Patrícia Isabel Pós-de-Mina Serrano

Degree in Biotechnology

Production of xylitol by the yeast *Komagataella pastoris*

Dissertation for the degree of Master in Biotechnology

Supervisor: Dr.ª Maria Filomena Andrade de Freitas,
Senior Researcher, Faculdade de Ciências e Tecnologia, UNL

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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À minha querida avó Bárbara.

Agradecimentos

Primeiramente, gostaria expressar o meu sincero agradecimento à minha orientadora, Filomena Freitas, por toda a ajuda, ensinamentos, apoio prestado, conhecimento transmitido, entusiasmo, paciência e amizade que teve comigo ao longo deste ano de investigação, encorajando-me sempre a continuar e a fazer melhor.

À Diana Araújo por todos os ensinamentos e "truques" adquiridos no laboratório, por todos os conselhos e "brainstromings", pela paciência, energia e pela boa disposição transmitida nos dias de trabalho.

À professora Maria de Ascenção Reis por me ter recebido no seu grupo de investigação e me ter possibilitado a oportunidade de crescer profissionalmente.

A todos do laboratório 407, por estarem prontos para ajudar sempre que era necessário e pelo bom ambiente de trabalho que me proporcionaram.

Aos meus pais, um especial agradecimento, por terem sido o meu pilar e maior fonte de força, por toda a amizade, amor, por nunca terem duvidado que era capaz e por fazerem tudo por mim, sem vocês nada disto seria possível. À minha restante família, em especial à minha avó Bárbara, que, apesar de já não estar comigo, foi sempre uma fonte de apoio e força e, um exemplo bondade e serenidade.

À Beatriz, Susana, Lara e Inês por toda a amizade sincera, pelas palavras de carinho, de apoio que sempre tiveram para mim. Pelos cafés e pelas gargalhadas, pelos jantares sempre descontraídos e de muita alegria, por serem a prova que com bons amigos a vida se torna mais fácil e bonita.

À Sofia, Ana Teresa A., Carmen, e Catarina, que apesar de estarem longe estiveram sempre incondicionalmente para mim, com palavras de carinho e apoio.

À Ana Catarina, Ana Teresa R. e Rita pela amizade nestes dois anos de mestrado, pelas sessões de estudo, pelos desabafos partilhados em dias mais stressantes, pela entre ajuda e compreensão, cafés, passeios e jantares que colocaram sempre um sorriso no rosto. Sem vocês este percurso não teria sido a mesma coisa.

A todos, o meu muito obrigada!

Resumo

O xilitol é um poliol que pode substituir a sacarose, é tolerado por diabéticos e tem várias aplicações clínicas. Atualmente, o xilitol é fabricado em larga escala através de um processo químico, mas há uma busca por processos alternativos que sejam ecologicamente corretos e mais económicos.

Nesta tese, a produção biotecnológica de xilitol foi investigada como uma alternativa ao processo químico, utilizando a levedura *Komagataella pastoris* para produzir xilitol a partir de misturas de glicose/xilose. Diferentes parâmetros, nomeadamente, pH, temperatura, concentração de xilose, presença de arabinose como substrato e oxigénio dissolvido foram testados.

Este trabalho demonstrou que o melhor valor de pH para a síntese de xilitol foi de 7,0 - 7,5, o que resultou em 4,04 g/L de xilitol, com uma produtividade volumétrica de 0,024 g/L.h e uma produtividade específica de 0,35 $g_{xilitol}$ / g_{CDW}.

O cultivo com pH inicial de 7,00 e temperatura 37 ºC, em condições totalmente aeróbicas, e alteração do pH para 6,4 às 72 h de cultivo, resultou na maior concentração de xilitol deste estudo: 12,00 g/L, concomitante com uma produtividade volumétrica de 0,071 g/L.h e uma produtividade específica de 1,41 $g_{xilitol}$ /g_{CDW}.

A limitação de oxigénio resultou numa concentração de xilitol de 5,81 g/ L, com uma produtividade específica de 0,33 $g_{xilitol}$/g_{CDW}, e uma produtividade volumétrica de 0,035 g/L.h.

Foram testadas várias concentrações de xilose nas misturas glucose/xilose. A maior produção de xilitol foi obtida com 60 g/L de xilose. A redução da concentração de xilose resultou numa menor produção de xilitol.

Curiosamente, foi observado pela primeira vez que *K. pastoris* foi capaz de sintetizar arabitol usando arabinose como substrato, atingindo a concentração de arabitol de 3,15 g/L em cultivo a 37 ºC sob limitação de oxigénio. Esta característica abre a possibilidade de utilizar este processo para a síntese de ambos os álcoois de açúcar, xilitol e arabitol, sendo aparentemente possível adaptar a síntese de cada produto alterando as condições de cultivo.

Palavras-chave

Xilitol, Produção biotecnológica de xilitol, Fermentação de xilose, Xilose Redutase (XR), levedura, *Komagataella pastoris*

Abstract

Xylitol is a sugar alcohol that can replace sucrose, is tolerated by diabetics and has several clinical applications. Currently, xylitol is manufactured on a large scale through a chemical process, but there is a search for alternative processes that are environmentally friendly and more cost-effective.

In this thesis, the biotechnological production of xylitol has been examined as an alternative to the chemical process, using the yeast *Komagataella pastoris* to produce xylitol from glucose/xylose mixtures. Different parameters, namely, pH, temperature, xylose concentration, the presence of arabinose as substrate and dissolved oxygen were tested.

This work demonstrated that the best pH value for xylitol synthesis was 7.0 - 7.5 that resulted in 4.04 g/L of xylitol, with a volumetric productivity of 0.024 g/L.h and a specific productivity 0.35 $g_{xylitol}$ /g_{CDW}.

Cultivation with initial pH value 7.00 and 37 ºC, in fully aerobic conditions, and changing the pH to 6.4 at 72 h of cultivation, resulted in the highest xylitol concentration of this study: 12.00 g/L, concomitant with a volumetric productivity of 0.071 g/L.h and a specific productivity of 1.41 $g_{xylitol}$/g_{CDW}.

Limiting oxygen conditions resulted in a xylitol concentration of 5.81 g/L, with a specific productivity of 0.33 $g_{xylitol}$/ $g_{CDW,}$ and a volumetric productivity, of 0.035 g/L.h.

Several concentrations of xylose in the glucose/xylose mixtures were tested. The highest xylitol production was obtained with 60 g/L of xylose. Reducing the xylose concentration resulted in lower xylitol production.

Interestingly, it was observed for the first time that *K. pastoris* was able to synthesize arabitol using arabinose as substrate, with the arabitol concentration reaching 3.15 g/L on cultivation at 37 ºC under oxygen limitation. This feature opens up the possibility of using this process for the synthesis of both sugar alcohols, xylitol and arabitol, being apparently possible to tune the synthesis of each product by altering the cultivation conditions.

Keywords

Xylitol, Xylose, Xylose reductase (XR), Yeast, *Komagataella pastoris*

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Abbreviations

AOX - Alcohol oxidase promoter

ATP - Adenosine triphosphate

CDW - Cell Dry Weight

HCl - Hydrochloric acid

HPLC - High Performance Liquid Chromatography

K. pastoris – *Komagataella pastoris*

kLa - Oxygen transfer coefficient

n.a. - data not available

NADH - Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

n.d. - not determined

OD_{600nm} - Optical density at 600 nm

OTR - Oxygen transfer rate

r_p - volumetric productivity (g/L.h)

rpm - rotation per minute

XDH - Xylitol Dehydrogenase

XR - Xylose Reductase

XK - Xylulokinase

$Y_{P/S}$ – Product yield ($g_{xylitol}$ / g_{xylose})

$Y_{P/X}$ – Specific productivity ($g_{xylitol}$ / g_{CDW})

Chapter 1. Introduction and Motivation

1. Introduction

1.1 Xylitol: Description, characteristics and properties

Xylitol is a natural sugar alcohol or polyol (Rafiqul and Sakinah, 2013), composed of five carbon atoms, each connected to a hydroxyl group (OH), as can be seen in Figure 1. It is classified as a pentahydroxy pentane of molecular form $C_5H_{12}O_5$, belonging to the carbohydrate class that also includes mannitol, maltitol and sorbitol (O'Donnell and Kearsley, 2012).

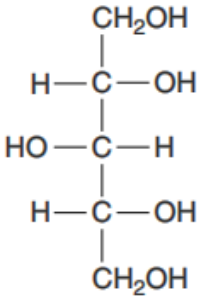

Figure 1. Chemical structure of Xylitol (adapted from O'Donnell and Kearsley, 2012)

This polyol was discovered by Hermann Emil Fisher in 1891, the compound was isolated for the first time, from beech wood. Fisher named the compound as *xylit*, the German word for xylitol. It is considered an example of sweetener with sweetening power resembling that of sucrose, but with a much lower caloric value (2.4 cal/g compared to 4 cal/g for sucrose) (Mohamad, Mustapa Kamal and Mokhtar, 2014).

The colour of this compound is white, has no odour, the melting point is between 92-96 ºC and the boiling point is 216 ºC (1 atm) (O'Donnell and Kearsley, 2012). Also, it does not caramelize if heated to temperatures near the boiling point during several minutes. The pH in aqueous solutions is between 5 and 7. On the other hand, the stability of xylitol is not affected by pH, hence, it can be utilized in a pH range of 1-11. The density of a 10% aqueous solution of xylitol is 1.03 g/mL and that of a 60% aqueous solution is 1.23 g/mL. Its solubility in water at 20 ºC is 64.2 g/100 g, in methanol and ethanol xylitol is slightly soluble, with solubility values of 6.0 g/100 g and 1.2 g/100 g, respectively (O'Donnell and Kearsley, 2012).

The water activity can influence the freshness and microbial stability of a particular product. Xylitol has less water activity than sucrose. This is because it has lower molecular weight, exerting a higher osmotic pressure and, consequently, providing a lower water activity. This means that xylitol has a preserving effect higher than sucrose (O'Donnell and Kearsley, 2012). Due to its microbial and chemical stability, even at low concentrations, it works as a preservative in food products, offering resistance to microbial growth and increasing the shelf life of those products (Rafiqul and Sakinah, 2013).

Xylitol is a natural component present in many vegetables, lichens, algae, mushrooms, fruits, namely plums, strawberries and bananas, cauliflowers, lettuces, carrots, onions, and other sources. It can be found in concentrations in the range 21 – 935 mg per 100 g dry solids (Washüttl, Riederer and Bancher, 1973).

Nowadays, it is a component of modern human's diet. A human adult produces approximately 5 to 15 g of xylitol per day during normal carbohydrate metabolism in the liver, and the concentration of xylitol in the blood is between 0.03 - 0.06 mg per 100 mL (Pepper and Olinger, 1988). Xylitol is capable of dissolving in human mouth and it can give a fresh sensation. This is due to its high endothermic heat of solution (34.8 cal/g), which provides an excellent taste and feeling of freshness particularly advantageous in certain food products, such as spicy, herbal and mint flavours (O'Donnell and Kearsley, 2012).

As xylitol, arabitol is a sugar alcohol. It used in the food and pharmaceutical industries as a natural sweetener, a sugar substitute for diabetic patients due to its low caloric, such as a dental caries reducer (Kordowska-Wiater et al., 2017).

1.1.1. Applications of xylitol

Xylitol was approved as legally permitted by law, for use in food in more than 50 countries worldwide. Countries such as Scandinavia and other parts of Europe are examples of locations where xylitol was used already for over 30 years. Xylitol is also used in oral health products, pharmaceuticals and cosmetics, shows in figure 2. (O'Donnell and Kearsley, 2012).

In 1983, the Joint Expert Committee on Food Additives (JECFA) belonging to the World Health Organisation (WHO) and the Food and Agricultural Organisation of the United Nations (FAO) recommended "Acceptable Daily Intake", for xylitol consumption, which reveals the safety of this compound and does not represent for Committee a hazard to health (O'Donnell and Kearsley, 2012).

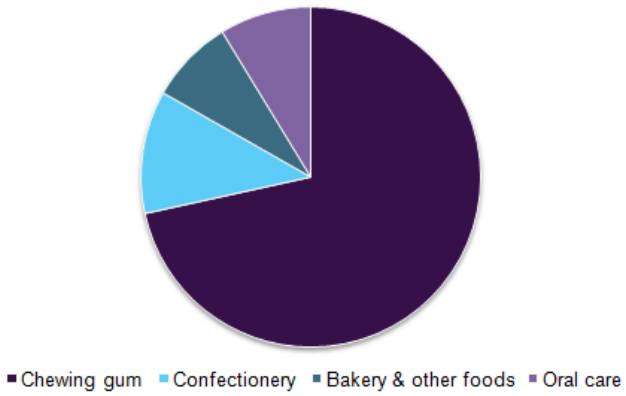

Figure 2. Global xylitol market volume by application, 2015 (Grandviewresearch.com, 2018).

1.1.2. Food applications of xylitol

Xylitol has been mostly utilized in food, in order to replace traditional sugars such as sucrose, because of its sweetening power and lower caloric value, also reducing obesity risk. As already mentioned, this natural sugar alcohol presents other advantages. For example, chemical and microbial stability, being used as preservative in food products (O'Donnell and Kearsley, 2012). On the other hand, the sweetener presents advantages relatively to other polyols such as sorbitol and mannitol since it has a sweetening power similar to sucrose and does not need to be used in combination with other sweeteners to obtain the desired taste (Gliemo et al., 2008).

In the last years, the number of products using xylitol as sweetener or additive has increased rapidly, in spite xylitol is used since the 1960s. Currently, in the market, the products containing xylitol are the following: jams, chocolates, frozen desserts, cookies, chewing gums, confectionery, puddings, ice creams, and soft drinks (Mäkinen, 2011).

The main application in foods of xylitol is in chewing gums. Xylitol can be used to create new textures in bubble gum products, as it makes the product more flexible than other polyols. Xylitol in yoghurts, jams, and frozen desserts contributes to improve the texture, colour and taste of these products, and gives greater stability to the products as well. In addition, xylitol has antioxidant, hydrant, stabilizer and cryoprotectant properties (Maia et al., 2008). Moreover, it can be used as humectant in cakes and muffins, as it promotes bonding to moisture inside the product, improving its texture, quality and longevity (Ronda et al., 2005).

To sum up, xylitol can replace sucrose as it has numerous advantages.

1.1.3. Benefits to health

Due to the growing number of people with metabolic diseases, there has been established an objective, which is the reduction of sugar consumption. Therefore, efforts are being made to find a substitute of the more traditional sugars. This substitute could be beneficial to health, nutritious and could prevent diseases. Xylitol satisfies those requisites with additional advantages and has many other applications: it is tolerated by diabetics and has diverse clinical applications, such as treating hemolytic anemia, renal and parenteral lesions, prevent dental caries, acute otitis media, osteoporosis and inflammatory processes (Mussatto and Roberto, 2002).

1.1.3.1. Diabetes and obesity

Xylitol can be consumed by diabetics, because it does not depend on the insulin to be metabolized in the organism, it is well tolerated by people with diabetes mellitus type I or type II (Pepper and Olinger, 1988). In people with this deficiency in the metabolism of carbohydrates, it is necessary control the level of glucose in the blood, to avoid health problems, such as for example, hyperglicemia, excessive thirst and hunger, or disturbances in the lipids' metabolism (Ylikahri, 1979). Xylitol has a minimal impact on blood glucose level (Ylikahri, 1979). Thereby, it is considered a sweetener suitable for diabetics. In addition, it provides fewer calories and it is used by people that need a controlled diet, as well as people that are concerned with having a healthy lifestyle (Mussatto and Roberto, 2002).

1.1.3.2. Prevent dental caries

In the early 1970s, the importance of xylitol as potential component in dental caries prevention was first recognised by Turku Sugar Studies. These studies showed that substituting sucrose in the diet with xylitol resulted in a large reduction in the occurrence of caries (O'Donnell and Kearsley, 2012). Xylitol is not fermented by most oral cavity microorganisms, being an example of sugar with cariostatic action. Streptococcus mutans bacterium, form bacterial plaque is the main cause of caries, however cariostatic action of xylitol is observed because this microorganism not able ferment xylitol as carbon source (instead of sucrose), preventing dental caries (O'Donnell and Kearsley, 2012). The sequence of hydroxyl groups (OH), characteristic of xylitol chemical structure can be responsible for the formation of stable complexes with ions as calcium. Amaechi, Higham and Edgar (1998), demonstrated in their studies that in vitro experiment that teeth surface erosion caused by consumption of acidic drinks as orange juice, that can be diminished due to the addition xylitol and fluoride. Xylitol forms a complex with calcium ions, being responsible to retard demineralization of tooth plaque (O'Donnell and Kearsley, 2012).

1.2. Market of xylitol

The market for xylitol has shown a great increase since the 1960s, the first time it was produced by a Finnish company (Silva and Chandel, 2012). The production of xylitol has increased more than forty times in four last decades. In 1978, the manufacture of xylitol and mannitol was reported together, with a total of 6000 ton (Arcaño et al., 2018). The market of xylitol was estimated at US $ 340 million per year, in 2009, being the price $ 4 per 5 kg (Prakasham, Rao and Hobbs, 2009). In 2016, global market for xylitol was estimated in 190.9 thousand metric tons, valued in US $ 725.9 million. In 2022 forecast that production of xylitol to

be 266.5 thousand metric tons, with estimated value an amount above US $ 1 billion (Arcaño et al., 2018).

Production of xylitol is concentrated in Asia Pacific region, followed by Europe, the United States and Australia. The cost of production of this sweetener may be the reason for the limited expansion for other countries.

At present, the Dupont Danish Company Danisco is the largest xylitol producer in the world (Franceschin et al., 2011). Other market participants include the DFI Corporation, Shandong Futaste Co., Ltd., Xylitol Canada, Inc., Zuchem Inc., and Roquette Freres (Grandviewresearch.com, 2018).

1.3. Chemical and Biological processes for xylitol production

Currently, the production of commercial xylitol occurs by chemical processes. Xylitol is manufactured by reducing pure xylose obtained from hardwood hemicellulosic hydrolysate with a catalyst (Rafiqul and Sakinah, 2013).

The chemical process to obtain xylitol by chemical processes involves four main steps. The first step is the hydrolysis of lignocellulosic biomass by mineral acid. The hydrolysate thus obtained is composed of an ample variety of sugars including xylose, arabinose, glucose, galactose and mannose, and their proportions depend on the materials from which they are withdrawn and the experimental conditions. In the second step, the hydrolyzate is purified and separated, with the objective to obtain pure xylose as a solution or in crystalline form. Posteriorly, the catalytic reduction of xylose to xylitol is performed at 80-140 ºC, at pressures up to 50 atm in the presence of metal catalysts, in this case, Raney nickel catalysts. The last step consists in the crystallization and separation of xylitol (Silva and Chandel, 2012).

The xylitol formed by this method requires a chromatographic purification, concentration, and crystallization of the product to obtain pure xylitol (Silva and Chandel, 2012). As a result of this method, the xylitol yield is only about 50-60% of the xylan fraction or 8-15% of the starting raw material. Consequently, xylitol production by this method is very expensive because several steps of separation and purification are required (Nigam and Singh, 1995). The disadvantages of this method are, for instance, the various steps and complicated processes necessary that have relatively low efficiency. One of the biggest problems consists in achieving complete separation of xylose from the other by-products as weak acids, for example formic acids and acetic acids, furan derivatives as 5-hydroxyl methyl furfural (5-HMF), phenolic compounds such as acid, and aldehyde, as well syringic acid, ferulic acid and guaiacol and heavy metal ions (nickel and copper) (Silva and Chandel, 2012). The complete purification is essential because the catalysts used in hydrogenation of xylose are very sensitive to by-products. In addition, the chemical method for xylitol production is not environmentally friendly

because it requires great energy expenditure and, consequently, carries great pollution for the environment (Rafiqul and Sakinah, 2013).

Lignocellulosic wastes are cheap, renewable, abundant and easily available materials. Most of lignocellulosic residues come from agricultural practices and agro industries. (Anwar, Gulfraz and Irshad, 2014)

Generally, lignocellulose is made up of three major components: cellulose (34-50%), hemicellulose (19-35%) and lignin (11-30%). Others components, although in smaller quantities, are ash, pectins, and proteins. In general, hemicellulose is composed by pentoses such as xylose (xylose as the major sugar component) and arabinose, hexoses for example glucose, galactose, and mannose, and uronic acids (Figure 3), (Ramos, 2003).

Figure 3. Generalized schematic representation of lignocellulosic materials conversion into xylose and other sugars.

The main materials that have been research as biomass for xylitol production are oak, corn cobs, rice straw and corn stover, being that presents a high concentration of xylose (Cheng et al., 2009) (Rao et al., 2006). Other materials with much potential are, for instance, oil palm empty fruit bunch, sugarcane bagasse, sago trunk, eucalyptus wood, brewery's spent grain, barley bran, corn leaves, oak and sorghum straw (Table 1) (Mohamad, Mustapa Kamal and Mokhtar, 2014).

In table 1, it can be seen the composition of hemicellulosic hydrolysate from various lignocellulose materials.

Acid hydrolysis it is quick and a simple method for lignocellulosic biomass. The hydrolysis conditions vary with raw material type, acid type and concentration, temperature and time reaction (Rafiqul and Sakinah, 2013).

Table 1. Composition of hemicellulosic hydrolysate from various lignocellulose materials, adapted (Mohamad, Mustapa Kamal and Mokhtar, 2014).

Material	Hydrolysis	Xylose (g/L)	Glucose (g/L)	Arabinose (g/L)	Non sugars (g/L)
Corn cobs	Dilute sulfuric acid	70.40	5.60	4.70	8.40
Sugarcane bagasse	Dilute sulfuric acid	24.30	1.00	-	2.60
Rice straw	Dilute sulfuric acid	91.15	15.26	18.30	1.77
Brewery spent grain	Dilute sulfuric acid	14.90	5.20	6.20	3.32
Oil palm empty fruit bunch	Dilute sulfuric acid	30.80	7.61	-	10.40
Eucalyptus wood	Dilute sulfuric acid	18.00	3.60	0.60	~ 6.0
Oak	Dilute sulfuric acid	106.00	13.20	1.60	31.30
Corn stover	Dilute sulfuric acid	67.30	14.30	11.80	-

1.3.1. Biological processes for xylitol production

In the last decade, many studies have been directed towards searching for alternative methods for xylitol production. The biotechnological processes (xylose fermentation to xylitol by microorganisms) are a promising method for its production, presenting many advantages relatively to the chemical method; the factors of biological and chemical processes for xylitol production are in table 2 (Mohamad, Mustapa Kamal and Mokhtar, 2014).

Table 2. Comparison between the biological and chemical processes for xylitol production, adapted from Mohamad, Mustapa Kamal and Mokhtar (2014).

Factor	Biological	Chemical
Carbon source	Xylose from lignocellulose	Pure xylose Xylose from lignocellulose
Catalyst	Yeast/ bacteria/fungi that require xylose reductase and xylitol dehydrogenase enzyme	Nickel and hydrogenation
Process steps	1.Acid or enzymatic hydrolysis of lignocellulose; 2.Detoxification of hydrolysate; 3. Hydrogenation of xylose to xylitol; 4.Xylitol purification and crystallization	1.Acid hydrolysis of lignocellulose; 2. Purification of hydrolysate to obtain pure xylose; 3. Hydrogenation of xylose to xylitol (80 ºC - 140ºC, pressures up to 50 atm in the presence of metal catalysts); 4.Xylitol purification and crystallization
Purification	Complex downstream process because of different microbial by-products.	Ion-exchange resins
Cost	Lower overall production costs, lower energy requirements and more eco friendly.	High: need two steps of purification process, high energy requirements, laborious, generates hazardous wastes.

1.3.2. Xylitol production by microorganisms

The biotechnological processes for the production of xylitol are based on the use of microorganisms such as yeasts, bacteria and fungi. (Rafiqul and Sakinah, 2013). For optimization of the fermentation process, the investigators considered two main aspects, in particular, strain and process development and optimization (Mohamad, Mustapa Kamal and Mokhtar, 2014). There is few information about xylitol production by bacteria, being mostly used xylose or xylulose solutions to produce xylitol. The examples of bacteria capable of xylitol production are *Corynebacterium* sp, *Enterobacter liquefaciens*, *Cellulomonas cellulans*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Serratia marcescens*, *Bacillus coagulans* and *Mycobacterium smegmatis*. In most of cases, the final concentrations of xylitol and productivities were very low (Silva and Chandel, 2012). Other microorganisms capable of xylitol production are filamentous fungi, namely, *Fusarium oxysporum*, *Petromyces albertensis* , *Penicillium roqueforti*, *P. crustosum*, *P. brevicompactum, P. crysogenum*, *P. purpurogenum*, *P. citrinum*, *P. janthinellum*, *P. griseorolsum*, *P. expansum*, *P. italicum* and *Aspergillus niger*, although the productivities and concentration of xylitol were very low. Sampaio et al. (2003)

screened 11 filamentous fungi belonging to the genera of Aspergillus and Penicillium to research on xylitol production. *P. crustosum* presented achieved xylitol production 0.52 g/L xylitol from 11.5 g/L xylose.

Yeasts are the microorganisms more often utilized for xylitol production, showing higher yields than other microorganisms (Mohamad, Mustapa Kamal and Mokhtar, 2014). Chen and Gong (1985) reported *Candida sp* as the best producer, with 204 g/L - 210 g/L xylitol from 250 g/L of xylose and production yield ($Y_{P/S}$= 0.84 g $_{xylitol}$/g $_{xylose}$). Barbosa et al. (1988) reported *C. guilliermondii* and *C. tropicalis* as the highest xylitol producers. These yeasts produced 77.2 g/L xylitol from 104 g/L D-xylose using high cell densities in medium under aerobic conditions. A volumetric productivity was 2.67 g/L.h xylitol with 172 g/L initial D-xylose as substrate was obtained using *C. tropicalis*.

As an alternative, xylitol production by genetically modified microorganisms carrying the gene Xylose Reductase (XR) responsible for xylitol production in yeasts has also been extensively studied. This method increased the productivity. A mutant, *Candida tropicalis* produced 220 g/L and 3.3 g/L. h and overall yield of 0.93 $g_{xylitol}$ of D-xylose. However, this process appears to have certain genetic instability and safety problems (Mohamad, Mustapa Kamal and Mokhtar, 2014).

In view of this, many researchers have conducted a lot of studies, investigations, in order to develop alternative processes for the production of xylitol, namely, the biological method to convert xylose into xylitol (Nigam and Singh, 1995).

1.3.3. Metabolic pathways

Winkelhausen and Kuzmanova (1998), discussed the D-xylose metabolism in yeasts, considering that it occurs in two main steps. The first step is the reduction of D-xylose from Xylose Reductase (XR). In the next step, occurs the oxidation of (XR) by xylitol dehidrogenase (XDH), before phosphorylation of xylulose-5-phosphate, catalysed by xylulokinase (XK). Reduction and oxidation are considered the limiting steps, and the enzymes XR and XDH are extremely important in D-xylose fermentation and xylitol production, respectively (figure 4). These enzymes require the pyridine nucleotide cofactors as nicotinamide adenine dinucleotide (NAD^+/NADH) or nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH), according to the type of yeast (Mohamad, Mustapa Kamal and Mokhtar, 2014).

Besides that, the function and regeneration of cofactors are extremely dependent on the oxygen level and the oxygen transfer rates.

For the yeasts with NADPH dependent XR activity, for example, *Candida mogii*, the dissolved O_2 level must be controlled. Optimal values of O_2 concentration for production of xylitol are usually micro-aerobic conditions that allow for NAD^+ regeneration, producing adenosine triphosphate (ATP) and NADPH that necessary to ensure cell growth and XR activity, respectively (Silva and Chandel, 2012).

At a low oxygen level, the electron transport system is incapable to oxidize intracellular NADH totally, increasing the NADH concentrations thus allowing the excretion of xylitol. At high oxygen level, occurs the oxidation of NADH to NAD^+, and the high concentration of NAD^+ relatively NADH favours xylitol oxidation to xylulose, where NAD^+ is regenerate by respiratory chain. When there is NADH in excess, the yeast cannot oxidize because it does not possess an enzyme with transhydrogenase activity (Mussatto and Roberto, 2003). Therefore, the oxygen transfer rate, oxygen transfer coefficient (kLa) and specific rate of oxygen, are critical parameters must be strictly controlled during the bioconversion xylose at xylitol (Silva and Chandel, 2012).

In the figure 4, it is observed the metabolic pathways for xylitol production by the yeast *Candida mogii*.

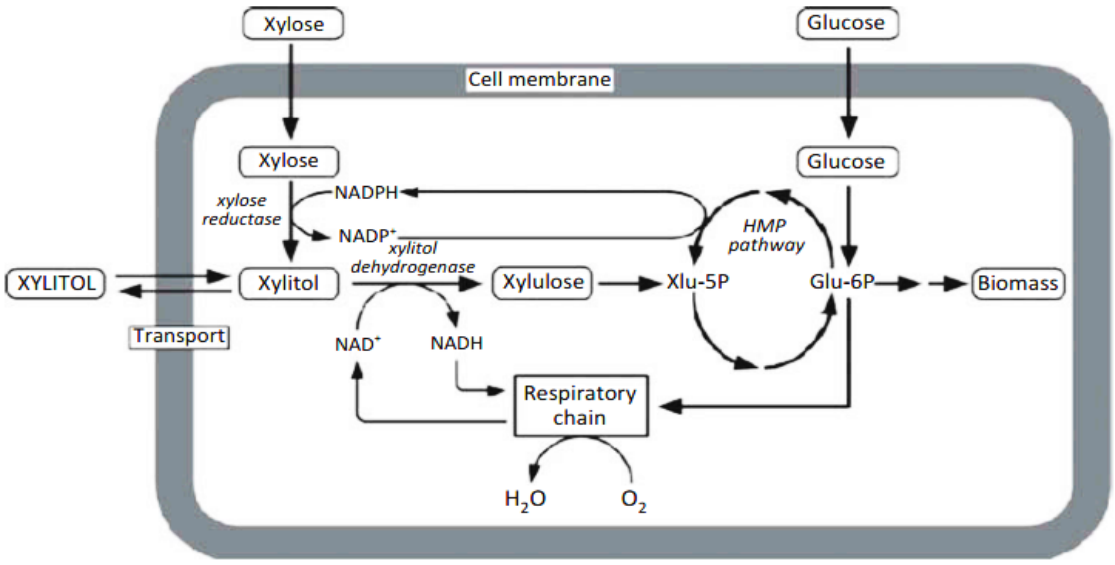

Figure 4. Metabolic pathways for xylitol production by *Candida mogii* (adapted from Silva and Chandel, 2012).

1.3.4. Fermentation parameters that influence the production of xylitol

The biotechnological production of xylitol is dependent of several operating variables and the economical viability of the process is influenced by the optimization of the various fermentation parameters (Branco et al., 2009). Xylitol bioproduction by yeasts is generally influenced by the cultivation conditions, namely pH, temperature, aeration, immobilization and

inoculum concentration, the nutritional composition of the medium (carbon source, nitrogen source, and micronutrients and their concentrations), the operation mode (batch, fed-batch or continuous), and the genetic nature of the microbial strains (wild type isolates, recombinant strains, and mutants) (Rafiqul and Sakinah, 2013).

The influence of pH was considered a potentially valuable in the production of xylitol, given that the xylose reduction by monospecific (msXR) and dual specific XR (dsXR) it is due to pH-dependent ionization of the groups involved in substrate binding and catalysis (Branco et al., 2009).

Yokoyama et al. (1995), reported that the optimum pH for the bioreduction of D-xylose by purified *Candida tropicalis* XR was 6.0. In another studies Verduyn et al. (1985), reported that pH range for the production of xylitol by XR from *Pichia stipitis* was 5.0–8.0, with an optimum value of 6.0 .

The temperature is a factor that influences xylitol production. In an experiment, Verduyn et al. (1985), observed that the optimum temperature for XR from *Pichia stipitis* was 38 ºC and that pH linked XR activity increased linearly from 20 ºC to 38 ºC.

1.4. The yeast Komagataella pastoris

The yeast *Pichia pastoris*, currently reclassified as *Komagataella pastoris* (*K. pastoris*), is a methylotrophic yeast given its capacity of growing with reduced carbon compounds, such as methanol and methane (Spohner et al, 2015).

K. pastoris was introduced by Phillips Petroleum Company more than forty years ago, as potential source of cell single protein (SCP) with the aim to be marketed principally as animal feed additive. The company developed media and protocols for growing *P. pastoris* on methanol in continuous culture, achieving over 130 g/L dry weight (Cereghino and Cregg, 2000). From that moment, it has become a substantial expression system in biotechnological processes, mainly for heterologous protein production (Spohner et al., 2015). In 1970s the price of methanol and methane increased due to the oil crisis, which made SCP production unattractive. (Spohner et al., 2015).

In the following decade, Phillips Petroleum contracted the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA) (Cereghino and Cregg, 2000), with the aim to develop *P. pastoris* as a protein expression system, using the tightly regulated alcohol oxidase promoter (AOX) which provided high levels of heterologous protein expression (Spohner et al., 2015).

Nowadays, more than 500 recombinant proteins and pharmaceutical compounds are produced by *P. pastoris* system (Cos et al., 2006). In the last decade, this system has been optimized with the focus being on genetics with the purpose of being used in drug and pharmaceutical industries (Spohner, 2015).

Taxonomically, *K. pastoris* belongs to the Kingdom Fungi, Phylum *Ascomycota*, Class *Saccharomycetes*, Order *Saccharomycetales*, Family *Saccharomycetacea*, and Genus *Komagataella*.

K. pastoris when cultivated in glucose/xylose mixtures use glucose as substrate for cell growth. After glucose completely depletion, xylose was consumed, however no cell growth was observed, that indicate that *K. pastoris* was unable use xylose to growth.

Recently, Araújo et al (2017) reported that *K. pastoris* as capable of producing xylitol. In a 5 L bioreactor operated under batch mode, in a BSM medium, with glucose/xylose mixtures, with controlled temperature (30 ± 0.1 ◦C) and pH (5.0 ± 0.02), with DO was controlled at 50% air saturation by the automatic variation of the stirring rate, between 300 and 2000 rpm.

K. pastoris reaching a production of 7.64 g/L of xylitol, with a yield on xylose of 0.52 g/g and a volumetric productivity of 0.10 g/L.h (Araújo et al., 2017).

1.5. Motivation

As previously mentioned, xylitol is a five carbon sugar alcohol, which has a great applicability in food and pharmaceutical industries, as well in dental related products (Silva and Chandel, 2012)

One of the biggest advantage of this natural sugar is that xylitol is an ideal sweetener for diabetic patients because its metabolism is independent of insulin. Thereby, xylitol has attracted global market mainly owing to use by diabetic patients, such as other applications due to it is of a number advantageous properties (Silva and Chandel, 2012).

The production of xylitol has increased more than forty times in four decades. Currently, production of xylitol is performed by chemical methods (Arcaño et al., 2018).

Biotechnological production of xylitol is possible by fermentation using xylose from renewable sources, by microorganisms (Silva and Chandel, 2012). Given that, chemical production of xylitol involves high temperature, pressure and expensive purification steps. So, biotechnological production of xylitol using microorganisms is gaining more interest over chemical process.

There is a high demand for xylitol and there is lack of biotechnological processes for xylitol production, on an industrial scale. First goal of this work focused on the optimization of biotechnological production of xylitol by the yeast *K. pastoris* (this yeast not yet studied for this purpose).

So, it were performed several assays with *K. Pastoris* in shake flask screening in order to evaluate the effect of several parameters, as pH, temperature, concentration of carbon source and different aeration conditions, on production of xylitol and arabitol.

Chapter 2. Materials and Methods

2. Materials and Methods

2.1. Microorganism

The yeast used in all assays was *Komagataella pastoris* DSM70877. The culture was cryopreserved at -80 ºC, in 20% (v/v) glycerol, in 1 mL aliquots.

2.2. Growth media

In all experiments, medium K was used, with the following composition (per litter of deionized water): KH_2PO_4, 28.0 g; $CaSO_4.2H_2O$, 0.125 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $(NH4)_2SO_4$, 13.55 g and 400µL of a trace mineral solution. The trace mineral solution had the following composition (per litter): $CuSO_4 \cdot 5H_2O$, 2 g; $MnSO_4 \cdot H2O$, 3 g; $ZnCl_2$, 7 g; $FeSO_4 \cdot 7H_2O$, 22 g; biotin, 0.2 g; H_2SO_4, 5 mL. Medium K, without the trace mineral solution, was autoclaved at 121 ºC for 20 minutes. The pH of the medium was set to the desired value by addition of NaOH 5 M (eka) or HCl 2 M (Sigma-aldrich). The trace mineral solution was filter-sterilized (0.2 µm, Sartorius Stedim Minisart), and added to medium K after cooling.

A mixture of glucose/xylose was used as substrate for the experiments. Glucose and xylose solutions were prepared individually, 12 g of D-xylose (Sigma-aldrich) in 20 mL deionized water, 4 g of D-glucose (Scharlau) in 20 mL deionized water, and sterilized separately at 121 ºC during 20 minutes. The solutions were added to medium K after cooling, to give glucose and xylose concentrations of 20 g/L and 60 g/L, respectively.

2.3. Inocula preparation

The inoculum was prepared, by inoculating 1 mL of the cryopreserved culture into 160 mL medium K, supplemented with 40 mL of the substrate mixture, as described above, in a 500 mL baffled shake flask. Also, 400 µL of the trace mineral solution was added. This procedure was performed in aseptic conditions, in a laminar flow chamber (Heraeus).

The inoculum was incubated in an orbital shaker (Lab Companion), at 30 ºC and 200 rpm, during 72 h.

2.4. Shake Flask Experiments

The experiments were performed in 500 mL baffled shake flasks with 160 mL of medium K, supplemented with 20 mL of glucose solution (to give a concentration of 20 g/L), 20 mL of xylose solution (to give a concentration of 60 g/L), 20 mL of arabinose (Panreac) solution (to give a concentration 30 g/L or 60 g/L) and 400 µL trace mineral solution.

The assays were run for 168 hours in an orbital shaker, with controlled temperature (23, 30, 37, 40, 43 ºC), and constant agitation (200 rpm). Samples of 5 mL were periodically (24 hours) taken for measurement of the optical density at 600 nm ($OD_{600\ nm}$) and pH, and determination of the concentration of glucose, xylose, xylitol and arabitol.

At the end of the assays, 15 mL of samples were collected for quantification of the cell dry weight (CDW) and the concentration of glucose, xylose and xylitol.

In order to select the best conditions for cellular growth and xylitol production, several assays were performed with different pH in the range 2.1 – 10.0, temperature 23, 30, 37, 40 and 43 ºC, substrate concentration 10, 20, 30, 40 and 60 g/L of xylose, and aeration: oxygen exhaustion. Oxygen exhaustion was achieved by replacing the filter cap of the flasks with a cap with no filter.

In order to select the best conditions for arabitol production assays were performed with different temperature 30 and 37 ºC and aeration: full aerobic conditions and oxygen exhaustion.

2.5. Analytical Techniques

2.5.1. Cell growth

Cell growth was monitored during the experiments by determining the optical density of the cultivation broth at 600 nm (OD600nm), in a UV-Vis spectrophotometer (Thermo Spectronic, Heλios α). The samples were diluted with deionised water so that the OD600nm value was below 0.3. Deionised water was used as zero reference. All measurements were done in replicate analysis.

2.5.2. Cell Dry Weight

The cell-free supernatant obtained by centrifugation of the broth samples (17 418 *g*, for 15 min, at 4 ºC). The cell pellet was used for the determination of the CDW. After being washed with deionized water by resuspension in deionized water and centrifugation, the pellet was freeze-dried (ScanVac CoolSafeTM, LaboGene) at -110 ˚C for 48 h. This analysis was done in triplicate.

2.5.3. Substrate and product concentration

For quantification of sugars, namely glucose, xylose, xylitol and arabitol the cell-free supernatant obtained by centrifugation of the culture broth samples, as described above, was diluted (dilution of 1:1000 for glucose and xylose quantification, dilution of 1:100 for xylitol and

arabitol). After dilution, the samples were filtered with 0.20 µm centrifuge filters (9 600 g for 10 minutes; VWR), before the analysis.

The concentration of glucose, xylose, xylitol and arabitol was determined by high performance liquid chromatography (HPLC), using a column (Thermo Carbopac PA10 250 mm x 4 mm + Aminotrap + Boratetrap) coupled to electrochemical detector – PAD - quadruple potential. The mobile phase was 52 mM NaOH solution, as eluent with a flow rate of 1 mL/min and the column was operated at 25 ºC. Standard solutions of glucose (Scharlau), xylose (Sigma-aldrich, 99%), xylitol (Sigma-aldrich, ≥ 99%) and L-arabitol (TCI), at concentrations ranging from 0.005 to 0.100 g/L were used to generate the calibration curves (Appendix 1).

2.6. Calculus

The product yield ($Y_{P/S}$, $g_{xylitol}/g_{xylose}$) was determined using equation 3.9.1:

$$Y\mathrm{p/s} = \frac{P_f - P_i}{S_i - S_f} \qquad \text{Equation 3.9.1}$$

Where, P_f and P_i correspond to the final and initial xylitol concentrations (g/L), respectively; S_f and S_i correspond to the final and initial xylose concentrations (g/L), respectively.

The specific productivity ($Y_{P/X}$, $g_{xylitol}/g_{CDW}$) was determined by the equation 3.9.2:

$$Y\mathrm{p/x} = \frac{P_f - P_i}{X_f - X_i} \qquad \text{Equation 3.9.2}$$

Where, P_f and P_i correspond to the final and initial xylitol concentration (g/L), respectively; X_f and X_i correspond to the final and initial CDW (g/L), respectively.

Xylitol volumetric productivity (r_p, g/L.h) was determined by Equation 3.9.3:

$$r\mathrm{p} = \frac{P_f - P_i}{t_f - t_i} \qquad \text{Equation 3.9.3}$$

Where P corresponds of xylitol concentration (g/L) produced at the time t (h), of the assay.

Chapter 3. Results and Discussion

3. Results and Discussion

In order to select the suitable conditions to maximize cellular growth and xylitol production, were performed in shake flasks screening, several assays with different pH values, temperature values, substrate concentration (glucose/xylose mixtures) and aeration.

3.1. Effect of pH

3.1.1. Effect of the initial pH on cell growth and xylitol production

In order to determine the most appropriate pH for *K. pastoris* cultivation and production of xylitol, a set of shake flask experiments (Figure 5), was performed wherein different values of pH were tested, in the range 2-10 (Table 3). All experiments were carried out at 30 ºC and 200 rpm, during 96 hours.

Figure 5. Photos of the shake flasks at the end of the experiments (96 h) with different initial pH values.

Figure 6, shows the results obtained in each assay in terms of CDW and production of xylitol.

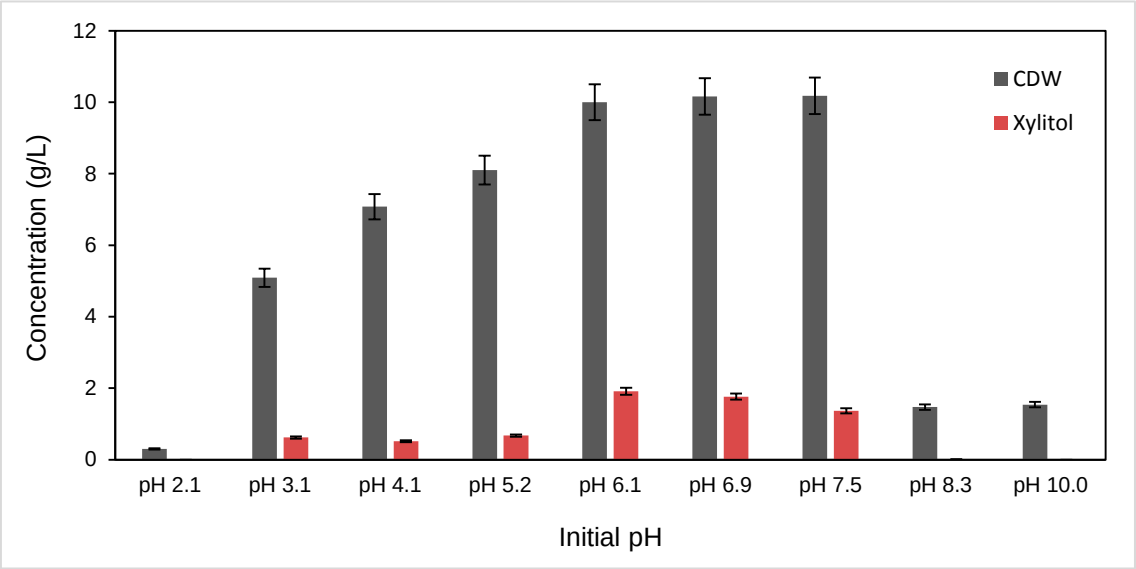

Figure 6. CDW and xylitol concentration obtained by cultivation of *K. pastoris* with different initial pH values (shake flask assays performed during 96 hours).

In this assay, were evaluated the effect of pH on cell growth and production of xylitol.

The culture was able to grow in the assays performed with initial pH values between 3.1 and 7.5, despite the CDW varying with the different pH values tested (Figure 6). The highest CDW values (10.00 g/L, 10.16 g/L and 10.18 g/L) were obtained for the assays performed with initial pH set at 6.1, 6.9 and 7.5, respectively (Figure 6). On the other hand, low CDW values were obtained for the assays performed with initial pH of 8.3 and 10 (1.47 and 1.54 g/L, respectively), while practically no growth was observed at pH 2.1 (0.30 g/L).

Chagas et al. (2014), also reported that cell growth of *K. pastoris* with initial pH 6.5 revealed the highest cell growth in the range tested 3.5 - 6.5, although a different cultivation medium, standard basal salts medium (BSM), was used in that study.

With an initial pH of 5.2, a CDW of 8.10 g/L was reached. This result is in accordance with Araújo et al. (2017), that reported a similar value, 8.80 g/L, using BSM medium in 175 hours shake flask cultivation.

Every microorganism has a pH optimum and a pH range in which they grow. Nevertheless, variations are reported in the literature. For instance, *C. parapsilosis* ATCC 28474 and *C. guilliermondii* NRC 5578 showed maximum cell growth at pH 6.0 (Prakasham, Rao and Hobbs, 2009).This fact can be explained because they are different types of yeasts, so they have different optimum growth conditions comparing with *K. pastoris.*

Relatively to the production of xylitol, (Figure 6), the highest values were also obtained in the assays performed with initial pH value of 6.1, 6.9 and 7.5 (1.91 g/L, 1.76 g/L and 1.37 g/L, respectively). This corresponds to volumetric productivities of 0.020, 0.018 and 0.014 g/L.h, respectively. Interestingly, the highest specific productivity values (0.19 and 0.17 and 0.13 $g_{xylitol}/g_{CDW}$) were attained for the same experiments (performed with initial pH of 6.1, 6.9 and 7.5, respectively).

Therefore, based on these results, the pH value of 7.0 was selected for the subsequent studies because it maximized both *K. pastoris* cell growth and xylitol production, under the conditions tested.

3.1.2. Effect of pH during xylitol synthesis

In order to determine the most appropriate pH for the synthesis of xylitol by *K. pastoris*, a set of shake flask experiments was performed, wherein the same initial pH value of 7.0 was used for cell growth. In all experiments, the same glucose and xylose concentrations were used (20 and 60 g/L, respectively). In each set of experiments, after growing for 72 hours, the pH of the broth was altered to different values in the range 2.5-9.2, in order to assess its impact on xylitol synthesis. The experiments were extended for a total cultivation time of 168 hours.

It was observed that at 72 hours the culture reached the maximum CDW (on average 11.51±2.02 g/L). Within that period of time (72 hours), the pH dropped from the initial value of 6.9±0.05 to 6.0±0.26. Then, the pH of each shake flask was altered as shown in Table 3. After that time, until the end of the experiments, no significant cell growth was observed and production of xylitol occurred.

Table 3. Concentration of xylitol, volumetric productivity and specific productivity ($\boldsymbol{Y_{P/X}}$ = $g_{xylitol}/g_{CDW}$) as a function of the initial pH in the cultivations.

pH*	Xylitol (g/L)	r_p (g/L.h)	$Y_{P/X}$ ($g_{xylitol}/g_{CDW}$)
2.5	3.28	0.020	0.29
4.3	1.85	0.011	0.16
5.1	1.57	0.009	0.14
6.1	2.44	0.015	0.21
7.0	3.93	0.023	0.34
7.5	4.04	0.024	0.35
9.2	3.15	0.019	0.27

* altered at 72 hours of cultivation

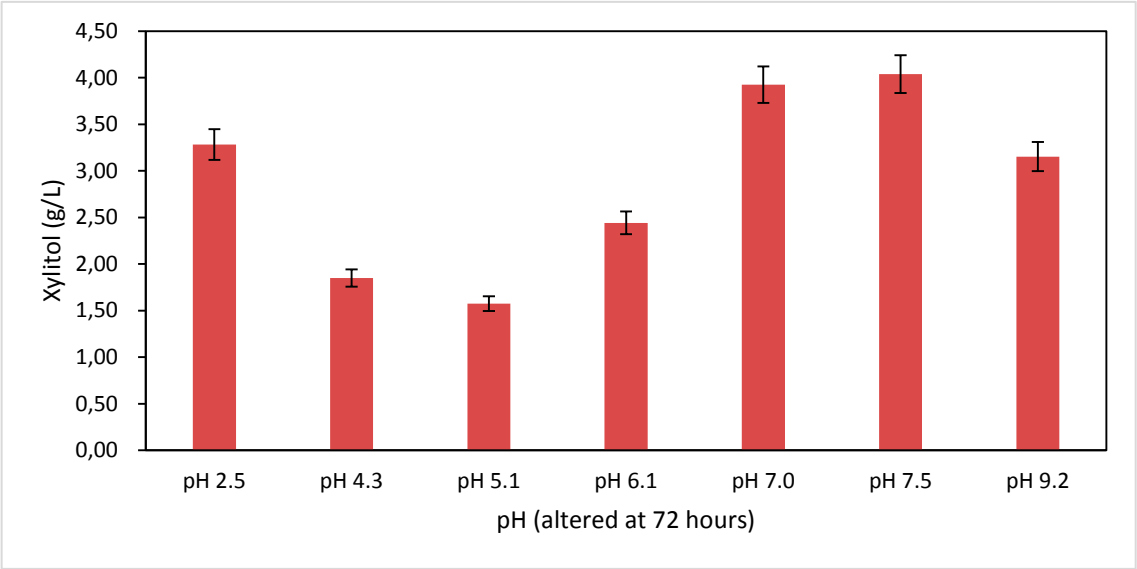

Figure 7. Concentration of xylitol at the end of 168 hours of cultivation of *K. pastoris* in the shake flask assays with initial pH of 6.9±0.05, altered at 72 hours to different pH values.

Figure 7, shows that the change of pH to 7.0 and 7.5 resulted in the highest xylitol concentration obtained in this set of experiments, 3.93 g/L and 4.04 g/L, respectively.

Interestingly, as can be seen in figure 7, a higher concentration of xylitol was observed for pH altered to 2.5 (3.28 g/L) compared to higher pH values such as 4.3, 5.1 and 6.1, reaching 1.85 g/L, 1.57 g/L and 2.44 g/L respectively.

Curiously, on the other hand, high production of xylitol (3.15 g/L) was observed by altering the pH to a high value of 9.2.

Concomitantly, the highest specific productivity values (0.34 and 0.35 $g_{xylitol}/g_{CDW}$) were attained for the experiments performed with change of pH to 7.0 and 7.5, respectively. By looking at table 3, it is possible to see that higher values of specific productivity correspond to higher concentrations of xylitol. These experiments shown that the values obtained of specific productivity (0.34 and 0.35 $g_{xylitol}/g_{CDW}$) were higher than in the first experiments mentioned in section 3.1.1, corresponds to (0.17 and 0.13 $g_{xylitol}/g_{CDW}$), on similar values of pH tested. Therefore, changing the pH at 72 hours of the assay resulted in a good strategy for xylitol production.

According to the table 3, it can be seen that regarding the volumetric productivity, experiments performed with change pH of 7.0 and 7.5, represented the best values obtained 0.023 g/L.h and 0.024 g/L.h respectively.

As previously referred in section 3.1.1, low CDW values were obtained for the assays performed with initial pH of 2.1, and pH value in the range 8.3 to 10, these pH values revealed unsuitable for growth, however, in stationary phase promote the synthesis of xylitol.

In the previous assay, mentioned in section 3.1.1, initial pH value of 6.1 was favourable for cell growth, in turn, in this assay this value result in lower values of xylitol concentration, as can be observed in table 3.

Araújo et al. (2017), using *K .pastoris*, reported in their studies 7.64 g/L concentration of xylitol, in batch bioreactor cultivation. However, the highest value of xylitol concentration obtained in the shake flask assay (4.04 g/L) is a little bit different than mentioned study, this might be because in a bioreactor the environmental conditions are better controlled.

D-Xylose is reduced to xylitol by xylose reductase. Literature describes examples of yeasts that present similar pH range at *K. pastoris*. In general, the optimum pH value for the best xylitol yield in *C. boidinii* was 7.0. (Prakasham, Rao and Hobbs, 2009). *Pachysolen tannophilus* has an optimum pH in the range between 6.0 and 7.5 while *Hansenula polymorpha* optimum pH is 7.0 (Silva and Chandel, 2012).

3.1.3. Conclusion

These results seem to indicate that cell growth was enhanced by cultivating *K. pastoris* with an initial pH in the range of 6.0 to 7.5, while the production of xylitol was enhanced at pH 6.9-7.5. Therefore, a pH of 7.0 was selected for the subsequent experiments.

3.2. Effect of Temperature

3.2.1. Effect of the initial temperature on cell growth and production of xylitol

All microorganisms have an optimum temperature for growth and different groups of microorganisms grow over different temperature ranges. *K. pastoris* is a mesophile microorganism. As for temperature optima, mesophilles have optimum cell growth temperatures in the range 20 ºC – 45 ºC (Waites et al., 2009).

In order to evaluate the most appropriate temperature for *K. pastoris* cultivation and xylitol synthesis, a set of shake flask experiments was performed, with an initial pH of 7.0, wherein different values of temperature were tested, namely 23, 30 and 37 ºC, as shown in table 4. Also, the combined effect of pH and temperature was assessed by altering the pH after 72 hours of cultivation. The same substrate concentrations were used, 20 g/L of glucose and 60 g/L of xylose. All assays took 168 hours, in an orbital shaker (200 rpm).

Table 4. CDW, concentration of xylitol, yield ($Y_{P/S}$), specific productivity ($Y_{P/X}$) and volumetric productivity (r_p) at different temperatures and pH values.

T (ºC)	pH	CDW (g/L)	Xylitol (g/L)	$Y_{P/S}$ ($g_{xylitol}/g_{xylose}$)	$Y_{P/X}$ ($g_{xylitol}/g_{CDW}$)	r_p (g/L.h)
23	4.0*	9.49	0.46	n.d.	0.05	0.003
	6.2	8.56	0.14	n.d.	0.01	0.001
	7.8*	9.56	0.48	n.d.	0.05	0.003
30	4.3*	10.29	4.86	0.17	0.47	0.029
	6.4	10.27	2.43	n.d.	0.24	0.014
	7.9*	9.83	2.41	n.d.	0.25	0.014
37	4.5*	9.76	11.30	0.28	1.16	0.067
	6.4	8.53	12.00	0.35	1.41	0.071
	7.8*	7.90	8.77	0.28	1.11	0.052

* pH altered at 72 hours; n.d. – not determined

Through the observation of table 4, it is easy to understand that the different values of temperature tested had a small impact on cell growth. However, it is possible to see that the CDW values were slightly higher at 30 ºC (9.83-10.29 g/L). Nevertheless, the CDW values obtained on cultivation at 23 ºC (8.56 - 9.56 g/L) and 37 ºC (7.90 - 9.76 g/L) were similar.

Figure 8, shows the concentration of xylitol obtained by the combined effect of pH and temperature, by altering the pH after 72 hours of cultivation.

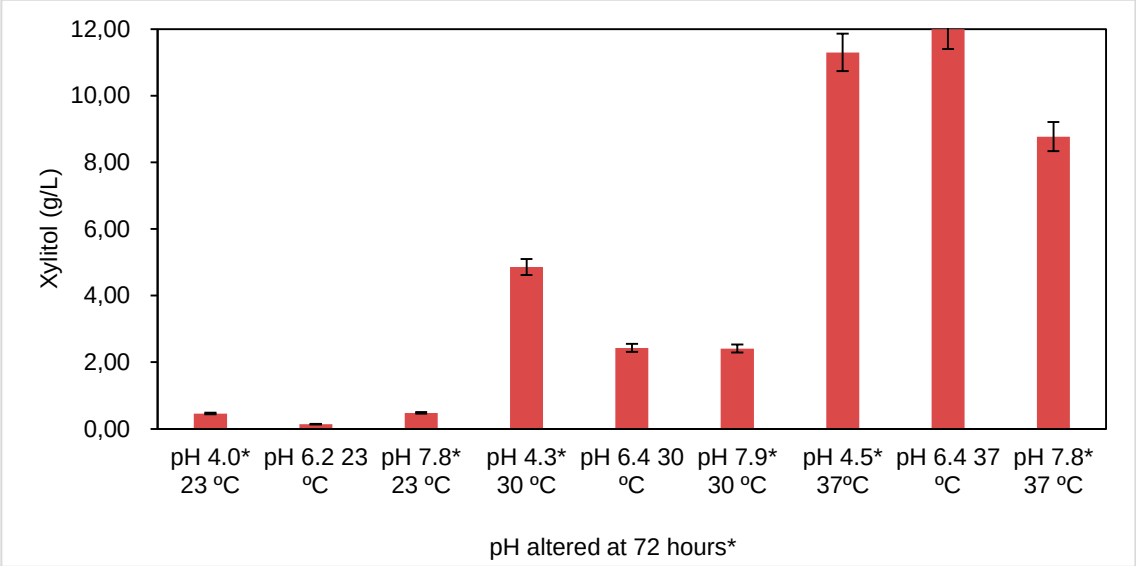

Figure 8. Concentration of xylitol at the end of 168 hours of cultivation of *K. pastoris* in the shake flask assays with different temperatures, initial pH 7.0 and pH altered at 72 hours.

By observation of figure 8, it remains clear that at 23 ºC, at any of the pH tested (4.0, 6.2 and 7.8), the concentration of xylitol (0.14 - 0.48 g/L), specific productivity (0.01-0.05 $g_{xylitol/}$ g_{CDW}) and volumetric productivity (0.001-0.003 g/L.h) were very low. As a result, this value of temperature proved to be unfavorable to xylitol production by *K. pastoris*.

As can be seen in table 4, cultivation at 30 ºC with pH 4.3, 6.4 and 7.9 resulted in concentration of xylitol 4.86 g/L, 2.43 g/L and 2.41 g/L, respectively.

Cultivation at 37ºC with at 4.5 resulted in a very high concentration of xylitol (11.3 g/L), as well as high values of productivity yield 0.28 $g_{xylitol}/g_{xylose}$, specific productivity (1.16 $g_{xylitol}/g_{CDW}$) and volumetric productivity 0.067 g/L.h.

Cultivation at 37 ºC with pH 6.4 resulted in the highest xylitol concentration obtained in this study (12.00 g/L). Curiously, the best values of productivity yield 0.35 $g_{xylitol}/g_{xylose}$, specific productivity 1.41 $g_{xylitol}$ / g_{CDW} and volumetric productivity 0.071 g/L.h in this study were obtained.

Interestingly, cultivation at 37 ºC with change pH to 7.8 resulted in 8.77 g/L of xylitol and the values of productivity yield 0.28 $g_{xylitol}/g_{xylose}$, specific productivity and volumetric productivity were 1.11 $g_{xylitol}/g_{CDW}$ and 0.052 g/L.h , respectively.

In this assay, values of xylitol concentration, productivity yield, specific productivity and volumetric productivity were higher comparatively at the assay mentioned in section 3.1.2, that reveals optimization of the parameters for production of xylitol by *K. pastoris*.

Comparing with Araújo et al. (2017), that reported 7.64 g/L of xylitol by *K. pastoris* in batch bioreactor, under controlled conditions, in this assay were achieved better results (12.00

g/L), in shake flask. This shows that it was possible improve the synthesis of xylitol by the yeast.

According to the table 4, and figure 8 it is clear to realize that at 37 ºC, in any pH value tested, the production of xylitol improves greatly.

Glucose, xylose and xylitol concentration were quantified during the assay's time. The profiles of cultivation at 37 ºC, it can be seen in figures 9, 10 and 11.

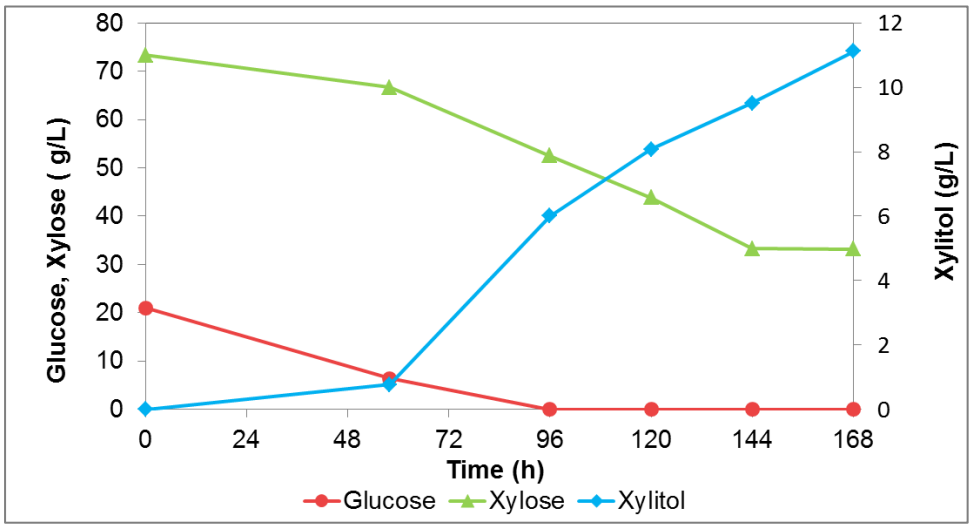

Figure 9. Cultivation profile of the shake flask experiment at 37 ºC with initial pH of 7.0 and altering the pH to 4.5 at 72 h of cultivation.
Glucose (–●–, g/L), xylose (–▲–, g/L) and xylitol (–♦–, g/L).

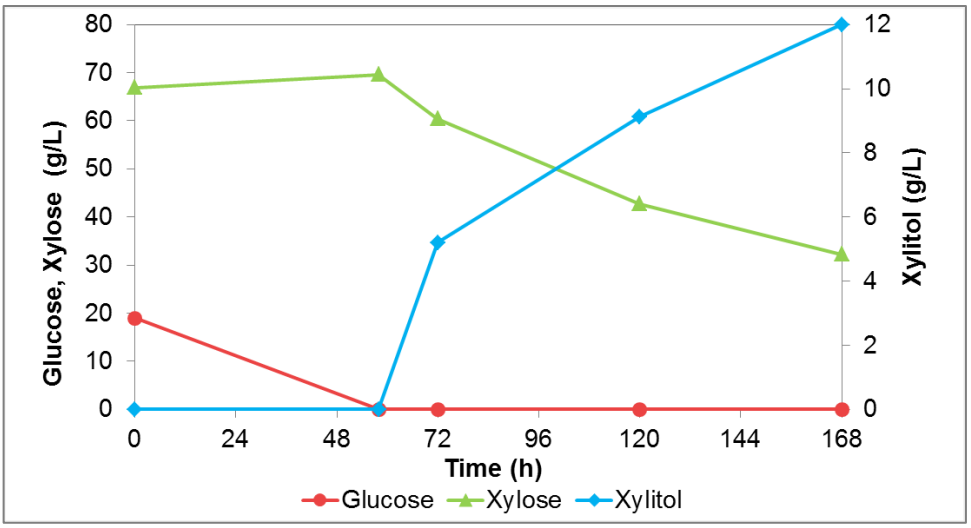

Figure 10. Cultivation profile of the shake flask experiment at 37 ºC with initial pH of 7.0 and altering the pH to 6.4 at 72 h of cultivation.
Glucose (–●–, g/L), xylose (–▲–, g/L) and xylitol (–♦–, g/L).

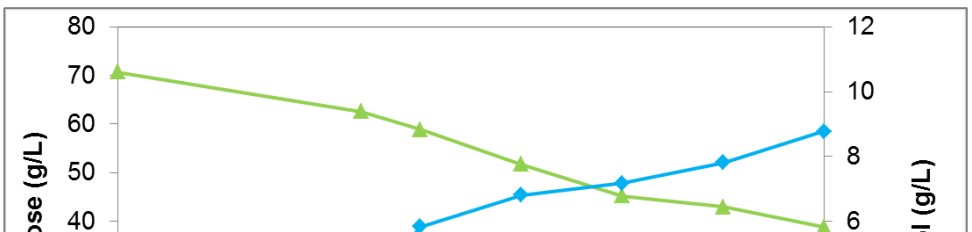

Figure 9, shows the cultivation profile of *K. pastoris* at 37 ºC with pH altered to 4.5 at 72 h of cultivation. It is possible to observe that the glucose available was exhausted during the first 96 hours (approximately). By the observation of figures 10 and 11, cultivation profile at 37ºC with pH 6.4 and cultivation profile at 37ºC with pH 7.8, respectively, it is possible to see that glucose was completely depleted between 48 – 72 hours of the assays. In all assays, this means that cell growth reached the maximum at that time.

On the other hand, in all assays, xylose was not completely consumed, however it can be observed that as the concentration of xylitol increased, xylose concentration decreased, which means that some of the available xylose was consumed for production of xylitol by the yeast.

Table 5. Fermentation conditions, values of xylitol concentration (g/L), production yield ($Y_{P/S}$ = $g_{xylitol}$ / g_{xylose}) and volumetric productivity (r_p $g/L_{xylitol}$ .h).

Microorganism	Fermentation conditions					Time (h)	Xylitol (g/L)	$Y_{P/S}$ ($g_{xylitol}$/ g_{xylose})	r_p (g/L.h)	Reference
	Cultivation mode	Agitation (rpm)	Raw material	Temperature (ºC)	pH					
K. pastoris	Shake flask 200 mL	200	60 g/L D-Xylose	37	7.0 - 6.4	168	12.00	0.35	0.071	This study
K. pastoris	Bioreactor	300 – 2000	60 g/L D-Xylose	30	5.0±0.02	96	7.64	0.52	0.100	Araújo et al., 2017
Candida tropicalis HPX 2	Shake flask 100 mL	200	20 g/L D-Xylose	30	n.a.	24	40.00	>0.90	1.670	Gong, Chen and Tsao, 1981
C. guilliermondi FTI-20037	Shake flask 100 mL	200	104 g/L D-Xylose	30	n.a.	78	77.20	0.74	0.990	Barbosa et al., 1988
D. hansenii UFV-170	Shake flask 25 mL	200	10 g/L D-Xylose	30	n.a.	24	5.84	0.54	0.240	Sampaio et al., 2008

n.a. - data not available

As it can be seen on table 5, the result of xylitol concentration reported by Araújo et al.

(2017), using *K. pastoris*, was lower comparatively than this study. The lower value of xylitol concentration probably can be due to different cultivation conditions, namely temperature and pH, as well as using a different culture medium (medium BSM). In contrast, presents values of production yield and volumetric productivity higher because of the longer cultivation time.

In turn, the concentration of xylitol produced in this study was lower than that obtained in studies reported by Gong, Chen and Tsao (1981), and Barbosa et al. (1988), which may be due to type of microorganism (*Candida tropicalis* HPX 2 was genetically modified to produce highest concentrations of xylitol), initial concentration of xylose (*C. guilliermondi* FTI-20037 had 104 g/L of initial xylose concentration).

In this study, the concentration of xylitol was higher than that reported by Sampaio et al. (2008). This might be because different yeast was used. In turn, the values of xylitol yield and volumetric productivity were higher.

Such as pH, the temperature also has impact in xylose reductase activity and consequently in production of xylitol by yeast. Thus, each yeast presents an optimum value of temperature for XR maximum activity.

The yeasts produce xylitol in the range of 24 ºC - 45 ºC, but the suitable temperature in general is between 28 - 30ºC for xylitol production. *C. guillermondii* presents 28 ºC as the favourable temperature (Silva and Chandel, 2012), whilst the optimum temperature for XR from *P. stipitis* was 38◦C (Rafiqul and Sakinah, 2013).

Based on previous assays, the best values of pH were selected (between 4.6-4.8 and 6.6-6.7) and different temperatures were tested (37, 40 and 43 ºC) with aim to observe the impact on xylitol production.

3.2.2. Effect of the change of temperature at 72 hours of cultivation on xylitol production

In order to observe the influence of altering the temperature during *K. pastoris* cultivation on the production of xylitol, another set of shake flask experiments was performed in which the initial pH value was 7.0 and the initial temperature was 30 ºC, being altered at 72 hours to 37, 40 and 43 ºC. All other cultivation conditions were kept, namely, agitation at 200 rpm, during 168 hours, substrate concentration were 20 g/L and 60 g/L glucose and xylose, respectively. The conditions it can be seen in table 6.

Table 6. Xylitol concentration, specific productivity ($Y_{P/X}$), and volumetric productivity (r_p) as a function of temperature and pH altered at 72 hours of cultivation

Temperature (ºC)*	pH*	Xylitol (g/L)	$Y_{P/X}$ ($g_{xylitol}/g_{CDW}$)	r_p (g/L.h)
37	4.8	2.54	0.27	0.015
37	6.6	3.58	0.36	0.021
40	4.6	0.18	0.02	0.001
40	6.7	0.00	0.00	0.000
43	4.7	0.00	0.00	0.000
43	6.6	0.00	0.00	0.000

***** temperature and pH altered at 72 hours of cultivation

The results obtained at 37 ºC, show that the concentration of xylitol produced by the yeast was 2.54 g/L and 3.58 g/L, for pH 4.8 and 6.6, respectively. The change of temperature in these assays was revealed to result in a lower xylitol production relatively to previous assay, when the temperature was maintained at 37 ºC during all assay, concentrations between 8.77 - 12.00 g/L. Values of specific productivity and this result might be explained as a result of the temperature change, seeing that the yeast *K. pastoris* had to adapt the metabolism to new conditions.

By looking at table 6, it is clear that *K. pastoris* was not able to produce xylitol at temperatures of 40 ºC and 43 ºC. Based on these results, can be compared with reported by Cao et al. (1994), in which the conversion to xylitol from D-xylose by *Candida sp* B-22 decrease at temperatures higher than 40 ºC. This fact it was probably due to loss of the activities of NADPH and NADPH - dependent xylose reductase associated with the temperature increase. At higher temperatures, the enzyme is rapidly inactivated, however at lower temperatures, the reaction rates diminish according to Arrhenius equation (Rafiqul and Sakinah, 2013).

Thereupon, it is suggested for optimization of xylitol production, initial value of temperature 30 ºC with change at 72 hours to 37 ºC. It is an optimization strategy to be carried out in future assays.

3.2.3. Conclusion

The results achieved with this set of experiments seem to indicate that the best value of temperature for cell growth was 30 ºC, while for production of xylitol it was 37 ºC.

3.3. Oxygen effect

3.3.1. Oxygen limited effect in synthesis of xylitol

It has been reported in the literature that oxygen is necessary for cell growth and activation of the mitochondrial functions, depending on the type of organism, aerobic or facultative.

Dissolved oxygen concentration is the most important environmental parameter to be considered in the production of xylitol given that influence operational aspects of yeast physiology (Silva and Chandel, 2012), in the same way as concentration, yields and volumetric productivity of xylitol. Mohamad, Mustapa Kamal and Mokhtar (2014), reported that the oxygen is a key element according as accumulation of xylitol always occurs under oxygen-limited conditions. Thus, it is very important, determine to the oxygen flux that will enable utilization of D-xylose for xylitol production.

In order to observe the impact of oxygen concentration on production of xylitol by *K. pastoris*, a set of shake flasks experiments was performed in oxygen exhaustion conditions. Oxygen exhaustion was achieved by replacing the filter cap of the flasks with a cap with no filter. The assay was performed at 200 rpm, during 168 hours, substrate concentration were 20 g/L and 60 g/L glucose and xylose, respectively, initial pH value 7 and temperature 30 ºC. Also, the combined effect of oxygen, pH and temperature was assessed by altering the pH and temperature after 72 hours of cultivation, shown in table 7.

Table 7. CDW (g/L), xylitol concentration, yield ($Y_{P/S}$), Specific productivity ($Y_{P/X}$) and volumetric productivity (rp) as a function of temperature and pH, under oxygen limiting conditions.

Temperature (ºC)	pH	CDW (g/L)	Xylitol (g/L)	$Y_{P/S}$ ($g_{xylitol}/g_{xylose}$)	$Y_{P/X}$ ($g_{xylitol}/g_{CDW}$)	r_p (g/L.h)
30	4.7*	16.17	2.48	0.95	0.15	0.015
30	6.6	16.88	2.59	0.32	0.15	0.015
30	7.8*	17.13	2.49	0.41	0.15	0.015
37*	4.5*	17.63	5.81	0.33	0.33	0.035
37*	6.6	16.55	2.85	0.10	0.17	0.017
37*	7.7*	15.65	2.48	0.27	0.16	0.015

* Temperature and pH altered at 72 hours of cultivation

Glucose, xylose and xylitol concentration were measured during the assay's time. In all experiments, glucose was depleted completely, despite xylose was not completely depleted in all assays. However, it was observed that the concentration of xylitol increased, xylose concentration was decreased, which means that some of the available xylose was consumed for production of xylitol by the yeast.

Oxygen exhaustion test was performed by replacing the filter cap of the flasks with a cap with no filter, so this strategy it seems to be improving the cell growth. The values of CDW obtained in this assay are between 15.65 - 17.63 g/L, as can be seen in table 7.

Figure 12, show concentration of xylitol obtained by combined effect of oxygen exhaustion, pH and temperature, by altering the pH and temperature after 72 hours of cultivation.

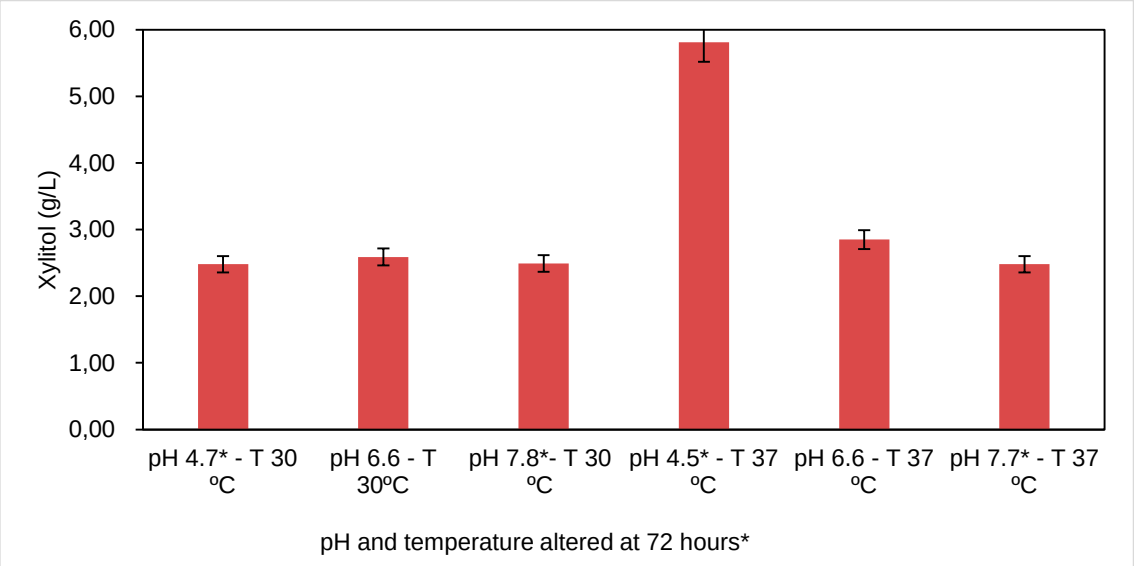

Figure 12. Concentration of xylitol at the end of 168 hours of cultivation of *K. pastoris* in the shake flask assays in oxygen exhaustion conditions with different pH and temperature altered at 72 hours of cultivation.

According to the figure 12, it can be seen that in cultivations at 30 ºC concentration of xylitol presented similar values 2.48 - 2.59 g/L.

By observation to the table 7, cultivation at 30 ºC with pH 4.7, 6.6 and 7.8 resulted in concentration of xylitol 2.48 g/L, 2.59 g/L and 2.49 g/L, respectively. In these experiments, values of specific productivity were obtained 0.15 $g_{xylitol}/g_{CDW}$ and volumetric productivity was attained 0.015 g/L.h. These results seem to demonstrate that the different values of pH tested had not significant impact on xylitol production. Nevertheless, cultivation at 37 ºC with pH 4.7 resulted in the highest value of productivity yield of this assay (0.95 $g_{xylitol}$ /g_{xylose}).

Curiously, cultivation at change temperature to 37 ºC with pH 4.5 resulted in the highest concentration of xylitol in this assay, resulted in 5.81 g/L. In addition, also was reached the highest value of specific productivity, resulting in 0.33 $g_{xylitol}$/ g_{CDW}, as well as the highest value of volumetric productivity 0.035 g/L.h, revealing a faster production of xylitol (table 7).

Regarding at the cultivation with temperature change to 37ºC with pH 6.6, were

obtained 2.85 g/L of xylitol, and specific productivity 0.17 $g_{xylitol}/g_{CDW}$. Relatively at cultivation with temperature change to 37ºC with pH 7.7, the concentration of xylitol achieved was 2.48 g/L and specific productivity was 0.16 $g_{xylitol}/g_{CDW}$.

From these results, it is possible to realize that different values of pH and temperature had not a significant impact on xylitol production, when performed on oxygen exhaustion conditions.

Comparing the results of xylitol concentration previously mentioned, with results obtained in section 3.2.1. (fully aerobic conditions) wherein was reached 11.3 g/L, 12 g/L and 8.77 of xylitol concentration, with pH 4.5, 6.4 and 7.8 at 37 ºC, respectively.

Therefore, it is possible to conclude that in oxygen exhaustion conditions, synthesis of xylitol by the yeast *K. pastoris* is not favoured, revealing a disadvantageous strategy for this process.

Walther, Hensirisak and Agblevor (2001), studied the influence of oxygen limitation and the initial xylose concentration using *C. tropicalis* under semi - aerobic conditions with an initial substrate concentration of 150 g/L, achieving 0.7 $g_{xylitol}/g_{xylose}$.

Literature reported that, in general, in strictly aerobic and anaerobic conditions xylitol is not produced or is produced in low rates. For instance, xylitol production by *Candida shehatae* was drastically affected when the culture was in under anaerobic conditions. It was observed that the xylitol production, yields and volumetric productivity, substantially improved with the change anaerobic conditions to aerobic conditions (Prakasham, Rao and Hobbs, 2009).

3.3.2. Conclusion

From this study, it was possible observe the impact of different oxygen conditions production of xylitol. Through this study was possible to observe that the limitation in oxygen for the yeast *K. pastoris* does not promote the synthesis of xylitol. In spite of literature reports that the accumulation of xylitol for other microorganisms always occurs under oxygen-limited conditions, however, for *K. pastoris* this is not a valid strategy.

3.4. Effect of substrate concentration

3.4.1. Effect of xylose concentration in cell growth and synthesis of xylitol

The initial concentration of xylose has a substantial impact on production of xylitol by yeasts. In yeasts that use xylose for cell growth, productivity yield are low, because xylose is mainly used for cell growth. Thus, increasing the concentration of xylose, higher percentage of substrate is used for production of xylitol, then increasing the production of xylitol and yields. In particular, this phenomenon was observed in several yeasts as *D. hansenii*, *C. parapsilosis* and *C. tropicalis* (Silva and Chandel, 2012).

In previous studies of this work, it was possible to observe that xylose was not completely depleted during the 168 hours of the assays.

In order to observe the impact of different concentrations of xylose in growth of *K. pastoris* cultivation and production of xylitol, were performed in shake flask screening at 200 rpm, during 168 hours, initial pH value 7, temperature value 30ºC with change to 37ºC at 72 hours, substrates concentration was 20 g/L of glucose and different values of xylose concentration were tested, as can be seen in table 8.

Table 8. CDW (g/L), xylitol concentration, Specific productivity ($Y_{P/X}$), yield ($Y_{P/S}$), and volumetric productivity (***rp***) as a function of concentration of xylose.

Concentration of xylose (g/L)	CDW (g/L)	Xylitol (g/L)	$Y_{P/X}$ ($g_{xylitol}/g_{CDW}$)	$Y_{P/S}$ ($g_{xylitol}/g_{xylose}$)	r_p (g/L.h)
40	9.65	1.85	0.19	0.08	0.011
30	9.34	1.82	0.19	0.07	0.011
20	9.67	1.27	0.13	0.06	0.008
10	9.44	0.54	0.06	0.05	0.003

The results demonstrated that glucose was consumed in first 72 hours in all assays (Figures 13, 14, 15 and 16). In all trials, after glucose depleted, culture reached the maximum growth. Xylose is consumed only after glucose is completely depleted, nevertheless no cell growth was observed, which seems to demonstrate that *K. pastoris* unable xylose to use, regardless concentration for growth.

As can be observed in table 8, it was not observed differences in CDW values, range between 9.34 g/L - 9.67 g/L. Thereby, different values of xylose concentrations had not impact on cell growth.

Araújo et al. (2017), reported in their study that *K. pastoris* in culture media glucose/xylose mixtures for production of chitin-glucan complex (CGC). After glucose completely depleted, xylose began consumed being, however no cell growth was observed, which indicates that *K. pastoris* was unable use xylose for growth.

Likewise, Agbogbo et al. (2006), reported in their studies, using *Pichia stipitis* that the highest cell growth rates were in culture media containing 100% glucose and 75% glucose / 25% xylose, while growing on a media containing 100% xylose had the lowest growth rate.

Bicho et al. (1988), reported this behaviour in their studies in the presence of glucose and xylose as substrate for yeasts. The preference of glucose relatively at xylose and other sugars may be due to catabolite repression, once the enzymes necessary for xylose reduction are not synthesized or synthesized at low rates. Xylose fermentation began after the glucose was consumed. *Pachysolen tannophilus* and *Pichia stipitis* are examples of yeasts in which this phenomenon is observed.

Concentrations of glucose, xylose and xylitol were quantified along the assay.

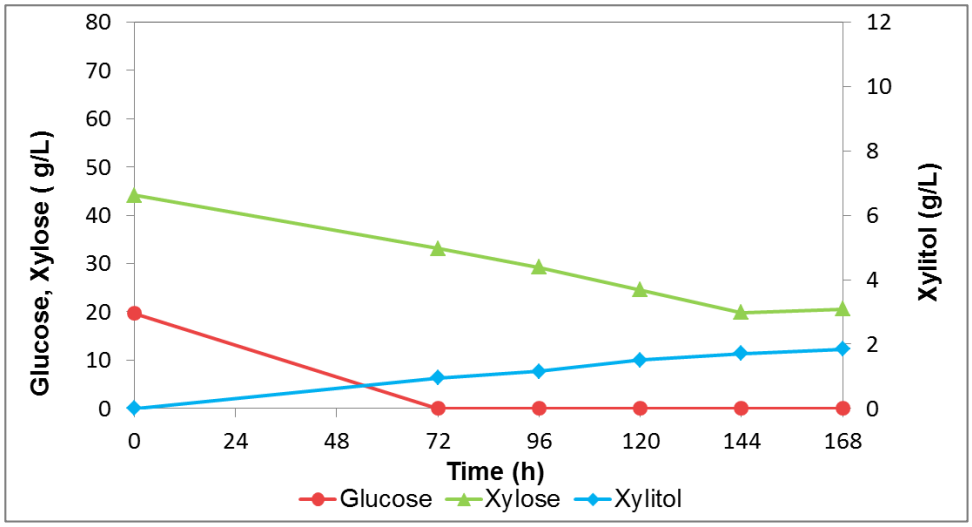

Figure 13. Cultivation profile of the shake flask experiment with initial xylose concentration of 40 g/L.
Glucose (–●– ,g/L), xylose (–▲– ,g/L) and xylitol (–♦–, g/L)

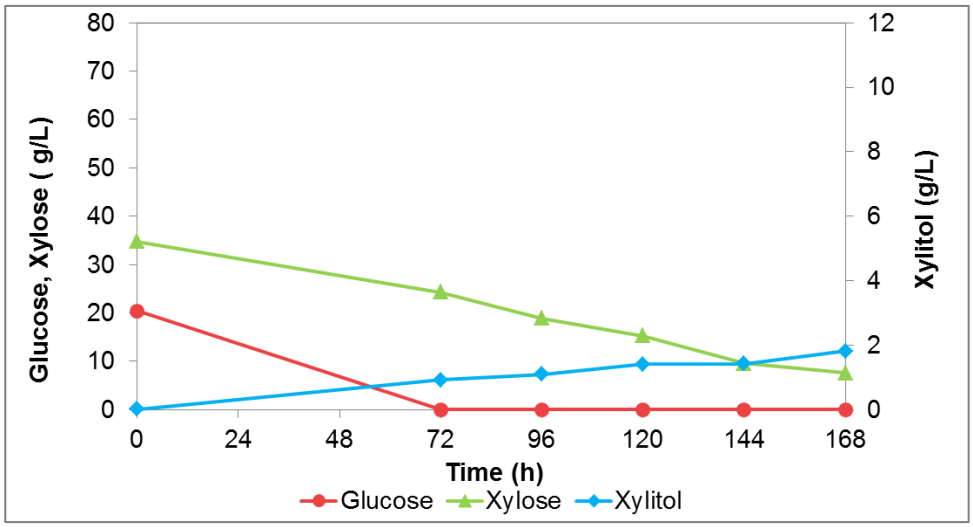

Figure 14. Cultivation profile of the shake flask experiment with initial xylose concentration of 30 g/L.
Glucose (–●– ,g/L), xylose (–▲– ,g/L) and xylitol (–♦–, g/L)

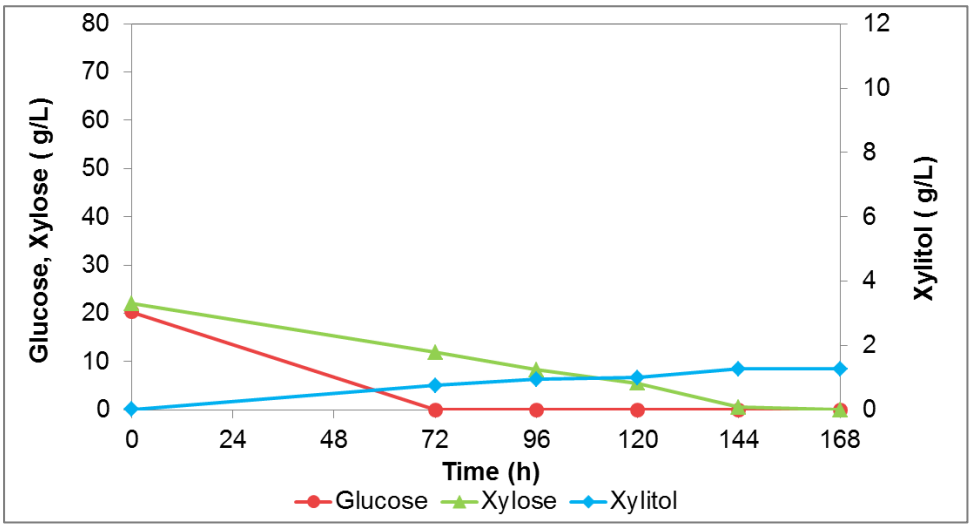

Figure 15. Cultivation profile of the shake flask experiment with initial xylose concentration of 20 g/L.
Glucose (–●– ,g/L), xylose (–▲– ,g/L) and xylitol (–♦–, g/L).

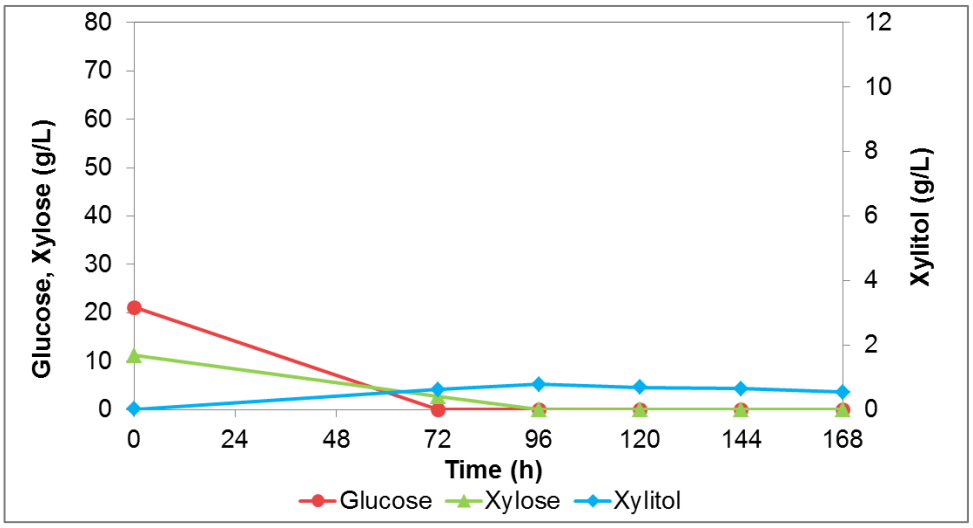

Figure 16. Cultivation profile of the shake flask experiment with initial xylose concentration 10 g/L.
Glucose (–●– ,g/L), xylose (–▲– ,g/L) and xylitol (–♦–, g/L)

In assays performed with initial xylose concentration (40 g/L) and (30 g/L), figure 13 and figure 14, xylose was not completely consumed. On the other hand, in assays performed with initial xylose concentration 20 g/L and 10 g/L, figure 15 and figure 16, this substrate was completely depleted at 144 and 96 hours of the assay, respectively.

It can be observed that as the concentration of xylitol increased, xylose concentration was decreased, which means that xylose was consumed for production of xylitol by the yeast.

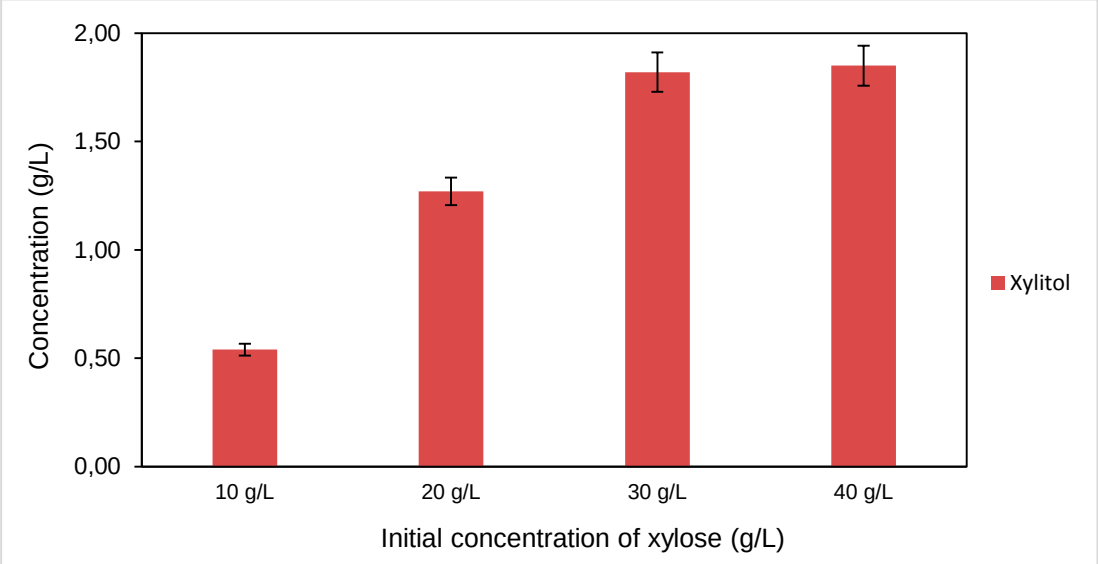

Figure 17. Concentration of xylitol at the end of 168 hours of cultivation of *K. pastoris* in the shake flask assays with initial concentration of xylose (g/L).

Through the observation of figure 17, it is possible to notice that the highest xylitol concentration (1.85 g/L) corresponds to the highest concentration of xylose provided in the medium (40 g/L). The lowest concentration of xylitol (0.54 g/L) was obtained for the assay performed with initial xylose concentration (10 g/L).

By looking to the table 8, it is possible to see that when the concentration of xylose supplied decreased, xylitol concentration also decreased, that way the specific productivity, production yield and volumetric productivity as well present lower values.

In terms of specific productivity, production yield, volumetric productivity the best assay corresponds at assay with initial concentration of 40 g/L xylose. So, relatively at concentrations of substrate should be used high concentrations of xylose in order to achieve high concentrations of xylitol.

Another point is that, comparing concentration of xylose 40 g/L with 60 g/L provided in all assays in this work, in same conditions, it is possible to conclude that the best concentration of xylitol was when applied 60 g/L, wherein obtained 12.0 g/L (section 3.2.1.) of this natural

sugar alcohol.

According with literature, the assays performed by Araújo et al. (2017), with *K. pastoris* in a bioreactor using glucose/xylose mixtures, shows that concentration of xylitol increased when percentage of xylose was increase. The percentages of glucose/xylose tested were 85% glucose/15 % xylose, 50% glucose/50% xylose and 25 % glucose/75 % xylose, corresponding at 1.40 g/L, 5.31 g/L and 7.64 g/L, respectively.

Ikeuchi et al. (1999), reported in their study, the effect of initial xylose concentration on xylitol production using the yeast *Candida sp*. 559-9. In this experiment were used initial concentrations of xylose in the range from 50 to 200 g/l. Providing 50, 100, 150 and 200 g/L of xylose were obtained 27.9±7.0, 71.2±2.0, 121.7±1.3 and 173.0±2.8 g/L of xylitol, respectively, and volumetric productivity were 0.39, 0.74, 1.01 and 1.44 g/L.h, respectively. So, was observed that the xylitol production and volumetric productivity increased with increasing xylose concentration. This may be due to the stress from the high sugar concentration (Ikeuchi et al., 1999).

3.4.2. Conclusions

Relatively at this study, it was possible observe that different xylose concentration used as substrate in culture media did not have any impact in cell growth, given that *K. pastoris* was unable to use xylose for growth. Regarding at impact of xylose concentration on xylitol production there seems to be a direct proportionality, in that xylose concentration provided increased, xylitol concentration increased similarly.

3.5. Assays with other substrates: Arabinose

As previously mentioned in section 1.3, hemicellulosic hydrolysate is composed by pentoses such as xylose and arabinose, and hexoses as glucose, galactose, and mannose. By observation of table 1, in section 1.3 it can be seen examples of lignocellulose materials that contain in its constitution glucose, xylose and arabinose.

In order to observe if *K. pastoris* was able to convert arabinose into arabitol, a shake flask screening was performed at 200 rpm, during 168 hours, initial pH value of 7.0±0.1, temperature value 30 ºC, substrates concentration was 20 g/L of glucose and different values of arabinose and xylose concentration, as can be observed in table 9.

Table 9. Concentration of arabinose (g/L), concentration of xylose (g/L), arabitol concentration, volumetric productivity of arabitol (***rp***), Xylitol concentration (g/L), volumetric productivity of xylitol (***rp***).

Concentration of arabinose (g/L)	Concentration of xylose (g/L)	Arabitol (g/L)	$r_{p\ \text{arabitol}}$ (g/L.h)	Xylitol (g/L)	$r_{p\ \text{xylitol}}$ (g/L.h)
60	-	0.86	0.005	0.05	0.000
30	30	1.36	0.008	0.87	0.005

Through the observation of the table 9, it is possible to see that the *K. pastoris* was able to production arabitol by synthesis of arabinose. Interestingly, there are no available reports on the ability of *K. pastoris* to use arabinose for arabitol production.

By looking to the table 9, it is possible to observe that the highest value of arabitol concentration (1.36 g/L) corresponds at cultivation with 30 g/L of arabinose and 30 g/L of xylose. A volumetric productivity of 0.008 g/L.h was obtained. Also, was noticed production of xylitol, concentration of 0.87 g/L, with volumetric productivity of 0.005 g/L.h.

Curiously, *K. pastoris* in shake flask cultivation with 30 g/L of arabinose and 30 g/L of xylose produces a higher concentration of arabitol relatively at xylitol 1.36 g/L and 0.87 g/L, respectively. This fact opens up the possibility that the yeast have a preference for arabinose over xylose.

On the other hand, on the shake flask with 60 g/L of arabinose, was observed a lower concentration of arabitol (0.86 g/L), and volumetric productivity 0.005 g/L.h. A low concentration of xylitol 0.05 g/L was also noticed.

Arabitol and xylitol concentration were quantified during the assay's time.

The profiles of cultivation, are demonstrated in figures 18 and 19.

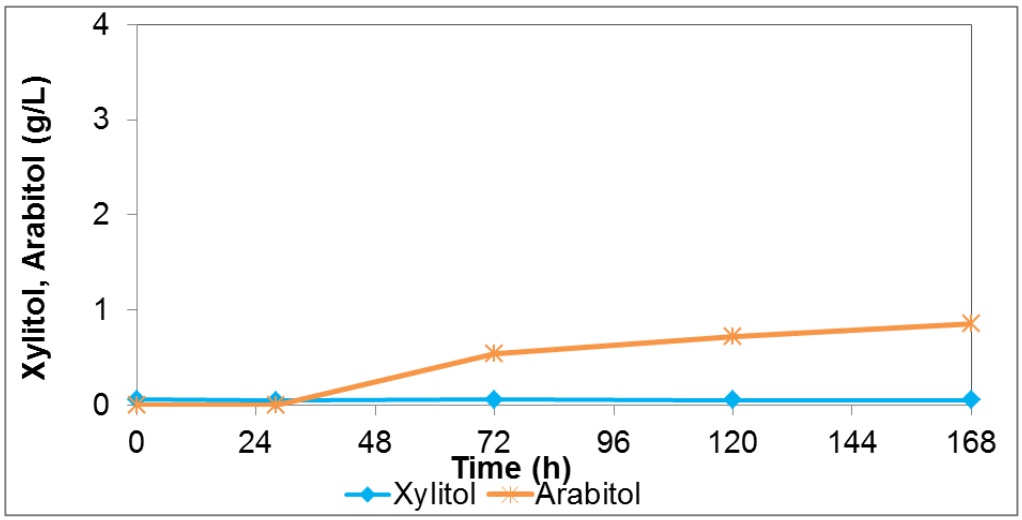

Figure 18. Cultivation profile of the shake flask experiment with initial arabinose concentration of 60 g/L.
Arabitol (-✳-, g/L) and xylitol (-♦-, g/L)

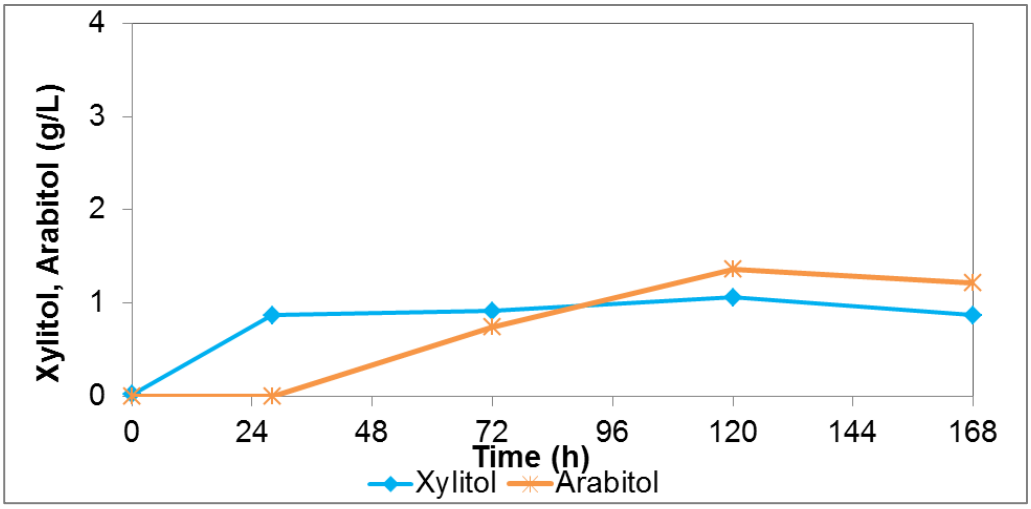

Figure 19. Cultivation profile of the shake flask experiment with initial arabinose concentration of 30 g/L and xylose concentration of 30 g/L.
Arabitol (-✳-, g/L) and xylitol (-♦-, g/L)

Figure 18, shows the cultivation profile of *K. pastoris* with 60 g/L of arabinose. It is possible to observe that the production of arabitol began from the 24 hours into cultivation.

Figure 19, shows the cultivation profile of *K. pastoris* with 30 g/L of arabinose and 30 g/L of xylose. It is possible to see that the production of arabitol began from 24 hours of the assay, nonetheless the production of xylitol was noticed in the first hours of the assay. Additionally, it can be seen that the production of arabitol increases relatively at xylitol production from 72 hours of the assays.

3.5.1. Combined effect of oxygen and temperature on arabitol synthesis

To observe the impact of oxygen concentration on production of arabitol by *K. pastoris*, a set of shake flasks experiments were performed in oxygen exhaustion and full aerobic conditions. Oxygen exhaustion was achieved by replacing the filter cap of the flasks with a cap with no filter. The assay was performed at 200 rpm, during 168 hours, substrate concentration were 20 g/L and 60 g/L glucose and arabinose, respectively. Initial pH value was 7.0±0.2 and initial temperature 30 ºC. Also, the combined effect of oxygen and temperature was assessed by altering temperature after 72 hours of cultivation to 37 ºC, shown in table 10.

Table 10. Concentration of arabinose (g/L), concentration of xylose (g/L), arabitol concentration, volumetric productivity of arabitol (***rp***), Xylitol concentration (g/L), volumetric productivity of xylitol (***rp***) under full aerobic conditions and oxygen exhaustion.

Oxygen conditions	Arabitol (g/L)	$r_{p\ \text{arabitol}}$ (g/L.h)	Xylitol (g/L)	$r_{p\ \text{xylitol}}$ (g/L.h)
Full aerobic	1.32	0.008	0.00	0.000
Oxygen exhaustion	3.15	0.019	0.60	0.000

Cultivation in full aerobic conditions and 30 ºC with change at 72 hours to exhaustion conditions and 37 ºC, as show table 10, resulted in the highest value of arabitol concentration produced by *K. pastoris* (3.15 g/L), and volumetric productivity corresponds at 0.019 g/L.h. Curiously, was observed a low concentration of xylitol 0.60 g/L.

In full aerobic conditions, value of arabitol concentration was 1.32 g/L and volumetric productivity was 0.008 g/L.h. In this assay the production of xylitol was not observed.

As previously referred in section 3.3.1, in oxygen exhaustion conditions, synthesis of xylitol by the yeast *K. pastoris* is not favoured, revealing a disadvantageous strategy for this process. Interestingly, oxygen exhaustion conditions it seems to promote the production of arabitol, resulting in a suitable strategy to improve production by the yeast.

Another point is that the cultivation at 30 ºC with change to 37 ºC at 72 hours of the assay, it is possible to observe that production of arabitol was higher relatively at first assay, wherein temperature was maintained at 30 ºC (as show in table 9). This strategy it seem suitable to improve the production of arabitol.

Arabitol and xylitol concentration were quantified during the assay's time. The profiles of cultivation, it can be seen in figures 20 and 21.

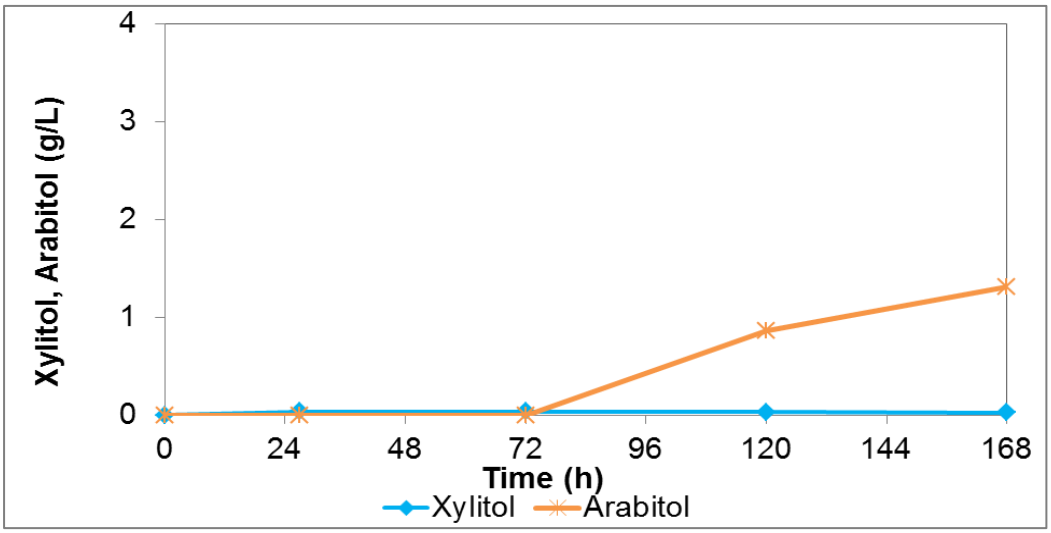

Figure 20. .Cultivation profile of the shake flask experiment at 30 ºC in full aerobic conditions and altering the temperature to 37 ºC at 72 h of cultivation. Arabitol (-✳-, g/L) and xylitol (–♦–, g/L)

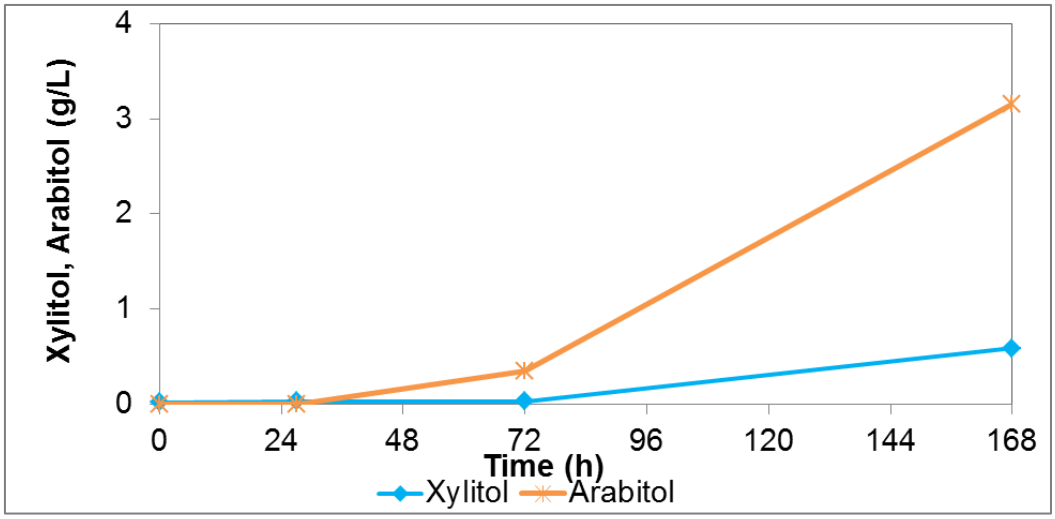

Figure 21. Cultivation profile of the shake flask experiment at 30 ºC in full aerobic conditions and altering the temperature to 37 ºC in oxygen exhaustion at 72 h of cultivation. Arabitol (-✳-, g/L) and xylitol (–♦–, g/L)

By observation of the figure 20, cultivation in full aerobic conditions, it is possible to see that the production of arabitol began from the 72 hours of the assay.

Figure 21, shows the cultivation of *K. pastoris* in full aerobic conditions with change at 72 hours to oxygen exhaustion. As can be seen in figure 21, the production of arabitol began about from 24 hours of the assay. In addition, it is easy to realize that when oxygen conditions were changed (full aerobic conditions to oxygen exhaustion), production of arabitol was drastically increased. This fact, seem to show that this strategy reveals suitable for increased production of arabitol.

Table 11. Fermentation conditions and values of arabitol concentration (g/L).

Microorganism	Fermentation conditions					Arabitol (g/L)	Reference
	Cultivation mode	Agitation (rpm)	Raw material	Temperature (ºC)	pH		
K. pastoris	Batch fermentation in shake flask	200	20 g/L D-Glucose and 60 g/L L-arabinose	37*	7.0±0.2	3.15	This study
Candida entomeae	Batch fermentation in shake flask	200	50 g/L L-arabinose	34	5.0	34.7	Saha and Bothast (1996)
Kodamae ohmeri NH-9	Batch fermentation in shake flask	220	200 g/L Glucose	37	7.0	81.2	Zhu et al., 2009
Debaryomyces nepalensis NCYC 3413	Batch fermentation in shake flasks	180	100 g/L Arabinose	30	6.0	24.6	Kumdam, Murthy and Gummadi (2012)

* temperature altered at 72 hours

As it can be seen on table 11, the result of arabitol concentration in this study, using *K. pastoris*, was lower (3.15 g/L) comparatively than studies performed by Saha and Bothast (1996), (34.7 g/L), Zhu et al. (2010), (81.2 g/L) and Kumdam et al. (2012), (24.6 g/L). This might be because in this work was used a different yeast with a lower concentration of substrate.

Moreover, studies by Saha and Bothast (1996), Zhu et al. (2009), and Kumdam, Murthy and Gummadi, (2012) refer at optimization studies wherein were tested several conditions and obtained the highest value of arabitol concentration, whereas this study is a preliminary study and still few conditions were tested.

3.5.2. Conclusions

Interestingly, it was observed that *K. pastoris* was able to production arabitol by synthesis of arabinose. In literature, was not reported that *K. pastoris* is capable to produce arabitol. Cultivation at 30 ºC in full aerobic conditions, with change at 72 hours to 37 ºC and oxygen exhaustion resulted in the highest concentration of arabitol produced by the yeast (3.15 g/L). Wherefore, these conditions may apply in future works. However, it is still necessary to develop many studies to improve production of arabitol by *K. pastoris*.

Chapter 4. Conclusions and Future work

4.1. Conclusions

In this thesis, the impact of several parameters namely, pH, temperature, glucose/xylose concentration and oxygen conditions, on production of xylitol by the yeast *K. pastoris* was investigated. Several assays were performed in batch shake flasks.

This work demonstrated that the best pH value for both cell growth and xylitol synthesis was 7.0 - 7.5 that resulted in the production of 4.04 g/L xylitol.

In the second part of this study, different temperatures were tested: 23, 30 and 37 ºC. The highest CDW was attained at 30 ºC (10.29 g/L), although the highest xylitol concentration of this study (12.00 g/L) was obtained at pH 6.4 and 37 ºC.

The oxygen effect on production of xylitol was also evaluated. The culture was maintained under limiting oxygen conditions at 37 ºC, after 72 hours of the assay. Results obtained in this part of this study revealed that the limitation in oxygen for the yeast *K. pastoris* does not promote the synthesis of xylitol.

Lastly, several concentrations of xylose in the glucose/xylose mixtures were tested. The results obtained show that xylose concentration provided increased xylitol production. Thereupon, in future assays to improve the xylitol production by yeast, higher concentrations of xylose should be provided.

Among all conditions tested in this study, the highest concentration of xylitol (12.0 g/L) was obtained by cultivation with initial pH value 7.00 and 37 ºC, 20 g/L of glucose and 60 g/L of xylose in fully aerobic conditions, at pH 6.4. It can be concluded that it was possible to improve the synthesis of xylitol by the yeast along this work. In this study were achieved better results (12.00 g/L), in shake flask, comparing with Araújo et al. (2017), that reported 7.64 g/L of xylitol in batch bioreactor.

Curiously, it was observed that *K. pastoris* was able to synthesize arabitol using arabinose as substrate. Moreover, the highest value of arabitol concentration was 3.15 g/L obtained by cultivation at 30 ºC in full aerobic conditions, with change at 72 hours to 37 ºC and oxygen exhaustion.

4.2. Future work

In this thesis, based on the results it remains clear that the process of xylitol production needs to be improved. So, the following suggestions can be implemented.

4.2.1. Optimization of arabitol production

As can be seen in this thesis, *K. pastoris* was able to produce arabitol by fermentation of arabinose, however preliminary tests were still performed. So, it is necessary to perform several tests, evaluate several parameters as pH, temperature, oxygen conditions, concentration of substrates, in order to improve concentration of arabitol. In subsequent work, might be apply the suitable conditions in bioreactor.

4.2.2. Scale up in Bioreactor of xylitol production

As described above, *K. pastoris* reached the maximum concentration of xylitol, on cultivation with initial pH value 7.00 and 37 ºC, 20 g/L of glucose and 60 g/L of xylose in fully aerobic conditions at pH 6.4. So, it is suggested that these conditions be applied in batch bioreactor, in order to maximize concentration of xylitol, once on bioreactor a more controlled environment can be achieved. In future works, also a fed - batch or a continuous mode could be applied. In this way, xylitol production could be improved.

4.2.3. Test with lignocellulosic - raw materials

In this study, it was possible demonstrate that *K. pastoris* grown in glucose and produced xylitol and arabitol by fermentation of xylose and arabinose, respectively, in spite this substrates to be synthetic.

Lignocellulosic wastes are cheap, renewable, abundant and easily available materials. So, in order to value lignocellulosic materials make the process economical and profitable, could be used as substrates for growth, production of xylitol and arabitol by *K. pastoris*. Examples of lignocellulosic materials could be used, specifically oak, rice straw, corn cobs, corn stover, brewery spent grain, due to constitution of glucose, xylose and arabinose (as mentioned in section 1.3, table 1). Another topic that could be interesting to test was the waste oil palm empty fruit bunch due to its composition in glucose and xylose. The utilization of these materials could increase the growth of *K. pastoris* and xylitol and arabitol production.

4.2.4. Downstream processes for xylitol recovery

After increasing xylitol production rate by optimization strategies, the downstream of xylitol should be tested in order to recover the final product. As literature reported, xylitol can be recovered using different approaches as chromatographic methods, membrane separation and crystallization (Silva and Chandel, 2012).

Crystallization has been seen as energetically advantageous and as an environmental friendly method as the final step to obtain highly purified xylitol.

4.2.5. Characterization of xylitol

Lastly, after extraction of xylitol, it would be necessary to proceed to the characterization of xylitol. So, there are many analytical methods available for the qualitative and quantitative measurement of D-xylitol.

The methods that could be used for the characterization of xylitol are, for example based in chromatography techniques, gas chromatography (GC), HPLC, and capillary electrophoresis (CE) methods with different detection options such as UV, refractive index (RI) and evaporative light scattering (ELS). In addition, also could be used biotechnology based methods as indirect competitive enzyme-linked immunosorbant assay (IC-ELISA) and enzymatic assays (Silva and Chandel, 2012).

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Chapter 6. Appendix

6.1. Appendix 1

Calibration curves

Calibration curve for determination of glucose, xylose, xylitol and arabitol concentration in shake flask experiments.

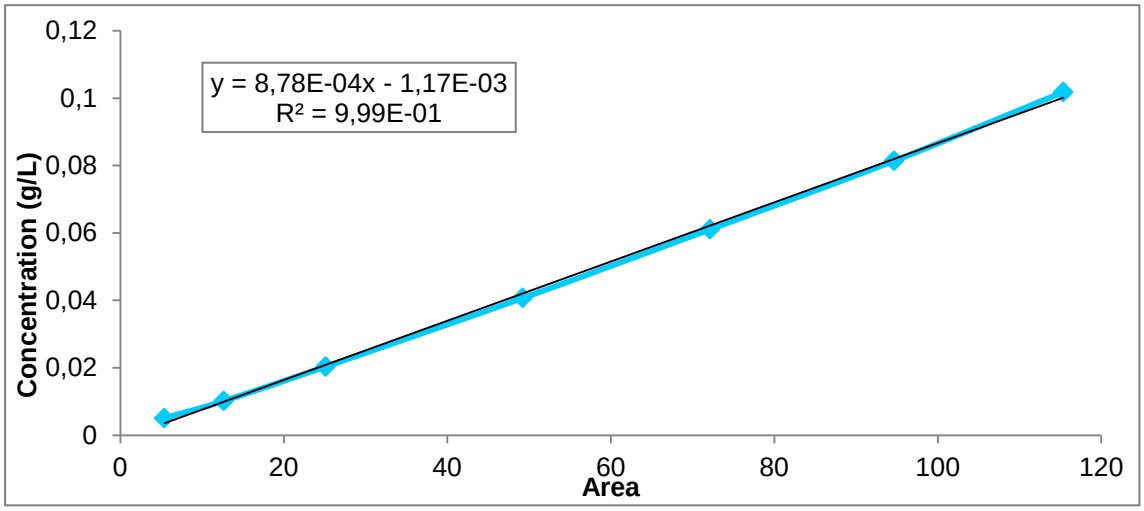

Figure 22. Calibration curve obtained for glucose, analysis was performed by HPLC.

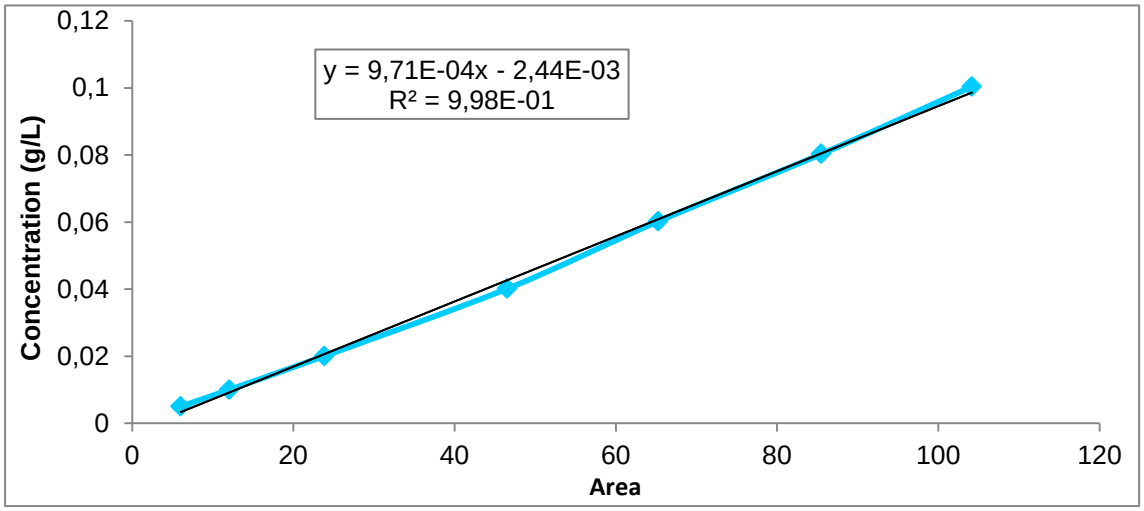

Figure 23. Calibration curve obtained for xylose, analysis was performed by HPLC

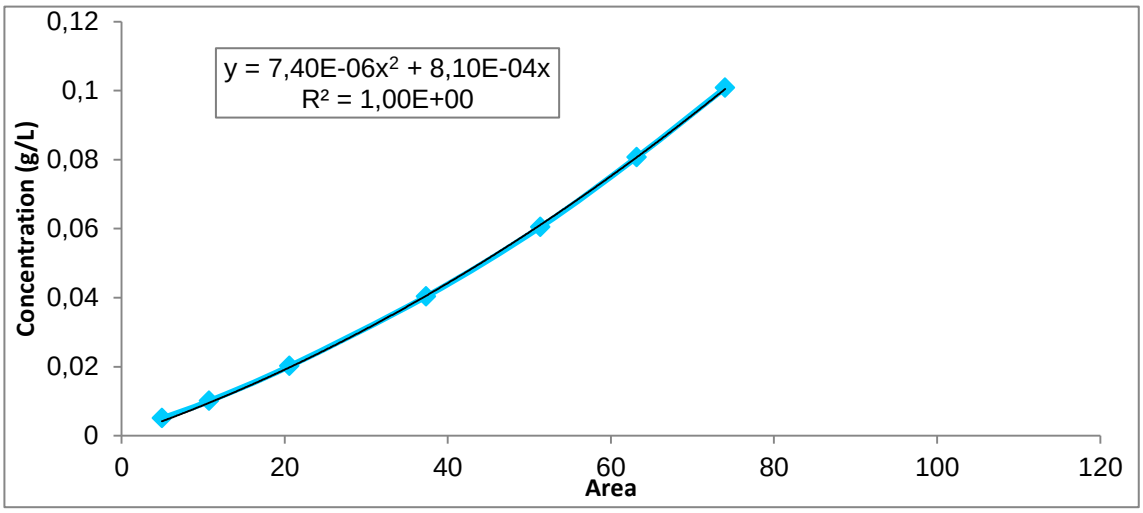

Figure 24. Calibration curve obtained for xylitol, analysis was performed by HPLC.

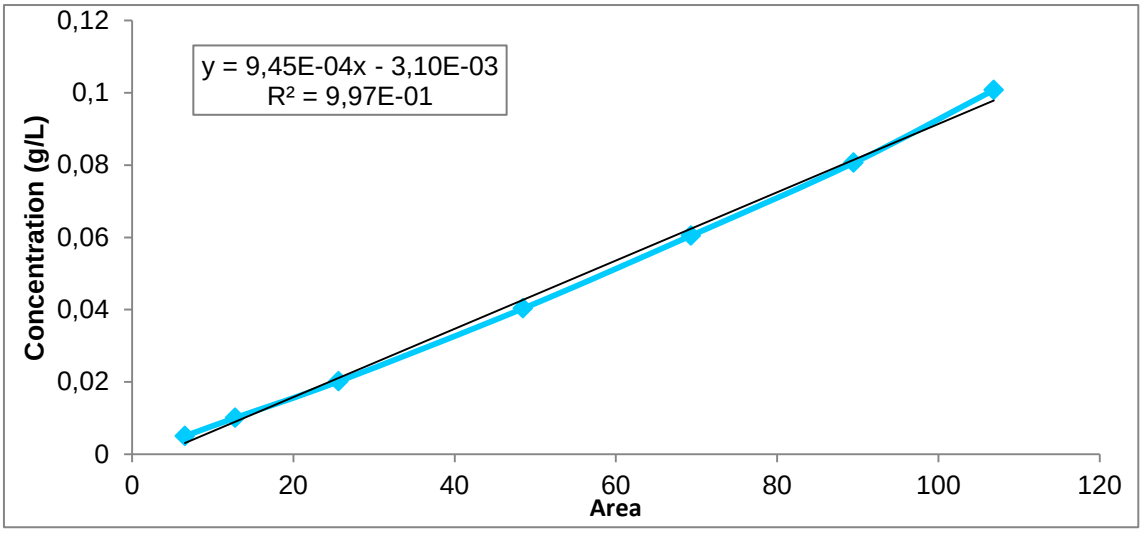

Figure 25. Calibration curve obtained for arabitol, analysis was performed by HPLC.