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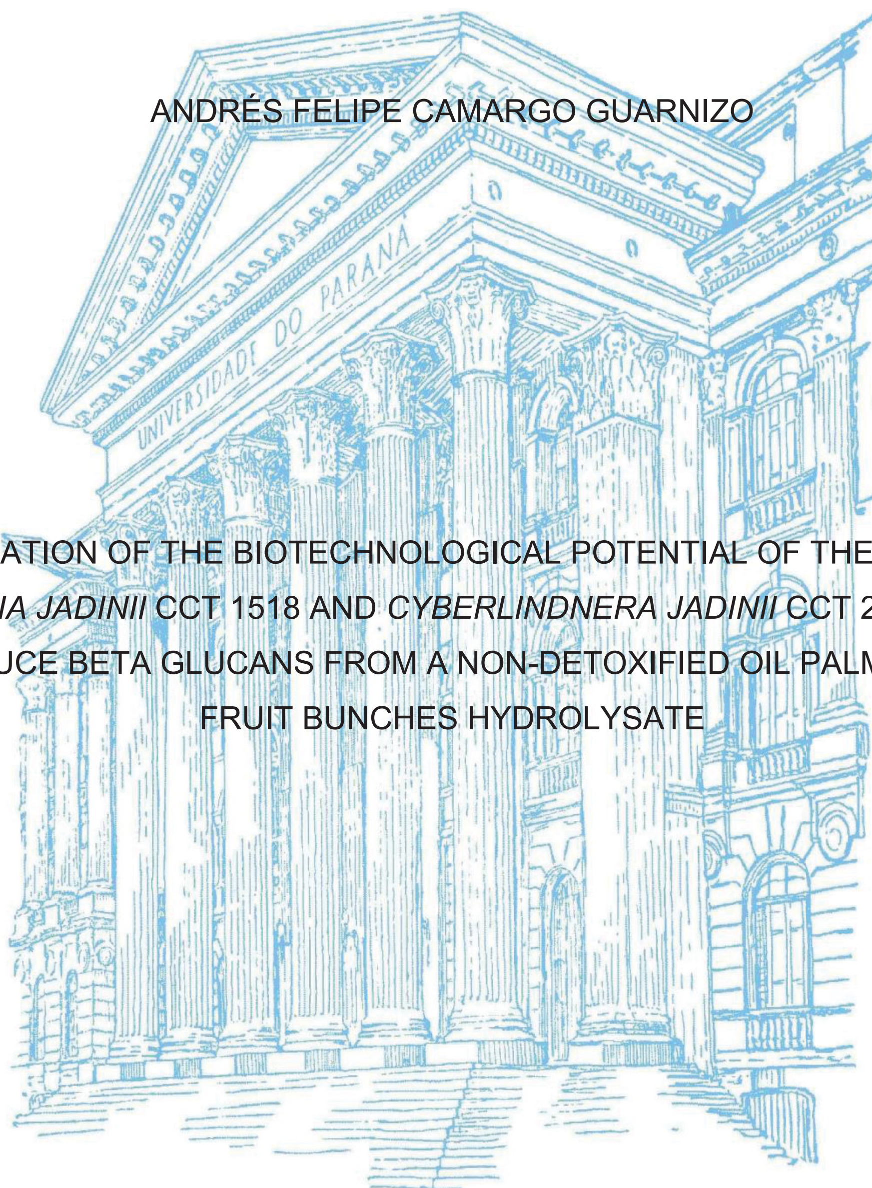


ANDRÉS FELIPE CAMARGO GUARNIZO

# EVALUATION OF THE BIOTECHNOLOGICAL POTENTIAL OF THE YEASTS *PICHIA JADINII* CCT 1518 AND *CYBERLINDNERA JADINII* CCT 2612 TO PRODUCE BETA GLUCANS FROM A NON-DETOXIFIED OIL PALM EMPTY FRUIT BUNCHES HYDROLYSATE

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2018

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Dissertação apresentada como requisito parcial para à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia, no curso de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, setor de Tecnologia, da Universidade Federal do Paraná

Orientadora: Profa. Dra. Adenise Lorenci Woiciechowski

Coorientador: Prof. Dr. Luiz Alberto Júnior Letti

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **ANDRÉS FELIPE CAMARGO GUARNIZO** intitulada: **EVALUATION OF THE BIOTECHNOLOGICAL POTENTIAL OF THE YEASTS *Pichia jadinii* CCT 1518 AND *Cyberlindnera jadinii* CCT 2612 TO PRODUCE BETA GLUCANS FROM A NON-DETOXIFYED OIL PALM EMPTY FRUIT BUNCHES HYDROLYSATE**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

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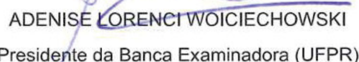

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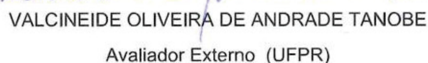

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# DEDICATORIA

Para os meus pais e minha irmã, pela ajuda, compreensão e dedicação em todo este processo de mestrado.

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God

My Family

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“An investiment in knowledge

pays the best interest”

Benjamin Franklin.

**RESUMO**

Os cachos vazios de palma de aceite (EFB) são um resíduo lignocelulósico abundante na produção de óleo de palma e possuem alta quantidade de açúcares hemicelulósicos (25-30% w/w Dry EFB); que pode ser usado para a produção de produtos de alto valor agregado, como os β-glucanas (polissacarídeos que têm potencial para prevenção de doenças) (PIZARRO et al., 2014; RAMAN; GNANSOUNOU, 2014). Para a liberação dos açúcares hemicelulósicos, utilizou-se o pretratamento ácido diluído e a metodologia da superfície de resposta como método de otimização. As variáveis avaliadas foram temperatura (°C), tempo (h) e concentração de ácido sulfúrico em três níveis. A maior liberação de açúcares hemicelulósicos foi de 24,18g/100g EFB na condição com 2,9% p/p de ácido sulfúrico, 125 °C e 25 minutos. Compostos tóxicos inibidores do crescimento microbiano foram gerados em concentrações semelhantes às relatadas na literatura (RAMAN; GNANSOUNOU, 2014). Este hidrolisado foi neutralizado com NaOH, não desintoxicado e suplementado com nitrogênio modificando a relação carbono/nitrogênio (C/N) em três níveis. Posteriormente foi inoculado com as leveduras *Pichia Jadinii* CCT 1518 e *Cyberlindnera jadinii* CCT 2612 e avaliou-se a produção de biomassa dessas leveduras resistentes. A levedura *Pichia Jadinii* apresentou uma biomassa de 5,87 g/L hidrolisado às 120 horas com uma relação C/N 14 e a *Cyberlindnera Jadinii* apresentou uma biomassa de 10,50 g/L hidrolisado às 96 horas com uma relação C/N 11,5. Adicionalmente, as fases lag e alguns parâmetros cinéticos foram determinados com a ajuda do modelo de Gompertz modificado para as duas leveduras. Uma análise proximal e isolamento de β-glucanas (método de Muller) foi realizada à biomassa das leveduras cultivadas nas relações C/N e nos tempos descritos (MULLER et al., 1996). Os β-glucanas isolados de cada levedura foram caracterizados. β-glucanas insolúveis (1-3) e β-glucanas solúveis (1-3) (1-6) foram obtidas para as duas leveduras com a presença de alguns grupos pertencentes à quitina. e/ou proteínas. Finalmente, o hidrolisado do EFB pode ser usado para a produção de β-glucanas, no entanto, métodos para reduzir os custos de produção devem ser estudados para uma escala industrial.



**Palavras chave:** EFB, Hidrolisado, Pretretamento ácido diluido, β glucanas, Relação C/N, *Pichia Jadinii* CCT 1518 e *Cyberlindnera jadinii* CCT 2612.

## ABSTRACT

The Empty Fruit Bunches (EFB) are a lignocellulosic residue abundant in the production of palm oil and have a high amount of hemicellulose sugars (25-30% w/w Dry EFB); which can be used for the production of high value-added products such as beta glucans (polysaccharides that have potential for disease prevention) (PIZARRO et al., 2014; RAMAN; GNANSOUNOU, 2014). For the release of the hemicellulose sugars, the diluted acid pretreatment was used, and the response surface methodology was used as an optimization method. The variables evaluated were temperature (°C), time (h) and concentration of sulfuric acid in three levels. The highest release of hemicellulose sugars was 24.18g/100g EFB in the condition with 2.9% w/w sulfuric acid, 125 °C and 25 minutes. Toxic compounds inhibiting microbial growth were generated in concentrations similar to those reported in the literature (RAMAN; GNANSOUNOU, 2014). This hydrolysate was neutralized with NaOH, not detoxified and supplemented with nitrogen modifying the carbon/nitrogen ratio in three levels. Later it was inoculated with the yeasts *Pichia Jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612 and the biomass production of these resistant yeasts was evaluated. The *Pichia Jadinii* yeast presented a biomass of 5.87 g/L hydrolyzed at 120 hours with a C/N 14 ratio and *Cyberlindnera Jadinii* presented a biomass of 10.50 g/L hydrolyzed at 96 with a C/N 11.5 ratio. Additionally, the lag phases and some kinetic parameters were determined with the help of the modified Gompertz model for the two yeasts. A proximal analysis and isolation of β-glucans (Muller's method) was performed on the biomass of the yeasts grown in the C/N ratios and times described (MULLER et al., 1996). The β-glucans isolated from each yeast were characterized, insoluble β-glucans (1-3) and soluble β-glucans (1-3) (1-6) were obtained for the two yeasts with the presence of some groups belonging to chitin and/or proteins. Finally, the hydrolysate of the EFB can be used for the production of β-glucans; however, methods to reduce production costs must be studied for a industrial scale.



**Key words:** EFB, Hydrolysate, Diluted Acid Pretreatment, β glucans, C/N ratio, *Pichia Jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612.

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# CHAPTER 1. INTRODUCTION

The oil palm "Elaeis guineenses" is a perennial crop, coming from Africa and one of the most economically important in the world. In Brazil, in the Pará region, more than 340000 tons of palm oil / year are produced. However, for every kilogram of palm oil produced, 1.1 Kg of Empty Fruit Bunches (EFB) are generated (LAI et al., 2012). The EFB is an abundant lignocellulosic residue in the production of palm oil and has a large amount of hemicellulose sugars (25-30% w / w dry EFB) and is currently not well exploited (RAMAN; GNANSOUNOU, 2014). For this reason, the palm oil industry is seeking different alternatives for the use of the EFB, such as the production of high value-added compounds.

For the release of sugars in a liquid phase from lignocellulosic material there are different techniques (physical, chemical, biological and physico-chemical) called pretreatments. Some of the characteristics to consider for the selection of a pretreatment are, the speed of reaction, the generation of toxic products, the cost, the ease of application, among others. This step is very important because it can define the economic viability of the project (ZABED et al., 2016).

One of the most used microorganisms for the generation of high added value compounds from hemicellulose sugars are yeasts. because they have a high resistance to toxic compounds present in hydrolysates, they grow rapidly and can generate different types of compounds such as proteins, enzymes, oil, carbohydrates, biomass, among others (RAJOKA et al., 2004; RAMACHANDRAN et al., 2005; NAESBY et al., 2009; ZHAO et al., 2010; KAMOLDEEN et al., 2017).

Currently, a product with high added value and biotechnological interest, food and pharmaceutical, are beta glucans from yeast. These polysaccharides have shown great potential for the prevention of human diseases such as antimicrobial, anti-inflammatory, prebiotic, among others (PIZARRO et al., 2014, RAMAN, GNANSOUNOU, 2014).

Therefore, the following bibliographical review will focus on

- Describe the current situation on the production of the EFB in Brazil and the characteristics of the lignocellulosic residues

- Specify the advantages and advantages of different pre-treatments for a later selection.

- To review the biotechnological potential of the use of yeasts for the utilization of sugars from lignocellulosic waste

# CHAPTER 2. BIBLIOGRAPHIC REVIEW

## 2.1. PALM

### 2.1.1 Oil palm in the world and Brazil

The oil palm "Elaeis guineenses" is a perennial crop, coming from Africa and one of the most economically important in the world. The palm is cultivated in more than 40 countries and data from the Food and Agriculture Organization (FAO) of 2014, show that, 57.328 million tons of palm oil were produced, on 17.32 million hectares worldwide (http://www.fao.org/faostat/en/#data/QC/visualize, 2014).

Indonesia and Malaysia are the countries with the highest oil palm production, in South America stand out Colombia, Ecuador and Brazil, in order of production from highest to lowest, respectively (CORAL MEDINA et al., 2015). Brazil for 2016 has produced 340000 tons of palm oil (www.indexmundi.com, 2016), but by different government programs and the creation of institutions has encouraged the production of palm oil. Programs such as incentives for the use of renewable energy in the 1980s, the program of sustainable palm oil production in Brazil in 2010, and the "ZEE" (50/50) law regulating crops in the Amazon in 2012 are some of the proposals to increase plantations in the country (http://www.agricultura.gov.br, 2017). Embrapa reports that Brazil in 2013 had 180000 hectares cultivated with palm, but has a capacity of 38 million hectares for the expansion of cultivation (Embrapa, 2013). In addition to proposals to increase palm plantations in the country, new plant species are being developed to increase oil production yields and the palm life cycle (ABIOVE et al., 2016).

### 2.1.2. Cultivation of oil palm

The oil palm is a perennial crop and its plantations reach up to 30 years, although they can last many more. However, after this period the yield of palm oil extraction may decrease, because it is strongly related to the age of the crop. The Palm Oil production begins after three years of starting the crop, when the palm gives its first clusters (LAI et al., 2012). After that, the fruits are sterilized and then the oil is extracted by compression. The residue of this extraction is called Empty fruit bunches (EFB) and it is very studied because it is a residue for this industry.

### 2.1.3. Processing of palm oil and co products

The oil palm clusters are cultivated after three years of planting, then the clusters are sterilized for a period between 65 to 90 minutes, to inactivate the lipases and soften the fruit. Then the treated bunches are squeezed to separate the rachis from the fruit. The rachis is used as a fertilizer for the plantation, while the fruit goes to a maceration process to form a paste, where the palm oil is extracted. After the oil is clarified to remove impurities, and the fiber generated with the nuts is dried to be separated later (LAI et al., 2012).

EFBs are used for the generation of energy in the boilers. The amount of EFB generated in the palm oil production process is 1.1 kg per kilogram of oil, according to data reported by Shinoj et al. (SHINOJ et al., 2011). In Brazil more than 350000 ton / year of EFB were generated, and this residue can not all be incinerated.

### 2.1.4. Empty fruit bunches

The EFB is a fibrous material that is composed of cellulose, hemicellulose, lignin and other minor compounds (ashes, proteins, extractive). This composition varies depending on the geographical position of the crop, the environmental conditions, the varieties of palm cultivation and agricultural management (TAN et al., 2013; RAMAN; GNANSOUNOU, 2014). Shinoj et al, report that EFBs have between 30-40% cellulose, 20-27% hemicellulose and lignin between 20-35% (SHINOJ et al., 2011). Raman and contributors in 2014, obtained a result according to the ranges described by Shinoj analyzing the EFBs of the Pará-Brazil region and determined that, the composition of the EFBs of Pará was 33.5% of glucans, 26.8% of xylans, 2% of arabinans, 0.2% galactans, 20.1% insoluble lignin, 1.1% soluble lignin, 12.9% extractives, 5.1% acetylated groups and 2.8% ash (RAMAN; GNANSOUNOU, 2014).

Due to the complex chemical composition of the EFB and its recalcitrance, it is difficult to make a biotechnological use of this product. For this reason, many investigations are carried out focusing on cellulose, lignin and hemicellulose separation processes. These separation processes for a later biotechnological use are called pretreatments, and depending on the nature of the pretreatments are classified as physical, chemical or biological pre-treatments.

## 2.2 PRETREATMENTS OF LIGNOCELLULOSIC SOURCES

Currently more than 200 billion tons of agricultural vegetable biomass are produced per year worldwide (HU; RAGAUSKAS, 2012), however, the low utilization and the negative environmental impacts caused by this lignocellulosic material in several cases, is one of the main problems to be solved. Different alternatives for the use have been proposed as, use for biofuels, fertilizers, generation of biocompounds of industrial interest and substrate for biomass generation.

One of the best alternatives for the use of plant biomass is as a substrate for growth of microbial biomass, which can be used later in animal and/or human food.

Among the advantages offered by the use of plant biomass for the generation of microbial biomass are its great abundance, the reduction of the environmental impact of these wastes generated by different crops, the biomass renewable, nutritional improvement of the animal ration and decrease in the prices said ration (SINGH et al., 2010; FES et al., 2016). However, biomass have disadvantages, which have not allowed it to be the perfect substitute for the generation of biomass, some of them are, a high processing value, complexity in the composition of biomass, microbiological and enzymatic inhibitors, among others. All these problems associated with biomass generate low profitability or economic infeasibility, which poses new challenges for the use of these biorecursos.

To improve the generation of products from vegetable biomass and increase economic profitability, different productive aspects have been investigated, such as the recalcitrant chemical composition of the biomass, the different species, the effect of the cultivation conditions, the genetic modifications, types of industrial treatments, among others.

The chemical composition of plant-based biomasses is the most important industrial constraint, because it is heterogeneous and has complex chemical structures that make processing difficult. Biomass of plant origin are composed of three macromolecules (cellulose, hemicellulose and lignin). Each of these macromolecules is composed of monomers classified as sugars or polyalcohols.

Cellulose is a complex of molecules that form a network and support plant cells, is formed by hexoses (glucose) and depending on their ligations, can be called amorphous or crystalline cellulose (weak or strongly bound, respectively). On the other hand, hemicellulose is a set of molecules that provides elasticity to plant cells and is composed mainly of pentoses (Xylose, Arabinose, among others) and some hexoses. Finally, lignin is an amorphous structure that confers rigidity to the plant cell, due to the union of various polyalcohols, and can be classified as soluble or insoluble lignin depending on its bonds (ZABED et al., 2016).

For a long time, the industry sought to separate the cellulose fraction of the lignin and the hemicellulose, by different chemical, physical and/or biological methods; due to the facility to ferment glucose by many microorganisms and transform it into different biocompounds with diverse added value. Later on, it was not only used the cellulose, but also the hemicellulose and the lignin. The sugars of five carbons, they are only fermented by some microorganisms and their products are limited compared to the derivatives of sugars of 6 carbons, however, there are several compounds of high added value that can be produced. In another hand, lignin was seen as the waste of downstream processes from plant biomass, but various investigations on the structural polyalcohols of lignin have shown that they can generate high value-added products such as antioxidants and antimicrobial compounds of degree GRAS (Generally Recognized As Safe). Although all the fractions of the biomass generate derivatives of high added value, the separation processes limit their implementation and the industry's approach to take advantage of only one or two of the fractions, decreases the profitability in the use of the selected biomass. For this reason, the concepts of biorefineria and optimization have become frequent when considering plant biomass as an alternative of solution, these concepts seek to increase the profitability of the biorefinery, reducing the losses of raw material, of time and economics. On the other hand, increasing the efficiency of the separation processes and the diversity of products.

### 2.2.1 Biomass composition

Vegetable biomass is a source of energy, high value-added chemical and biological products widely available (200 billion tons per year) and renewable worldwide (HU; RAGAUSKAS, 2012). Due to this, biomass derived from food,

energy or industrial crops are investigated for the production of biocompounds, biofuels and microbial biomass, because they do not compromise food security. However, the great difference of sources of biomass, age, part of the plant, harvest and environmental factors make the organic chemical composition has a large variation in its main fractions (hemicellulose, cellulose, lignin and proteins)(KLASS, 1998).

#### 2.2.1.1 Cellulose

Cellulose is the most abundant polysaccharide on the planet, because it is part of the plant cell wall. The function of this polysaccharide is structural and its percentage varies between species, in young plants it can be 40% and in cotton, greater than 90%. This polysaccharide is made up of hundreds or thousands of units of beta D-glucose with glycosidic bonds 1-4, general formula ($C_6H_{10}O_5$) n and molecular weight between 300,000 and 500,000 Da, structurally it can be linear or fibrous (multiple hydrogen bridges). Regarding its organization, cellulose can be classified as amorphous and crystalline (disordered and ordered, respectively). Amorphous cellulose is weakly bound to one another, which facilitates hydrolysis and is enzymatically more accessible than crystalline cellulose (ZHAO; CHEN, 2013).

In another hand, cellulose is an intermediate product where important aspects such as production yield and digestibility must be analyzed. Because obtaining large amounts of cellulose with low digestibility can generate low yields for subsequent harvesting processes, therefore, it is important to achieve a good relationship between these parameters (HU; RAGAUSKAS, 2012).

The partial hydrolysis of cellulose generates cellobiose, cellotriose and celotetrosa, which can be saccharified in a later step more easily (DELGENES; PENAUD, V. Y MOLETTA, 2003; NADEEM AKHTAR, KANIKA GUPTA, DINESH GOYAL, 2015) and its total hydrolysis generates glucose, which can be transformed into different products such as ethanol, organic acids and other biocompounds of high added value (HAYES et al., 2008; LIMAYEM; RICKE, 2012)

#### 2.2.1.2 Hemicellulose

Hemicellulose is a heteropolysaccharide that bound cellulose with lignin in the cell wall of the plant, it has a generic formula ($C_5H_8O_4$) n and is made up of different polymers, which in turn are composed in a greater percentage by units of sugar residues such as mannose, glucose, galactose, xylose and arabinose, and sometimes fructose and rhamnose monomers are found. In a lower percentage, acidic sugars can be found α-D–glucuronic, α-D–galacturonic and α-D-4-O-methylgalacturonic acid. These polymers have a variable length between 500 to 3000 units, depending on the type of plant and the processes to which they were subjected. The most important polymers in hemicellulose are, xylan (1 → 4 β- D-xylose) present in hardwoods and agroindustrial waste, glucomanans (β- D-glucose y β- D-manose) present in softwoods and galactoglucomanans (α-D-galactose, β-D-glucose y β-D manose) (KLASS, 1998; ZABED et al., 2016).

Table 1. Amount of hemicellulose in lignocellulosic products

| Name | Hemicellulose (% w/w) |
| --- | --- |
| Switch grass | 30-50 |
| Kraft paper | 9.9 |
| Newspaper | 25-40 |
| Corn stover | 24-26 |
| Rice straw | 23-28 |
| Softwoods | 35-40 |
| Hardwoods | 45-50 |

Adapted from Zabed and contributors (ZABED et al., 2016).

The processing of hemicellulose as an intermediate is more limited compared to cellulose, because not many microorganisms have the capacity to use five-carbon sugars as a substrate. Some of the most important compounds generated by the hydrolysis of hemicellulose are xylose that can be used as a final product in the food and cosmetic industry, or as an intermediate product for the generation of furfural, xylitol or hydrogen (RAMAN; GNANSOUNOU, 2014).

#### 2.2.1.3 Lignin

It is an aromatic heteropolymer (benzene rings joined with methoxy, hydroxy and propyl groups) in matrix form, constituent of the plant cell wall and the second most abundant after cellulose, its function is to unite the plant and provide resistance. The constituent monomers of the lignin matrix are, the coniferyl

alcohol, sinapyl alcohol and p-coumaryl alcohol. When these alcohols are polymerized they form the guaiacyl, syringyl, and p-hydroxyphenyl molecules (WONG, 2009). The proportion of lignin in the plant cell on dry basis varies depending on the species and processes, the softwoods have between 30-60% w / w, the hardwoods have between 30-55% w/w, while non-woody species they have between 10 - 30% w/w and agro-industrial waste 3 - 15% w / w (LIMAYEM; RICKE, 2012; ZABED et al., 2016). Unlike hemicellulose and cellulose, the biological processing of lignin is more complex and very few microorganisms can biotransform this substrate, for this reason, lignin is used as a source of electrical or thermal energy by direct incineration (DAVIS et al., 2016).

The products derived from lignin currently are not found on an industrial scale, however, the high estimated value of these makes them attractive for the pharmaceutical and food industry. Some of its most important functions is as carbon fiber, antioxidant, antimicrobial (BECKHAM et al., 2016).

### 2.2.2 Biomass pretreatment

For the use of different products (energy and bioproducts of high added value) from the lignocellulosic materials, there are different steps and configurations of them; These involve chemical, physical, biological processes and / or their combinations, to obtain the highest industrial yields. However, although several steps are necessary to obtain the different fractions or bioproducts in the processing of lignocellulosic biomass, the most important step is pretreatment. Which seeks to separate hemicellulose and lignin from the lignocellulosic matrix, increase the degradability of cellulose (redistribution of lignin), avoid the generation of toxic compounds for microbial growth (furfural, hydroxymethyl furfural, acetic acid and fumaric acid), reduce energy and economic costs (because it is the most expensive step in the use of biomass). There are several pretreatments which vary depending on the reaction conditions, each with advantages and disadvantages (ZABED et al., 2016). These pretreatments can be classified into physical, chemical, physical - chemical and biological. And the selection of pretreatment depends on the type of biomass, pretreatment value, environmental impact, inhibition compounds generated, initial investment, among others.

#### 2.2.2.1 Physical pretreatment

These pretreatments are based on the modification of the physical conditions (reduction of size, temperature or unconventional treatments) to which the biomass is subjected and seek to facilitate the separation of its constituent polymers, reduce the crystallinity of the cellulose (the crystallinity is determined for the percentage of crystalline material and is called crystallinity index), increase the surface area and the size of the pores in the fibers, all with the purpose of increasing the yield of hydrolyzable sugars and lignin, trying to preserve their biological characteristics for a future use (SUN et al., 2016). There are different physical pre-treatments, then the most important ones and some applications of them are named.

**Comminution**

This physical pretreatment is an upstream process of particle size reduction of the lignocellulosic material and is usually accompanied by another pretreatment. The reduction of the particle size is based on variables such as milling methods (ball, compression, hammers, among others) the process time and the nature of the lignocellulosic matrix (ZHANG et al., 2011).

For the application of the communition a previous or simultaneous drying is used by natural or forced convection depending on the biomass and the energy and economic expenses. Then the size reduction of the biomass is made at the macroscopic level, at a microscopic level there is a rupture of the cellulose microfibrils or crystallinity (3-6 nm) to increase the surface area and the efficiency in the generation of bioproducts (DELGENES; PENAUD, V. Y MOLETTA, 2003; ZHANG et al., 2011). After the comminution step, the material is classified and selected by granulometry. However, in spite of increasing production yields, the non-separation of any of the fractions, the time and energy expenditures for its large-scale implementation must be analyzed. (FAN et al., 1988).

**Non-conventional physical treatments**

There are different non-conventional methods for the pre-treatment of biomass, which share as a common characteristic the use of radiant energy to achieve specific objectives with defined mechanisms, according to the technology used. These pretreatments present advantages such as effectiveness in heating and

heat transfer, homogeneous distribution of heat, require low temperatures and low production of toxic compounds. Among the most important unconventional pretreatments are the electron beam, gamma ray, and ultrasound.

**Electron beam**

This pretreatment uses ionizing radiation that causes the breakdown of β-glycosidic bonds and increases the digestibility of cellulose in the saccharification process and partially degrades lignin, avoiding the negative effects of excess temperature on the biomass (GRYCZKA et al., 2014). This technique generally complements other pretreatments to more efficiently separate the constituent polymers from the biomass and its application is rapid. However, some of the variables to take into account to apply the Electron Beam are the high costs of application, partial loss of hemicellulose by depolymerization and partial binding with lignin and cellulose, increased cellulose crystallinity by the formation from a para-crystalline phase between crystalline and amorphous cellulose, high radiation dose and low penetration of the fibers by radiation (DUARTE et al., 2012; KARTHIKA et al., 2013; SUNDAR et al., 2014).

**Gamma Rays**

The technique with gamma rays consists of the application of photons of high energy, short wavelength and high frequency on the biomass, which break the links β-1-4 glicosidics and decreases the crystallinity index of cellulose in the presence of water. In addition, it forms intermediates such as phenoxy radicals in the cellulose hydrolyzing process, increases the amount of reducing sugars and breaks other bonds by the degradation of secondary molecules (YOON et al., 2012). This is due to the weakening of the van der Waals forces between the molecules and the generation of free radicals. Generating greater delignification and depolymerization, when the radiation dose is increased (YOON et al., 2012). This process increases the separation effect of molecules of industrial interest when mixed with other pretreatments (HYUN HONG et al., 2014).

**Ultrasound**

This type of pretreatment is based on the application of energy in the form of sound waves with a frequency higher than 20,000 Hz. When the biomass is

suspended in an aqueous medium and the ultrasound is applied, the energy is transmitted to the lignocellulosic matrix by the phenomena, cavitation and warming (REHMAN et al., 2014). These phenomena produce hydromechanical shear forces due to the formation of bubbles and explosion of them, which can break some bonds, generate localized heating and increase the surface area in the fibers of the matrix, however, this pretreatment must be used with other pretreatments to achieve sugar saccharification with good yields (LUO et al., 2014).

#### 2.2.2.2 Physicochemical pretreatment

The physical-chemical pretreatments seek to remove fractions other than cellulose in plant biomass, applying specific conditions of temperature and pressure, with or without the use of organic or inorganic catalysts. Decreasing the crystallinity of the cellulose and generating the least amount of furfural, hydroxymethyl furfural and acetic acid. This type of pretreatment has as advantages a low environmental negative impact and high selectivity in cellulose separation. As disadvantages have the high cost for implementation. The most important physical-chemical pretreatments are steam explosion, ammonia fiber explosion and $CO_2$ explosion.

**Steam explosion**

It is a pretreatment that causes the rupture of the structure of the biomass, due to the degradation of the hemicellulose and the transformation of the lignin, by breaking the inter and intra molecular bonds in the matrix by means of a pressure change to increase the accessibility enzymatic in cellulose. This process is generally applied to biomass of vegetable origin due to its recalcitrance. The effectiveness in the application of the steam explosion depends on the operating conditions, the particle size of the biomass, the design technology, the decompression speed and the water content in the reactor. (VOCHOZKA et al., 2016). The operating conditions in this pretreatment are, temperatures between 160 to 260 ºC and pressures between 690 to 4830 kPa which are then reduced by a decompression to atmospheric pressure (SUN; CHENG, 2002). The disadvantages of this pretreatment are, the high economic value for its operation and the amount of lignin retained in the solid fraction after pretreatment (DATAR et al., 2007).

### Ammonia fiber explosion (AFEX)

This process uses high pressures during the reaction (injecting ammonium) and then quickly releases this pressure inside the reactor until reaching atmospheric pressure to degrade the hemicellulose to oligomers, release the largest amount of lignin and increase the surface area of the cellulose for later saccharification. The independent variables to be controlled in this pretreatment are the temperature, the moisture content in the biomass, the amount of ammonium and the residence time. The most frequently used operating conditions for the pretreatment are the injection of dry biomass and anhydrous ammonium in a 1: 2 ratios, reaction pressures between 17 to 20 bar and temperatures between 60 to 100 ° C for periods of time ranging from 5 to 30 minutes. The ammonia fiber explosion has as advantages a reduction in the energy demand in the process, no generation of toxic compounds and has better effects in biomass of agroindustrial type compared to the steam explosion. It has economic disadvantages when injecting ammonium for the reaction, it does not generate two easily separable phases in downstream processes and it does not totally degrade the hemicellulose (CAPOLUPO; FARACO, 2016)

### Carbon Dioxide explosion

The pretreatment is investigated since the nineties, seeking to increase the permeability of cellulose and decrease its crystallinity, to facilitate saccharification. The process involves injecting carbon dioxide (a molecule similar in size to water and ammonium) in the reactor, increasing temperature and pressure, which in some cases reaches the super critical point of the substance, to take advantage of the gaseous behavior of the molecule and its high penetration power in lignocellulosic fibers. Unlike other substances, carbon dioxide reaches its super critical point at a low temperature and a moderate pressure (31ºC and a pressure of 73.8 bars). Sometimes different organic solvents such as ethanol are used to improve the removal of lignin and hemicellulose. Some important parameters in carbon dioxide explosion are the radius of carbon dioxide / biomass, the reaction time and the moisture content in the lignocellulosic matrix. The advantages of this pretreatment are, a low energy consumption compared to the steam explosion, it does not generate toxic degradation compounds and a high yield of saccharification. On the other hand,

the disadvantages are the high economic value of the pretreatment compared to chemical hydrolysis, the use of carbon dioxide and the high resistance required by the reactor to withstand high pressures (CAPOLUPO; FARACO, 2016).

#### 2.2.2.3 Biological pretreatments

This set of pretreatments seeks to remove lignin by degradation processes, using microorganisms; also, in some cases, release of polysaccharides from the partial digestion of cellulose and hemicellulose occurs. To improve the effects of pretreatment, it can be accompanied by a hydrolysis process, following the degradation of lignin. The advantages of biological pretreatment are, low energy cost, easy implementation on an industrial scale (fermentation in solid state), reduction of the degree of crystallinity of cellulose, partial hydrolysis of hemicellulose, does not require the use of chemicals and does not generate pollution. However, it has as disadvantages the low degradation speed of the lignocellulosic material, the adequacy of the conditions for the fermentation for a prolonged time (pH, temperature and humidity) and the consumption of monosaccharides. (SUN; CHENG, 2002; PRASAD; ANKIT, 2015).

The microorganisms used for the decomposition of lignocellulosic material can be fungi or bacteria. However, fungi are the most effective and studied. The fungi can be classified according to the colonization time as, soft rot fungi (ascomycetes - filamentous fungi), brown rot fungi and white rot fungi (basidiomycetes) (see the table 2) (FAN et al., 1988). The bacteria used for the degradation of biomass are the actinomycetes, which have shown effectiveness in the hydrolysis of grasses. On the other hand, brown rot fungi have the ability to hydrolyze cellulose, and soft and white rot fungi degrade lignin and cellulose (PRASAD et al., 2007; PRASAD; ANKIT, 2015).

Brown rot fungi are common in conifers and in structural wood. These fungi degrade holocellulose with their hyphae by enzymatic and non-enzymatic mechanisms (GOODELL et al., 2008). The degradation process begins with the release of non-enzymatic compounds of low molecular weight, which cross the cell wall of the cells of the lignocellulosic matrix and generate hydroxyl radicals in the Fenton reaction. Then, these radicals attack different molecules within the plant cell such as the cell wall, which allow the extraction of cytoplasmic nutrients (GOODELL et al., 2008).

The fungi of white rot (basidiomycetes) are the most studied for their effectiveness in the degradation processes of hardwoods (GOODELL et al., 2008). These have the ability to hydrolyze low molecular weight lignin and hemicellulose, due to the production of degrading enzymes such as Lignin peroxidases and manganese-dependent peroxidases (PRASAD; ANKIT, 2015).
Soft rot fungi are used for degradation of lignocellulose with high moisture content and low lignin content. These microorganisms are characterized by degrading the wood by forming cavities in the sheet S2 of the cell wall of the wood and then, causing erosion from the lumen of the S3 interface outwards. To perform the degradative processes, these fungi generate different types of glucanases that attack cellulose, and low molecular weight compounds and oxidases to degrade lignin (GOODELL et al., 2008).

Table 2. Classification of some species of microorganisms used for biological pretreatment

| Bacterias | Fungi | | |
|---|---|---|---|
| | Brown Rots | Soft Rots | White Rots |
| Actomicetos | Piptoporus betulinus<br>Laetiporus sulphureus<br>Trametes quercina<br>Fomitopsis pinicola<br>Gloephyllum saepiarium<br>Gloeophyllum trabeum | Paecilomyces Sp<br>Graphium Sp | Fomes Jomentarius<br>Phellinus igniarius<br>Ganoderma appalanatum<br>Armillaria mellea<br>Pleurotus ostreatus<br>Phanerochaete chrysosporium<br>Ceriporia lacerata<br>Cyathus stercolerus<br>Ceriporiopsis subvermispora<br>Pycnoporus cinnarbarinus |

**2.2.2.4 Chemical pretreatment**

They are the most commonly used because of the effective separation of hemicellulose or lignin. Among them are pretreatments, concentrated or diluted acid, alkaline, wet oxidation, ionic liquids, wet oxidation and organo solvents. All these pretreatments seek to separate the fractions of the biomass, decrease the crystallinity index of the cellulose, the degree of polymerization in the lignocellulosic matrix and decrease the generation of toxic compounds (XU et al.,

2016). The advantages of chemical pretreatments are its low cost, they can be used with different biomasses, high reaction speed and easy industrial application. However, they have disadvantages such as the value of two reagents and unfavorable environmental effects. The most commonly used and some related studies will be briefly described below.

**Acid concentrated pretreatment**

The concentrated acid pretreatment uses concentrated inorganic acids as a catalyst, among them, sulfuric, hydrochloric or phosphoric acid. The pretreatment conditions are generally, inorganic acid concentration between 37 - 86% w / w, temperature between 30-60ºC, moderate pressures and continuous flow (BENSAH; MENSAH, 2013). Among the advantages of concentrated acid pretreatment are high yield in the recovery of sugar monomers and little presence of toxic compounds derived from the degradation of sugars by the low temperatures used. On the other hand, it has some disadvantages such as the neutralization of the solid phase before saccharification, high catalyst utilization, difficult recovery of the catalyst, corrosion of equipment (SATHITSUKSANOH et al., 2010).

**Alkaline pretreatment**

The pretreatment is widely used in the paper industry and is characterized by using as a catalyst a base to hydrolyze the ester and glycosidic bonds within the lignocellulosic matrices, it also breaks the lignin bonds and increases the crystallization rate of the cellulose by the removal of amorphous substances. This pretreatment uses moderate temperatures and pressures, compared to other pretreatments. The most used bases are sodium, potassium, calcium and ammonium hydroxides (KUMAR et al., 2009). After the pretreatment, two phases are formed, the wet phase which is mainly composed of cellulose and the liquid phase, which is composed of dissolved lignin and hemicellulose. (BENSAH; MENSAH, 2013). Among the advantages of this pretreatment are the removal of lignin, the decrease of acetyl groups, moderate process conditions (except in processes with ammonium catalyst), little investment in machinery (except with ammonium catalyst) and recovery in the form of salts of the bases. The disadvantages of alkaline pretreatment are, the long times when working at

moderate conditions, the neutralization of the fibers after pretreatment, and the generation of degradation compounds. (KIM et al., 2016).

**Pretreatment with ionic liquids**

Ionic liquids are salts that are in a liquid state at room temperature. These salts have long chains of cations and a lower proportion of anions. Other characteristics of ionic liquids, is the selective solubility (can dissolve one of the compounds of the lignocellulosic matrix, can dissolve two compounds of the biomass without importing which are or the three), they are not very volatile and have their melting point by below 100ºC (BENSAH; MENSAH, 2013). The cation most commonly used as the ionic liquid is imidazolium, which has the ability to react with various anions such as nitrate and sulfate. Ionic liquids can be designed (varying length and branches) by combining cations and anions for specific biomasses. As a disadvantage, the ionic liquids can be corrosive, hygroscopic, viscous and can interact with the active sites of the enzymes (BENSAH; MENSAH, 2013).

This pretreatment consists of mixing the ionic liquids with the biomass, plus water or acids. The reaction can last a few minutes or hours, and the operating temperature varies between 80 to 160 ° C. After the reaction, anti-solvents are used to precipitate to recover the ionic liquids, accompanied by a downstream process to separate the solid phase from the liquid phase. The polarities of the ionic liquids influence the solubilization of the lignocellulosic matrix, the anions solubilize the cellulose better, while the dissolution of the lignin is related to the cations (QIDONG HOU, MEITING JU, WEIZUN LI, LE LIU, 2017).

**Wet Oxidation**

This pretreatment uses water with air or oxygen, to release as much lignin and hemicellulose from the matrix. The variables to be controlled in this pre-treatment are the air pressure, the temperature and the reaction time. When wet oxidation is applied all the constitutional fractions of the lignocellulosic material are affected. Hemicellulose is solubilized in water by high temperatures, lignin is hydrolyzed and oxidized, and cellulose is partially degraded. Sometimes this process is complemented with some bases to improve the results. Among the advantages of pretreatment are the high solubilization of lignin and hemicellulose.

The disadvantages are the generation of toxic degradation compounds and the high value of the injected gases (PRASAD; ANKIT, 2015).

**Organosolv**

The pretreatment consists in the use of organic solvents, polar or protrotic, at certain temperatures and pressures, mixed with or without acid catalysts, alkalis or salts (BENSAH; MENSAH, 2013). Among the advantages of the use of organo solvents is the high selectivity in obtaining the main components of the biomass. In contrast, this pretreatment has high prices compared (solvents and recovery) against acid pretreatment and presents corrosion problems, so its application on an industrial scale has not been possible. Among the most used organo-solvents are alcohols, polyhydric alcohols, alkylene carbonates, organic acids, methyl isobutyl ketone, tetrahydrofuran, ethyl acetate, acetone, among others (ZHANG et al., 2016).

The mechanism of the organo-solvents begins with the breaking of the bonds of the lignin of low molecular weight. When the process with organo-solvents is applied without the use of catalysts, the water is ionized and hydronium and acetic acid ions are released from the degradation of hemicellulose, which break the alpha and beta bonds of the aryl ether groups of lignin , causing its dissolution (ZHANG et al., 2016). On other hand, when catalysts are used the process is similar, however, the amount of acid in the reaction generates the solubilization of the hemicellulose and the amorphous cellulose  (ZHANG et al., 2016).

**Diluted acid pretreatment**

The pretreatment with diluted acid is the most reported worldwide. It consists in the application of a diluted inorganic acid catalyst on a lignocellulosic matrix under established temperature conditions, where the hemicellulose is diluted by the breaking of the covalent bonds, the hydrogen bridges and the Van der Waals forces of this fraction of the biomass and the two remaining fractions (LI et al., 2010), which generates greater porosity in the cellulose fiber and accessibility of the enzymes for its subsequent saccharification. Generally, the most used acid is sulfuric acid; however, other acids have been investigated such as nitric acid, hydrochloric acid, phosphoric acid among others. This pretreatment has as advantages the high solubilization of the hemicellulose, the moderate operating

costs compared with other treatments, short reaction time, high saccharification yields of the hemicellulose, among others. On the other hand, it has disadvantages such as the production of furfural, hydroxymethylfurfural (HMF), the condensation of the non-soluble lignin on the surface of the cellulose after the process and the difficult recovery of the catalyst (LI et al., 2010).

The most important process variables are the inorganic acid concentration, temperature and time. Generally, the concentration of the inorganic acid used in the process varies between 0.5 - 4% w / w, the temperatures worked are between 120 - 210ºC and the time is usually less than 1 hour (RAHMAN; CHOUDHURY; AHMAD; et al., 2006; BADIEI et al., 2014; TA et al., 2016). This pretreatment has been used successfully in the separation process of hemicellulose from EFB by Rahman and contributors. With hemicellulose recovery yields above 90% w/w and with few toxic compounds (RAHMAN; CHOUDHURY; AHMAD; et al., 2006).

## 2.3 PRETREATMENTS USED IN EMPTY FRUIT BUNCHES AND CONSIDERATIONS

The pretreatments are fundamental in the development of efficient processes for the use of lignocellulosic materials. The selection of a pretreatment is a very important and complex step, which defines the viability of a process based on many technological variables, intrinsic to the raw material, investment capital, environment, among others.

For the above, taking into account the ease of application, low cost, low environmental impact, speed, the experience of different investigations, in this work the pretreatment with diluted acid was selected.

## 2.4 BIOTECHNOLOGIC POTENCIAL OF YEAST

Taxonomically the yeasts are unicellular microorganisms belonging to the Fungi kingdom, with two phylum, Ascomycota and Basidiomycota; and more than 1500 species. Some of its most important characteristics are chemorganotrophs (they usually get their energy from hexoses, however, in some cases they use other sources such as pentoses, organic acids and alcohols). They can be aerobic, anaerobic or facultative anaerobes, they grow in media with different pH (however they prefer slightly acidic medium pH <7), the growth temperature varies between -2 to 45°C (depending on each species). The yeasts can grow in liquid and solid

media, the size varies between 3 to 40 micrometers, and their reproduction can be done by mitosis or budding (ASHBEE. H; BIGNELL. ELAINE, 2010).

The yeasts are widely used in industries such as food (bakery, wine and beer), pharmaceutical, chemical, environmental, biotechnology, energy, among others; for the ability to generate different products of interest such as biomass, chromosomes, primary metabolites, enzymes, fatty acids, amino acids and carbohydrates (RAJOKA et al., 2004; RAMACHANDRAN et al., 2005; NAESBY et al., 2009; ZHAO et al., 2010; KAMOLDEEN et al., 2017). Below are some uses of yeasts in different industries, highlighting *Saccharomyces cerevisiae* and the genus Ciberlindnera.

### 2.4.1 Cyberlindnera genre

Among the most important yeasts for bioprocesses, there is the genus Cyberlindnera, belonging to the class *Saccharomycetes* and the phylum *Ascomycota*, which is used in the food industry, both human and animal, because it is classified as GRAS (Generally recognized as safe) by the FDA ( Food Drug Administration). In this genus (*Cyberlindnera*), there are two species recently classified by molecular techniques such as *Pichia Jadinii* and *Cyberlindnera Jadinii*, with food, agroindustrial, environmental and pharmaceutical potential. These two yeasts, formerly considered as *Candida utilis* and the literature and studies on their biotechnological potential is strongly linked (all yeasts with certain biochemical and morphological characteristics were considered *Candida utilis*) with this last yeast (BUERTH et al., 2016). However, there is not much information about these yeasts, such as kinetic parameters in different substrates and conditions, possible mathematical models to simulate and optimize their growth and product generation, which would allow a more simple industrialization in the future.

### 2.4.2 Biotechnologic Potencial of the *Ciberlindnera jadinii* and *Pichia Jadinii*

*Candida utilis* has been reported by different researchers as important yeasts for the production of biomass, proteins, vitamins, acceptor yeasts with the capacity to express different genes and possible source of polysaccharides with relevance at the pharmaceutical level. In addition, it has been published that they have the ability to use different sources of monosaccharides as an energy source (glucose

or xylose) and tolerate highly toxic environments such as natural substrates pre-treated (substrates with acetic acid, furfural and hydroxymethyl furfural*)*. Due to the above, various researches are being carried out in the *Cyberlindnera* genre to take advantage of these advantages and, in addition to this, to determine specifically the biotechnological potential of each species based on the molecular classification of the species (BUERTH et al., 2016).

#### 2.4.2.1 Single cell Protein and biomass

The evident lack of food at the global level, triggered by the increase in the world population and the increase in life expectancy, has forced the food industry, scientists and countries, to look for different alternatives to supply the amount of nutrients necessary to the population. Of the nutrients to be supplied, the most scarce and complicated to produce are the proteins; due to the abundance of nitrogen (with respect to carbon, hydrogen and oxygen), trophic chains, social customs and limited knowledge about new sources of amino acids (NASSERI et al., 2011).

To solve the problem of global food, scientists have proposed several alternatives such as genetic modification of crops and animals, creation of food supplements, increase in the variety of animals for human consumption (insects) and the use of microbial biomass (called single cell protein ) for animal or human consumption. Of the above alternatives, the most promising is the use of microbial biomass because it can be produced quickly, and with low costs (NASSERI et al., 2011).

There are different types of single cell protein due to the diversity of microorganisms, however, one of the most important is the biomass generated by the yeasts. The production of yeast biomass has different advantages such as growth rate, adaptation to different substrates (natural and synthetic), the amount and composition of proteins, among others (NASSERI et al., 2011).

Several yeasts have been used for the production of single cell protein, among them are, *Candida tropicalis*, *Saccharomyces cereviciae* and *Candida utilis* (NASSERI et al., 2011). Ibrahim et al. 2004. Report that Candida utilis presents 32.75% w / w of protein grown in the polished rice residue and provides essential amino acids such as lysine, phenylalanine, methinine, leucine, among others.

Which are necessary for cellular operation and maintenance (RAJOKA et al., 2004). Martin and colleagues also used *Candida utilis* grown on synthetic medium and report a greater amount of protein around 48% w / w and the production of 18 amino acids (essential and non-essential)(MARTIN et al., 1993). The amount of proteins can be regulated through the C/N ratio (EGLI; QUAYLE, 1986).

Additionally, different investigations are being carried out for the production of Single cell protein from yeasts, not only for the nutritional contribution of amino acids, but also for the possible benefits to the food industry as emulsifiers and antioxidants (FERRACINI-SANTOS; HARUMI, 2009).

#### 2.4.2.2 Single cell Oil

The lipids in yeast are generally in the form of triacylglycerols, with fatty acids of 16 to 20 carbons, which vary depending on the phylogeny of the yeast, the substrate and the growth conditions. The lipids in the yeasts vary between 1.5 to 30% w / w cell dry, approximately (EL-FADALY et al., 2009; ZHAO et al., 2010; NASSERI et al., 2011). *Yarrowia lipolytica* and *Cryptococcus curvatus* are among the yeasts important for the production of lipids (EL-FADALY et al., 2009; ZHAO et al., 2010).

Depending on the proportion of insaturations of the fatty acids of the yeasts, they can have two purposes. If the amount of unsaturated fatty acids is low, they can be used for the production of biodiesel by the transestirification process. On the other hand, if the proportion of unsaturated fatty acids is high, they can be used in the food or pharmaceutical industry. The number of unsaturations depends on the substrate, the growing conditions and the type of yeast (ZHAO et al., 2010). If they have an unsaturation they are called MUFAS (Monounsaturated fatty acids) and if they have more than one unsaturation they are called PUFAS (Polyunsaturated fatty acids).

Among the unsaturated fatty acids of interest to the food industry are linoleic acid (C18:2), linolenic acid (C18:3), omega acids (3, 6 and 9), eicosapentaenoic acid and docosa- Hexaenoic These acids have been related to regulation of insulin secretion, antitumor, regulate low density lipoproteins, among other benefits (KELLY, 2001). The amount of lipids can be regulated through the C/N ratio

(EGLI; QUAYLE, 1986).

#### 2.4.2.3 Carbohydrates

Yeasts are widely used in different industries such as food, pharmaceutical, among others. The carbohydrates in the yeast as well as the proteins or lipids vary a wide range depending on the type of yeast, the substrate and the growth conditions. Unlike plants and algae, yeasts do not have the capacity to generate glucose through photosynthesis, but can store carbohydrates that were not used for the generation of primary or secondary metabolites. The main molecules to store carbohydrates in yeasts are glycogen and trehalose, however, they are not the only molecules with carbohydrates present in yeasts, they are also found, the mannanas, free glucose and glucans. Berke and contributors affirm that, the amount of mannanas and glucans does not vary much in spite of the stress generated on the cells. The amount of carbohydrates varies in storage, bone in the amount of glycogen and trehalose (BERKE; ROTHSTEIN, 1957).

Among the most important carbohydrates in yeasts are the mananas and the glucans. Mananas are usually linked to proteins and are called mannoproteins and these mannoproteins are used as emulsifiers and possible antioxidants (FERRACINI-SANTOS; HARUMI, 2009). In another hand, glucans can be α-glucans and β-glucans (the latter are considered the most important). α-glucans are used for the production of films and β-glucans are related to anti-inflammatory, antitumor, cholesterol regulating activity, among other health benefits (PIZARRO et al., 2014).

##### 2.4.2.3.1 β-glucans

Beta glucans are structural polysaccharides present in the cell membranes of different plants, yeasts, fungi and algae. These polysaccharides have functional properties such as cholesterol regulation, antimicrobial, anti-inflammatory, antioxidant, prebiotic, emulsifier, among others (PIZARRO et al., 2014; BZDUCHA et al., 2015).

Beta glucans are linear homopolysaccharides formed by glucose linked in the β-position (1-2), (1-3), (1-4) and/or (1-6). Sometimes they are branched as beta glucan (1-3)/(1-6) present in yeast. β-glucans of type (1-3) / (1-6) are found and their percentage varies between 2 to 5% w / w (MULLER et al., 1996). Depending

on the degree of polymerization of beta glucans, they can be more or less soluble. The size of the branches also varies among microorganisms, beta glucans of long or short branches can occur (PIZARRO et al., 2014). Beta-glucans (1-3)/(1-6) have different types of three-dimensional structures, they can be simple or triple helix. These conformations depend on the isolation processes of beta glucans (SAITÔ et al., 1989). The size of beta glucans varies widely within an organism and between organisms, the average sizes range from 5 to 2000 kDa in fungi (SYNYTSYA et al., 2009).

The structural conformation of the two main types of beta glucans (1-3)/(1-4) or (1-3) / (1-6) determines the functional properties of each polysaccharide. Beta glucans (1-3) / (1-4) present in vegetables, have been recognized in reducing the risk of coronary heart disease and help control the level of blood sugar by the food and drug administration (FDA), when 3g/day is consumed (PIZARRO et al., 2014). In another hand, beta glucans (1-3)/(1-6) have been associated with modulation of the immune system (PIZARRO et al., 2014). Data reported by Talbott and contributors reported that, the consumption of 250 to 500 mg of (1-3) (1-6) beta glucans for 4 weeks reduces the frequency of infections in the respiratory tract (TALBOTT et al., 2013). For the abundance of cereals as oats, the ingestion of beta-glucans of type (1-3) / (1-4) can be supplemented with the consumption of about 60 grams of oats / day. However, beta glucans (1-3) / (1-6) can not be easily delivered in an ordinary diet. For this reason, the pharmaceutical industry isolates, purifies and concentrates them in food supplements, which have a price ranging from 7.97 to 31.87 US$ depending on the purification processes (www.evitamins.com.br, 2018).

**2.4.3 Technical and tools to improve the production of biocompounds**

There are different techniques to improve (generate faster or greater quantity) the production of biocompounds (proteins, carbohydrates and/or lipids). All these techniques are based on the modification of the cellular metabolism, silencing some routes and enhancing other. The techniques can be of two types, by direct genetic modifications, or by some type of external stress (EGLI; QUAYLE, 1986). In the case of the food industry, the direct genetic modifications of some matrix or microorganism used in the manufacturing process of a food are not very accepted by the current trend towards the natural. For this reason, this food

industry seeks to improve the production of biomass or biocompounds generating some type of external stress and evaluating the adaptation response of the organism to the variation of its environment.

Within the types of stress most commonly used in microorganisms is the modification in nutrient concentrations (excess or limitation of nutrients), changes in temperature, pH, volume of inoculum, agitation, aeration, amount of inhibitory compounds in the medium, type of process, etc. For the above, one of the most used to modify the cellular composition is the change in the concentration of nutrients, with this type of stress different authors report variations in the relationship of the macromolecules and/or the generation of biomass (EGLI; QUAYLE, 1986).

#### 2.4.3.1 Effect of the carbon/nitrogen ratio on the production of biocompounds and biomass

One of the most important factors for microbial growth is the composition of the culture medium. Microorganisms have the ability to adapt to the limitations or excesses of nutrients in various substrates, changing their cellular composition (EGLI; QUAYLE, 1986). Several studies have shown that, a modification in the amount of carbon and/or nitrogen, influences the production of biomass, proteins, carbohydrates and lipids, by the modification of metabolic routes (blocking of some routes and activation of others) (LÓPEZ et al., 2003).

Studies carried out by Egli and collaborators, reported that, ratios C/N greater than 11.32 enhanced the production of carbohydrates and lipids in microorganisms, however, when the ratio was less than 11.32, a greater amount of biomass and protein were generated (EGLI; QUAYLE, 1986). Similarly Arous et al. Report a similar effect with C/N ratios around 10 for *Schwanniomyces etchellsii* (AROUS et al., 2015). This phenomenon of biomass generation and increase in protein production is explained by the immobilization and accumulation of nitrogen in media with low C/N ratio (RITTMANN; MCCARTY, 2001).

Therefore, in an industrial production of some biocomposite or biomass, it is important to determine the appropriate carbon/nitrogen ratio to obtain the highest

ratio of the compound of interest and/or the greatest amount of biomass, using the least amount of resources (nutrients, energy, time, etc.) and generating the least environmental impact. And it should be evaluated more quickly, when there are little studied microorganisms and whose biotechnological potential is not known, such as *Pichia jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612.

#### 2.4.3.2 Effect of the inhibition compounds of a hydrolyzate on microbial growth

The aim of the pretreatment with diluted acid is to release the greatest amount of hemicellulose from the lignocellulosic matrix. However, due to the severity of the pretreatment as a function of temperature, reaction time and acid concentration, toxic compounds are produced by the degradation of the 5 and 6 carbon sugars present in the lignocellulosic matrix (RAHMAN; CHOUDHURY; AHMAD, 2006).

The main toxic compounds that inhibit microbial growth generated in the pretreatment with diluted acid are hydroxymethylfurfural, furfural and acetic acid. However, under very severe pretreatment conditions other inhibition compounds such as formic acid, levulinic acid and lignin degradation products can be generated (LAVARACK et al., 2002).

Acetic acid is produced by the degradation of pentoses, following first-degree kinetics (LAVARACK et al., 2002). Delgenes et al report that concentrations of acetic acid above $3 g.L^{-1}$ generate partial or total inhibition of microbial growth, depending on the microorganism (DELGENES; PENAUD, V. Y MOLETTA, 2003).

Furfural is generated by the degradation of pentoses such as arabinose and xylose, following first order kinetics as described by Lavarack and contributors (LAVARACK et al., 2002). Data from Delgenes and contributors, affirm that furfural concentrations above $0.5 g.L^{-1}$ generate partial or total inhibition of microbial growth (DELGENES, PENAUD, V. AND MOLETTA, 2003). On the other hand, hydroxymethylfurfural (HMF) generated by the degradation of glucose present in hemicellulose or from amorphous cellulose, also presents a first-order kinetics (LAVARACK et al., 2002) and generates inhibition in

concentrations greater than 1 $g.L^{-1}$ (DELGENES, PENAUD, V. AND MOLETTA, 2003).

The production of secondary toxic compounds such as the phenolic derivatives of lignin, generate partial inhibition of microbial growth from concentrations higher than 0.1 $g.L^{-1}$. However, for the generation of these compounds very severe process conditions must be applied (DELGENES; PENAUD, V. Y MOLETTA, 2003).

Therefore, when using a hydrolyzate it is important to determine the quantities of toxic compounds in said aqueous phase and apply techniques such as dilution, detoxification of the medium or microorganisms resistant to these inhibiting compounds (LAVARACK et al., 2002; DELGENES; PENAUD, V. Y MOLETTA, 2003).

#### 2.4.3.4 Prediction of biomass and biocomposite production

The effect on the response variable (production of a biocompounds or biomass) of the application of controlled external stress in microorganisms is evaluated and predict by the application of statistical and mathematical techniques that allow determining if, occurred or not it will occur, any significant change in the production of some molecule of interest or biomass. Among the techniques associated with the evaluation and prediction of production of biocompounds are growth kinetics, production of metabolites and consumption of substrates, mathematical models for the kinetics described, optimization processes and selection of statistical variables, among others (TAYLOR; BLANCH, 1981).

##### 2.4.3.4.1 Kinetics of microbial growth and application of mathematical models

The microbial growth is the result of hundreds of metabolic reactions, ideally, it should be study each metabolic reaction, however, the variables to study are many, which makes this process difficult. On the other hand, several studies have reported that the study of a few variables and their behavior over time, may be sufficient to optimize the production of some compound or biomass quickly (TAYLOR; BLANCH, 1981). According to Mcmeekin and contributors, microbial

growth is a function of simple parameters such as the total growth of the microorganism and the phases of exponential growth and adaptation (MCMEEKIN; ROSS, 2002).

The microbial growth curve is a simple tool to determine the 4 phases of microbial development (adaptation phase, exponential, stationary and death) under controlled conditions of temperature, pH, agitation, aeration, among other variables. The kinetic studies try to diminish the phase of adaptation (phase of little cellular growth and little production of biocompounds) and reach the exponential phase quickly, because this generates a greater amount of biomass and generally some metabolites of industrial interest (MCMEEKIN; ROSS, 2002).

The microbial growth curve is determined by specific samples in a specific period of time. It is possible to apply mathematical models that allow to obtain some kinetic parameters of interest with greater precision, they help to forecast changes in the response variable in a specific time, they allow to save time and resources (MCMEEKIN; ROSS, 2002).

The most studied kinetic responses by mathematical models are the latency time (duration time of the latency phase), the generation time (time for the duplication of the initial microorganisms in the fermentation process) and the growth rate ( is the number of cells produced per unit of time). These can be modeled by the effect of the type of stress exerted on them. On the other hand, the kinetic variables most studied for their easy relation with economic balance are biomass yields and hourly productivity (TOBAJAS; GARCIA-CALVO, 1999).

The mathematical models of microbial growth can be of two types, segregated and not segregated. The segregated models consider a microbial cell different from another microbial cell. On the other hand, non-segregated models consider that the microbial population of a crop has no difference (it is an average). These two models can be in turn, structured or unstructured, where the unstructured models consider the reactions of the microorganism, from a single component, on the other hand, the structured models consider the metabolism of the microorganism as a function of several compounds. Another level to consider in microbial growth models is, whether they are deterministic or stochastic. Stochastic models take into account the distribution of cellular characteristics,

while deterministic models do not consider variations in a population (TAYLOR; BLANCH, 1981)

To select a model should take into account some features such as model accuracy, simplicity, growth conditions, among others. Among the most used models are the Monod model, the Gompertz model, the Richards model, the Standar model, the logistic model, etc (TAYLOR; BLANCH, 1981).

#### 2.4.3.4.2 Modified Gompertz model

It is a sigmoid function proposed by Benjamin Gompertz in 1825 and applied to several biological processes, including the growth of animals, plants, microorganisms and cancer cells. In 1987 this equation for the growth of microorganisms was applied and they found that it had great precision in the determination of the exponential and stationary phases, however, it was not very precise to determine the phase latency. After this finding, Zwietering and colleague modified the function, this increased the precision to determine the first three phases of the microbial growth, and likewise increased its use in agencies as the USDA (Department of agriculture of the United States). With time and applications, the Gompertz model has been modified, for that reason, not only is there a Gompertz equation (TJ; TJ, 2017). One advantage that the Gompertz model has over other models is the meaning of the constituent variables of the model. Below is the Gompertz model and the meaning of the variables. Taking into account the above, the modified model of Gompertz presents sufficient characteristics to be applied in yeasts of food type.

## 2.5 FINAL CONSIDERATIONS

Taking into account the high generation of EFB from the process of obtaining palm oil and the economic impact for the region of Pará and Brazil. It should look for alternatives for the production of high added value products from this co-product such as Beta glucanas, single cell protein or single cell oil.

## 2.6 OBJECTIVES

### 2.6.1 General Objective

Produce beta glucans from non-detoxified EFB hydrolysate using the yeasts *Pichia jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612.

#### 2.6.1.1 Specific objectives

- Produce and optimize the production of hemicellulose from the EFB, using diluted acid pretreatment and optimizing the variables acid concentration, temperature and time

- Evaluate and select the kinetic parameters of the biomass production of the yeasts *Pichia jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612 in the optimized hydrolysate of EFB varying the ratio C/N.

- Apply a microbial growth model that determines with precision the microbial growth phases as a first step for the optimization of the process.

- Determine the proximate composition of the yeasts *Pichia jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612.

- Isolate and characterize beta glucans from yeasts *Pichia jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612.

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## CHAPTER 3. OPTIMIZATION OF DILUTED ACID HYDROLYSIS APPLIED TO OIL PALM EMPTY FRUIT BUNCHES TO OBTAIN HEMICELLULOSE


**Abstract**

The EFB is a lignocellulosic waste generated in large quantities in the region of Pará Brazil (around 370000 tonnes/year) and with a high amount of pentoses, that can be used for the production of high added value products such as xylitol, cell protein, cell oil, others. The objective of this work was to determine the conditions of the variables temperature (° C), sulfuric acid concentration (% w/w) and reaction time (hours) to release the highest amount of hemicellulose sugars in the diluted acid pretreatment. The response surface methodology was used to optimize the hydrolysis process of the variables evaluated. The highest amount of hemicellulose sugars released was 24.18 g/100 g of EFB, in the condition with 2.9% w/w of sulfuric acid, 125 °C and 25 minutes.


### 3.1 INTRODUCTION

Currently most bioproducts of industrial interest are produced by petrochemical or biotechnology industries (based on the use of first generation raw materials). However, this type of production presents disadvantages such as contamination (20% of greenhouse gases), dependence on oil and the decrease in food production (BIDDY et al., 2016).

An alternative in the production of biocomposites or microbial biomass of industrial interest is the implementation of biorefineries that could make use of lignocellulosic residues generated from local agroindustrial processes, which are low cost, abundant, help to reduce the environmental problems generated by the petrochemical industry and are not used as food (CORAL MEDINA et al., 2015).

In Brazil, one of the most important agro-industries economically and presenting high production of lignocellulose residues is the oil palm industry. Brazil in 2016 produced 340 million tons of palm oil (www.indexmundi.com, 2016). For this reason, the palm oil industry is looking for process that could make for the use of these co-products. One of the residues of the palm oil industry are empty fruit bunches, which are generated after sterilization of the palm fruit and extraction of the oil. Data reported by Shinoj and contributors show that for every kilogram of oil produced, 1.1 kg of empty fruit bunches are generated. (SHINOJ et al., 2011).

The oil palm empty fruit bunches are a complex lignocellulosic matrix to take advantage of, by the chemical composition they present. They are made up of lignin, hemicellulose, cellulose and other compounds to a lesser proportion, and for their biotechnological use it is necessary to separate these compounds (RAHMAN; CHOUDHURY; AHMAD, 2006).

There are different methods (pretreatments) for separating the major fractions of the lignocellulosic materials. One of the most used pretreatments is the hydrolysis using a dilute inorganic acid, also known as acid hydrolysis, because it is an economical, efficient and few negative effects on the environment. In this type of hydrolysis, the plant material is subjected to specific conditions of temperature, time, pressure, agitation and amount of acid to solubilize the hemicellulose. The obtained hemicellulose exhibits an heterogeneous composition conformed by monosaccharides of 5 and 6 carbons, being xylose and arabinose the main ones (CHIANG et al., 2008).

Xylose and arabinose are 5-carbon sugars, which can be transformed by microorganisms into different bioproducts such as xylitol, ethanol, lactic acid, formic acid, biomass, among others. For this reason, many investigations are made in different lignocellulosic matrices to recover the largest amount of hemicellulose using acid hydrolysis as pretreatment and different statistical optimization tools (ZHANG et al., 2012).

For this reason, the aim of this work is to optimize the recovery of hemicellulose (liquid phase with all sugars obtained after acid hydrolysis) using acid hydrolysis as pretreatment and modifying process conditions such time, temperature and amount of acid.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Adequacy Process of Empty Fruit Bunches

**Raw material**

The different batches of empty fruit bunches (EFB) were obtained from the Biopalm company located in the state of Pará, North of Brazil in the year 2014.

**Drying**

Subsequently, the EFB were sun dried for a few days and then dried in a air-circuling oven for 48 hours until a moisture content lower than 10% w/w was obtained.

**Milling, sifting and selection of EFB particle size**

Having dried the EFB, its surface area was increased in a mill Marconi. Subsequently, the milled biomass was divided into 4 fractions in a Bertel sieve. The fraction of EFB used in the optimization process of the acid pretreatment was retained between the Tyler reference screens 42 and 80, and the size of these particles was between 0.177 and 0.355 mm. This fraction was selected according to previous studies on acid pretreatment for EFB (CORAL MEDINA et al., 2015).

**Homogenization of EFB and storage**

The different batches of EFB with the selected particle size were all mixed to have a homogeneous sample and they were stored in a dry place at room temperature until further use.

### 3.2.2 Composition Analysis of EFB

After increasing the surface area and reducing the humidity in the EFB, its composition was analyzed using the protocol of the National Laboratory of Renewable Energies (NREL) for determination of structural carbohydrates and lignin in biomass - version 07-08-2011 (SLUITER et al. , 2012). For the calculation of soluble lignin, the sugar cane parameters defined in the same protocol were used.

### 3.2.3 Diluted Acid pretreatment

The acid pretreatment was done using a Parr reactor model 4848. The medium consisted of 10 g of dry EFB / 100 g of liquor - the mass ratio of EFB and acid solution used was according to the procedure of Coral and Collaborators (CORAL MEDINA et al., 2015), and 1.5 - 3.5 g of sulfuric acid / 100 g liquor. The operating time ranged from 20 to 60 min and the temperature was evaluated at 110 to 130 ° C. After the process was complete, the medium was vacuum filtered and the liquid and solid phases were recupered. The solid phase was dried at 45 ° C for 24 hours, weighed and stored in an airtight vessel. On the other hand, the liquid

phase was analyzed for xylose, arabinose, cellobiose, glucose, acetic acid, hydroxymethyl furfural (HMF) and furfural by high performance liquid chromatography (HPLC) and then stored in refrigeration until its later use.

### 3.2.4 Liquid phase processing and analysis

A 5 ml aliquot of each pretreatment centrifuged at 10,000 rpm for 10 min, microfiltered using a 0.22 µm pore membrane and deposited in a vial for HPLC. The HPLC runs were done on a chromatograph (1200, Agilent Technologies, USA) with IR detectors (Agilent, HP1047A) for sugars detection and UV detector (Agilent, G1315D) for the other components analyzed. For the analysis, an animex HPX-87 column (300x7.8mm, Bio-Rad, USA) with $H_2SO_4$ (5mM) as mobile phase, a flow rate of 0.6 ml/min and 65 ºC were used.

### 3.2.5 Combined Severity Factor (CSF)

In function of the acid hydrolysis parameters used in each experiment (time, temperature and acid concentration), the corresponding combined severity factor was calculated according the equation 1 (HSU et al., 2010).

$$CSF = log\left(t\,.Exp\,(Th - Tr)/_{14,75}\right) - pH$$

*Equation 1. Combined severity factor (CSF)*

t= reaction time in minutes

Th= reaction temperature in Celsius degree

Tr= reference temperature (100°C by reference)

pH= hidrogenionic potencial of solution

### 3.2.6 Statistical analysis

For the pretreatment optimization the response surface methodology was used $3^{3-1}$ and as a statistical design a fractional factorial design with 11 runs were made. The samples were evaluated in function to 3 independent variables with three levels according to Rahman and contributors (RAHMAN; CHOUDHURY; AHMAD; et al., 2006), the first variable evaluated was the acid concentration expressed as mass percentage (% w / w). The second was the time (min) and the last variable was the temperature (°C). The table number 3 shows the coding of the levels per each variable and the table 4 shows the runs sequence of the

acid pretreatment. The statistical software used was Statistica 7. The prediction model for the estimation of the optimal point was the one expressed by equation 2:

$$Y = \beta o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11}(X_1)^2 + \beta_{22}(X_2)^2 + \beta_{33}(X_3)^2$$

*Equation 2. Regression Model for the xylose release*

Where:

$Y$ = Xylose quantity (g xilose/L licor)

βo = Intercept

β1, β2 e β3 = Linear coefficients

$X_1$, $X_2$ e $X_3$= Independent variables

$\beta_{11}$, $\beta_{22}$ e $\beta_{33}$= Quadratic coefficients

*Table 3. Variable codification*

| Indepent variables | Symbol | Level | | |
|---|---|---|---|---|
| | | Low | Medium | High |
| | | -1 | 0 | 1 |
| Acid concentration (% w/w) | $X_1$ | 1,5 | 2,5 | 3,5 |
| Time (min) | $X_2$ | 20 | 40 | 60 |
| Temperature (ºC) | $X_3$ | 110 | 120 | 130 |

*Table 4. Runs sequence*

| Sequence | Acid concentration | Time | Temperature |
|---|---|---|---|
| 1 | -1,0 | -1,0 | -1,0 |
| 2 | -1,0 | 0,0 | 1,0 |
| 3 | -1,0 | 1,0 | 0,0 |
| 4 | 0,0 | -1,0 | 1,0 |
| 5 | 0,0 | 0,0 | 0,0 |
| 6 | 0,0 | 1,0 | -1,0 |
| 7 | 1,0 | -1,0 | 0,0 |
| 8 | 1,0 | 0,0 | -1,0 |
| 9 | 0,0 | 0,0 | 0,0 |
| 10 | 0,0 | 0,0 | 0,0 |
| 11 | 1,0 | 1,0 | 1,0 |

### 3.3 RESULTS AND DISCUSSIONS:

#### 3.3.1 EFB Composition

The main macromolecules present in EFB were analyzed on a dry basis according to the NREL methodology, which is based on the quantification of monosaccharides in concentrated and diluted acid medium to fully hydrolyze cellulose and hemicellulose. The table 5 shows the composition of EFB. Previous analysis of EFB from Pará region exhibited the following mass composition: 32.01% glucan, 23.24% xylan, 2.2% arabinan, 5.4% Acetyl groups, 26.78% of lignin and ashes, the remainder being extractives and other molecules (RAMAN; GNANSOUNOU, 2014).

*Table 5. EFB Composition on Dry basis*

| Main fraction | Composition % w/w | |
|---|---|---|
| | Raman | Current work |
| Glucan | 33,5 | 32,01 |
| Xylan | 26,8 | 23,24 |
| Arabinan | 2 | 2,4 |
| Acetyl Groups | 5,1 | 5,4 |
| Lignin and others | 21,2 | 23,57 |
| Ash | 2,8 | 3,21 |

In general, it can be observed in the table 5 that, the total carbohydrate quantity was around 50 to 60% of the dry weight of the EFB, where the fraction of cellulose formed by glucose and it was equivalent to a percentage around 30 to 35%. On the other hand, the fraction of hemicellulose formed by xylose and arabinose, it was equivalent to 21 to 28% of the dry mass of the EFB. By this, it is an agro-industrial coproduct of interest as a carbon source for various bioprocesses with the purpose of generating products of high commercial value. The sugar composition of the EFB compared to other biomass derived from agro-industrial processes such as corn stover, Straw rice, Straw Strawberry and Barley Straw, is similar with a slightly lower percentage of 6-carbon sugars and a higher percentage of pentoses. However, when the EFBs are compared against the sugarcane bagasse the main agroindustrial biomass generated in the country, the amount of hexoses is lower (between 7 to 13%) and the amount of pentoses is equal or superior in 5%.

The insoluble lignin fraction represented 20% of the dry mass of the EFB, and the soluble lignin varied between 1 to 2%. The insoluble lignin is a promising raw material, which is beginning to be used on a laboratory scale and due to its composition, it is an important fraction from the economic point of view (DAVIS et al., 2016). Comparing this agroindustrial waste with others, EFBs exhibits more lignin than most co-products reported in the table 6 and is only comparable with wood (HU; RAGAUSKAS, 2012; LIMAYEM; RICKE, 2012; ZABED et al., 2016). On other hand, although the soluble lignin was present in small concentration, it could be a problem for future biotechnological processes because it affects the digestibility of sugars and therefore, it must be removed from the liquid fraction obtained from the diluted acid pretreatment using any separation technique (HENDRIKS; ZEEMAN, 2009). The data obtained are similar to those obtained in other biomasses such as rice straw or sugarcane bagasse, 1.9 and 2 g / 100 g dry biomass, respectively (LAVARACK et al., 2002; HSU et al., 2010).

Futhermore, the fraction corresponding to ashes is an important fraction, from an environmental and industrial point view. The researches seeks the least amount of this fraction, because it is an important pollutant for the effluents in the downstream processes to which the phytobiomass is subjected. The ash fraction in the EFB was 3.21 g / 100 g dry biomass and compared to the amount of ash reported as a percentage of dry basis in of sugarcane bagasse (4.8) and Rice Straw (11.8). It can be said that the amount of ash in the EFB is lower, which increases the use of main fractions and decreases the economic and energy expenditure in order to avoid effluent pollution (JIN; CHEN, 2007; KIM; DAY, 2011; RAMAN; GNANSOUNOU, 2014; LIU et al., 2015).

*Table 6. Composition of differents agroindustrial residues and woods.*

| Main fraction | Composition (% w compound/w dry biomass) | | |
|---|---|---|---|
| | Cellulose | Hemicellulose | Lignin |
| Empty fruit bunches | 30-35 | 25-30 | 20-25 |
| Corn Stover* | 30-40 | 24-26 | 7-19 |
| Sugarcane bagasse** | 42-48 | 19-25 | 20-25 |
| Rice Straw* | 28-36 | 23-28 | 12-14 |
| Wheat Straw* | 33-38 | 26-32 | 17-19 |
| Barley Straw* | 31-45 | 27-38 | 14-19 |
| Sweet Sorghum Bagasse* | 34-45 | 18-27 | 14-21 |
| Softwood*** | 40-45 | 25-29 | 25-30 |
| Hardwood*** | 45-47 | 25-40 | 30-60 |

*Data reported by (ZABED et al., 2016)
**Data reported by (HU; RAGAUSKAS, 2012)
*** Data reported by (LIMAYEM; RICKE, 2012)

The composition of the EFB was used to determine the recovery yield of the liquid fraction after acid hydrolysis.

### 3.3.2 Mass balance in the diluted acid pretreatment

A mass balance was made to each of the operational runs of the statistical model in the diluted acid pretreatment of the EFB on dry basis, to quantity the solid mass recuperated (cellulose and acid lignin) and determinate the sugars release in the aqueous phase (see the table 7).

*Table 7. Mass Balance of the diluted acid pretreatment of the EFB on dry basis*

| Run | Operational conditions | | | CSF (Ro) | Initial Mass of EFB (g) | Acid solution (g) | Mass of aqueous phase (g) | Final Mass of EFB (g)* | Solid recovery (% w/w) |
|---|---|---|---|---|---|---|---|---|---|
| | Acid concentration (% w/w) | Time (minutes) | Temperature (ºC) | | | | | | |
| 1 | 1,5 | 20 | 110 | 0,815 | 60 | 535,62 | 480,32 | 38,49 | 64,16 |
| 2 | 1,5 | 40 | 130 | 1,705 | 60 | 537,25 | 485,46 | 41,84 | 69,74 |
| 3 | 1,5 | 60 | 120 | 1,587 | 60 | 537,63 | 479,53 | 41,95 | 69.92 |
| 4 | 2,5 | 20 | 130 | 1,524 | 60 | 538,12 | 483,58 | 37,99 | 63,32 |
| 5 | 2,5 | 40 | 120 | 1,531 | 60 | 537,41 | 486,23 | 37,82 | 63,03 |
| 6 | 2,5 | 60 | 110 | 1,413 | 60 | 537,38 | 483,8 | 39,33 | 65,54 |
| 7 | 3,5 | 20 | 120 | 1,390 | 60 | 538,18 | 484,11 | 40,77 | 67,95 |
| 8 | 3,5 | 40 | 110 | 1,396 | 60 | 536,39 | 481,74 | 38,93 | 64,89 |
| 9 | 2,5 | 40 | 120 | 1,531 | 60 | 537,76 | 486,21 | 38,32 | 63,87 |
| 10 | 2,5 | 40 | 120 | 1,531 | 60 | 535,96 | 484,91 | 39,64 | 66,07 |
| 11 | 3,5 | 60 | 130 | 2,161 | 60 | 538,23 | 478,39 | 39,57 | 65,95 |

*biomass obtained next of the filtration process and the drying in an oven by 24 hours at 40ºC.

The solid fraction of the EFB obtained after the diluted acid pretreatment is composed of Cellulose and acid insoluble lignin and it represented between 63 to 69% w/w of the initial biomass. This value of recuperation agreed with the compositional analysis of the EFB, where the glucans and the acid insoluble lignin represented almost 55 g/100 g EFB. Hong and contributors applied a diluted acid pretreatment in EFB with a low CFS (1.95) like in this study (0.81 – 2.161), and the results obtained were a slightly better because the recoveries of the solid phase were lower than those in the current study (≈ 60% w/w) (HONG et al., 2013). On other hand, the 28 % w/w of diluted compounds approximately in the aqueous phase also correspond to the sum of the xylan, soluble acid soluble lignin and amorphous cellulose described above in the compositional analysis of the EFB.

On other hand, the table 7 doesn`t show a defined trend between the solid phase recovery and the CSF, this can explained because the recovery of the solid phase was difficult and there were significant losses of mass in the PARR reactor before filtration step. In the same table, it can be observed the loss of 10% of the initial mass (initial mass of EFB + Acid solution) compared to the mass obtained after the pretreatment (solid recovery + mass of aqueous phase). This loss can be explained by the difficult recovery of the solid phase after the pretreatment and due to the evaporation of the aqueous phase in the drying process of the cellulose and lignin, which increased the losses of mass in the hydrolysis process.

### 3.3.3 Sugar release and degradation products

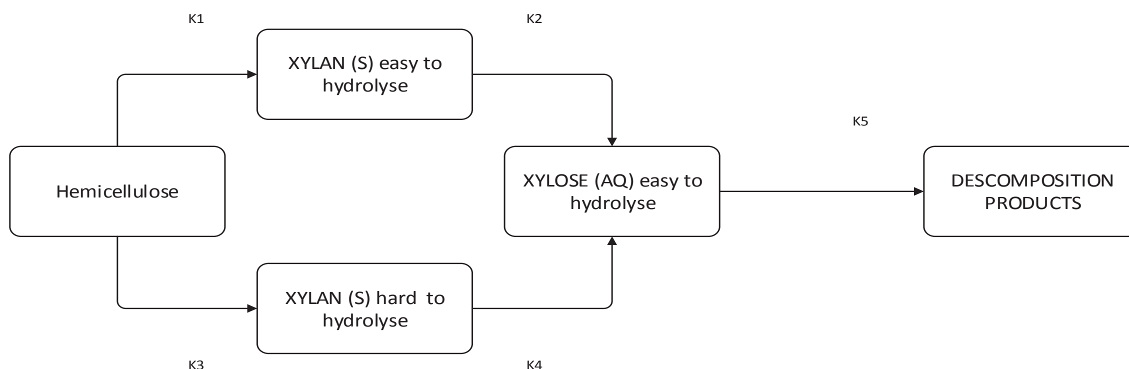
The concentrations of the different sugars and others compounds present in the liquid phase after the diluted acid pretreatment are shown in table 8. Additionally, the CSF was calculate like the combination of the independent variables. The CSF value varied between 0.815 and 2.16, corresponding to the boundary conditions (lowest condition: 1.5% w/w acid, 20 minutes and temperature 110°C; highest condition: 3.5% w/w acid, 60 minutes and 130°C), respectively.

In the liquid phase obtained, the xylose was the main compound with more than 43% w/w in all the pretreatments tested (see Table 8). The xylose extraction from the EFB under the different conditions tested presented a directly proportional behavior, where, at the lowest CSF condition (1.5% acid concentration, 20 minutes and 110°C) approximately 5.276 g/100 g Dry EFB were extracted. Then,

when the CSF increased until 1.5, the xylose concentration increased reaching a maximum recovery of 18.24 g/100 g Dry EFB. After that, for CFS values higher than value 1.5, the xylose recovery in the liquid phase decrease down to 18.08 g/100 g Dry EFB at the highest CSF value (see Figure 8). This parabolic behavior in the xylose release has also been reported by different studies (RAHMAN; CHOUDHURY; AHMAD, 2006; THAMSEE et al., 2017). There are different explanations for this phenomenon, however, the biphasic kinetical model is the one widely accepted. In this model, at the lowest condition of CSF, the acid pretreatment does only reach to break the easy hydrolysable sugar linkages of the xylose, and the harder hydrolysable sugar linkages of the xylose are not broken. Additionally, others compounds like cellulose and lignin are not affected. With the CSF increasing in the pretreatments, the glicosidic linkages of the hemicellulose in form of xylan are broken in function of the severity, and the xylose is release in the liquid phase. However, when the CSF reaches a value higher than 1.7, the xylose concentration decreases due to degradations of xylose into furfural. All the Hydrolysis and degradative processes of xylan until furfural formation follow a first order irreversible kinetic (LAVARACK et al., 2002). The xylan degradation is shown in the Scheme 1.

*Table 8. Aqueous phase composition after the acid hydrolysis and determination of the combined severity coefficient*

| Hydrolysis Conditions | | | Liquid phase recuperation of acid pretreatment of EFB (g compound/100 g EFB) | | | | | | | | Combined Severity Factor (CSF) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| % Acid (%w/w) | Time (min) | Temperature (°C) | Cellobiose | Glucose | Xylose | Arabinose | Sum | Acetic Acid | HMF (254 nm) | F (254 nm) | |
| 1,5 | 20 | 110 | 2,3395 | 0,019 | 5,2759 | 1,8854 | 9,5198 | 2,6501 | 0,0275 | 0 | 0,8155 |
| 1,5 | 40 | 130 | 1,1446 | 0,6774 | 15,064 | 2,0166 | 18,9031 | 4,3499 | 0,0625 | 0,4109 | 1,7054 |
| 1,5 | 60 | 120 | 0,9579 | 0,5432 | 17,517 | 1,8132 | 20,8314 | 4,2321 | 0,0568 | 0,2054 | 1,5870 |
| 2,5 | 20 | 130 | 0,9847 | 0,4221 | 18,241 | 1,7864 | 21,4345 | 4,3435 | 0,0403 | 0,3459 | 1,5243 |
| 2,5 | 40 | 120 | 0,5555 | 0,4642 | 16,382 | 2,2359 | 19,6379 | 3,9229 | 0,0583 | 0,2895 | 1,5309 |
| 2,5 | 60 | 110 | 1,4427 | 0,1473 | 16,415 | 1,9347 | 19,94 | 4,4309 | 0,0508 | 0,1278 | 1,4126 |
| 3,5 | 20 | 120 | 1,4056 | 0,1218 | 18,210 | 1,7619 | 21,4999 | 4,8129 | 0,0532 | 0,2118 | 1,3899 |
| 3,5 | 40 | 110 | 1,4243 | 0,1271 | 13,555 | 1,6887 | 16,7955 | 3,9059 | 0,0513 | 0,1353 | 1,3965 |
| 2,5 | 40 | 120 | 1,1036 | 0,4232 | 16,676 | 1,9783 | 20,1817 | 4,3931 | 0,0592 | 0,2528 | 1,5309 |
| 2,5 | 40 | 120 | 0,5752 | 0,5335 | 16,508 | 1,9632 | 19,5805 | 5,2668 | 0,0564 | 0,1654 | 1,5309 |
| 3,5 | 60 | 130 | 0 | 0,8228 | 18,086 | 0,3759 | 19,2854 | 5,3498 | 0,0683 | 1,3484 | 2,1615 |
| 2,9 | 25 | 125 | 0,9838 | 0,6326 | 20,843 | 1,7244 | 24,1842 | 4,0884 | 0,0598 | 0,1739 | 1,5540 |




Scheme 1. Degradatives processes of the xylan. Adapted of Lavarack and contributors (LAVARACK et al., 2002).

In this Scheme, K1, K2, K3, K4 and K5 represent the first order constant of the kinectics of the differents hydrolysis processes in the transformation of xylan to decomposition products.

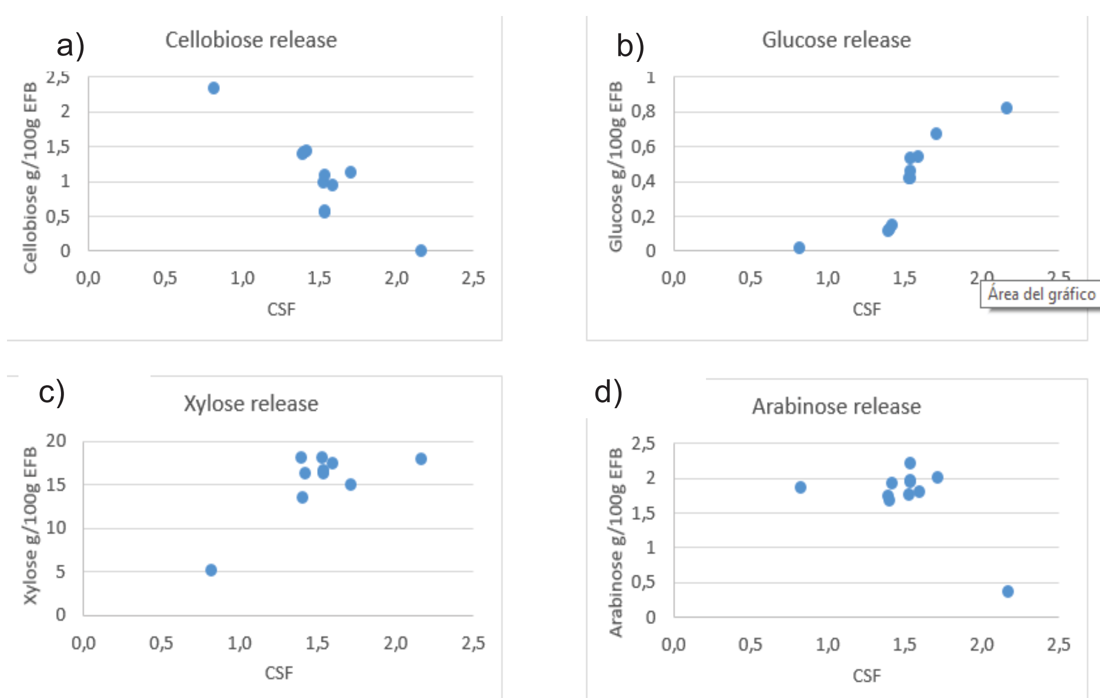



*Figure 1. Sugars released in the liquid phase after diluted acid pretreatment against CSF*

The acetic acid released during the pretreatment was quantified in the aqueous phase because it was the main degradation product of xylose. Different investigations about the diluted acid pretreatment of the EFB reported a significant production of acetic acid in function of CSF (0.4 -8 g/100 g of EFB)

(RAHMAN; CHOUDHURY; AHMAD; et al., 2006; HONG et al., 2013). In the current study, the furfural generation was proportional to the CSF (see figure 2b). The acetic acid values were in the range of 2.65 and 5.35 g/100 g of EFB (see figure 2a), and corresponding to the CSF values of 0.8155 and 2.1615, respectively. These results are similar to the literature. However, if the aqueous phase will be used as carbon source, it should be detoxified because an acetic acid concentration higher than 3 g/L produces partial inhibition and this inhibition increases with the acetic acid concentration (DELGENES; PENAUD, V. Y MOLETTA, 2003).

The furfural was quantified in the aqueous phase because it is also a degradation product of the xylose and a strong inhibitor of cell growth (see figure 2b). The furfural generation is proportional with the increase of the CSF. In the pretreatment with the lowest CSF value, furfural was not detected furfural, and in the pretreatment with the highest CSF value, furfural was detected in maximum concentration (1.3183 g/100 g EFB). This furfural quantity is in concordance to the results of Rahman and contributors in EFB under similar conditions (≈1.5 g/L phase aqueous; acid concentration 4% w/w, reaction time 60 min and temperature 115°C) (RAHMAN; CHOUDHURY; AHMAD; et al., 2006). On the other hand, Delgenes and contributors tested the effect of furfural added on the growth of *Candida shehatae* and *Pichia stipitis* in a medium rich in xylose, they showed that, the furfural concentrations higher than 0.5 (g/L of medium) affect the growth of the microorganisms and the generation of secondary metabolites . In this case, the amount of furfural in the aqueous phase was high and it is not advisable to use with some microorganism without a previous detoxification process.

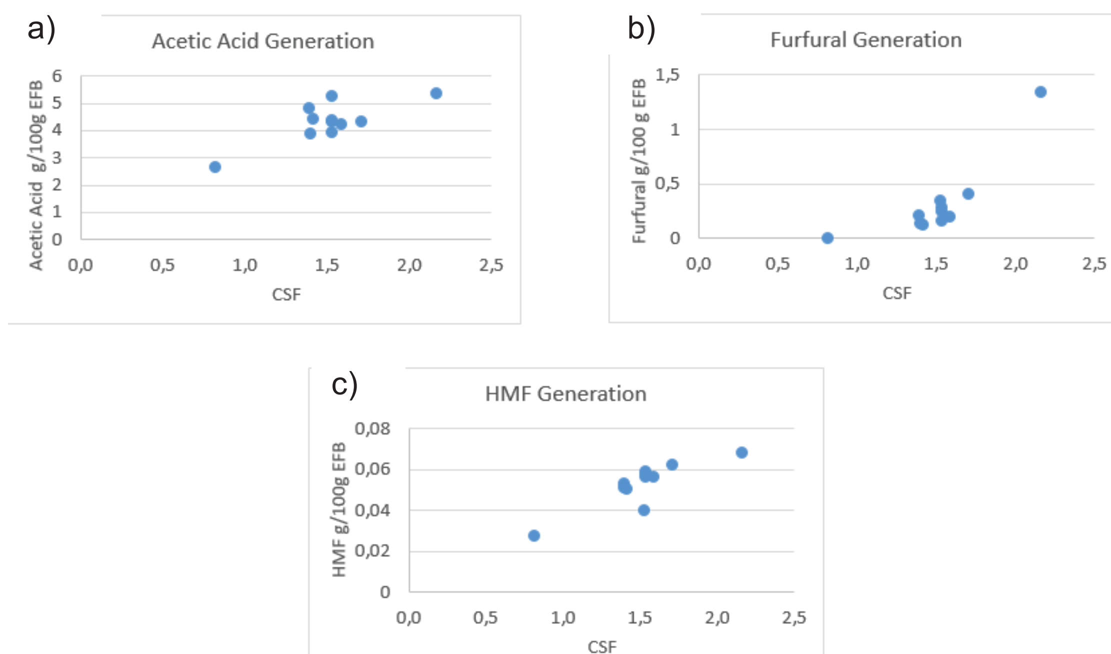
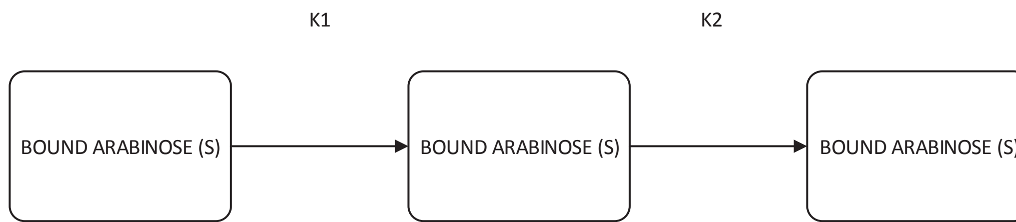



*Figure 2. Generation of degradation products*

In the case of arabinose, its release from EFB is shown in the Figure 1 d, the arabinose amount increased up to 2.236 g/100 g dry EFB for a CSF value of 1.5309. However, the arabinose amount didn`t vary a lot compared with the lowest condition of the CSF (1.8854 g/100 g dry EFB). Additionally, the arabinose amount decreased significantly with the highest CSF value (0.3759 g/100 g dry EFB). The recuperation of arabinose was 93 %(w/w) compared with the arabinose total obtained with the NREL method. On the other hand, in this hydrolysis process, the arabinose release from the dry EFB exhibits a parabolic trend as the xylose releasing process. Nevertheless, the arabinose release is easier that the xylose release because at the lowest CSF value the arabinose amount do not vary a lot compared with the maximum arabinose amount recuperated during all the tests. Additionally, the arabinose amount decreased a lot with CSF higher than 1.5, probably due to degradation processes producing furfural and others compounds. The degradation process is shown in the scheme 2.




Scheme 2. General reaction of the degradatives processes of the Arabinan. Lavarack and contributors. (LAVARACK et al., 2002).

In this Scheme, K1 AND K2 represent the first order constant of the kinectics of the different hydrolysis processes during the transformation of Arabinose into decomposition products.

The cellobiose release is shown in the Figure 1 a. and it presents a trend inversely proportional to the CSF, the maximum concentration of cellobiose in the liquid phase was 2.3395 g/100 g of EFB for a CSF value equal to 0.8155 and the disaccharide wasn`t detect at the highest value of CSF. This trend was related with the initial depolymerization of the amorphous cellulose under soft severity conditions, and then, the cellobiose concentration decreased through the break and formation of two units of glucose by the effect of the increase of the acid catalyzer concentration (RINALDI; SCHÜTH, 2009). The presence of cellobiose in the liquid phase for CSF values $x<1.7$ indicated an incomplete hydrolysis.

Other monosaccharide released in the diluted acid pretreatment is glucose. The quantity of glucose released is smaller than xylose, and it represents the 2.57% of the total glucose present in the EFB. The glucose obtained in the liquid phase is part of the hexoses in the hemicelulose and the amorphous cellulose. The releasing process is directly proportional with the CSF. For the lowest CSF value the glucose release was minimum (0.019 g/100 g of EFB) and it increased up to approximately 0.82 g/100 g of EFB at the maximum (see the figure 1 b). This behavior is similar to the reported by Raman and contributors, where, the glucose in glucan form was released with CSFs higher than 1.36 and then it reached a limit in function of the glucose decomposition reactions and the fitocomposition (RAMAN; GNANSOUNOU, 2014). On the other hand, the degradation process of glucose generates 5 –Hydroxymethylfurfural (HMF), and its concentration increases from the lowest to the maximum CSF value maximum (0.0268 and 0.0683 g/100 g of EFB, respectively) (see the figure 2 c). The quantity of HMF

released is in accordance with the results obtained by Rahman and contributors (RAHMAN; CHOUDHURY; AHMAD; et al., 2006). Additionally, the HMF quantity in the aqueous phase was lower than the inhibition limit for all conditions evaluated (the growth inhibition begin with HMF concentration higher than 1 g/L) (DELGENES; PENAUD, V. Y MOLETTA, 2003).

### 3.3.4 Statistical optimization of total sugar release in phase aqueous

For the diluted acid pretreatment applied to EFB, a $3^{3-1}$ factorial design was used with time (min), acid concentration (% w/w) and temperature (°C) as independent variables and total sugars concentration as response variable. Total sugars release was select as response variable and not the xylose released, because the microorganisms further used to take advantage of this fraction, can metabolize different sugars (glucose, arabinose and cellobiose). The statistic software used was Statistic 8.0 and the Analysis of variance (ANOVA) was used as hypothesis test and confidence level of 95% (see table 9). The influence of the lineal and quadratic variables was evaluated with the correlation coefficient ($R^2$).

Then of the eleven experimental runs, a regression analysis was done and the experimental model for the response variable (total sugar release) was determinated (see the equation 3).

$$Y = 18.4533 + 2.7755X_1 + 2.5342\,X_2 + 4.45590\,X_3 + 1.94236\,{X_1}^2 - 0.89559\,{X_2}^2 + 2.42076\,{X_3}^2$$

*Equation 3. Regression model of xylose release after of the pretreatments*

The regression equation of the total sugar released presented a determination coefficient of 0.71186 where the 71.186% of results can be explained by this equation. This coefficient is low compared with others optimizations studies (RAHMAN; CHOUDHURY; AHMAD; et al., 2006), moreover, the others studies emphasized the recuperation of xylose in the aqueous phase, however, xylose isn`t the unique fermentable sugar in the aqueous phase.

*Table 9.* ANOVA

| Factor | Anova, Var: sum; Adj= 0, 27966. 3 3 level factors, 1 blocks, 11 runs, MS residual= 8,154519. DV: sum | | | | |
|---|---|---|---|---|---|
| | **SS** | **df** | **MS** | **F** | **p** |
| Acid concentration (%w/w) (L) | 11,5551 | 1 | 11,5551 | 1,417018 | 0,299712 |
| Acid concentration (%w/w) (Q) | 9,14 | 1 | 9,14 | 1,120121 | 0,349572 |
| Time (min) (L) | 9,6333 | 1 | 9,6333 | 1,181339 | 0,338195 |
| Time (min) (Q) | 1,9419 | 1 | 1,9419 | 0,238136 | 0,651097 |
| Temperature (ºC) (L) | 29,7826 | 1 | 29,7826 | 3,652278 | 0,128584 |
| Temperature (ºC) (Q) | 14,1875 | 1 | 14,1875 | 1,739838 | 0,257602 |
| Error | 32,6181 | 4 | 8,154525 | | |
| Total SS | 113,2034 | 10 | | | |
| R^2 | 0,71186 | | | | |

In the table 9, the influence of the independent variables on the total sugar release in the aqueous phase can be seen. The temperature is the more important variable follow the acid concentration and time, respectively. The P-value of three variables was bigger than 0.05, this meaning that, none of the three variables under the evaluated conditions separate have a significant effect on the total sugar release. The P values were 0.128, 0.299 and 0.338 for temperature, acid concentration and time, respectively.

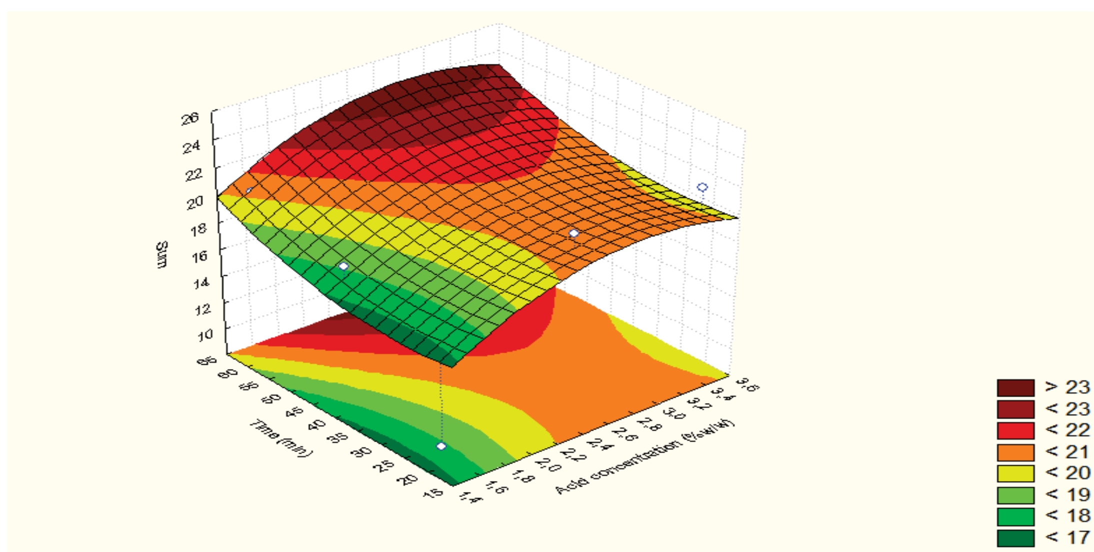



*Figure 3.* Effect of time and Acid concentration on the total sugar release in the diluted acid pretreatment when the temperature was maintained at 120ºC

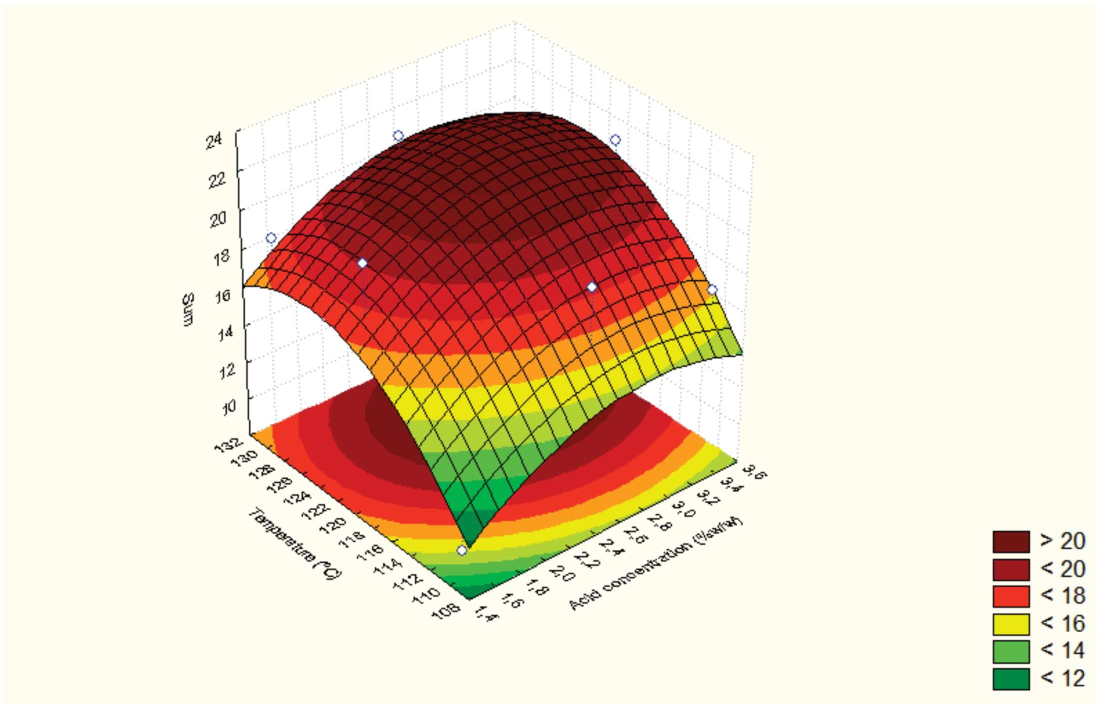



Figure 4. Effect of temperature and Acid concentration on the total sugar release in the diluted acid pretreatment when the time was maintained at 40 minutes.

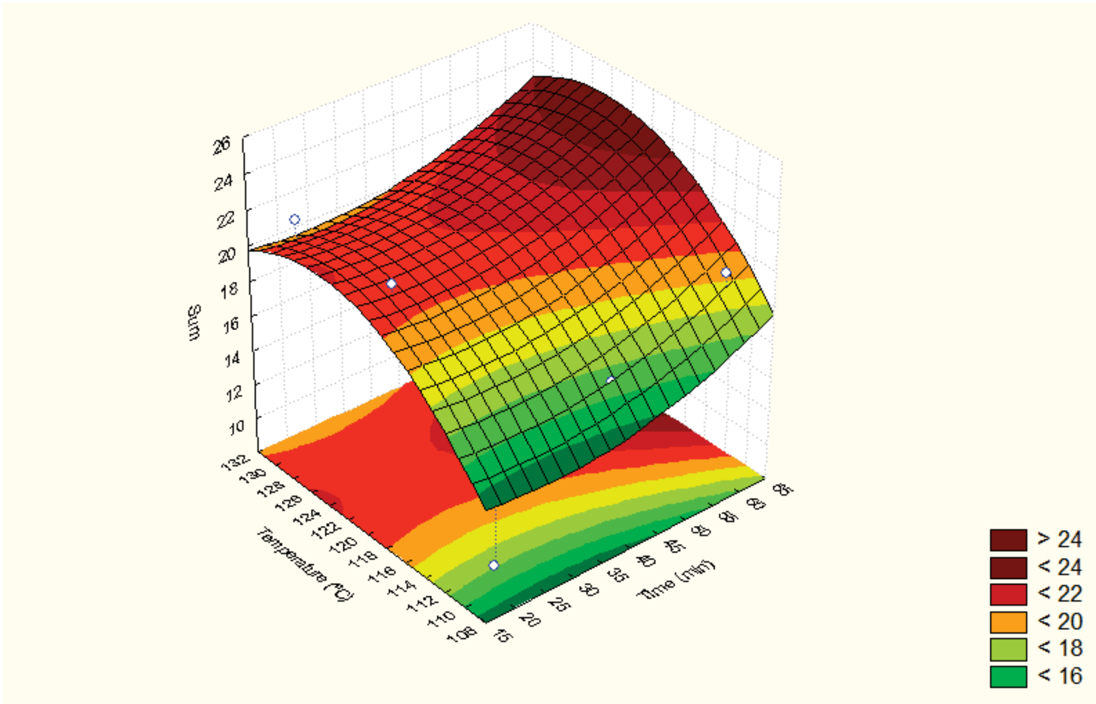



Figure 5. Effect of temperature and reaction time on the total sugar release in the diluted acid pretreatment when the Acid concentration was maintained at 2.5 % w/w.

The figure 3 shows the effect of the time and the acid concentration on the total sugars released in the diluted acid pretreatment when the temperature was maintained at 120°C. Here, the response surface didn`t present a dome shape, meaning that the response variable wasn`t optimized under the evaluated conditions. However, the figure 3 shows a parabolic trend where the possible value of the acid concentration will present the maximum value for total sugars (between the values 2.8 and 3). About the time variable, the figure shows that the possible value to recover the maximum quantity of total sugars is higher than 65 minutes (outside the evaluated limits).

The figure 4 shows the effect of the temperature and the Acid concentration on the total sugar release in the diluted acid pretreatment when the time was maintained in 40 minutes. In this case, the figure presented a dome shape, meaning that the response variable reached a statistic maximum under the evaluated conditions. The intercept values were 2.8572 and 124.60, for acid concentration and temperature, respectively. Additionally, this result is in concordance with the figure 3, where the predicted value of acid concentration (% w/w) was between 2.8 and 3. On the other hand, the figure 5 shows the effect of temperature and reaction time on the total sugar released in the diluted acid pretreatment when the Acid concentration was maintained at 2.5 % w/w. This figure is similar to the figure 4, however, the parabolic trend is stronger and the possible optimum value to the temperature would be between 124 to 128 °C, confirmed by the figure 4.

In contrast to the information about the possible optimum time value, supplied by the figures 3 and 5, the statistical model supplied another time value under the evaluated conditions. The time was 25.85 minutes. So, the critical values of the independent variables were 2.8572 % w/w of sulphuric acid, 124.6 °C and 25.85 minutes for 21.07 g of total sugars release/100 g of Dry EFB as response variable. To apply of the critical values in the PARR reactor, the value were approximated to 2.9% w/w of sulphuric acid, 125°C and 25 minutes. The optimal conditions were evaluated in triplicated, in the table 9 is showed the average value.

With the critical statistical values, 0.9838 to cellobiose, 0.6326 to glucose, 20.84 to xylose and 1.7244 to arabinose in g/100g of EFB were obtained. From that data, it was extracted important information, the cellobiose value is an average value compared with the others runs, moreover, it indicated that, the hydrolysis process was incompleted, yet. The glucose and arabinose values were average values compared with the others operational runs.  Finally, the xylose value was 15% higher than any other operational run, the xylose recovery compared with the xylose of the compositional analysis of EFB was 89.67% (w/w). The xylose release is explained because the total sugars increased. Despite that, the release of the others sugars was into the average compare with other operational runs. On the other hand, the degradation products generated with the critical values were 4.0884 for acetic acid, 0.0598 for HMF and 0.1739 for furfural (the above values are expressed in g/100 g of EFB). This values are within the averages values compared against the others operating runs. The acetic acid was the unique degradation product with a high value that could produce inhibition of the cell growth, for this reason, the best option to use the hydrolysate as carbon source is by its detoxification.

The amount of total sugars released in the aqueous phase was 24.1842 g/100 g of EFB, which is the maximum value of total sugars recovery, however, it is 14.7% higher than the statistical value proposed by the model. This can be explained by the limited number the interactions in the mathematical model and its low determinate coefficient, which permitted to find a point with higher total sugars recovery, but, not the approximate quantity.

## 3.4 CONCLUSION

The diluted acid pretreatment was carried out with the selected operating conditions. A fractionated factorial statistical model was choose to optimize the total sugars release from EFB. The critical values to recovery a higher quantity of the total sugars (24.18 g/100 g of EFB), the conditions were 2.9% w/w of sulphuric acid, 125°C and 25 minutes. The unique degradation product with inhibition power was acetic acid (4.0884 g/100 g of dry EFB). The aqueous phase obtained under the selected operating conditions can be used source carbon, previously detoxifying it or using tolerant microorganisms to high concentration of acetic acid.

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## CHAPTER 4.Growth kinetics of the Pichia jadinii CTT 1518 and Cyberlindnera jadinii CTT 2612 in EFB hydrolysate

**Abstract**

An alternative for the use of hemicellulose sugars with a high content of compounds toxic for microbial growth, it is the use of microorganisms resistant to these toxic compounds. The objective of this work was to evaluate the growth capacity of the microorganisms (number of cell and biomass weight) *Pichia Jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612 in the hydrolysate obtained from chapter 2, and supplemented with three carbon/nitrogen ratios.Then, it was selected the carbon/nitrogen ratio that generated the highest production of biomass (w/L), and with this relationship, a proximal characterization of the biomass of each microorganism was made. The C/N ratios that generated the highest amount of biomass were 11.5 and 14, for the microorganisms *Cyberlindnera Jadinii* CCT 2612 and *Pichia Jadinii* CCT 1518, respectively. The proximal characterization in percent (w compound / w of the yeast in dry basis) for *Pichia Jadinii* CCT 1518 was 35.9, 3.29, 37.28 and 6.3 of protein, lipids, sugars and ashes, respectively. The proximal characterization in percent (w compound / w of the yeast in dry basis) for Cyberlindnera Jadinii CCT 2612 was 44.43, 2.23, 33.13 and 5.9 of protein, lipids, sugars and ashes, respectively.

### 4.1 INTRODUCTION

The current trend in the world is to take advantage of the co-products generated by different agroindustries to reduce their environmental impact. In Brazil, different agroindustrial coproducts are obtained in a high quantity, such as sugarcane bagasse, empty fruit bunches (EFB), among others. Nevertheless, the use of them is complex by its composition and is traditionally done by dividing the lignocellulosic matrices into their main compounds (lignin, hemicellulose and cellulose)(ABIOVE et al., 2016).

The use of hemicellulose as a source of carbon for microorganisms depends on its composition. Mainly the amount of sugar in the aqueous phase, the most abundant type of sugar and the concentrations of degradation compounds and nitrogen. In the case of the aqueous phase of the empty fruit bunches, it contains a good amount of reducing sugars, the most abundant sugar and xylose that is

metabolized by few microorganisms, has high amount of acetic acid as the main growth inhibitor compound and nitrogen concentration is low. Hinders the development of any microorganism (DELGENES; PENAUD, V. Y MOLETTA, 2003).

For the use of this type of hydrolyzate there are two options, detoxify it and supplement it with nitrogen that increases the production costs of any biotechnology compound or make a nitrogen supplementation, but use a microorganism tolerant to toxic compounds, which has the ability to metabolize pentoses and that is of biotechnological utility. The latter option economically increases the viability of industrialization, however, it is complex to find a microorganism with these characteristics.

Candida utilis is an interesting yeast for the food industry, its use as a food additive (classified as Generally Recognized as safe - GRAS), an important source of carbon and nitrogen, a host for expression of genes of interest, possible antimicrobial and possessing effect of beta-glucans which is a polysaccharide with functional properties (PIZARRO et al., 2014). Parallel to the interest in Candida utilis, other genetically related yeasts and formerly classified as Torulas (including Candida Utilis), are being studied from different points of view like genetics, composition, among others. An example of this are *Pichia Jadinii* and *Cyberlindnera Jadinii*, which differ from Candida utilis in the number of genetic pools (ploidia) and have presented some advantages over Candida utilis in gene expression and in the ability to use alternative sources of carbon as the xylose (BUERTH et al., 2016). However, there is not much information on these yeasts, such as kinetic parameters in different substrates and conditions, possible mathematical models to simulate and optimize their growth and product generation, which would allow easier industrialization in the future.

A Cell growth mathematical model widely used at the food and the biotechnology industry, it is the Gompertz model. This model explains with precision the lag and exponential phases of the cell growth. Additionally, it presents information about bioprocess variables as the cell growth rate and generation time. On the other hand, it is only required of the cell account and the reaction time, for its application.

Due to the possible food importance of the yeasts *Pichia Jadinii* and *Cyberlindnera Jadinii* as possible sources of nitrogen, carbon and functional molecules as β-glucans (PIZARRO et al., 2014). The aims in this chapter were to evaluate the kinetic parameters for the biomass production of *Pichia Jadinii* and *Cyberlindnera Jadinii*, in the hydrolyzate of EFB obtained in chapter two, supplementing it with nitrogen at three different levels and applying the modified Gompertz model (CASTRO et al., 2008) to predict biomass generation under experimental conditions. Additionally, it was determinated the quantification of lipids, total carbohydrates, crude protein and ashes.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Strains

The yeasts evaluated were *Pichia jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612 described in the literature as consumers of pentoses, resistant to toxic compounds such as acetic acid, furfural and hydroxymethylfurfural and classified as GRAS (http://www.mycobank.org/BioloMICS.aspx?TableKey=14682616000000089&Rec=769&Fields=All, 2017), purchased from the tropical culture collection André Tosello Foundation.

The yeasts were kept for storage at -20ºC in tubes with inclined Yeast Medium (YM) agar (1.5% w/v Agar, 1% w/v Glicose, 0.5% w/v bacteriological peptone, 0.3% w/v extract of yeast and 0.3% w/v malt extract). The yeasts were kept at refrigeration temperature in YM broth (1% w/v Glicose, 0.5% w/v bacteriological peptone, 0.3% w/v yeast extract and 0.3% w/v malt extract) for yeast selection tests and their subsequent optimization of kinetic parameters.

### 4.2.2 Evaluation of the growth kinetics of the yeasts Pichia jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612 in synthetic médium

The growth kinetics of the yeasts Pichia jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612 in YM medium were evaluated for the determination of the time before inoculation in the non-detoxified hydrolyzate of the EFB and the final mass concentration. As a parameter for selecting the time before repeating the hydrolyzate, the growth curves of the two yeasts and the literature on related microorganisms were used.

The two yeasts were repicated in three phases. The first repique was performed in 5 mL of YM broth taking a roast of inoculum from solid medium and incubated at 30°C for 72 hours. The second repique was made using 10% v/v of inoculum and 90% v/v of YM medium (this step was carried out using a ratio of 1: 5 total volume of the inoculated medium: total volume of the erlenmayer) and subsequently incubated at 30ºC, 120 RPM for 72 hours. Finally, the third repique was made using 10% v/v of inoculum and 90% v/v of YM medium (this step was carried out using a ratio of 1:5 total volume of the inoculated medium: total volume of the erlenmayer) and subsequently incubated at 30°C, 120 RPM for 72 hours (the last ring was made in triplicate with each one of the yeasts).

**4.2.2.1 Measurement of biomass and biomass yield in YM médium**

For the measurement of biomass, samples of 5 mL were collected at times 0, 24, 48 and 72 hours, from each of the 6 erlenmayers with inoculated YM medium. The samples were deposited in 15 mL falcon tubes (previously dried and weighed), then the tubes were centrifuged at 4000 RPM for 10 minutes in a Fanem Centrifuge. Centrifuged the samples, the supernatant and the biomass were separated. The supernatant was analyzed for reducing sugars with the colorimetric method of dinitrosalicylic acid 3-5 (DNS). In another hand, the biomass was resuspended using 10 mL of distilled water and centrifuged again under the conditions described above. The new supernatants were discarded and the biomass was drying oven at 80ºC for 24 hours. Next, the falcons with biomass were cooled in a desiccator and weighed until reaching constant weight. The biomass generated was determined by weight difference (AOAC, 1990), and with this result the microbial growth curves were made. In addition to this, the biomass yield (x/s) and the overall productivity were determined as kinetic parameters. Equations 4 and 5, respectively.

$$Y(^{X}/_{S}) = \frac{X\text{- }Xo}{So\text{-}S} \quad [gg^{-1}]$$

*Equation 4. Biomass yield*

Where Xo corresponds to the initial biomass concentration (g / L), X is the final biomass concentration (g / L), So is the initial concentration of reducing sugars (g / L) and S is the final concentration of reducing sugars. (g / L).

$$OP = \frac{X\text{- }Xo}{t\text{-}to} \quad [gh^{-1}]$$

Equation 5. Overall Productivity

Where Xo corresponds to the initial biomass concentration (g / L), X is the final biomass concentration (g / L), t is the initial time (h) and t is the final time (h).

### 4.2.2.2 Reducing Sugar determination

The reducing sugars were determined through spectrophotometry and using the 3,5- dinitrosalicylic acid (DNS) method (MILLER, 1959). To determine the reducing sugars, 1 ml of each supernatant obtained in the determination of biomass was taken and mixed with 1 ml of DNS in a test tube, then the mixture was heated to 96 °C for 5 minutes and then cooled for 10 minutes. Then, 5 mL of distilled water were added and the measurements were made in a spectrophotometer with a wavelength of 540 nm. The equation 6 represents the standard curve with $R^2$ = 0.989 (the reference sugar was glucose).

$$Y = 0.0121\,x\text{-}0.1087 \quad [A]$$

*Equation 6. Reducing Sugars using glucose as parameter*

Where Y represents the absorbance as a function of the concentration of sugar (A) and X represents the concentration of sugar in (g / L).

The sugar consumption for each of the yeasts was also determined.

$$SC = \left(\frac{(So\text{-}S)}{So}\right) x\,100 \quad [\%]$$

*Equation 7. Sugar consumption*

Where So is the initial concentration of reducing sugars (g / L) and S is the final concentration of reducing sugars (g / L).

### 4.2.3 Analysis of the hydrolysate of the EFB and nitrogen supplementation

The hydrolysate of the EFB used for the microbial growth test, was obtained in a Parr reactor according to the following operating conditions, 10 g of dry EFB (with particle diameter between 0.177 - 0.355 mm) / 100 g of liquor, 2.5 g of sulfuric acid / 100g of liquor, 125ºC of temperature and a residence time of 25 min. After the hydrolysis process, the liquid phase was neutralized with 10M sodium

hydroxide until reaching a pH between 5.8 and 6. Afterwards, the neutralized liquid phase was filtered and analyzed elementally for carbon, hydrogen, sulfur, nitrogen and oxygen. Next, the neutralized hydrolysate was supplemented with yeast extract, malt extract and bacteriological peptone (previously analyzed elementally) in a fixed amount initially to determine the ratio C/N (the amount applied was 5g bacteriological peptone, 3 g of extract of yeast and 3 g malt extract). Determined the ratio C/N of the hydrolysate supplemented (C/N = 17.985) and taking constant the proportion of the YM medium (0.5% w/v bacteriological peptone, 0.3% w/v yeast extract and 0.3% w/v malt extract ), the C/N ratio was varied in three levels 9, 11.5 and 14 (the C/N ratios was selected in agreement with the literature) (AROUS et al., 2015). Then the hydrolysate was dosed in erlens mayers in a radius of 1:5 (volume of liquid phase supplemented: total volume of erlen mayer), sterilized, analyzed for reducing sugars by the method of 3,5-dinitrosalicylic acid (DNS) and finally stored under refrigeration until its later use.

**4.2.4 Kinetics of microbial growth in hydrolysate with different C/N ratios**

After selecting the appropriate time of the two yeasts for the inoculation of the hydrolyzate, the kinetics of microbial growth in the liquid phase of the EFB were performed with the three proportions of organic nitrogen previously described, in order to evaluate the differences in production of biomass.

The growth kinetics of the yeasts was carried out in the following way, the repique was done in three phases. The first repique was made in 5 ml of YM broth taking a roast of inoculum from solid medium and incubated at 30ºC for the time selected above, the second repique was made using 10% v / v of inoculum and 90% v / v of YM medium (this step was carried out using a ratio of 1:5 total volume of the inoculated medium: total volume of the erlenmayer) and subsequently incubated at 30ºC, 120 RPM for the previously selected time. Finally, the third repique was performed in the hydrolysate of EFB using 10% v/v of inoculum and 90% v/v of hydrolysate (this step was carried out using a ratio of 1:5 total volume of the inoculated medium: total volume of the erlenmayer) and subsequently incubated at 30 ° C, 120 RPM for 144 hours. The final ring in the hydrolysate was made in duplicate for each of the three C/N ratios of each of the yeasts (see figure 6).

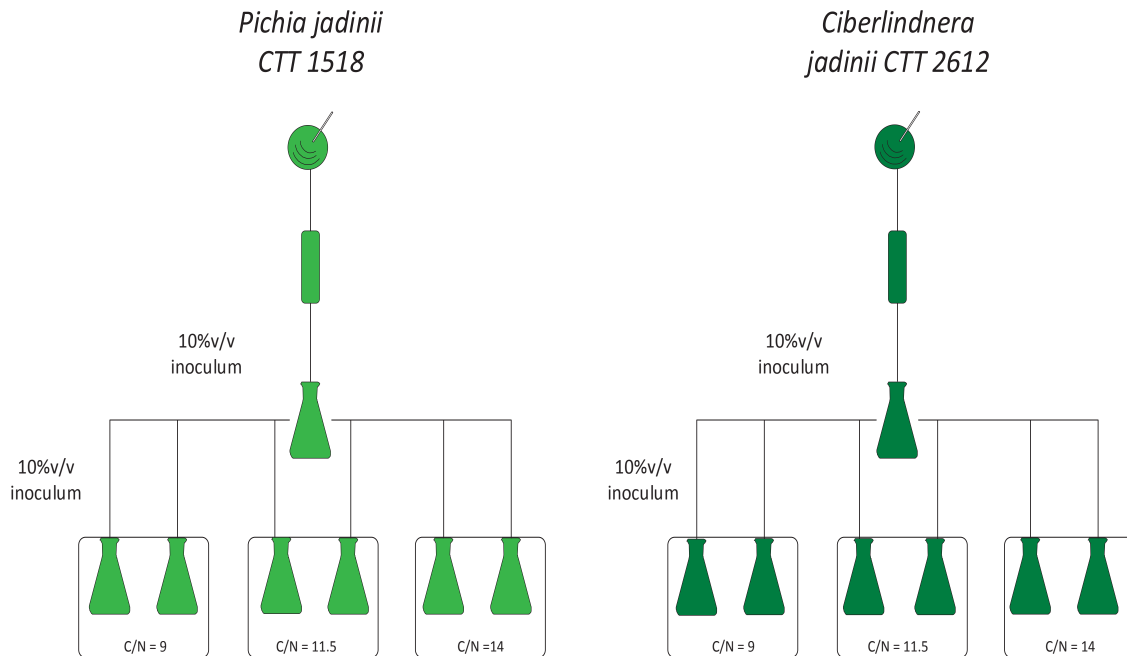


Figure 6. Inoculation diagram

#### 4.2.4.1 Microbial growth determination by dry weight

For the measurement of biomass by dry weight, a sample of 5 mL of inoculated hydrolyzate (corresponding to times 0, 24, 48, 72, 96, 120 and 144 hours) of each of the erlenmayers and deposited in falcon tubes was collected. of 15 mL (previously dried and weighed), subsequently the tubes were centrifuged at 4000 RPM for 10 minutes. Centrifuged the samples, the supernatant and the biomass were separated. The supernatant was analyzed for reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method. In another hand, the biomass was resuspended using 10 mL of distilled water (X2) and centrifuged again (4000 RPM for 10 minutes). The supernatants of the two washes were discarded and the biomass in the falcons was deposited in an oven at 80 ° C for 24 hours. Next, the falcons with biomass were cooled in a desiccator and weighed. The biomass generated was determined by weight difference. In addition to these measurements, the Biomass yield (see equation 4), and the overall productivity of the two biomasses were determined as kinetic parameters.

#### 4.2.4.2 Microbial growth determination by cell count

For the measurement of biomass by cell count, a sample of 1 mL of inoculated hydrolysate (corresponding to times 0, 24, 48, 72, 96, 120 and 144 hours) of each of the erlenmayers and deposited in eppendorf tubes was collected (previously

clean and dry). Then, the samples were diluted with distilled water in different proportions depending on the cell concentration. Then, 100 ul of one of the diluted samples was taken, placed in a Neubauer chamber (this step was repeated for each of the 12 samples) and the cell count was performed in a microscope at the 40X objective. For the count in the neubauer chamber, only 13 squares of the general picture were counted (see figure 7); then averaged and the calculation of the number of yeasts per milliliter was made (see equation 8). Additionally, the Gompertz model was used as a possible tool for the prediction of growth of yeasts in hydrolysates with different C/N ratios, and with this model the kinetic parameters, growth rate, latency time and duplication time were determined. (see equation 9). Finally, the $R^2$ of the adjustment of the Gompertz model to the real data was found.

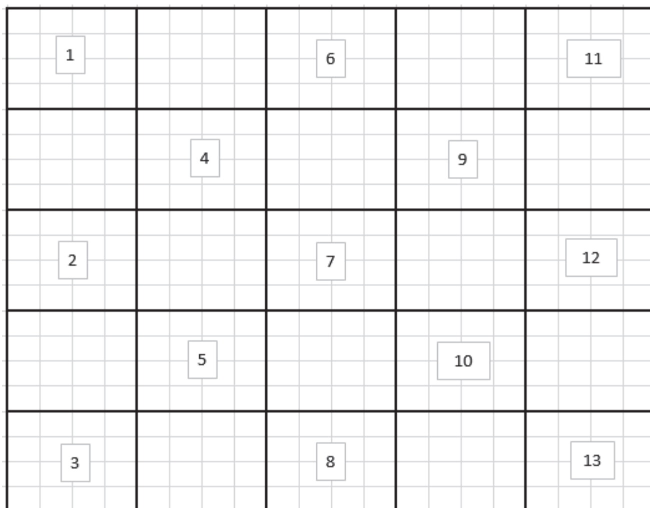


*Figure 7. Counting in Neubauer's camera*

$$\text{Cell counting} = \left(\frac{\text{cuadriculo average (cells)x 1ml}}{\text{cuadriculo volume ml}}\right) \text{x dilution factor} \ \ [\text{cells/ml}]$$

Equation 8. Cell counting

$$\text{YA} = \text{ae}^{-\text{e}^{(\text{b-c*TA})}}$$

Equation 9. Gompertz model

Where YA = log (N / No), N is the number of yeasts (number of yeasts / milliliter) at a given time, It is not the number of yeasts at time zero. a, b, c are constants determined by Statistica 7 and TA is the time (hours). The kinetic parameters

were determined from the model were lag phase (equation 10), specific growth rate (equation 11), and generation time (equation 12).

$$\lambda = {}^{(b\text{-}1)}/_{c} \qquad [\text{hours}]$$

*Equation 10. Lag Phase*

$$\mu max = a.c \qquad [\text{hours}^{-1}]$$

Equation 11. specific growth rate

$$G = {}^{\ln 2}/_{\mu max} \qquad [\text{hours}]$$

Equation 12. Generation Time

#### 4.2.4.3 Sugar determination in the hidrolizate

The reducing sugars in the hydrolyzate were determined by method 3,5 - dinitrosalicylic acid (DNS). To determine the reducing sugars, 1 mL of each supernatant obtained in the determination of biomass was taken and mixed with 1 mL of DNS in a test tube, then the mixture was heated to 96 ° C for 5 minutes and then cooled for 10 minutes. Then, 5 mL of distilled water were added and the measurements were made in a spectrophotometer with a wavelength of 540 nm. The equation 13 represents the standard curve with $R^2$ = 0.9974 (the reference sugar was xylose). The consumption of sugar for each of the yeasts was also determined using the equation 7.

$$Y = 0.7574\,x\text{-}0.0109 \quad [A]$$

Equation 13. Reducing sugar using xylose as parameter

Where Y represents the absorbance as a function of the concentration of sugar (A) and X represents the concentration of sugar in (g / L).

#### 4.2.4.4 Statistical analysis of the growth kinetics in the hydrolysate with different C/N ratios

For the statistical analysis, a completely randomized design with 2 repetitions was used. The generation of biomass in the hydrolyzate was determined as a response variable using three factors (C/N ratios). An ANOVA was used as a means comparison technique and Tukey as a test to compare treatments. As statistical software was used Statistica 7.

### 4.2.5 Yeast characterization

After selecting the C/N ratio that provided the highest amount of biomass per yeast, the biomass was characterized.

#### 4.2.5.1 Organic Nitrogen Quantification

The nitrogen quantification was realized by the micro Kjeldahl method (MILLER; OUGHTON, 1945) and each yeast was analysed by quadruplicate. 0.2 g of each dried-freeze yeasts was mixed with 1 g of the mixture of copper sulfate and potassium sulfate (1:9, respectively). Then, 5 mL of sulfuric acid (96.5 %) was added and the mixture was stirred until the homogenization of the compounds. The mixture was in the digester at 420°C and 3 hours. Later, the samples was cooled at room temperature. Then, the samples was a distillated process with 30 mL distillated water, 20 mL boric acid and 40 mL sodium hydroxide. The distillated was titrated with sulfuric acid 0.02 N. Finally, the values obtained was replaced in the equation 14.

$$\text{Protein (\%)} = \frac{(\text{Va-Vb}) \text{ x Fc x N x F x } 0.014 \text{ x } 100}{\text{P}}$$

Equation 14. Protein percent (w/w)

Where Va is the acid volume that was spent in the sample, Vb is the acid volume that was spent in the blank, Fc is the correlation factor of acid (1.036), N is the normality of the acid (0.02), F is the conversion factor of nitrogen in protein, 0.014 is the milliequivalent gram of nitrogen, P sample weight (g). As conversion factor was used 6.25 to animal protein.

#### 4.2.5.2 Cellular breakdown and Total Sugars Quantification

It was weighted 20 mg of freeze dried of each yeast. Then, it was added 1 mL of $H_2SO_4$ (80%v/v) and this reaction lasted 20 hours (in the first 4 hours the reaction was in ice bath). Later, it was added 9 ml of distillated water and it was homogenized. Finished the homogenization, 1.5 ml of samples were centrifugated to 10000 RPM and 10 minutes (this process was made by triplicate for each yeasts).

The total sugars was quantified for the sulfuric phenol method (DUBOIS et al., 1956). 0.5 µL of sample was mixed with 450 µL of distillated water, 500 µL of phenol and 2.5 mL of sulfuric acid. Then, the release sugars was reading in a

spectrophotometer to 490 nm. The equation 6 represent the standard curve with a $R^2$=0.989 (the sugar standard was glucose).

#### 4.2.5.3 Lipids Quantification

The lipid quantification was made by the bligh and dyer method. to 0.5 g of each freeze dried yeast was added 3.2 ml of hydrochloric acid (4m). then, the mixture was put to 70°c by 1 hour. later, it was 8 ml of absolute methanol and with 4ml of chloroform and this mixture was stirred to 100 rpm and 25°c by 1 hour. finished the stirred process, it was added 3 ml of sodium sulfate (1.5% p/v) and the samples was centrifuged to 2000 rpm by 5 minutes. the mixture was divided in three phases (in the low phase was the chloroform with the non-polar molecules, in the medium phase was the cellular debris and in the high phase was the methanol, water and polar molecules) (breil et al., 2017). the low phase was extracted and it was put in the dried flasks (previously weight) at 60°c by 1 hour in gas cab. then the flasks were weighted and the results was used in the equation 15, lipid percent.

$$\% \text{ of lipids} = \left(\frac{\text{weight of residue x total volume of organic phase}}{\text{weight of yeast x evaporated volume}}\right) \text{x } 100$$

Equation 15. Lipids Percent (w/w)

#### 4.2.5.4 Fatty Acids determination

To the fatty acid determination, 1 mg of the dry lipids was mixed with 1,5 mL of heptane, then, this mixture was injected in Gas Chromatography. FAME were analyzed in a Varian® 450-GC gas chromatograph coupled to a Varian® 320-MS mass spectrometer in which the sample ionization was carried out by electron impact. The VF-5MS chromatographic column (30 m x 0.25 mm x 0.25 µm) was eluted with helium as the carrier gas at 0.8 mL $min^{-1}$ and the pressure at the tip of the column was 2 mmTorr. The injection volume was 2.0 µL and the split ratio was 1:50. The injector, manifold, transfer line, and ion source temperatures were set at 40, 280 and 300 °C, respectively. The column oven was adjusted to the following temperature program: isothermal at 100 °C for 1 min, heating at 10°C $min^{-1}$ until 200 °C, isothermal at 200 °C for 2 min, heating at 3.5°C $min^{-1}$ until 260 °C, and isothermal at 260 °C until a total running time of 37 min was reached. The mass spectra were acquired at every 0.5 s in the 32 to 380 m/z mass range.

FAME components were identified either by comparison of their retention times with analytical standards (C4-C24 FAME mixture, Supelco®) or by interpretation of their mass spectra, whose similarity index was investigated using the NIST (National Institute of Standards and Technology) spectral database. FAME quantification was carried out by area normalization.

#### 4.2.5.5 Ash Determination

The ash content was measured gravimetrically, according to AOAC (1990). The samples in the crucibles were incinerated in a muffle (Quimis, Brazil) at 550 °C for 6 h.

## 4.3 RESULTS AND DISCUSSIONS

### 4.3.1 Growth kinetic in YM medium

In the table 10 is shown the biomass production in synthetic medium YM, the sugar consumption, and the kinetics parameters biomass yield, substrate consumption and specific growth rate.

The cell growth is shown in the figure 8 and it can be evidenced that, the lag phase was small to both yeasts. The exponential phase begins little hours after the initial time and end at 48 hours. In this period, the biomass weight reached the maximum value in the two yeasts, the values were 2.91 g/L and 3.25 g/L to pichia jadinii CCT 1518 and Cyberlindnera jadinii CCT 2612, respectively. Similar results was obtained by Rosma and contributors with Candida utilis, they reached a biomass quantity around of 2.25 g/L at 30ºC, 100 RPM and 30 hours (ROSMA, A. AND OOI, 2006). Added to this, the substrate consumption was 75% and 92% in the first 24 and 48 hours approximately, to both yeast. This confirmed the high use of carbon source to the cell division in the exponential phase. The biomass yield was calculated to each yeast at 48 hours, pichia jadinii CCT 1518 presented a biomass yield a sight small than cyberlindnera jadinii CCT 2612 (0.225 against 0.251 g/g); which meaning that, the two yeasts only could transform around of 22 to 25 % of the carbon source in biomass, the remaining carbon source was used to other cellular functions. Then of 48 hours, the yeasts begin the stationary phase by the depletion of the carbon source and a small loss of the weight, in this moment, it was calculated the substrate consumption. The substrate consumption was similar for the both yeasts; the results were 92.97 and 95.41,

to *Pichia jadinii* and *Cyberlindnera jadinii*, respectively. The remaining sugar was lower to 8% after 72 hours, in both case. Finally, it was determinated the global productivity in a work volume of 100 ml. The global productivity of Pichia Jadinii was 0.0036 g/L and for Cyberlindnera jadinii was 0.0039 g/L.

Table 10. Kinetics of Cell growth

| Yeast | Time (h) | Biomass (g/L) | Glucose quantity (g/l) | BiomassYield (g/g) | Substrate consumption (%) | Global productivity (g/L) |
|---|---|---|---|---|---|---|
| Pichia Jadinii 1518 | 0<br>24<br>48<br>72 | 0,28<br>1,37<br>2,91<br>2,84 | 12,53<br>3,22<br>0,91<br>0,88 | 0,2259 | 92,97 | 0,0036 |
| Cyberlindnera Jadinii 2612 | 0<br>24<br>48<br>72 | 0,30<br>1,47<br>3,25<br>3,13 | 12,68<br>3,14<br>0,92<br>0,57 | 0,251 | 95,46 | 0,0039 |

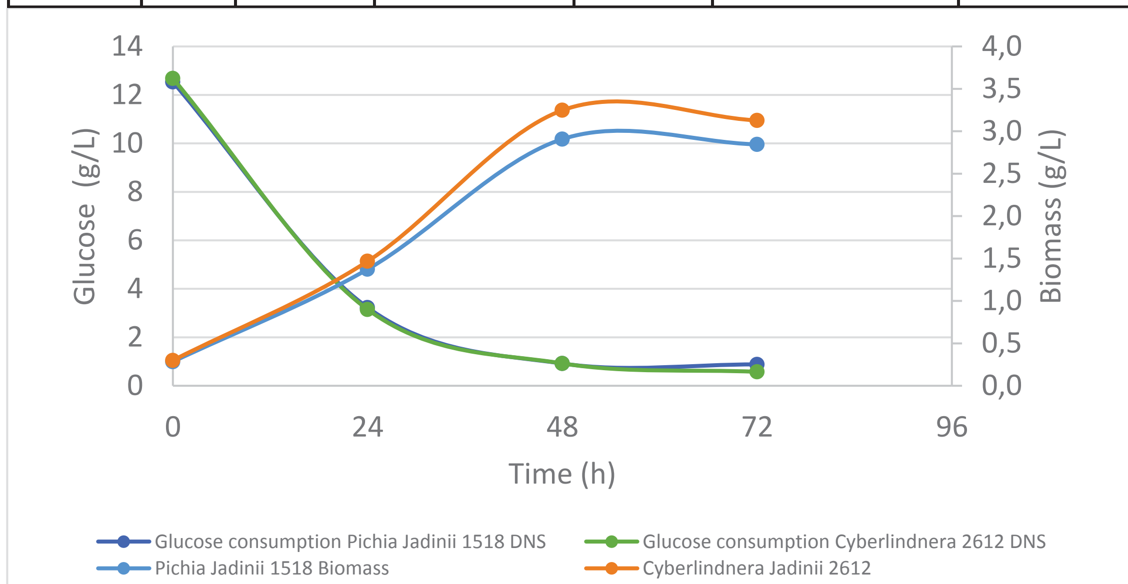


*Figure 8. Growth Kinetics and sugar consumption of Pichia jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612 in YM medium*

For the above, the exponential phase was presented in the first 48 hours and the 75% of this phase is around of the 36 hours. This value has been reported by others authors like the adequate time to a inoculum of the same genus (CADAVID

VILLALBA et al., 2009). For these reasons, 36 hours was selected like the time before to inoculate the aqueous phase of EFB.

### 4.3.2 Composition of the EFB Hydrolysate and supplementation with nitrogen

The table 11 shown the elemental composition of the EFB hydrolysate in percent, and the annexed 3 shown the elemental composition of the nitrogen sources.

*Table 11.* Elemental composition of the Hydrolysed of the EFB (%)

| **Product** | **N** | **C** | **H** | **S** | **O** |
|---|---|---|---|---|---|
| EFB Hydrolysate | 0.076 | 1.643 | 9.631 | 1.464 | 87.18 |

The oxygen value was calculated like the difference between 100% minus the others components sum
The EFB Hydrolysate wasn`t determinated in dry basis as the others products.

The composition of the substrate is an important parameter in the cell growth, where the nitrogen quantity generally is adjusted by the C/N ratio, and this variable can determinate the generation of some compounds as biomass, proteins, carbohydrates, lipids and others (AROUS et al., 2015). The carbon source in the EFB Hydrolysate is approximately 1.65 %. This valor was expected because the HPLC analysis of the chapter 2 was evidenced that, the sum of all analyzed compounds in the hydrolysed was around of 28-30 g/L and the carbon source is almost 50 – 60%. The carbon quantity in the EFB hydrolysate is almost three times more than the carbon source supplied by the glucose in the YM medium; which ensure the sufficient quantity of carbon source for the cell growth. On the other hand, the quantity of nitrogen is 0.076%, which is a very low level for the biomass production (GUTIÉRREZ et al., 2012). For this reason, the nitrogen supplementation is necessary, and some investigations reported that a C/N ratio between 10 to 11.5 can be generated a higher biomass production, without to have nitrogen in excess. Accordingly, the EFB hydrolysate was supplied with nitrogen until to reach a C/N ratio of 11.5 and it was selected two levels more of C/N ratios (9 and 14) to evaluate the effect in the biomass production of the yeast Cyberlindnera Jadinii CCT 2612 and Pichia Jadinii CCT 1518.

### 4.3.3 Effect of the C/N ratio on the biomass production and sugar consumption of the *Pichia jadinii* yeast.

The table 18 shown the data of the biomass production and others kinetics parameters of *Pichia Jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612 in the EFB Hydrolysate supplemented with different C/N ratios (Annexed 1).

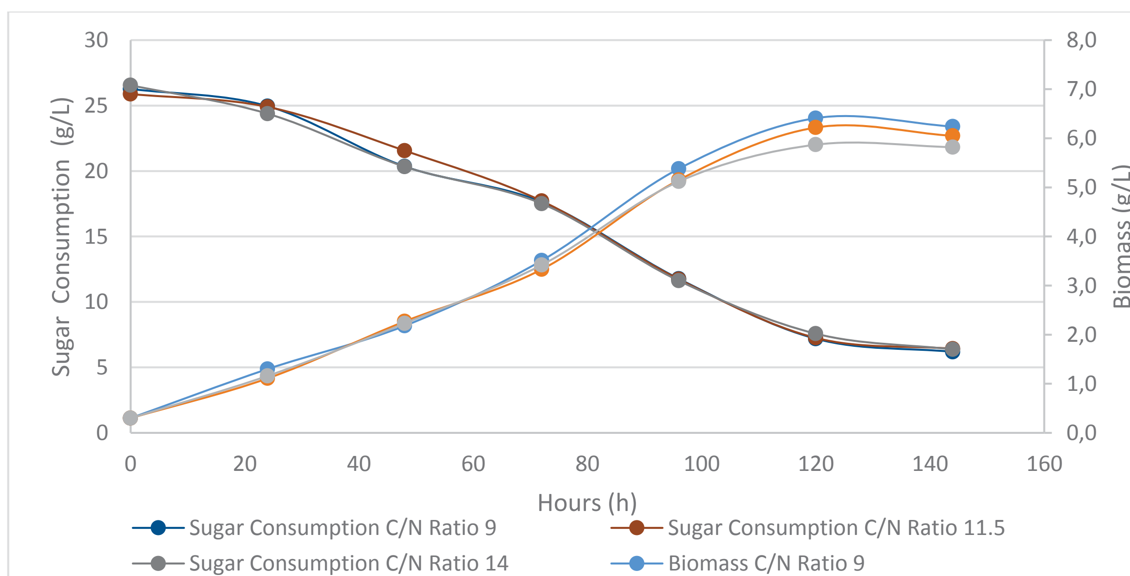


*Figure 9. The biomass production and the substrate consumption of Pichia Jadinii CCT 1518 in the hydrolyzed of the EFB*

The biomass production and the substrate consumption of Pichia Jadinii is shown in the figure 9. The lag phase in the three C/N ratios was 20 hours approximately, in this period cann`t be seen a difference between the three C/N ratios and the sugar consumption was around of 10% of the initial sugars . Add to that, this period was longer than the lag phase in the YM medium of the same yeast, but, this was expected by complexity of the medium (xylose as carbon source and toxic compounds for microbial growth) and by the synergic effect of the toxic compounds as it`s reported by others authors in hydrolyses (CADAVID VILLALBA et al., 2009).

The exponential phase began at 20 hours and ended at 120 hours. The exponential phase was very long time and here, the biomass production was maximum (6 g/L, approximately), at 120 hours can be seen a slight difference in the biomass production between the C/N ratios; the biomass production was 5.9, 6.2 and 6.4 g/L, for the c/n ratios 14, 11.5 and 9, respectively. The ANOVA

analysis in the point with highest biomass production didn`t show significant difference between the three C/N ratios (P>0.05) (see table 12). This result wasn`t agree with Egli and contributors, which obtained the highest biomass concentration of the yeast Hansenula polymorpha in ratios C/N <11.32 (EGLI; QUAYLE, 1986).

The biomass yields was determinated at 120 hours to each C/N ratio, the values were 0.293, 0.318 and 0.32, to the C/N ratio 14, 11.5 and 9, respectively. This result meaning that, the transformation of carbon source in biomass was around of 31% and this value was higher compared with kinetic of the same yeast in the YM medium. On other hand, the sugar consumption in the final of this phase was around of 71%, and compared with the kinetic in YM medium, the difference was big (in the YM medium was around 90%). Probably this behavior some nutrient was depleted and the yeasts couldn`t continue generating biomass or processing the toxic compounds of the hydrolyzed.

*Table 12. ANOVA to biomass production of Pichia jadinii CCT 1518 in the EFB Hydrolysate*

| Factor | SS | df | MS | F | p |
|---|---|---|---|---|---|
| Treatment | 0.1233 | 2 | 0.0617 | 0.943 | 0.481143 |
| Error | 0.1962 | 3 | 0.0654 | | |
| Total | 0.3195 | 5 | | | |

Finally, the stationary phase began after of the 120 hours and ended at 144 hours with a significate change in the slope of the growth function. At 144 hours was determinated the global production and the substrate consumption. The substrate consumption at 144 hours was around of 76% for all cases; this means that, more than 20% was not taken advantage to produce biomass and in the kinetic with YM medium the remaining sugar was lower than 8%. On the other hand, the global production value varied between 0.0038 and 0.0041 g/L h (see annexed 1) this value is slightly high that the value reported in the kinetic with YM medium. In spite of the greater amount of time in the kinetics with hydrolyzed compared against the kinetics with YM medium, the amount of sugar remaining is greater in the kinetics with hydrolyzate and therefore, the productivity is higher.

**4.3.4 Effect of the C/N ratio on the biomass production and sugar consumption of the *Cyberlindnera jadinii* yeast**

The biomass production and sugar consumption of *Cyberlindnera jadinii* CCT 2612 is shown in the figure 10. The lag phase was around of 20 hours for the three C/N ratios used; the duration of this phase compared with the fermentation of the same yeast in the YM medium, it was higher. The duration of the lag phase of *Cyberlindnera jadinii* was similar to the lag phase of *Pichia jadinii* CCT 1518 in the EFB hydrolysate supplied with nitrogen and it was the double compared with the reports of Rosma and contributors with *Candida utilis* in a pineapple waste(ROSMA, A. AND OOI, 2006).

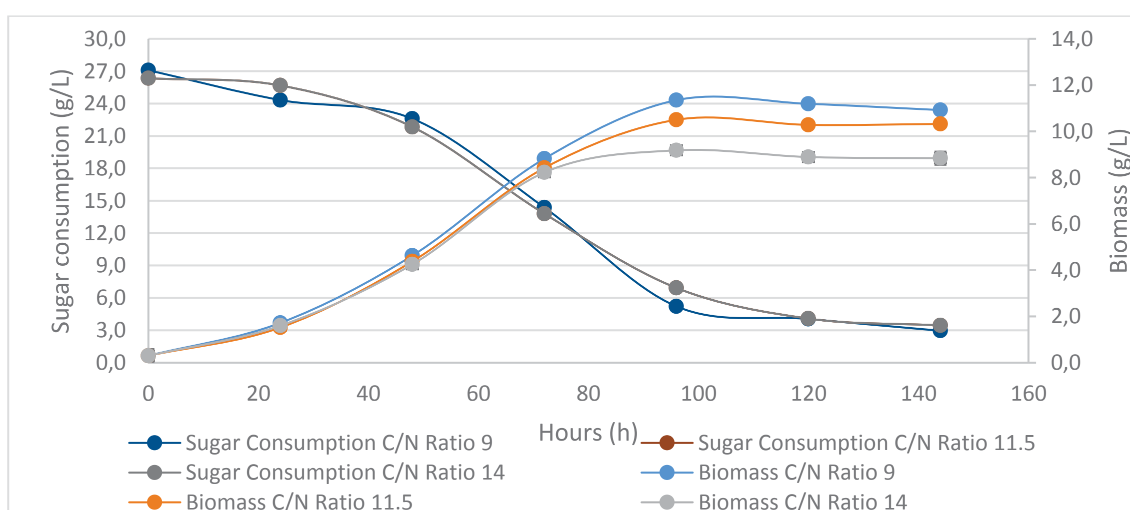


*Figure 10. Effect of the C/N ratio on the biomass production and sugar consumption of Cyberlindnera jadinii CCT 2612*

The exponential phase began after of 20 hours and ended at 96 hours. The duration of the exponential phase was around of 76 hours for all C/N ratios; this value is lower than the exponential phase of *Pichia jadinii* CTT 1518 (≈100 hours). The biomass production was 9.18, 10.5 and 11.2 g/L, for the c/n ratios 14, 11.5 and 9, respectively. This result is agree with Arous and contributors using *Candida pararugosa and Schwanniomyces etchellsii* in olive mill waste water with different c/n ratios (AROUS et al., 2015). On the other hand, the ANOVA analysis showed significate difference between the C/N ratios ($p<0.05$) (see the table 13). The C/N ratios 14 and 9 are significate different ($P=0.0255$), but, these c/n ratios didn`t present significate difference with the C/N ratio 11.5 (see the table 14). This result is very important from the economic point of view. Additionally, the biomass

production of the *Cyberlindnera jadinii* CCT 2612 was the double compared against *Pichia jadinii* CCT 1518.

*Table 13. ANOVA to biomass production of Cyberlindnera jadinii CCT 2612 in EFB hydrolysate*

| Factor | SS | df | MS | F | p |
|---|---|---|---|---|---|
| Treatment | 4.7825 | 2 | 2.3913 | 14.694 | 0.02819 |
| Error | 0.4882 | 3 | 0.1627 | | |
| Total | 5.2707 | 5 | | | |

*Table 14. Tukey HDS test to biomass production of Cyberlindnera jadinii CCT 2612 in EFB hydrolysate*

| **Treatment** | **Tukey HDS test, Variable: biomass (g/L), MS= 0.16273, df=3** | | |
|---|---|---|---|
| **c/n ratio** | **9 (11.35)** | **11.5 (10.5)** | **14 (9.18)** |
| 9 | | 0.2355 | 0.0255 |
| 11.5 | 0.2355 | | 0.0920 |
| 14 | 0.0255 | 0.0920 | |

The biomass yield was determinate at 96 hours, the values were 0.505, 0.509 and 0.457, to the c/n ratio 14, 11.5 and 9, respectively. In this case, the transformation of carbon source in biomass was around of 47% in general, and compared against the biomass yield of the same yeast in YM medium, it was the double. In comparison against *Pichia jadinii* CTT 1518, the biomass yield was higher in 16%. Moreover, the sugar consumption in this phase was around of 77% to all C/N ratios. This sugar consumption was lower compared against of sugar consumption the same yeast in YM medium in the final of exponential phase (95.46%). In comparison with the sugar consumption of Pichia jadinii in EFB hydrolysate supplied with nitrogen, it was a little higher.

The stationary phase of *Cyberlindnera jadinii* CCT 2612 began at 96 hours, in this phase; there was a little loss of weight. At 144 hours was determinated the global production and the substrate consumption. The substrate consumption was around of 87% in general, this value was lower in comparison against the same yeast in YM medium; however, this value was higher in comparison against the *Pichia jadinii* CCT 1518 in the same conditions. *Cyberlindnera jadinii* CCT

2612 didn`t waste so much substrate as *Pichia jadinii* CCT 1518, 13% against 20%. By last, the global productivity was calculated, this value varied between 0.0059 and 0.0073 (g/h L), to the C/N ratios 14 and 9, respectively. This global productivity was the double in comparison with *Pichia jadinii* CCT 1518, and it can explained by the high biomass production.

#### 4.3.5 Effect of the C/N ratio on the cell counting of the *Pichia jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612.

The table 19 shown the effect of the c/n ratio on the cell counting of the work yeasts (see annexed 2). The data of this table was used in the modified Gompertz model.

The logarithm of the number of cell of the *Pichia jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612 is shown in the figure 11. In this figure can be see the difference in the concentration cellular of the two yeasts in the time zero. The *Cyberlindnera jadinii* CCT 2612 yeast present an initial concentration around of 22 x $10^6$ cell/mL for all C/N ratios (7.3 in log scale), while that, the Pichia jadinii CCT 1518 yeast had an initial concentration around of 11 x $10^6$ cell/mL for all C/N ratios (7 in log scale). The initial concentration of Cyberlindnera jadinii CCT 2612 is almost the double compared with Pichia jadinii CCT 1518, however, this difference wasn`t very evident in weight.

The lag phase can be see more clearly. The difference in the number of cell between the initial time and the time equal to 20 hours wasn`t so much, the approximate values for Pichia Jadinii CCT were 11 x $10^6$ and 12x $10^6$ cell/mL (7 and 7.1 in log scale). In the case of *Cyberlindnera jadinii* CCT 2612, it were 22 x $10^6$ and 32x $10^6$ (7.3 and 7.5 in log scale). The two yeasts didn`t present a lot difference in the cell concentration between the three C/N ratios. This behavior is agree with the cell growth of bacteria and yeasts in batch production (TORTORA et al., 2013).

The first difference between *Pichia jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612 was the duration time, approximately 100 and 75 hours, respectively. The cell concentration of *Pichia Jadinii* CCT 1518 changed of 12x $10^6$ to 12x $10^7$ cell/mL in this phase, in other terms, it`s cell concentration multiplied by 12 times for all C/N ratios. In the case of *Cyberlindnera jadinii* CCT 2612 multiplied it`s cell

concentration close to 16 times for all C/N ratios (changed of 7.5 to 8.7 in log scale). On other hand, the cell concentration between the two yeast in the final of exponential phase is approximately 5 times, Pichia Jadinii CCT 1518 presented a cell concentration of 12x $10^7$ cell/mL against 57.7x $10^7$ cell/mL of *Cyberlindnera jadinii* CCT 2612. Additionally, in this point the difference in the cell concentration between the C/N ratios 9 and 11.5 against the C/N ratio 14, for both yeasts, but it´s more clear in *Cyberlindnera jadinii* CCT 2612. This can support the difference in the biomass production between the C/N 9 and 11.5 against the C/N ratio 14.

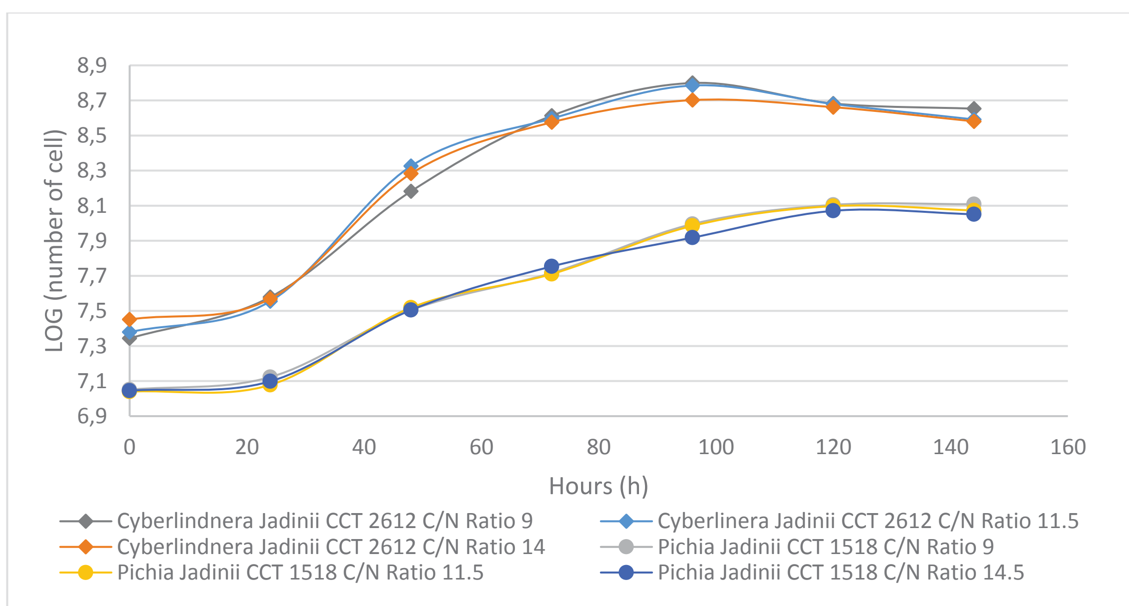


Figure 11. Effect of the C/N ratio on the cell counting of the Pichia jadinii CCT 1518 and Cyberlindnera jadinii CCT 2612.

According to the data obtained previously, the biomass production decreased a little after the exponential phase and the cell concentration, too. In the *Cyberlindnera jadinii* CCT 2612 yeast the cell concentration decreased slight more than the cell concentration of Pichia jadinii CCT 1518. This behavior can be for the duration of the exponential phase, in the case of *Pichia Jadinii* CCT 1518 lasted until 144 hours against the 96 hours of *Cyberlindnera jadinii* CCT 2612. At 144 hours after of the inoculation of the yeast, the effect of c/n ratio wasn`t very clear, the C/N ratio 9 in both yeasts presented a slightly higher cell concentration in comparison with C/N ratio 11.5 and 14. But, it`s not much.

**4.3.6 Application of the modified Gompertz model and data adjustment**

After the cell counting in the neubauer camera, the experimental data was runs

in the modified Gompertz model. The table 19 shown the data for the application of the modified Gompertz model and data adjustment (See annexed 2). The column 5 shown the experimental values of the cell account, the column 7 the predicted values with the modified Gompertz model and the column 8 the adjustment between the experimental values and the predicted values. According to the adjustment data, all predicted data for the cell growth kinetics curves have an adjustment higher to 90%. However, the datas presented a higher adjustment with Pichia Jadinii CCT 1518 kinetics curves compared against Cyberlindnera jadinii kinetics curves (around 3-8). This is good result because in the modified Gompertz model only was used the cell counting values and the time. generally, to reach high adjustment in alternative substrates and with little-known microorganisms is necessary more information. In this way, the modified Gompertz model is a strong tool to begin the optimization process because describes accurately the growth kinetics in an easy way.

The table 15 shown the Gompertz parameters (determined by Statistica 7) and the kinetics parameters (determined by the equations $10$, $11$ and $12$). The value a express the initial number of viable microorganisms, and this values are agree with the cell account values, where the Cyberlindnera jadinii CCT 2612 yeast began with a higher cell concentration compared with the other work yeast. The value b indicate the relative velocity of growth expressed in units of 1/maximum velocity in the work conditions. The value c meaning the difference between the initial number and the maximum accounting (RAMÍREZ, 2016).

*Table 15.* Gompertz parameters and kinetics parameters

| Gompertz parameters | | | | Kinetics parameters | | |
|---|---|---|---|---|---|---|
| **Yeast** | **C/N Ratio** | **Constant** | **Constant value** | **Lag phase (h)** | **Specific velocity of growth ($h^{-1}$)** | **Generation time (h)** |
| Pichia jadinii CTT 1518 | 9 | a<br>b<br>c | 1,111872<br>1,722124<br>0,035598 | 20,28 | 0,039580 | 17,51 |
| | 11,5 | a<br>b<br>c | 1,078400<br>1,859779<br>0,039439 | 21,80 | 0,042531 | 16,29 |
| | 14 | a<br>b<br>c | 1,031651<br>1,861006<br>0,040733 | 21,13 | 0,042022 | 16,49 |
| Cyberlindnera Jadinii CTT 2612 | 9 | a<br>b<br>c | 1,382869<br>2,061168<br>0,059535 | 17,82 | 0,082329 | 8,41 |
| | 11,5 | a<br>b<br>c | 1,302663<br>2,550150<br>0,077061 | 20,11 | 0,100384 | 6,90 |
| | 14 | a<br>b<br>c | 1,197222<br>2,723795<br>0,078002 | 22,09 | 0,093385 | 7,42 |

The duration in lag phase was determined with the modified Gompertz model, these results were similar to the values estimated in the biomass production and the cell counting figures. The lag estimated by the model varied in function of the c/n ratios for each yeast, when the C/N ratio decrease, the lag phase decrease, too. The specific velocity of growth was higher in the Cyberlindnera jadinii CCT 2612 yeast compared with the other work yeast (almost 2 times). The yeasts in the EFB hydrolysate with the C/N ratio 9 presented the lowest specific velocity, however, the model assigns for the same C/N ratio, the highest number of viable microorganisms. Additionally, the C/N ratio 11.5 presented the highest specific velocity of the growth, this may explain why there is no significant difference in the biomass production and cell counting with the C/N ratio 9. On other hand, the generation time was according with the biomass production between the two yeasts. The generation time of Pichia jadinii CCT 1518 was the double. Moreover, these results was according the specific velocity of the growth, the treatments with higher specific velocity of the growth presented the lower generation time.

### 4.3.7 Yeast Composition

The data presented on table 16 shown the macromolecular composition of the yeasts Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612.

*Table 16.* Yeast characterization

| Yeast Composition | | | | | |
|---|---|---|---|---|---|
| Compound | Percent (% w/w) | | | | Method |
| | PJ CCT 1518 | SD | CJ CCT 2612 | SD | |
| Crude Protein * | 35,972 | ±1,022 | 44,434 | ±0,21 | Micro Kjedahl |
| Lipids | 3,29 | ±0,222 | 2,234 | ±0,279 | Bligh and Dyer |
| Sugars | 37,28 | ±2,1 | 33,13 | ±1,6 | Sulfuric Phenol |
| B glucans | 3,142 | ±0,002 | 3,003 | ±0,001 | Mueller et al. |
| Ash | 6,3 | ±0 | 5,9 | ±0,003 | AOAC.1990 |

#### 4.3.7.1 Crude protein

The crude protein was determinated for the two work yeasts in the substrate specific conditions. Pichia jadinii CCT 1518 reported 35.97 % (w/w), while, Cyberlindnera jadinii CCT 2612 presented 44.434 % (w/w). The difference between the two yeast in the percent of crude protein is around of 10%, however, the C/N ratio in the EFB hydrolysate for the Pichia Jadinii CCT 1518 was lower compared with C/N ratio in the EFB Hydrolysate used with Cyberlindnera Jadinii CCT 2612. Ibrahim and contributors evaluated the crude protein quantity in *Candida utilis* PY 12 using as substrate Rice Polishing supplied with yeast extract and urea; they obtained 32.75% (w/w) (RAJOKA et al., 2004). This result is similar to crude protein quantity obtained with the Pichia Jadinii yeast. Other investigation realized by Olvera and contributors evaluated the use of *Candida utilis* in the tilapia growth. The *Candida utilis* growth in synthetic medium and it presented around of 46.11 % (w/w) of crude protein (OLVERA-NOVOA et al., 2002), such as, *cyberlindnera jadinii* in the EFB hydrolysate supplied with c/n ratio 11.5. The results obtained with the working yeasts show that, the two reasons C/N can generate levels similar to the literature in the amount of crude protein.

#### 4.3.7.2 Lipid quantification and fatty acids determination

Pichia Jadinii CCT 1518 presented 3.28% (w/w) of lipids in dry basis, while, Cyberlindnera jadinii CCT 2612 presented 2.234 % (w/w). These values are agree with the Nasseri and contributors; they reported a lipid quantity in yeasts around of 2 and 6 % (w/w). On other hand, Olvera and contributors, obtained with *candida utilis* 1.51 % (w/w) and Ibrahim et al, obtained 4.68% (w/w). Moreover, in the table 17 can be show the fatty acids composition of each one yeasts. The quantity of fatty acid generated by Pichia Jadinii CCT 1518 is slightly bigger compared against Cyberlindnera jadinii CCT 2612. Pichia jadinii CCT 1518 presented fatty acids with 16 and 18 carbons, with ramifications and without insaturations. In the case of Cyberlindnera jadinii, the fatty acids presented 16 and 18 carbons, with ramifications and some fatty acids with insaturations. Since of economic point of view Pichia jadinii presented a bigger yield compared with Cyberlindnera jadinii CCT 2612, however, Cyberlindnera jadinii presented some unsaturated fatty acids and with ramifications that they can be used as biofuels.

*Table 17.* Fatty Acid composition of *Pichia Jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612

| ***Pichia Jadinii* CCT 1518** | | |
|---|---|---|
| **Molecular Formula** | **Compound Name** | **Retention Time** |
| C17H34O2 | Me. C16:0 (Methyl hexadecanoate, Methyl palmitate, C16:0 Methyl ester) | 15.079 |
| C18H39O2 | Et. C16:0 (Hexadecanoic acid Ethyl ester) | 16.332 |
| C19H34O2 | Me. C18:2n6 (Linoleic acid, methyl ester) | 18.363 |
| C19H36O2 | 9-Octadecenoic acid, methyl ester, (E)- | 18.484 |
| C20H36O2 | Linoleic acid ethyl ester | 19.731 |
| C20H38O2 | Ethyl Oleate | 19.854 |
| C22H43NO | 13-Docosenamide, (Z)- | 33.102 |
| ***Cyberlindnera Jadinii* CCT 2612** | | |
| **Molecular Formula** | **Compound Name** | **Retention Time** |
| C19H34O6 | Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester | 14.700 |
| C17H34O2 | Me. C16:0 (Methyl hexadecanoate, Methyl palmitate, C16:0 Methyl ester) | 15.089 |
| C18H34O2 | Ethyl 9-hexadecenoate (Palmitelaidic acid ethyl ester) | 15.955 |
| C18H39O2 | Et. C16:0 (Hexadecanoic acid Ethyl ester) | 16.344 |
| C19H36O2 | Me. C18:1n9 (octadecenoic acid Methyl ester) | 18.512 |
| C19H38O2 | Me. C18:0 (octadecanoic acid Methyl ester) | 19.015 |
| C20H38O2 | (E)-9-Octadecenoic acid ethyl ester | 19.872 |
| C20H40O2 | Et. C18:0 (octadecanoic acid Ethyl ester) | 20.418 |

#### 4.3.7.3 Sugar quantification

Pichia Jadinii CCT 1518 presented 37.28 % (w/w) of total carbohydrates in dry basis, while, Cyberlindnera jadinii CCT 2612 presented 33.13 % (w/w). These values are higher than the results reported by Alves do Nascimento, He reported a quantity of total carbohydrate in *Candida utilis* around of 16 % (w/w). However, the protein quantity of Alves was higher compared with the current study (61% against 44%). It is probably that, he modified some metabolic route to increase the protein yield, but, decreased the quantity of the total carbohydrates (NASCIMENTO, 2008). on the other hand, the beta glucan yield was agree with the Muller report`s (MULLER et al., 1996). Aditionally, the beta glucan yield was slightly bigher in Pichia Jadinii CCT 1518. Of the same mode, the quantity of total carbohydrate was higher in this yeast compared with cyberlindnera Jadinii CCT 2612.

#### 4.3.7.4 Ashes

The ashes of the two working yeasts were determined as shown in the table 16. the amount of ash from Pichia Jadinii CCT 1518 was greater than, the amount of ash from Cyberlindnera Jadinii CCT 2612. the amount of ashes is in accordance with the reports of Nasseri and collaborators (NASSERI et al., 2011). They report an amount of ash for yeasts of 5 to 10%. On the other hand, the amount of ash was lower than the Alves report (NASCIMENTO, 2008).

## 4.4 CONCLUSIONS

It was proven that, *Pichia jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612 can to growth in the non- detoxified EFB hydrolisate. Additionally, it was determinated the cell growth kinetics of the both yeasts in the non- detoxified EFB hydrolisate supplied with nitrogen in different C/N ratios (9, 11.5 and 14) using as response variables the biomass production and the cell counting. The biomass production to *Pichia jadinii* CCT 1518 was 6.41, 6.22 and 5.87, for the C/N ratios 9, 11.5 and 14, respectivamente. In this case, the ANOVA analysis determined that didn´t presented significate difference. The biomass production to *Cyberlindnera jadinii* CCT 2612 was 11.35, 10.50 and 9.18, for the C/N ratios 9, 11.5 and 14, respectivamente. For *Cyberlindnera jadinii* didn`t presented significate difference between the C/N ratios 9 and 11.5,which it`s very important since economic point view. On other hand, With the cell counting data was

applied the modified Gompertz model which it be adjustmented in more of 90% to each treatment evaluated and it can help a next optimization process.

The amount of lipids, crude protein, total carbohydrates, beta glucans and ashes was determined for *Pichia jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612. In the crude protein parameter, the yeast Cyberlindnera Jadinii CCT 2612 presented the highest yield, nevertheless, the relationship c / n of the hydrolyzate was greater. On the other hand, *Pichia Jadinii* CCT 1518 presented more lipids, carbohydrates, beta glucans and ashes. however, the differences with the other yeast analyzed were not very large.

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*Table 18. Cell Growth kinetic of Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CTT 2612 in the hydrolysed of the EFB*

| Yeast | C/N Ratio | Time (h) | Biomass (g/L) | Reducing sugars | Biomass Yield (g /g) | Global productivity (g/h*L) |
|---|---|---|---|---|---|---|
| Pichia Jadinii 1518 | 9 | 0 | 0,28 | 26,27 | 0,320 | 0,0041 |
| | | 24 | 1,30 | 24,96 | | |
| | | 48 | 2,18 | 20,35 | | |
| | | 72 | 3,51 | 17,64 | | |
| | | 96 | 5,38 | 11,77 | | |
| | | 120 | 6,41 | 7,19 | | |
| | | 144 | 6,24 | 6,19 | | |
| | 11.5 | 0 | 0,28 | 25,88 | 0,318 | 0,0039 |
| | | 24 | 1,11 | 24,90 | | |
| | | 48 | 2,27 | 21,56 | | |
| | | 72 | 3,33 | 17,71 | | |
| | | 96 | 5,15 | 11,73 | | |
| | | 120 | 6,22 | 7,25 | | |
| | | 144 | 6,05 | 6,42 | | |
| | 14 | 0 | 0,28 | 26,55 | 0,293 | 0,0038 |
| | | 24 | 1,16 | 24,38 | | |
| | | 48 | 2,23 | 20,33 | | |
| | | 72 | 3,42 | 17,51 | | |
| | | 96 | 5,12 | 11,62 | | |
| | | 120 | 5,87 | 7,57 | | |
| | | 144 | 5,82 | 6,37 | | |

*Table 18. Continuation*

| Yeast | C/N Ratio | Time (h) | Biomass (g/L) | Reducing sugars | Yield (g /g) | Global productivity (g/t*L) |
|---|---|---|---|---|---|---|
| Cyberlindnera Jadinii 2612 | 9 | 0 | 0,30 | 27,09 | 0,505 | 0,0073 |
| | | 24 | 1,72 | 24,32 | | |
| | | 48 | 4,63 | 22,60 | | |
| | | 72 | 8,82 | 14,37 | | |
| | | 96 | 11,35 | 5,21 | | |
| | | 120 | 11,19 | 4,05 | | |
| | | 144 | 10,92 | 2,96 | | |
| | 11.5 | 0 | 0,30 | 25,36 | 0,509 | 0,0069 |
| | | 24 | 1,52 | 24,75 | | |
| | | 48 | 4,38 | 22,11 | | |
| | | 72 | 8,42 | 13,89 | | |
| | | 96 | 10,50 | 5,33 | | |
| | | 120 | 10,28 | 4,11 | | |
| | | 144 | 10,32 | 3,15 | | |
| | 14 | 0 | 0,30 | 26,35 | 0,457 | 0,0059 |
| | | 24 | 1,60 | 25,68 | | |
| | | 48 | 4,25 | 21,84 | | |
| | | 72 | 8,23 | 13,79 | | |
| | | 96 | 9,18 | 6,92 | | |
| | | 120 | 8,89 | 4,08 | | |
| | | 144 | 8,84 | 3,45 | | |

*The global productivity was determinated in a work volume of 100 ml.

## Chapter 4. Annexed 2

*Table 19. Data for the application of the modified Gompertz model and data adjustment*

| Yeast | C/N Ratio | Time (h) | Average number of cell | LOG | LOG (N/No) | N=(10^YA)/No* | $R^{2**}$ |
|---|---|---|---|---|---|---|---|
| Pichia Jadinii 1518 | 9 | 0 | 11250000 | 7,051 | 0,000 | 11357398,91 | 0.99 |
| | | 24 | 13269230,77 | 7,123 | 0,072 | 14252482,66 | |
| | | 48 | 32019230,77 | 7,505 | 0,454 | 28489952,6 | |
| | | 72 | 52115384,62 | 7,717 | 0,666 | 59358397,71 | |
| | | 96 | 98942307,69 | 7,995 | 0,944 | 94748454,26 | |
| | | 120 | 127211538,5 | 8,105 | 1,053 | 120082007,2 | |
| | | 144 | 128365384,6 | 8,108 | 1,057 | 133866204,6 | |
| | 11.5 | 0 | 10961538,46 | 7,040 | 0,000 | 11005855,01 | 0.98 |
| | | 24 | 11971153,85 | 7,078 | 0,038 | 13460706,23 | |
| | | 48 | 33028846,15 | 7,519 | 0,479 | 28170721,19 | |
| | | 72 | 51346153,85 | 7,711 | 0,671 | 60362321,14 | |
| | | 96 | 96634615,38 | 7,985 | 0,945 | 93773567,76 | |
| | | 120 | 125192307,7 | 8,098 | 1,058 | 114550246 | |
| | | 144 | 118269230,8 | 8,073 | 1,033 | 124413977,7 | |
| | 14 | 0 | 11105769,23 | 7,046 | 0,000 | 11148380,86 | 0.98 |
| | | 24 | 12548076,92 | 7,099 | 0,053 | 13720274,01 | |
| | | 48 | 32019230,77 | 7,505 | 0,460 | 28890929,82 | |
| | | 72 | 56730769,23 | 7,754 | 0,708 | 59990739,51 | |
| | | 96 | 82788461,54 | 7,918 | 0,872 | 89641321,28 | |
| | | 120 | 117692307,7 | 8,071 | 1,025 | 106756605,1 | |
| | | 144 | 112500000 | 8,051 | 1,006 | 114435459,5 | |
| Cyberlindnera Jadinii 2612 | 9 | 0 | 22067307,69 | 7,344 | 0,000 | 22094570,55 | 0.92 |
| | | 24 | 37932692,31 | 7,579 | 0,235 | 35837993,56 | |
| | | 48 | 152182692,3 | 8,182 | 0,839 | 167770359,3 | |
| | | 72 | 410000000 | 8,613 | 1,269 | 384605327 | |
| | | 96 | 630288461,5 | 8,800 | 1,456 | 491231287,9 | |
| | | 120 | 481250000 | 8,682 | 1,339 | 522480151,1 | |
| | | 144 | 449519230,8 | 8,653 | 1,309 | 530353542,9 | |
| | 11.5 | 0 | 23942307,69 | 7,379 | 0,000 | 23942504,2 | 0.91 |
| | | 24 | 35913461,54 | 7,555 | 0,176 | 35711667,3 | |
| | | 48 | 211740384,6 | 8,326 | 0,947 | 212767697,6 | |
| | | 72 | 395000000 | 8,597 | 1,217 | 415384492,1 | |
| | | 96 | 609615384,6 | 8,785 | 1,406 | 469512418,4 | |
| | | 120 | 478365384,6 | 8,680 | 1,301 | 478874381,1 | |
| | | 144 | 390384615,4 | 8,591 | 1,212 | 480369853,1 | |
| | 14.5 | 0 | 28269230,77 | 7,451 | 0,000 | 28269249,6 | 0.96 |
| | | 24 | 37067307,69 | 7,569 | 0,118 | 36830507,1 | |
| | | 48 | 192009615,4 | 8,283 | 0,832 | 193275870,7 | |
| | | 72 | 376153846,2 | 8,575 | 1,124 | 383672560,2 | |
| | | 96 | 503365384,6 | 8,702 | 1,251 | 434879924,4 | |
| | | 120 | 458653846,2 | 8,661 | 1,210 | 443574684,0 | |
| | | 144 | 380769230,8 | 8,581 | 1,129 | 444933007,1 | |

## Chapter 4. Annexed 3

*Table 20. Elemental composition of nitrogen sources (%)*

| Product | N | C | H | S | O |
|---|---|---|---|---|---|
| Yeast extract | 11,41 | 40,19 | 8,33 | 0,81 | 39,26 |
| Malt extract | 1,39 | 38,77 | 8,53 | 1,37 | 49,95 |
| Bacteriological peptone | 14,15 | 41,69 | 8,11 | 0,82 | 35,23 |

The values in this table were calculated in dry basis, in the EMBRAPA institute.

The oxygen value was calculated like the difference between 100% minus the others components sum

## CHAPTER 5. Β GLUCAN ISOLATION AND CHARACTERIZATION OF YEASTS PICHIA JADINII CCT 1518 AND CIBERLINDNERA JADINII CCT 2612


**Abstract**

β-glucans are polysaccharides present in the cells of different organisms, such as plants, fungi, yeasts, among others. The (1-3) (1-6) β-glucans present only in yeast, have presented several beneficial effects on health, such as regulation as antiflamatory and antimicrobial effect. For this reason, it is sought to produce these biomolecules with lignocellulosic substrates to reduce the costs production. The objective of this work was to isolate (method of Muller et al.) and characterize (HPLC, NMR and FTIR) the β-glucans generated by the yeasts *Pichia Jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612, using as substrate the hydrolysate of Chapter 2 and the C/N ratio that generated more microbial biomass. It was possible to isolate β- glucans from both yeasts, the β-glucan yield for *Pichia Jadinii* CCT 1518 was 3.14% (w/w dry basis) and for *Cyberlindnera jadinii* 3% (w/w dry basis). They were isolated (1-3) β-glucans were insoluble and showed residues of other types of molecules such as proteins or chitin. Soluble (1-3) (1-6) β-glucans were isolated from both yeasts with the same method, but with fewer purification steps, however, they presented a greater amount of impurities.


### 5.1 INTRODUCTION

β glucans are structural polysaccharides present in the cell membranes of different plants, yeasts, fungi and algae. These polysaccharides have functional properties such as cholesterol regulation, have antimicrobial, anti-inflammatory, antioxidant, prebiotic, emulsifier, among others (PIZARRO et al., 2014; BZDUCHA et al., 2015).

β glucans are composed of repetitive glucose units bound in the beta position of their anomeric carbon. The anomeric carbon of β glucans can be linked to carbons 2,3,4 and/or 6 of another glucose unit. The structural conformation of β glucans is variable depending on the source, the growth conditions, the isolation and purification processes (treatment severity). The size of β glucans in fungi is

between 5 and 2000 daltons, can be linear or branched, and depending on the degree of branching (DB) of β glucan, will have greater or lesser solubility (SYNYTSYA et al., 2009). β glucans in some cases may be linked to other types of glucans, such as α glucans, forming α-β glucans. From its three-dimensional conformation, β glucans can be linear or be composed of one or three helices (this three-dimensional conformation confers different biological properties) (SAITÔ et al., 1987). Like its structure, the amount of β glucans varies according to the source, the β glucans (1-3)/(1-4) are found in cereals and their percentage is between 1.8 to 20% w/w (PIZARRO et al., 2014). In the case of yeasts, β-glucans of type (1-3)/(1-6) are found and their percentage varies between 2 to 5% w/w (MULLER et al., 1996).

The structural conformation of the two main types of β glucans (1-3)/(1-4) or (1-3)/(1-6) determines the functional properties of each polysaccharide. β glucans (1-3)/(1-4) present in vegetables, have been recognized in reducing the risk of coronary heart disease and help control the level of blood sugar by the food and drug administration (FDA), when 3g/day is consumed (PIZARRO et al., 2014). In another hand, β glucans (1-3)/(1-6) have been associated with modulation of the immune system (PIZARRO et al., 2014). For the abundance of cereals like oats, the ingestion of β glucans of type (1-3)/(1-4) can be supplemented with the consumption of about 60 grams of oats/day. However, β glucans (1-3)/(1-6) can not be easily delivered in an ordinary diet. For this reason, the pharmaceutical industry isolates, purifies and concentrates them in food supplements, which have a price ranging from 7.74 to 48.75 dollars depending on the purification processes (www.evitamins.com.br, 2018).

Taking into account the potential of the yeasts *Pichia jadinii* CTT 1518 and *Cyberlidnera jadinii* CTT 2612 for the production of biomass in toxic substrates and its GRASS certificate given by the FDA. The aims of this chapter, it was to isolate, characterize and quantify the β glucans of these two strains of yeast grown in a non-detoxified hydrolysate of EFB as a possible product of high added value for this residue.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Biomass generation

For the generation of biomass, 1 ml of each of the yeasts conserved in refrigeration was taken, and then they were placed in test tubes with YM broth (1% w/v Glicose, 0.5% w/v bacteriological peptone, 0.3% p/v yeast extract and 0.3% w/v malt extract) each separately, maintaining the inoculum ratio previously used (10% v/v) and incubated at a temperature of 30 °C for 36 hours. The second repique consisted of depositing each fermented broth for each one of the yeasts, in erlens mayers with YM broth, conserving the inoculum/work volume ratio (10% v/v), after inoculated, they were incubated at 30°C, 120 RPM for 36 hours. For the third repique, each yeast was inoculated into erlen mayers containing hydrolyzed EFB with the C/N ratio selected in chapter 3 (C/N ratio 14 to Pichia Jadinii CCT 1518 and C/N ratio 11 to Cyberlindnera jadinii CCT 2612) and using 10% v/v inoculum. In addition to this, the ratio of work volume/total volume of the reactor was 1/5. The conditions of incubation were 30°C, 120 RPM and two times of collection (96 hours for the yeast Cyberlindnera Jadinii CCT 2612 and 120 hours for Pichia jadinii CCT 1518). Subsequently, the biomass was centrifuged at 3000 RPM, 10°C for 10 minutes in a Fanem centrifuge, then the biomass was washed with distilled water, this process of centrifugation and washing of biomass was done 3 times. Once the biomass the process was completed, the yeasts were lyophilized. Finally, the dry biomass was weighed and divided for the different characterization processes.

### 5.2.2 Isolation of Insoluble B glucans

The (1-3) β - D glucan isolation was realized according the Williams and coworkers method, and the modifications of Mueller and coworkers (WILLIAMS et al., 1991; MULLER et al., 1996). 2 g of each yeast were dispersed in 15.56 mL of (0.75 M) NaOH and heated until 96°C. The mixtures were cooled to room temperature and let stand overnight, the supernatant was stored in refrigeration. The NaOH digestion was realized twice more in same conditions, the supernatants were mixed, and freeze dried to subsequent analyzes. Each cell debris was mixed with 15.56 mL of HCl (2.45M) and heated until boiling. The mixtures with HCl were cooled to room temperature and let stand overnight, the supernatants was stored in refrigeration. The digestion with HCL was realized

twice more, but, the HCl concentration in the two remaining cycles decreased at 1.75 M and 0.94 M, respectively. The supernatants of the acid digestion was mixed and freeze dried to subsequent analyzes. Further, the solid residue was extracted with 6.67 mL of absolute ethanol mix with 1% of HCl. The last mixture was heated until boiling, and then, cooled to room temperature and let stand overnight, and the supernatant was discard. This step was repeated 5 times more. To continuation, the residue was extracted with 6.67 mL of absolute ethanol mix with 1% of NaOH. Following, of heating until boiling, and then, cooled to room temperature and let stand overnight, the supernatant was discard. Subsequently, the residue was washed with 8.89 mL of distillated water and the supernatant was discard, the washed cycle was repeated twice more. Thereafter, the residue was filtrated with a filter of carbon fiber and the mass was freeze dried. Finally, the β glucans was weighted and the value obtained was replaced in the equation 16, to the determination of the β glucans yield.

$$\beta \text{ glucans Yield} = \left( {}^{\beta \text{ glucans liophilizated mass}} / {}_{\text{Yeast initial mass}} \right) \text{x } 100$$

*Equation 16. B glucans Yield*

### 5.2.3 Solubilization of B glucans and freeze dried supernatants

The insoluble B glucans and the freeze-dried supernatants of the digestions were subjected to several solubilization processes described in the table 21.

*Table 21. Solubilization methods*

| Method | Solvent and temperature |
|---|---|
| 1 | DMSOd at Room temperature |
| 2 | DMSOd at 80°C |
| 3 | $D_2O$ at Room temperature |
| 4 | NaOH solution (0.25 M) at Room temperature |
| 5 | NaOH solution (0.5 M) at Room temperature |
| 6 | S1 solution (NaOH 8%/thiourea 6.5%/ Ureia 8%/D2O) at 80°C |
| 7 | Williams solution (DMSO/$H_3PO_4$ at 100°C by 6 hours) |

#### 5.2.4 β Glucan Characterization

##### 5.2.4.1 Solid State $^{13}C$ CP MAS Nuclear Magnetic Resonance spectroscopy ($^{13}C$ CP MAS NMR)

To elucidate the molecular structure of each insoluble sample was used the $^{13}C$ CP MAS NMR. For this analyzed were used two types of samples, freeze dried samples and hydrated samples (SAITÔ et al., 1989). For the hydration of b glucans, the freeze-dried samples were deposited in a desiccator with a high relative humidity (≥ 80%) were deposited by one week.

The solid state $^{13}C$ CP MAS NMR measurement were carry out on the 400MHz Bruker solid-state NMR spectrometer. The high-resolution 13C CP/MAS NMR spectra were recorded at the resonance frequency of approximately 100MHz with the use of 7 mm rotors and MAS frequency of 5000Hz and $\pi/2$ pulse duration of 1.9 $\mu$s. In the CP experiments the CP.AV condition was achieved with the radio frequency field strength of 58 kHz, a contact time of 1ms, and relaxation delay of 4s between two consecutive scans. A high-power proton-decoupling field of 92 kHz was applied during data acquisition (FRICOVÁ; KOVAL'AKOVÁ, 2013). The spectra were at ambient prove temperature with 539 to 3000 scans.

##### 5.2.4.2 $^{13}C$ Nuclear Magnetic Resonance spectroscopy ($^{13}C$ NMR)

The soluble samples was analyzed by $^{13}C$ NMR, to elucidate the molecular structure and the type of linkages. For the analyzes was used at least 40 mg of sample and recorded at 70°C in a Bruker Avance DRX400 NMR spectrometer, as internal reference were used acetone deuterated for the water soluble samples (29.92 of chemical shift) and $DMSOd_6$ for the $DMSOd_6$ soluble samples (39.51 of chemical shift). Using a spectral width of 31.7 kHz, an acquisition time of 0.5 s, no relaxation delay, and a pulse width of 4.5 ms. Spectra were accumulated for 8 to 24 h (RODRIGUEZ et al., 1999).

##### 5.2.4.3 Fourier transformed infrared (FTIR) spectroscopy

The insoluble B glucans were carried out on the vertex equipment with KBR disks (Each sample was carried out by duplicate). The measures was realized in the 650 – 1800 $Cm^{-1}$ region. The concentration of the insoluble β glucans was 0.1% (w/w).

#### 5.2.4.4 Monosaccharide composition

The purity of the β glucans and freeze-dried supernatants was determinated with the monosaccharide composition. The analysis was carried out on HPLC. To release the sugars, 1 mg of the each sample (Insoluble B glucan and freeze-dried supernatants) was mixed with 0.5 mL of trifluoroacetic acid (2M). This mixture was heat at 100°C by 2 hours for the total hydrolysis of the polysaccharides. Then, the trifluoroacetic acid was evaporated with vacuum pump by 40 minutes in a gas cab. Later, the residue was mixed with sulfuric acid (5 mM). The HPLC runs were done on a chromatograph (1200, Agilent Technologies, USA) with IR detectors (Agilent, HP1047A) for sugars detection and UV detector (Agilent, G1315D) for other compounds like aminoacid residues. For the analysis, an animex HPX-87 column (300x7.8mm, Bio-Rad, USA) with $H_2SO_4$ (5mM) as mobile phase, a flow rate of 0.6 mL/min and 65 °C were used.

## 5.3 RESULTS AND DICUSSION

### 5.3.1 Isolation of Insoluble β glucan

The insoluble β glucans were isolated with the Williams protocol with the Mueller modifications (MULLER et al., 1996). The isolation yields were 3.14 % (w/w) to Pichia Jadinii CCT 1518 and 3 % (w/w) to Cyberlindnera Jadinii CCT 2612. Despite that, the biomass production of Pichia Jadinii CCT 1518 was lower than Cyberlindnera jadinii CCT 2612, the insoluble β glucan yield of Pichia jadinii was slight higher than Cyberlindnera jadinii CCT 2612. This result is similar at the reports of Mueller and coworkers (MULLER et al., 1996). On other hand, the freeze-dried supernatants of the basic digestion and acid, presented a phase solid of 104 and 8 mg, respectively. For this reason, only the freeze-dried supertnatants of the basic digestion was used for the subsequent analyses.

### 5.3.2 Solubilization of β glucans and freeze dried supernatants

The table 22 show the solubilization percent of the β glucans and freeze dried supernatants of the basic digestion.

*Table 22. Solubility percentage of isolated beta glucans and supernatants of basic digestion*

| Method* | B glucan CCT 1518 (B CCT 1518) | B glucan CCT 2612 (B CCT 2612) | Freeze dried supernatants of Basic Digestion (CCT 1518) | Freeze dried supernatants of Basic Digestion (CCT 2612) |
|---|---|---|---|---|
| 1 | No soluble | No soluble | 47% | 36% |
| 2 | No soluble | No soluble | | |
| 3 | No soluble | No soluble | 53% | 64% |
| 4 | No soluble | No soluble | | |
| 5 | No soluble | No soluble | | |
| 6 | No soluble | No soluble | | |
| 7 | No soluble | No soluble | | |

- The methods are described in the table 21.

The supernatants of the Basic digestion presented a high solubility in two solvents. The 53% was soluble in D2O and the 47% (w/w) of the remaining biomass was soluble in $DMSOd_6$ to *Pichia Jadinii* CCT 1518. In the case of the supernatants of the Basic digestion of *Cyberlindnera Jadinii* CCT 2612, the 64% it was soluble in D2O and the 36% (w/w) of the remaining biomass was soluble in $DMSOd_6$. Because of this, it was not necessary the use of the others solubilization methods. The high solubility of this fraction can be explained because these polysaccharides was branches polysaccharides and the short chains (SYNYTSYA et al., 2009). On other hand, the B glucans of the two work yeasts presented a low solubility in all the solubilization methods employed. The highest solubility reached was 22% to *Cyberlindnera jadinii* CCT 2612 and 15 % to *Pichia Jadinii* CCT 1518. The little branch of these polysaccharides and the long chain of the same can explain this phenomenon. The low solubility of the β glucans is agree with the results of Williams and contributors, which one isolated β glucans of the *Sacharomyces cerevisiae*, and your solubilization was lower (WILLIAMS et al., 1991).

### 5.3.3 β glucan characterization

#### 5.3.3.1 FTIR

Figure 12. shows the FTIR spectra of the yeasts *Pichia jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612 in the range of 650 - 1800 $cm^{-1}$. The FTIR spectrum for *Pichia jadinii* CCT 1518 can be divided into 5 regions. The first, centered at 1620.62 $cm^{-1}$, indicating the presence of proteins with functional group amide type 1 (mainly by the signals of the Carbonyl group and the flexion

of the N-H group). This band confirms protein residues evidenced in the NMR spectra of lyophilized and hydrated β glucans. Galichet and contributors has been found the same signal in β glucans isolated from *Saccharomyces cerevisiae* (GALICHET et al., 2001). The second region, centered at 1369 $cm^{-1}$, belongs to β glucans. Synytsya and colleagues report this signal in different extracts derived from *Pleurotos ostreatus and Pleurotus eryngii* (SYNYTSYA et al., 2009). The third region between (1150-1160), has been described as the glycosidic bond C-O-C belonging to α glucan residues (SYNYTSYA et al., 2009). The fourth region (1150 - 950 $cm^{-1}$) has different specific signals of β and α glucans (see table 23). These signals refer to stretches between the C-C and C-O bonds in glycopyranose rings (GALICHET et al., 2001; SYNYTSYA et al., 2009; SYNYTSYA; NOVAK, 2014). The last band centered on 891.44 refers to the glycosidic linkage of the beta glucans, in the insoluble β glucans and the lyophilized supernatants of the basic digestion (GALICHET et al., 2001; SYNYTSYA et al., 2009).

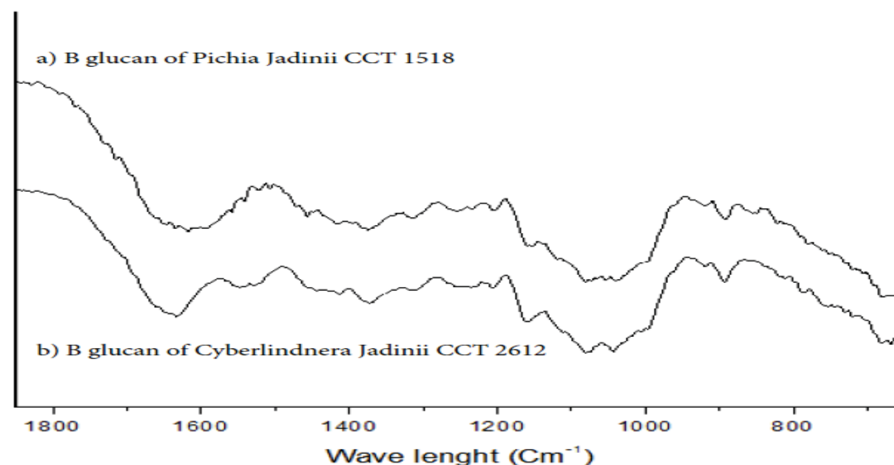


*Figure 12. FTIR spectra. A) B glucan Pichia Jadinii CCT 1518. B) B glucan Cyberlindnera Jadinii CCT 2612*

*Table 23. FTIR spectrum of beta glucans isolated from Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612*

| Assignments of bands resulting of the FTIR spectra of *Pichia Jadinii* CCT 1518 and *Cyberlindnera Jadinii* CCT 2612. | | | |
|---|---|---|---|
| Pichia Jadinii CCT 1518 | Cyberlindnera Jadinii CCT 2612 | Assignment | Author |
| Wave lenght ($cm^{-1}$) | Wave lenght ($cm^{-1}$) | | |
| 891,44 | 894,41 | (1-3)(1-6)-β-Dglucans | Galichet and Synytsya |
| 919,2 | 920,74 | Mannans | Galichet |
| 939,39 | | (1-3)-α-Dglucan | Synytsya |
| 994,79 | 1000,88 | (1-6) Beta glucans | Galichet |
| 1052,84 | 1057,44 | (1-3)(1-6)-β-Dglucans | Synytsya |
| 1074,23 | 1079,76 | (1-3)(1-6)-β-Dglucans | Galichet and Synytsya |
| 1119,54 | | (1-3) Beta glucans | Galichet |
| 1139,73 | 1136,32 | (1-3) Beta glucans | Galichet |
| 1152,35 | 1157,38 | α-D-glucans | Synytsya |
| 1369,15 | 1373,08 | B-D glucan | Synytsya |
| 1398,12 | | B-glucan | Synytsya |
| 1521,66 | 1529,59 | Residues of proteins | Galichet and Cameli |
| | 1544,01 | Proteins | Galichet |
| 1620,62 | 1630,79 | Residues of proteins | Synytsya and Galichet |
| 1636,31 | | | |

Figure 12 shows the FTIR spectrum of β glucan extracted from *Cyberlindera Jadinii* CCT 2612. The FTIR spectrum of *Cyberlindnera Jadinii* CCT 2612 is very similar to the FTIR spectrum of *Pichia Jadinii* CCT 1518, however, it has one more band and the intensity of the bands is greater. The first band is centered at 1630.79 cm $^{-1}$ and corresponds to proteins with the amide group I. The second band, centered at 1544.01 $cm^{-1}$, is characteristic of protein proteins with the amide group II. These two bands have been found in β glycans extracted from *Pleurotus* as reported by Synytsya (SYNYTSYA et al., 2009). On the other hand, it may indicate a greater amount of protein from *Cyberlindnera Jadinii* CCT 2612 compared to *Pichia Jadinii* CCT 1518. The third band centered on 1373.08 $cm^{-1}$ is present in β -D-Glucans and the fourth band centered on 1157.38 $cm^{-1}$ is characteristic of α-D-glucans, the signals of the 4 band are attributed to CC and COC links as they are reported by Zechner. The fifth band (1150 - 950 $cm^{-1}$), presented different signals belonging to β glucans type (1-3) and type (1-3) (1-6).

The sixth band centered on 894.41 clearly shows the type of beta anomer, corroborating the β glucan isolation of the yeast *Cyberlindnera Jadinii* CCT 2612.

#### 5.3.3.2 Monosaccharide composition

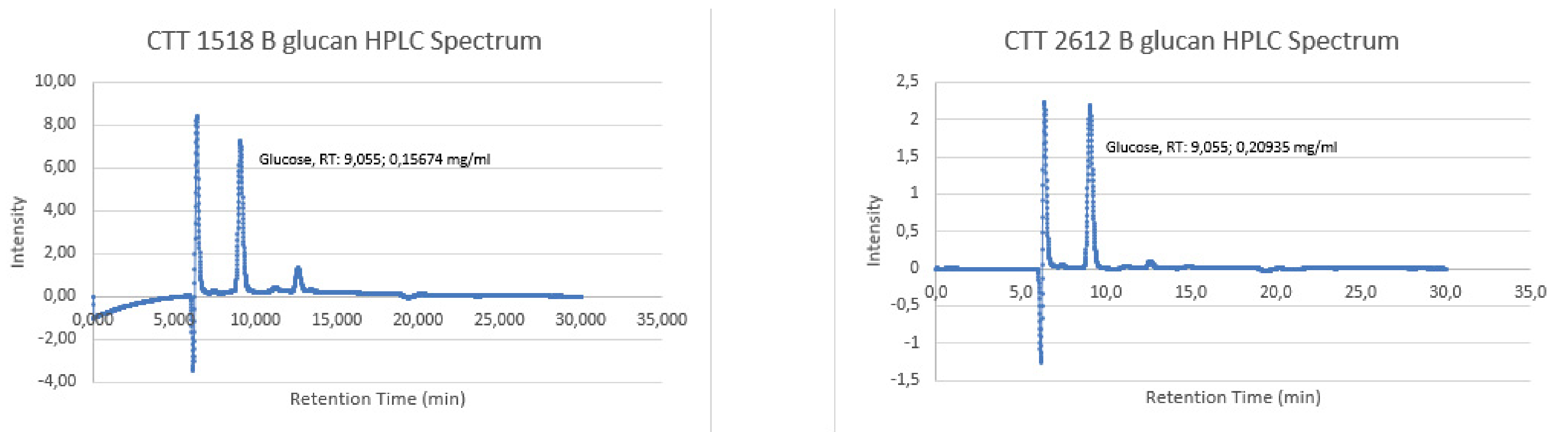


*Figure 13. Monosaccharide composition of β glucan isolated of Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612*

The figure 13 shows the monosaccharide composition of β glucans isolated from *Pichia Jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612. The two figures show 3 peaks corresponding to retention times, 9,055, 11.42 and 12.7 min. The first and most intense signal (9,055 min) corresponds to glucose as expected by the repetition of this monosaccharide in β glucans. However, there are 2 small signs (11.42 and 12.7) that may correspond to uronic acids or sugar residues, such as glucosamine (deacetylated form of chitin). With these results, the presence of glucans in the insoluble fraction of the isolation is confirmed. Additionally, signals belonging to other molecules were found, corroborating the results obtained in the FTIR.

**5.3.3.3 $^{13}$C CP MAS NMR**

The non-hydrate β glucan of yeast *Pichia Jadinii* CCT 1518 (B CCT 1518 A) was analysed with $^{13}$C CP MAS NMR and the spectrum is show in the figure 14. This spectra presented 10 peaks to analyze. There are three signals not very well defined around of 23.9 ppm ($CH_3$), 56 ppm (C2) and 175,3 ppm (C=O) that probably belongs to chitin residues (FRICOVÁ; KOVAL'AKOVÁ, 2013). The peak on 39.51 ppm belong to $DMSOd_6$ from the dissolution process. The signal in 62.5 ppm is common of the C6 non-linked of a glucose. The peak on 105 ppm with left hand side shoulder probably belong to the anomeric carbon (C1) of a β glucan (KIM et al., 2000), generally the (SYNYTSYA; NOVAK, 2014)carbon anomeric of glucose is find between 102-104 ppm, however, the application of urea can modified the chemical shift to the left (SAITÔ et al., 1987). On other hand, the left hand side shoulder could be the presence of others compounds or low resolution of the spectrum (SAITÔ et al., 1989). The small signal at 88.3 ppm can be the C3 linked of Curdlan Anhydrous with the single helix conformation of β glucan (SAITÔ et al., 1989). The peak at 91 ppm probably can be C1 of alpha glucosamine a precursor of chitin (RENAULT et al., 2012). The signal at 84.6 can be residues of C3 of β glucans in the triple helix conformation (SAITÔ et al., 1987). This information may suggest that a percentage of β glucans is in triple helix conformation and another percentage in simple helix conformation. The tridimensional conformation varied in function of some process like lyophilization and solubilization with NaOH or Urea. Finally, the big signal between 66.5 and

81.4 ppm is the overlapping of the peaks belonging at unlinked carbons of β glucans (C2, C4 and C5).

The hydrate β glucan of yeast *Pichia Jadinii* CCT 1518 spectrum (B CCT 1518 B) is show in the figure 14. This spectrum have a higher resolution compared with the same sample non-hydrate. This spectra have 11 clear signals. Newly presented four related signals with chitin or chitin precursors at 24 ppm (CH3), 56.2 ppm (C2), 91.37 ppm (probably it`s the C1 of alpha glucosamine), and 175.2 ppm (C=O) (RENAULT et al., 2012; FRICOVÁ; KOVAL'AKOVÁ, 2013). At 105 ppm a well-defined peak was observed, this signal belongs to the anomeric carbon of the β glucans with slight chemical shift to the left (SAITÔ et al., 1989). The signal of C3 linked was more defined too, at 88.26 ppm. This peak indicate the presence of the single helix conformation (SAITÔ et al., 1989). The big peak between 66.5 and 81.4 ppm was divided in three signals belong to carbons non-linked C4, C2 and C5 of β glucans. The peaks formed were at 70, 74.6 and 77.27 ppm probably belong to the C4, C2 and C5, respectively. These signals are agree with the annealed curdlan obtained by Saito and contributors (SAITÔ et al., 1989). In the case of C1, your signal was more defined at 62.58 ppm as the hydrate β glucans analyzed by Fricová and contributors (FRICOVÁ; KOVAL'AKOVÁ, 2013). By the results of $^{13}C$ CP MAS NMR of dried-freeze sample and hydrate, the β glucan molecular structure corresponding to (1→3) β glucan with the single helix conformation and triple. Moreover, probably this β glucan have some residues of (1→4) α glucosamine presented in fungus. The figure 15 shown the structure proposed.

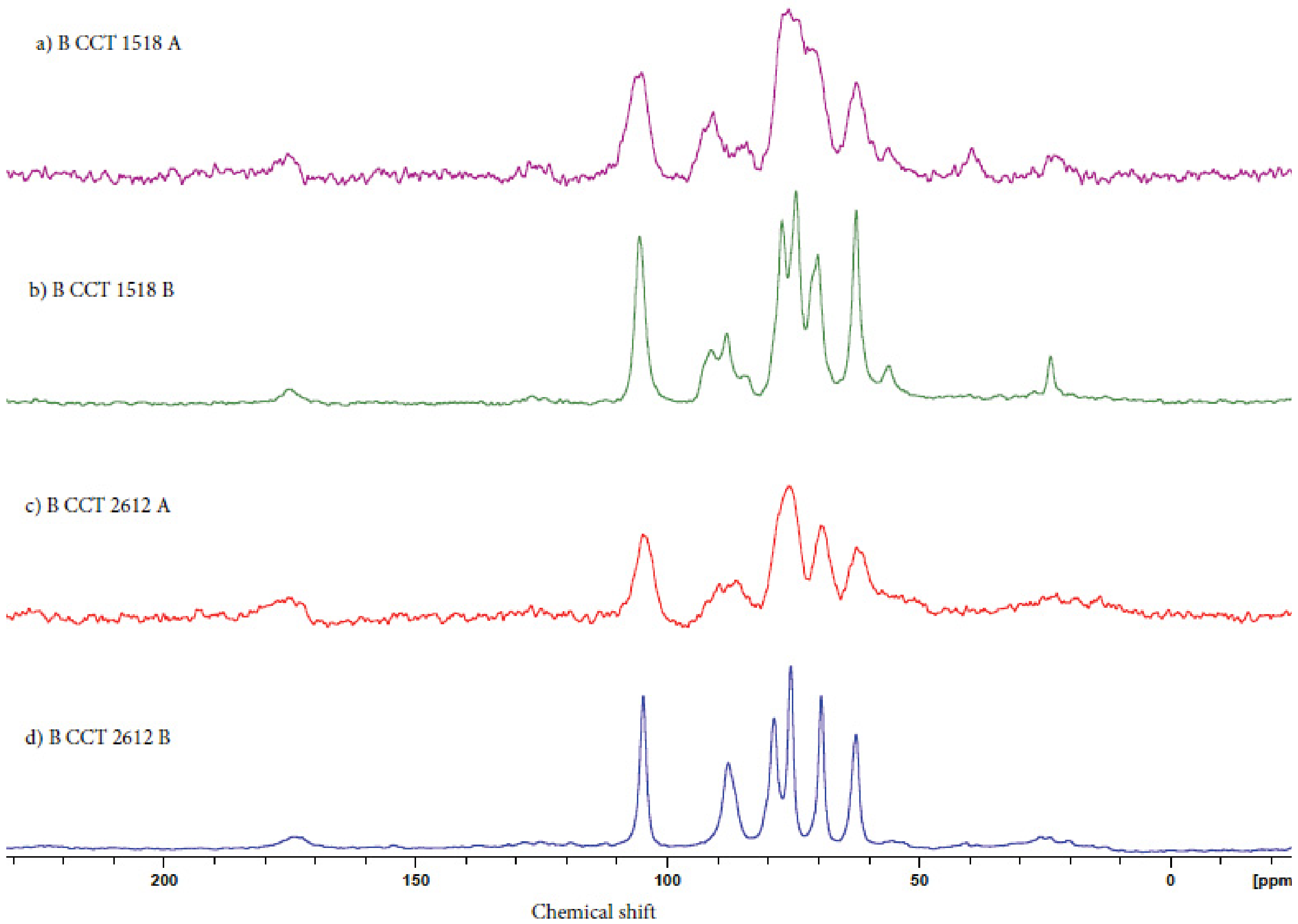


*Figure 14. $^{13}C$ CP MAS NMR Spectrum of B glucans isolated of Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612.A) Pichia Jadinii CCT 1518 non-hydrate (B CCT 1518 a), B) Pichia Jadinii CCT 1518 hydrate (B CCT 1518 b), C) Cyberlindnera Jadinii CCT 2612 non-hydrate (B CCT 2612 a) and D) Cyberlindnera Jadinii CCT 2612 hydrate (B CCT 2612 b).*

The spectra of non-hydrate β glucan of yeast *Cyberlindnera Jadinii* CCT 2612 (B CCT 2612 A) is show in the figure 14. The β CCT 2612 A spectra presented seven signals to analyze. Equal to the β glucans of *Pichia jadinii* CCT 1518 spectra, the B CCT 2612 a spectra have small peaks non well defined, these peaks probably belong to chitin residues and by their intensity, the chitin quantity is more lower than on β glucans of *Pichia Jadinii* CCT 1518. The signals are at 22 ppm (CH3), 54.7 ppm (C2), and 175.2 ppm (C=O) (FRICOVÁ; KOVAL'AKOVÁ, 2013) (SAITÔ et al., 1989) (SYNYTSYA; NOVAK, 2014). The signal at 104.84 ppm is characteristic of anomeric carbon of β glucan (SAITÔ et al., 1989; KIM et al., 2000), moreover, this pick presented one left hand side shoulder which can belong to other compound. The signal at 89.78 ppm probably it`s the residues of C3 in single helix conformation (SAITÔ et al., 1987). The peak in 86.5 ppm related (1-3) linked residues of C3 like the laminarin with the triple helix conformation found in some microorganisms (WILLIAMS et al., 1991). The B CCT 2612 A sprectrum presented two peak in the region from 65.6 until 81.3 ppm, while the B CCT 1518 A spectrum presented one big peak by signal overlapping; probably the two peaks of B CCT 2612 A belong to unlinked carbons of the glucose (C4, C2 and C5). The signal in 62.5 represent the residues of unsubstituted C6 of β glucan found in fungus (SYNYTSYA et al., 2009).

On other hand, the of hydrate β glucan of yeast Cyberlindnera Jadinii CCT 2612 (B CCT 2612 B) is show in the lower part of the figure 4.7.1. This spectrum have a highest resolution compared with others spectra. The B CCT 2612 B spectrum show 6 peaks well definite belonging to the glucose and 3 small peaks without high resolution. The signal of anomeric carbon at 104.74 ppm didn`t present any side shoulder compared with the B CCT 2612 a spectrum. The peak of residue of C3 at 87.8 didn`t present side shoulder with the others three spectra. This peak presented the chemical shift characteristic of the hydrate curdlan (SAITÔ et al., 1989), but, it doesn`t give a lot information about its tridimensional conformation. The signals at 78.79, 75.45 and 69.32 ppm, belong the unliked carbons C5, C2 and C4. The peak at 62.51 ppm is the signal of unliked C6 of β glucans (SYNYTSYA et al., 2009). The results of $^{13}C$ CP MAS NMR of dried-freeze sample and hydrate of *Cyberlindnera jadinii* CCT 2612 presented a molecular structure of (1-3) β glucan, , with single helix conformation and triple to the sample

B CCT 2612 A, but, the B CCT 2612 B didn´t present the two characteristic peaks of the tridimensional conformation. Additionally, many authors reported (1-3) (1-6) β glucan as molecular structure of β glucans isolated of yeasts, however, it wasn`t found evidence of the linked C6 (1-6), this can be by the severity of the isolation process, that it can break the linked between the main chain and the ramifications (see figure 15).

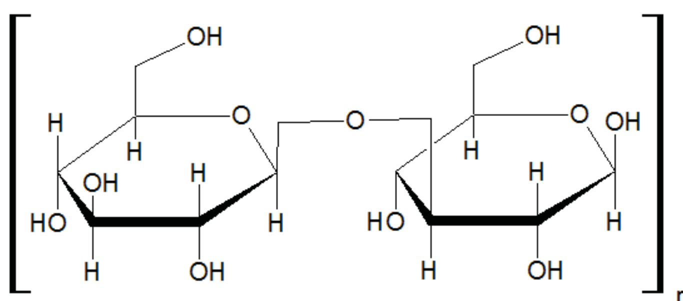


*Figure 15. (1→3) β glucan proposed for Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612*

#### 5.3.3.4 Freeze-dried supernatants characterization

The freeze-dried supernatants of the basic digestion with high soluble in $DMSOd_6$ and $D_2O$ was analyzed by $^{13}C$ NMR to know that sugars types can be recovery during the isolation process, the Spectra are show in the figure 16.

The supernatant sample soluble in $D_2O$ of *Pichia Jadinii* CCT 1518 was called S CCT 1518 A. In the figure 16. S CCT 1518 A present a higher number of signals compared with the above $^{13}C$ CP MAS NMR spectra, because this fraction was less purified and it has a high resolution. The S CCT 1518 A show around of 11 regions with signals defined and 5 regions with low resolution. The signals in the region 1 (16 to 27 ppm) can be attributed to proteins and/or chitin residues. The peaks in the region 2 (53 to 55 ppm) probably belong at residue of C2 of chitin. The peaks in the region 3 (115 – 131 ppm) could belong the aromatic molecules like proteins. The signals in the region 4 (170 – 175 ppm) belong to residues of molecules with the carbonyl group (proteins or chitin). The signals in the region 5 (215-216 ppm) belong to molecules with the carbonyl group unit a ketones (SYNYTSYA; NOVAK, 2014). On the other hand, the signals with higher resolution are described below. The region with signals defined are region 6 with

a peak at 29.92 ppm, this signal corresponds to acetone deuterated as internal reference. The peak at 61.09 ppm (region 7) may belong to residues of C6 carbon in (1-3) β glucan. The signals in the region 8 (65.2 – 67.3 ppm) may correspond to linked C6 in α glucans (CÔTÉ; SKORY, 2016). The small peak at 68.9 ppm may be to linked C6 in β glucans (SUGAWARA et al., 2004; FRICOVÁ; KOVAL'AKOVÁ, 2013). The region 11 (69.6 - 71.5 ppm), the peak at 73.37 and the region 13 (77.6 – 79.11 ppm) may represent the C4, C2 and C5 carbons of (1-3) β glucan (SAITÔ et al., 1989), this result could corroborate the isolation of the insoluble (1-3) β glucan of *Pichia Jadinii* CCT 1518 described in the $^{13}C$ CP MAS NMR. The region 15 (97.9 – 103.09 ppm) presented some peaks characteristic of the anomeric carbon of α and β glucans (SYNYTSYA; NOVAK, 2014).

The soluble supernatant in $DMSOd_6$ correspondent to *Pichia jadinii* CCT 1518 was called S CCT 1518 B (see figure 16). The signals in this sample was lower intensity than the S CCT 1518 A and probably a lower concentration of compounds. The big signal around 39 ppm correspond to DMSO non-deuterated. The signals in the region 1 (13- 33 ppm) and region 9 (170 – 175 ppm) can be attributed to proteins or chitin residues (SYNYTSYA et al., 2009). The region around of 127 – 130 ppm are generally associate to proteins (SYNYTSYA et al., 2009; SYNYTSYA; NOVAK, 2014). The peaks at 61, 70.5, 72.9, 76.4 ppm are characteristic of the unliked C6, C4, C2 and C5 carbon of the b glucans (SAITÔ et al., 1987; SYNYTSYA; NOVAK, 2014). The signals at 68.3, 86.1 and 102.8 may belong to the linked C6, C3 and C1 carbon of the β glucan (SAITÔ et al., 1987; WILLIAMS et al., 1991; LOWMAN et al., 2011). These results show the presence of β glucans as was described in the analysis in $^{13}C$ CP MAS NMR. In addition, the branch can be seen in carbon 6, which it did not appear at the end of the isolation process.

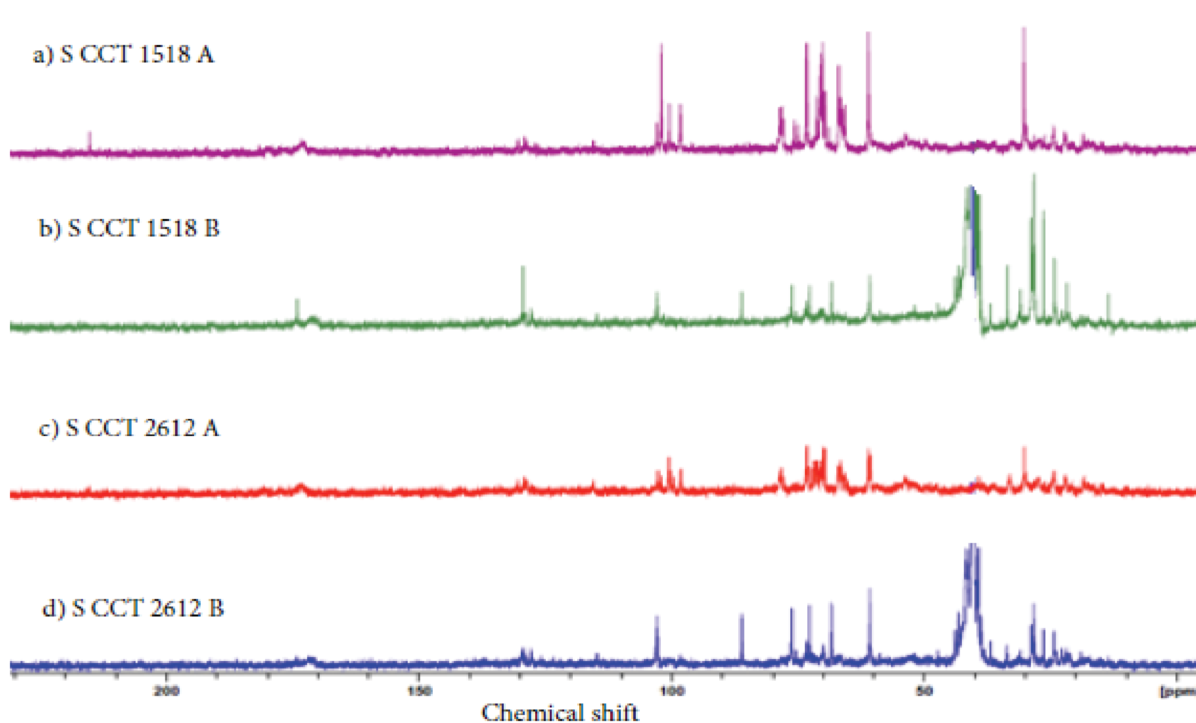


Figure 16. $^{13}C$ NMR Spectra of Freeze-dried supernatants of basic digestion of Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612. A) supernatant sample soluble in D2O of Pichia Jadinii CCT 1518 (S CCT 1518 A). B) supernatant sample soluble in $DMSOd_6$ of Pichia Jadinii CCT 1518 (S CCT 1518 B). C) supernatant sample soluble in D2O of Cyberlindnera Jadinii CCT 2612 (S CCT 2612 A) and D) supernatant sample soluble in $DMSOd_6$ of Pichia Jadinii CCT 1518 (S CCT 1518 B).

The soluble supernatant in $D_2O$ of *Cyberlindnera Jadinii* CCT 2612 Spectra (S CCT 2612 A) is show in the figure 16. The signals in the region 1 (13 – 29 ppm), the region 2 (53 – 54 ppm), the region 4 (126 – 130 ppm) and the region 5 (171.4 – 175 ppm) probably belong to chitin residues or proteins (FRICOVÁ; KOVAL'AKOVÁ, 2013). The peak at 115.7 ppm may be attributed to proteins (SYNYTSYA; NOVAK, 2014). The signal at 29.92 ppm belong to acetone deuterated as internal reference. The signals at 61.2, 70.1, 73.2, 78.4 ppm relates to residues unliked of C6, C4, C2 and C5 of the β glucans, this results are similar of the insoluble β glucans of *Cyberlindnera jadinii* CCT 2612 (SAITÔ et al., 1987; WILLIAMS et al., 1991; LOWMAN et al., 2011). The signals in the region 6 (64.6 – 67.5 ppm) may correspond to linked C6 in α glucans (CÔTÉ; SKORY, 2016). The signals in the region 6 (98.2 – 103.4 ppm) belong to residues of anomeric carbon of α and β glucans (SAITÔ et al., 1989).

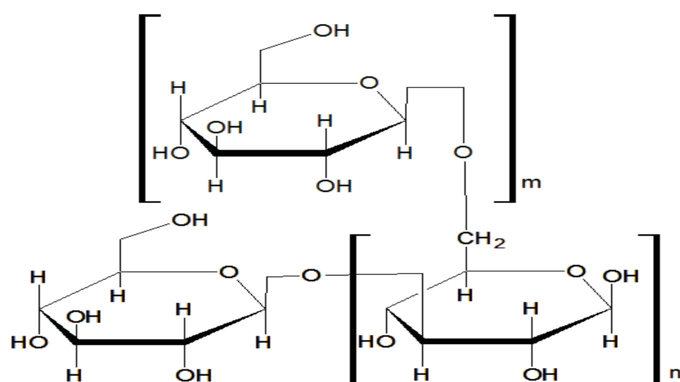


*Figure 17. (1→3) (1→6) β glucan proposed for the freeze-dried supernatants of the basic digestion of Pichia Jadinii CCT 1518 and Cyberlindnera Jadinii CCT 2612*

The soluble supernatant in $DMSOd_6$ of *Cyberlindnera Jadinii* CCT 2612 spectrum (S CCT 2612 B) is show in the figure 16. The big signal around 39 ppm correspond to DMSO non-deuterated. The S CCT 2612 B spectrum show two regions with possible presence of residues of chitin and/or proteins (18.5- 33.5 ppm) and (170 – 173 ppm) (SYNYTSYA; NOVAK, 2014). The signals around (127 – 130 ppm) probably belong to aromatic aminoacids and the peaks between (51 – 54 ppm) may be chitin residues (SYNYTSYA et al., 2009). The signals at 61.2, 70.1, 72.9, and 76.2 ppm correspond to unlinked carbons C6, C4, C2 and C5. S CC 2612 B show three defined signals usually found in linked carbons of (1-3)(1-6) β glucans (the signals was 68.3, 86.1 and 102.8, to C6, C3 and C1 carbons) (KIM et al., 2000). Like, the other supernatants spectra analyzed, S CCT 2612 B evidenced all characteristic signals of the (1-3)(1-6) β glucans and corroborates the results obtained with the $^{13}C$ CP MAS NMR analysis applied to *Cyberlindnera Jadinii* CCT 2612. The figure 17 show the possible structure of the freeze-dried supernatants of the basic digestion to both work yeasts.

### 5.4 CONCLUSION

Insoluble β-glucans were isolated (1-3) from the *Pichia Jadinii* CCT 1518 and *Cyberlindnera jadinii* CCT 2612, using the method of Williams and collaborators, with the modifications of Muller et al. The β glucans yields was 3.14% w/w for *Pichia Jadinii* CCT 1518 and 3% w/w *Cyberlindnera jadinii* CCT 2612. The β glucans obtained were confirmed by $^{13}C$ CP MAS NMR, FTIR and monosaccharide composition. The resolution of the insoluble hydrated samples presents higher resolution than the untreated samples. The carbon peaks of the NMR showed chemical shifts according to all the solubilization processes and the hydration process. In addition, it was possible to observe 2 signals in the $^{13}C$ CP MAS NMR of hydrated samples, possibly belonging to carbon 3 with two three-dimensional configurations (simple helix and triple helix). In another hand, the presence of molecules other than beta glucans (proteins and sugar residues) is evidenced to identify other techniques.

From the supernatants of the basic digestion of the isolation process, (1-3) (1-6) β glucans were obtained. These results corroborate the isolation of β glucans by the Williams method. Additionally, they may suggest that the isolation process is quite severe by breaking the ramifications (1-6).

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