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SweetBiotech - Biotechnological processes for xylitol production from corn cob autohydrolysates

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RESUMO

A transformação da dieta humana nos últimos dez mil anos tem levado ao aumento da ingestão média global de energia, traduzindo-se num incremento da obesidade e diabetes por todo o mundo. Um dos maiores problemas da dieta atual é a quantidade de açúcares que possui. Esforços têm sido feitos para reverter a situação, como o uso de substitutos do açúcar em produtos alimentares. Estes adoçantes podem ser produzidos artificialmente por processos biotecnológicos ou naturalmente presentes nas plantas. Os polióis são um exemplo do segundo e o xilitol representa uma alternativa à sacarose devido a ter 40 % menos calorias que esta e ao seu metabolismo não induzir a libertação de insulina.

Adicionalmente, a produção biotecnológica de xilitol tem sido atingida com o uso de resíduos agro-industriais, como materiais linhocelulósicos. A sua composição rica em celulose e hemicelulose oferece a possibilidade de utilizar xilose como um percursor do xilitol.

Neste estudo, a produção de xilitol a partir de caroço de milho pré-tratrado hidrotermicamente foi avaliada e investigada por sacarificação e fermentação simultâneas (SSF) usando uma estirpe recombinante de *Saccharomyces cerevisiae*. Em termos de condições do pré-tratamento, 25 % (p/p) de caroço de milho durante a auto-hidrólise apresentou a maior recuperação de xilano nas fases líquidas e sólidas. Nesta condição o xilano foi solubilizado no licor da auto-hidrólise como xilose e xilo-oligossacáridos. Ainda, a hidrólise enzimática das duas frações atingiu uma concentração final de xilose de 53.8 g/L, mostrando uma eficiente conversão dos xilo-oligossacáridos em xilose.

Um processo de SSF foi realizado para avaliar e otimizar várias condições operacionais, como a temperatura, a pré-sacarificação, a concentração do inóculo, de enzima e de sólidos, para a produção de xilitol. Os resultados revelaram que a concentração de sólido ótima foi 6.76 % (p/p) e de enzima foi 24 FPU/g de substrato, com uma produção potencial de 46.5 g/L de xilitol e 0.32 g/L/h de produtividade. Estas condições foram realizadas para validar o modelo proposto e este ensaio produziu 42.9 g/L de xilitol e 0.30 g/L/h de produtividade, um erro de 7.66 % e 6.66 % dos valores teóricos, o que valida o modelo. Finalmente, a destoxificação completa do hidrolisado foi feita para melhorar a produtividade de xilitol sob condições previamente otimizadas. Um processo de SSF usando licor destoxificado e com 22 g/L de massa fresca de células resultou em 71.7 g/L de xilitol, 0.60 g/L/h de produtividade e com

praticamente conversão completa da xilose. De forma geral, neste estudo, a conversão biotecnológica de xilose em xilitol foi atingida com sucesso.

Palavras-Chave: *SACCHAROMYCES CEREVISIAE*, XILITOL, AUTO-HIDRÓLISE, CAROÇO DE MILHO

ABSTRACT

The transformation of the human diet in the last ten thousand years has led to an increase of the global average energy intake, translating into an upsurge of obesity and diabetes all over the world. One of the major problems with the current diet is the amount of sugars that it has. Efforts have been made to reverse the situation, such as the use of sugar substitutes in food products. These sweeteners can be artificially produced by biotechnology processes or naturally present in plants. Polyols are an example of the latter and xylitol represents a great alternative to sucrose due to having 40 % less calories than sucrose and its metabolism not inducing the release of insulin into the bloodstream.

Additionally, the biotechnological production of xylitol may be accomplished with the use renewable lignocellulosic materials, such as agro-industrial residues. Their composition rich in cellulose and hemicellulose offers the possibility to utilize xylose as a precursor of xylitol.

In this study, the xylitol production from hydrothermally pretreated corn cob was evaluated and investigated by simultaneous saccharification and fermentation (SSF) process using a recombinant *Saccharomyces cerevisiae* strain. In terms of pretreatment conditions, 25 % (w/w) of corn cob of solid loading in the autohydrolysis treatment presented the highest xylan recovery in liquid and solid phases. In this condition, the xylan was solubilized in the autohydrolysis liquor as xylose and xylooligosaccharides. Moreover, the enzymatic hydrolysis of whole slurry corn cob achieved a xylose final concentration of 53.8 g/L, showing an efficient conversion of xylooligosaccharides into xylose.

SSF process was performed to evaluate and optimize several operational conditions, such as temperature, pre-saccharification, size of inoculum, enzyme loading and percentage of solids, for the production of xylitol. The results revealed that the optimal solid concentration was 6.76 % (w/w) and the enzyme loading was 24 FPU/g of substrate, with a potential production of 46.5 g/L of xylitol and 0.32 g/L/h of productivity. These optimized conditions were carried out to validate the proposed model and this assay produced 42.9 g/L of xylitol and 0.30 g/L/h of productivity, an error of 7.66 % and 6.66% of the theoretical values, which validated the model. Finally, complete detoxification of hydrolysate was carried out to improve xylitol productivity under previous optimized conditions. SSF process using detoxified autohydrolysis liquor and 22 g/L of wet cells resulted in 71.7 g/L of xylitol, 0.60 g/L/h of

productivity and practically full conversion of xylose. Overall, in this study, the biotechnological conversion of xylose into xylitol was successful achieved.
KEYWORDS: *SACCHAROMYCES CEREVISIAE*, XYLITOL, AUTOHYDROLYSIS, CORN COB

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LIST OF ABBREVIATIONS

AFEX – Ammonia fibre explosion

BSG – Brewer's Spent Grain

$\mathbf{C_{GH}}$ - glucan concentration in hydrolysate

$\mathbf{C_{LH}}$ - lignin concentration in hydrolysate

$\mathbf{C_{XH}}$ - xylan concentration in hydrolysate

$\mathbf{C_{GRM}}$ - glucan concentration in raw material

$\mathbf{C_{LRM}}$ - lignin concentration in raw material

$\mathbf{C_X}$ – xylitol concentration at t

$\mathbf{C_{XRM}}$ - xylan concentration in raw material

EGW – *Eucalyptus globulus* wood

FGS - final glucose concentration with Solid

FGWS - final glucose concentration with Whole Slurry

FPU - Filter Paper Unit

FXS - final xylose concentration with solid

FXWS - final xylose concentration with whole slurry

HMF - Hydroxymethylfurfural

HPLC - High Performance Liquid Chromatography

$\mathbf{H_{RM}}$ - humidity of raw material

$\mathbf{H_{SF}}$ - humidity of solid fraction

LCM - Lignocellulosic materials

LSR – Liquid to Solid Ratio

$\mathbf{M_{HRM}}$ - mass of humid raw material

$\mathbf{M_{HSF}}$ - mass of humid solid fraction

$\mathbf{M_{PX}}$ – mass of potential xylitol

$\mathbf{M_X}$ – mass of xylitol produced

PSSF – Pre-Saccharification and Simultaneous Fermentation

$\mathbf{P_X}$ – xylitol productivity

$\mathbf{R_G}$ - glucan recovery

$\mathbf{R_L}$ - lignin recovery

$\mathbf{R_X}$ - xylan recovery

SSF – Simultaneous Saccharification and Fermentation

SY - Solid Yield

t – incubation time when xylitol concentration stabilized

USFDA – United States Food and Drug Administration

$\mathbf{X_1}$ - solid concentration factor

$\mathbf{X_2}$ - enzyme concentration factor

$\mathbf{Y_{GS}}$ - glucose yield with solid

$\mathbf{Y_{GWS}}$ - glucose yield with whole slurry

$\mathbf{Y_{GS}}$ - xylose yield with solid

$\mathbf{Y_{GWS}}$ - xylose yield with whole slurry

$\mathbf{Y_X}$ – xylitol yield

YPD - Yeast extract, Peptone and Dextrose medium

1. INTRODUCTION

1. INTRODUCTION

1.1. Contextualization

Over millennia the human species has had a very different diet from the current standard Western diet. Although what humans ate about 50 thousand years ago was dependent of their geographic position, the main form of food consisted of naturally present vegetation, wild animals or aquatic resources (Cordain *et al.*, 2005; Eaton, 2006). About 10 thousand years ago this started to change as agriculture and animal domestication were introduced and the industrial revolution, which occurred around 200 years ago, led to novel foods entering the human diet. These foods included cereals, dairy products and fatty meats from the agricultural and animal domestication period and refined sugars, refined vegetable oils, alcohol, salt and combinations of these (processed foods) and this altered the nutritional composition of a person's diet and slowly displaced the formerly wild plant and wild animal foods in hunter-gatherer societies. This amount of time is not sufficient for the human population to totally adapt to these different sources of nutrients (certainly not 200 years), that now represent most of the calories ingested and affect several health and diet indicators, such as the macronutrient composition, micronutrient density, acid-base balance, glycemic load, fatty acid composition and fibre content (Cordain *et al.*, 2005; Jew *et al.*, 2009).

The transformation of the human diet and the increased consumption of cereals, refined vegetable oils and sugar, have led to an increase of the global average energy intake, which translates to an upsurge of obese and overweight people around the world (Swinburn *et al.*, 2004). In fact, currently chronic diseases cause more deaths than every other cause combined and 4 of them (cardiovascular diseases, cancer, chronic respiratory diseases and diabetes) are responsible for 82% of chronic disease deaths (World Health Organization, 2014a). Most of these are dietary diseases, which means that they originated from diet related issues.

With the global surge of obesity over the last decades, efforts have been made to reverse the tables. Nowadays, people seem to be more interested in the characteristics of the food they eat, being because of the health problems said people may already possess or the direct effects that it may generate, like weight gain, cardiovascular diseases or *diabetes mellitus* (Swinburn *et al.*, 2004). One of the big problems of the current standard diet is the amount of sugars that

it has. These compounds, according to the World Health Organization (WHO), represent "intrinsic sugars, which are those incorporated within the structure of intact fruit and vegetables; sugars from milk (lactose and galactose); and free sugars, which are monosaccharides and disaccharides added to foods and beverages by the manufacturer, cook or consumer, and sugars naturally present in honey, syrups, fruit juices and fruit juice concentrates" (World Health Organization, 2014b).

It is known that the human consumption of added sugar has been consistently increasing over the last decades and a provable reason for that seems to be the fast drop in its cost, putting it directly in competition with cereals to be the cheapest ingredient in the market (Mooradian *et al.*, 2017; Swinburn *et al.*, 2004). With this decrease in the price of sugar, the proportion of food that is derived from grains has also decreased and the world average energy consumption has highly increased (Swinburn *et al.*, 2004). Therefore, and according to the fist law of thermodynamics that states that energy cannot be created nor destroyed, if we consume food with more energy than the energy that we require, that energy has to be stored, in this case in the form of triacylglycerols, also known as fatty acids or fat, in adipose tissue. Consequently, if we consistently ingest more energy than we spend, the trend is the accumulation of this compound in our body and thus the increase of our overall body weight (Frayn, 2010). To decrease this amount of energy, several approaches can be taken, one being the search for compounds that offer the taste of sweetness without the energy intake (Sadler and Stowell, 2012).

1.2 Sugar substitutes

Low caloric and/or high intensity sweeteners may represent an interesting part of our diet, providing the sweet taste to food without the addition of extra calories and the increase of the glycemic index (Grembecka, 2015). Therefore, using these substitutes to sugar may lead to an energy reduction in sweet foods and beverages, and most likely in an overall lower energy intake (Sadler and Stowell, 2012). With this knowledge, many sugar alternatives, or sweeteners, have been discovered and produced over the years, possessing different characteristics.

A type of sweeteners represents substances that are non-nutritive sweeteners (NNS), used in small amounts to replace large quantities of sugar and thus consumed as a means to reduce body weight and improve glucose management (Chattopadhyay *et al.*, 2011; Lê *et al.*,

2016). These molecules are used primarily in liquid and semi-liquid products and can be from an artificial or natural source. However, taste, stability, and economic reasons limit their application and, because of that, only a small number of NNS are utilized by the food industry, for instance, aspartame, acesulfame-K, sucralose, cyclamate, neotame, alitame, saccharin, which are artificial, and steviol glycosides and mogrosides, which are natural (Lê *et al.*, 2016; Chattopadhyay *et al.*, 2011). These compounds produce a sweet taste that ranges from 200 to 13000 times the sweetness that sucrose provides (Lê *et al.*, 2016).

1.2.1. Artificial sweeteners

Nutritive sweeteners from carbohydrate sources have been utilized for many years as alternatives to sugar (Poldermans and Matt, 1991; Smith, 1991; Well, 1993). Non-nutritive sweeteners are also able to be crucial as food additives, particularly to those people who cannot consume nutritive sweeteners. However, despite non-nutritive sweeteners being much sweeter than nutritive sweeteners and moderately cheaper, the artificial ones, which are produced chemically, must pass the safety assessments and should be consumed only within the allowed amount (Higginbotham, 1979;; Gibbs *et al.*, 1996).

Aspartame is technically a nutritive sweetener, containing the same number of calories that sucrose does (4 per gram). However, because it is much sweeter than the traditional sugar (160 – 220 times) and it is used in minor quantities, the calories that it provides are not significant, being thus placed in the non-nutritive category (Vaclavik and Christian, 2014). It was discovered by accident by GD Searle and James Schlatter in 1965 and then tested thoroughly until it was released in the market in 1981 under the brand NutraSweet (Butchko *et al.*, 2001; O'Donnel, 2012). This sweetener is an artificial one because it can't be found in nature, being created with the chemical combination of two amino acids (L-aspartic acid and L-phenylalanine) (Chattopadhyay *et al*., 2011).

Another artificial sweetener is acesulfame-K, which is a high intensity sweetener that contains approximately 120 times the sweetness of sucrose and is more soluble in water, was developed by Hoechst. It is a heat stable molecule, having the ability to be applied in cooking and baking products, but it can have a bitter after taste, which is the reason it is used together with other sweeteners. This substance is approved by the USFDA for consumption and it is not metabolized, providing no calories to the consumer (Chattopadhyay *et al*., 2011).

Sucralose, an artificial sweetener discovered in 1976, is produced by replacing 3 chloride atoms for 3 hydroxyl groups from sucrose and it contains 450 to 650 times the sweetness of the conventional sugar. It has high water solubility, as well as high pH and temperature range stability. Despite being produced from sucrose, our body does not metabolize this molecule, which means it does not provide any calories (Chattopadhyay *et al*., 2011).

Saccharin is an artificial sweetener that was discovered in 1878 by Remson and Fahlberg. It contains 300 times the sweetness of the traditional sugar and presents an unpleasant bitter or metallic off taste. With many studies being focused on this molecule's safety, no study showed a clear relationship with its consumption and health risks, but some studies revealed a correlation between consumption and cancer incidence. Its use is currently allowed under certain regulations that specifies and limits its use on food and beverage products (Chattopadhyay *et al*., 2011).

Cyclamate is a non-nutritive sweetener that is 30 times sweeter than the table sugar, presents a bitter off taste, but with good synergy with saccharin. This substance was discovered in 1937 and is chemically produced. Its toxicity is being researched, due to being metabolized into cyclohexylamine by the gut bacteria, which is more toxic than its precursor (Chattopadhyay *et al*., 2011).

Artificial sweeteners such as saccharin, aspartame, cyclamate and acesulfame-K are consumed around the world as low calorie sweeteners by patients with diseases linked to the consumption of sugar, for instance diabetes, hyperlipemia, caries and obesity, but these substitutes may possess side effects such as psychological problems, mental disorders, bladder cancer, heart failure and brain tumours (Kant, 2005). Natural sources are, therefore, safer than the artificial ones and, the consumption of natural, non-nutritive and intense sweeteners may be a feasible and stable source of the sweeteners (Daniell *et al.*, 2000; Saumi *et al.*, 1997).

Artificial sweeteners may be replaced by sweet proteins, due to being natural, low caloric alternatives to sweeteners and do not activate a demand for insulin in our body, whereas sucrose does. Despite most of these proteins not sharing sequence homology or structural similarity, these proteins have been extracted from plants that grow in the same habitat, tropical rainforests. The possible applications for these molecules include the low calorie sweetener industry and the soda, snacks and chocolate industries (Kant, 2005).

1.2.2. Natural sweeteners

Natural sweeteners, which are molecules naturally present in foods, are another form of substances that offer a sweet taste and include, in addition to steviol glycosides and mogrosides, sweet proteins and sugar alcohols (Mooradian *et al*., 2017). The first provide low caloric alternatives to sugar, do not require an insulin response and include molecules such as monellin, thaumatin and brazzein. The latter, also known as rare sugars or polyols, are a type of molecules that provide less energy than sucrose to our bodies and are used as bulking replacers (Kant, 2005; Lê *et al*., 2016). The most common ones are erythritol, isomalt, xylitol, maltitol, sorbitol, mannitol, and lactitol. These polyols, alcohols with multiple hydroxyl groups, generally provide less sweetness compared to sucrose but offer a clean, non-lingering sweet taste, close to the profile of the traditional sugar (Lê *et al.*, 2016).

Monellin and thaumatin are among the best-known sweet proteins within the eight that are identified, including brazzein, miraculin, curculin, mabinlin, pentadin and hen egg white lysozyme. One of the first proteins to be related to intense sweet taste was monellin, which is a protein that is 3000 times sweeter than sucrose on a weight basis and 100000 times on a molar basis (Temussi, 2006). It was discovered by George E. Inglett and Joaan F. May, and isolated by James A. Morris and Robert H. Cagan from *Dioscoreophyllum cumminsii*, a plant taxonomically close to the sweet potato (Temussi, 2006; Faus and Sisniega, 2005). This sweet protein is a junction of two subunits (A and B) held together by secondary forces and the sweet taste it is only reported when the whole molecule is present, whilst the separate chains do not establish a sweet taste (Bohak and Li, 1975; Faus and Sisniega, 2005).

Thaumatin comprises a mixture of sweet proteins that exist naturally in the arils of the fruits of the West African plant *Thaumatococcus daniellii* (Lipinski, 2013; Moralejo *et al.*, 1999). The most abundant components are thaumatin I and II and the lesser ones are thaumatin a, b and c, all having a similar molecular mass of 22 kDa (Szwacka *et al.*, 2012).

The accessibility of thaumatin from the plant source is largely limited, so, the cultivation of *T. daniellii* has been made in different habitats from the natural one that the plant is found,

for instance greenhouses, however the plant grew but did not produce fruits (Moralejo *et al.*, 1999). Consequently, as an alternative method to the production of thaumatin from its natural source, its production outside of the plant was attempted by transferring its gene to another host such as the bacteria *Escherichia coli*, *Bacillus subtilis* and *Streptomyces levidans* (Gibbs *et al*., 1996*;* Moralejo *et al.*, 1999; Masuda, 2016).

Sugar alcohols, also known as polyols, are acyclic polyols where the sugar's carbonyl group is reduced to a hydroxyl group (Loescher, 2016; Park *et al.*, 2016). Mannitol, sorbitol, erythritol, isomalt, maltitol, lactitol and xylitol, the main polyols, are common constituents of many organisms, such as plants and microorganisms, and are similar to sugar, being utilized to improve the nutritional profile of food products because of their properties like noncariogenicity, the low caloric content and low glycemic index and insulin response (Akinterinwa *et al*., 2008; Loescher, 2016). Due to these benefits as sugar replacements, they are produced by the food and pharmaceutical industries, being utilized in dietic and diabetic food products as food additives (**Table 1**). Additionally, they are not absorbed by the small intestine, being metabolized by specific bacteria, releasing fatty acids that provide health benefits to the consumer (Ortiz *et al.*, 2013; Park *et al.*, 2016). Lastly, their characteristics allow them not to participate in the Maillard reaction and to be texturizing agents, colour stabilizers and humectants (Ortiz *et al.*, 2013).

Table 1 – Different polyols described as food additives numbers.

Polyol	**Food additive number**
Mannitol	E421
Sorbitol	E420
Erythritol	E968
Isomalt	E953
Maltitol	E965
Lactitol	E966
Xylitol	E967

The conventional industrial production of most sugar alcohols is achieved through hydrogenation of sugars with nickel catalysts under high pressure and temperature. However,

these processes need tough conditions and expensive purification procedures, leading to low yields (Park *et al.*, 2016). On the other hand, the biotechnological path provides the possibility for an environmentally friendly and safer production and the potential to use agricultural waste residues (Akinterinwa *et al*., 2008; Park *et al.*, 2016). Additionally, the conventional methods to optimize the strain and the culture conditions have been less resorted to, while the metabolic engineering procedures to make the organism consume cheap substrates or diminish the repression by compounds present in those materials have been the primary focus (Park *et al.*, 2016).

Mannitol is a polyol derived from fructose and part of the composition of certain plants, algae, bacteria, mushrooms and yeasts. It is a substance that it is utilized by the pharmaceutical, chemical and food industries, and in medicine. It possesses half the sweetness of sucrose, does not have cariogenic properties and it is produced in the food industry to act as a natural sweetener and used in chewing gum (Ortiz *et al.*, 2013).

Sorbitol is a sugar alcohol derived from glucose and it is naturally present in specific vegetables and in fruits like berries, grapes, plums, pears, cherries and apples. Its sweetness is around 60 % of the sucrose's and it is applied in the food industry in products such as chewing gum, deserts, candies, ice creams and foods for diabetics. Additionally, it can be used to produce certain pharmaceutical substances such as sorbose and ascorbic acid (Ortiz *et al.*, 2013).

Erythritol, or 1,2,3,4-Butanetetrol, is a polyol derived from erythrose, which is a compound of fruits such as melons, pears, watermelons and grapes, mushrooms and yeast fermented products, for instance, beer, soy sauce and wine. It is very stable at high temperatures and acid or alkaline media and does not take part in Maillard-type browning reactions. This substance is produced industrially by osmophilic yeasts' (*Moniliella pollinis* and *Trichosporonoides megachiliensis*) fermentation and by lactic acid bacteria (*Oenococcus oeni, Leuconostoc mesenteroides, and Lactobacillus sanfranciscencis*) (Ortiz *et al.*, 2013; Grembecka, 2015).

Isomalt, or isomaltose or isomaltitol, is a mixture of two isomeric disaccharide alcohols: gluco-mannitol and gluco-sorbitol, is produced through an enzymatic conversion of sucrose, making it more stable than the traditional sugar, becoming a substance that is stable at high temperature. It is a molecule that contains 50 % of the calories present in sucrose and is utilized

in bubble gums, gelatins, chocolate, coatings, baked goods and yogurts. It is combined with NNS due to its ability to disguise the bitter aftertaste of some sweeteners and bulking agents. This polyol also contains anti-cariogenic properties, does not increase blood glucose and insulin levels (Grembecka, 2015; Carocho *et al*., 2017).

Maltitol, or 4-O-α-d-glucopyranosyl-d-glucitol, is a disaccharide polyol containing glucose and sorbitol and can be produced by hydrogenating maltose from starch or high maltose glucose syrup. It contains 90 % of the sweetness of sucrose, but with only 52.5 % to 60 % of its calories. This polyol also contains non-cariogenic properties, reduced insulin response, due to its slow absorption, and it does not take part in browning effects (Grembecka, 2015).

Lactitol, is also a disaccharide polyol, but composed of sorbitol and galactose, and can be produced by a catalytic hydrogenation of lactose. It contains a 40 % sweetening power compared to sucrose. It is soluble at lower temperatures than the conventional sugar, does not take part in browning reactions, it is able to be stored for long periods, stable at high temperatures and in acidic and alkaline conditions. Its blood glucose levels and insulin response are negligible, and it is non-cariogenic. Due to this, this polyol it is applied in pharmaceutical products and in foods, such as ice cream, chocolate, candies, baked foods and chewing gums (Grembecka, 2015).

1.3. Xylitol

1.3.1. Characteristics

Xylitol is a sugar alcohol derived from xylose and is a substance found in fruits and vegetables, for instance, berries, oats and mushrooms. It is a sweet product with 40 % less calories than sucrose, with the same sweetness as the traditional sugar, and it does not possess cariogenic characteristics, which means it can be applied in dental products and chewing gums (Hyvönen *et al*., 1982; Ortiz *et al.*, 2013).

Additionally, it does not take part in the Maillard reactions, not reducing the nutritional value of the available proteins, and it can reduce the risk of tooth decay, inhibiting the growth of *Streptococcus mutans* and *Streptococcus sobrinus*, major components in the lack of dental health, by increasing the pH in the oral cavity and contributing to teeth remineralization. It also

contains high chemical and biological stabilities, making it useful as a food preservative, extending the shelf life of food products (Parajo *et al*., 1998).

However, perhaps the most important characteristics of this substance is that it does not induce the production of insulin, the molecule associated with diabetes, due to entering the cells independently of this hormone and is gradually converted to glucose-6-phosphate. This results in every glucose molecule derived from the metabolism of xylitol being mainly stored as glycogen in the liver, as being released slowly to the bloodstream (Cocate *et al.*, 2011). This signifies that xylitol is a great alternative for sugar to reduce the predominance of diabetes (Tada *et al.*, 2004). All of these properties allow this substance to be utilized in the food industry, in chocolate, candy, caramel, jelly, ice cream and beverage products (Ortiz *et al.*, 2013; Albuquerque, Ivanildo José Da Silva, *et al.*, 2014).

1.3.2. Xylitol Production

The production of xylitol has been accomplished chemically by a catalytic hydrogenation process to a purified solution of xylose, under a temperature between 80 and 140 ºC and a pressure of 50 atm (Albuquerque *et al.*, 2014). However, this process involves the use of harsh physical conditions and expensive purification procedures, which led to the search of biotechnological methods to replace the chemical one. Furthermore, in order to diminish the production costs, the use of agro-industrial wastes, that can release sugars like xylose after a pre-treatment (an hydrolysis with diluted acids and submission to a temperature above 100 ºC), is also a possibility (Albuquerque *et al.*, 2014).

Bacteria could also be applied to this process and provide some potential advantages, such as their high growth rate, their ability to metabolize inexpensive growth media and to be easily genetically manipulated. An heterologous xylose reductase was utilized, with a strain that could simultaneously metabolize glucose and xylose with no catabolic repression, and the intensification of the production of NADPH (Park *et al.*, 2016). Furthermore, xylitol production was also attempted by Nyyssölä *et al.*, 2005 with the expression of xylose reductase and xylose transporter genes from *S. stipitis* (formerly known as *Pichia stipitis*) CBS 5773 and *Lactobacillus brevis* ATCC 8287, respectively, with a plasmid that possessed the nisA promoter, inserted in *Lactococcus lactis* NZ9800.

However, the most efficient natural producers of xylitol are yeasts, with the genus *Candida* having the best producing strains, but these species cannot be added to food products due to their pathogenic characteristics (Ortiz *et al.*, 2013; Albuquerque *et al.*, 2014). A xylose reductase/xylitol dehydrogenase (XR/XDH) pathway is utilized for the conversion of xylose into xylitol and then xylulose, but xylitol is a by-product due to the co-factor imbalance that exists. This disparity happens because xylose reductase utilizes NAD(P)H- (preferring NADPH) and xylitol dehydrogenase uses NAD+ (Albuquerque *et al.*, 2014). However, this accumulation of xylitol is associated with reduced conversion yields due to the capacity of these yeasts to metabolize xylose for energy requirements (Kim *et al.*, 2017). Therefore, the use of genetically engineered strains that are GRAS (Generally Recognized as Safe) and unable to metabolize xylitol is associated with increased xylitol yields (Kogje and Ghosalkar, 2016; Kim *et al.*, 2017).

The production of xylitol by genetically engineered strains was attempted in the GRAS yeast *Saccharomyces cerevisiae* by Lee *et al.*, 2002 with the insertion of the XYL1 gene that translates to the xylose reductase from *Scheffersomyces stipitis*. Furthermore, due to not being able to produce the enzyme xylitol dehydrogenase to metabolize xylitol, the yeast had to be provided with additional sugars in order to grow (glucose, galactose, fructose and mannose). The process was attempted within a batch reactor, with glucose, xylose and the recombinant yeast expressing the inserted gene. However, only at low concentrations of glucose *S. cerevisiae* was able to uptake xylose through the glucose transporters (Lee *et al.*, 2002). Additionally, after the exhaustion of glucose, xylose was not metabolized into xylitol because there was no NADPH, necessary to the xylose reductase conversion. To surpass this problem the utilization of a fed-batch reactor and the addition of non-catabolite repression sugars (for instance, cellobiose) were implemented, which allowed for a slow release of glucose into the medium and the uptake of cellobiose to the cell to then be hydrolysed into glucose, respectively (Lee, *et al*., 2000; Park *et al.*, 2016). Despite the latter having 40 % higher productivity, the first was able to maintain the glucose concentration lower than 0.35 g/L and offer up to 30 g/L of xylose, resulting in the conversion rate of 0.95 grams of xylitol per gram of xylose, the final concentration of 105.2 g/L of this polyol and the productivity of 1.7 g/L/h (Lee *et al*., 2000).

Aldose reductase catalyses a reaction that NADPH-dependently converts aldoses, such as xylose, into polyalcohols, like xylitol (**Figure 1**). This enzyme occurs naturally in yeasts that metabolize xylose, such as *S. stipitis, Candida shehatae* and *Pachysolen tannophilus*. By overexpressing the GRE3 gene, which codifies to a non-specific endogenous aldose reductase capable of converting xylose into xylitol, Baptista *et al.*, 2018 was also able to produce xylitol in a reactor, using *S. cerevisiae* PE-2 GRE3, to produce this polyol from a synthetic medium, and it was able to obtain 148.5 g/L of xylitol with 95 % of conversion. Additionally, in the same study, xylitol production was achieved with a corn cob hydrolysate, obtaining 29.61 g/L and 93 % of conversion.

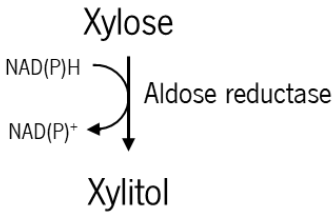


Figure 1 – Xylitol production pathway with the enzyme aldose reductase.

The utilization of laboratory yeast strains has been a developed system to produce added-value compounds. However, the insertion of hydrolysed lignocellulosic materials as a carbon source also implies the addition of other undesirable substances formed by the pre-treatment (for instance, acetic acid, furan derived compounds, mainly furfural and 5-hydroxymethylfurfural and several phenolic molecules, like vanillin), that are inhibitors to microbial growth (Pereira *et al.*, 2011, 2014). This suggests that the success of lignocellulosic biomass fermentations is highly reliant on the aptitude of the present yeast strain to deal with the numerous inhibiting compounds in the culture medium (Pereira *et al.*, 2011). An alternative to the use of laboratory strains is the utilization of industrial ones, which are naturally very robust, displaying the ability to obtain higher fermentations and tolerate stress conditions, including the presence of toxic compounds (Mussatto *et al.*, 2010). In fact, as shown by Pereira *et al.*, 2014, the strains of *S. cerevisiae* with industrial distilleries background presented a higher lignocellulosic hydrolysate fermentative aptitude than industrial *Kluyveromyces marxianus* isolates, *S. cerevisiae* from a beer isolate and laboratory strains. Specifically, in the mentioned work, the industrial *S. cerevisiae* strains PE-2, derived from first generation bioethanol plant, and CCUG53310, derived from second generation bioethanol plant, were the most efficient in producing ethanol from real hydrolysates, which may suggest being robust yeas chassis to use

in the production of other substances of interest, such as sweeteners, from agro-industrial renewable wastes.

1.4. Agro-industrial residues

Environmental concerns related to fossil fuel sources have been increasing and the use of renewable resources as lignocellulosic biomass for the manufacturing of biofuels and chemicals aims to create a sustainable resource ecosystem and a circular economy. Lignocellulosic biomass is the most abundant renewable resource on the Earth, widely spread. The use of these biomasses for the biotechnological production of xylitol is shown as an interesting alternative, which have to be addressed (Ninawe and Kuhad, 2005; Singh *et al*., 2012; da Silva, 2016).

A major part of the agricultural waste produced every year possesses lignocellulose, which represents half of the plant material and it is formed by photosynthesis, and these residues include corn, wheat, rice and other cereals, which can be inserted into fermentative processes (Vandamme, 2009; Sharma *et al.*, 2016). Lignocellulose contains three types of polymers that cannot be metabolized by most microorganisms, lignin, cellulose and hemicellulose, linked together by covalent cross-linkages and non-covalent forces (Singh *et al*., 2012).

Cellulose is a polymer constituted of D-glucose molecule linked by β(1,4)-glycosidic bonds. It is the most abundant polymer on the planet and the most present component of lignocellulosic biomass. Chains of cellulose form cellulose fibrils, which are linked by hydrogen bonds and, because of that, are insoluble in water and many organic solvents. However, this polymer can be chemically hydrolysed with acid (whether concentrated or diluted) or with enzymes (Swatloski *et al.*, 2002; Cheng *et al.*, 2010).

Another component of lignocellulosic materials, hemicellulose, is a branched polymer which contains a varied quantity of xylan and, in hardwood and agro-industrial residues, arabinoxylan. This varied composition is dependent on the source and species of the raw material. Arabinoxylan contains polymers of L-arabinose and D-xylose, which cannot be naturally metabolized by *S. cerevisiae* (Costa *et al.*, 2017). Xylan makes up 20 to 40 % of the total plant biomass and can be hydrolysed for appropriate utilization of this lignocellulosic material. This process can be made chemically or with the use of enzymes (xylanases) (Ninawe and Kuhad, 2005). The resulting lignocellulosic materials have plenty of applications in the

bioenergy industry, with the production of bioethanol, biogas and biohydrogen and in the chemical industry, with the formation of substances that include acetone-butanol, lactic acid, polylactic acid, levulinic acid. Additionally, they can also be utilized to produce lubricants, softeners, solvents, polymers and other products such as furfural, β-galactosidase, xylanase and xylitol (Nasr *et al.*, 2013; Raol *et al.*, 2015; da Silva, 2016; Rane *et al.*, 2017).

Lignin, an aromatic polymer, is also another main component of lignocellulosic materials. Its presence adds strength and rigidity to the cell wall of plants due to its phenylpropane monomers (such as synapyl alcohols, p-courmaryl and coniferyl) being linked together (Kang *et al.*, 2014).

In order to process these agro-industrial residues to be utilized in the production of added-value compounds, treatments that release the sugar monomers into an aqueous environment are required. These pretreatments, or fractionation processes, break down the structure of the raw materials and originate polysaccharides. This process facilitates the posthydrolysis of these molecules to simple and able to be metabolized sugars. Pretreatments can be physical (by milling, freeze treatments, etc.), physico-chemical (steam explosion or ammonia fibre explosion (AFEX) and chemical (with acid, liquid hot water or autohydrolysis) (Haghighi Mood *et al.*, 2013).

In the case of the fractionation process being an autohydrolysis, it presents an ecologically considered procedure for the pretreatment of lignocellulosic materials. In this process the only reagents are the raw material and water, the temperature is risen up to 220 ºC, and the selective solubilization of hemicellulose is accomplished with the catalytic action of hydronium ions from the heated water and the generated substances, such as acetic acid, uronic acids and phenolic acid (B. Rivas *et al.*, 2002; Gullón *et al.*, 2012). This results in a solid residue, composed of cellulose and lignin, and a liquid fraction that contains multiple products of the degradation of cellulose, such as xylose, glucose, mannose, galactose, arabinose and rhamnose. It also originates sugar degradation products like furfural and hydroxymethylfurfural. Lastly, acetic acid, uronic acids and phenolics (from the degradation of lignin) are also produced (B Rivas *et al.*, 2002).

Some of the most relevant agro-industrial residues include brewer's spent grain (BSG), *Eucalyptus globulus* wood (EGW), and corn cob, with cellulose, non-cellulosic polysaccharides and lignin in their composition. The non-cellulosic polysaccharides component includes

hemicelluloses, such as xylan, glucan and mannan (Mussatto *et al*., 2006). Corn cob is an agricultural waste product rich in xylan, and consequently, through a pre-treatment, high quantities of xylose may be extracted (Pointner *et al.*, 2014; Samanta *et al*., 2014). Furthermore, corn cob and EGW are lignocellulosic materials already utilized in the production of bioethanol (Cai *et al*., 2016). In this study, corn cob was selected for its high xylan composition. **Table 2** shows the results obtained by different studies that utilized lignocellulosic materials, and specifically corn cob, to produce xylitol.

For this study, autohydrolysis was selected and, in this process, the raw material is mixed with water to stainless steel reactor and the temperature is risen to solubilize the hemicellulose fraction mainly by xylooligosaccharides. During the autohydrolysis the acetyl groups connected to the hemicellulose are released to the liquid fraction and autocatalyze the reaction. After this process, an enzymatic hydrolysis of both solid and liquid fractions must be performed. The added enzymes most importantly convert glucan into glucose and xylan in xylose so that these monomers may then be metabolized by the cells (Gullón *et al.*, 2012; Baptista *et al.,* 2018). The addition of this enzyme mixture may be performed simultaneously as the insertion of the inoculum or multiple hours previous to it. The first represents a system with simultaneous saccharification and fermentation (SSF) and the latter system of pre-saccharification and simultaneous fermentation (PSSF).

In SSF, cellulose and xylan are converted by cellulases and xylanases into fermentable sugars. This process demands lower enzyme concentration because the product inhibitor (glucose) that is formed during enzymatic hydrolysis is immediately consumed by the yeast (Chandel *et al.,* 2007). SSF presents multiple additional advantages, such as an increase in the hydrolysis rate, higher conversion yields, the non-accumulation of sugars for the microorganism fermentation and shorter process time. However, a compromise between the pH, temperature and substrate concentration must be achieved, due to the need of a compatibility of the saccharification and fermentation processes occurring at the same time.

Table 2 – Studies performed with corn cob autohydrolysates with multiple strains for the production of xylitol. LSR: liquid to solid ration.

Microorganism	Pretreatment	Xylitol (g/L)	Xylitol yield (g/g)	Reference
a) Fermentation using detoxified corn cob hydrolysates for xylitol production				
Debaryomyces hansenii NRRL Y-7426	Autohydrolysis (Tmax 202ºC, LSR 8g/g). Posthydrolysis (125 ºC, 0.5 % (w/w) H_2SO_4, 165 min)	62	0.73	Rivas *et al.*, 2002
Immobilized *Debaryomyces hansenii* NRRL Y-7426	Dilute acid treatment (2% (w/w) H_2SO_4, 15 min, 130 ºC, LSR: 8 g/g)	13.5	0.57	Pérez-Bibbins *et al.*, 2014
Candida tropicalis	Dilute acid treatment (1 % (w/w) H_2SO_4, 121 ºC, 30 min, LSR 8 w/v)	11.89	0.58	Misra *et al*., 2013
C. tropicalis MTCC 6192	Dilute acid treatment (0.5% (w/w) HNO_3, 121 ºC, 30 min, LSR 10 g/g)	33.4	0.66	Kumar *et al.*, 2018
C. tropicalis As 2.1776	Dilute acid treatment (1 % (w/w) H_2SO_4, 121 ºC, 40 min, LSR 10 g/g)	96.5	0.83	Li *et al.*, 2012
C. tropicalis HDY-02	Dilute acid treatment (1 % (w/w) H_2SO_4, 120 ºC, 60 min) LSR 10 g/g	58	0.73	Ling *et al.*, 2011
C. tropicalis W103	Dilute acid treatment (1% (w/w) H_2SO_4), 120 ºC and 60 min; Liquid to solid ratio 10 g/g	68.4	0.7	Cheng *et al.*, 2009
C. magnoliae FERMP-16522	Dilute acid treatment (1-3 % (w/w) H_2SO_4, 60 min, LSR 10 g/g)	49	0.62	Tada *et al*., 2012
Candida sp. ZU04	Dilute acid treatment (1 % w/w (w/w) H_2SO_4, 110 ºC, 180 min, LSR 8 g/g)	34.5	0.60	Ding and Xia, 2006
K. marxianus YZB001	Data not supplied	11.32	0.60	Zhang *et al.*, 2013
b) Fermentation using non-detoxified hemiceullulosic hydrolysates for xylitol production				
S. cerevisiae YRH 396 (XYL1/XYL2 genes+XKS1 overexpression)	Dilute acid treatment (1% (w/w) H_2SO_4, 121 ºC, 40 min, LSR 10 g/g)	8.17	0.45	Cortivo *et al.*, 2018
S. cerevisiae XP-RTK (GRE3 overexpression and STU1 expression)	Steam acid treatment (2 % Sulfuric acid, 2% oxalic acid) in continuous digester (6 bar 160 ºC) LSR 5-5.5 g/g. Diluted non-detoxified hydrolysate	16.3	~ 1	Kogje and Ghosalkar, 2017
S. cerevisiae ScpGT (GRE3 overexpression and STU1 expression)	Steam acid treatment (2 % Sulfuric acid, 2% oxalic acid) in continuous digester (6 bar 160 ºC) LSR 5-5.5 g/g. Diluted non-detoxified hydrolysate	21	0.6	Kogje and Ghosalkar, 2016
C. tropicalis CCTCC M2012462	Dilute acid treatment (1% H_2SO_4, 125 ºC 60 min, LSR 6.6 g/g. Non-detoxified hydrolysate added in fed-batch mode	38.8	0.7	Ping et al., 2013
S. *cerevisiae* PE-2 GRE3 (GRE3 overexpression)	Autohydrolysis (Tmax of 205 ºC, LSR 8 g/g). Posthydrolysis (125 ºC, 0.5 % H_2SO_4, 165 min). Non-detoxified hydrolysate added in fed-batch mode	24.3	0.88	(Baptista *et al.*, 2018)

2. OBJTECTIVES

2. OBJECTIVES

Environmental concern related to the use of fossil resources have been increasing and the use of agro-industrial residues for the production of added-value compounds aims to create a sustainable recourse ecosystem. Among these compounds, xylitol is considered an important alternative to sucrose, mainly for not involving the secretion of insulin and containing a lower quantity of calories than the conventional sugar.

In this study, the main objective was to develop strategies for the biotechnological production of xylitol, a low-calorie sweetener, utilizing an agro-industrial residue. This process was performed using a recombinant *S. cerevisiae* (able to convert xylose into xylitol by aldose reductase) in hydrolysed corn cob, which is a lignocellulosic material rich in xylose. In order to optimize the production of xylitol in the fermentation of corn cob hydrolysates, the following specific objectives were proposed:

- Evaluation of different operational conditions (solid loading and temperature) of autohydrolysis treatment in order to maximize xylan solubilisation into hemicellulose derived compounds (namely xylose and xylooligosaccharides).

- Evaluation of enzymatic saccharification of corn cob whole slurry after different pretreatment conditions in order to obtain an efficient conversion of polysaccharides into xylose and glucose.

- Optimization of simultaneous saccharification and fermentation process for the bioconversion of xylose into xylitol using a *S. cerevisiae* PE-2 GRE3

- Comparison of enzymatic hydrolysis and dilute acid hydrolysis in a SSF process for xylitol production of whole slurry corn cob.

- Development of strategies to increase xylitol productivity: evaluation of presaccharification process and inoculum size on SSF process.

3. MATHERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Yeast strain

The strain utilized in this study for the production of xylitol was *Saccharomyces cerevisiae* PE-2-GRE3, constructed by Baptista *el al*., 2018, with the GRE3 gene overexpressed and with the geneticin (G418) resistance gene. The GRE3 gene contains the information for the cell to produce an unspecific aldose reductase, which includes the first reaction the polyol pathway, the metabolism of d-xylose into xylitol, and this reaction is strictly NADPH dependent (Kuhn *et al.*, 1995). A plasmid was constructed by homologous recombination from the plasmid p417-kan (**Figure 2**). After AatII digestion the plasmid was co-transformated with the GRE3 gene and amplified to create the plasmid p417-kan-GRE3 (Baptista *et al.*, 2018).

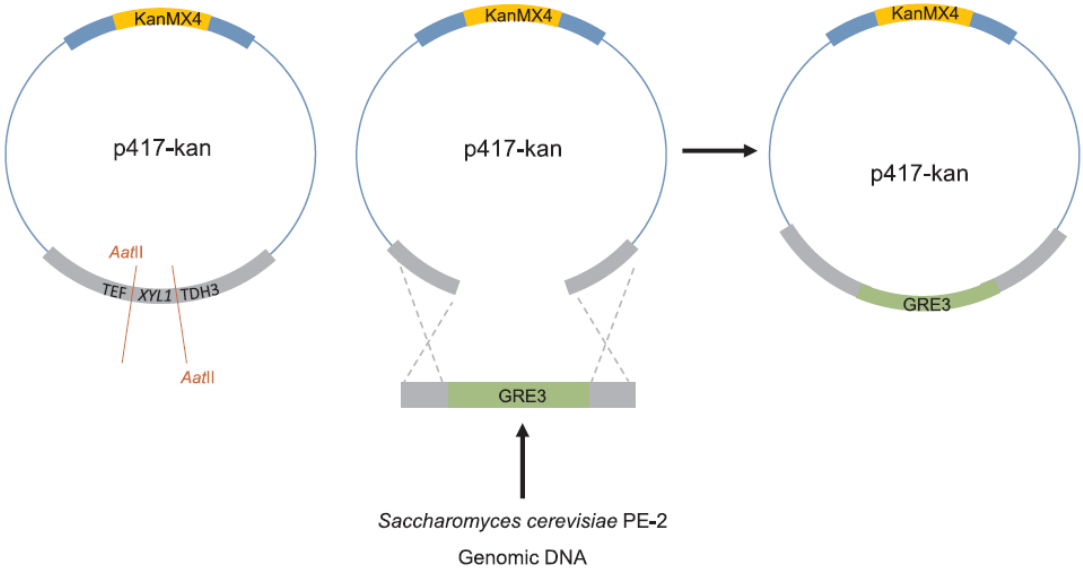


Figure 2 – Schematic representation of the p417-kan plasmid construction with the GRE3 gene (Source: Baptista *et al.*, 2018).

3.2. Raw material

The raw material used in this work was corn cob, provided by a local producer from the Minho region (Northern Portugal). It was selected due to its high content in xylose (Baptista *et al.*, 2018). Corn cob was collected and milled to reduce the particle size to less than 1 cm of

diameter. This was accomplished with a mill located in the CVR (Centro para a Valorização de Resíduos) in the Azurém Campus of the University of Minho. After milled, corn cob was homogenized in a single lot to avoid differences and stored at room temperature in a dark and dry place until its use. Raw material was analysed to determine its chemical composition, as described in the section **3.4**.

3.3. Corn cob fractionation: Autohydrolysis pretreatment

Corn cob was submitted to an autohydrolysis treatment to solubilize hemicellulose fraction composed mainly by xylooligosaccharides. For that, corn cob was mixed with water at different percentages (20, 25 and 30 g of corn cob solid dry weight for 100 g) and heated at temperature in the range of 205-211ºC in non-isothermal regimen as presented by **Figure 3**. Additionally, **Figure 4** shows heating profiles of experiments carried out. Autohydrolysis treatments were carried out in a stirred, stainless steel reactor (Parr Instrument Co) of 2 L volume. The reactor was heated to the desired temperature and then it was cooled by internal refrigeration. After treatment, solid and liquid phases were separated by filtration. Solid was washed with distilled water. Both phases were analysed for determination of sugars content.

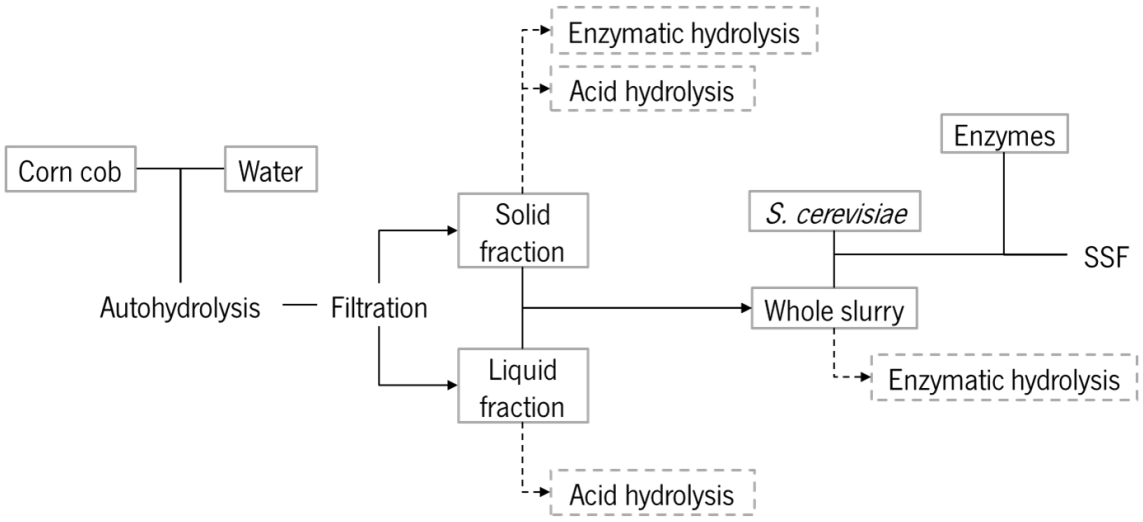


Figure 3 – Schematic representation of the autohydrolysis process of corn cob and the enzymatic hydrolysis procedure.

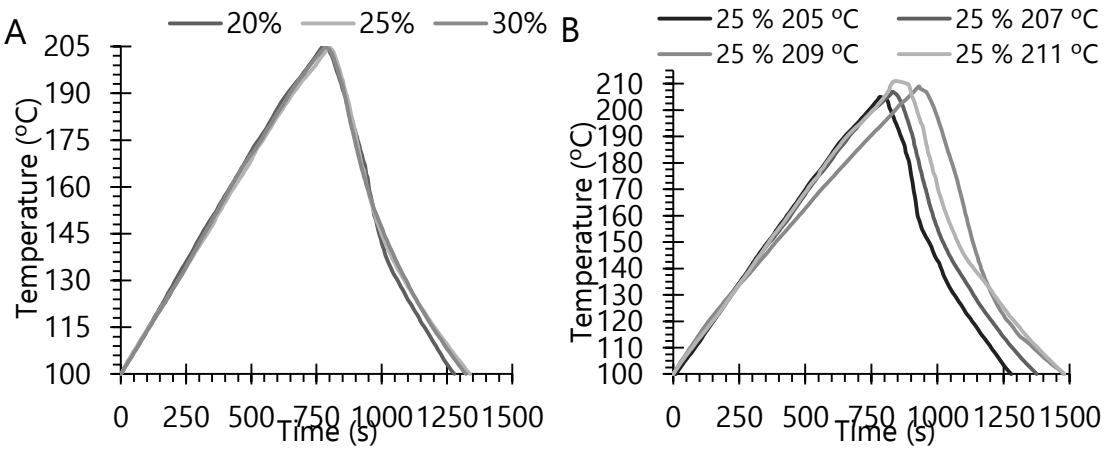


Figure 4 – Temperature profiles of: A - 20, 25 and 30 % (w/w) of corn cob during autohydrolysis at Tmax of 205 ºC; B – 25 % (w/w) of corn cob during autohydrolysis at Tmax of 205, 207, 209 and 211 ºC.

The liquid phase was stored at 4 ºC until further use, while the solid fraction was then washed with distilled water to remove the sugars present in the liquid fraction that did not pass through the filter. To verify the solid yield, the solid fraction was dried for several days at 60 ºC and it was weighed and analysed for humidity percentage. Solid yield was calculated with equation (1):

$$SY = \frac{M_{HSF} \times (1 - H_{SF})}{M_{HRM} \times (1 - H_{RM})} \times 100 \qquad \text{Equation (1)}$$

where SY: solid yield (%); M_{HSF}: mass of wet solid fraction recovered after treatment (g); M_{HRM}: mass of humid raw material before treatment(g); H_{SF}: humidity of solid fraction (%); H_{RM}: humidity of raw material (%).

Glucan, xylan and lignin recovery were also determined and were calculated with equation (2):

$$R_G = \frac{C_{GH} \times SY}{C_{GRM}}; R_X = \frac{C_{XH} \times SY}{C_{XRM}}; R_L = \frac{C_{LH} \times SY}{C_{LRM}} \qquad \text{Equation (2)}$$

Where R_G: glucan recovery (%); C_{GH}: glucan concentration in hydrolysate (%); SY: solid yield (%); C_{GRM}: glucan concentration in raw material; R_X: xylan recovery (%); C_{XH}: xylan concentration in hydrolysate (%); C_{XRM}: xylan concentration in raw material; R_L: lignin recovery (%); C_{LH}: lignin concentration in hydrolysate (%); C_{LRM}: lignin concentration in raw material;

3.4. Analytical methods

Raw material, and solid phase obtained after autohydrolysis treatments were analysed for chemical composition by quantitative acid hydrolysis as described by NREL protocols (NREL/TP-510-42618-42622-4218). For that, 0.5 g of sample and 5 mL of H_2SO_4 at 72 % (w/w) were added to a test tube and the tube was inserted in a water bath at 30 ºC for 1 hour with periodic agitation. After this period, the content was transferred to a Schott flask and 148.7 g of distilled water were added to dilute the sulphuric acid to 4% (w/w). The flask was weighed, went through an autoclave cycle at 121 ºC for 60 minutes and then weighed again to verify any volume losses. The content of the flask was put through a number 3 filter Gooch flask of known weight, dried at 105 ºC and weighed again to analyse the Klason lignin content. The filtered liquid was analysed through High Performance Liquid Chromatography (HPLC) to determine sugar content.

To analyse the sugar content in the liquid phase a NREL protocol was followed (NREL/TP-510-42623), where the liquor was subjected to a second hydrolysis, where 4 g of liquor, 19.93 g of distilled water and 1.07 g of 96.5 % (w/w) H_2SO_4 were added to a Schott flask to obtain a 4 % (w/w) H_2SO_4 solution, which then went through an autoclave cycle at 121 ºC for 20 minutes and the resulting content was examined through HPLC.

The concentration of multiple compounds was determined throughout this work by HPLC, including glucose, xylose, arabinose, xylitol, acetic acid, ethanol, furfural and hydroxymethylfurfural. The device contained a refracted index and ultraviolet detectors, BioRad Aminex HPX-87H (300 x 7.8 mm) column and was eluted by an aqueous solution of 0.005 M of H_2SO_4 at a flowing rate of 0.6 mL/min and a temperature of 60 ºC.

3.5. Acid posthydrolysis

After the autohydrolysis, with the aim of preparing the liquid fraction of the hydrolysed material to be utilized as a culture medium, by releasing the simple sugars present in the form oligosaccharides, this fraction was added into a Schott flask to obtain 0.05 % (w/w) of H_2SO_4 and was put through an autoclave cycle at 121 ºC for 165 minutes (Rivas *et al.*, 2002). The resulting liquid was neutralized with $CaCO_3$. Then, a sample was analysed with HPLC to assess its sugar content.

3.6. Enzymatic saccharification of pretreated corn cob

In order to hydrolyse xylooligosaccharides into xylose, after the autohydrolysis process, instead of an acid hydrolysis it was applied an enzymatic hydrolysis to degrade the glucan into glucose and to hydrolyse xylan and xylooligosaccharides into xylose. This was achieved by adding a solution with a mixture of enzymes (Cellic CTec2, provided by Novozymes, Bagsvaerd, Denmark) several hours before the inoculation or as the inoculum is transferred to the Erlenmeyer flasks. This procedure was not performed at 50 ºC despite being the optimal temperature of hydrolysis, due the activity of the cellulase being significantly affected by continuous exposure to this temperature. Therefore, as the temperature of 45 ºC was not associated with a decrease in activity and it was used in this procedure (Novoenzymes A/S, 2010; Gomes *et al*., 2016). However, for a fermentation, when the cells are introduced the temperature was lowered to either 30 or 35 ºC.

3.7. Liquid fraction detoxification

During the autohydrolysis process the acetyl groups connected to the hemicellulose are released to the liquid fraction and autocatalyze the reaction. This originates a high accumulation of acetic acid in the liquid fraction. For the elimination of this acetic acid [i]an ionic exchange detoxification was performed, adding, for every 10 grams of liquor utilized, 1 gram of dry Amberlite IR120 hydrogen form (strongly acidic) by Sigma-Aldrich to the liquid fraction and stirred for 1 hour. The pH was verified before and after this procedure. Then, the solution was filtered to remove the resin and 9,45 grams of dry anionic resin (HiMedia Laboratories) was added per every gram of acetic acid present in the liquor. This was stirred for 3 hours, verifying the pH after the time and an aliquot was taken for HPLC sugar content analysis.

3.8. Corn cob autohydrolysate fermentation through Pre-Saccharification and Fermentation and Simultaneous Saccharification and Fermentation

The *S. cerevisiae* PE-2 GRE3 used for the preparation of the inoculum were grown and preserved in a YPD agar plate (containing 10 g/L of yeast extract (Oxoid), 20 g/L of peptone (HiMedia Laboratories), 20 g/L of D-glucose (AMRESCO, Inc.), 0,15 g/L of G418 antibiotic and 20 g/L of agar) with regular replating. For a fermentation, a small colony was removed from the plate and added to an Erlenmeyer flask with YPD medium (with 10 g/L of yeast extract, 20 g/L of peptone and 20 g/L of D-glucose) and the antibiotic G418 and the yeasts were

incubated overnight at 30 ºC and 200 rpm. Before the fermentation the cells were centrifuged and the medium was discarded. A calculated volume of 0.9 % (w/w) NaCl (VWR International, LLC) solution was added to create the starting inoculum of 11 g of wet cells/L.

With the objective of producing xylitol, the conversion of the xylose present in corn cob to this sugar alcohol was performed by starting to add 5 % (w/w) of solid auto-hydrolysed corn cob to an Erlenmeyer flask and then putting it through an autoclave cycle at 121 ºC for 15 minutes to sterilize the solid particles. After that, 2 mL of a stock solution of 300 g/L of peptone and 150 g/L of yeast extract was added to put the final medium at 20 g/L and 10 g/L, respectively, as well as 45 µL of a solution with 1 mg/mL antibiotic G418 (geneticin). Furthermore, a controlled volume of auto-hydrolysed liquor (that had been sterilized by filtration through a 0.2 µm membrane) was transferred, followed by 1 mL of inoculum to obtain an initial cell concentration of 11 mg of wet cells/L. Also, a selected volume of Cellic CTec2 enzyme mixture was added before 24 hours, 12 h (PSSF) or right after the rest of the components were transferred (SSF) to release the sugars to be utilized by the yeast.

In order to analyse the results of these experiments, several parameters were calculated, such as xylitol productivity and xylitol yield. The xylitol productivity (in g/L/h) was calculated by the equation (3):

$$P_X = \frac{C_X}{t} \qquad \text{Equation (3)}$$

where P_X: xylitol productivity; C_X: xylitol concentration at t; t: incubation time when xylitol concentration stabilized.

The xylitol yield was calculated by equation (4):

$$Y_X = \frac{M_X}{M_{PX}} \qquad \text{Equation (4)}$$

Where Y_X: xylitol yield; M_X: mass of xylitol produced; M_{PX}: mass of potential xylose.

3.9. Experimental design for optimization of SSF process

An experimental design was constructed to evaluate the independent variables solid loading (x_1) and enzyme loading (x_2) in the objective of optimizing the xylitol concentration through SSF. Dependent variables (y) were correlated with the independent variables by empirical models, following the equation (5):

$$y_j = b_{0j} + \sum_{i=1}^{2} b_{ij} x_i + \sum_{i=1}^{2} \sum_{k \geq i}^{2} b_{ikj} x_i x_k \qquad \text{Equation (1)}$$

where y_j (j=1 to 3) is the dependent variable; xi or x_k (i or k: 1 to 2, $k \geq i$) are the normalized, independent variables and $b_{0j} \ldots b_{ikj}$ are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed models using commercial software (Microsoft Excel, Microsoft Office 365 ProPlus).

3.10. Statistical analysis

In order to compare the results obtained by fermentations with different conditions a model was constructed using the software Microsoft Excel and the supplements Data Analysis and Solver were applied to assess if the model fitted the experimental results.

4. RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Raw material

Corn cob was analysed following the standard procedures described in Material and methods. **Table 3** shows the chemical composition of corn cob used in this study and composition of corn cob samples reported in literature. As observed, the hemicellulose fraction was the main fraction of the raw material utilized (mainly composed by xylan) followed by cellulose. Comparing to other results reported in literature, its composition was the most similar to the material described by Rivas *et al.*, 2002. It is also notable that this residue, corn cob, was selected due to its similar percentage in xylan when compared with the literature (34.8 % (w/w)), which will be hydrolysed into xylose and bioconverted into xylitol by a recombinant *S. cerevisiae* strain (Baptista et al., 2018). Considering the recalcitrant structure of lignocellulosic materials, a biomass processing is necessary to obtain fermentable sugars for the bioconversion of xylose into xylitol. In this sense, autohydrolysis treatment was selected for the fractionation of lignocellulosic biomass and recovery of sugars present in corn cob.

Table 3 – Chemical composition of corn cob raw materials derived from different studies. NA – Not available

Glucan (% (w/w))	Xylan (% (w/w))	Arabinan (% (w/w))	Lignin (% (w/w))	Acetyl groups (% (w/w))	Reference
32.3	34.8	3.4	22.9	2.0	This study
38.8	44.4	NA	11.9	NA	Pointner *et al.*, 2014
34.0	29.0	NA	25.0	2.4	Costa *et al.*, 2017
31.7	34.7	NA	20.3	3.4	Rivas *et al.*, 2002
37.1	27.9	2.2	13.9	NA	Lee *et al.*, 2011

4.1.1. Effect of solid loading of corn cob in autohydrolysis treatment on biomass fractionation

Autohydrolysis treatment was selected for the treatment of corn cob to solubilize the xylan into hemicellulose derived compounds (mainly xylose and xylooligosaccharides). In this sense, the solid loading of biomass in the pretreatment was evaluated with the goal of saving

water consumption in the process and to concentrate xylose in the liquid phase (or hydrolysate). Thus, with this purpose, several corn cob concentrations were tested, ranging from 20 to 30 % (w/w) of solids at 205 º C, the severity of treatment was selected in basis of previous works (Rivas *et al.*, 2006; Garrote *et al.*, 2008)). After treatment, liquid and solid phases were separated by filtration and analysed.

Considering the liquid fraction (**Table 4**), the concentration of hemicellulose-derived compounds was superior with the condition that contained 25 % (w/w) of corn cob during autohydrolysis, resulting in 47.0 g/L of potential xylose. Regarding other minor components in the liquid fraction, the glucose concentration is also higher with 25 % (w/w) of corn cob autohydrolysate, containing 5.53 g/L of potential glucose (**Table 4**). The increase of corn cob loading in the autohydrolysis treatment allows to obtain a liquor enriched in sugars. Nevertheless, inhibitory compounds (furfural, hydroxymethylfurfural and acetic acid) also increased. These compounds have a negative effect on microbial growth (Palmqvist *et al*., 1999). As shown in **Table 4**, the hydrolysed material with 30 % (w/w) of corn cob originated the highest concentration of inhibitory compounds, with 9.02 g/L of acetic acid. Then, the assays with 25 and 20 % (w/w) of corn cob obtained 7.37 and 4.57 g/L, respectively. The highest corn cob concentration during autohydrolysis also originated more furfural, at 4.30 g/L. Then 25 % (w/w) of corn cob obtained 1.29 g/L and with 20 % (w/w) corn cob 1.34 g/L of furfural were present. The combination of these molecules causes lower specific growth rate and the biomass yield in fermentation processes being then harmful to the yeast growth, (Palmqvist *et al.*, 1999). Therefore, a minimal concentration of these compounds is desired, while maintaining a high amount of potential xylose to be converted into xylitol. 25 % (w/w) of corn cob during autohydrolysis can provide with the highest xylose concentration, and with lower acetic acid and furfural than the ones present in the hydrolysate from 30 % of corn cob during the pretreatment. When comparing these results with the obtained by Baptista *et al.*, 2018, in which 27.82 g/L of xylose and 2.83 g/L of acetic acid were obtained, the potential xylose concentration was superior, as expected, however the concentration of acetic acid was also substantially higher.

Table 4 - Autohydrolysed corn cob fraction composition with multiple solid concentrations during the autohydrolysis and assessment of potential compounds present after a second hydrolysis of the liquid fraction. ND – Not detected.

Solid concentration during autohydrolysis	**20 %(w/w)**	**25 % (w/w)**	**30 % (w/w)**
Solid Fraction			
Solid yield (%)	57.7	60.0	57.7
Glucan (% (w/w))	48.6 ± 0.6	43.8 ± 0.6	44.4 ± 0.1
Xylan (% (w/w))	16.8 ± 0.1	17.0 ± 0.2	18.3 ± 0.3
Arabinan (% (w/w))	1.21 ± 0.10	1.29 ± 0.11	1.21 ± 0.02
Acetyl groups (% (w/w))	0.53 ± 0.01	ND	ND
Klason Lignin (% (w/w))	19.4 ± 0.6	22.2 ± 0.2	21.2 ± 0.1
Liquid Fraction			
Glucose (g/L)	0.73 ± 0.04	0.94 ± 0.05	0.71 ± 0.04
Xylose (g/L)	2.87 ± 0.14	8.88 ± 0.44	8.80 ± 0.44
Arabinose (g/L)	1.31 ± 0.07	2.81 ± 0.14	2.09 ± 0.1
Acetic acid (g/L)	1.56 ± 0.08	2.26 ± 0.11	3.65 ± 0.18
HMF (g/L)	0.44 ± 0.02	0.26 ± 0.01	0.75 ± 0.04
Furfural (g/L)	1.34 ± 0.07	1.29 ± 0.06	4.30 ± 0.21
Gluco-oligosaccharides (g/L)	1.28 ± 0.3	4.59 ± 0.01	2.31 ± 0.01
Xylo-oligosaccharides (g/L)	25.1 ± 1.4	38.1 ± 1.10	31.9 ± 0.08
Arabino-oligosaccharides (g/L)	1.05 ± 0.22	ND	0.50 ± 0.03
Acetil-oligosaccharides (g/L)	3.01 ± 0.16	5.11 ± 0.02	5.37 ± 0.43
Sum of liquid components*			
Potential glucose (g/L)	2.01	5.53	3.02
Potential xylose (g/L)	27.9	47.0	40.7
Potential arabinose (g/L)	2.35	2.81	2.59
Potential acetic acid (g/L)	4.57	7.37	9.02

*potential sugars were calculated as the sum of oligosaccharides and monosaccharides.

The chemical composition of each autohydrolysis treatments was listed in **Table 4**. Relatively to the glucan concentration of the pretreated corn cob (or solid phase), 20 % (w/w) of corn cob loading in the autohydrolysis treatment contained 48.61 % (w/w) of this polysaccharide, the highest concentration among the samples. This result, as well as the recovery data, imply that increasing the corn cob concentration during the pretreatment, starting at 20 % (w/w), does not influence the glucan concentration in the solid fraction in a positive way.

Glucan, xylan and Klason lignin recoveries after autohydrolysis treatments in the solid phase are present in **Table 5**. In it, it is possible to observe that 86.9 % of glucan were recovered, relatively to the glucan present in the raw material in the experiment using 20 % (w/w) of corn cob solids. This represents the highest glucan recovery of the three tested corn cob percentages and when this concentration was increased this parameter was lower, reaching 79.3 % with 30 % (w/w) of corn cob. This result implies that increasing the concentration of the raw material used during the pretreatment leads to a decrease in glucan recovery. This fact can be due to increase of acetic acid concentration in the liquid phase being the severity of treatment also increased.

On the other side, xylan recovery follows the opposite trend, where the highest recovery was obtained using 30 % (w/w) of corn cob during the pretreatment and recovered 30.4 % of xylan from the raw material. The lowest xylan recovery was originated from the condition with 20 % (w/w) of corn cob, resulting in 27.9 %. It is notable that these values are not near totality (100 %) because most of the xylan was extracted from the solid to the liquid fraction.

Relatively to the Klason lignin, 56.4 % remained while using 25 % (w/w) of corn cob during the pretreatment and this was the highest recovery out of the three tested concentrations. The lowest lignin recovery was obtained with 20 % (w/w) of raw material during autohydrolysis, resulting in 47.3 %, and with 30 % (w/w) of corn cob during the pretreatment 51.7 % of lignin were preserved.

Table 5 – Solid fraction recovery data of different concentrations of raw material (corn cob) during autohydrolysis pretreatment at 205 ºC.

Corn cob during autohydrolysis	20 % (w/w)	25 % (w/w)	30 % (w/w)
Glucan recovery (%)	86.9	81.3	79.3
Xylan recovery (%)	27.9	29.4	30.4
Klason lignin recovery (%)	47.3	56.4	51.7

In order to remove inhibitory compounds, detoxification processes have been evaluated in the lignocellulosic hydrolysates. Therefore, an ionic exchange detoxification of the liquid fraction can be performed in order to eliminate the acetic acid present. However, this process can only eliminate with acetic acid present in the liquor, not the acetyl groups linked to hemicellulose, which means the acetic acid from the liquid fraction of the 25 % (w/w) of corn cob condition can be lowered to 4.63 g/L. Total removal of acetic acid from the liquid fraction can be performed with an acid hydrolysis before the detoxification.

4.1.2. Effect of solid loading of corn cob in autohydrolysis treatment on enzymatic saccharification

In order to evaluate the effect of pretreament at different solid loadings on the cellulose saccharification, pretreated corn cob was used as a substrate in enzymatic hydrolysis assays using the commercial enzyme Cellic CTec2 (**Figure 3**). Moreover, the liquid phase (or hydrolysate) obtained from autohydrolysis treatment was also used as a reaction medium (conforming the whole slurry). The hydrolysate on enzymatic saccharification has an inhibitory effect on enzymatic activity (Oliveira et al., 2018). For this reason and for comparative purposes, enzymatic assays were also carried out using water as reaction media. Thus, enzymatic assays were carried out at 45 ºC with 24 FPU of CellicCtec2/g of substrate, an enzyme cocktail that contained cellulases and xylanases. The xylanase activity could also convert the oligosaccharides present into glucose and xylose respectively. The enzymatic hydrolysis could substitute the dilute acid hydrolysis that is normally used to obtain hydrolysates enriched in xylose.

In fact, the enzymatic hydrolysis of pretreated corn cob in water (using 5 % (w/w) of solids) (**Figure 5**) showed different ranges of glucose and xylose concentrations. Although the highest amount of glucose was observed using a solid fraction obtained from the autohydrolysis using 20 % (w/w) of corn cob loading, which was 35.2 g/L, the concentration of xylose was obtained with a solid from 25 % (w/w) of corn cob during the autohydrolysis: 13.2 g/L. It is also notable that in every assay the enzymatic reactions were successful, meaning that the glucose and xylose concentrations reached the potential calculated values.

The enzymatic hydrolysis (using 5 % (w/w) solids) from 20, 25 and 30 % (w/w) of corn cob during autohydrolysis resulted in, at 96 h of incubation at 45 ºC, an acetic acid concentration of 1.1, 1.1 and 0.9 g/L, respectively. These results are very similar between them and signify that the addition of a solid loading to a liquid fraction not only provides with glucose and xylose, but also transfers a small amount of acetic acid. This may have to be considered if the final concentration can achieve values higher than the concentration that start to inhibit the growth of the utilized microorganism.

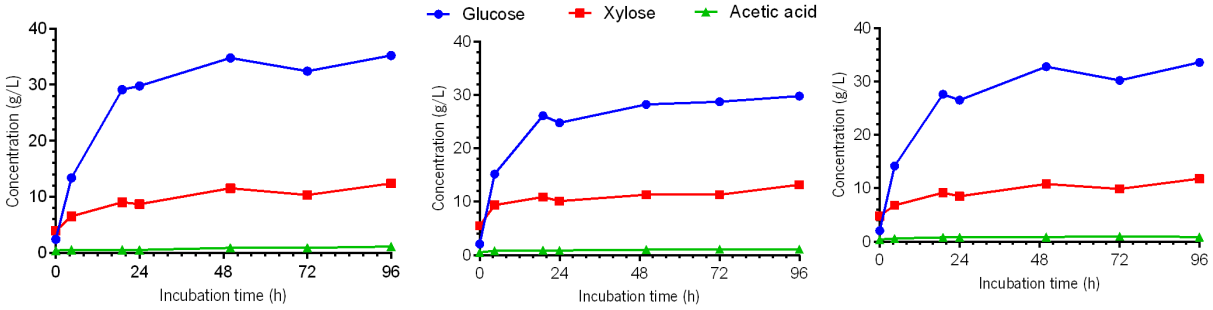


Figure 5 – Enzymatic hydrolysis at 45 ºC of 5 % (w/w) of solid fraction, with water as the reaction medium, of autohydrolysed corn cob at different concentrations during autohydrolysis with 205 ºC of Tmax and using 24 FPU of CellicCtec2/g of substrate. A: autohydrolysate from 20 % (w/w) corn cob; B: autohydrolysate from 25 % (w/w) corn cob; C: autohydrolysate from 30 % (w/w) corn cob.

When 5 % (w/w) of solid fraction (pretreated corncob) was mixed with the liquid fraction (hydrolysate) (**Figure 6 and Table 6**), together with the enzyme mixture, it is possible to observe that the highest concentration of glucose was obtained with 20 % (w/w) of corn cob in the autohydrolysis, which is the lowest concentration of corn cob used in this experiment. Furthermore, the concentration of xylose obtained was the lowest with 20 % (w/w) of corn cob in the autohydrolysis, which was to be expected due to the liquid fraction providing a lower amount of xylo-oligosaccharides to be hydrolysed. Additionally, the concentration of xylose was similar with 25 and 30 % (w/w) of corn cob in the autohydrolysis, being 53.8 and 53.4 g/L of xylose at 96 hours. This means that 25 and 30 % of corn cob obtain the same concentration, which provides the same result as the quantitative acid hydrolysis (**Table 4**), and that adding the final 5 % (w/w) of corn cob does not increase the xylose concentration.

The enzymatic hydrolysis of different concentration corn cob autohydrolysates with the liquid fraction present, in the beginning of the assay, from 20, 25 and 30 % (w/w) of corn cob originated 3.9 g/L, 5.8 and 6.4 g/L of acetic acid, respectively, and at 96 h of experiment there were 5.6, 7.9 and 8.9 g/L, respectively. During this enzymatic hydrolysis, the acetic acid obtained was higher when the hydrolysate and 5 % (w/w) of solid fraction of autohydrolysed corn cob was more concentrated (30 % (w/w) solids). That is, when the concentration of corn cob utilized in the autohydrolysis was higher, it originated a more concentrated liquid fraction and in more production of acetic acid during the actual enzymatic hydrolysis, which resulted in

more total acetic acid. These acetic acid concentrations are similar to the values obtained when analysing the liquid fraction through acid hydrolysis (**Table 4**), which implies that with an enzymatic hydrolysis all the acetic acid is released.

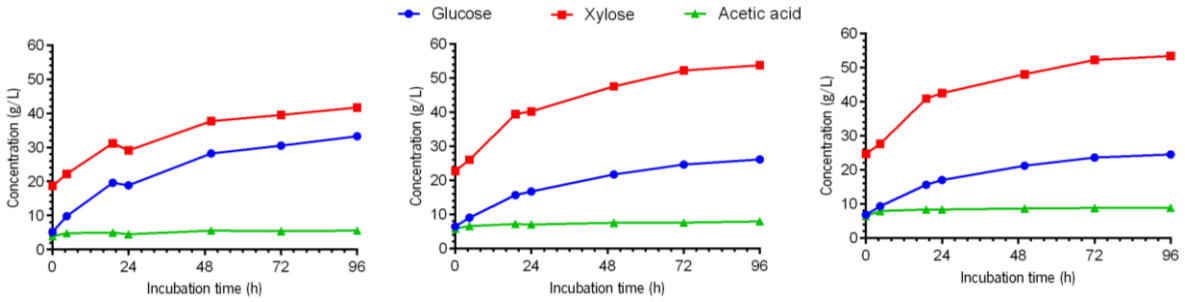


Figure 6 – Time course of enzymatic hydrolysis at 45 ºC using 5 % (w/w) of pretreated corn cob with autohydrolysis liquor as reaction media (whole slurry) and 24 FPU/g of CellicCTec2 enzyme, with 205 ºC of Tmax during pretreatment. A: from autohydrolysis with 20 % (w/w) of corn cob; B: from autohydrolysis with 25 % (w/w) of corn cob; C: from autohydrolysis with 30 % (w/w) of corn cob.

When comparing the sugar concentration obtained during enzymatic hydrolysis with only solids and with the whole slurry (**Table 6**), the first originated glucose yields slightly superior when compared to the second, in spite of a small amount of glucose present in the liquid fraction. This is the result of a enzymatic hydrolysis inhibition of the cellulase activity possibly due to a high presence of xylooligosaccharides and another degradation products from the autohydrolysis process (Kovacs *et al.*, 2009). On the other hand, the xylose yields of the hydrolysis of only solid fraction were similar to the assays that contained both solid and liquid fractions, which implies that the hydrolysis of xylooligosaccharides was not compromised by the addition of the liquid fraction. In these experiments, the condition that present the highest xylitol yield contained 25 % (w/w) corn cob during the pretreatment.

When analysing the xylan recovery considering both fractions of the hydrolysate it is visible that the condition that preserved more xylan of the provided in the raw material, at 61.8 %, contained 25 % (w/w) of corn cob during the pretreatment. The condition with 20 and 30 % (w/w) solids during autohydrolysis had a xylan recovery of 59.4 % and 57.5 %, respectively. This result signifies that the hydrolysate with 25 % (w/w) corn cob maintains the highest amount of xylan during the autohydrolysis.

Table 6 – Final glucose and xylose concentrations after enzymatic hydrolysis assays with 24 FPU of CellicCtec2/g of substrate of solid autohydrolysed at 205 ºC but at different concentrations of 5 % (w/w) solids or whole slurry with 5 % (w/w) solids. FGS: final glucose concentration with solid; FGWS: final glucose concentration with whole slurry; Y_{GS}: glucose yield with solid; Y_{GWS}: glucose yield with whole slurry; FXS: final xylose concentration with solid; FXWS: final xylose concentration with whole slurry; Y_{GS}: xylose yield with solid; Y_{GWS}: xylose yield with whole slurry.

Corn cob concentration during autohydrolysis	20 % (w/w)	25 % (w/w)	30 % (w/w)
FGS (g/L)	35.2	29.8	33.6
FGWS (g/L)	33.3	26.1	24.5
Y_{GS} (%)	100	95	100
Y_{GWS} (%)	91	71	70
FXS (g/L)	12.4	13.2	11.8
FXWS (g/L)	41.7	53.8	53.4
Y_{XS} (%)	93	98	91
Y_{XWS} (%)	92	96	89

When analysing these results, the condition that originates the culture medium with the desired composition for microbial growth contained 25 % (w/w) of corn cob during autohydrolysis pretreatment, and presented, after an enzymatic hydrolysis assay, 53.8 g/L of potential xylose, 26.1 g/L of potential glucose and 7.9 g/L of acetic acid. This condition was selected for further research of operational conditions during the pretreatment. It is notable that the acetic acid of the liquid fraction can be eliminated with an ionic exchange detoxification that eliminates the free acetyl groups within the liquor, removing up to 2.26 g/L of the potential acetic acid.

4.1.3. Effect of temperature of autohydrolysis treatment on biomass fractionation

In the previous section, different concentrations of corn cob was evaluated to obtain a hydrolysate enriched in hemicellulose-derived compounds (mainly xylose and xylooligosaccharides), in which treatment with 25% of solids was shown as the most promising condition taking into account the xylan mass balance. Nevertheless, 17 % of xylan (**Table 4**) remain in solid phase after autohydrolysis treatment, which represents 30 % of xylan in the raw material. Other strategy to increase the xylan extraction implies the rise of temperature of treatment. Therefore, an evaluation of the increase of temperature during autohydrolysis was

performed. In this assay, the treatment was carried out in non-isothermal regimen at Tmax between 205-211ºC.

The chemical composition of pretreated corn cob are present in the **Table 7** and it can be observed that the amount of glucan obtained in the solid fraction is altered as the Tmax increases. In fact, the concentration increases as the temperature increases, up to 209 ºC (53.7 %) and then, with 211 ºC, the glucan concentration decreases. This result implies that the use of a Tmax of 209 ºC provides with the highest amount of glucan in the solid fraction.

Relatively to the xylan concentration in the solid fraction, the Tmax of 205 ºC originated the highest concentration of xylan, with 18.1 % (w/w). It is observed that xylan decreases with of the temperature, achieving 4 % (w/w) of xylan at 211 ºC.

Additionally, xylose concentration in the autohydrolysis liquor increased with the incremented temperature. However, the concentration of xylo-oligosaccharides does not follow the same pattern, decreasing with the temperature increment, which leads to the potential liquid fraction concentration of xylose to be superior with the temperature of 205 ºC at 46.99 g/L. When comparting to the literature, Garrote *et al.*, 2008 obtained autohydrolysates that followed the same pattern, in which the increase in temperature presented lower concentrations of oligosaccharides but higher concentration of xylose, furfural and HMF in the liquid fraction. The increase of autohydrolysis temperature implied a higher solubilisation of xylan, however, the recovery of xylan as xylose and xylooligosaccharides was maximal at 205 ºC. In this sense, this temperature was selected as the most suitable for xylitol production.

Furthermore, with the increase of the temperature of autohydrolysis the potential concentration of acetic acid in the liquid fraction also rises, reaching 8.95 g/L at a Tmax of 211 ºC, possibly due to the increase in severity of the process, leading to more acetyl groups being released from the solid fraction and to be converted into acetic acid.

Glucan, xylan and Klason lignin recoveries of different treatments are present in **Table 8** and the results reveal that an increase in temperature leads to an increase in glucan recovery, up to 209 ºC, which maintained 92.2 % of glucan after autohydrolysis. In contrast, the recovery of xylan at the solid fraction was superior with lower Tmax, with the maximum of recovery at 205 ºC, at 29.4 %, and the lowest at a Tmax of 211 ºC, with 6.3 % of xylan recovered. This

may imply that a higher maximum temperature is able to extract more xylan of the solid fraction to the liquid fraction.

Table 7 – Autohydrolysed corn cob fraction composition with several maximum temperatures (Tmax) of autohydrolysis and assessment of potential compounds present after a second hydrolysis of the liquid fraction. ND – Not detected.

Tmax (ºC)	**205**	**207**	**209**	**211**
Solid Fraction				
Solid yield (% (w/w))	60.0	53.5	55.4	54.1
Glucan (% (w/w))	43.8 ± 0.6	53.5 ± 0.01	53.7 ± 2.7	44.2 ± 0.4
Xylan (% (w/w))	17.0 ± 0.2	10.3 ± 0.3	10.4 ± 1.1	4.05 ± 0.07
Arabinan (% (w/w))	1.29 ± 0.11	0.63 ± 0.16	ND	ND
Acetyl groups (% (w/w))	ND	ND	ND	ND
Klason Lignin (% (w/w))	22.2 ± 0.2	24.2 ± 0.8	23.6 ± 0.9	26.3 ± 0.7
Liquid Fraction				
Glucose (g/L)	0.94 ± 0.05	1.70 ± 0	0.70 ± 0	1.67 ± 0
Xylose (g/L)	8.88 ± 0.44	10.8 ± 0	11.9 ± 0	17.2 ± 0
Arabinose (g/L)	2.81 ± 0	2.72 ± 0	3.28 ± 0	2.78 ± 0
Acetic acid (g/L)	2.26 ± 0	2.38 ± 0	3.15 ± 0	3.62 ± 0
HMF (g/L)	0.26 ± 0	0.31 ± 0	0.39 ± 0	0.42 ± 0
Furfural (g/L)	1.29 ± 0	1.50 ± 0	1.68 ± 0	1.93 ± 0
Gluco-oligosaccharides (g/L)	4.59 ± 0	1.68 ± 0	4.10 ± 0	1.49 ± 0
Xylo-oligosaccharides (g/L)	38.1 ± 0	31.7 ± 0	20.4 ± 0	20.1 ± 0
Arabino-oligosaccharides (g/L)	0.00 ± 0	1.26 ± 0	4.45 ± 0	0.15 ± 0
Acetil-oligosaccharides (g/L)	5.11 ± 0	3.56 ± 0	5.54 ± 0	3.71 ± 0
Sum of liquid components				
Potential glucose (g/L)	5.53	3.38	4.80	3.16
Potential xylose (g/L)	47.0	42.5	32.3	37.3
Potential arabinose (g/L)	2.81	3.98	7.73	2.93
Potential acetic acid (g/L)	7.37	7.70	8.68	8.95

Table 8 – Solid fraction recovery data of 25 % (w/w) of raw material (corn cob) during autohydrolysis pretreatment at different Tmax.

Corn cob during autohydrolysis	205 ºC	207 ºC	209 ºC	211 ºC
Glucan recovery (%)	81.3	88.8	92.2	74.0
Xylan recovery (%)	29.4	15.9	16.5	6.3
Klason lignin recovery (%)	56.4	54.7	55.3	60.1

4.1.4. Effect of temperature of autohydrolysis treatment on enzymatic hydrolysis

In order to evaluate the influence of autohydrolysis temperature on enzymatic hydrolysis of pretreated biomass, the solid phases obtained from pretreatements shown in **Table 7** using 25 % (w/w) of corn cob. The enzymatic hydrolysis assays were performed at 45 ºC with 5 % (w/w) of solid fractions with 24 FPU/g of Cellic Ctec2 (under conditions used in the previous section) using autohydrolysis liquors or water.

Figure 7 shows enzymatic hydrolysis assays using water as reaction water for 96 h of saccharification. The concentration of glucose at 96 hours of incubation was the highest in assays using Tmax of 209 ºC obtaining 43.2 g/L and the lowest concentration (29.8 g/L of glucose) was obtained in the enzymatic hydrolysis using pretreated corn cob at 205 ºC. These results reveal the influence of severity of treatment on enzymatic susceptibility of cellulose, which increases with the severity. These results can be compared to those obtained by other authors, such as Garrote *et al.*, 2008 and Rivas *et al.*, 2006, in which the glucose concentration also increased with the increment in temperature, reaching a maximum glucose concentration with a Tmax 212 ºC, and with 216 ºC the concentration was lower.

On the other hand, the highest concentration of xylose in enzymatic hydrolysis assays was 13.2 g/L, obtained from the pretreated corn cob at 205 ºC. Otherwise, the lowest xylose concentration (5.1 g/L) was obtained in the enzymatic hydrolysis of pretreated corn cob from autohydrolysis treatment at 211 ºC. The xylose concentration is related to content of xylan in pretreated samples of corn cob.

Relatively to the concentration of acetic acid in the solid fractions, after enzymatic hydrolysis, there were released 1.1, 0.6, 0.6 and 0.3 g/L of this compound to the water, implying that the highest amount of acetic acid originated from a peak temperature of 205 ºC.

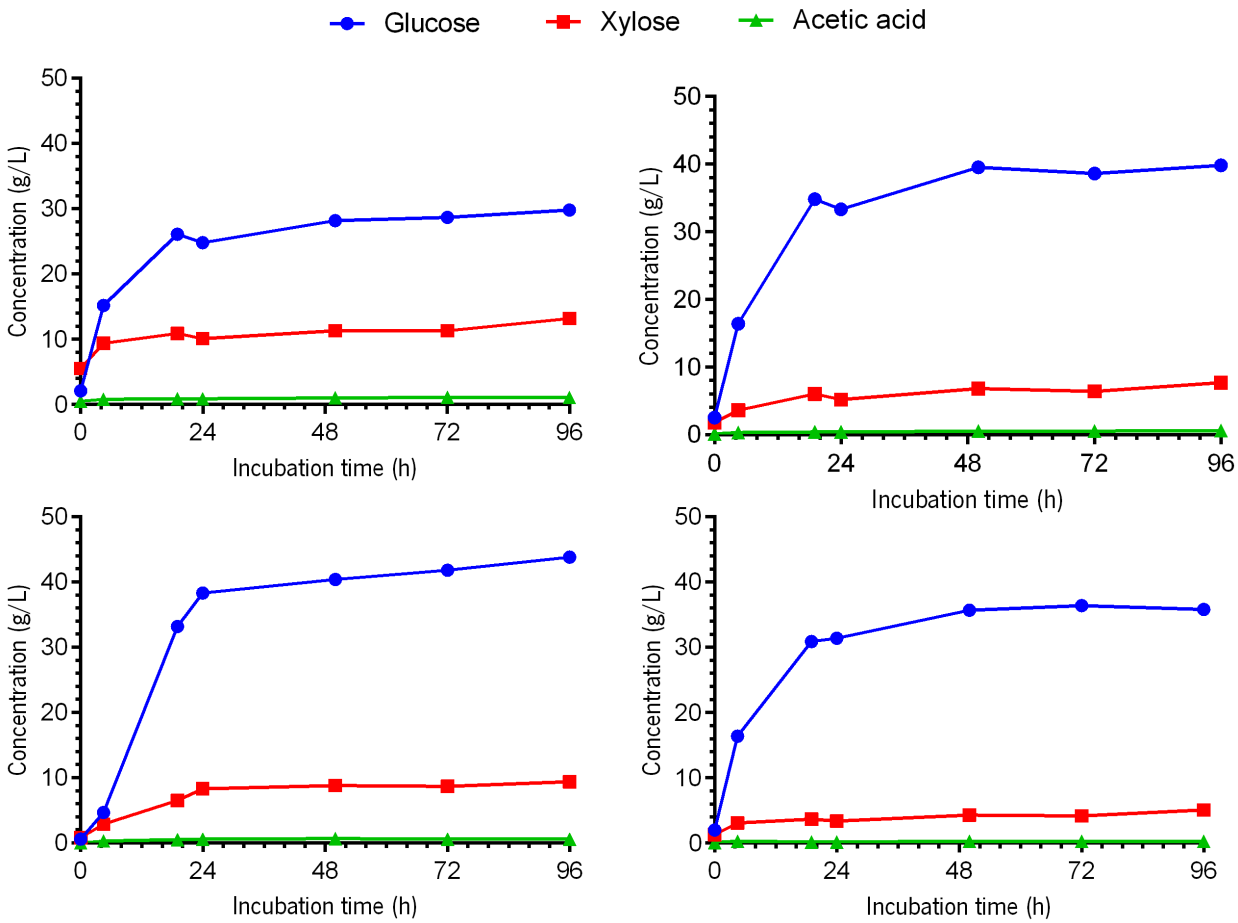


Figure 7 – Enzymatic hydrolysis of 5 % (w/w) solid fraction, with water as the reaction medium, of autohydrolysed corn cob at different peak temperatures during autohydrolysis using 24 FPU of CellicCtec2/g of substrate. A: autohydrolysate from 25 % (w/w) corn cob with 205 ºC of Tmax; B: autohydrolysate from 25 % (w/w) corn cob with 207 ºC of Tmax; C: autohydrolysate from 25 % (w/w) corn cob with 209 ºC of Tmax; D: autohydrolysate from 25 % (w/w) corn cob with 211 ºC of Tmax.

In the enzymatic hydrolysis of corn cob whole slurry at different autohydrolysis temperatures (**Figure 8**), the highest concentration of glucose (28.1 g/L) was obtained at 207 ºC, followed by 211 ºC (27.3 g/L) and by 205 ºC (26.1 g/L). The lowest concentration of glucose (23.1 g/L) was obtained at 209 ºC. When comparing with the results obtained with the enzymatic saccharification using water as the reaction medium, the assays with liquid fraction presented a lower maximal concentration, due to the presence of products of the raw material degradation, which possesses an inhibitor effect on the enzymatic hydrolysis (Kovacs *et al.*, 2009).

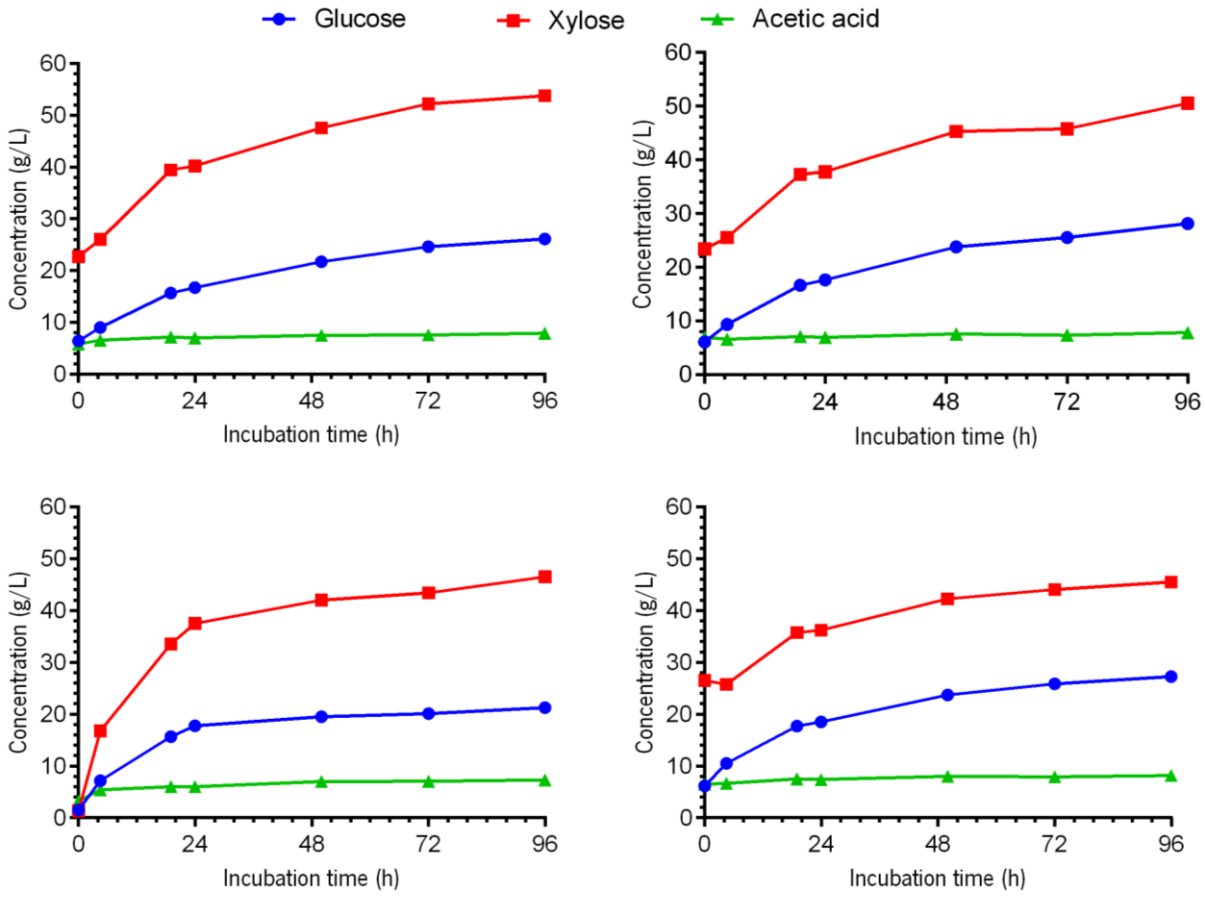


Figure 8 - Enzymatic hydrolysis of 5 % (w/w) solid fraction in liquid fraction of autohydrolysed corn cob at different peak temperatures during autohydrolysis using 24 FPU of CellicCtec2/g of substrate. A: autohydrolysate from 25 % (w/w) corn cob with 205 ºC of peak temperature; B: autohydrolysate from 25% (w/w) corn cob with 207 ºC of peak temperature; C: autohydrolysate from 25 % (w/w) corn cob with 209 ºC of peak temperature; D: autohydrolysate from 25 % (w/w) corn cob with 211 ºC of peak temperature.

Regarding the concentration of xylose after the enzymatic hydrolysis (**Table 9**), the highest concentration of xylose (53.8 g/L) was obtained at 205 ºC, at, followed by 207, 209 and 211 ºC, with 50.5, 46.5 and 45.5 g/L of this sugar, respectively. Comparing these results with the ones obtained from a hydrolysis with solids in water, the enzymatic hydrolysis followed the same pattern, where an increase in the temperature of the treatment led to a decrease in produced xylose. However, the values are superior to the ones previously obtained, which is due to the addition of the liquid fraction in the enzymatic hydrolysis assays. This fraction is mainly composed of xylooligosaccharides and xylose, which means it provides with an increase of the xylose concentration compared to a hydrolysis that only contained solid fraction. Considering the addition of xylan prevenient from the solid fraction, in every assay the conversion into xylose is complete, with the exception of the Tmax of 205 ºC, in which the xylose yield was 0.95 g xylose/ g xylan. It is notable that this condition originated the highest final xylose

concentration in the medium. These results reveal that the enzymatic hydrolysis of the liquid and solid fractions was not compromised by the addition of the liquor.

Table 9 – Final glucose and xylose concentrations after enzymatic hydrolysis assays with 24 FPU of CellicCtec2/g of substrate of 25 % (w/w) solid during autohydrolysis with 5 % (w/w) solids or 5 % (w/w) solids in the liquid fraction but at different Tmax during pretreatment. FGS: final glucose concentration with solid; FGWS: final glucose concentration with whole slurry; Y_{GS}: glucose yield with solid; Y_{GWS}: glucose yield with whole slurry; FXS: final xylose concentration with solid; FXWS: final xylose concentration with whole slurry; Y_{GS}: xylose yield with solid; Y_{GWS}: xylose yield with whole slurry.

Tmax during autohydrolysis	205 ºC	207 ºC	209 ºC	211 ºC
FGS (g/L)	29.8	39.8	43.8	35.8
FGWS (g/L)	26.1	28.1	21.3	27.3
Y_{GS} (%)	95	100	100	100
Y_{GWS} (%)	71	67	49	79
FXS (g/L)	13.2	7.7	9.4	5.1
FXWS (g/L)	53.8	50.5	46.5	45.5
Y_{XS} (%)	98	99	97	99
Y_{XWS} (%)	95	100	100	100

Considering its higher potential xylose concentration, the condition with 205 ºC during autohydrolysis was selected for further assays. However, relatively to the concentration of acetic acid produced during this enzymatic hydrolysis, the results ranged from 7.3 g/L and 8.2 of acetic acid present at the end of incubation, which are values superior to the concentration that inhibits the growth of the yeast, and this is not desirable for a fermentation that utilizes these materials. This inhibition results in the lack of capacity of the ATPase to counteract the inflow of protons into the cell and high intracellular acid concentrations result in cell death (Palmqvist *et al*., 1999). With this in mind, a detoxification of the liquid fraction had to be performed in order to utilize this culture medium through enzymatic hydrolysis, microbial growth and conversion, which was accomplished by the addition of two ionic resins to remove the free acetyl groups.

4.2. Xylitol production from corn cob whole slurry

Previous results carried out in our research group showed an interesting strategy for the production of xylitol by simultaneous saccharification and fermentation process (Baptista et al. 2018). However, operational conditions such as temperature, enzyme loading and percentage of substrate were not optimized. In this work, these operational conditions were evaluated and a complete factorial design was proposed for the optimization of xylitol production. The

recombinant *S. cerevisiae* PE2 GRE-3 strain (overexpressing the endogenous GRE3 gene from the yeast) was selected for the following assays. This strain was developed by Baptista *et al.*, 2018, in which three different strategies were carried out to express three different enzymes: a xylose reductase wild-type, a xylose reductase mutant and a non-specific aldose reductase. The latter presented superior productivity and produced a higher xylitol concentration.

4.2.1. Temperature effect on xylitol production

Traditionally, in a study where *S. cerevisiae* is cultivated, the selected temperature is 30 ºC, due to being close to the optimum temperature of growth for the yeast. However, a hydrolysis of xylan and xylooligosaccharides present in the liquid fraction must be performed in order to maximize the xylose concentration. This process is accomplished by the use of an enzyme mixture named CellicCtec2, which contains enzymes with an optimal temperature from 45 to 50 ºC (Novoenzymes A/S, 2010). Additionally, the specific growth of this yeast is optimal within the range of 29 to 35 ºC. Both of these factors may mean that increasing the incubation temperature might increase the xylitol produced without inhibiting its growth (Walsh and Martin, 1977). Therefore, duplicate cultivations at 30 and 35 ºC of *S. cerevisiae* with autohydrolysed and partially detoxified liquor (with a cationic and an anionic resin) of corn cob were performed in order to verify this hypothesis (**Figure 9**) and, at 30 ºC, the final concentration of xylitol was 48.7 g/L (**Figure 9A**) whereas at 35 ºC it was 51.7 g/L (**Figure 9B**). The xylitol yield was also superior in the assay performed at 35 ºC with total conversion with 35 ºC and 0.95 g xylitol/ g xylose with the 30 ºC assay. Additionally, the productivity was also slightly higher when 35 ºC was utilized, at 0.36 g/L/h, versus 0.34 g/L/h obtained with 30 ºC. Although this may not be a substantial increase, further cultivations were performed at 35 ºC in order to maximize the production of xylitol.

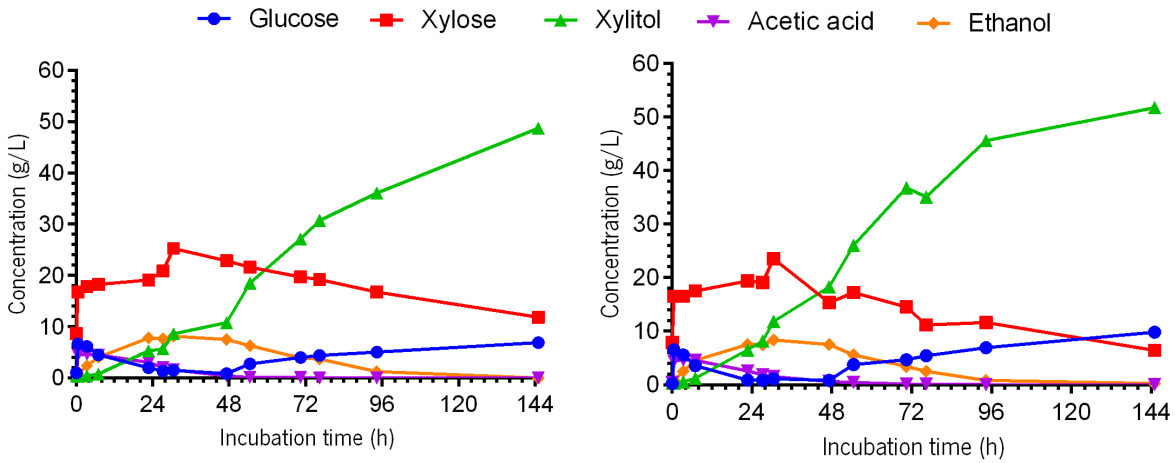


Figure 9 – Cultivation of *S. cerevisiae* PE-2-GRE3 with 86.53 % (v/v) detoxified liquid fraction and 5% (w/w) solid fraction of autohydrolysed raw material at 25 % (w/w) corn cob at 205 ºC of Tmax during pretreatment, 20 g/L of peptone, 10 g/L of yeast extract, 150 mg/L of geneticin, 11 g/L of cells and 24 FPU CellicCtec2/g of substrate inserted at 0 hours of incubation time. A: 30 ºC; B: 35 ºC.

4.2.2. Use of pre-saccharification on xylitol production

In this work, the growth of *S. cerevisiae* was previously achieved with SSF, in which the glucose released is immediately consumed, which signifies that the sugar concentration in the medium when the inoculum was added is low. Increasing the initial concentration of those sugars might be more beneficial to the growth of the yeast, as it may be able to utilize more nutrients and, consequently, have more cells converting xylose into xylitol. Therefore, with the purpose of increasing the amount of xylitol produced, the yeast was cultivated with the addition enzymes inserted 24 hours before the inoculum, maintaining the flasks at 45 ºC, and then decreased the temperature up to 35 ºC to introduce the cells (**Figure 10**). By observing the results with the presence of the 24 h pre-saccharification, where it is possible to verify that with 8 FPU of CellicCtec2/g of substrate (**Figure 10A**) where obtained 32.4 g/L of xylitol with the 24-hour pre-saccharification and 39.0 g/L without it (**Figure 10B**). Additionally, with 24 FPU/g and the pre-saccharification resulted in 38.0 g/L of xylitol (**Figure 10C**), whereas 44.4 g/L were obtained with only the simultaneous saccharification and fermentation (**Figure 10D**). This implies that the use of a pre-saccharification is not beneficial for the growth of the yeast, having obtained the lowest production of the studied polyol, perhaps, due to the catabolite repression that is induced by the high concentration of glucose which does not allow the uptake of xylose through the glucose transporters (Lee *et al.*, 2000). The use of a SSF process was, therefore, selected for further assays.

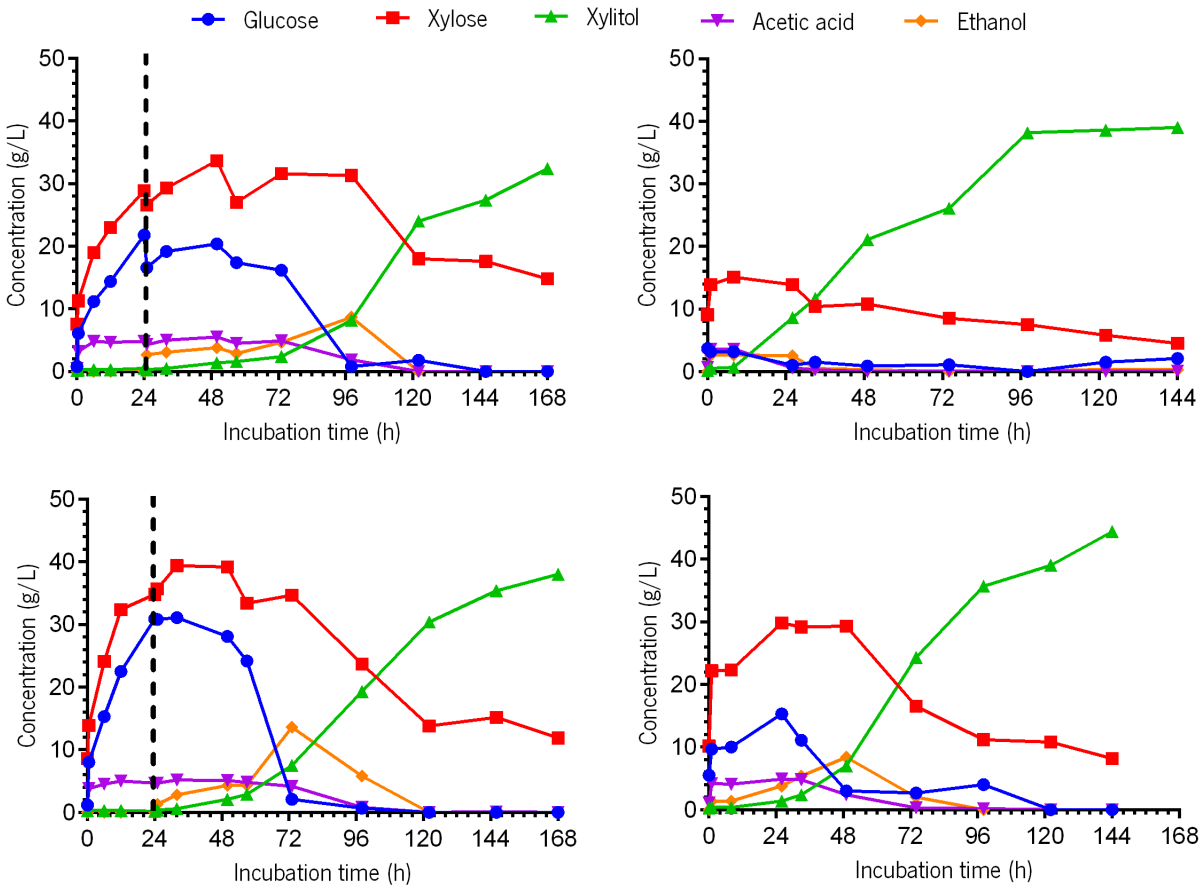


Figure 10 – Cultivation of *S. cerevisiae* PE-2-GRE3 at 35 ºC with 86,53 % (v/v) detoxified liquid fraction and 8 % (w/w) solid fraction of autohydrolysed corn cob at 25 % (w/w) corn cob and 205 ºC of Tmax during pretreatment, 20 g/L of peptone, 10 g/L of yeast extract, 150 mg/L of geneticin, 24 FPU CellicCtec2/g of substrate, 11 g/L of cells. A: 24 hours of PSSF, 8 enzyme FPU/ g substrate; B: SSF, 8 enzyme FPU/ g substrate; C: 24 hours of PSSF, 24 enzyme FPU/ g substrate; D: SSF, 24 enzyme FPU/ g substrate; The dashed line represents the time where the inoculum was added.

4.2.3. Optimization of xylitol production by experimental design

In this work, the pretreated corn cob from autohydrolysis treatment provides the main source of carbon of the culture medium and it is hydrolysed by an enzyme mixture during the incubation. Therefore, these two components may be manipulated to possibly increase the concentration of xylitol produced. So, an experimental design that provided a solid loading from 4 to 12 % (w/w) and an enzyme concentration from 8 to 24 FPU/ g of substrate was constructed with equation (3) from Materials and Methods.

With the purpose of optimizing the solid fraction and enzyme concentration, an incubation with the conditions present in **Table 10** was performed and the results are present in **Table 10 and Figure 11**. The results reveal that every combination of conditions were able to produce xylitol, even if a small amount or only after more than 100 hours. The settings that

obtained the lowest concentration of xylitol at 143 hours contained 12 % (w/w) solids and 16 FPU/g of enzyme (with 11.1 g/L of xylitol), 12 % (w/w) solids and 24 FPU/g (with 12.3 g/L), followed by 4 % (w/w) solids and 8 FPU/g (with 22.3 g/L) and 12 % (w/w) solids and 8 FPU/g (with 24.6 g/L). The conditions that originated the highest xylitol concentrations contained 8 % (w/w) solids and 16 FPU/g (with 43.7 and 40.3 g/L), 4 % (w/w) solids and 24 FPU/g (with 40.4 g/L), 8 % (w/w) solids and 16 FPU/g (with 40.0 g/L) and 8 % (w/w) solids and 8 FPU/g (with 39.0 g/L). These results reveal that a high solid concentration influences the production of xylitol negatively and this may be explained by the amount of acetic acid released to the medium when the solid loading is superior, increasing the inhibition of the yeast growth even more than the other conditions with less solid concentration. Furthermore, when both conditions have elevated values combined it also effects the xylitol production in a negative way, which may happen due to the release of the acetic acid reaching higher levels, but also being released more rapidly giving less time for the cells to adapt.

Table 10 – Xylitol production optimization design factors and real conditions and response factors: final xylitol concentration, xylitol yield and productivity. X_1: solid concentration factor; X_2: Enzyme concentration factor.

Sample	x_1	x_2	Solid concentration (% (w/w)) (x_1)	Enzyme concentration (FPU/ g substrate) (x_2)	Final xylitol concentration (g/L) (y_1)	Xylitol yield (g xylitol/ g potential xylose) (y_2)	Productivity (g/L/h) (y_3)
1	-1	-1	4	8	22.8	0.46	0.19
2	0	-1	8	8	39.0	0.67	0.27
3	1	-1	12	8	24.6	0.37	0.17
4	-1	0	4	16	16.0	0.32	0.11
5	0	0	8	16	40.3	0.69	0.28
6	0	0	8	16	43.7	0.75	0.31
7	1	0	12	16	11.1	0.17	0.08
8	-1	1	4	24	40.4	0.81	0.28
9	0	1	8	24	40.0	0.69	0.28
10	1	1	12	24	12.3	0.19	0.09

The mathematical models that were proposed permitted to obtain a prediction of the effect of operational conditions in the response variables obtained using the SSF processes. In

fact, **Table 11** presents the regression coefficients and their statistical significance (obtained using Regression from Result Analysis of Microsoft Excel). These coefficients, with all negative values when connected to solid loading, reveal with a significance level superior to 90 %, that higher solid loadings are not beneficial to any response variable. Additionally, the coefficients associated with enzyme concentration contain generally positive coefficient, although without any significance level superior to 90 %.

Table 11 – Regression coefficients and statistical parameters measuring the correlation between responses and significance of the models. b: intercept coefficient; b_1: coefficient of solid loading; b_2: coefficient of enzyme loading; b_{11}: coefficient of solid loading squared; b_{22}: coefficient of enzyme loading squared; b_{12}: coefficient of solid and enzyme loadings.

Response variable	Xylitol concentration		Xylitol yield		Xylitol productivity	
	Coefficient	P value	Coefficient	P value	Coefficient	P value
b	38.63	0.0003	0.65	0.0007	0.26	0.0007
b_1	-5.20	0.073	-0.14	0.039	-0.04	0.091
b_2	1.78	0.455	0.03	0.554	0.002	0.924
b_{12}	-7.45	0.047	-0.13	0.082	-0.04	0.129
b_{12}	-21.71	0.003	-0.33	0.012	-0.14	0.010
b_{22}	6.44	0.135	0.10	0.248	0.04	0.227
R^2	0.93		0.90		0.88	
F	11.0		6.9		6.0	
Significance level	98 %		96 %		95 %	

Figure 11 presents the response surface data relatively to the fermentations that contained the conditions present in **Table 10**. As it is possible to be observed, xylitol concentration above 40 g/L was obtained with middle of the range values of solid loading but with either the minimal or maximal enzyme loading. Both the highest and the lowest solid concentration were not the most beneficial for xylitol production, independently of enzyme loading. On the other hand, the maximal xylitol concentration was obtained with 24 FPU/ g substrate. Therefore, the enzyme loading had a positive effect on the production of this polyol. The xylitol yield and productivity follow a similar pattern, in which the maximal conversion (values superior to 0.8 g xylitol/ g potential xylose and 0.3 g/L/h, respectively) was performed with solid loading in the middle of the range and maximal enzyme loading. The latter therefore affects positively the xylitol yield and productivity.

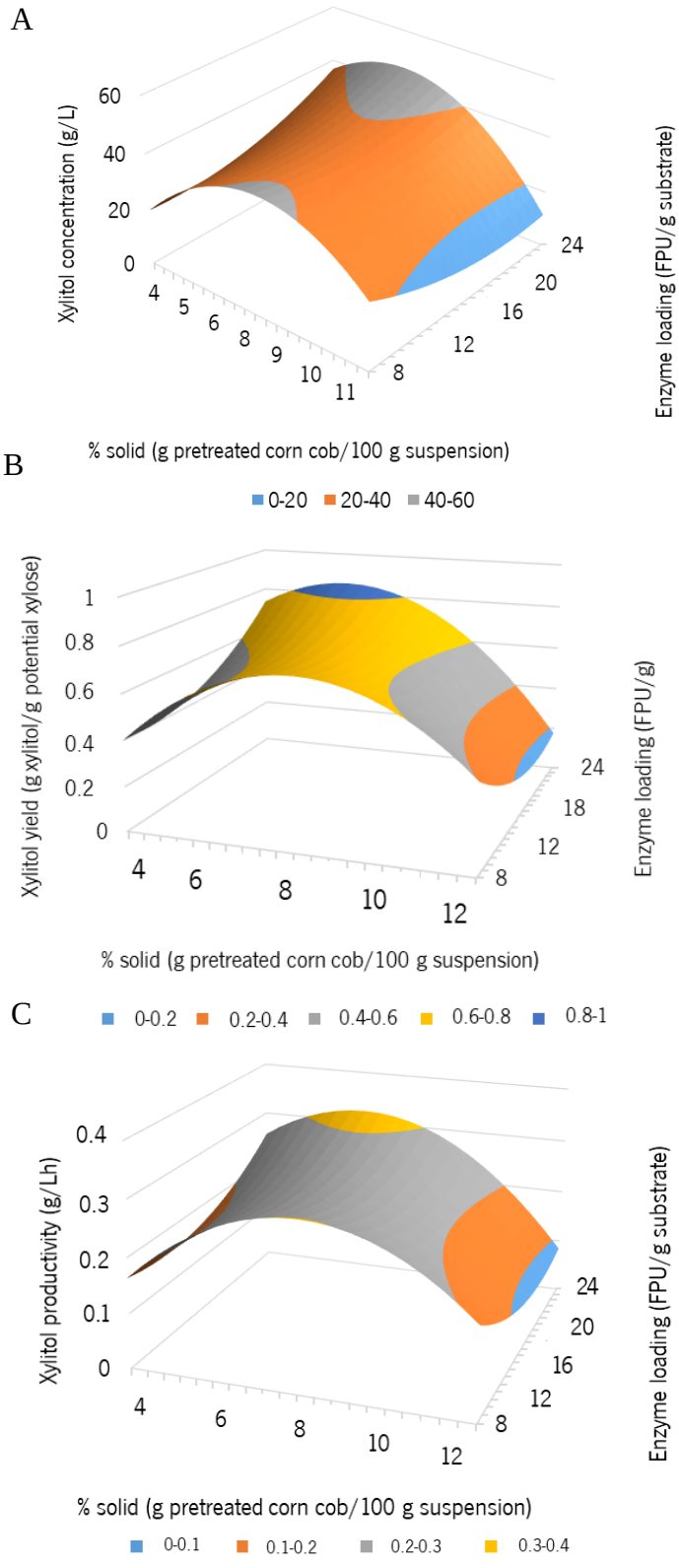


Figure 11 –Response surface as function of solid loading and enzyme loading. A: Xylitol concentration (g/L) ; B: Xylitol yield (g xylitol/ g potential xylose); C: Xylitol productivity (g/L/h).

Lastly, in order to optimize the SSF process, the design of this experiment was able to be mathematically optimized, reaching an optimum point with 6.76 % (w/w) of solid fraction and 24 FPU/ g of substrate, which would theoretically be able to produce 46.5 g/L of xylitol and 0.32 g/L/h of productivity, and these conditions were created in order to validate this model.

4.2.4. Validation of the model

The optimized conditions previously mentioned were recreated and a fermentation in duplicate was performed in order to validate these optimal conditions (**Figure 12**). This assay, after 144 hours of incubation, obtained a 42.9 g/L of xylitol, resulting in an error of 7.66 % of the expected value, and 0.30 g/L/h of productivity, an error of 6.66 % from the theoretical value. Both the xylitol concentration and the productivity possess an error within 10 % of the expected value, which verifies the sustainability of the model for reproducing and predicting experimental results, meaning that these conditions were utilized in further assays in this work.

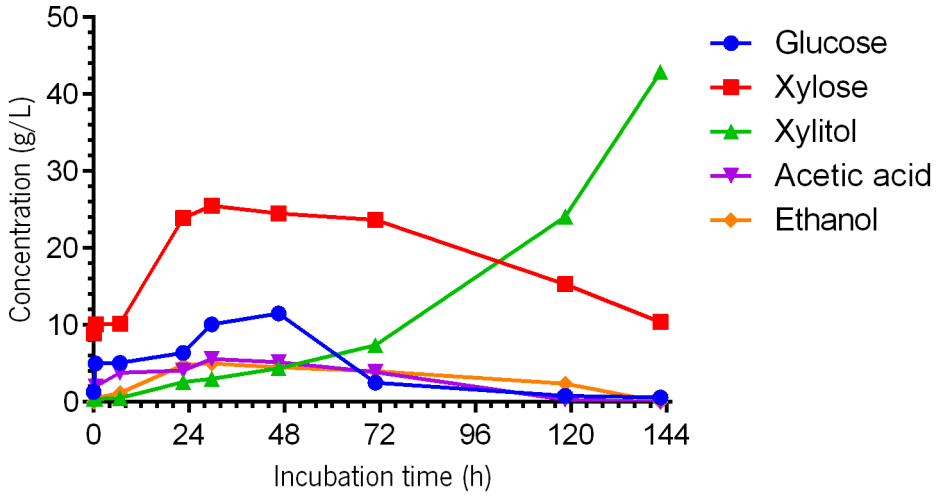


Figure 12 - Cultivation of *S. cerevisiae* PE-2-GRE3 at 35 ºC with 86,53 % (v/v) liquid fraction and 6,76 % (w/w) solid fraction of autohydrolysed corn cob at 25 % (w/w) corn cob, 20 g/L of peptone, 10 g/L of yeast extract, 150 mg/L of geneticin, 24 FPU of CellicCtec2/ g of substrate, 11 g/L of cells.

4.2.5. Acid hydrolysis and detoxification's effect on xylitol production

Previously in this work the production of xylitol has been performed with detoxified liquor by two ionic resins, without any hydrolysis before the detoxification, due to the gradual release of sugars during the cultivation being more suitable for growth than a high initial glucose concentration. However, acetic acid has shown to be a worrying factor due to its ability

to inhibit microbial growth when joined by furfural, as described by Palmqvist *et al.*, 1999. Therefore, some of the acetic acid had been removed by an ionic exchange detoxification before the enzymatic hydrolysis of the culture medium, but this process still releases several grams per litre of this substance to the liquid, which means that an acid hydrolysis of the liquor can be performed, to remove every acetyl group present into the liquid, followed by the detoxification. This would only allow a small amount of acetic acid to be released to the culture medium from the solid fraction and possibly increase the productivity and/or final concentration of xylitol.

The results of this assay are present in **Figure 13**. In the first place, comparing directly the results obtained with the same conditions but with an acid hydrolysis of the liquid fraction and a complete detoxification of the acetic acid previous to an enzymatic hydrolysis. It is possible to observe that the final xylitol concentration was 67.0 g/L (**Figure 13B**), which is 1.56 fold the concentration obtained with the optimum result of the previous assay with the partial liquor detoxification, at 42.9 g/L. Additionally, the xylitol yield (0.93 g xylitol/ g potential xylose) and productivity (0.56 g xylitol/L/h) were 1.21 and 1.85 times the obtained with the enzymatic hydrolysis at 120 hours of incubation. This implies that, not only does the bioconversion is more successful, producing more xylitol from the potential xylose, but the time that is takes to perform the conversion is also shorter, potentially saving a substantial amount of time in a large scale process of xylitol production.

Relatively to the use of a pre-hydrolysis in this assay (**Figure 13A**), it revealed, consistently with previous experiments in this study, not to be beneficial to the production of xylitol, achieving 63.6 g/L in the end of the incubation time. Furthermore, the xylitol yield and productivity were also lower, at 0.89 g xylitol/ g potential xylose and 0.44 g xylitol/L/h, respectively. This result strengthens the hypothesis that the use of pre-saccharification for 24 hours does not lead to an advantageous condition for the production of xylitol.

Lastly, the quantity of inoculum added in the beginning of the incubation was doubled (from 11 g/L to 22 g/L of wet cells) to evaluate if this alteration would lead to a superior xylose bioconversion into xylitol (**Figure 13**). By comparing the incubations with the same conditions but only different inoculum concentrations it was revealed that an increase in the inoculum lead to an increase in the production of xylitol, at 71.7 g/L, xylitol yield, at 0.998 g xylitol/ g potential xylose, and productivity, at 0.6 g xylitol/L/h, which was 1.07 fold higher the results obtained in the inoculum of 11 g of cells per litre. With this result it can be observed that practically all

the xylose is converted into xylitol at the end of the incubation and that increasing the quantity of inoculum at the beginning leads to an increase in the conversion of xylose into xylitol.

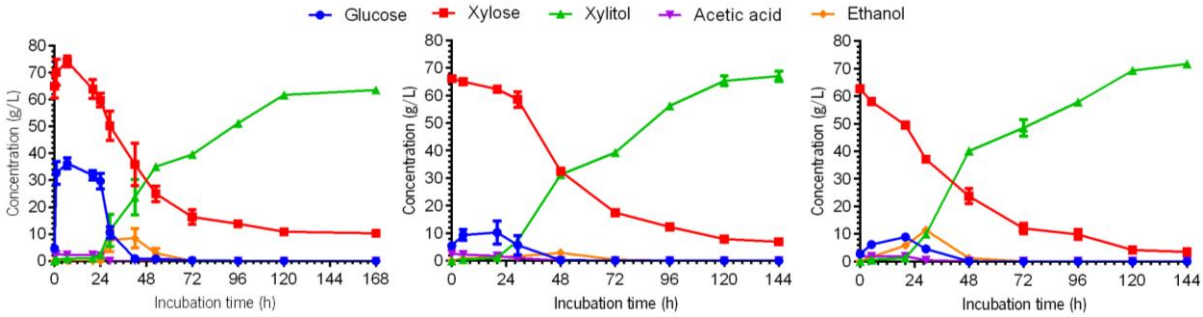


Figure 13 - Cultivation of *S. cerevisiae* PE-2-GRE3 at 35 ºC with 86,5 3% (v/v) liquid fraction previously acid hydrolysed and detoxified and 6,76 % (w/w) solid fraction of autohydrolysed corn cob at 25 % (w/w) corn cob, 20 g/L of peptone, 10 g/L of yeast extract, 150 mg/L of geneticin, 24 FPU of CellicCtec2/g of substrate. A – 24 hours of PSSF and 11 g/L of initial cells; B – SSF and 11 g/L of initial cells; C – SSF and 22 g/L of initial cells.

In this study the best results obtained were 71.7 g/L of xylitol, 0.60 g/L/h of productivity and 0.998 g xylitol/ g potential xylose of xylitol yield, obtained using an SSF process with no fed-batch supply of nutrients, in an Erlenmeyer flask, with a raw material of 25 % (w/w) of corn cob autohydrolysed at 205 ºC and with the liquid fraction being posthydrolysed and completely detached of acetic acid. When comparing it with the literature present in **Table 2** it is possible to verify that the xylitol conversion was near complete, which is a parameter that does not reach totality in any study, with the exception of Kogje and Ghosalkar, 2017, where the xylitol yield was approximately 1. Furthermore, the xylitol concentration was only inferior to the obtained with Li *et al.*, 2012, at 96.5 g/L of this polyol, however, it is notable that it involved a vacuum concentration of the liquid fraction to obtain an initial xylose concentration of 160 g/L, leading to a xylose conversion of 83 %. This result implies that, due to the conversion of xylitol with the utilized operational conditions being near totality, the xylitol concentration may be improved with further processes, such as the concentration of the liquid fraction. When comparing with the results previously obtained in this work group, the xylitol production was significantly superior to the obtained by Baptista *et al.*, 2018, in which were produced 24.3 g/L of this polyol with 0.88 g xylitol/g of potential xylose, probably due to the utilization of a corn cob concentration during autohydrolysis that is double the concentration used in that study and to the complete acid acetic detoxification of the liquid fraction in this study.

5. CONCLUSION

5. CONCLUSION

In this study, several strategies were carried out for the biotechnological production of xylitol from hydrothermally pretreated corn cob using a genetic modified industrial *S. cerevisiae* strain. Autohydrolysis treatment was proposed for the solubilisation of xylan into xylose and xylooligosaccharides and for the enhancement of cellulose saccharification by cellulolytic enzymes. High xylan solubilisation was achieved operating with high solid loadings (25 % (w/w) of corn cob) and 205 ºC of Tmax during autohydrolysis obtaining 47.0 g/L of potential xylose in the liquid fraction. Both phases (solid and liquid) conforming the whole slurry were used for the biotechnological production of xylitol.

The results obtained showed that the use of whole slurry corn cob was successful for the production of xylitol in a simultaneous saccharification and fermentation process, even with the presence of inhibitory compounds, such as acetic acid, furfural and hydroxymethylfurfural.

- Two incubation temperatures were tested with the previously optimized conditions and 35 ºC was the temperature that obtained higher xylitol production, despite similar concentration and productivity. The use of a partially detoxified liquor produced a satisfactory xylitol concentration (up to 51.8 g/L) with a relative low xylitol productivity (0.36 g/L/h).
- A comparison between the use of pre-saccharification and fermentation or simultaneous saccharification and fermentation processes was also performed under the previous conditions. SSF showed a superior xylitol production and this assay revealed that the use of a pre-saccharification was not beneficial for this fermentation.
- A complete factorial design was proposed to optimize the concentration of solids added to the medium and the enzyme loading, and the optimal conditions obtained with this study were 6.76 % solids and 24 FPU/g of substrate. This model was validated with the theoretical optimal conditions replicated and presenting a xylitol concentration (42.9 g/L) and a productivity (0.30 g/L/h).
- The utilization of an acid hydrolysis of the liquid fraction followed by a complete detoxification and the increase of the inoculum concentration to 22 g/L of wet cells was tested, obtaining the best results in this study. Xylitol concentration and productivity were superior (71.7 g/L and 0.60 g/L/h, respectively), with practically full xylose conversion, showing that the complete removal of the acetic acid from the liquid fraction was beneficial to the production of the polyol.

- Overall, the optimization of operational conditions was successful and in future studies other parameters may be further optimized, such as the exact inoculum concentration or the insertion of solid loads in fed-batch to potentially increase the production of this polyol and supply nutrients through the fermentation. Additionally, the study of the scale-up process may also be performed utilizing a bioreactor to produce xylitol.

Lastly, with this study, a protocol for the production of xylitol with the utilization of corn cob autohydrolysates is proposed, in which the operational conditions during the pretreatment and during the fermentation are described in **Figure 14**.

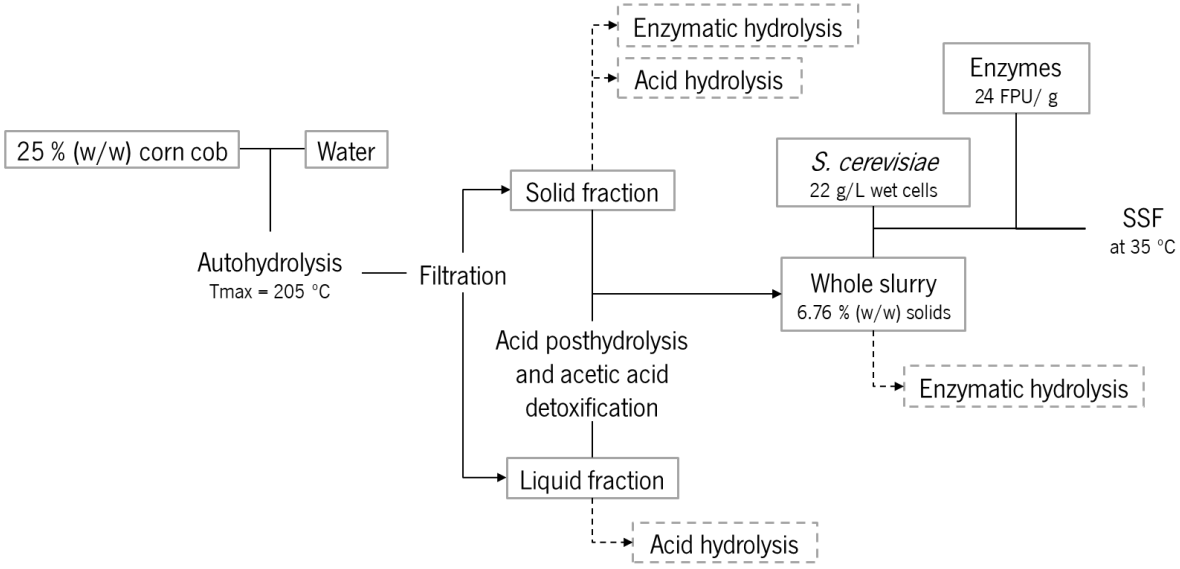


Figure 14 – Corn cob treatment and SSF process with optimized conditions.

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