature, pH, ion concentration etc. Proteins have complex structures that are important to their function. This structure has numerous levels including primary, secondary, tertiary, and quaternary. The last two were related to the enzymes function. When this structure is changed or altered, the protein is unable to carry out its specific function, for example by denaturation. Besides that, the movement of electrons and protons is so important to enzyme activity, enzymes are very sensitive to the pH of reaction solution. The pH dependence is usually due to the side groups of the amino acids and a change in pH changes the protonation pattern and can, in extreme cases, result in protein denaturation [53; 54].

The enzymatic extract produced by *T. reesei* QM9414 cultivated in SS medium (12 culture days) showed high activity in pH 5 (100 U/ml) (Fig. 7). In this optimum pH the low range (pH 3-4) and basic pH (pH 8-11) strongly decreased the enzymatic activity. In spite of this, when basic pH is desired, is possible to choose to work with Tris-HCl buffer than sodium phosphate, due to the higher activity in the same pH 8, respectively 65 and 20 U/mL. Xylanase residual activities linearly decreased after the incubation time (20, 40 and 60 min) for all pH ranges (Fig. 8). The loss of activity varied from 20-95% in contrast to the control, and a higher loss was verified at pH 8, after 60 min of incubation. In the range of pH 5-6, the enzyme remained with 80% active after all incubation times tested. Xylanases from other *Trichoderma* species also have been found by some authors with optimum pH 5-6, but with broader pH ranges (Tab. 4).

Figure 7 - Effect of pH on Xylanase activity from the crude extract produced by *T. reesei* QM9414 cultivated with SS (pH 4.5, 28 ºC, 288 h)*.

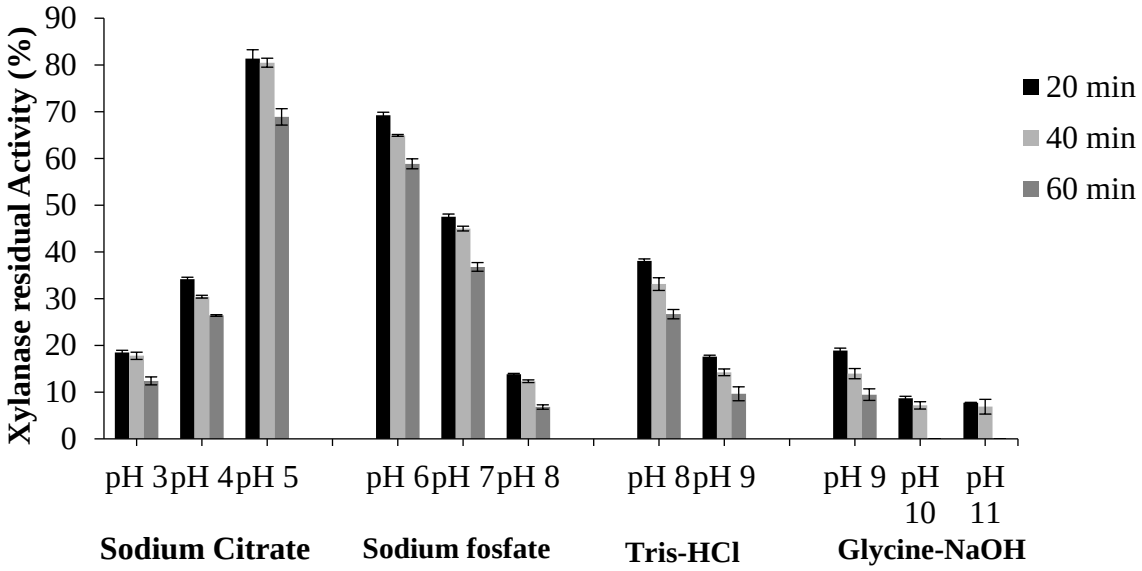


*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Table 4 - Comparative xylanase characteristics produced by different *Trichoderma* species.

Microorganism	Optimum pH	Stability range pH	Optimum temperature (ºC)	Reference
T. reesei QM9414	**5.0**	**5.0-6.0**	**50**	**This work**
T. inhamatum	5.5	4.0-11.0	50	41
T. inhamatum	Xyl I: 5-5.5 Xyl II: 5	Xyl I: 4.5-6.5 Xyl II: 5.0	50 (both)	55
Trichoderma sp SC9	6.0	3.5-9.0	42.5	56
T. harzianum 1073 D3	5.0	3.0-7.0	60	57
T. reesei	6.0	3.0-8.0		58

Figure 8 - Effect of pH on Xylanase activity stability from the crude extract produced by *T. reesei* QM9414 cultivated with SS (pH 4.5, 28 ºC, 288 h)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

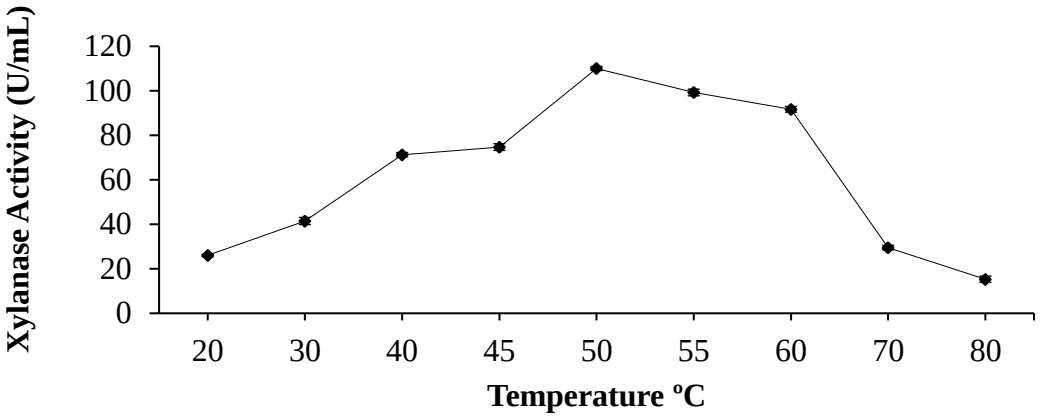
To the optimum pH founded (pH 5) two xylanase applications can be proposed. Zhang et al [30] proposed the use of xylanases as an additive in bird fed, due to the pH range used in this food is 5.5-6.5. In addition, the use of xylanases that operating in the conditions described in this work, for pH and temperature, can be used industrially in juice mills [59; 60], bleaching of kraft pulp [61] and in bioethanol from fiber [62].

The xylanase optimum activity was also observed at 50 ºC (Fig.9). This temperature is commonly reported in papers to *Trichoderma SY and T. inhamatum* (Tab. 4) beyond microorganisms from other genera: *Bacillus* BD-23 [63]; *Paenibacillus macquariensis* [64]; *Penicillium janczewskii* (Terrasan et al., 2013); *Bacillus pumilus* SV-85S [51].

The Fig. 10 depicts the thermostability. In temperatures of 20-30 ºC, and after 20, 40 and 60 min, the enzyme maintains almost 80% of its activity. On the other hand, in temperatures higher than 50 ºC, a linear decrease in enzymatic activity was observed, except in the point that represents the incubation at 50 ºC for 20 min, in which the activity just improves a little and after decrease again. The best indication is that the xylanase enzyme produced by *T. reesei* QM9414 using SS as substrate be used in 50º C incubation temperatures and short times. The low stability of xylanase by another microorganism from *Trichoderma* genus was also observed in literature. The thermostability of *T. inhamatum* xylanase presented a half-life of 2.2 h at 40 ºC, and subsequently when the temperature reaches 50 ºC this time dropped drastically to 2 min [42]. In another work, the stability of *T. reesei* RUT C-30 xylanase was 94% in 50 ºC after 30 min of incubation [58].

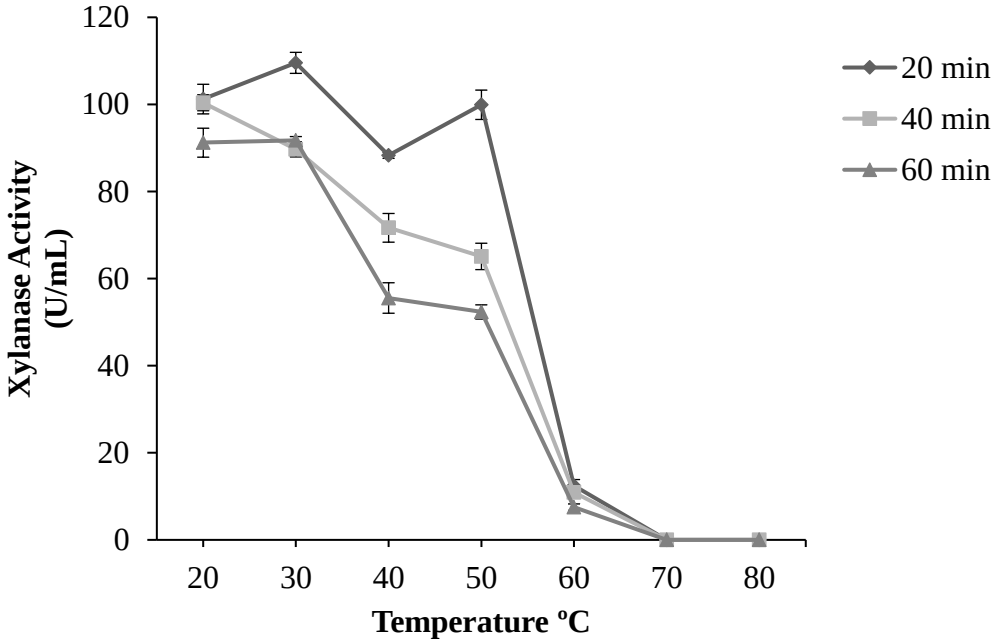
The thermostability loss from xylanase from *Trichoderma* genus in temperatures higher than 50 ºC can be explained by a conformational structure change [65], as well as the loss of secondary structure at 58.8 ºC and tertiary one in 56.3 ºC, reflecting in activity decrease [66]. Some treatments in xylanases can be applied to solve the thermostability loss, such as polyhydroxylic co-solvents addiction [67] and mutations in bisulfide bounds [56].

Figure 9 - Effect of temperature on the activity of xylanase produced from *T. reesei* QM9414 (pH 4.5, 28 ºC, 288 h)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Figure 10 - Effect of temperature in xylanase, produced by *T. reesei* QM9414 (28 ºC, 288 h), thermostability*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

The activation or inhibition of xylanases, using ions and EDTA, were tested and considering the two ions solution concentrations, 5 and 10 mM. When the Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions were added, there was an increase in the enzymatic activity (Table 5). The most expressive result was with Mn^{2+}, 39 and 49%, for the respective concentrations. The presence of Mn^{2+} and Zn^{2+} also increased xylanase activity produced by *T. harzianum* 1073 D3, whereas in the presence of Mg^{2+} and Cu^{2+} an activity was not affected [57]. 10 mM of the ions Cu^{2+} and Ag^{1+} resulted in a strong inhibition of xylanase, 21 and 18% respectively. According to Blanco et al. (1995) [63] Mn^{2+} and Cu^{2+} did not affect the xylanases, while Mg^{2+} had a stimulatory effect on this. The Mn^{2+} also stimulated the enzymatic activity for xylanases from *Paenibacillus macquariensis* [51], as well as for recombinant *Penicillium oxalicum GZ-2* xylanases [58]. In this last work Cu^{2+} and Fe^{3+} caused inhibition of the enzymatic activity, whereas Mn^{2+} and Mg^{2+} presented no difference to the control. EDTA caused a small decrease in the enzymatic activity of xylanase at concentrations of 5 and 10 mM, 10 and 0.8%, respectively (Table 5). The explanation of the authors [59] for this fact is that an enzyme needs divalent ions for a catalysis. In other works, EDTA caused inhibition of the enzymatic activity of xylanases in the concentrations of 1 [58], 2 and 10 mM [59].

Table 5 – Ions and EDTA effect on xylanase activity produced by *T. reesei* QM9414 in SS medium.

	Xylanase Activity (%)	
	5 mM	**10 mM**
Cu^{2+}	106.2	78.7
Mg^{2+}	106.5	108.7
Mn^{2+}	138.8	148.7
Zn^{2+}	104.4	111.9
Fe^{3+}	89.8	93.6
Ag^{1+}	98.9	82.0
EDTA	89.9	99.2

Obs: Culture parameters: pH 4.5, 28 ºC, 288 h

4. CONCLUSION

Sugarcane straw (SS) is an important solid residue from sugar and bioethanol industry which can be used as the main carbon source in an axenic submerged culture of *T. reesei* QM 9414 to produce fibrolytic enzymes, mainly xylanases. The mixed culture of *T. reesei* QM 9414 with *A. fumigatus* M51 and/or *T. harzianum* FS09 did not increase the enzymes production when compared with the axenic culture. The mixture of SS with other agroindustrial residues (citrus pulp and/or wheat bran) in the culture medium did not show any advantage to improve the fibrolytic enzymes production. The production can be scaled-up from 50 mL in shaker flaks to 1.5 L in a 3L bioreactor, maintaining the same temperature and medium, without loss of enzyme production. The enzyme is thermostable in pH 5-6 and 50 ºC, interesting conditions for some industrial applications. Besides this, Cu^{+2} and Ag^{+1} results in a strong inhibition of xylanase, while Zn^{2+}, Mg^{2+} and mainly Mn^{2+} increase this enzyme activity in both concentration used.

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CHAPTER 3- Biobleaching of Kraft pulp using fungal xylanases produced from sugarcane straw and the subsequent decrease of chlorine consumption

Abstract

Xylanase extract, produced by *T. reesei* QM9414 in a culture with Sugarcane Straw (SS) as substrate, was studied in the kraft pulp biobleaching associated with chemical bleaching. The biobleaching parameters optimization showed that 30 U/g of xylanases, in pH 5, at 50 ºC, during 30 min, are the best conditions to bleach the crude kraft pulp (3% of consistency). A decrease of 12.5% of Kappa number was observed at these conditions, as well as the release of 1 g/L of sugar and the presence of chromophores compounds (237 nm). After this xylanase biobleaching the final chlorine dioxide consumption decrease in 10%, maintaining the same brightness as in the control on the subsequent chemical process. Scanning electron micrographs shows the effect on the pulp surface. These results clearly demonstrated that the *T. reesei* QM9414 xylanase is an eco-friendly alternative for paper production.

Key-words: Biobleaching. Fungal xylanase. Kraft pulp. Chlorine dioxide consumption. Scanning Electron Micrographs.

1. INTRODUCTION

Tons of lignocellulosic residues are wasted per year in the world with potential use in renewable fuels, chemical products, and energy [1]. Brazil will reach 646.5 million tons of produced sugarcane in 2017/2018 [2], and, following the São Paulo state legislation, the major sugarcane producer, Law number 11.241 (2002), established that after 2017 80% of sugarcane harvesting should be mechanized, and after 2021 no more burning will be allowed to mechanizable areas. Straw represents around one third of the total primary energy of the sugarcane as a crop and it has very similar characteristics to the bagasse [3; 4].

The amount of sugarcane straw recovered depends on the its variety and sugarcane production yield [5]. According to the reported case, the amount of straw varies from 10 to 18 t/ha (dry basis), and the ratio of straw (db) and stalk (wet basis) is 11-17%. The straw resulted from this process will be a residue that can be left in the soil, ranging from positive impacts such as increasing the macro fauna (mainly worms and ants) to negative impacts such as increases in pest populations [3], or collected to produced energy or co-products such as xylanolytic enzymes [6] or fermentable sugars [7; 8]. In this sense, agroindustrial residues used to produce enzymes are important to the bioprocess, since this substrate has a low cost [9; 10; 11]. In addition, filamentous fungi, bacteria and yeast, could be used to produce complete xylanoytic enzyme systems [9]. The ability of these microorganisms to produce extracellular enzymes is the main industrial interest. However, different species of *Trichoderma genera* are potential producers of xylanases: *T. inhamatum* [12] *Trichoderma sp.* SY [13], *Trichoderma sp*. SC9 [14], *T. harzianum* 1073 D3 [15], and *T. reesei* QM9414 [16].

Pulp and paper mills process huge quantities of woody materials every year. During the paper manufacturing it is important that lignin is removed from all woody plants and agroresidues used as raw materials to obtain a whiter paper, and crystallinity and brightness are some important paper properties [17-19]. The break and subsequent removal of all lignin present are based on the following steps: a) pulping processes in which lignin is removed during the heating process; b) the residual lignin remains blocked on the cellulose fiber surface (mainly linked to short-chains xylan), and is then removed by a multistep bleaching process using large amounts of elemental chlorine (Cl_2) and chlorine dioxide (ClO_2) chemicals [20]; [21]. Recently, studies along the replacement of Cl_2 bleaching with ClO_2 is the better available techniques for organic pollutants, as adsorbable organic halides (AOX), reduction in non-wood pulp and paper mills, considering the lower environmental pollution compared to Cl_2 bleaching and the superior bleaching effect [22- 24]. The difficulty to remove lignin from the cellulose fibers consists

in that xylan forms a complex with lignin named lignin-carbohydrate complex, acting as a physical barrier that prevents the bleaching agent's action on the lignin [19; 25]. Products generated from the use of these chemicals are chlorinated organic substances, some of which are toxic, mutagenic, persistent, and bioaccumulating, cause numerous harmful disturbances in biological systems [21]. Due to these effluent-based problems, legislative and environmental pressures have suggested that these mills should change some parameters in bleaching technologies, making it greener [25; 26]. Because of these interventions, some modifications, and/or capital investments should be done. A biotechnological route of pulps bleaching using xylanases is one suitable alternative to be used in the pulp and paper industry to reduce and/or eliminate the use of chlorine and chlorine dioxide, and other chemical bleaching chemicals, as well the operational costs related to water, electricity, and fuels [18; 19; 21; 26].

As hemicellulose is easier to depolymerize than lignin, biobleaching of pulp appears to be more effective with the use of xylanases than with lignin-degrading enzymes. This is due to the fact that the removal of even a small portion of the hemicellulose could be sufficient to open up the polymer, which facilitates removal of the residual lignin by mild oxidants [27]. A moderate dosage of xylanase prior to alkali extraction can thus be used to make the hemicellulose removal easier while simultaneously protecting the native structure of cellulose [28]. Other factors are also reported to affect the biobleaching process using enzymes, such as: the physical pulp characteristics, enzymatic extract, and cooking process [25; 29].

The xylanolytic enzyme system that carries out the xylan hydrolysis is normally composed of a repertoire of hydrolytic enzymes, including endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72). All of these enzymes act cooperatively to convert xylan into its constituent sugars [27; 30].

Thus, the present paper was designed to achieve the following objectives: utilization of agricultural wastes/by-products for xylanase production; application of xylanase produced by *Trichoderma reesei* QM9414 in biobleaching of kraft pulp, evaluation of chemicals decreases in bleaching process, and some characteristics of paper production by previous biobleaching. Keeping in view the important chemicals decrease, economy and the role of xylanases in pulp and paper industry, the efficiency of friendly-environment cellulose pulp bleaching was evaluated in this work based in a biotechnological process using fungal enzymes produced by waste.

2. MATERIAL AND METHODS

2.1 Microorganism

The microorganism *Trichoderma reesei* QM9414 was obtained from Tropical Culture Collection (CCT), André Tosello Foundation, Campinas, SP, Brazil, and it was cultivated on Potato Dextrose Agar (PDA) medium and stored at 4 ºC.

2.2 Kraft Pulp

The pulp from Eucalyptus was donated by one cellulose mill, LWARCEL Cellulose, in São Paulo State (Brazil). The sample showed 70% humidity.

2.3 Analytical methods

2.3.1 Xylanase production

The strain *T. reesei* QM9414 was cultivated in Erlenmeyer flasks (250 mL) by submerged fermentation (SmF) in a formulated culture medium containing 80 mL medium (m/v): 3.0% sugarcane straw previously autoclaved at 121 ºC, 15 min, 1 atm, 14 mesh), 0.1% $(NH_4)_2SO_4$, 0.0017% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.0028% $ZnSO_4$, 0.1% $NH_4H_2PO_4$,0.06% KCl, 0.1% yeast extract and 0.1% sucrose at pH 4.5 [31]. The culture medium was inoculated with 10^6 spores/mL counted by microscopy in a Neubauer chamber. The flasks were incubated at 28 ºC, in an orbital shaker (Tecnal mod. 421, Piracicaba, Brazil) at 180 rpm for 144 h. The biomass was separated by centrifugation in 15 min, 4 ºC and 2900 x *g*. The liquid fraction was used as a raw extract of xylanase enzymes.

2.3.2 Enzyme assay

Endoxylanase activity was assayed at 50 °C in a reaction with 0.1 mL raw extracts and 0.65 mL of 0.5% (m/v) xylan Birchwood solution (Sigma-Aldrich) in 250 mM sodium acetate buffer, at pH 5 [32]. The amount of reducing sugar was quantified by the dinitrosalicylic acid (DNS) method [33]. One unit (U) of xylanase activity was defined as the amount of enzyme to release 1 μmol of reducing sugar per minute per mL of reaction.

2.3.3 Biobleaching of kraft pulp

Xylanase produced by *T. reesei* QM9414 under submerged fermentation was used for pre-biobleaching process of kraft pulp as well as to evaluate its potential use as biobleaching agent.

Pulp samples were incubated in a plastic bag with sodium acetate buffer, pH 5.0, in a thermostatic bath at 50 ºC for 10 min (soaking stage). Treatment was started by diluting the enzyme in the same buffer (pre-heated at 50 ºC) to reach the concentration to be used in the experiments and then adding the solution to pulp in sealed plastic polyethylene bags. The final pulp content in the reaction mixture was 3%. Controls were performed in a similar manner but with water substituted by enzyme.

2.3.4 Optimization of biobleaching

Three enzyme dosages were used to perform the pulp enzymatic hydrolysis, 10, 30 and 50 U/g of pulp. Each experiment was performed in triplicate in five different times 15, 30, 60, 120 and 360 min. The pulps were periodically mixed by kneading the bags during 30 seg.

After the enzymatic hydrolysis period, the bags were boiled at 100 ºC for 5 min to disable the enzymes, cooled, and then the pulp was filtered on a Buchner funnel and washed thoroughly with distilled water. An aliquot of the filtrate separated from the enzymatic pretreated pulp was reserved for further analyses. The sheets formed during the filtration were used to perform the physical (brightness and yellowness) and chemicals analyses (HexA and Kappa number). Tests were made in triplicate and the difference between the treatments was evaluated by Anova and Dunnet´s Test (confidence $p<0.05$) (GraphPad Prism 5).

2.3.5 Release of chromophores compounds and sugar

Chromophores released from the biobleaching treatment, present in the liquid phase, were qualitatively analyzed at 237 and 280 nm in a Bel Photonics UV-M51 spectrophotometer. The amount of reducing sugar present in this filtrate was quantified by the DNS method [33].

The control of sugar content was performed to discount the amount present previously to hydrolysis process.

2.3.6 Hexenuronic acid content of chemical pulp

The content of hexenuronic acid (HexA) in the pulp was determined using the spectrophotometric method reported by Tappi T 282 pm-07 [34]. The pulp was hydrolyzed using a sodium acetate and mercury chloride solution at 70 ºC for 30 min. Absorbance was measured at 260 nm and 290 nm. The HexA content was calculated according to the Eq. 1:

$$C_{Hexa}\left(\mu\frac{mol}{g}\right) = 0.287 \times \frac{(A_{260} - 1.2A_{290}).V\ (mL)}{w\ (g)} \quad Eq.(1)$$

where C_{HexA} is the HexA molar fraction in the pulp expressed as (µmol HexA /g dry pulp); A_{260} and A_{290} are absorbance values determined at 260 and 290 nm, respectively; V is hydrolysis solution volume in mL, and *w* is the dry mass of pulp (g); 0.287 is a calibration constant obtained using a standard pulp; 1.2 is the ratio between lignin absorption at 260 and 290 nm that is used to correct lignin absorption on HexA determination.

2.3.7 Physical and chemical characterization of kraft pulps

The prepared sheets were evaluated for some properties following the ISO (International Standards Organization) protocols. All the measurements were conducted in triplicate, with their averages and Standard Derivation (SD) calculated. Kappa number of pulps was measured according to ISO 302:2015. Kappa number is defined as the amount (mL) of 0.1 N $KMnO_4$ solution consumed by 1g moisture-free pulp under the specified conditions. The brightness and yellowness were determined by ISO 2470-1: 2008 and ISO 17223:2014, respectively, in the testing machine (Color Touch PC, Technidyne, USA). The Equation 2 shows the brightness increase.

$$Brightness\ increase = B(\%ISO)_T - B(\%ISO)_{NT} \quad \text{Eq. (2)}$$

Where, “T” represents the brightness in the pulp chemically bleached after an enzyme pretreatment, and “NT” is the control of bleached pulp.

2.3.8 Chemical bleaching

The bleaching process was performed according to A/Do methodology, where A means the acid step and Do is the subsequent followed step using chlorine dioxide. Pulps pre-bleached with enzymes (30 U/g at 15, 30 and 60 min) at 10% consistency had the pH adjusted to 2.8-3.0 with sulfuric acid 2M and allocated in sealed propylene bags for 95 min in a thermostatic bath at 90 ºC. The sequent treatment was performed with addition of 4.5 kg ClO_2/ton of pulp in the bags. The bags were sealed again and maintained for 15 min in a bath at 85 ºC. Pulp was filtered on a Buchner funnel and washed thoroughly with distillated water. The sheets formed during the filtration were used to perform the physical and chemicals analyses.

2.3.9 Scanning electron micrograph (SEM)

For the Scanning Electron Microscope (SEM) the samples were coated with gold (30 nm) using a sputter coater (Quorum equipment model Q 150R ES), and observed under a scanning electron microscope (Zeiss-EVO LS15 microscope -Carl Zeiss Ltd), operating in extended pressure (EP) and constant temperature mode.

3. RESULTS AND DISCUSSION

3.1 Enzyme production

The crude extract, obtained after 6 days of submerged fermentation, presented 60 U/mL of xylanase activity and 0.3 FPU/mL of cellulase activity. Cellulase-free xylanase hydrolysis of unbleached pulp have been related as necessary for the maximum pentosans removal and to preserve the physical properties of the pulp [17-19; 25]. However, in the produced xylanases, the cellulase activity was considered low, since the ratio between activity units of xylanases and cellulases was 200 to 1, respectively.

3.2 Optimization of pulp biobleaching using xylanases

The efficiency of xylanase enzyme produced using sugarcane straw as substrate, by *T. reesei* QM9414, was observed in all applied treatments in kraft pulp, which was also confirmed by sugar and chromophores compounds release, Kappa number and HexA reduction (Table 1). These parameters were compared to the control, which was not treated with the fungal xylanase.

Table 1 - Different treatments for bio-bleaching kraft pulp using xylanase by *T. reesei* QM 9414*.

	HexA (µmol/g)	**Brightness (% ISO)**	**Yellowness**	**Sugar released (g/L)**	**237 nm**	**280 nm**	**Kappa number**
10 U/g							
15 min	47.2±1.4 ^{ns}	32.9±0.3*	14.1±0.2	0	0.3	0.1	21.9±1.5^{ns}
30 min	40.8±2.8*	33.8±0.3^{ns}	14.3±0.1	0.1±0	0.3	0.1	21.1±0.9^{ns}
60 min	38.9±2.9*	33.0±0.3*	14.2±0.1	0.1±0	0.3	0.1	22.9±1.6^{ns}
160 min	42.0±0.7*	33.4±0.2^{ns}	14.3±0.1	0.2±0	0.3	0.1	22.6±1.3^{ns}
360 min	39.7±1.9*	32.8±0.1*	14.3±0.2	0.4±0.1	0.3	0.1	20.6±0.5^{ns}
30 U/g							
15 min	39.8±1.7*	32.9±0.4*	14.2±0.1	0.6±0.2	0.4	0.1	20.3±0.4^{ns}
30 min	40.0±1.9*	33.7±0.4^{ns}	14.3±0.2	1.0±0.1	0.5	0.1	19.6±0.5*
60 min	40.2±2.3*	31.6±0.3*	14.6±0.2	1.1±0.1	0.5	0.2	20.0±0.3^{ns}
160 min	37.3±2.3*	33.4±0.7^{ns}	15.0±0.4	1.4±0.1	0.5	0.1	20.5±0.3^{ns}
360 min	38.7±1.9*	32.9±0.4*	14.5±0.1	1.5±0.2	0.5	0.1	21.2±0.8^{ns}
50 U/g							
15 min	43.3±0.4*	32.5±0.3*	14.2±0.1	0.4±0	0.5	0.2	21.1±0.8^{ns}
30 min	42.8±2.0*	33.4±0.5^{ns}	14.2±0.2	0.6±0	0.6	0.2	21.1±0.6^{ns}
60 min	42.1±1.0*	33.1±0*	14.9±0.2	0.7±0	0.5	0.2	22.0±0.7^{ns}
160 min	40.7±2.0*	33.2±0.4*	15.0±0.6	1.3±0.1	0.6	0.2	21.9±0.7^{ns}
360 min	39.9±0.6*	32.0±0.7*	14.8±0.5	2.0±0.2	0.6	0.2	21.2±0.8^{ns}
Control	48.9±0.3	34.5±0.3	15.4±0.1	0.1±0	0.1	0.0	22.4±0.7

*The results are related with the average and standard deviation of three experiments.

The optimization of enzyme dosage and time of enzyme reaction of kraft pulp biobleaching process using xylanases showed that there was no significant difference ($p<0.05$) between the treatments performed and the control, regarding the Kappa number, except to the treatment performed with 30 U/g of enzyme dosage and 30 min of hydrolysis time (Table 1). This reported treatment showed 12.5% of Kappa number reduction. High enzymatic dosages, however, did not help turn the pulp more bleached, based on the kappa number assay, showing that the use of an appropriated enzyme dosage in biobleaching saves time and enzyme extract (Table 1). In addition, Salles and co-workers [35] reported that xylanase pretreatment of kraft pulp at higher enzyme dose are not interesting because it decreases the bonding of the pulp fiber.

In fact, the reduction of kappa number by xylanases of other microorganisms was also verified in low dosages. Battan and co-workers [18] using 5 IU/g xylanase from *Bacillus pumilus* ASH reduced the Kappa number in 16% of kraft pulp, and Guimarães [36] reduced kappa number in 25 and 36% using 10 U/g of xylanases from *A. niger* and *A. flavus*, respectively. The enzyme dosage uncovered that 30 U/g xylanase was reported as the most appropriated dosage to pre-bleaching kraft pulp and ensure the pulp quality [37]. Low dosages of xylanases are also important due to its effect in the pulp strength [19; 38]. Actually, the ability of xylanase in hydrolyzing the xylan chains deposited on the surface of the fiber is known as boosting effect of xylanase on biobleaching process [17].

The enzymatic hydrolysis time showed that long periods of enzyme treatment tested, 60-120-360 h, do not increase the brightness nor reduce the kappa number, important parameters to obtain a final chlorine reduction. When an enzyme loading of 50 U/g was performed at 15 min and 6 h of hydrolysis, an increase of 80% in the release of reducing sugar was observed. Under the same conditions (time) but using 30 U/g, the increase was about 57%. To the enzyme concentration of 10 U/g, an improvement in sugar liberation of 57% occurred between 120 and 360 min of hydrolysis. The remaining hydrolysis time tested, 60, 30 and 15 min, presented less or no sugar released than the hydrolysis with long time.

Enzyme dosage was related to the concentration of sugar, proving the enzymatic activity and also to the chromophores compounds release (237 nm) in a directly proportional ratio (enzyme dosage x media of these data in all times), r^2=0.9854 and r^2=0.953 (237 nm), respectively. The reduced sugar concentration also had a directly proportional increase to time, resulting in a r^2= 0.9418; 0.6106 and 0.9339 for 10, 30 and 50 U/g, respectively. However, long periods of enzyme treatment (1-6 h) did not modify neither the brightness nor the kappa number and HexA, important parameters to obtain a final chlorine reduction. Therefore, the pre-

bleaching of kraft pulp is interesting just for a specific time (Table 1). In this case 30 min was enough.

The presence of HexA in the control pulp, 49 µmol/g, is due to the pulp cooking that forms these unsaturated compounds as a result of methanol elimination from 4-O-methylglucuronic acid groups bonded to side groups xylans [39]. HexA compounds contributed to kappa number as well as to consume a portion of bleaching reagents [40].

All the treatments performed with xylanase enzyme from *T. reesei* QM 9414 showed a significant decrease of HexA content compared to the control. Regarding to the treatment using 30 U/g and 30 min of hydrolysis, the HexA reduction reached 18%. Sharma and co-workers [41] in their work reported a reduction of almost half of the HexA present in the kraft pulp using 25 UI/g xylanase from *Bacillus subtilis* during 2 h. Andreu and Vidal [42] removed 21% of HexA of kneaf pulp using 3 U/g of pulp and commercial xylanase Pulpzyme® HC, in 2 h of hydrolysis. The HexA content is reduced after xylanase pretreatment, consequently the absorbable organic halides (AOX) are also reduced after chlorine dioxide bleaching [23];[41].

All the treatments showed chromophores release at 237 and 280 nm, however, the time does not influence the liberation among the enzyme loads tested, since the values do not represent a directly proportion ratio. This result is contrary to the findings by Salles [35] using xylanases enzymes by *Acrophialophora nainiana* and *Humicola grisea* var. *thermoidea* to biobleached kraft pulp, where prolonged time of incubation and higher doses of enzyme lead to an increase in chromophore release and increased absorbance at 237 nm attributed to lignin depolymerisation.

The xylanase action on the reduction of the Kappa number and HexA, besides the liberation of chromophores compounds and sugars were also observed by some works [18; 19]. The increase of chromophores compounds release and not in the Kappa number can be explained by the sugar degradation, which contribute also to the color formation [43]. These last authors and also Guimarães and co-workers [36], showed that the material released during enzyme treatment absorbs strongly at 205 and 237 nm. During the time optimization, an increase was observed on sugar and chromophores compounds liberation of 70 and 20%, respectively, when the hydrolysis time increased from 1h-5h [44]. Biobleaching using xylanases of *A. niger* also showed chromophores compounds liberation at 237 nm [45].

The brightness is also observed after the treatments. There are some differences in the brightness %ISO between the control and the treatments related to the brightness in Table 1; some treatments were non-statistically significant showing a little decrease in bio-bleaching

with tested xylanase. In the treatments using 50 and 30 U/g, in hydrolysis times of 360 and 60 min was found a decrease of 2.5 and 2.9 %ISO, respectively. These results were not expected since that other authors such as [19], reported an increase in brightness of 1.9 %ISO using xylanase from *Bacillus pumilus* SV-85S, 5 %ISO for xylanases by *Bacillus* ASH [18], and 3.1 %ISO for *Aspergillus niger* [45], during the biobleaching kraft pulp.

The importance of the application of crude xylanase extract in biobleaching of kraft pulp is because the further sequences of chemical bleaching are more eco-friendly and cheaper [41]. In fact, the reduction of chlorine compounds in bleaching process is the important consequence of biobleaching resulted by the enzymatic hydrolysis using fungal xylanases.

Considering that the Kappa number is an important parameter to be considered in the next steps, the essay with 30 U/g and 30 min was the most effective treatment to receive the chemical bleaching due to its better result compared to the control. The brightness was not so affected (only 0.8 %ISO of difference). Treatments with longer (1h) and shorter (15min) hydrolysis time than the standard (30 min) were also considered to receive the chemical bleaching. From these results, this enzymatic biobleaching sets were used to receive the chemical bleaching and to make the further analysis.

3.3 Chemical bleaching

In all essays cited in Table 2, the xylanase treatment is shown to be effective for an increase in brightness after the A/Do treatment. An increase of brightness is expected at the end of the chemical bleaching process, regardless of the different chemical bleaching techniques. The essay with a hydrolysis time of 30 min and 30 U/g showed the highest increase, 4.5 points %ISO on pulp brightness, followed by 15 and 60 min respectively. This hydrolysis time (30 min) was elected as the best condition due to its good results in the whole process and was applied in the subsequent chlorine consumption experiment. The brightness improvement was higher than other authors using different xylanases (Table 2).

The A/Do chemical process was applied decreasing the chlorine concentration in the biobleached kraf pulp, and Table 3 shows that almost 10% of chlorine dioxide is saved using a pre-bleaching treatment with xylanase from *T. reesei* QM9414, 30 U/g for 30 min of hydrolysis, due to the brightness being equal to the control with no enzyme applied.

Table 2 - Final brightness improvement and other comparatives on kraft pulp after bleaching.

Enzyme dosage (U/g)	Time (min)	HexA	Brightness (%ISO)	Brightness increase (%ISO)	Yellowness	Chemical Bleachment System	Reference
30	60	11.6±1.4	59.6±1.6	3.8	12.7 ±0.4	A/D_0	This work
30	30	12.0±1.0	60.3±1.66	4.5	12.9±0.4	A/D_0	This work
30	15	13.8±0.2	60.0±0.9	4.2	13.5±0.3	A/D_0	This work
0	Control	13.9±0.2	55.8±0.9	-	14.0±0.65	A/D_0	This work
10	120	-	83.0	2.4	1.41±0.2	$CDE_1D_1D_2$	[19]
25	300		29.42	4.1	-	Chlorination+H_2O_2	[44]
5	180	-	87.7	0.9	-	$CDED_1D_2$	[18]

Table 3 - Effect of *T. reesei* QM9414 xylanase treatment (30 U/g; 30 min) on chlorine dioxide consumption*.

	Brightness (%ISO)
Control**	55.8±1.87
100% ClO_2	60.3±1.68
90% ClO_2	55.36± 1.55
80% ClO_2	48.68 ±2.3

* The results are related with the average and standard deviation of three experiments.

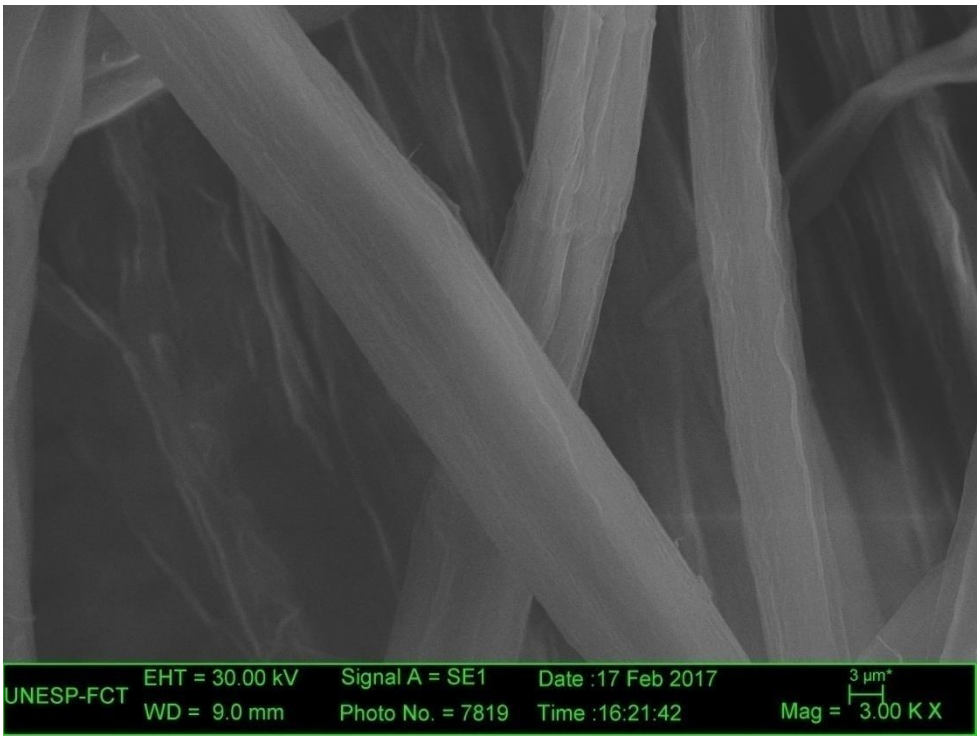
** no-enzymatically treated.

3.4 SEM of enzyme-treated pulp

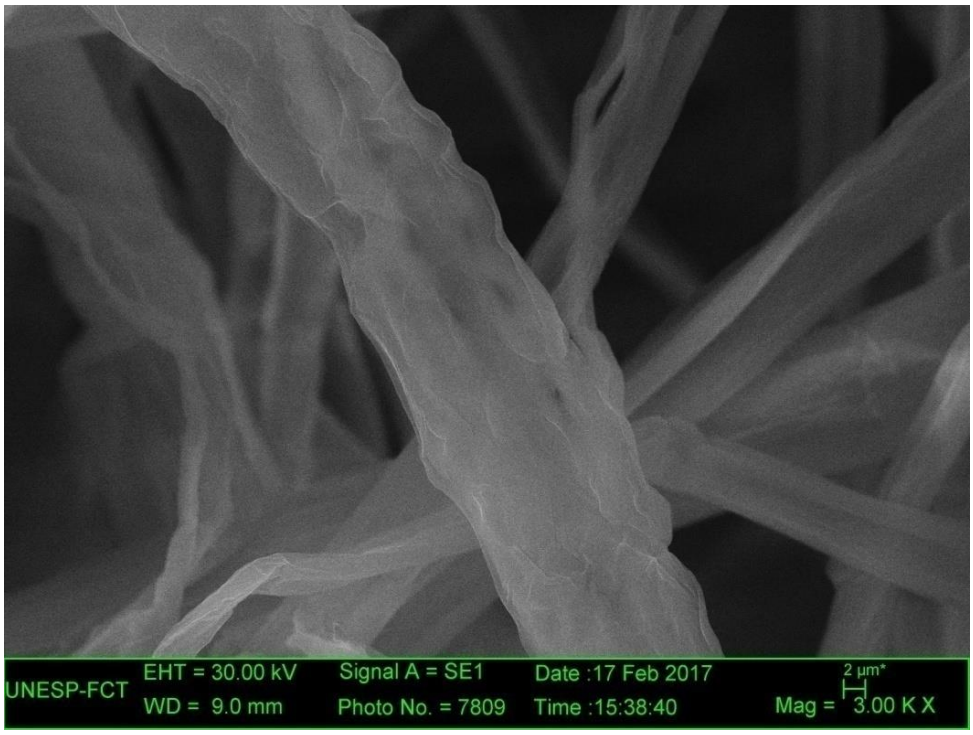
SEM analysis, Figures 1 and 2, clearly revealed morphological changes in the kraft pulp fiber after treatment using crude xylanase. The comparison of SEM images with the control and *T. reesei* QM9414 enzyme-treated samples were less straight and their surface was rough and striated, indicating that they were modified. The raw fiber showed uniform fibers and no sign of external fibrillation.

Figure 1 - A) SEM of crude kraft pulp surface structure, 3000X; B) pretreated kraft pulp surface, using xylanase crude extract from *T. reesei* QM9414 (30 U/g; 30 min).

A)



B)



This result can be related to Viikari [46] since they showed that the enzymatic treatments, based on xylanases and mannanases, introduced modifications in the carbohydrate structures, leading to enhanced delignification. The mechanism is based on the partial depolymerization of hemicelluloses, which block the chemical removal of residual lignin from pulp fibers. The effect of xylanase enzyme on the kraft pulp surface was also founded by other authors [17; 19; 35; 47].

4. CONCLUSIONS

T. reesei QM9414 has been shown to be efficient in xylanase production using SS residue as carbon source. The characteristics of pulp hydrolyzed by xylanases ensures that the enzyme acts on the fiber surface (SEM), releasing sugars and chromophores compounds, decreasing the Kappa number, and showing its potential as "bioagent" in kraft pulp biobleaching process. The enzymatic method can be combined to various types of kraft pulping processes and bleaching sequences. Among the benefits obtained with this fast-biological treatment, using relatively little enzyme obtained from residues we can highlight the decrease of almost 10% in the consumption of chemicals in the traditional bleaching of the pulp, maintaining the brightness of the product.

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CHAPTER 4: Enzymatic and acid hydrolysis of xylan from sugarcane straw and xylooligosaccharides production

Abstract

Sugarcane straw (SS), one of the main residue from sugarcane industry after the crop mechanization, was studied as a viable raw material for bioprocesses. This waste is source of xylan and its extraction and hydrolysis were evaluated for xylooligosaccharides (XOS) production. Subsequently, some tests with probiotic cultures were performed to prove the XOS fermentability. The chemical analysis performed in SS revealed the presence of hemicelluloses (21.3%), cellulose (30.3) and lignin (27.5). The alkaline pretreatment of the SS, with 4% (w/v) NaOH in water solution by autoclavation process in 45 min at 121^{o} C, enabled the xylan extraction (63% of efficiency). Xylan was subsequently hydrolyzed by two routes for XOS production: acid (in boiling water bath at 95 ºC or oil bath at 120 ºC) or enzymatic process (50 ºC). The best results for XOS production from enzymatic (24 h/50 ºC) or acid xylan hydrolysis (0.25 M H_3PO_4/2 h/120 ºC) were respectively 5.34 and 5.94 g/L corresponding to 16 and 17.45% XOS yields. The acid treatment produced furfural which increased with the hydrolysis time and acid concentration. The hydrolysate produced from enzymatic xylan degradation, containing XOS and xylose, was assimilated by *Bifidobacterium longum* BL-05 (BL-05), *B. breve* BB-03 (BB-03), *Lactobacillus brevis* ATCC 367 (LB 367), *L. acidophilus* ATCC 4356, and *Wickerhamomyces onychis*, in axenic cultures. These results showed the SS potential for the production of special biomolecules, as XOS, in an integrated biorefinery, since a great amount of cellulose and lignin also is left over from the process.

Keywords: Fungal xylanase. Xylan. Acid and enzymatic hydrolysis. XOS. Probiotic cultures.

1. INTRODUCTION

Lignocellulosic materials (LCM), also known as agricultural renewable sources, are currently studied for biofuels or other value-add products production, such as healthier foods, mainly due to they represent an important alternative to decrease the environmental pollution caused by the petroleum fuels dependence, using cleaner technologies [1-3]. On the other hand, the demand of food production using functional ingredients is increasing in special to food market uses, and LCM can be used as a sustainable and cheap source for these purposes [4; 5]. For the reasons cited above, technology must advance to improve the use of agricultural and agroindustrial residues to obtain high-value products in the nutritional field, biofuels and others [6].

Sugarcane Straw (SS), an example of LCM residue from sugarcane and bioethanol production, represents around one-third of the total primary energy of the sugarcane crop. The composition of SS is similar to the widely used bagasse and other LCM materials. It is chemically consisted by mainly cellulose, hemicellulose and lignin, respectively 30, 30 and 25% [7- 9]. Currently, this agricultural residue is being accumulated in the soil since the mechanical harvesting was implanted, besides that, this accumulating results in some problems such as insects increase on the field, risk of fire, delay in sprouting, among others [10].

In the most of processes that reuse LCM residues, their isolated components cellulose, hemicellulose, and lignin, were used. The main drawback to turn these processes more available is due to the recalcitrance that these three compounds are found in LCM vegetal cellular wall [1; 3]. There are many types of pretreatments for LCM xylan extraction, as auto-hydrolysis, acid, and alkaline treatments, using chemicals (KOH, NaOH, hydrogen peroxide, H_2SO_4), temperature and pressure [3]. The alkaline treatment has the advantages of better lignin removal and low carbohydrate loss, but some disadvantages are the cost with chemicals, neutralization of pretreated slurry and longtime process to hemicellulose solubilization [1; 11; 12].

One promising LCM component is xylan, a heteropolysaccharide with a backbone formed by xylose subunits, that is the main sugarcane bagasse hemicellulose constituent [1; 13; 14; 15] and also sugarcane straw as cited above. In this way, the xylan from sugarcane bagasse has been recently studied to obtain: Xylooligosaccharides (XOS) [14; 15], enzymes [6], xylitol [16], among others. Until our knowledge, few studies of xylan from sugarcane straw to XOS production are found the literature. These special sugars are formed by oligomers 1,4-linked β-xylose backbone, produced during the xylan hydrolysis, previously extracted from hemicellulose [6; 15].

XOS are not digested by humans since the human body lacks the enzymes required to hydrolyze the β-links, so they are considered prebiotics and soluble fiber because they are not degraded in the stomach and reach the large intestine intact. With the increased interest in new prebiotics, XOS have been studied as a new one, mainly because it can be produced by renewable sources. They are indicated for the consumption due to: are not carcinogenic, have a low calorific value and stimulate the growth of beneficial bacteria in the colon. XOS have been shown to have important prebiotic properties and thus great potential for use in pharmaceutical industry, food and feed [3; 5; 17; 18].

The XOS natural sources can be fruits, vegetable and honey [17], or they can be produced from LCM hemicellulose, by enzymatic or acid hydrolysis [11; 14; 19]. During the acid hydrolysis, the xylan depolymerization is performed randomly by the breakdown of the glycosidic bonds between adjacent xylose units. In the enzymatic route, the breaks occurred generally by endo-β-1, 4-xylanases which degrades xylan by attacking the β-1, 4-bonds between xylose units to produce XOS, and β-xylosidase converts the lower-DP (degree of polymerization) XOS into monomeric xylose. In both treatments, the DP of released XOS are not possible to be controlled, but the enzymatic process did not produce furfural from pentoses degradation [15; 19; 20]. Another important issue in the XOS composition and production is the source since many LCM can be used: birchwood, beechwood, corncob, sugarcane bagasse, brewery's spent grain and coconut husks, soybean waste. In addition, the use of residues can contribute to XOS decrease cost, an important parameter to industrial scale, due to its low price [6; 18; 21-23].

The use of dilute phosphoric acid (H_3PO_4) as a catalyst to biomass hydrolysis has been investigated and compared to strong acids such as sulfuric acid (H_2SO_4). One disadvantage of H_3PO_4 related is the price, almost twice higher when compared to H_2SO_4. On the other hand, some interesting characteristics are related to: the process using H_3PO_4 results in lower sugar losses and less accumulation of furfural compounds in the reaction medium, this acid is less corrosive, and it can act as an additional source of nutrients for microbial growth, particularly as ammonium phosphate source [24-26].

Concerning the most effective xylan hydrolysis to XOS production, the present work evaluated the effect of two different methods, enzymatic and acid hydrolysis, considering the obtained XOS yields on each one. The objective of the present work was to compare these both types of xylan hydrolysis, analyzing its products, and the possible furfural production in these proposed conditions. In addition, the fermentability of the produced XOS was also evaluated

by some probiotic cultures, *Bifidobacterium* and *Lactobacillus* strains, and with the fibrolytic yeast, *Wickerhamomyes onychis* LABI2.

2. MATERIALS AND METHODS

2.1 Microorganisms

The microorganism *Trichoderma reesei* QM9414 was obtained from Tropical Culture Collection (CCT), André Tosello Foundation, Campinas, SP, Brazil, and cultivated on Potato Dextrose Agar (PDA) medium and stored at 4 ºC.

Lactobacillus brevis strains ATCC 367 and *Lactobacillus acidophilus* ATCC 4356 were obtained from Collection of Reference Microorganisms on Health Surveillance (CMRVS-Oswaldo Cruz Foundation - FIOCRUZ, RJ, Brazil). The cultures were maintained in Laboratory of Industrial Biotechnology (LABI - UNESP - Assis/SP) in frozen liquid nitrogen samples (-270 °C) in De Man, Rogosa and Sharpe (MRS) medium, pH 6.0, with 10% glycerol in solution for cell preservation. The microorganisms of the genus *Bifidobacterium* used in this study were: *Bifidobacterium longum* BL-05 (Danisco - Madison, USA) and *Bifidobacterium breve* BB-03 (Danisco - Madison, USA). These strains were kept in the LABI in samples frozen in liquid nitrogen (-270 ° C) in MRS medium more cysteine, pH 6.0 and 10% of glycerol in the solution for cell preservation. The yeast *Wickerhamomyces onychis* LABI2 characterized as *Wickerhamomyes* (*Pichia*) *onychis* that belongs to the Family Phaffomycetaceae (NCBI, 2016), was isolated from rice bran, identified by Shinya [27] and stored in PDA medium at 4 ºC.

2.2 Materials

Sugarcane straw residue was obtained from the crop in the Água Bonita Mill, located in Tarumã city, São Paulo State, Brazil.

Xylooligosaccharides (XOS) standards, xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexose (X6) were obtained from Megazyme, Ireland. Furfural, xylose (X1), glucose, and mannose standards were obtained from Sigma Aldrich.

2.3 Xylanase production

The strain *T. reesei* QM9414 was cultivated in Erlenmeyer flasks (250 mL) by submerged fermentation (SmF) in a formulated culture medium containing 80 mL medium (m/v): 3.0% of grounded sugarcane straw (previously autoclaved at 121 ºC, 15 min, 1 atm, 14 mesh) , 0.1% $(NH_4)_2SO_4$, 0.0017% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.0028% $ZnSO_4$, 0.1% $NH_4H_2PO_4$,0.06% KCl, 0.1% yeast extract and 0.1% sucrose at pH 4.5 [28]. The culture medium was inoculated with 10^6 spores/mL counted by microscopy in a Neubauer chamber. The flasks were incubated at 28 ºC, in an orbital shaker (Tecnal mod. 421, Piracicaba, Brazil) at 180 rpm for 144 h. The biomass was separated by centrifugation in 15 min, 4 ºC and 2900 x *g*. The liquid phase from centrifugation was used as a raw extract of xylanases.

2.4 Arabinoxylan extraction

The arabinoxylan extraction process was performed in triplicate, the sugarcane straw (10% m/v) was previously soaked in 4% (m/v) NaOH solution in a glass vessel (1 L total volume) and heated in an autoclave at 121 ºC, for 45 min. The liquid fraction was filtered with gauze until no solids were observed in the liquid phase. The arabinoxylan in the liquid fraction was precipitated by the mixture 1.8 volume of 98% (v/v) ethanol + 2% (v/v) acetic acid to 0.2 volume of arabinoxylan solution provided by the pretreatment. The precipitated material (arabinoxylan) was centrifuged at 4000 x *g* for 15 min. This material was washed twice using the proportion: 1 volume of ethanol solution (50% of 98% (v/v) ethanol + 50% of EDTA 0.5% (m/v) water solution): 1 volume arabinoxylan, before being dried at 40 ºC [11]. Part of this material was characterized (item 2.9.2) and the rest submitted to grinding and hydrolysis (enzymatic and acid).

The Yield of Extracted Material (Ym), Yield of Xylan (Yx) and Efficiency in Xylan Extraction (Ex), expressed in percentage, based on dry mass were calculated by equations [15]:

$$Ym = (Me/Mb) \times 100 \qquad \text{Eq. (1)}$$

Where: Me = mass of extracted material (g) and Mb = mass of sugarcane straw (g).

$$Yx = (Mx/Mb) \times 100 \qquad \text{Eq. (2)}$$

Where: Mx = xylan mass (g) (Me x Cx) and Cx = concentration of xylan in the Me (g x 100 mL).

$$Ex = (Mx/Mxb) \times 100 \qquad \text{Eq. (3)}$$

Where: Mxb = mass of xylan quantified in sugarcane straw (g) (Mb x Cxb) and Cxb = concentration of xylan in the Mb (g x 100 mL).

2.5 Enzymatic hydrolysis of arabinoxylan

The enzymatic hydrolysis was conducted in triplicate, applying 100 U of endoxylanase (produced as described in 2.3 item) per gram of extracted arabinoxylan (Table 1). The substrate was 50 mL of 7% (m/v) arabinoxylan in 100 mM acetate buffer at pH 5.0 [6]. The reaction was incubated in a shaker (Tecnal TE 421, Piracicaba, Brazil) at 50 ºC with agitation of 100 rpm during 72 h. Samples of 2 mL were collected at 0, 3, 6, 12 24, 48 and 72 h. Hydrolysis reactions were stopped by heating the samples during 10 min in a boiling water bath. After centrifugation 4000 x *g*, 10 min, the supernatant was analyzed by xylose and XOS production (item 2.9.3).

2.6 Acid hydrolysis of arabinoxylan

In order to optimize acid concentration on the XOS production, the reaction was conducted by the mixture of arabinoxylan in phosphoric acid 7% (m/v) using the following acid concentrations: 0.125, 0.25, 0.5 mol/L, in a glass sealed tube to avoid the liquid loss. Triplicated assay of each mixture was incubated in a boiling water bath at 95 ºC, or in an oil bath at 120 ºC, at different hydrolysis time 0.5, 1, 2 and 3 h. The reaction was stopped in each glass tube by cooling water. After cooling down, the reaction mixture was neutralized with calcium carbonate, and the resulting precipitate was removed by centrifugation (4000 x *g*, 10 min), and the final supernatant was taken to xylose and XOS analysis (item 2.9.3) [4; 19].

2.7 *W. onychis* LABI2 culture in enzymatic xylan hydrolysate

The submerged culture with the yeast *Wickerhamomyces onychis* LABI2 was performed in triplicate, for 72 h, 180 rpm at 28 °C in flasks (TE421, Tecnal, Piracicaba/SP, Brazil) containing 100 mL of culture medium. The medium followed the composition (w/v): 0.13% $(NH_4)_3PO_4$, 0.10% $(NH_4)_2SO_4$, 0.031% K_2SO_4, 0.0028% $ZnSO_4$, 0.0012% $MnSO_4$, 0.024% $MgSO_4$ modified [29], and 2.3% carbohydrates (XOS and monosaccharides) obtained by the enzymatic hydrolysis of xylan from SS (item 2.5). Control flasks were absent of carbohydrates. Samples were collected and evaluated regarding cell concentration and total reducing sugars

(TRS). The growth curve was performed and the cells concentration were microscopically measured by Neubauer counting chamber (7301-1B, New Optics). Dried biomass at the end of the fermentation was performed after centrifugation at 5000 x *g* for 30 min and 4 °C (Megafuge 16R, Heraeus, Thermo Fisher, Massachusets, USA) [30]. TRS quantification occurred in samples after acid hydrolysis with H_2SO_4 (2.0 M) at the proportion 1 v per 1 v of NaOH (2.0 M) (1:1:1) and quantification through the DNS method [31]. The kinetics parameters as maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$) and biomass productivity (g/L.h) were determined [32].

2.8 Oligosaccharides "in vitro" fermentation: growth medium for the *Lactobacillus* and *Bifidobacterium* strains

The MRS medium was used to growth the microorganisms, which consisted of (g/L): glucose or xylose 20 g, proteose peptone 10 g, meat extract 10 g, yeast extract 5 g, sodium acetate ($C_2H_3NaO_2$) 5 g, disodium phosphate (Na_2HPO_4) 2 g, ammonium citrate ($C_6H_{14}N_2O_7$) 2 g, Tween ®80 1 g, magnesium sulfate ($MgSO_4$) 0.1 g and manganese sulfate (M_nSO_4) 0.05 g. The medium was prepared at pH 6 and autoclaved for 20 min at 121 °C. *L. Acidophilus* strains were grown at 37 °C for 72 h and *L. brevis* strains at 30 °C for 72 h, in triplicate. For *Bifidobacterium* cultures, modified MRS medium [33] was applied, with L-cysteine addition. The cysteine solution was filtered on Millipore membrane (0.22 μm) and added to the previously autoclaved medium (121 °C for 20 min). Strains were grown at 37 °C for 72 h. To both microorganisms used the inoculum rate was 10^6 cells/mL.

The produced XOS in conditions defined at item 2.5 were used for the in vitro fermentation test using as carbon source 2% (m/v) of the mixture of xylose and XOS according to the enzymatic reaction of xylan. For comparison, tests were performed with medium containing 2% (m/v) D-xylose and another medium containing 2% (m/v) D-glucose as the only carbon source. Growth experiments on media containing different carbon sources were performed in triplicate.

2.9 Analytical methods

2.9.1 Enzyme assay and reducing sugars determination

Endoxylanase activity was assayed at 50 °C in a reaction with 0.1 mL raw extracts and 0.65 mL of 0.5% (m/v) xylan Birchwood solution (Sigma-Aldrich) in 250 mM sodium acetate buffer, at pH 5 [34]. The amount of reducing sugar was quantified by the dinitrosalicylic acid (DNS) method [31]. One unit (U) of xylanase activity was defined as the amount of enzyme to release 1 μmol of reducing sugar per minute per mL of reaction.

2.9.2 Chemical composition of sugarcane straw, lignocellulose and arabinoxylan

The moisture content of SS and the extracted xylan were determined by an oven at 105 ºC. The percentages of total lignin, arabinoxylan, and cellulose (glucan) were determined by NREL procedures [35]. Following these methodologies, the samples were oven dried and ground in a hammer mill to pass through a 20-mesh screen. The amount of inorganic material, either structural or extractable, in the SS, hereafter referred to simply as ash was determinate by the percentage of the residue of the straw remaining after dry oxidation at 500-700 °C in a muffle furnace (Marconi MA385, Piracicaba, Brazil). The material was then subjected to acid hydrolysis with H_2SO_4 for conversion of the insoluble carbohydrates into soluble monomeric sugars. The solid residue was washed with deionized water, oven dried and used to quantify insoluble lignin gravimetrically. The filtrate was analyzed for monomeric sugars by High-Performance Liquid Chromatograph (HPLC-Agilent 1100 system with a refractive index detector, with Bio-Rad HPX-87C column at 65 ºC) and the samples were eluted with (H_2SO_4-5 μM) at a flow rate of 0.6 mL/min. The filtrated liquid was also analyzed for soluble lignin by measuring the absorbance at 215 and 280 nm [36].

2.9.3 Xylooligosaccharides and Furfural determination

The released XOS in the enzymatic and acid hydrolysis were quantified using High-Performance Liquid Chromatography (HPLC). The analyzes were performed with an Agilent

1100 system with a refractive index detector. A Bio-Rad HPX-87C column at 65 ºC was eluted with purified water (Milli-Q). The elution time was 20 min with a flow rate of 0.5 mL/min. The standards used were, xylose, xylobiose, xylotriose, and xilotetraose. Total XOS is the sum of oligosaccharides detected.

The yield of total XOS and X1 produced from, arabinoxylan degradation were determined by the following equations [15]:

$$\text{X1 yield (\%)} = (\text{X1 (g)/pure arabinoxylan (g)}) \times 100 \quad \text{Eq. (4)}$$

$$\text{Total XOS yield (\%)} = (\text{total XOS (g)/pure arabinoxylan (g)}) \times 100 \quad \text{Eq. (5)}$$

Furfural compound was determined using HPLC, Agilent 1100 System, UV detector, and Sorbax C18 collumn, at 25ºC of temperature, eleuted with methanol 85% (v/v). The elution time was 2.5 min with a flow rate of 0.5 mL/min. The standad was furfural from Sigma Aldrich.

2.10 Determination of the *Lactobacillus* and *Bifidobacterium* strains growth

Growth curves of probiotic bacteria were qualitatively determined by optical density analysis (O.D.). The absorbance was determined at 600 nm in a spectrophotometer (Fento 700 Plus) [18]. Aliquots of probiotic bacteria were taken at 0, 4, 8, 12, 24, 48 and 72 h and immediately read.

3. RESULTS AND DISCUSSION

3.1 SS chemical characterization and arabinoxylan extraction

The arabinoxylan content in SS was 21.3%, cellulose (30.3%), lignin (27.5%) and ashes (10.8 %) (Table 1). The arabinoxylan is similar to other important xylan sources of XOS production, as example: coconut husks (26%)[23], sugarcane bagasse 25% [11], tobacco stalk 21.8%, cotton stalk 21.3%, sunflower stalk 18.9% and wheat straw and 20.6% [37].

In addition, SS also have other important components, which were not used for XOS production, but can be destined to some integrated processes. For example: the cellulose can be used in second generation ethanol production [1; 13], lignin as a renewable aromatic resource for

chemical industries as phenols, aldehydes, carboxylic acids and alkanes and [38], and ashes for cement-based building materials [39].

The NaOH (4% m/v) method of arabinoxylan extraction from SS showed an Extraction efficiency (Ex) of 63% (m/m), based on the calculation using the yield of extracted material from SS (27.7% m/m), the purity of arabinoxylan in the extracted material (48.7% m/m) and the content of arabinoxylan in SS, 21.3% (Tables 1 and 2). Figueiredo [11] also reach 63% Ex of arabinoxylan using 5% (m/v) of KOH, 30 min, 121 ºC, but the yield of arabinoxylan extraction from sugarcane straw was lower (23% m/m). On the other hand, better results of Ex were founded, 93 and 90 %, using NaOH, but in very high concentrations, 20 and 16%, using brown coconut husk and corn cobs, respectively [23; 40].

Table 1 - Chemical composition of sugarcane straw and extracted material by NaOH hydrolysis*.

	Cellulose (%**)	Arabinoxylan (%**)	Total Lignin *** (%)	Ashes (%**)
Sugarcane straw	30.3±2.0	21.3±2.4	27.5±0.5	10.8±0.56
Extracted material	5.9±0.1	48.7±1.7	23.2±0.4	12.2±0.23

*The results are related with the average and standard deviation of three experiments.
** % is based on dried mass; ***(Soluble +insoluble)

Table 2 - Yield of Extracted Material (Ym), Yield of Arabinoxylan (Yx) and Efficiency in Xylan Extraction (Ex) from sugarcane straw*.

	Yield of extracted material (Ym %)**	Yield of Xylan (Yx %)**	Efficiency in xylan Extraction (Ex %)**
Extracted material	27.7 ± 0.6	13.5 ± 0.8	63.3± 0.5

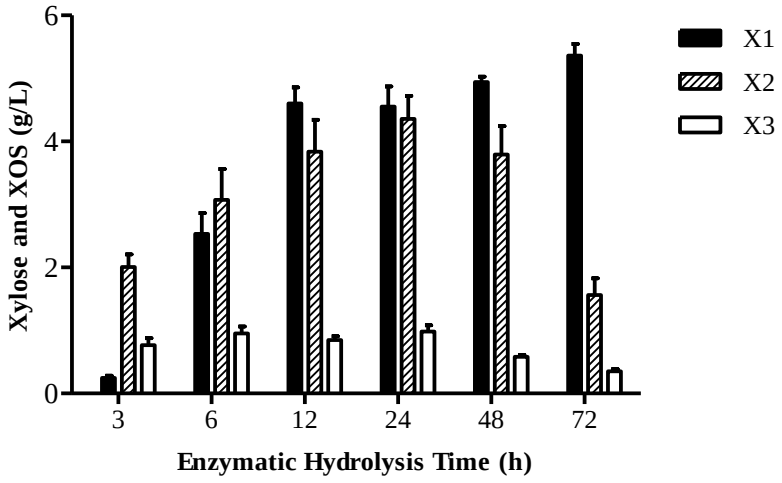
*The results are related with the average and standard deviation of three experiments.
** Following item 2.4

3.2 XOS production from enzymatic and acid arabinoxylan hydrolysis

3.2.1 Enzymatic hydrolysis yields

The evaluation of the hydrolysis of xylanases from *T. reesei* cultures converting extracted arabinoxylan in xylose and XOS were performed. In the Enzymatic Hydrolysis (EH) was produced more xylose (X1) than isolated oligosaccharides (X2 and X3), except on the beginning of the process, 3 and 6 h (Figure 1). Higher XOS production occurred in 24 h, after this time, X2 and X3 concentration started to decrease, probably due to the XOS degradation in monomers, detect by X1 concentration increase. Besides, the high presence of xylose is related to the inhibition of xylanase activity to convert xylan into XOS [3; 17; 19] justifying a lower XOS yields. In this sense, xylose detected in EH reached the maximum production in 72 h, 5.36 g/L (Fig. 1). The β-xylosidase can be cited as an enzyme responsible for hydrolysis of XOS in monomer sugar (X1) and the action of this enzyme can explain the great sum of reduced sugars (pentoses) in contrast with XOS.

Figure 1 – XOS production from 7% (m/v) arabinoxylan enzymatic hydrolysis in 72 h at 50 ºC, with *T. reesei* QM9414 xylanase (100 U/g substrate)*.



X1 (Xylose); X2 (Xylobiose); X3 (Xylotriose)

*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

X2 production in 24 h, 4.35 g/L, was better than other works, for example, Jnawali [23] obtained only 1.69 g/L using xylan from Coconut Husk, 4.50% (v/v) of enzyme concentration, using crude xylanase enzyme, and 18 h incubation. Jayapal and co-workers [41] using xylan from sugarcane bagasse obtained 1.153 ± 0.13 g/L X2, in 8 h of reaction time, and enzyme dosage of 2.65 U/g. Using corn cobs, the X2 production was also lower to Samanta et al [40], 1.2 g/L, with an enzyme dosage of 7.28 U/g for 17.3 h of hydrolysis. However, these authors used only 2% (m/v) of extracted xylan as substrate while in the present work was used 7% of crude arabinoxylan corresponding to 3.4% (m/v) pure xylan.

The X2 production until 24 h of EH showed a gradual increase, after this time, the production started to decrease, probably because the X2 degradation in xyloses (Fig. 1). A similar effect on the xylobiose yield has been reported by Samanta and co-works [42]. However, X3 production was not affected by the EH time, since in 3 h the production reaches the maximum, 1 g/L, and remained until 48 h when this sugar started to decrease (Fig. 1).

Since no trace of XOS with a higher degree of polymerization (DP), or more than X3, was detected in the arabinoxylan hydrolysis by xylanases of *T. reesei*, the XOS yield were represented only by X2 (xylobiose) and X3 (xylotriose). The type of substrate affects the XOS DP in enzymatic hydrolysis, but in a similar work with arabinoxylan from sugarcane bagasse and corn cobs, only X1, X2 and X3 were also described [11; 40]. The relationship between DP rate in XOS production and its prebiotic effect is a controversial issue. Some authors show the importance of a wide range XOS DP for bifidobacteria growth [43; 44] while others show that XOS with lower DP (2-3) is related to higher prebiotic activity than XOS with higher DP [18]. If this last statement is correct the XOS produced in the conditions showed in the present work would be more available as prebiotic.

Some authors showed the Xylanase of the family 10 produces different XOS of the family 11, and xylanase of family 10 produces lower DP than 11 [5; 15; 20]. The characterization of xylanase from *T. reesei* QM9414 was not performed but is reported in the literature that both families are produced by *T. reesei* [45].

The XOS yield, calculated by the pure arabinoxylan degradation, reached the maximum in 24 h, 15.67% (Fig. 2). Considering this condition, the total XOS concentration, 5.34 g/L, was better than Brienzo et al [15] (3.7 g/L), obtained using xylan from sugarcane bagasse and xylanase from *T. aurantiacus* in 24 h. This result is higher compared to a similar work, that performed the sugarcane bagasse xylan (7% m/v) hydrolysis with *T. reesei* QM9414 xylanase, and obtained 15% of XOS yield [11]. Despite this result were reached in 12 h, the authors used

treatments to xylose extraction base on KOH, 24 and 10% (m/v), and this chemical is more expensive than NaOH and its use is controled by the government (ex. in Brazil). A lower XOS yield, 11.4%, was also obtained from tobacco stalk xylan EH, also extracted by KOH 24 % (m/v), in 24 h using commercial enzyme hydrolysis [19]. When Figueiredo et al [11] decreased the KOH content in xylan extraction, a similar XOS yield was obtained, 15.7%, but the EH time increase to 24 h. So, the treatment applied on LCM to xylan extraction is important to XOS production and the enzymatic performance on xylan EH depends the improvement of several parameters [11; 46]. Considering the yields of XOS and xylose produced in the arabinoxylan enzymatic hydrolysis in 24 h was possible to hydrolyze 29% of xylan (Fig. 2). Thus, this enzymatic process should be improved to increase the XOS yields, probably using purer enzymes and/or xylan.

Figure 2 – XOS yields production from arabinoxylan 7% (m/v) enzymatic hydrolysis, 72 h at 50 ºC, with *T. reesei* QM9414 xylanase (100 U/g substrate)*.

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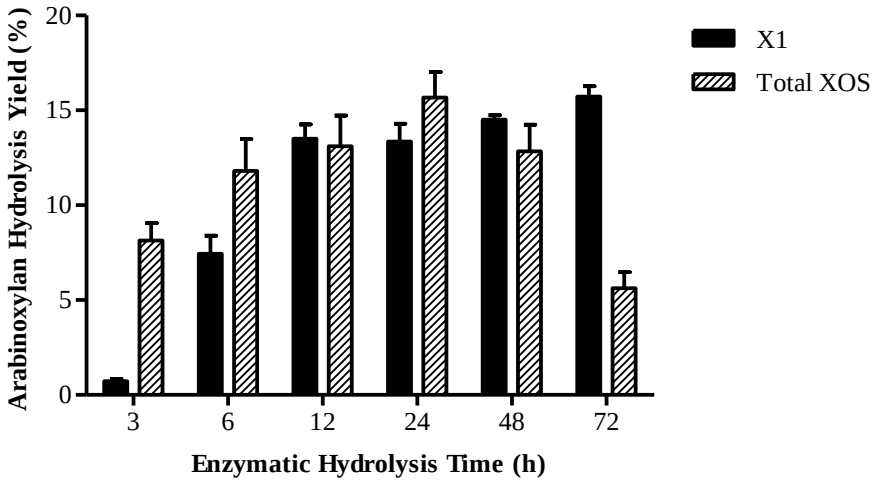


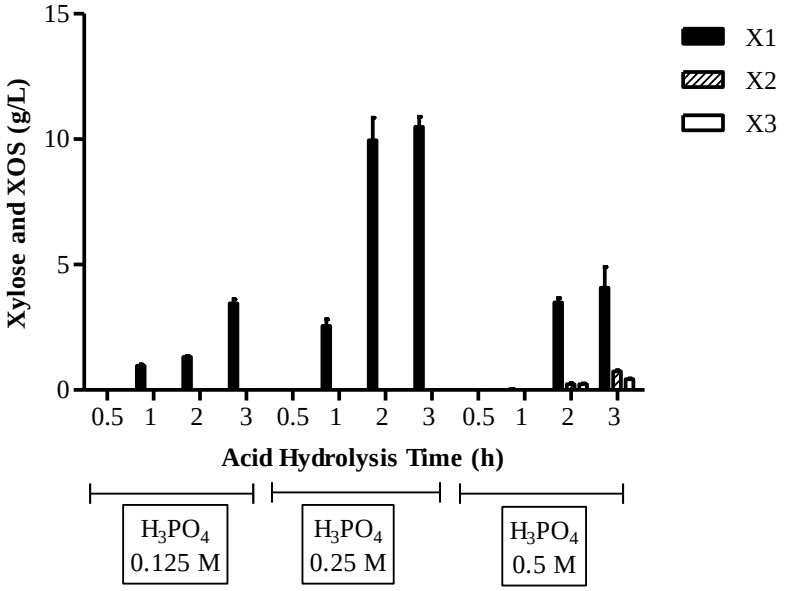
X1 (Xylose); Total XOS = X2 (Xylobiose) + X3 (Xylotriose)

*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

3.2.2 Acid hydrolysis

The arabinoxylan Acid Hydrolysis (AH), was performed using H_3PO_4 as a catalyst, at 0.125, 0.25, and 0.5 M. Two isolated temperatures were tested: 95º C in boiling water bath (AH $_{95\ ºC}$) and 120 ºC in oil bath (AH $_{120\ ºC}$) (Fig. 3 and 4).

Figure 3 – Effect of time on XOS production from arabinoxylan (7% m/v) acid hydrolysis (95 ºC - water bath, H_3PO_4 0.5-0.25 and 0.125M)*.



X1 (Xylose); X2 (Xylobiose); X3 (Xylotriose)

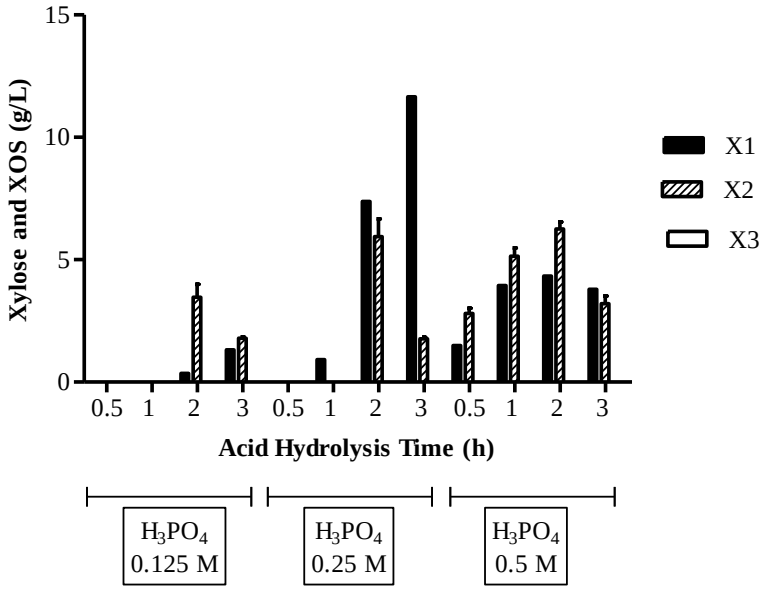
*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Initially, analyzing the xylan products fragmentation from both acid processes (Fig. 3 and 4), were produced mainly xylose (X1) than oligosaccharides. In the AH $_{95\ ºC}$ almost only xylose was produced (Fig 3) and its higher concentrations, almost 10 g/L, were obtained with 0.25 M H_3PO_4 in 2 or 3 h of reaction, since more than 0.25 M H_3PO_4 concentrations decreased the xylose level. This fact is probably due to xylose degradation in furfural, more evident for the both temperatures tested (Fig. 5). The XOS production was not favored in AH $_{95\ ºC}$ process due to using H_3PO_4, at the lower proposed concentrations, 0.125 and 0.25 M, since no XOS was detected. In this temperature, XOS production was achieved after 3 hours of hydrolysis, 0.5 M,

0.75 g/L X2 and 0.45 g/L X3, reaching low XOS yields, 3.46% (Fig. 6), when compared with the same assay in AH $_{120\ ^oC}$ (Fig. 7). Therefore, this temperature is not possible to work for XOS production considering the type of acid and its concentration applied in this work.

The XOS produced in all AH $_{120\ ^oC}$ assays were only X2, and this sugar was detected in lower amount than xylose, except to 0.5 M H_3PO_4 in 0.5, 1 and 2 h of process, when more XOS was produced than xylose. In addition, the highest level of XOS (5.9 g/L) was reached at this temperature in 2 h reaction time using 0.25M or 0.5 M, but the xylose was also more degraded at the highest concentration of this acid. In fact, this degradation was evident by the conversion in furfural, since more than three times higher level of this toxic compound was quantified when 0.5 M was used in AH $_{120\ ^oC}$ after 2 h of reaction time (Fig. 5). After 2 h the X2 concentrations started decrease, thus this time is more convenient for this process. Besides that, in these situations was not observed the X1 increase to affirm that the XOS were fragmented in monomers, as observed with 0.125 M. The xylan degradation, in this case, could be transformed in furfural as detected in Fig.5. In addition, no X3 were detected in the AH $_{120\ ^oC}$ process.

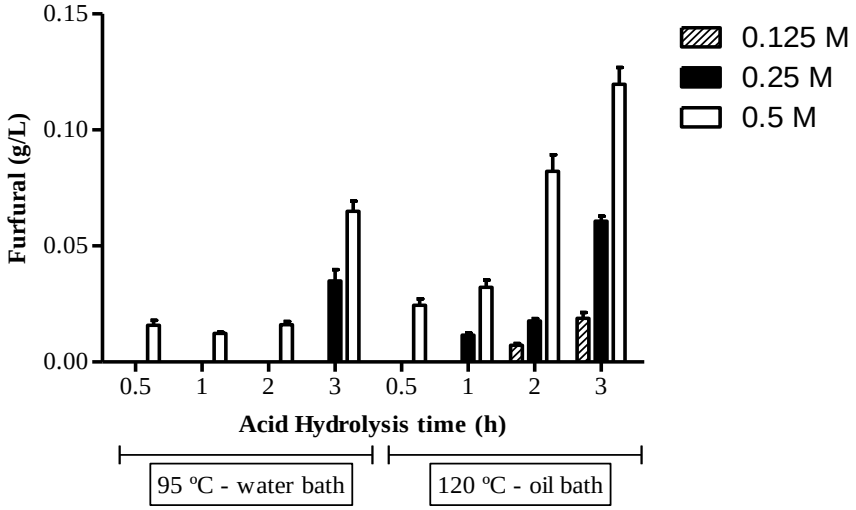
Figure 4 – Effect of time on XOS production from arabinoxylan (7% m/v) acid hydrolysis (120 ºC - oil bath, H_3PO_4 0.5-0.25 and 0.125M)*.



X1 (Xylose); X2 (Xylobiose); X3 (Xylotriose)

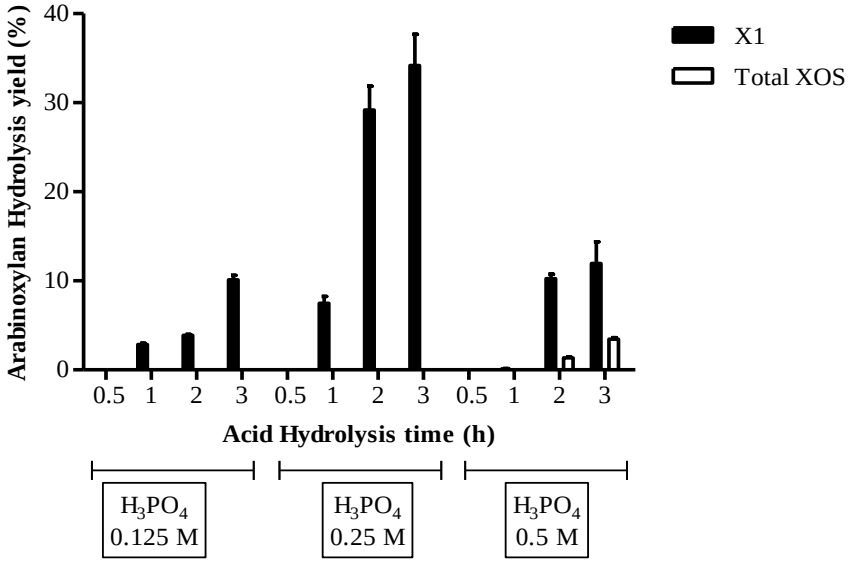
*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Figure 5 – Effect of time on furfural production from acid hydrolysis (95 ºC - water bath and 120 ºC- oil bath, H_3PO_4 0.5-0.25 and 0.125M) of arabinoxylan (7% m/v)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

Figure 6 – XOS yields from arabinoxylan (7% m/v) acid hydrolysis (95 ºC - water bath, H_3PO_4 0.5-0.25 and 0.125M)*.



X1 (Xylose); Total XOS: X2 (Xylobiose) + X3 (Xylotriose)

*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

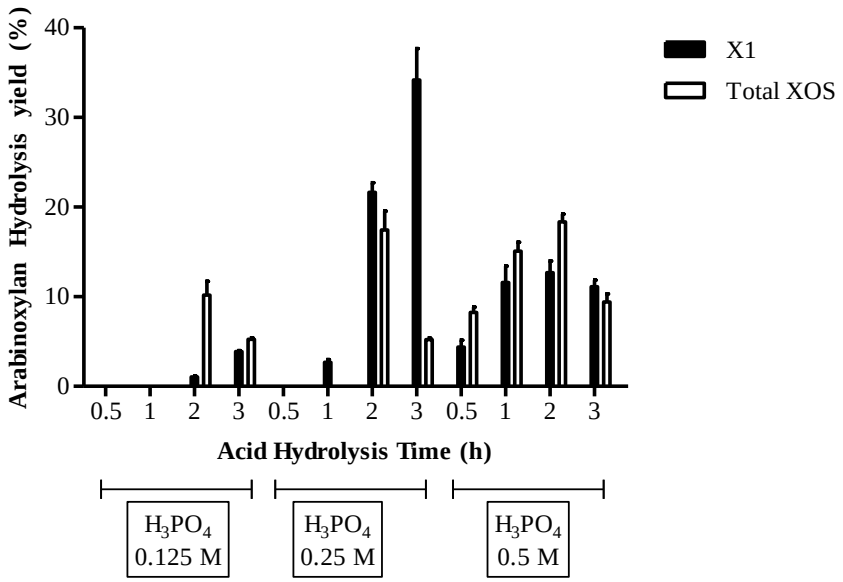
Almost 35% of xylose yield was obtained by AH $_{95\ ^oC}$, 0.25 M H_3PO_4 in 3 h reaction time (Fig. 6), and this result was obtained in AH $_{120\ ^oC}$ using the same level of acid and time (Fig. 7). However, the best conditions for the highest XOs yield (about 17% m/m) was in AH $_{120\ ^oC}$, 2 h reaction time and 0.25 M or 0.5 M H_3PO_4. If the furfural reached the highest level when the concentration of 0.5 M H_3PO_4 was used, for both acid process, then the best conditions to obtain higher level of XOS with lower furfural level are 0.25 M H_3PO_4, 2 h reaction time at 120 ^oC. The pure xylan conversion yield to XOS in this sample was 17.45% (Fig 7). Carvalho et al obtained the XOS yield of 40% XOS from xylan, but using a more elaborated xylan extraction (190 °C, 5 min and 0.5% H_2SO_4 as catalyst in a steam explosion process) [14]. The XOS yields obtained in the present work were higher than Akpinar and co-workers[4], using other sources like: tobacco stalk (13%), cotton stalk (7.5%), sunflower stalk (12.6%) and wheat straw (10.2%). The differences between this work in comparison with the present work are: they used 24% KOH for xylan extraction, and the proposed conditions were H_2SO_4, 2.5 M, 30 min, besides that, the xylan concentration was low (2% m/v).

The X1 and XOS increased, in $AH_{95\ ^oC}$ and $AH_{120\ ^oC}$ process, with time and acid catalyst concentrations, but no more than 2 h and 0.25 M H_3PO_4 was necessary to X1 and X2 highest yields. In fact, these results with the increase of reaction time were verified in other works and they were followed by a great decrease in the selectivity towards XOS [4; 14; 19]. Thus, long time and higher acid concentration were not considered. In the conditions proposed in the $AH_{120\ ^oC}$, higher temperatures and/or time were not to apply, since severe treatment with high temperature and acid concentration resulted in high sugar-degradation [14]. Despite these results, an optimization of parameters of xylan hydrolysis in the oil bath is necessary to adequate the X1 and the DP of XOS produced depending on the subsequent application, as well as the improvement of the respective yields. In addition, other catalysts with an appropriate time and temperature of reaction need to be tested to improve this reaction.

The temperature was essential to the XOS production, however, the furfural, an inhibitor, was also produced (Fig. 5). The maximum furfural production reached 0.13 g/L or 0.00135 M using the most severe condition in this work (0.5 M H_3PO_4, 3h, 120 ^oC). The presence of furfural after the acid hydrolysis of arabinoxylan is expected, since furfural is derived from xylose degradation, and it was also detected by other researchers[19] with H_2SO_4 0.25 M (3.89 mg/L) in boiling water bath. This compound increases with time and acid concentration, but its concentration is relatively low. Furfural causes growth inhibition of the microorganisms and only its presence in lignocellulosic hydrolysates in concentrations close to

20 mM or even lower are toxic for them [13; 47]. Therefore, a purification step is advised in order to remove the furfural compounds since it is considered inhibitors for many fermenting microorganisms [3; 13; 19].

Figure 7 – XOS yields from arabinoxylan (7% m/v) acid hydrolysis (120 ºC - oil bath, H_3PO_4 0.5-0.25 and 0.125M)*.



X1 (Xylose); Total XOS: X2 (Xylobiose) + X3 (Xylotriose)

*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

3.2.3 Comparison of Acid and Enzymatic xylan hydrolysis yields

Analyzing the Table 3 is possible to resume the acid and enzymatic route in the xylan hydrolysis. The XOS DP production were different. To EH X2 and X3 were detected, and to AH $_{120\,^{o}C}$ only X2. Besides that, monosaccharides, main xylose, were formed in acid hydrolysis, about twice more than in enzymatic method. Xylan hydrolysate containing xylose can be interesting for important industrial uses. However, the presence of this sugar in the xylan

hydrolysis with acid can form an undesirable product, the furfural, as reported in other works [14;19].

In spite of the XOS yields, the AH $_{120\ ^{o}C}$, in 2 h (17.45%) faced the best enzymatic hydrolysis assay in 24h (16%) but no significant difference (ANOVA+Tuckey, $p>0.05$) was verified. Then, analyzing the Table 3 we can conclude that EH was a safer treatment than AH $_{120^{o}C}$ for XOS production considering food purpose, since no toxic chemicals was generated, as furfural, and no neutralization step was necessary. On the other hand, if considered some additional purification steps, a faster xylan hydrolysis for XOS production and legal permission to use this method, the acid method is more adequate.

Table 3 – Comparison between enzymatic and acid hydrolysis of arabinoxylan extract from sugarcane straw.

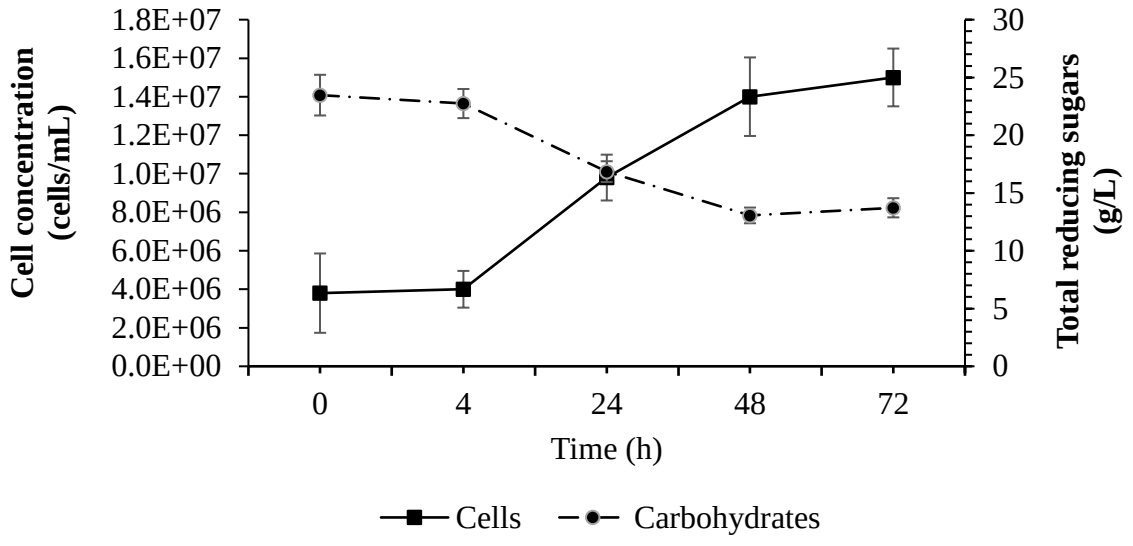
	Enzymatic Hydrolysis (xylanase from *T. reesei* Qm 9414	Acid Hydrolysis
Hydrolytic reagent	Xylanase (100 U/g)	H_3PO_4 (0.25 M)
Time (h)	24	2
Temperature	50 ºC	120 ºC
Neutralization	No	Yes
XOS (g/L)	5.34	5.94
Conversion of xylan to XOS (%)	15.67	17.45
Conversion of xylan to X1 (%)	13.35	21.56
DP Range	2-3	2
Furfural (g/L)	0	0.08

3.3 *W. onychis* LABI2 fermentability

The enzymatic hydrolysate containing xylose and XOS (produced according the item 2.5) was used as carbon source to *W. onychis* LABI2 grown, as objective to evaluate its fermentability. Submerged culture with *W. onychis* LABI2, with 2.3% of total reducing sugars initially present, achieved a low specific growth rate (μ_{max} = 0.02 h^{-1}) with a log phase ranging the culture time of 4-48 h (Fig. 8) Regarding the total volume in the flasks at the end of fermentation, after 72 h, the dried biomass present reached 3.7±0.2 g/L of yeast with a

productivity of 0.08 g/L.h and good level of growth yield (Yx/s = 0.29±0.07 g/g). In these conditions the total reducing sugars remained were 18±1.7 g/L. In contrast, in the control flasks, with no carbon source presence, after 72 h of fermentation, only 0.37±0.1 g/L of dried biomass was obtained or just 10% of the result obtained with the xylan hydrolysate. According these results *W. onychis* LABI2 was able to grow in culture medium with XOS and xylose from xylan EH. Total reducing sugars were not entirely consumed remaining 13.7 g/L (Fig. 8) may be due to some difficult in the assimilation of all sugars present in hydrolysate. This result can be compared with this yeast culture using only xylose as a carbon source (2% w/v), 1.7 g/L of biomass production, lower than 3.7 g/L obtained from xylose+XOS hydrolysate [27]. According to Kurtzman and co-workers [48] *W. onychis* is capable to grow in submerged cultures using xylose and many other sugars, but there is no reference of *W. onychis* culture with only XOS. Díaz et al. (2005) evaluated the biomass production of the same yeast in flasks, at 110 rpm and pH 6.0, using different carbon and nitrogen sources (molasses, soy hydrolysate, yeast hydrolysate and corn hydrolysate), and produced up to 6.3 g/L (or $8.9x10^9$ cells/mL), when the carbon concentration, molasses, was 43.4 g/L [47].

Figure 8 – *Wickerhamomyces onychis* LABI2 growth curve during submerged fermentation in flasks using carbohydrates obtained by the enzymatic hydrolysis of sugarcane straw*.

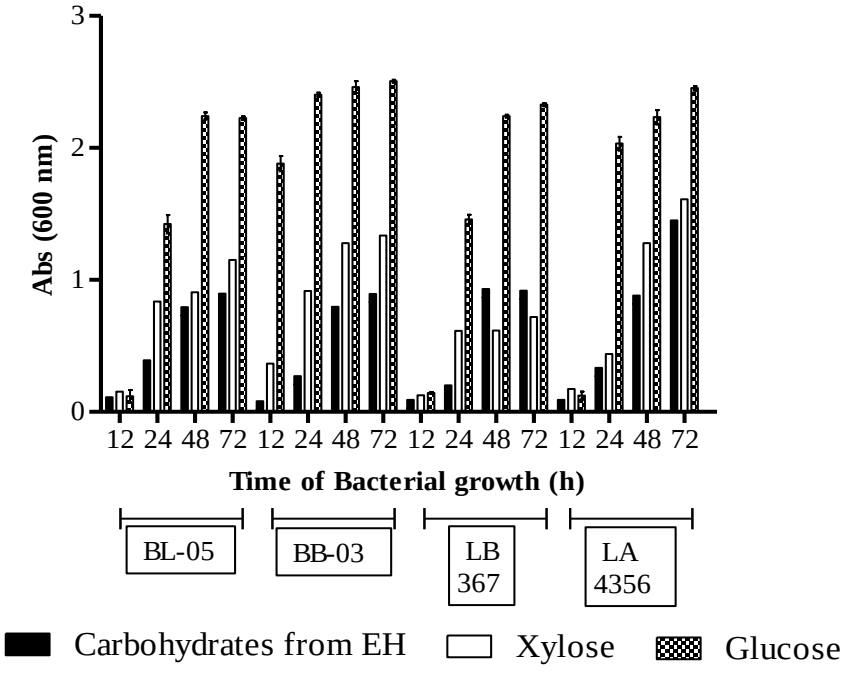


*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

3.4 XOS "*In vitro*" bacteria fermentation

In vitro fermentation tests were designed to prove, the ability to stimulate the growth of beneficial bacteria from xylose and XOS hydrolysate, obtained by enzymatic hydrolysis of arabinoxylan extracted from SS (item 2.5), comparing with commercial sugars glucose and xylose. The enzymatic hydrolysate was effective in promoting the growth of all probiotic bacteria tested. However, when the bacterial growth in glucose was compared, there was a faster and more complete assimilation of this sugar in all experiments. In addition, all bacterial cultures in xylose medium in 24 h were faster than the hydrolysate (XOS +xylose) (Fig. 9). However, the culture of *Bifidobacterium longum* BL 05 in 48 h in the hydrolysate (XOS +xylose) showed a similar growth to that obtained by xylose medium. In fact, *B. Longum* showed a lower growth with XOS [19; 49], but in the present work this happen only in a short time cultures.

Figure 9 - Probiotic growth profile during 72 h of *Bifidobacterium longum* BL-05 (BL-05); *Bifidobacterium breve* BB-03 (BB-03); *Lactobacillus brevis* strains ATCC 367 (LB 367) and *Lactobacillus acidophilus* ATCC 4356. The microorganisms were cultivated using different carbon sources (2% m/v)*.



*Each bar value was the average of three replicate experiments, and the error bars show the data ranges.

On the other hand, the culture in 48 h of *Lactobacillus brevis* strains ATCC 367 (LB 367) showed a higher growth in the medium with hydrolysate (XOS +xylose) than with pure xylose (fig. 9). In fact, a great growth of *L. brevis* in commercial XOS medium was proved and this strain showed preference for xylobiose assimilation [18].

The xylose medium cultures of *Bifidobacterium breve* BB-03 and *Lactobacillus acidophilus* ATCC 4356 showed higher growth than hydrolysate (XOS +xylose) in all times. Probably XOS are not very well assimilated for some lactic acid bacteria. Additional step of purification with low molecular compounds for oligosaccharide filtration is necessary to obtain a purer XOS liquid [48-50; 51]. The use of probiotic microorganisms with low growth rate in pure xylose was interesting to obtain a good sugars assimilation from no pure enzymatic hydrolysates obtained from xylan wastes [18]. The use of acid xylan hydrolysate were not tested in this work due to the furfural presence, since its absence is important to probiotic growth [10,21]. The main probiotic action is to promote the lactic bacterial growth in the intestine, improving gut function, besides to control the *Salmonella* and *E. coli* proliferation [51]. Therefore, in a next step this hydrolysate will be tested with some pathogenic bacteria in order to evaluate an inhibition in their growth.

4. CONCLUSION

The sugarcane straw as a feedstock for some important biotechnological process was studied. The alkaline extraction of xylan from sugarcane straw was efficient in a relatively low level of NaOH. Enzymatic and acid (H_3PO_4) hydrolysis of the xylan extracted from sugarcane straw were performed. If considered some additional purification step, the acid method is more adequate since is faster. Only 2 h of arabinoxylan acid hydrolysis, 0.25 M H_3PO_4 at 120 ^oC, produces the highest results of X1 and X2 production. This hydrolysis at 120 ^oC produced similar XOS yield to enzymatic hydrolysis but the last one does not produce furfural. In both types of hydrolysis xylose was produced. The probiotic bacteria *Bifidobacterium longum* BL-05, *B. breve* BB-03, *Lactobacillus brevis* ATCC 367 and *L. acidophilus* ATCC 4356 and the yeast *W. onychis* were able to grow in the enzymatic hydrolysate containing xylose and XOS.

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GENERAL CONCLUSIONS

The integrated bioprocesses used in this work using sugarcane straw show different manners to reutilize this waste in some biotechnological routes. In Chapter 1 was present a review about fibrolytic enzymes, including its production and applications, that were important to plan our work. Besides that, a comparison between the widely used sugarcane bagasse and sugarcane straw was performed to prove the former can be replaced for the second and the importance to promote strategies to use this abundant residue. In Chapter 2, The optimization of the enzyme production selecting straw as the main carbon source to produce xylanase and cellulase fungal enzymes was performed with high hydrolytic activity, as well the *T. reesei* QM9414 as the best producer. The biochemical characterization was also important to us to plan and execute easily the next steps of enzyme application. In Chapter 3, the crude extract, mainly constituted by xylanases, shows specific activity on hemicellulose from kraft pulp, being easily the lignin removal from pulp after cooking, and consequently almost 10% of chlorine dioxide could be saved in the chemical step of bleaching. The enzyme action could be seen in SEM images. In Chapter 4, the xylan was extract from sugarcane straw and hydrolyzed by two different processes, acid and enzymatic, to produce XOS. The enzymatic route was more interesting due to the good results obtained that were very similar to the acid one and no chemical and neutralization is necessary, despite the need a longer treatment period. Besides that, no furfural was detected in this hydrolyzed. Probiotic bacteria and a yeast was feed by the sugars (xylose and XOS) produced by enzymatic route and these microorganisms tested were capable to grow on enzymatic xylan hydrolisate. The probiotic bacteria *Bifidobacterium longum* BL-05, *B. breve* BB-03, *Lactobacillus brevis* ATCC 367 and *L. acidophilus* ATCC 4356 and the yeast *W. onychis* were able to grow in the enzymatic hydrolysate containing xylose and XOS.

SUGGESTIONS FOR FUTURE RESEARCHES

Based on our results we can considerate the following actions in the future:

- Scale-up the enzymatic production to more than 1.5 L;
- Scale-up the Kraft pulp biobleaching in a pilot scale with the study of economic viability
- Improvement of the xylan extraction, using other chemicals and physical processes, to improve the yields;
- Optimization the xylan enzymatic hydrolysis conditions aiming to produce more XOS with different DP and less xylose and separation of XOS and Xylose;
- Use the cellulose from xylan extraction for the biotechnological processes aiming the integrated bioprocess and biorefinery purpose;
- Study the purification step of the xylose and XOS hydrolysate produced by acid hydrolysis to remove furfural and the evaluation of the growth of probiotic microorganism